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This proposal aims to exploit advances in biotechnology and informatics to develop a genetics resource termed the Prostate Expression Database (PEDB) (http://www.pedb.org). The foundation of PEDB is the identification and characterization of a prostate transcriptome, the intermediary between the genome and the proteome that represents that portion of the human genome actively used or transcribed in the prostate. The research accomplished to date has assembled a working virtual prostate transcriptome that defines the genes used or transcribed in prostate cell types and tissues. This transcriptome has a physical counterpart of 6,000 cDNAs arrayed in cDNA microarray format for large-scale expression studies. This transcriptome has been used as a foundation for studies of the prostate proteome, the working counterpart to the genome and transcriptome. Our results show that these approaches are complementary. Analysis of the virtual transcriptome of LNCaP cells has identified 13 new androgen-regulated genes to date. Characterization of these genes is in progress. One gene, PSDR1, exhibits sequence homology with a family of proteins involved in steroid hormone metabolism, and may modulate steroid activity within the prostate.

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FOREWORD

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INTRODUCTION

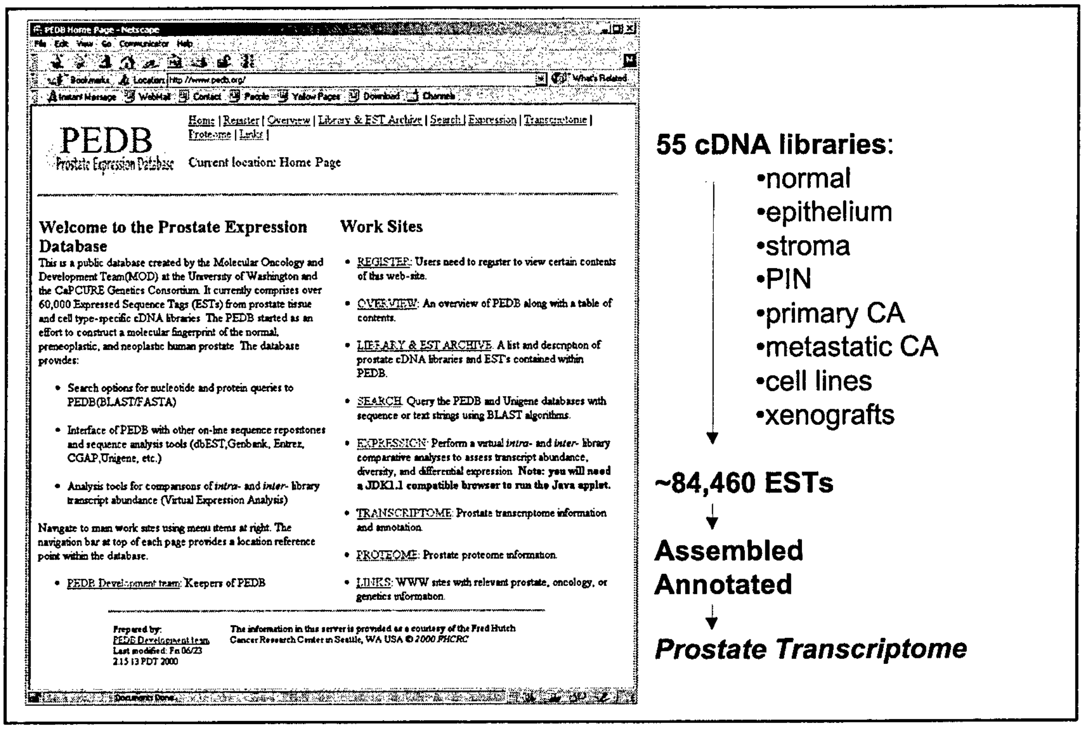
This proposal aims to exploit advances in biotechnology and informatics to develop a genetics resource termed the Prostate Expression Database (PEDB) (http://www.pedb.org). PEDB is an integrated resource focused exclusively on prostate cancer that incorporates public DNA and protein sequence and informatics resources where applicable. The foundation of PEDB is the identification and characterization of a prostate transcriptome, the intermediary between the genome and the proteome that represents that portion of the human genome actively used or transcribed in the prostate. This proposal will extend PEDB capabilities by accomplishing the following specific objectives: 1) assemble and annotate a working prostate transcriptome; 2) develop a suite of database tools to facilitate investigator-initiated database queries; 3) extend the prostate transcriptome in 3 dimensions: acquiring rare transcripts, assembling sequences representing full-length genes, and mapping the locations of interesting and novel prostate genes; and 4) assemble a solid-phase nonredundant archive of prostate-derived cDNA clones for distribution to investigators and to the Image Consortium sites.

BODY

The following summarizes the technical objectives for the proposal and the work accomplished during the 8-month interval between the last report (07/14/00) and the preparation of this report (03/14/01).

Technical objective 1: *To assemble and annotate a working prostate transcriptome* (months 1-16)

- *Task 1: Install Phrap, d2-cluster, and CAP3 software and test on small, known genomic sequence assemblies (months 1-3).* Completed. Phrap is the selected assembly algorithm of choice.
- *Task 2: Assemble UniGene and prostate EST test sets. Compare with previous assemblies performed with CAP2. Manually review assembly discrepancies. Compare assemblies with UniGene and CGAP clusters (months 1-6).* Completed.
- *Task 3: Assemble and annotate all PEDB sequences using best available algorithm (months 6-12).* Completed. We have completed the download of 10,000 additional chromatograms from the Washington University web site and added an additional 4,000 ESTs from our own sequencing project. The assembly of these ESTs with phrap has been completed and the contigs have been annotated against sequences housed in Genbank and Unigene. The latest assembly statistics are shown in Figures 1 and 2 with the assembly schema and results.



PEDB Home Page - Netscape

File Edit View Go Communicator Help
Bookmarks Location: http://www.pedb.org/ What's Related
Instant Message WebMail Contact People Yellow Pages Download Channels

PEDB
Prostate Expression Database

Home | Register | Overview | Library & EST Archive | Search | Expression | Transcriptome | Proteome | Links |

Current location: Home Page

Welcome to the Prostate Expression Database

This is a public database created by the Molecular Oncology and Development Team(MOD) at the University of Washington and the CaPCURE Genetics Consortium. It currently comprises over 60,000 Expressed Sequence Tags (ESTs) from prostate tissue and cell type-specific cDNA libraries The PEDB started as an effort to construct a molecular fingerprint of the normal, preneoplastic, and neoplastic human prostate The database provides:

- Search options for nucleotide and protein queries to PEDB(BLAST/FASTA)
- Interface of PEDB with other on-line sequence repositories and sequence analysis tools (dbEST,Genbank, Entrez, CGAP,Unigene, etc.)
- Analysis tools for comparisons of *intra*- and *inter*- library transcript abundance (Virtual Expression Analysis)

Navigate to main work sites using menu items at right. The navigation bar at top of each page provides a location reference point within the database.

- PEDB Development team: Keepers of PEDB

Work Sites

- REGISTER: Users need to register to view certain contents of this web-site.
- OVERVIEW: An overview of PEDB along with a table of contents.
- LIBRARY & EST ARCHIVE. A list and description of prostate cDNA libraries and EST's contained within PEDB.
- SEARCH. Query the PEDB and Unigene databases with sequence or text strings using BLAST algorithms.
- EXPRESSION: Perform a virtual *intra*- and *inter*- library comparative analyses to assess transcript abundance, diversity, and differential expression Note: **you will need a JDK1.1 compatible browser to run the Java applet.**
- TRANSCRIPTOME: Prostate transcriptome information and annotation.
- PROTEOME: Prostate proteome information.
- LINKS: WWW sites with relevant prostate, oncology, or genetics information.

Prepared by:
PEDB Development team
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55 cDNA libraries:

- normal
- epithelium
- stroma
- PIN
- primary CA
- metastatic CA
- cell lines
- xenografts

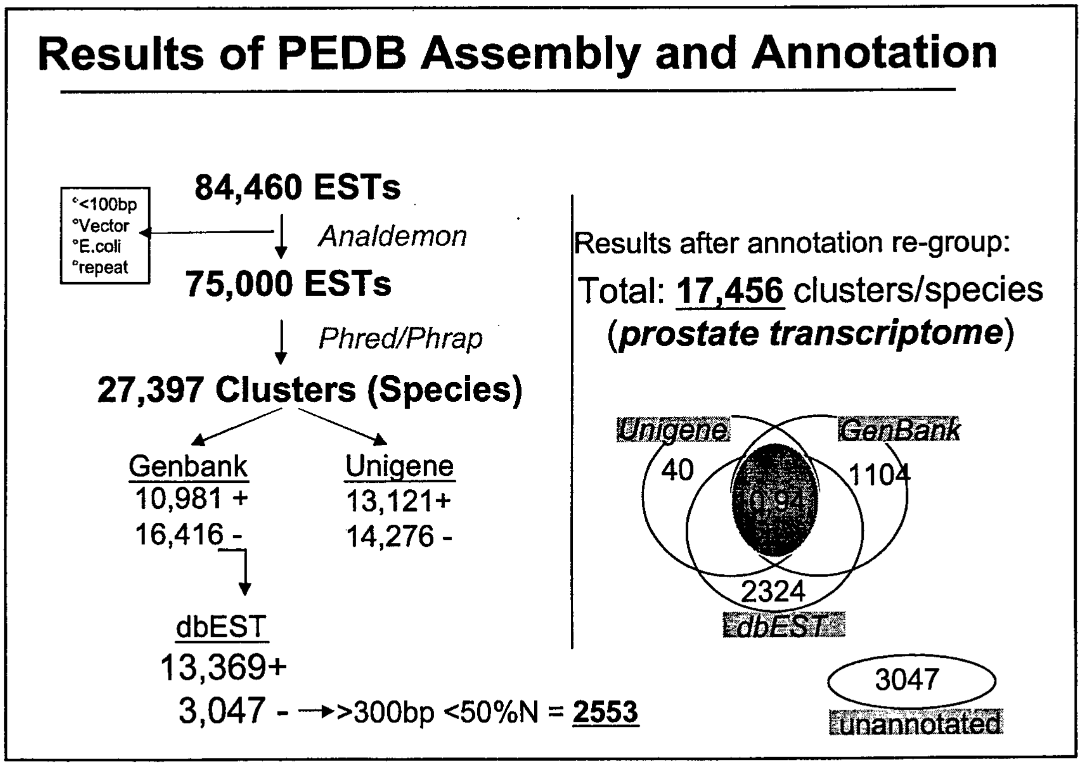
~84,460 ESTs

Assembled
Annotated

Prostate Transcriptome

Figure 1. (above) WWW Interface for the Prostate Expression Database (PEDB). 55 libraries containing ~85,000 EST have been processed, assembled, and annotated to comprise a Prostate Transcriptome.

