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Identification and Characterization of MYC Regulatory Elements: Links to Prostate Cancer

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Prostate cancer is the most common cancer diagnosed in males in the developed world. Genome-wide association studies (GWAS) have greatly helped in the identification of common risk variants associated with complex diseases such as cancer; routinely, these associated polymorphisms are located within gene deserts and other type of non-coding DNA. A striking example of GWAS implicating non-coding variants in the etiology of cancer can be seen on chromosome 8q24, where numerous studies have reported associations between prostate (and other) cancer and variants concentrated within a 1.2Mb gene desert. Although there are no genes within the interval, the proto-oncogene MYC lies just downstream of the gene desert, raising the possibility that the associated risk regions may harbor long-range cis-regulatory elements – such as enhancers – involved in the tissue-specific transcriptional regulation of MYC. To date, we have located and characterized an in vivo prostate enhancer encompassing the prostate cancer associated SNP rs6983267. Furthermore, we demonstrated that this enhancer exhibits allele-specific activity in developing and mature mouse prostates, mimicking MYC expression. Our findings help advance the field’s understanding of the mechanistic reason for the overwhelming association seen between this 8q24 gene desert and prostate cancer.

No subject terms provided.
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INTRODUCTION:

Prostate cancer is the most common cancer diagnosed in males in the developed world. While risk factors suggest a genetic basis for the disease, the search for causal genes has yielded few results. In the last decade, genome-wide association studies (GWAS) have greatly helped in the identification of common risk variants associated with complex diseases such as cancer; routinely, these associated polymorphisms are located within gene deserts and other type of non-coding DNA (1). A striking example of GWAS implicating non-coding variants in the etiology of cancer can be seen on chromosome 8q24, where numerous studies have reported associations between prostate, colorectal, breast and urinary bladder cancer and variants concentrated within a 1.2Mb gene desert (2-12). Evidence for prostate cancer association is particularly strong, with five distinct linkage disequilibrium (LD) blocks spanning a 440Kb interval harboring risk variants. Although there are no well-characterized genes within the interval, the proto-oncogene MYC lies just downstream of the gene desert, raising the possibility that the associated risk regions may harbor long-range cis-regulatory elements – such as enhancers – involved in the tissue-specific transcriptional regulation of MYC. Under this hypothesis, each distinct prostate cancer association interval would contain a functional element involved in regulating MYC expression in the prostate. The purpose of this proposal is to identify and characterize prostate enhancers within the prostate cancer associated intervals on 8q24 using a combination of in vivo and in vitro reporter assays.

BODY:

In the course of the past year, we have located and characterized a prostate enhancer that encompasses the prostate cancer associated SNP rs6983267. Furthermore, we have demonstrated that this enhancer element exhibits allele-specific in vivo enhancer activity in developing and mature mouse prostates (13) (see Appendix for full publication). These achievements support the hypothesis put forth in my initial proposal and represent significant progress towards the execution of my research Aims. The following section addresses each specific task within the Statement of Work by detailing the progress and accomplishments of the last year.

Task 1a: Perform in situ hybridization for all genes within a 1.0Mb interval surrounding the prostate cancer-associated region.

Because of its status as an ideal positional and functional target gene, we began our in situ hybridizations by assessing Myc expression in the genitourinary apparatus of male mice. Digoxigenin-labeled Myc antisense and sense riboprobes were generated from a full-length mouse Myc cDNA clone. Staining was performed on whole P8 and P21 mouse prostates for 48 hours. As expected, we observed Myc expression in the developing and mature prostate, as well as in the coagulating glands, seminal vesicles, and ductus deferens (13) (Appendix, Figure 2 of paper). This expression correlated very well with the reporter gene expression pattern driven by the rs6983267-containing enhancer we identified (described below).

While the parallel between the enhancer’s domain and Myc expression in the prostate is compelling, it does not directly show that MYC is the target gene for the 8q24 cis-regulatory element. In theory, other genes within the enhancer’s potential range of influence – including...
FAM84B, POU5F1P1, and PVT1 – could also exhibit prostate expression and be the target for the element’s regulatory influence. However, work published since the submission of this proposal has convincingly demonstrated that the 8q24 prostate cancer associated regions (including the rs6983267-containing enhancer we describe) physically interact with the MYC promoter in prostate cancer cell lines (14-16). These studies all employed chromosomal conformation capture (3C), a technique that assesses whether a specific fragment can loop over large genomic distances to physically connect with another DNA region (17). This direct link between the prostate cancer associated regions and the MYC promoter –located between 250kb and 650kb away – shows that MYC is the target gene of the cancer associated cis-regulatory regions. This obviates the need to investigate the expression patterns of other nearby genes.

