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13. ABSTRACT *(Maximum 200 words)*

We have developed an approach to isolate new tumor markers of prostate cancer. In order to identify genes encoding secreted protein, we have modified the signal sequence trap technique, using the SUC 2 gene encoding a secreted protein of yeast. Only the yeast which have been transformed with cDNAs containing secretory signal sequences can grow into colonies on the plates which contain sucrose as the only source of sugar. Early difficulties identifying positive clones with our initial vector system were problematic; we obtained a new vector system to reduce the possibility of enzymatically inactive fusion invertase and enhance the sensitivity of this signal trap system which allowed us to obtain positive clones. We successfully constructed and subtracted a prostate specific cDNA library. We will continue to pursue the selection of positive clones, make RNA dot blots from various tissues to establish tissue specificity of the cDNA and perform homology searches to determine if we have found a novel gene. Unique genes will be cloned into a GST vector and fusion proteins will be made and used to make antibody against the protein. Serum sample assays will be developed from antibodies that identify prostate specific genes. Additionally, structure/function analysis and sense and antisense expression vectors will be employed to elucidate further the function of these genes.

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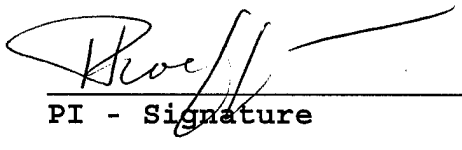

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Date

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Introduction

The subject of the grant is identifying secreted proteins made by prostate cancer. The purpose of the grant is to develop a unique assay of early detection of prostate cancer. The scope of the research includes a prostate cancer cDNA library hooked-up to the yeast invertase cDNA with the invertase gene having a deletion of the signal sequence. This chimeric cDNA is placed in yeast that have had the endogenous invertase gene removed. The yeast are grown in agar containing sucrose, and only those yeast that contain a chimeric cDNA which codes for a signal secreted protein having a signal sequence will grow. The yeast clone will be isolated, grown-up and the recombinant gene will be purified and sequenced. The interesting gene will be further studied including development of antibodies and use of these antibodies to look at levels of the protein in serum of individuals with prostate cancer.

Body

Task 1. Confirm preliminary experiments: We introduced a cloning site into plasmid pRB576 for the signal sequence trap system (pRB576L). This vector carries an invertase (SUC2) gene lacking both its initiation codon and its signal peptide. We subcloned a synthetic linker encoding a hydrophobic amino acid stretch into this vector (positive control plasmid). Yeast YT455 ($SUC2^{\Delta 9}$, ade2-101, URA3-52) lack SUC2, the invertase gene. So the yeast can not convert sucrose to glucose, and cannot grow in the absence of glucose. When we transformed this yeast with the positive control vector, the yeast grew on the plates containing sucrose but not glucose.

Task 2. Construct and subtract a prostate specific cDNA library. We made a cDNA library from LNCaP prostate cancer cells (see Figure 1) and subtracted a library with mRNA from MCF-7 breast cancer cells in order to enrich for prostate specific genes. We then used PCR based enrichment for the 5' fragments (see Figure 2 and 3) of the cDNA library and subcloned the fragments into PRB 5767. This was then amplified. We then amplified the library by transformation of E.coli, purified the plasmid and transformed into the yeast YT455. On this initial attempt, the library gave us two positive clones, but we failed to recover the plasmid from them. This was repeated again; and in this case, we got no positive clones. One reason that we could not get any positive clone might be insufficient number of clones screened. Another possible explanation of this result is that the inserted amino acid sequence at the amino terminus of the SUC2 gene may abrogate the function of invertase. To avoid this possibility, we got another vector system (pSUC2T7M13ORI, from Genetics Institute, Cambridge, MA) to solve this problem. This vector contains a hybrid human serum albumin-alpha factor KEX2 site between the cDNA cloning site and the invertase lacking both initiation codon and the signal peptide. Kex2 is an endogenous yeast protease, and the

Fig. 1. Procedures for making cDNA library enriched for prostate-specific transcript: cDNAs from a prostate cancer cell line, LNCaP, were subtracted with mRNA from MCF7 breast cancer cells.