Figure 2. (below) Assembly process for PEDB ESTs. ~85,000 ESTs were processed for low quality, vector, *e coli*, and repetitive sequences. The remaining ESTs were assembled using Phrap and annotated against sequences in the public nucleotide databases. Following re-assembly, a prostate transcriptome of 17,456 distinct species was entered into PEDB.



Results of PEDB Assembly and Annotation

84,460 ESTs

Analdemon

°<100bp
°Vector
°E.coli
°repeat

75,000 ESTs

Phred/Phrap

27,397 Clusters (Species)

Genbank
10,981 +
16,416 -

Unigene
13,121+
14,276 -

dbEST
13,369+
3,047 - →>300bp <50%N = **2553**

Results after annotation re-group:

Total: **17,456** clusters/species
(***prostate transcriptome***)

- *Task 4: Develop a gene classification schema based upon function, and automate assignments of clusters to functional groups (months 8-16).* We have completed a functional annotation scheme modeled after the TIGR annotation scheme for partitioning genes into cellular functional groups. This has been applied to the LNCaP dataset and is available for viewing and analysis on the PEDB website.
- *Task 5: Develop a Graphical User Interface for viewing and navigating between sequence, functional group, and expression data (months 3-12).* A user interface has been developed for the viewing of sequence chromatograms and for searching the database with keywords in addition to BLAST queries.
- *Task 6: Write scripts to automate the input of new prostate ESTs, processing of new ESTs, clustering of the database sequences and annotation of the entire cluster complement on a monthly basis. (months 12-16).* Completed.

Technical objective 2: *To develop a suite of database tools to facilitate investigator-initiated database queries. (months 1-18).* In progress.

- *Task 7: Evaluate potential sequence cluster/assembly viewing tools: DrawMap, Consed, Phrapview, CloneView, and AlignView (months 6-12).*
- *Task 8: Design a client application (ContigView) extending the functionality of the Virtual Expression Analysis Tool to view the cluster output produced by the best algorithm as identified in specific aim 1. (months 8-14)*
- *Task 9: Design GUI to support high level viewing of clustered data with graphical maps incorporating zoom features for viewing nucleotide sequence traces and assemblies (8-14).* A tool for viewing individual sequence traces has been developed and implemented into PEDB. A tool for viewing sequence assemblies is in progress.
- *Task 10: Write Java code for ContigView and test on datasets representing assemblies of few and many ESTs with both short and long consensus sequences. (months 10-24)*
- *Task 11: Test (and modify if necessary) ContigView on Windows/NT/MacIntosh/Unix operating systems (months 24-30)*
- *Task 12: Write applications to link cluster consensus to relevant public databases (Genbank, etc) (months 20-24)*
- *Task 13: Write applications for integrating gene analysis tools: exon prediction, promoter finders, transcription factor binding site ID, protein motif ID (months 18-28).*
- *Task 14: Evaluate the incorporation of software for SNP detection (PolyPhred) in client-selected PEDB clusters (26-30).*

Technical objective 3: To *extend the prostate transcriptome in 3 dimensions: 1) acquire rare transcripts 2) assemble sequences representing full-length genes and 3) map the location to EST clusters to specific chromosomal sites.* (months 12-25)

- *Task 15: construct LNCaP random primed library and CAP-finder library (months 6-7).* We have constructed one prostate cDNA library from androgen stimulated LNCaP and one cDNA library from androgen-starved LNCaP. A total of 3,000 ESTs have now been generated from each library to date. EST assemblies from these libraries have been used to virtually determine the gene expression network regulated by androgenic hormones. These datasets have been compared to profiles produced by the Serial Analysis of Gene Expression (see Clegg et al in reportable outcomes). Several genes previously not recognized to be under androgen regulation were identified.

- *Task 16: partially sequence 1,600 cDNAs from each library and enter ESTs into PEDB. (months 8-12)* See above. All 6,000 ESTs have been entered into PEDB, assembled using phrap, and annotated against sequences present in the public nucleotide databases.
- *Task 17: as above with normal prostate tissue (months 13-18).* We have constructed cDNA libraries from microdissected luminal cell, basal cell, and stromal tissue. A total of 1,500 ESTs have been produced from these libraries.
- *Task 18: as above with microdissected primary prostate cancer tissue (months 25-30)*
- *Task 19: "Negative Select" 10,000 cDNAs from normal prostate cDNA array (months 19-20)*
- *Task 20: partially sequence 10,000 negatively selected, low abundance cDNAs and submit ESTs into PEDB (months 21-25)*
- *Task 21: Identify 60 interesting uncharacterized prostate ESTs/cDNAs based upon a) homology to known physiologically important genes or b) novelty, to directly obtain full-length cDNA sequence using RACE, library screening, genomic assembly, and primer-directed sequencing. A total of 15 full-length cDNAs per year will be obtained (ongoing throughout period of award).* We have cloned and sequenced 10 full-length genes expressed in prostate. One of these, Prostate Short-Chain Dehydrogenase Reductase 1 (PSDR1) has been extensively characterized and a manuscript published in Cancer Research (see Lin et al in reportable outcomes). The remaining genes are under further evaluation.
- *Task 22: Map interesting prostate cDNAs described above using radiation hybrid panel mapping. (ongoing throughout period of award).* We have now mapped 8 novel prostate genes using radiation hybrid panel mapping. Future work will automate mapping procedures using the available human genome sequence annotation.
- *Task 23: submit data to PEDB and public databases* (ongoing throughout period of award).

Technical objective 4: *To assemble a solid phase nonredundant archive of prostate-derived cDNA clones.*

- *Task 24: identify a cohort consisting of 3,000 distinct, unique prostate clusters from year 1 PEDB assembly (month 10).* We have assembled a non-redundant set of 6,000 cDNAs from LNCaP, normal and neoplastic prostate cDNA libraries. These have now been re-arrayed into 96-well and 384-well microtiter plates. The clone set has been replicated. PCR amplification has been performed.
- *Task 25: cross-reference cluster sequences with PEDB clone archive to determine the clones physically available for biological studies (month 11).* Completed.
- *Task 26 determine the longest physical clone for each cluster and consolidate bacterial transformants into 96-well plates using the Genetix Q-bot. Preserve for storage (months 12-13).* Completed.
- *Task 27: annotate and ship to IMAGE consortium clone distributors (month 14).* In progress.
- *Task 28: repeat Tasks 24-27 for 3,000 additional unique clusters at the end of month 24.* In progress.
- *Task 29: repeat Tasks 24-27 for 3,000 additional unique clusters at the end of month 35.*

- *Task 30: plan for incorporation and integration of PEDB with microarray data and proteomics data (months 24-36).* Currently in planning stages. We have obtained database software for archiving and analyzing microarray data from Stanford University. We are currently testing for compatibility with PEDB.
- *Task 31: analyze/compile data and prepare formal report (month 36).*

KEY RESEARCH ACCOMPLISHMENTS

- Selected phrap as the sequence assembly algorithm for PEDB. Assembled and annotated 85,000 PEDB ESTs.
- Constructed cDNA libraries from microdissected luminal epithelial cells, basal epithelial cells, and stromal cells.
- Sequenced 3,000 cDNAs from LNCaP, luminal cell, basal cell and stromal cell cDNA libraries (total 4,000 ESTs) and assembled the ESTs into clusters/contigs. The data indicate the libraries are of good quality with significant diversity.
- Virtual comparison of the LNCaP libraries identified 3 additional new androgen-regulated genes. Northern analysis confirmed androgen-regulation for these genes.
- Constructed a cDNA library of prostate small cell carcinoma. 3,000 cDNA clones have been sequenced and deposited into PEDB.
- Compiled a non-redundant virtual and physical archive of prostate ESTs/cDNAs comprising 6,000 distinct species. These clones have been consolidated, replicated, and arrayed for cDNA microarray analysis.
- Identified a novel gene with prostate expression specificity, PSDR1, that is hypothesized to function in steroid metabolism. PSDR1 is highly expressed in primary and metastatic prostate carcinoma.

REPORTABLE OUTCOMES

Nelson PS, Han D, Rochon Y, Corthals G, Lin B, Monson A, Nguyen V, Franza BR, Plymate SR, Aebersold R, and Hood L. (2000) Comprehensive analyses of prostate gene expression: convergence of EST databases, transcript profiling and proteomics. *Electrophoresis* 21:1823-31.

Lin B, White JT, Ferguson C, Wang S, Vessella R, Bumgarner R, True LD, Hood L, and **Nelson PS**. (2001) *PSDR1*: a prostate-localized member of the short-chain steroid dehydrogenase/reductase (SDR) family highly expressed in normal and neoplastic prostate epithelium. *Cancer Research* 61:1611-8.

Grouse LH, Munson PJ, and **Nelson PS**. (2001) Sequence Databases and Microarrays as Tools for Identifying Prostate Cancer Biomarkers. *J. Urology* 57 (Suppl 4A): 154-159.

Clegg N, Erolgu B, Ferguson C, Arnold H, Moorman A, and **Nelson PS**. Digital Expression Profiles of the Prostate Cell Transcriptome (Submitted: *Genomics*)

Liu AY, **Nelson PS**, Ligner CL, van den Engh G, Hood L. Human Prostate Epithelial Cell-Type cDNA Libraries and Expression Pattern in Prostate Cancer. (Submitted: *Cancer Research*)

Clegg N, Eroglu B, Ferguson C, Arnold H, Moorman A, True L, Vessella R, and **Nelson PS**, *Transcript analysis of prostate small cell carcinoma.* (In preparation).

CONCLUSIONS

The research accomplished to date has assembled a working virtual prostate transcriptome that defines the genes used or transcribed in prostate cell types and tissues. This transcriptome has a physical counterpart of 6,000 cDNAs arrayed in cDNA microarray format for large-scale expression studies. This transcriptome has been used as a foundation for studies of the prostate proteome, the working counterpart to the genome and transcriptome. Our results show that these approaches are complementary. Analysis of the virtual transcriptome of LNCaP cells has identified 13 new androgen-regulated genes to date. Characterization of these genes is in progress. One gene, PSDR1, exhibits sequence homology with a family of proteins involved in steroid hormone metabolism, and may modulate steroid activity within the prostate.

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None

APPENDICES

Nelson PS, Han D, Rochon Y, Corthals G, Lin B, Monson A, Nguyen V, Franza BR, Plymate SR, Aebersold R, and Hood L. (2000) Comprehensive analyses of prostate gene expression: convergence of EST databases, transcript profiling and proteomics. *Electrophoresis* 21:1823-31.