**Task 1b, 1c and 1d: Use progressively smaller DNA fragments in in vivo mouse transgenic assays to identify and localize enhancers within the 8q24 region.**

To initially examine the 8q24 gene desert for regulatory elements, we surveyed the region using a broad-scale BAC scan approach. We identified three overlapping human BACs encompassing the prostate cancer risk regions (CTD-2506D10, RP11-124F15, and CTD-2533C10), which together span 480kb of non-coding DNA (Appendix, Figure 2 of paper). Each BAC carried the prostate cancer-associated risk haplotype and was tagged through a Tn7 transposon-mediated random insertion of a β-galactosidase (lacZ) gene driven by a β–globin minimal promoter (18). The lacZ cassette integration converts the BACs into enhancer trapping systems, whereby any long-range enhancer(s) contained within each ~180kb BAC can act upon the reporter gene to drive tissue- and temporal-specific β-galactosidase expression. The design of overlapping BACs aids in the efficiency of the system to narrow the critical region of interest, as expression profiles unique to only one BAC must be due to uniquely contained sequences; conversely, identical expression patterns present in overlapping BACs suggest that the functional element driving β-galactosidase expression must be contained in the shared genomic region. A detailed account of these experiments can be found in the attached manuscript (Appendix, Results and Methods).

The in vivo BAC transgenic reporter assays identified prostate enhancer activity contained within the 8q24 gene desert (Appendix, Figure 1 of paper). While we did not observe β-galactosidase prostate expression in BAC CTD-2506D10 transgenic mice (12 independent transgenics), animals harboring BACs CTD-2533C10 and RP11-124F15 displayed β-galactosidase prostate expression at days P0, P8 and P21 (13). Because of the highly similar reporter expression patterns obtained from BACs RP11-124F15 and CTD-2533C10, including prostate, coagulating gland, and urethral/bladder lining, we hypothesized that our BAC transgenic assays were identifying a single prostate enhancer within the 59kb shared genomic segment of these two BACs. Interestingly, one of the most strongly associated prostate cancer risk SNPs, rs6983267, is contained within this 59kb overlapping interval and disrupts an evolutionarily conserved sequence (Appendix, Figure 1 of paper).

Rather than using fosmids as an intermediate means to localize the putative prostate enhancer element, we directly tested the rs6983267-containing evolutionarily conserved element for regulatory potential in vivo. A 5kb DNA fragment containing each allele of this SNP was cloned into a lacZ reporter cassette using Invitrogen’s Gateway cloning system and transgenic mice harboring either the risk or the non-risk variant of rs6983267 were generated and analyzed. We determined that the conserved sequence containing the prostate cancer GWAS SNP displayed allele-specific in vivo prostate enhancer properties(13) (Appendix, Figure 2 of paper).
Specifically, the risk allele, rs6983267-G, led to consistent, stronger β-galactosidase expression in prostates and coagulating glands than the non-risk allele, rs6983267-T, in P0, P8 and P21 transgenic mice (Appendix, Figures 2 and 3). The expression pattern driven by the rs6983267-G risk allele in 3 independent mouse transgenic lines closely resembled that observed in BACs RP11-124F15 and CTD-2533C10 – both of which also harbor the risk allele. In contrast, the rs6983267-T non-risk allele led to weakened prostate and coagulating gland expression in 3 independent transgenic lines. For each allelic variant evaluated, those transgenic founders exhibiting enhancer activity showed highly concordant β-galactosidase expression in the prostate, with a clear qualitative difference between the risk and non-risk variants.

These results demonstrate that our BAC-based enhancer trapping screen is a powerful resource to rapidly uncover cis-regulatory regions in large DNA segments. We plan to use this screening tool to further refine our findings, uncovering other prostate-specific regulatory elements in this locus. As other non-coding SNPs within this 8q24 gene desert are associated with increased prostate cancer risk – independently of the SNP we already characterized – we anticipate discovering other prostate enhancers with allele-specific function upstream of MYC.

Task 2a: Construct a reporter plasmid for luciferase assay analysis in prostate cancer cell lines.

