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13. ABSTRACT (Maximum 200 Words) Seven 3' untranslated region(UTR) single nucleotide polymorphisms (SNPs) and one double mutant from the human steroid 5-alpha reductase I gene were functionally tested indirectly for mRNA stability using a luciferase construct in a human kidney cell line. There was no difference in luciferase signal using any of the variants, but the 3'UTR did double luciferase signal suggesting a stabilization effect. It was hypothesized that the secondary structure of the luciferase+SRD5A1 3'-UTR mRNA was so different from the native SRD5A1 message, that the 3'-UTR variants should be tested in the context of a native mRNA. A eukaryotic expression vector containing 850bp of the human SRD5A1 promoter was seamlessly cloned onto the 5' end of the full length SRD5A1 cDNA (containing the full length UTR). Human beta-globin intron 2 was inserted into exon 4 using a native blunt restriction site to ensure proper mRNA processing and export. This vector has been constructed and is being evaluated for expression via northern blotting and RT-PCR. There are no studies evaluating how SNPs in 'RNA haplotypes' alter RNA half-life, stability, or protein translatability.					
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Introduction:

Since the 1940s, a link between androgens and prostate cancer was established that demonstrated the effect castration had on reducing or eliminating prostate carcinoma, while injections of testosterone (a steroid hormone) had the opposite effect (Huggins, C.B., Hodges, C.V. 1941). Later work found that steroid hormones bind to receptors and regulate nuclear gene regulation, thus altering cell and tissue phenotypes (Lefkowitz, R.J., et al. 1970; Attramadal, A., et al. 1976). Enzymes that control androgen metabolic flux are of interest to understanding prostate cancer predisposition studies because they participate in the regulation, distribution, and concentration of the various types of steroid hormones.

Prostate cancer is strongly influenced by genetics, with the estimated contribution being 42% of the risk (Gann et al. 1995; Lichtenstein, et al. 2001). Although most sporadic cancers are caused by environmental factors and de novo mutations, the relatively large genetic contribution in prostate cancer demonstrates how wide are the gaps in our knowledge and understanding of cancer genetics (Lichtenstein, P., et al. 2000). Therefore, an examination of the different enzymes that catalyze the anabolism of testosterone into dihydrotestosterone (DHT) should be useful at elucidating how increased metabolic flux through these pathways impact prostate growth, and therefore, the likelihood of being afflicted with tumors (Davies, et al. 1991; George, et al. 1991; Wilson, et al. 1975). There are two known steroid 5- α reductases in humans, types I and II. The two genes lie on different chromosomes, have similar gene structure, and encode steroid 5- α reductases, but show different tissue expression. The type II reductase has been studied in relation to prostate cancer and certain SNPs play a role in prostate cancer predisposition for some populations.

The steroid 5- α reductase type II enzyme (E.C. 1.3.99.5), which catalyzes the reduction of testosterone (and other steroids) into the physiologically more active dihydrotestosterone (DHT) was shown to have at least ten single amino acid substitutions and three double mutations, all of which occur in normal, healthy males (Makridakis, et al. 2000). The kinetic properties of these mutant enzymes have been measured, with some showing larger or smaller values than the wild-type enzyme (Makridakis, N., et al. 1999; Makridakis, N., di Salle, E., Reichardt, J.K.V. 2000). One mutation, which changes an alanine at amino acid 49 into a threonine, boosts African-American men's chances of developing prostate cancer 7.2 fold, while the same mutation imparted Latinos with 3.2 times the likelihood of developing the disease (Makridakis, et al. 2000). Mutant enzymes also demonstrated a 32-fold range of inhibition to the selective steroid 5- α reductase inhibitor, finasteride (Makridakis, M, et al. 2004). In the prostate cancer prevention trial, daily oral dosing of finasteride decreased prostate cancer risk by roughly 25%, while those that did develop cancer and who had taken finasteride were at a 6 fold higher risk for more aggressive, higher grade cancers (Thompson, IM, et al. 2003).

This poses the question: Does the related type I reductase possess variants in human populations which can mutate the enzyme thus predisposing/protecting those men to prostate cancer or benign prostatic hyperplasia (BPH)?

Specific Aims

The SRD5A1 gene encodes the steroid 5-alpha reductase enzyme type I which reduces testosterone into a physiologically more active chemical form, dihydrotestosterone (DHT). I hypothesize that the human steroid 5-alpha reductase type I enzyme plays a role in prostate cancer development, and that gain-of-function single nucleotide polymorphisms (SNPs) in the SRD5A1 gene can increase androgen metabolic flux to the prostate, increasing its size, and thus predisposing to prostate cancer over decades of life.