Grouse LH, Munson PJ, and **Nelson PS**. (2001) Sequence Databases and Microarrays as Tools for Identifying Prostate Cancer Biomarkers. *J. Urology* 57 (Suppl 4A): 154-159.

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Comprehensive analyses of prostate gene expression: Convergence of expressed sequence tag databases, transcript profiling and proteomics

Several methods have been developed for the comprehensive analysis of gene expression in complex biological systems. Generally these procedures assess either a portion of the cellular transcriptome or a portion of the cellular proteome. Each approach has distinct conceptual and methodological advantages and disadvantages. We have investigated the application of both methods to characterize the gene expression pathway mediated by androgens and the androgen receptor in prostate cancer cells. This pathway is of critical importance for the development and progression of prostate cancer. Of clinical importance, modulation of androgens remains the mainstay of treatment for patients with advanced disease. To facilitate global gene expression studies we have first sought to define the prostate transcriptome by assembling and annotating prostate-derived expressed sequence tags (ESTs). A total of 55 000 prostate ESTs were assembled into a set of 15 953 clusters putatively representing 15 953 distinct transcripts. These clusters were used to construct cDNA microarrays suitable for examining the androgen-response pathway at the level of transcription. The expression of 20 genes was found to be induced by androgens. This cohort included known androgen-regulated genes such as prostate-specific antigen (PSA) and several novel complementary DNAs (cDNAs). Protein expression profiles of androgen-stimulated prostate cancer cells were generated by two-dimensional electrophoresis (2-DE). Mass spectrometric analysis of androgen-regulated proteins in these cells identified the metastasis-suppressor gene NDKA/nm23, a finding that may explain a marked reduction in metastatic potential when these cells express a functional androgen receptor pathway.

Keywords: Prostate / Transcriptome / Proteome / Androgen / Microarray EL 3957

1 Introduction

The development and subsequent progression of human prostate carcinoma is propelled by the accumulation of genetic alterations and influenced by environmental factors. One pivotal mediator of prostate carcinogenesis is the androgen receptor (AR) pathway. The majority of prostate cancers initially require androgens for growth, and the elimination of AR-ligands by surgical or chemical castration leads to marked tumor regression through a mechanism of programmed cell death [1]. The manipulation of the AR pathway has been used in clinical medicine since the 1940s as the primary treatment of advanced prostate cancer. However, this therapy is palliative and eliminates the potential beneficial effects of androgen-induced cellular differentiation. Surviving cancer cells lose their dependence on androgens over time and are capable of prolifertion in the absence of serum androgens. The molecular events leading to androgen independence (AI) have not been defined, but potential mechanisms include overexpression of the AR, mutations in the AR gene leading to promiscuous ligand binding, and the activation of the AR or downstream regulatory molecules by other endocrine or paracrine growth factors [2, 3].

Until recently, biological investigations have almost entirely focused on the study of individual genes and proteins. This has partly been due to the submicroscopic nature and transient existence of relevant molecules, combined with a lack of quantitative technology capable of providing accurate comprehensive views of biological complexity. Significant advances have been achieved studying individual genes, proteins and small numbers of molecular interactions. However, conclusions made on the basis of the study of an individual gene may have limited relevance as to how the gene and gene product function in the context of the whole cell, tissue, or organism. Progress in understanding complex molecular processes

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Abbreviations: AR, androgen receptor; **cDNA**, complementary DNA; **µLC**, microcapillary LC; **PEDB**, prostate expression database; **PSA**, prostate specific antigen

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has been hampered by the lack of a complete inventory or "tool-set" of all genes and their cognate proteins that are necessary for defining phenotypes of normal and pathological cellular states.

The completion of the Human Genome Project will provide a foundation for a thorough description of this molecular inventory. More specifically, the gene inventory or tool set required for studies of prostate carcinogenesis is that portion of the human genome used or expressed in the human prostate gland. The subset of genes transcribed or expressed in a given cell or tissue type such as the prostate may be defined as the "transcriptome", the dynamic link between the genome, the proteome, and the cellular phenotype associated with physical characteristics [4]. Once a transcriptome has been described, the next objective is to understand the relationships of the genes and their protein products in terms of a complex system, *e.g.*, biological pathways and networks, that may define health and disease. With this aim, novel technologies for comprehensively assessing genomes and patterns of gene expression have recently been developed.

Our initial efforts have focused on defining the prostate transcriptome through the production and assembly of expressed sequence tags (ESTs) derived from prostate complementary DNA (cDNA) libraries representing a wide sprectrum of normal and neoplastic states. These EST assemblies have been used to construct cDNA microarrays suitable for interrogating the transcriptome in experiments designed to examine specific biological pathways that may be involved in prostate carcinogenesis. The molecular pathway mediating androgenic hormone action on prostate cells is a specific focus of our work. The functional architecture of prostate gene networks is furth elucidated by our next level of analysis that incorporates studies of the prostate proteome. Analysis of the transcriptome facilitates proteome studies by providing a comprehensive prostate sequence database for identifying and annotating known and unknown proteins displayed by two-dimensional gel electrophoresis (2-DE) and analyzed by mass spectrometry (MS). Our objectives for delineating the molecular network(s) influenced by AR activation are to identify specific targets that promote the differentiation and apoptotic potential of prostate cancer cells while reducing their ability to proliferate.

2 Materials and methods

2.1 Assembly of a prostate transcriptome: Prostate Expression Database (PEDB)

A prostate transcriptome was assembled from ESTs derived from cDNA libraries representing a wide sprectrum of normal, benign, and malignant prostate tissues. A detailed description of the assembly and annotation procedure is described elsewhere [5]. Briefly, individual ESTs, detailed cDNA library information, and sequence annotations were loaded into a relational database (Oracle Corp.) termed the Prostate Expression Database (PEDB). Prostate ESTs used for the assembly were generated in our laboratory as previously described [6]. Additional public domain ESTs of prostate origin were obtained from Genbank (http://www.ncbi.nlm.nih.gov/Entrez/batch.html), the NCI Cancer Genome Anatomy Project (CGAP) [7], and The Institute for Genome Research (TIGR) (http://www.tigr.org). Each EST was examined for sequence homology to cloning vectors, *Escherichia coli*, and repetitive DNA sequences using a core program called AnalDemon (http://www.mbt.washington.edu/PEDB/software). AnalDemon first employs Cross_Match (http://bozeman.mbt.washington.edu/phrap.docs/general.html); a program based on the Smith-Waterman-Gotoh algorithm, to screen for vector and *E. coli* genome contamination. ESTs are then examined for interspersed repeats and regions of low sequence complexity using Repeatmasker (http://ftp.genome.washington.edu/RM/RepeatMasker.html). Specific portions of EST sequences exhibiting homology to any of these unwanted elements are masked in order to eliminate the sequence from contributing to an assembly process. CAP2 [8], a multiple alignment program based on a variant of the Smith and Waterman algorithm, was used for assembling ESTs into homologous groups or clusters. Clustering is based on maximal scoring of overlapping alignments and allows for general substitutions resulting from sequencing errors, insertions, and deletions. CAP2 produces a consensus sequence and allows varying sensitivity and overlap parameters. Each group or cluster of ESTs exhibiting significant homology with one another is termed a species. Thus, a species is a sequence or group of sequences that is unique relative to the nucleotide sequence of other groups of sequences, and each is given a unique PEDB Species Identification number (SID). The SID provides a means to analyze gene expression across the entire set of assemblies, and can be used to provide a library-by-library species-specific differential expression profile. Each distinct species from the clustering process was annotated by searching the Unigene (ncbi.nlm.nih.gov in /pub/schuler/unigene), Genbank (ncbi.nlm.nih.gov/blast/db/nt.Z), and EST databases (ncbi.nlm.nih.gov/blast/db/est.Z) using BLASTN (http://blast.wustl.edu). Annotations were assigned automatically using the program SmartBlast (http://www.mbt.washington.edu/PEDB/software) by selecting the database match with the lowest p value and the highest blast score where the maximum p value is e^{-20} and the minimum blast score is 500.

2.2 Prostate transcriptome analyses by cDNA microarray

2.2.1 Microarray fabrication

A nonredundant set of 1500 prostate-derived cDNA clones was identified from the prostate transcriptome archived in PEDB. Individual clone inserts were amplified by the PCR using 2 µL of bacterial transformant culture as template with primers BL_m13F (5′-GTAAAACGA-CGGCCAGTGAATTG-3′) and BL_m13R (5′-ACACAGG-AAACAGCTATGACCATG-3′ as previously described [6]. PCR products were purified through Sephacryl S500 (Amersham Pharmacia Biotech, Uppsala, Sweden), mixed 1:1 with dimethylsulfoxide, and spotted in duplicate onto coated Type VII glass microscope slides (Amersham Pharmacia Biotech) using a Molecular Dynamics (Sunnyvale, CA, USA) GenII robotic spotting tool. After spotting, the glass slides were air-dried and UV-cross-linked with 500 mJ of energy and then baked at 95°C for 30 min.

2.2.2 Probe construction and microarray hybridization

Total RNA was isolated from the androgen-responsive LNCaP prostate cancer cells [9] at time points of 0, 4, 8, 24, and 72 h after androgen depletion or supplementation using TRIzol (Life Technologies, Paisley, UK) according to the manufacturer's directions. Fluorescence-labeled probes were made from 30 µg of total RNA in a reaction volume of 20 µL containing 1 µL anchored oligo-dT primer (Amersham Pharmacia Biotech), 0.05 mM Cy3-dCTP (Amersham Pharmacia Biotech), 0.05 mM dCTP, 0.1 mM each dGTP, dATP, dTTP, and 200 U Superscript II reverse transcriptase (Life Technologies). Reactants were incubated at 42°C for 120 min followed by heating to 94°C for 3 min. Unlabeled RNA was hydrolyzed by the addition of 1 µL of 5 N NaOH and heating to 37°C for 10 min. One µL of 5 M HCl and 5 µL of 1 M Tris-HCl, pH 7.5, were added to neutralize the base. Unincorporated nucleotides and salts were removed by chromatography (Qiagen, Chatsworth, CA, USA), and the cDNA was eluted in 30 µL dH2O. One µg of dA/dT 12–18 (Amersham Pharmacia Biotech) and 1 µg of human Cot1 DNA (Life Technologies) were added to the probe, heat-denatured at 94°C for 5 min, combined with an equal volume of 2 × microarray hybridization solution (Amersham Pharmacia Biotech) and prehybridized at 50°C for 1 h. The mixture was then placed onto a microarray slide with a coverslip and hybridized in a humid chamber at 52°C for 16 h. The slides were washed once with 1 × sodium chloride and sodium citrate (SSC), 0.2% SDS at room temperature for 5 min and then twice with 0.1 × SSC, 0.2% SDS at room temperature for 10 min. After washing, the slide was rinsed in distilled water to remove trace salts and dried.