Two luciferase assay report plasmids have been constructed for the quantitative analysis of enhancer potential in prostate cancer cell lines. Both make use of Promega’s pGL4 vectors. The first uses the minimum promoter present in the pGL4.23, with the only alteration being the addition of Invitrogen’s Gateway cassette into the multiple cloning site. This allows for the easy shuttling of multiple elements into the vector without the need for traditional cloning. The second vector began with Promega’s promoterless pGL4.10, into which the MYC promoter and the Gateway cassette were both inserted. MYC is known to be expressed from numerous promoters, with the majority of transcripts initiating from promoter 2 (P2); as its proximal regulation is still not entirely understood, we wished to be overly conservative in the definition of “promoter” to ensure that all necessary elements were present in our reporter construct (19). To that end, 1.7kb of sequence upstream of the MYC transcriptional start site was cloned into the pGL4.10 vector. This element will be tested prior to use with putative enhancer elements to determine its basal regulatory potential in prostate cancer cell lines.

KEY RESEARCH ACCOMPLISHMENTS:

- We identified a prostate enhancer located within a prostate cancer associated region capable of driving in vivo reporter gene expression in the developing and mature mouse prostate. Furthermore, we showed that the genotype of the cancer associated SNP rs6983267 – contained within this enhancer – conveys allele-specific regulatory potential to the enhancer element, with the risk variant possessing stronger enhancer abilities than the protective allele. These findings were published in the high impact journal Genome Research (13).
- Our broad-scale BAC scan of the 8q24 gene desert showed that there are other regulatory sequences within the interval of interest; specifically, we uncovered a mammary gland enhancer within a region that has been associated with risk to breast cancer (13) (Appendix, Figure 1 of paper). These results demonstrate that we have generated a powerful tool to experimentally interrogate genomic regions showing association to multiple types of cancer, and that this tool can be widely disseminated among the cancer genetics research community.
Our results, combined with those of other researchers working with colorectal cancer (14-16, 20, 21), demonstrate that the same genetic variation – known to increases risk to both prostate and colorectal cancer – functions in both cases by altering the spatial, temporal, and/or quantitative fine tuning of MYC expression through allele-specific enhancer activity.

As our in vivo enhancer reporter assays allow for the interrogation of regulatory potential over developmental time, we were able to demonstrate that the rs6983267-containing enhancer is active throughout prostate organogenesis (13) (Appendix, Figure 2 and 3 of paper). These results pose the intriguing possibility that the increased risk to prostate cancer might result from a misregulation of MYC’s expression early in development, long before the onset of tumorigenesis.

Based on these accomplishments, I have been invited to write a book chapter discussing cis-regulatory mechanisms underlying cancer risk. This chapter will be included in an upcoming book edited by Nadav Ahituv, Ph.D, and will focus with prominence on prostate cancer genetics.

REPORTABLE OUTCOMES:

Publications:

Presentations at Scientific Meetings:
Wasserman NF, Nobrega MA. An 8q24 gene desert variant associated with prostate cancer risk confers differential in vivo activity to a MYC enhancer. Poster. IMPaCT, 2011.

CONCLUSION:

The BAC enhancer trapping strategy that we employed allowed us to rapidly interrogate the 440kb of 8q24 prostate cancer-associated non-coding DNA for cis-regulatory elements. We effectively screened a half-megabase genomic interval in vivo using only three constructs, and succeeded in identifying a prostate enhancer within an interval strongly associated with prostate cancer. In addition, we localized a specific prostate enhancer contained within the overlapping region of two of our BACs and showed that it possessed in vivo allele-specific regulatory abilities contingent on the genotype of the prostate cancer associated SNP rs6983267. These results – showing the cancer risk allele demonstrating stronger enhancer potential than the non-risk allele – are concordant with MYC’s known role as a proto-oncogene. Finally, we demonstrated that the rs6983267-containing enhancer exhibits differential in vivo activity throughout prostate organogenesis. As no association has been seen between rs6983267 genotype and steady-state MYC mRNA levels in normal prostate cells or prostate tumors (22), our results raise the possibility that this variant asserts its influence on prostate cancer risk before tumorigenesis actually occurs. Our findings contribute to the field’s understanding of the mechanistic reason for the overwhelming association seen between this 8q24 gene desert and
prostate cancer. By explaining the genetic basis for disease risk, progress towards clinical applications can be made.

