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**13. Abstract** ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Although treatment of prostate cancer by androgen withdrawal has been an established treatment for decades, the molecular basis for androgen-dependent prostate growth is unclear. Since androgen effects are mediated through changes in gene expression, the molecular basis for prostate cancer regression upon androgen withdrawal and eventual escape from withdrawal would be better understood if the genes regulated by androgen in the prostate were known. This laboratory had developed a method of identifying hormone-regulated genes by "trapping" (Harrison & Miller, Endocrinology 137:2758, 1996), using a selectable transgene lacking a functional promoter. After stable transfection, a two-stage selection is performed and only cells containing a transgene that has usurped the functions of a native, regulated promoter are left alive. The flanking, native DNA is then sequenced by gene-walking (Harrison et al, Biotechniques. 22:650-3, 1997). Over 100 LNCXAP clones have been isolated of which ~80 contain trapped, down-regulated genes and ~30 contain trapped, up-regulated genes. Initial walks have been performed on a total 20 DNA samples of which one is homologous to a gene not previously described as androgen-regulated. This gene has homology with known protein synthesis initiation factors and thus, may play a supporting role in androgen stimulation of prostate protein synthesis.

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R W Harris 5/18/00

PI - Signature Date

## Introduction:

Although treatment of prostate cancer by androgen withdrawal has been an established treatment for decades, the molecular basis for androgen-dependent prostate growth is unclear. Since androgen effects are mediated through changes in gene expression, the molecular basis for prostate cancer regression upon androgen withdrawal and eventual escape from withdrawal would be better understood if the genes regulated by androgen in the prostate were known. The purpose of this project is to identify the androgen-regulated genes in LnCaP cells that might play a role in the regulation of prostate cancer growth. This laboratory has developed a method of identifying hormone-regulated genes by "trapping" (Harrison & Miller, Endocrinology 137:2758, 1996). The "trap is a selectable transgene lacking a functional promoter. After stable transfection, a two-stage selection is performed in which only cells containing a transgene that has usurped the functions of a native, regulated promoter are left alive. The flanking, native DNA is then sequenced by gene-walking (Harrison et al, Biotechniques. 22:650-3, 1997). The specific gene could then be identified by gene walking from the transgene into the surrounding endogenous DNA. Over 100 LnCaP cell clones have been identified on which ~80 contain trapped, androgen down-regulated genes promoter elements and ~30 contain trapped, up-regulated genes.. Initial walks have been performed on a total 20 DNA samples Two genes have been identified by gene walking thus far. One is the gene for a casein kinase previously described as expressed in the prostate and androgen-regulated. The other is the gene for a peptide elongation factor EIF4A2. EIF4A2 is described as more highly expressed in skeletal muscle and prostate but has not been previously reported as androgen-regulated. This gene has homology with known protein synthesis initiation factors and thus, may play a supporting role in androgen stimulation of prostate protein synthesis. These results suggest that gene trapping is a viable method of identifying hormone regulated genes and that there remain androgen-regulated genes to be discovered.

**Progress (Heading are from Statement of Work):**

**Produce ~90 LnCaP cell clones containing trapped up-regulated genes and 190 LnCaP cell clones containing trapped down-regulated genes.**

(1 Jan 99 - 31 Dec 99) This objective has been accomplished. LnCaP cells have been successfully transfected with the pHyTKpcs transgene and over 50 cell clones isolated.

(1 Jan 00 - 31 Dec 00) This objective has been accomplished. LnCaP cells have been successfully transfected with the pHyTKpcs transgene and 60 cell clones were isolated.

**Using "Easy Gene Walking" and lysate from each clone, sequence at least 200 bp of the DNA 5' of the trap transgene to eliminate any duplicate clones.**

(1 Jan 99 - 31 Dec 99) DNA adjacent to the inserted transgene was sequenced by gene walking (Harrison, et al, 1997). The results are summarized below. More negatively-regulated colonies were recovered than positively regulated ones. Of the negatively-regulated clones, PCR of 60 DNA samples produced 30 bands that could be isolated and sequenced. In 15 cases, the adjacent DNA contained plasmid sequences suggesting that the vector had recircularized before insertion into the genome. Another eight bands provided three unique sequences.

(1 Jan 00 - 31 Dec 00) Previous studies have indicated that gene walking of PCR products from LnCaP genomic DNA is not always successful. The results occasionally exhibited no product, many nonsp0ecific bands, or a smear. We have tried to redesign the unknown primer by adding 5 random bases to 10 degenerate bases in the RSO primer. This approach appeared to yield better results. Through dilution (102-105) of genomic DNA used as DNA template and use of higher concentrations (1-5 μM) of the primers, the smear problem has been prevented and the PCR product exhibited single and larger bp bands. However, sequencing of these single bands by the Big Dye Terminator method still only produced 15% good sequence.