2.2.3 Image acquisition and data analyses

Fluorescence intensities of the immobilized targets were measured using a laser confocal microscope (Molecular Dynamics). Intensity data were integrated at a pixel resolution of 10 µm using approximately 20 pixels per spot, and recorded at 16 bits. Quantitative data were obtained with the SpotFinder Version 2.4 program written at the University of Washington. Local background hybridization signals were subtracted prior to comparing spot intensities and dtermining expression ratios. For each experiment, each cDNA was represented twice on each slide, and the experiments were performed in duplicate producing four data points per cDNA clone and hybridization probe. Intensity ratios for each cDNA clone hybridized with probes derived from androgen-stimulated LNCaP and androgen-starved LNCaP cells were calculated (stimulated intensity/starved intensity). Gene expression levels were considered significantly different between the two conditions if all four replicate spots for a given cDNA demonstrated a ratio > 2 or < 0.5, and the signal intensity was greater than two standard deviations above the image background. We have previously determined that expression ratios less than 1.5 are not reproducible in our system (datas not shown).

2.3 Prostate proteome analyses by 2-DE and MS

2.3.1 2-DE

LNCaP prostate cancer cells were grown under conditions of androgen stimulation or androgen starvation as described above. M12AR cells, a highly metastatic prostate cancer cell line derived from the serial passaging of SV40 immortalized prostate epithelial cells [10] and transfected with the AR were grown in serum-free DMEM high-glucose media (Life Technologies) supplemented with insulin, transferrin, selenium, and dexamethasone as previously described [11]. Cells were allowed to reach 80% confluency and then treated for 24 h with the same media supplemented with 10 nM R1881. Cells were washed once with PBS, scraped from plates with a rubber policeman and pelleted by centrifugation. Protein was harvested as described by Garrels and Franza [12]. Briefly, cell pellets were lysed in a buffer containing 0.3% SDS, 1% β-mercaptoethanol, and 50 mM Tris-HCl, pH 8.0, 100 µg/mL DNAase I, 50 µg/mL RNAase A, 5 mM $MgCl_2$, and heated for 1 min at 100°C. Harvested protein was lyophilized, resuspended in isoelectric focusing (IEF) gel rehydration solution, and stored at –80°C. Soluble proteins were run in the first dimension by using a commercial flatbed electrophoresis system (Multiphor II; Amersham Pharmacia Biotech). Nonlinear immobilized pH gradient (IPG) dry strips ranging from 3.0 to 10.0 (Amersham Pharmacia Biotech) were used for the first-dimensional separation. Forty micrograms of protein from whole-cell lysates were mixed with IPG strip rehydration buffer (8 M urea, 2% Nonidet P-40, 10 mM dithiothreitol), and 250–380 µL of solution (13 and 18 cm IPGs, respectively) was added to individual lanes of an IPG strip rehydration tray (Amersham Pharmacia Biotech). The strips were rehydrated at room temperature for 1 h. The samples were run at 300 V, 10 mA, 5 W for 2 h, ramped to 3500 V, 10 mA, 5 W over a period of 3 h, and then kept at 3500 V, 10 mA, 5 W for 15–19 h. Following IEF (60–70 kVh), the IPG strips were first reequilibrated for 8 min in a solution of 2% w/v dithiothreitol, 2% w/v SDS, 6 M urea, 30% w/v glycerol, 0.05 M Tris-HCl (pH 6.8) and subsequently for 4 min in a solution of 2.5% w/v iodoacetamide, 2% w/v SDS, 6 M urea, 30% w/v glycerol, 0.05 M Tris-HCl (pH 6.8) with a trace of bromophenol blue added for color. Following reequilibration, the strips were transferred and apposed to 10% polyacrylamide second-dimensional gels. Polyacrylamide gels were poured in casting stand with 10% acrylamide-2.67% piperazine diacrylamide-0.375 M Tris, pH 8.8, 0.1% w/v SDS, 0.05% w/v ammonium persulfate, 0.05% TEMED (*N,N,N′,N′*-tetramethylethylenediamine) in Milli-Q water (Millipore, Bedford, MA, USA). Second-dimensional gels (0.1 × 20 × 20 cm) were run in an apparatus supplied by Oxford Glycosciences (Abington, UK). Once the IPG strips were apposed to the second-dimensional gels, they were immediately run at a constant current of 50 mA at 500 V and 85 W for 20 min, followed by a constant current of 200 mA at 500 V and 85 W until the buffer front was 10–15 mm from the bottom of the gel. Gels were removed and silver stained according to the procedure of Blum *et al.* [13].

2.3.2 Protein identification by tandem mass spectrometry

Protein spots from gels were identified by tandem mass spectrometry (MS/MS) as previously described [14]. Spots from silver-stained gels were excised and in-gel tryptic peptides were separated by microcapillary LC (µLC) coupled to a tandem mass spectrometer (TSQ 7000; Finnigan, San Jose, CA). Peptide fragmentation spectra were generated in a data-dependent fashion. Spectra were searched against the composite OWL protein sequence database by using the computer program SEQUEST [15] and against the PEDB. A protein match was determined by comparing the number of peptides identified and their respective cross-correlation scores. Protein identifications were verified by comparison with theoretical molecular weights and isoelectric points.

3 Results and discussion

3.1 Prostate gene expression analyses: EST assemblies and annotation

ESTs produced from cDNA libraries derived from normal and neoplastic human prostate tissue samples were entered into the PEDB, an Oracle relational database running on a Sun SPARC workstation. The most recent PEDB build was assembled starting with 55 000 prostate ESTs produced from 42 cDNA libraries. Portions of EST sequences with homology to cloning vector, *E. coli* genomic DNA, and human repetitive DNA sequences

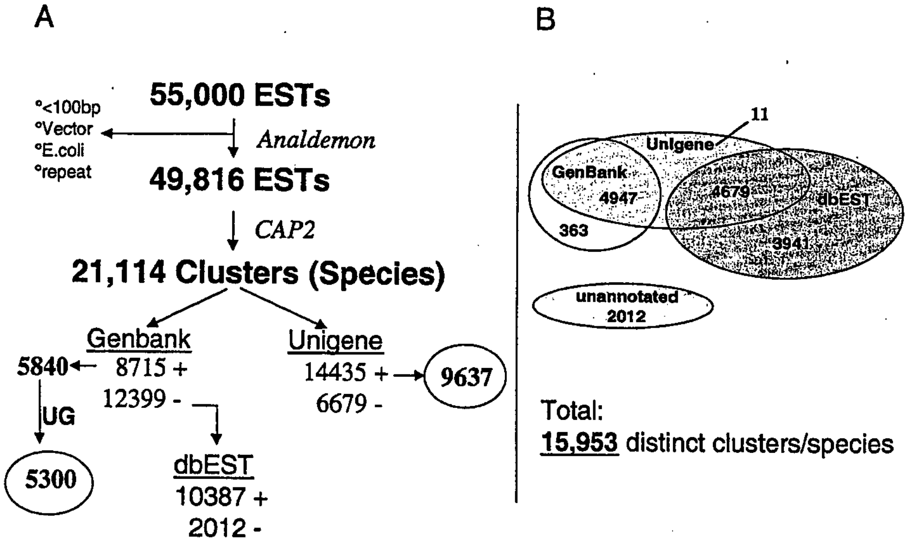


Figure 1. Assembly of a prostate transcriptome. (A) 55 000 prostate ESTs were examined for "junk" sequences leaving 49 816 high quality ESTs suitable for assembly. Clustering the ESTs into groups of high homology produced a set of 21 114 clusters that were annotated against nucleotide and protein sequences in the public sequence databases. Clusters exhibiting homology to Genbank sequences were also examined for homology to Unigene sequences (UG) to further collapse clusters into homologous groups. (B) Following clustering, database annotations and reclustering, a total of 15 953 distinct prostate EST species were identified. More than 2000 prostate species did not have homology to nonprostate-derived sequences in the public databases (unannotated).

were masked and ESTs with > 100 bp of high quality sequence were admitted to the assembly process (Fig. 1A). A total of 49 816 high quality ESTs were assembled using the sequence assembly program CAP2 to produce 21 114 clusters. Each cluster was annotated by searching the Unigene, Genbank, and dbEST databases with the CAP2-generated cluster consensus sequences using BLASTN. Clusters annotating to the same database sequence were joined to further reduce the number of distinct clusters to 15 953 (Fig. 1B).

Studies in the 1970s using reassociation kinetics to estimate the number of different transcripts indicate that between 10 000 and 30 000 distinct mRNAs are present in mammalian cells or organs [16, 17]. Recent data produced using the method of Serial Analysis of Gene Expression (SAGE) suport these estimates of transcript diversity in mammalian epithelial cells with estimates of 14 000–20 000 different mRNAs per cell [18]. Although the identification of alternatively spliced transcripts and highly homolgous gene family members may increase or decrease these estimates slightly, they nevertheless provide a rough estimate of the complexity of cellular gene activity. Based upon these data, the 15 953 prostate EST clusters that we have assembled should characterize roughly 50–75% of the prostate transcriptome. It is likely that this assembled dataset comprises all of the abundant and most of the moderately abundant prostate transcripts [6]. Ongoing work involves the acquisition of the remaining low abundance transcripts. Approaches to achieving this goal involve the construction of cDNA libraries from highly selected purified cell populations such as luminal epithelial and neuroendocrine cells, and from prostate tissues at different stages of development (*e.g.*, fetal prostate) or under different hormonal influences (*e.g.*, androgen stimulation). Another useful strategy involves the iterative removal of abundant and previously identified cDNAs in order to select for rare species. A high-throughput method using cDNA array-based technology has been developed to facilitate this process [19].