The specific aims in year two seek to answer fundamental questions as to how 'RNA haplotypes' which occur naturally in a screened human population, may affect RNA and protein levels, and thus SRD5A1 biochemistry. The average human mRNA is over 2000 bp long, which means that on average, there are 1-2 SNPs/mRNA (Celera Genomics, 2001). There are no systematic studies of RNA haplotype combinations even in artificial constructs, much less along a native mRNA. At best, one or two SNPs that occur in a 3'UTR are tested for affect in an artificial expression vector (e.g. CAT, luciferase), but the mRNA structure is completely different from the native mRNA of interest. There are no published studies on if or how combinations of SNPs at the RNA level affect RNA half-life, RNA steady state, or protein translatability. We hypothesize that 'RNA haplotypes' constitute a large class of molecules that play an important role in gene expression and consequently metabolic and cellular flux that can attenuate biological phenotypes.

Specific Aim #2 entails identifying how SNPs alter steroid 5-a reductase type I function in biochemical and kinetic assays.

Body:

- **Table 1.** This table contains human SRD5A1 haplotype blocks that compose the initial population sequenced in Specific Aim #1. Haplotypes and blocks are useful to minimize the number of variants that need to be functionally tested. Each block is composed of more than 1 sequence combination (e.g. Block A has 5 different sequence combinations that extend from the promoter to the 3'UTR). For the third column, each of the 25 characters represents one polymorphic locus as they occur from the 5'-to-3' end of the human SRD5A1 gene (there are 12 SNPs in the promoter region, and 13 in the cDNA+3'UTR). Hyphens indicate nucleotide identity between haplotypes within a block, while letters indicate IUPAC nomenclature. E.g. In block A, there is only 1 nucleotide difference between all 5 haplotypes in the promoter region (~900 bp), and two variant sites in the remainder of the gene (~2,300 bp). The % frequency is the frequency of men whose exact genotype at all 25 loci fall into a particular haplotype block. The five haplotypes of block A cover about 42% of the initial population that was screened. By testing just the three polymorphic loci in haplotype A, one would cover approximately 42% of genotypes present in the population. If all three blocks can be tested, then over 62% of the population's genotypes would have been tested. It is important to note that the DNA sequences between haplotypes also vary. One SNP in the 3'UTR, at nucleotide 1578 is predicted to create an enhancer binding protein, c/EBP-1, while another variant in the putative promoter is predicted to create a transcription factor-binding site. IUPAC code: Y=C or T, S=C or G, R=A or G.

	# haplotypes/block	PROMOTER	cDNA+UTR	% frequency
Block A	5	-----Y	S-----Y-----	41.65
Block B	2	-----	-----Y-----	8.68
Block C	3	-----Y-----	-RR-R--Y--Y-	12.65
SUM=				62.98
1578 Creates c/EBP-1 site-----Y-				
-648 Creates Elk-1 site ---Y-----				

Table 1. Human SRD5A1 haplotype blocks and frequencies.

* **Figure 1 and Table 2.** Figure shows the plasmid constructs that were lipofected into human embryonic kidney cells (HEK-293). Normally, the addition of a 3'UTR into luciferase leads to a reduction in luciferase signal. Transfected vectors include those with no SRD5A1 UTR (empty), 3-UTR in the native orientation (A), and opposite orientation (UTR-). Table 2 contains the luciferase data from lipofected 293 cells. The 'not transfected' lane is endogenous background, pGL3P is the empty luciferase vector, wt is the 3'UTR containing the sequence that is most common in the screened population, the numbers indicate the location and base of each SNP tested, and 'DM' is a double mutant containing 1368T/1757C. UTR- contains the UTR in an inverted order, and its expression is decreased, consistent with nonsense mediated decay.