## LnCaP Gene Trapping Results

| | Negatively-Regulated | Positively-Regulated |
|---|---|---|
| Total Colonies | 60 | 25 |
| Sequenceable DNA Bands | 58 | 12 |
| Plasmid Sequences | 15 | 2 |
| Identifiable Sequences | 4 | 1 |

(1 Jan 99 - 31 Dec 99) None of the six unique sequences appeared fully homologous to a human Genebank entry although some matches were found. In instance A, a sequence of 531 nucleotides contained a 411 bp stretch that had an 86% homology with the mouse gene for protein synthesis initiation factor 4A. In instance B, 194 of 343 bp had a 96% homology with a rat casein kinase and a 100% match to 44 bases of a patented human sequence. Lastly, instance C had only a 75.8% identity in a 120 nt overlap.

(1 Jan 00 - 31 Dec 00) During this period, we used the above improved methods to perform gene walking. PCR of 28 (out of 60) of Net (androgen down-regulated and 5 (out of 25) Pet (androgen up0regiulated) DNA samples produced 39 sequenceable bands. Only 6 or 39 samples have an 80-90% readable sequence. Two 100% readable bands are plasmids. FASTA search for these 6 sequences indicated that none of the sequences was fully homologous to any gene in the human Gene bank. They are randomized matched with some human and some mouse genes. And they didn't significantly identify with any known human gene.
Updated results follow.

### FASTA Search Results for Three LnCaP Genes Found by Trapping

| | |
|---|---|
| | **GB_RO:MMEIF4AII, M.** musculus eIF-4AII gene for protein synthesis initiation factor, 85.6% identity in 411 nt overlap |
| A | **GB_PR2:S79942,** eIFA2=human protein synthesis initiation factor 4A-II homolog, 98.9% identity in 88 nt overlap |
| | **GB_RO:RATCKID**, Rat casein Kinase I delta mRNA, complete cds, 95.4% identity in 194 nt |
| B | overlap |
| | **GB_PAT:I192777**, Sequence 43 from patent US 5728806, 100% identity in 44 nt overlap |
| | **GB_PR3:HUAC002990,** Human Chromosome 16 BAC clone CIT987SK-A-1000D7, complete |
| C | sequence, 75.8% identity in 120 nt overlap |

### A. The ARelong Gene

(1 Jan 99 - 31 Dec 99) The PCR fragment produced by gene walking using DNA from the LnCaP/N12 clone was over 400 bp in length. Nearly the entire length demonstrated significant homology to a highly conserved mouse gene (eIF-4AII) for protein synthesis initiation factor 4A and there was a 98.9% identity in a 88 nt overlap for the human gene, GB_PR2:S79942. This suggests that LnCaP cells may contain an androgen-regulated initiation factor that is similar, but not identical, to protein synthesis initiation factor 4A-II. Protein initiation factors have been previously shown to be involved in androgen-regulated prostate growth and many of the genes from this family encode a DEAD box protein/RNA helicase and are involved in cell cycle regulation (Kim et al, 1993; Nielsen and Trachsel, 1988; Sudo et al, 1995).

B. The ARkinase Gene

The sequence matches obtained using the FASTA search are shown below. The PCR fragment produced by walking using DNA from the LnCaP/N12 cell clone was ~343 bases long and consisted of 79 bases of the POMC promoter, followed by ~70 bases of plasmid sequence and ending in ~200 bases that have extensive homology with the Rat casein Kinase I delta clone designated GB_RO:RATCKID. The sequence lacking the POMC and plasmid sequence is shown below, matched to the rat casein kinase. Only 10 bases differ out of 193. Since there was not homology with any components of the trap transgene, we conclude that this gene, that we have termed *ARkinase* is a newly discovered, androgen-regulated kinase expressed in the prostate epithelial cell.

**Are any clones observed to grow more slowly than others do? If so, perform growth curves using charcoal stripped serum containing 0-$10^{-7}$ M dihydrotestosterone and compare growth of the trapped gene clone with parental LnCaP cells. Otherwise, perform steps 4 & 5. for clones exhibiting defective growth, perform step 4 followed by 9-12.**

(1 Jan 99 - 31 Dec 99) Slow growing clones were observed but could not be harvested in sufficient amounts to allow sequencing by our standard methods. If time permits, this objective may be revisited.

**Obtain a total of 1000-2000 bp of sequence of each trapped gene. Use this information to search data banks for matches and, with PCR, to produce Northern blot probes based on the gene walk sequence.**

(1 Jan 99 - 31 Dec 99) Two of three clones match open reading frames of genes archived in GenBank indicating that the sequences obtained by us contain open reading frames as well. Therefore, the ARkinase and ARelong sequences will be used to probe LnCaP mRNA. Sequence "C" will be extended.