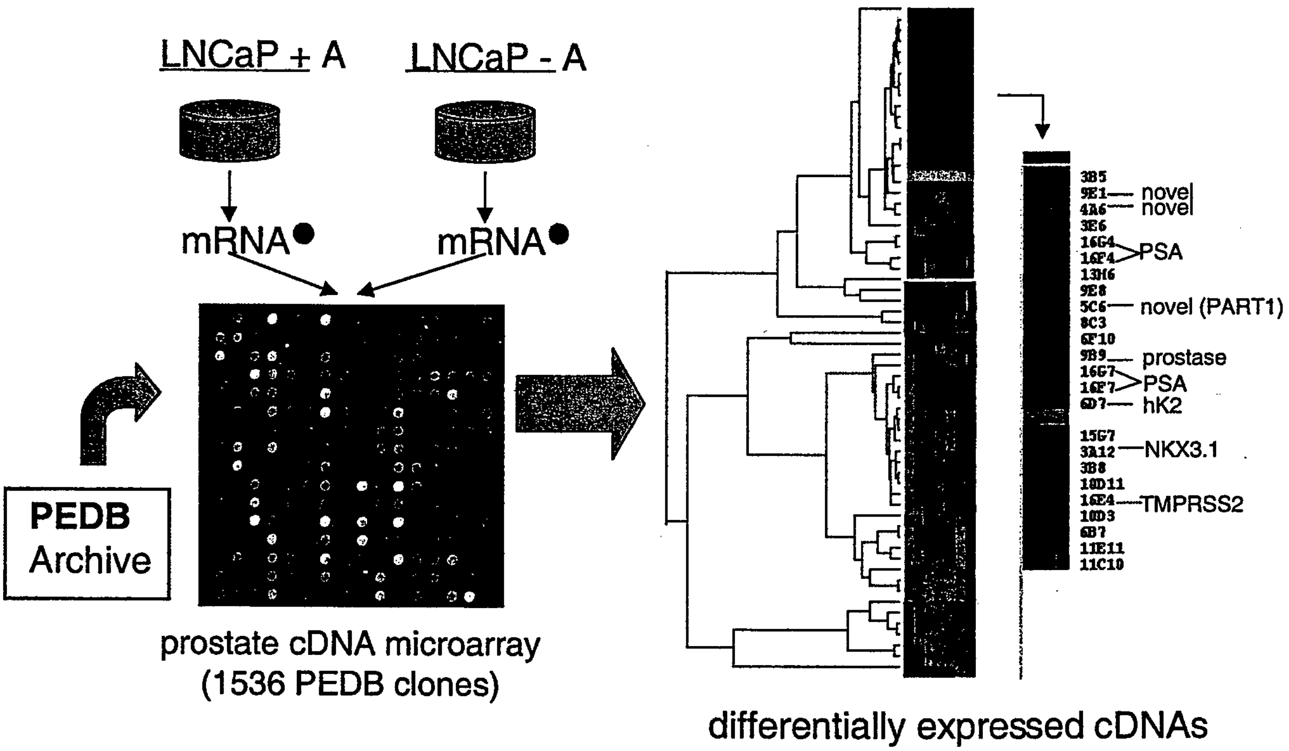


Figure 2. cDNA microarray analysis of prostate androgen-regulated gene expression. A nonredundant clone set comprised of 1536 cDNAs was hybridized with Cy3-labeled (red) cDNA from androgen-stimulated LNCaP cells and Cy5 labeled (green) cDNA from androgen-starved LNCaP cells. The expression ratio for each cDNA was determined and the ratios for all cDNAs with signal intensities 2.33-fold above the standard deviation of the background signal were clustered according to transcript levels over time. The Cluster and TreeView software programs availabe at the Stanford genome web site was used for the analysis (http://rana.Stanford.EDU/software/). Twenty genes were identified with increased expression after androgen stimulation.

3.2 Prostate gene expression analyses: cDNA microarray

Microarrays comprised of 1500 distinct prostate-derived cDNAs were hybridized with fluorescently labeled total cDNA probes produced from androgen-stimulated and androgen-starved LNCaP prostate cancer cells. No cDNAs were identified whose expression level decreased with androgen stimulation. In contrast, the hybridization ratios of 20 different cDNAs were consistently increased by > 2-fold in androgen-stimulated relative to androgen-starved cells (Fig. 2). This group included cDNAs encoding the human glandular kallikrein 2 (hK2) and human glandular kallikrein 3 (hK3), also known as prostate-specific antigen (PSA). The regulation of hK2 and PSA has previously been shown to be mediated by androgens through a mechanism involving androgen-response element (ARE) binding sites in the promoter regions of these genes [20, 21].

In addition to hK2 and PSA, we identified several other genes previously shown to be androgen-regulated, including the prostate homeobox gene NKX3.1 [22], the serine protease prostase/PRSS17 [23], and two genes involved in lipid metabolism. The microarray analysis also indicated that the expression of the membrane-bound serine protease TMPRSS2 [24] was regulated by androgen. We subsequently confirmed the androgen regulation of TMPRSS2 by Northern analysis, identified a putative ARE in the TMPRSS2 promoter region, and demonstrated that TMPRSS2 is highly expressed in the prostate gland relative to other human tissues [25]. Several cDNAs corresponding to uncharacterized genes also exhibited transcriptional regulation by androgen (Fig. 2). We have cloned the full-length cDNA and confirmed the androgen regulation of one of these novel sequences and designated it as PART-1, for Prostate Androgen-Regulated Transcript-1, as it lacks significant homology to nucleotide or protein sequences in the nonredundant subdivision of the GenBank and SWISS-Prot databases [26]. Interestingly, the tissue pattern of PART-1 expression is also essentially restricted to the prostate. The cloning and characterization of the other identified androgen-regulated cDNAs is in progress.

We anticipate that expanding these studies to include a greater portion of the prostate transcriptome coupled with experiments designed to determine direct *versus* indirect transcriptional regulation, and ultimately translational and post-translational regulation of these genes, will establish a framework for understanding the cellular functions mediated by androgens. Despite the important influence of androgenic hormones on prostate cancer growth, relatively few downstream targets of the AR pathway have been described. Studies designed to identify genes regu-

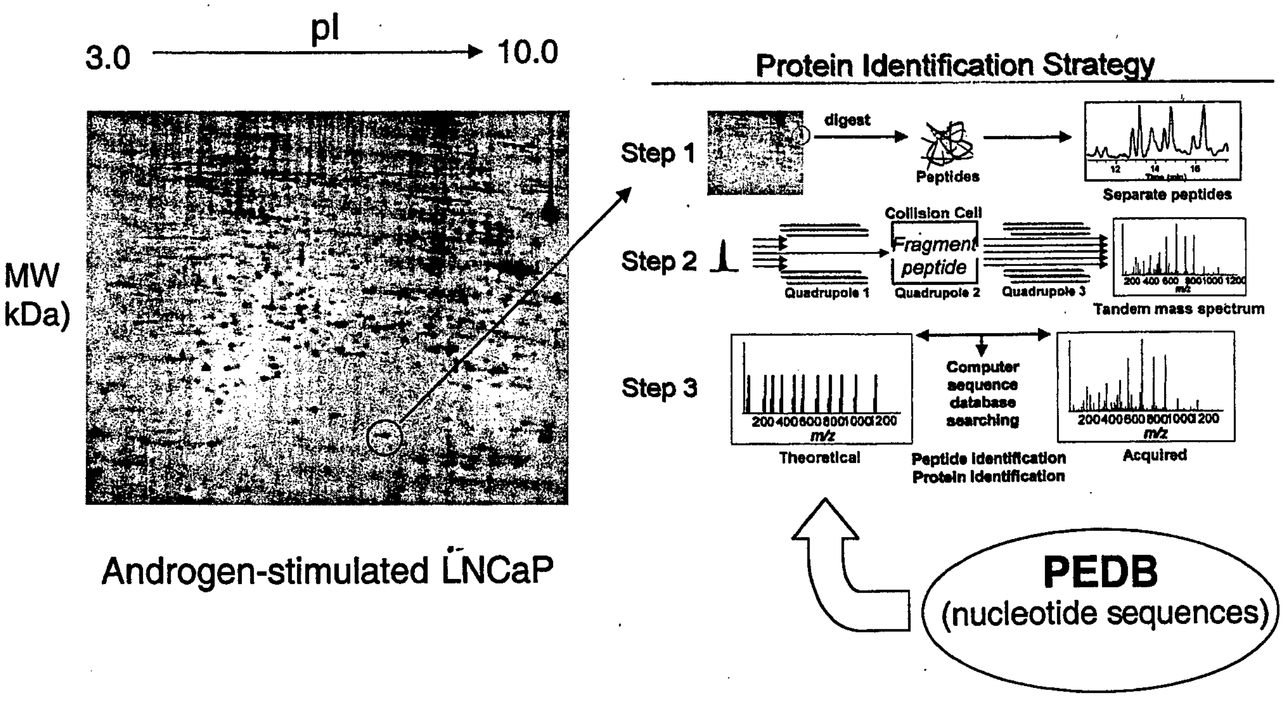
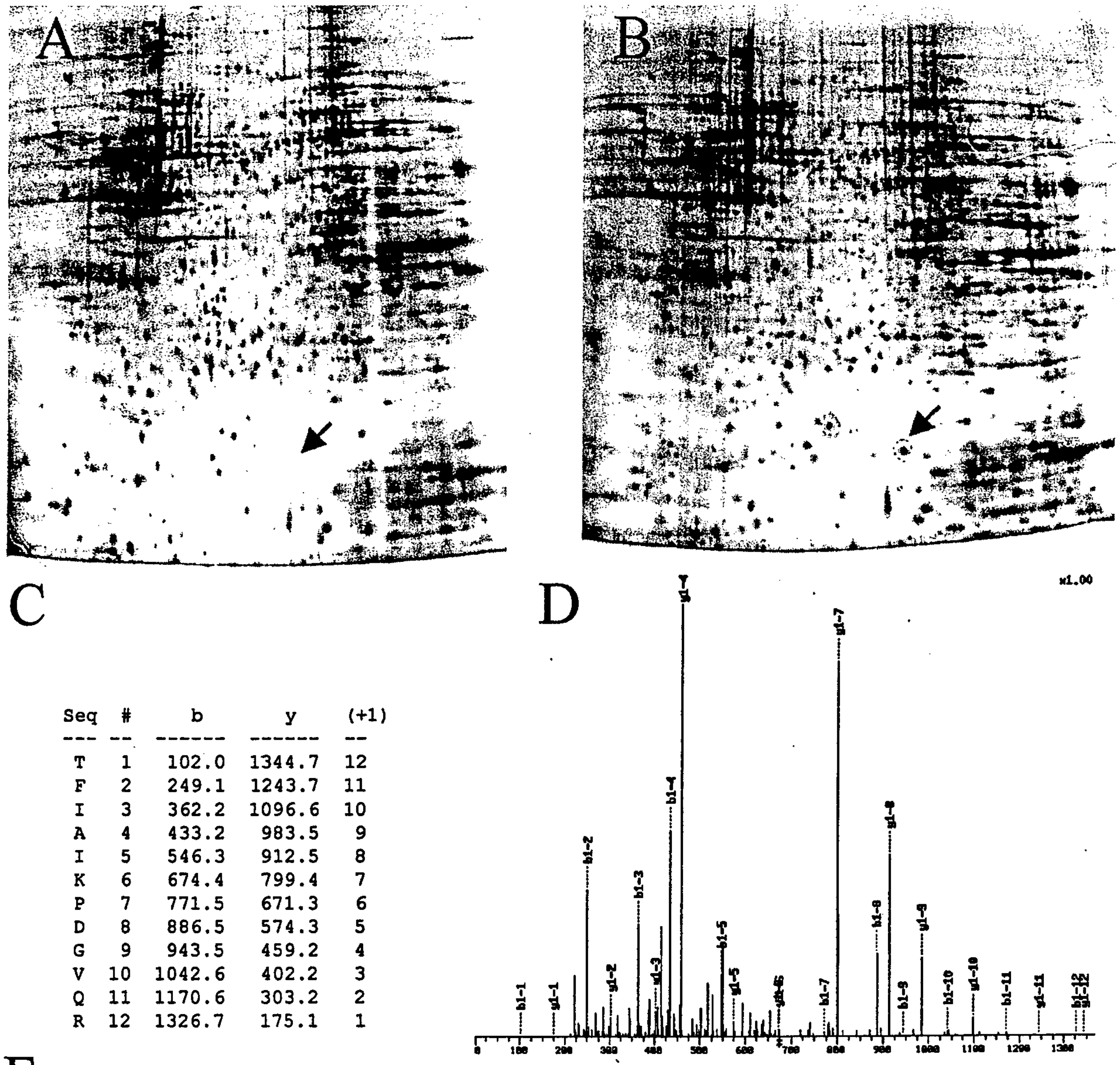