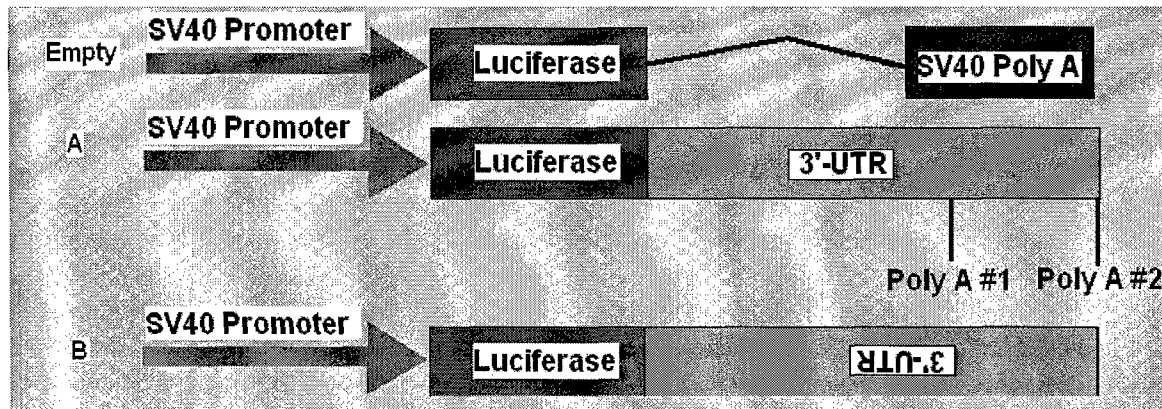


Figure 1. Cartoon of the luciferase constructs used to transfect human embryonic kidney cells (HEK-293). Empty=empty pGL3P vector backbone. A=pGL3P+full length human SRD5A1 3'UTR with both poly A sites. B=pGL3P+inverted human SRD5A1 3'UTR with both poly A sites(denoted as UTR- in Table 2).

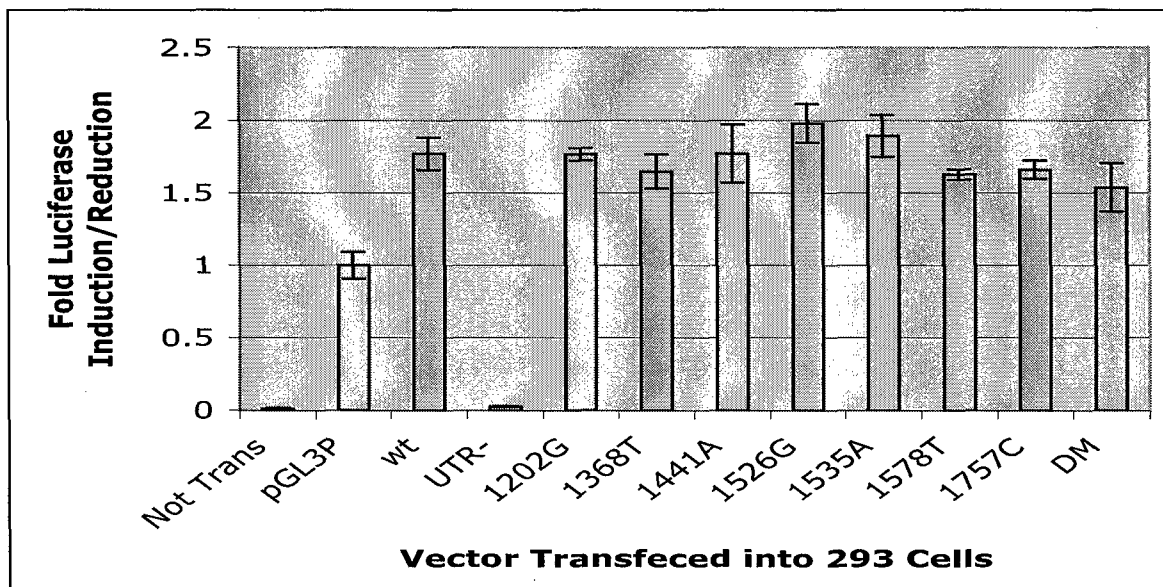


Table 2. Corrected luciferase experiment of three replicates per plasmid (N=3) in 293 cells. The fold induction/reduction as compared to the mean pGL3P (empty) vector is displayed on the y-axis.

- **Figure 2 and Table 3.** Prior to cloning a 'native' human SRD5A1 mRNA, it was important to determine if the promoter fragment to be used was actively expressed in human cells. To this end, a 895 bp fragment 5' of the SRD5A1 gene was placed 5' of the luciferase gene in a mammalian expression vector (pGL3 enhancer). The promoter in native and non-native orientation along with appropriate controls was lipofected into a metastatic prostate carcinoma cell line (PC-3).
- **Figure 2** shows the vectors used, and Table 3 shows the results. Vectors A and B both induced luciferase, with A giving the stronger signal. The SRD5A1 gene lies in a head-to-head orientation with another gene, with transcription from opposite strands. It was hypothesized prior to this experiment that the inverted fragment would also contain robust transcription as it is likely to be the promoter for the second gene.