**Statement of Work Items Remaining to be Done** (1 Jan 99 - 31 Dec 99)

1. Perform Northern Blots using RNA from the androgen-responsive LnCaP cells and RNA from the androgen-insensitive cells PC-3 and DU145. Probe the blots using the gene fragments created by PCR.
2. For probes that do not identify an RNA band that is not dihydrotestosterone regulated: discontinue evaluation of the cell clone.
3. For probes that identify an RNA band that is dihydrotestosterone regulated in insensitive as well as sensitive cells: retain for possible future evaluation in other androgen-responsive tissues.
4. For probes that identify an RNA band that is differently regulated in LnCaP cells vs the insensitive cells: use Northern blots of human tissues to determine the tissue distribution of the trapped gene and use the probe to identify and isolate clones from a prostate cDNA library giving highest priority to regulated genes expressed in few tissues.
5. Using one or more isolated cDNA clones from #9, obtain a full-length sequence and construct a full-length clone.
6. Use the protein sequence derived from the cDNA to design one or more peptides to produce antisera for use in Western blotting experiments to confirm that the gene product is regulated similarly to its mRNA.
7. Insert the cDNA into an expression vector driven by the CMV promoter or synthesize antisense oligomers to the candidate gene. Use androgen-independent expression of the gene or reduction of the gene's expression to test the effect on growth.

**Statement of Work Items Remaining to be Done** (1 Jan 00 - 31 Dec 00)

Previous study has indicated that the Net 12 cell contains a sequence of 531 nucleotides in which 411 bp had 86% homology with the mouse gene for protein synthesis initiation factor 4A (IF4A). We may use

the primers of mouse IF4A to seize the Northern blot probe from LnCaP genomic DNA after treating the cells with and without androgen.

The same can be done for Net 13, since 194 of 343 bp had a 96% homology with rat casein kinase gene. Our gene trap and gene walking strategy was used successfully in other lab projects and identified a messenger repressor regulated by GR mRNA. Therefore this strategy can be applied in future studies to identify androgen-regulated genes in LnCaP cells

## KEY RESEARCH ACCOMPLISHMENTS:

- Gene Trapping of androgen-regulated genes in LnCaP cells was accomplished
- At least two genes, not previously identified as expressed or regulated in LnCaP cells have been identified.

**REPORTABLE OUTCOMES:**

(1 Jan 99 - 31 Dec 99) Two genes have been identified by gene walking thus far. One is the gene for a casein kinase previously described as expressed in the prostate and androgen-regulated. The other is the gene for a peptide elongation factor EIF4A2. EIF4A2 is described as more highly expressed in skeletal muscle and prostate but has not been previously reported as androgen-regulated. These results suggest that gene trapping is a viable method of identifying hormone regulated genes and that there remain androgen-regulated genes to be discovered.

Previous studies have indicated that gene walking of PCR products from LnCaP genomic DNA is not always successful. The results occasionally exhibited no product, many nonsp0ecific bands, or a smear. We have tried to redesign the unknown primer by adding 5 random bases to 10 degenerate bases in the RSO primer. This approach appeared to yield better results. Through dilution (102-105) of genomic DNA used as DNA template and use of higher concentrations (1-5 μM) of the primers, the smear problem has been prevented and the PCR product exhibited single and larger bp bands. However, sequencing of these single bands by the Big Dye Terminator method still only produced 15% good sequence. (8/00-11/00).

(11/00-12/00) During this period, we used the above improved methods to perform gene walking. PCR of 28 (out of 60) of Net (androgen down-regulated and 5 (out of 25) Pet (androgen up0regiulated) DNA samples produced 39 sequenceable bands. Only 6 or 39 samples have an 80-90% readable sequence. Two 100% readable bands are plasmids. FASTA search for these 6 sequences indicated that none of the sequences was fully homologous to any gene in the human Gene bank. They are randomized matched with some human and some mouse genes. And they didn't significantly identify with any known human gene.

**Description of Androgen Regulated Genes Trapped in LnCaP Cells.**

- 3TN11.seq is all rat POMC
- 3SIN12.seq is a peptide initiation factor 4A4II.
- 3TN13.seq is POMC from 0-114, partial match to (GB_PR3:HUAC002990) from human Chromosome 16 BAC clone CIT9087SK-A-1000D7 from 274-390.Also get match from 259-399 with (GB_AF004854) Mus musculus MmRad52 gene, complete cds. (MmRad52; yeast DNA repair protein Rad562)
- 4B2NI12.seq starts as POMC,, has a short piece of plasmid and then casein kinase ! delta (GB_RO:RATCKID).
- 4E2P70.seq no real match on FASTA
- 4S2P70.seq matches (-91) to human H19 gene on chromosome 11.

- 4SN52 matches 338 nt-95.R11, unordered human sequencing. Keyword "HTG, HTGS"
- 4TP73.seq no match after modification removing CMV.
- 6S1P59.seq has no real match. (-81).

**CONCLUSIONS:**

- Gene trapping is a viable method of identifying hormone regulated genes
- Androgen-regulated genes exist in LnCaP cells that have not been previously described.
- The gene trapping procedure may preferentially identify exonic sequences.
- Gene trapping is a viable method of identifying hormone regulated genes and that there remain androgen-regulated genes to be discovered.

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