Figure 3. (Left) LNCaP 2-DE protein expression profile with androgen stimulation. (Right) Three-step schema for protein identification using MS and computer sequence database searching.



Seq	#	b	y	(+1)
T	1	102.0	1344.7	12
F	2	249.1	1243.7	11
I	3	362.2	1096.6	10
A	4	433.2	983.5	9
I	5	546.3	912.5	8
K	6	674.4	799.4	7
P	7	771.5	671.3	6
D	8	886.5	574.3	5
G	9	943.5	459.2	4
V	10	1042.6	402.2	3
Q	11	1170.6	303.2	2
R	12	1326.7	175.1	1

E

#	Rank/Sp	(M+H)+	Cn	deltCn	C*10^4	Ions	Reference	Peptide
1.	1 / 1	1344.8	1.0000	0.0000	2.9296.	14/22	NDKA_HUMAN	(R)TFIAIKPDGVQR
2.	2 / 30	1344.6	0.4673	0.5327	1.3689	9/20	RELB_HUMAN	(R)GMPFRYECEGR
3.	3 / 6	1344.8	0.4663	0.5337	1.3661	9/18	VE2_HPV25	(K)RRSRSRSRRK
4.	4 / 12	1344.8	0.4590	0.5410	1.3448	9/24	HIV1U16769	(R)GTKALTEVIALTK
5.	5 / 69	1345.7	0.4519	0.5481	1.3240	7/24	RU2A_HUMAN	(K)TFNPGAGLPTDKK
6.	6 / 8	1345.7	0.4189	0.5811	1.2273	9/22	HUMPRA	(K)GESGQSWPRLTK

Figure 4. Identification of an androgen-regulated protein from metastatic prostate cancer cells by 2-DE and MS. M12AR cells were (A) starved or (B) stimulated for 24 h with the synthetic androgen R1881 and total cell lysates (40 μg each) were subjected to 2-DE. Protein expression profiles were compared and proteins demonstrating a qualitative expression level differences were subjected to in-gel trypsin digestion, and identified by μLC-MS/MS analysis. (C), (D), MS/MS spectrum of identified peptide, peptide sequence, and identified ion series. (E) Results from correlation of acquired peptide fragmentation spectra with database entries (using SEQUEST software). The MS/MS spectrum in (D) was identified as NDKA_HUMAN (nm23) taken from the selected 2-D gel spot. Two additional peptides were identified from this protein in a single run.

lated by androgens in the rat prostate determined that androgens increase the transcription of about 56 genes and decrease the transcription of less than 10 genes [27]. From a therapeutic standpoint, it would be extremely useful to distinguish and subsequently modulate the relevant molecules in the AR program that mediate the divergent processes of cellular proliferation, cellular differentiation, and apoptosis.

3.3 Prostate gene expression analyses: 2-DE and MS

To complement our prostate transcriptional data and provide a more complete picture of prostate gene expression, we have undertaken a comprehensive analysis of that portion of the prostate proteome regulated by androgenic hormones. Reference protein expression profiles were produced for the LNCaP and M12AR prostate cancer cell lines using 2-DE protein separation techniques under steady-state conditions (Fig. 3). Protein expression profiles from cell lysates under conditions of androgen stimulation and androgen starvation have also been generated. A comparison of 2-DE protein profiles under these various conditions yielded a proteomic signature characterized by a subset of proteins with qualitative and quantitative changes. Individual proteins were identified using a sequential process of in-gel trypsin digestion and extraction, peptide separation by µLC, generation of MS/MS spectra, and database correlation with the acquired peptide fragmentation pattern (Fig. 3).

A comprehensive analysis of androgen-induced proteomic signatures is ongoing and our initial experiments demonstrate the utility of this approach in identifying molecules of potential importance in understanding androgen-mediated regulation of prostate cancer progression and metastasis. Figure 4 depicts a portion of the 2-DE protein profile from androgen-starved and androgen-stimulated M12AR prostate cancer cells with a differentially expressed protein spot that is upregulated in M12AR cells after exposure to androgens. This protein was identified as human nucleoside diphosphate kinase A (NDKA/nm23), a well-characterized gene with tumor metastasis suppressor activity in several different human tumors including melanoma, breast, ovary and prostate [28, 29]. Transfection of the DU-145 prostate cancer cell line with NDKA/nm23 inhibited the adhesion to cell matrix and impaired colony growth in soft agar [29].

The M12 prostate cancer cell line is highly tumorigenic when implanted into nude mice and metastasizes to different anatomical sites. Transfection of these cells with a functional androgen receptor (M12AR) markedly decreases the proliferation rate, tumor growth, invasiveness, and *in vivo* metastatic potential when these cells are injected into the prostate glands of nude mice (S. Plymate, unpublished observation). NDKA/nm23 transcripts have been shown to increase rapidly in prostate cancer cell lines after the administration of androgens, though no functional ramifications of this increased expression were described [30].

A possible mechanism for the decreased tumorigenic and metastatic capability of M12AR cells compared with M12 cells lacking the AR involves the upregulation of NDKA/nm23 by androgens through a functional androgen-response program restored by the AR transfection and expression. Such an observation has direct clinical relevance. Both human and *in vitro* studies suggest that there may be a survival benefit from maintaining an androgen responsive cohort of prostate tumor cells [31–33]. This concept has been studied in the LNCaP cell system by comparing the rate of tumor growth in castrated mice implanted with LNCaP cells with subsequent tumor growth (i) without further therapy, or (ii) followed by intermittent androgen replacement. The rate of tumor growth as measured by serum PSA was slower in animals treated with intermittent androgen supplementation compared to those maintained in the castrated state [31].

4 Concluding remarks

The results presented here demonstrate the utility of global expression studies to simultaneously identify multiple genes and gene products of biological relevance that participate in specific metabolic pathways. Both known and unknown genes are rapidly identified. Notable advantages of the microarray-based transcript profiling approach include the ability to perform detailed time-course or variable drug-dose experiments in a robust economical fashion. Controlled replicate experiments can determine system and procedural errors. However, this approach is absolutely dependent upon the identification of diverse clone sets for array construction that are biologically relevant to the system under study. In addition, a significant limitation of transcript profiling methods is the lack of a tight correlation between gene activity as measured by mRNA level, and protein abundance [34]. Global protein analyses focus on the actual biological effector molecules, but are restricted by difficulties in detecting low abundance proteins, accurately measuring the differences in protein levels between two samples, and a dependency on comprehensive annotated sequence databases for protein identification.

Integrating the assembly and annotation of sequence databases with transcript profiling and proteome analyses combines complementary robust approaches that capitalize on the strengths and avoid the limitations of relying on one method. The further expansion of this work to include the analysis of the entire prostate transcriptome coupled with quantitative proteome studies should enable the characterization of gene networks and cellular pathways that can be exploited for therapeutic intervention.

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SEQUENCE DATABASES AND MICROARRAYS AS TOOLS FOR IDENTIFYING PROSTATE CANCER BIOMARKERS

LYNETTE H. GROUSE, PETER J. MUNSON, AND PETER S. NELSON

ABSTRACT

Identification, acquisition, and assessment of molecular markers that could be adopted as surrogate endpoints for evaluating a response to prostate cancer intervention strategies is highly desirable. Recent advances in the fields of genomics and biotechnology have dramatically increased the quantity and accessibility of molecular information that is relevant to the study of prostate carcinogenesis. One major advance involves the construction of comprehensive databases that archive gene sequences and gene expression data. This information is in a format suitable for virtual queries designed to distinguish the molecular differences between normal and cancer cells. A second major advance uses robotic tools to construct microarrays comprising thousands of distinct genes expressed in prostate tissues. Such arrays offer a powerful approach for monitoring the expression of thousands of genes simultaneously and provide access for techniques designed to assess patterns or "fingerprints" of gene expression that may ultimately be used as signatures of response to therapeutic intervention. UROLOGY **57** (Suppl 4A): 154–159, 2001. © 2001, Elsevier Science Inc.

The human genome is estimated to comprise approximately 30,000 to 100,000 genes. To confer developmental and functional specificity, only a fraction of this total is active in a given cell type at a given time, and these expressed genes essentially define the state of that cell. The molecular profile of normal and cancer cells, ie, their set of expressed genes, differs in both qualitative (alternative forms of a gene) and quantitative fashions. Measurement of this profile may predict the phenotypic behavior of such cells more accurately than traditional histologic approaches.

To identify informative biomarkers and suitable intermediate endpoints of disease, it would be advantageous to have a catalog or index of all genes and their cognate proteins that are expressed in normal and neoplastic prostate tissues. This resource could then be rapidly exploited to identify candidate biomarkers for evaluation based on homology to known genes of importance in prostate cancer, gene polymorphisms and mutations, or alterations in gene expression. This review will focus particularly on the use of tissue-specific expressed sequence tag (EST) databases, the development and use of cDNA microarrays, and statistical issues related to microarray analyses. These approaches may become essential for identifying new biomarkers in prostate cancer.