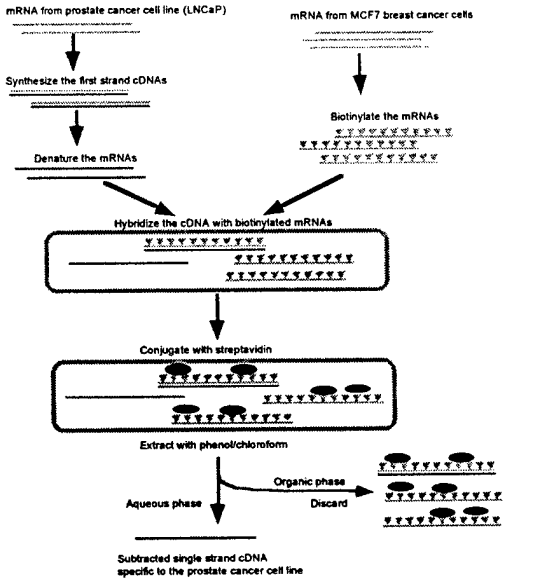
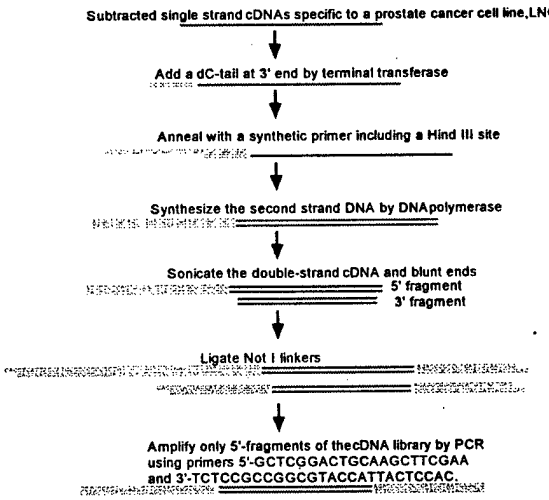
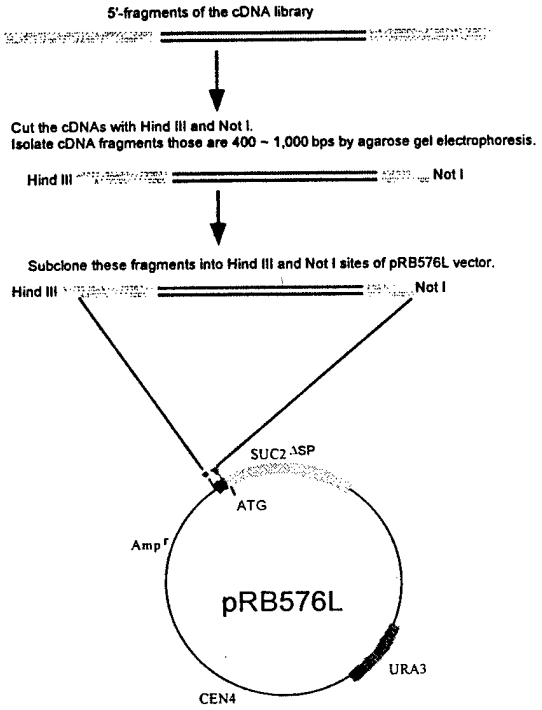


Fig. 2. PCR-based enrichment for the 5'-fragments of thecDNA library. During these procedures, Hind III sites and Not I sites are attached at the 5'-ends and the 3'-ends of cDNAs, respectively.



introduced Kex2 site will be cleaved during secretion. This modification will reduce the possibility of enzymatically inactive fusion invertase, which will enhance the sensitivity of this signal trap system. We place the synthetic linker encoding a hydrophobic amino acid stretch pSUC2T7M13ORI. We have repeated task one with the new vectors and found it to be successful and have begun on Task 3 using the pre-made cDNA library of LNCaP cells.

Fig. 3. Subcloning of the 5'-fragments of cDNAs into pRB576L.



Task 3. Further selection of positive clones: As we get positive clones, we will do RNA dot blots made from various tissues to look at tissue-specificity of the cDNA. Those cDNAs that appear to be prostate-specific will be sequenced and homology searches will be performed to determine if they are novel genes. Again, if a unique gene is isolated, it will be cloned into a GST vector and fusion protein will be purified and used to make antibody against the protein.

Task 4. For a gene that appears to identify a prostate-specific secreted protein, an antibody will be developed to examine serum samples from appropriate individuals.

Task 5. The function of the novel, secreted protein will be pursued by a variety of structure-function analyses, and the use of sense and antisense expression vectors.