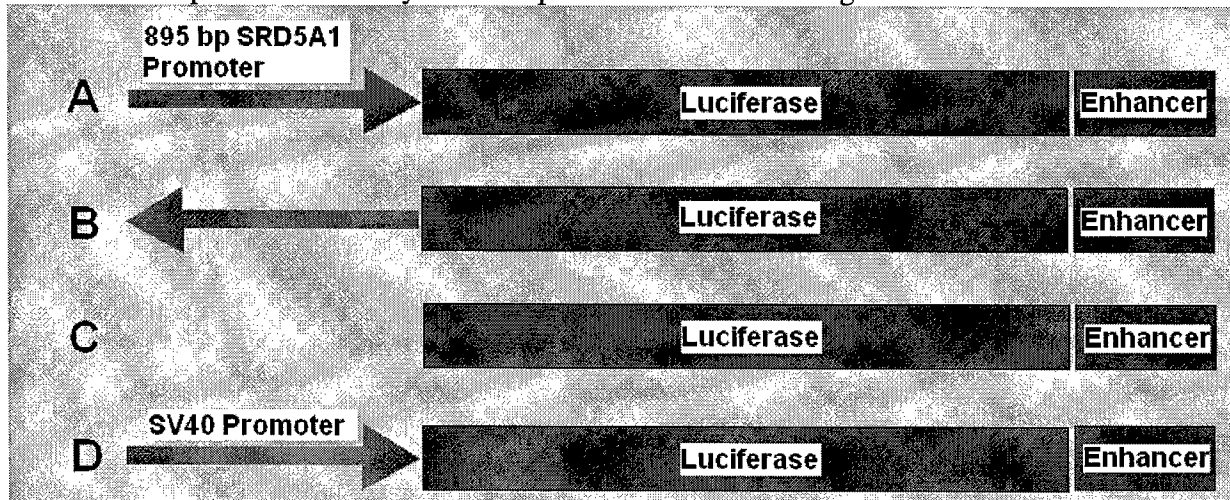


Figure 2. Luciferase vectors used for lipofecting a PC-3 cell line (prostate carcinoma). A=895 bp of the putative 5'-regulatory region from the human SRD5A1 gene. B=895 bp putative regulatory region in the opposite orientation. C=empty vector without a promoter, and D=pGL3P vector using the SV40 promoter.

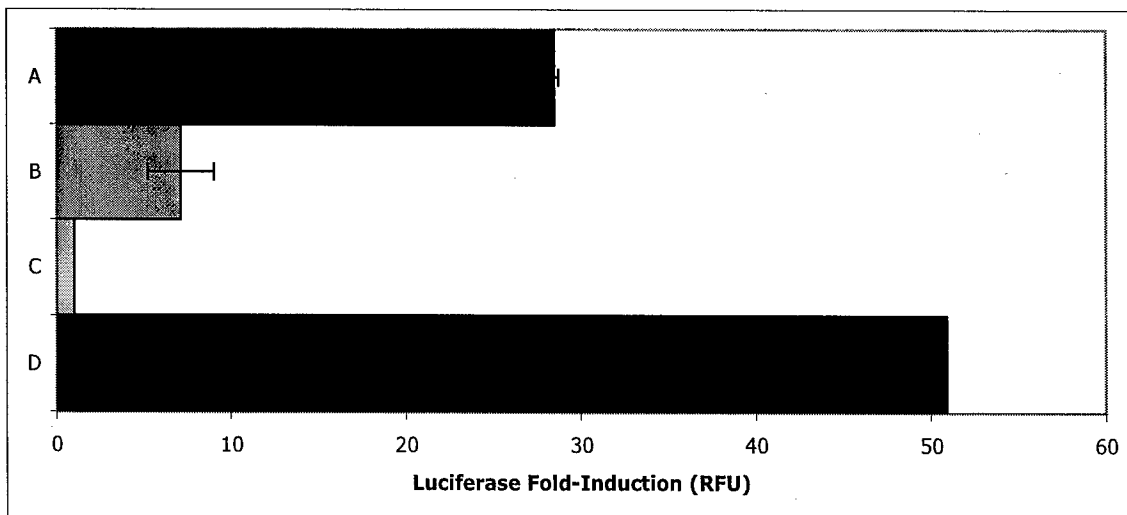


Table 3. PC-3 cell line lipofected in triplicate (N=3) using vectors in Figure 2.

***Figure 3.** Based on data from Table 2, a 'native' human SRD5A1 expression vector was created to mimic the normal mRNA expressed in man. To this end, ~850 bp promoter region was seamlessly cloned onto the full-length cDNA using naturally occurring restriction enzyme sites. Since all of the native SRD5A1 introns exceeded 4kb, it was decided to insert a human beta-globin intron 2 into the middle of exon4. Introns are needed for proper mRNA splicing, polyadenylation site choice, and nuclear export. The intron was cloned into a natural blunt site. The vector was transfected into human 293 cells and the mRNA intron junction site was sequenced after RT-PCR. Correct splicing at the GT/AG introns was observed (data not shown). A=DNA vector that was constructed, B=expected mRNA from transcribed A. Since the message contains only native SRD5A1 5'/3'UTRs, and cDNA, the secondary structure of the mRNA should be identical to the message expressed from the chromosome.