DATABASES AS TOOLS FOR BIOMARKER IDENTIFICATION

In 1997, the National Cancer Institute announced a bold new initiative, the Cancer Genome Anatomy Project (CGAP), with the overall goal of achieving the comprehensive molecular characterization of normal, precancerous, and cancerous cells.[1–3] The CGAP is an interdisciplinary program that uses National Institutes of Health intramural research teams, academic centers, and commercial resources to establish an index of genes expressed in tumors. The CGAP serves as an interface between genomics and cancer research. The new technologies supported by this initiative, and the products resulting from these technologies, will be accessible to the public through an Internet website (http://www.cgap.nci.nih.gov). This Internet site provides information about cDNA libraries of

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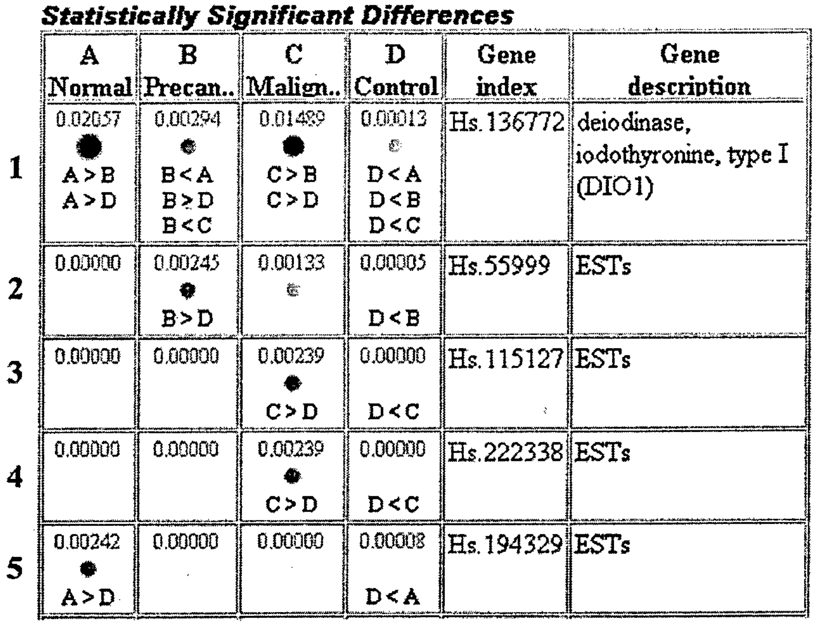

Statistically Significant Differences

	A Normal	B Precan..	C Malign..	D Control	Gene index	Gene description
1	0.02057 ● A>B A>D	0.00294 ● B<A B≥D B<C	0.01489 ● C>B C>D	0.00013 ● D<A D<B D<C	Hs.136772	deiodinase, iodothyronine, type I (DIO1)
2	0.00000	0.00245 ● B>D	0.00133 ●	0.00005 D<B	Hs.55999	ESTs
3	0.00000	0.00000	0.00239 ● C>D	0.00000 D<C	Hs.115127	ESTs
4	0.00000	0.00000	0.00239 ● C>D	0.00000 D<C	Hs.222338	ESTs
5	0.00242 ● A>D	0.00000	0.00000	0.00008 D<A	Hs.194329	ESTs

FIGURE 1. *Gene expression profiles in normal, precancerous, and malignant prostate tissue. Differences in gene expression between cDNA libraries prepared from various types of prostate tissues can be analyzed by using the Digital Differential Display (DDD) software program. Library A is prepared from normal prostate epithelium, Library B from precancerous prostate tissue, Library C from malignant prostate cancer, and Library D is from a control library prepared from a pool of brain, liver, and spleen tissue. The Gene Index contains the UniGene Cluster Identifier, and Gene Description lists the gene name. In each box, the number at the top represents the fraction of sequences in that cDNA library that expresses the gene or EST. The dot is a visual aid, which reflects the numerical values. Each library is compared with each of the other libraries in pairwise analysis. If the difference in gene expression between two libraries is statistically significant, it is indicated by a greater than or less than symbol.*

normal and cancerous tissue, description of the methods used in preparing each library, and informatics tools to perform analyses of gene expression using cDNA library data.

A goal of the CGAP is to facilitate the identification of possible molecular biomarkers for various types of cancer. To enable investigators to analyze molecular databases that are very large and complex, CGAP has developed software tools in collaboration with the National Center for Biotechnology Information at the National Institutes of Health. These software tools aid in the analysis and comparison of gene expression in a variety of tissues and stages of cancer. All of these tools are available on the CGAP Internet website.

An example of a software analysis tool is Digital Differential Display (DDD).[4] DDD is used to compare sequence-based gene expression profiles among individual cDNA libraries or pools of libraries from the same or different tissues. Analysis of different gene expression profiles may identify genes that contribute to a cell's unique characteristics. Such genes, when expressed at different levels in normal and cancer cells, may be considered as candidate biomarkers for use in cancer screening. DDD uses a statistical comparison of genes expressed in each cDNA library to determine which differences are statistically significant. The statistical analysis is based on the Fisher exact test.[5] Differences in gene expression values are presented both visually and numerically.

An example of a DDD analysis of three cDNA libraries made from prostate tissue is shown in Figure 1. Row 1 shows an expression profile of a gene that has a known function, whereas rows 2 to 5 show expression differences between genes of unknown function, referred to as ESTs. Row 1, column D, shows that all three prostate cDNA libraries have increased expression of the DIO1 gene compared with that of control. Within the prostate libraries, column A shows increased gene expression compared with that of the precancerous library in column B, but was not shown to be statistically significantly increased when compared with malignant prostate cancer tissue libraries. The power of this analysis is in identification of possible biomarkers within anonymous EST sequences. In row 2, the expression of this EST is increased over control in only the precancerous prostate cDNA library, whereas the EST in rows 3 and 4 is increased only in cancerous prostate tissues. These genes could be evaluated as candidate biomarkers to identify prostate cancer disease progression. The Prostate Expression Database (PEDB) (http://www.mbt.washington.edu/PEDB)[6] is another online resource of prostate genetic information. The PEDB is a curated relational database and suite of analysis tools designed specifically for the study of prostate gene expression in normal and diseased states. The ESTs, derived from more than 40 human prostate cDNA libraries, are assembled into distinct species groups that are annotated with information from the GenBank, dbEST, and Unigene public sequence databases. The expression pattern of each gene can be viewed across all libraries or tissues using the Virtual Expression Analysis Tool (VEAT), a graphical user interface written in Java for intra- and interlibrary gene expression analyses.

cDNA EXPRESSION ARRAYS FOR BIOMARKER IDENTIFICATION

The inherent heterogeneity of prostate cancers and the diversity of therapeutic interventions suggest that it is unlikely that a single biomarker or intermediate endpoint that will provide sufficient sensitivity or specificity for assessing a treatment response can be identified. Efforts have been directed toward methods of simultaneously measuring multiple biomarkers at the DNA, RNA, or protein levels. Such a multiplexed approach will greatly expand the information gained from each patient sample and clinical trial. In addition, patterns in biomarker data may be identified that together exceed the sum of individual measurements.

Recent developments in informatics, miniaturization, and robotics have provided new extremely powerful approaches for comprehensive measurements of genetic alterations that occur in neoplasia. These measured alterations could also reflect a response (or lack of response) to a chemopreventive or therapeutic agent. One such comprehensive approach involves the use of DNA arrays, a technique that combines the proven chemistry of nucleic acid hybridization with advanced automation and imaging technology to quantitatively detect changes in the expression levels of thousands of genes simultaneously. DNA arrays have been assembled in several configurations, including oligonucleotide arrays,[7] microarrays of cDNA spotted on glass slides,[8] and DNA spotted onto nylon membranes.[9] The basic method is straightforward: DNA representing a particular gene of interest is either spotted (printed) or synthesized onto a solid support, such as a glass microscope slide, silicon wafer, or nylon membrane (Figure 2). The procedure is repeated in an automated fashion with thousands of different genes, such that each is deposited in a precise spatial location that allows for the subsequent identification of any individual spot. Probes representing the expressed genetic information in a tissue sample are labeled with radioactive or fluorescent markers that can be quantified by sensitive detectors and used for comparative analyses. A limitation on the number of individual elements that can be placed on the area of a given "chip" array places a premium on efficient construction. This is accomplished by eliminating redundancy (maximizing diversity), and incorporating DNA sequences that are relevant for the biological system under study.

Gene expression catalogs, such as the CGAP and the PEDB,[6,10] can be exploited for the construction and analysis of cDNA expression arrays by providing a virtual archive of thousands of genes expressed in prostate tissue. Coupling this virtual repository with the physical clones representing the corresponding DNA molecules allows for the construction of comprehensive arrays. The continued expansion of this resource to encompass all prostate transcripts will allow for the simultaneous analysis of all genes expressed in normal and neoplastic prostate cells. This effort will require extensive testing on prostate tumors and a further refinement of the methods to include statistical measures of biological and experimental variance.