If we are successful, we will have developed a technique that may rapidly allow isolation of unique prostate-specific secreted proteins which can then form the basis of determining if this protein can be used as a marker of clinical care for prostate cancer patients. It could be used for early diagnosis, early recognition of relapse, and rapid identification of resistance to various chemotherapies.

Additional Task: In addition, we pursued one additional task which was not supported by this grant, but which also focuses on prostate cancer.

Survey of protein-tyrosine phosphatase expression in prostate: Protein tyrosine phosphatases and protein tyrosine kinases work together to regulate tyrosine phosphorylation in their various target proteins. Vital cellular functions such as cell proliferation and signal transduction are regulated in part by the balance between the activities of protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPases). Nonreceptor PTPases have one or two intracellular PTPase domains that afford enough homology to allow development of PTPase-

domain - specific PCR primer sets.

To investigate a potential role of protein tyrosine phosphatases (PTPases) in prostate cancer, a degenerate primer-based reverse transcription-polymerase chain reaction approach was used to isolate cDNAs for proteins that contain a PTPase catalytic domain. To isolate and identify a wide range of known and potentially novel PTPases, we have developed a two-step PCR strategy and several sets of PCR primers. Primer design was based on regions of high amino acid sequence homology of known PTPases: "box 0" (SDYINASP), "box A" (DFW{E/K/R/Q}M), "oox C" (KC{A/V} QYWPDEGS) and "box B" (HCSAGVP). Prostate cancer samples were used as a source of polyadenylated mRNA, and cDNA was synthesized using Superscript RT from Gibco-BRL. The cDNA samples were then amplified using pairs of degenerate primers representing box O - box B, box A - box B, and box C - box B in low-stringency PCR conditions as described by Cao L, et al. *J Biol Chem* 1998 Aug 14;273(33):21077-83. PCR products were then size-fractionated using LMP agarose gel electrophoresis, fragments in the range 200-300 bp (box C- specific primers), 300-400 bp (box A - specific primers), and 400-500 bp (box O - specific primer set) were purified and subjected to a second round of amplification using 20 cycles and the same primer sets. Resulting PCR fragments were likewise size-fractionated in agarose gels, purified, and cloned into Stratagene TOPO PCR cloning vector system. Clone products were then sequenced using an automated ABI system.

Approximately 50% of analyzed clones contained sequences derived from various PTPases. Among the 12 distinct PTPases isolated by reverse transcription-polymerase chain reaction from the initial prostate cancer samples (Table 1), one designated PTP-HSP, was previously unidentified. Subsequent computer analysis identified this novel PTPase as a possible human homologue of the murine cytoplasmic sperm-specific PTPase Typ, isolated by Ohsugi et al. *J Biol Chem* 1997 Dec 26;272(52):33092-9. In addition, a number of unique cDNA sequences were isolated, that show homology to known phosphatases, analysis of these sequences is currently underway. It is unclear at present time, what role some of the following PTPases that we isolated from human prostate cancer samples play in prostate cancer cell proliferation and differentiation:

Table 1: PTPases isolated from human prostate cancer

PTPase Lyp1	PTPase sigma	SH - type PTPase 1A, C
PEST PTPase	PTPase delta	Receptor-type gamma PTPase
D1-2E PTPase	PTPase BAS	PTP HSP
LCA (T200)	MEG PTPase	
LAR PTPase	PTPase U2	

While some of these PTPase are known to be expressed in a variety of tissues, others

(e.g., MEG PTPase BAS, Lyp1 PTPase and a murine homologue of PTP HSP) have a highly restricted pattern of expression. In addition, ubiquitously-expressed PTPases are known to have tissue-specific splicing variants. The PCR based method that we developed allows for rapid and reliable identification of a wide range of human PTPases, including novel genes that may play a role in prostate neoplasia.

Key Research Accomplishments

Key Research Accomplishments: Developed a novel approach to the isolation of prostate specific secreted proteins.

Reportable Outcomes

Not applicable

Conclusions

We have constructed and subtracted a prostate specific cDNA library. It has been inserted into a newly acquired vector system (pSUC2T7M130RI) obtained from Genetics Institute, Cambridge, MA. This vector contains a hybrid human serum albumin-alpha factor Kex2 site between the cDNA cloning site and the invertase cDNA which lacks the initiation codon and the signal peptide. The Kex 2 is an endogenous yeast protease and the Kex 2 site will be cleaved during secretion. This modification reduces the opportunity of the formation of enzymatically inactive fusion invertase and therefore will enhance the sensitivity of our signal TRAP system.

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