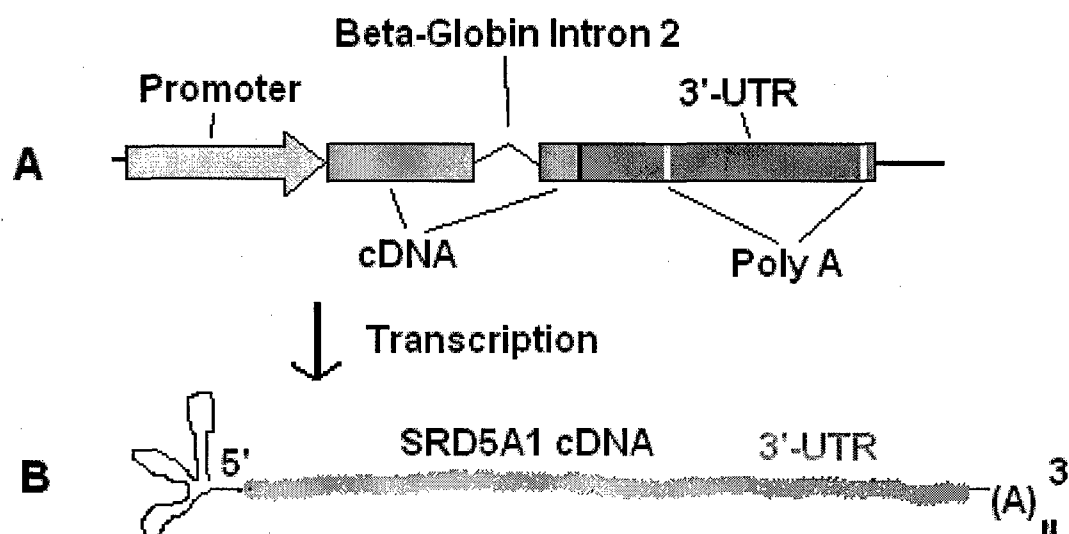


Figure 3. 'Native' SRD5A1 mammalian expression vector and its transcript.

Specific Aim #3 involves a case-cohort study to determine if the polymorphisms increase or decrease the risk of developing prostate cancer in African-Americans, Japanese-Americans, Caucasians, and Latinos, and if there is an interaction between the gene and environmental factors such as dietary fat and obesity.

Case and control DNA samples are expected next month. At that time, specific aim #3 will begin.

Key Research Accomplishments:

- 1) delineation of natural SRD5A1 haplotypes in human males.
- 2) Completed functional testing of SRD5A1 3'UTR SNPs in a luciferase reporter, showing that the human SRD5A1 3'UTR stabilizes luciferase signal, while a random piece of DNA (UTR-) ablates this effect to background.
- 3) Completed construction of a native mammalian expression vector that is driven by the human SRD5A1 promoter, also giving proper splicing. Currently testing expression and mRNA levels with various mRNA mutants.

Reportable Outcomes:

There are no reportable outcomes yet.

Conclusions:

Luciferase constructs containing the human SRD5A1 3'UTR demonstrated luciferase signal increases, but there was no significant difference using any one SNP, or combination. Thus, a 'native' mRNA structure was proposed at being more sensitive for testing subtle SNP changes on RNA biochemistry. Prior to cloning the 'native' expression vector, a promoter fragment of the SRD5A1 gene was tested for its ability to drive luciferase expression. The promoter proved to express strongly in a human prostate cell line. Therefore, in an attempt to mimic a natural mRNA secondary structure, the promoter, cDNA, and 3'UTRs from the human SRD5A1 gene were cloned together using endogenous restriction sites. A human beta-globin intron 2 was dropped into the middle of exon 4 so that mRNA poly A choice and RNA trafficking would not be altered. When transcribed, this message should have the identical number of nucleotides, length, and structure as the chromosomally expressed gene. Do date, there are no systematic studies of human RNA haplotypes and biochemistry in the literature. On-going efforts are attempting to determine if molecular 'rules' for RNA haplotypes exist. If so, these lessons can be extended to other messages and are likely to help substantively add to our collective knowledge about prostate cancer etiology via RNA biochemistry and metabolism.

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