STATISTICAL ISSUES IN THE ANALYSIS OF cDNA MICROARRAYS

Special-purpose, tissue-specific cDNA microarrays can now be routinely generated using commercially available spotting robots, either using glass-based or nylon-based substrate. A growing number of commercial cDNA microarrays are also available, giving smaller labs the opportunity to use this technology. Careful attention to the design and statistical analysis of each experiment is essential, especially given the high cost of microarrays. As with any assay procedure, microarray data are subject to three major sources of random and systematic error: reagent quality, sample preparation, and laboratory technique. The most important reagent is the microarray itself, which may be subject to significant batch-to-batch variability. The array may include clones of questionable quality, possibly including troublesome repetitive DNA or another contaminating sequence. Variability of the substrate, either nylon or glass, can have a marked effect on the uniformity of the array image. Sample preparation includes all tissue handling, cell isolation, RNA extraction, and labeling steps. Unintended variation in RNA content may easily result from poor temperature control, heat shock, degra-

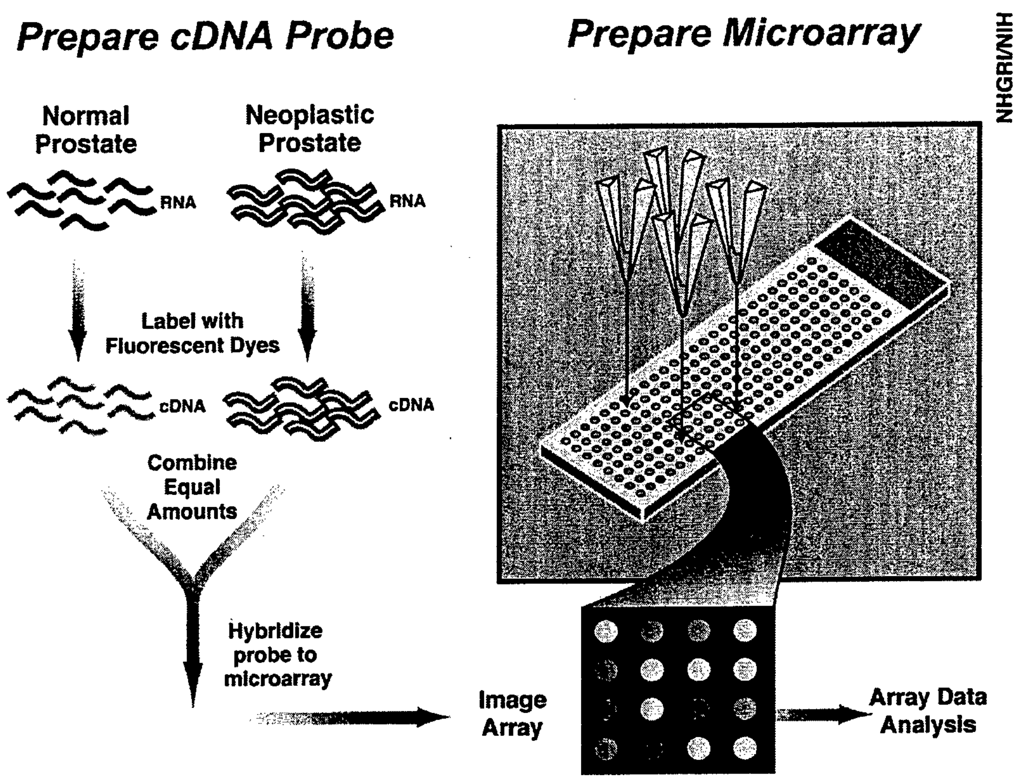

FIGURE 2. *cDNA microarray construction and analysis. Microarray assays are performed in a multistep process. First, microarrays are prepared by assembling sets of cDNA clones in 96- or 384-well microtiter plates. Small tweezer tips or needles attached to a robotic arm are used to withdraw small amounts of the DNA solution from the microtiter plates and print them onto glass microscope slides in a precise spatial orientation with high replicative fidelity. cDNA probes are prepared from two distinct tissue sources (eg, normal tissue and neoplastic tissue) by first extracting RNA followed by a conversion step to cDNA that incorporates a different fluorescent dye into the different tissue source cDNA (eg, green for normal and red for neoplastic). These labeled cDNA probes are then combined and hybridized to the microarray such that cDNAs in the probe will attach to their complementary cDNA spot on the microarray surface. Nonhybridizing cDNAs are removed by a washing step, and the remaining bound cDNA molecules are quantitated by measuring the fluorescent intensity at every spot location. Array analyses determine the ratio of intensities at each spot and thus identify specific genes that are overexpressed in normal tissue relative to neoplastic (green spot), overexpressed in neoplastic relative to normal (red spot), or expressed at equivalent levels (yellow spot).*

dation, sample handling, etc. Fluorescence or radioactive label incorporation may also be subject to variation and can strongly influence the results. During the hybridization of labeled probe to the target cDNA on the array, carefully controlled time, temperature, and agitation conditions should prevail. Issues of saturation and dynamic range compression may arise during image acquisition and storage.

By far the most straightforward way to address each of these issues is by use of independent replicated experiments. Apparent gene expression changes that persist through such repeated experiments can correctly be ascribed to interesting biological changes rather than artifacts of the assay itself. The following illustrative analysis of duplicate experiments easily screens out many artifactual expression changes. We compared a melanoma cell line to a prostate tumor cell line for expression differences on a prostate-specific, nylon-based cDNA array.[11] Spot intensities were quantified using the P-SCAN software (available at http://abs.cit.nih.gov/PSCAN). The intensities of each spot were compared in Figure 3A, which at first seems to indicate that a large number of genes have greater than fourfold changes in relative expression levels between the two cell types. Analysis of a duplicate experiment gave a different picture. Figure 3B shows that a much smaller number of genes undergo greater than fourfold changes consistently in both experiments, meaning that many of the apparent fourfold changes in the first experiment were "false-positives." A family of differen-

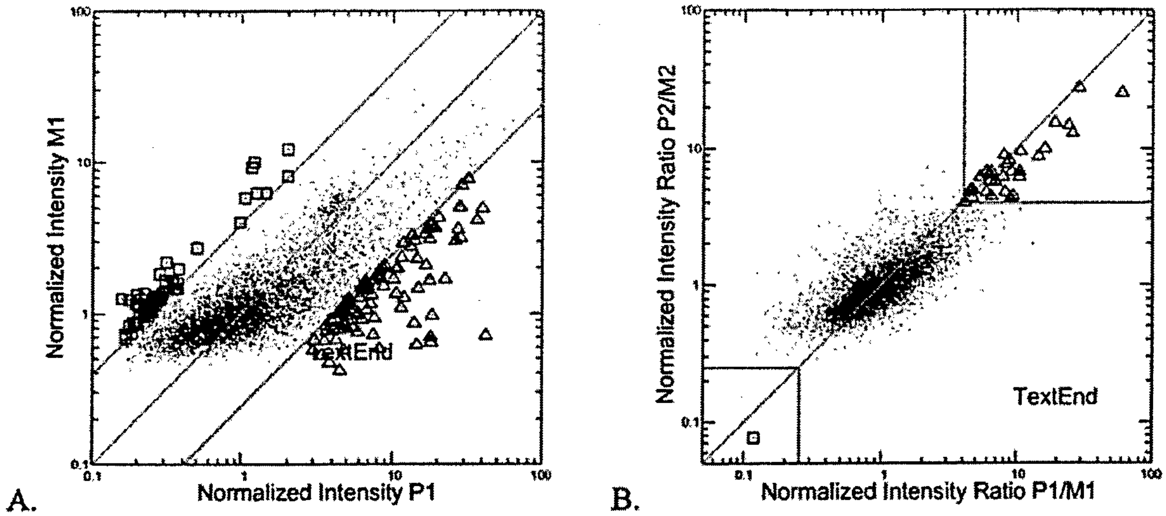

FIGURE 3. *Comparison of melanoma (M) expression levels to that from a prostate tumor cell line (P).[11] (A) Normalized intensities show more than 148 genes with apparent expression change over four-fold (up, squares or down, triangles). (B) Expression ratios are compared for duplicate experiments (P1/M1, P2/M2). Only 31 genes are consistently over- or underexpressed by greater than fourfold in both experiments.*

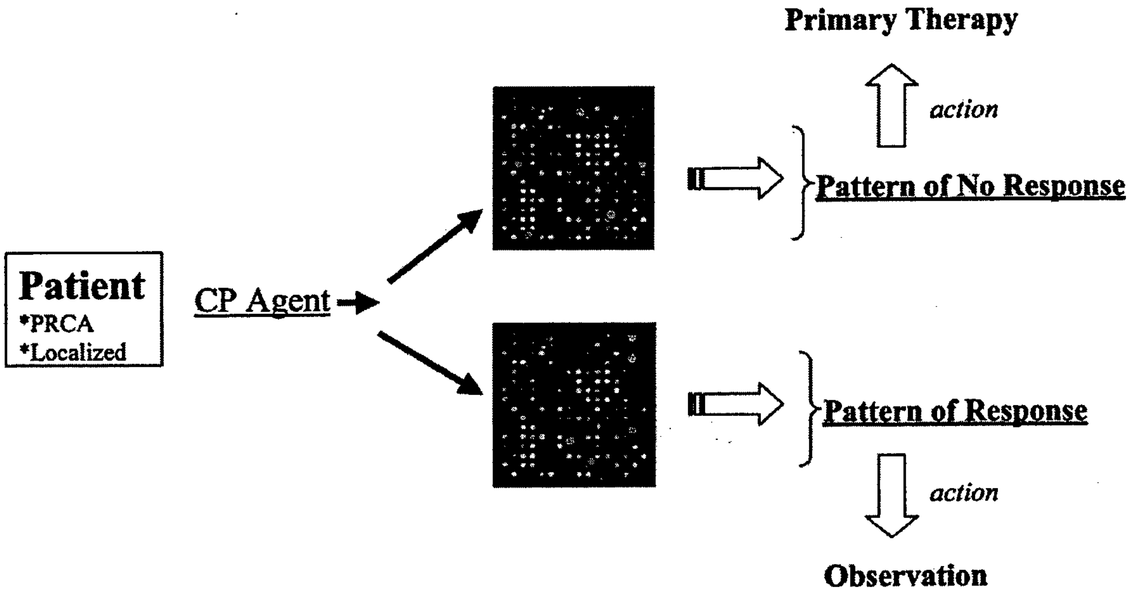

FIGURE 4. *Molecular profiles as surrogate endpoint biomarkers. Patients with localized prostate cancer are treated with a chemopreventive (CP) agent. A biopsy is performed and subjected to molecular profiling by cDNA microarray analysis. The pattern of expression is compared with reference patterns previously shown to correlate with a tumor response or lack of tumor response to the CP agent. These data are used to guide further therapeutic intervention.*

tially expressed genes also clearly emerged and was later confirmed by Northern blot analysis. Reduction in the number of false-positives can be essential when using microarray technology to look for new cancer markers, as tens of thousands of clones must be screened.

APPLICATION TO CHEMOPREVENTION

Among their many applications, database and array-based methods of genetic analysis can be useful for the identification, acquisition, and assessment of candidate molecular markers that could be adopted as surrogate endpoints for assessing preventive strategies (chemoprevention or nutritional intervention). One scenario involves a cohort of patients diagnosed with low- or intermediate-grade prostate cancers by needle biopsy. Patients who elect to forgo primary therapy (radical prostatectomy or radiotherapy) could be offered a chemopreventive agent aimed at halting cancer progression. Gene expression profiles of tumor tissue before and after the chemopreventive agent would be assessed for expression patterns correlating with a propensity for the cancer to progress, indicating that a primary therapy should be offered, or for the cancer to respond to the chemopreventive agent and thus require no further intervention (Figure 4). The development of this type of assay is clearly desirable, but defining predictive patterns of expression is not a trivial task.

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