REFERENCES:

An 8q24 gene desert variant associated with prostate cancer risk confers differential in vivo activity to a MYC enhancer

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Genome-wide association studies (GWAS) routinely identify risk variants in noncoding DNA, as exemplified by reports of multiple single nucleotide polymorphisms (SNPs) associated with prostate cancer in five independent regions in a gene desert on 8q24. Two of these regions also have been associated with breast and colorectal cancer. These findings implicate functional variation within long-range cis-regulatory elements in disease etiology. We used an in vivo bacterial artificial chromosome (BAC) enhancer-trapping strategy in mice to scan a half-megabase of the 8q24 gene desert encompassing the prostate cancer-associated regions for long-range cis-regulatory elements. These BAC assays identified both prostate and mammary gland enhancer activities within the region. We demonstrate that the 8q24 cancer-associated variant rs6983267 lies within an in vivo prostate enhancer whose expression mimics that of the nearby MYC proto-oncogene. Additionally, we show that the cancer risk allele increases prostate enhancer activity in vivo relative to the non-risk allele. This allele-specific enhancer activity is detectable during early prostate development and throughout prostate maturation, raising the possibility that this SNP could assert its influence on prostate cancer risk before tumorigenesis occurs. Our study represents an efficient strategy to build experimentally on GWAS findings with an in vivo method for rapidly scanning large regions of noncoding DNA for functional cis-regulatory sequences harboring variation implicated in complex diseases.

[Supplemental material is available online at http://www.genome.org.]

Genome-wide association studies (GWAS) routinely implicate variation within gene deserts and other types of noncoding DNA in the etiology of disease (Houlston et al. 2008; Silverberg et al. 2009; Yang et al. 2009; Liu et al. 2010). A recent meta-analysis of ~1200 disease-associated single nucleotide polymorphisms (SNPs) found that in 40% of cases, known exonic sequences were absent from the associated linkage disequilibrium (LD) blocks (Visel et al. 2009). While the presence of nonannotated transcripts or noncoding RNAs may explain some of the noncoding disease associations, these observations also have been interpreted as evidence that many of the associated noncoding regions harbor variants that alter the activity of long-range cis-regulatory elements controlling gene expression. Enhancers are one such type of long-range element, functioning over up to megabase-long genomic distances to regulate the temporal and tissue-specific expression patterns of their target gene(s) (Nobrega et al. 2003). A large number of genes with tissue- and temporal-specific expression patterns are known to be controlled by an array of enhancers, with each individual cis-regulatory element driving a subset of its gene’s entire expression profile (Carroll 2008). This modular nature of enhancer activity makes them ideal candidates for involvement in complex diseases, as functional variants in an individual cis-element would result in changes to gene expression only in specific organs/tissue types.

Despite the plethora of GWAS signals implicating noncoding regions in complex disease risk, strategies to experimentally follow up on such findings are lacking. This deficiency stems principally from the difficulty in identifying functional noncoding sequences that map remotely from their target genes. Programs such as ENCODE have been addressing this deficiency by developing and applying technologies to identify these elusive types of long-range regulatory elements (The ENCODE Project Consortium 2007). While these technologies have been invaluable in the identification of putative functional noncoding sequences, they rely heavily on cell culture and other in vitro and in silico methodology to identify and experimentally validate enhancers and other elements. Thus, although these techniques are ideal for functionally following up on noncoding GWAS results when the relevant cell type of interest is obvious and accessible, problems can arise if the putative element under investigation imparts its transcriptional regulatory effects in a cell type of unpredicted origin or one that is not amenable to routine culture. Necessary, but lagging, is the development of simpler in vivo strategies that can concurrently query the spatial and temporal properties of functional cis-regulatory sequences within large segments of noncoding DNA. Our goal in this study is to describe one such strategy for following up on GWAS results, and to test its ability to uncover noncoding risk variants in loci associated with complex diseases.

A striking example of GWAS implicating noncoding variants in the etiology of complex diseases can be seen on chromosome 8q24, where numerous studies have reported associations between multiple types of cancer—including prostate, colorectal, breast, and urinary bladder—and variants concentrated within 620 kb of a 1.2-Mb gene desert (Amundadottir et al. 2006; Easton et al. 2007; Gudmundsson et al. 2007; Haiman et al. 2007; Ghoussaini et al. 2008; Al Olama et al. 2009). Evidence for prostate cancer association within the region is particularly strong, with five distinct LD blocks spanning a 440-kb interval on 8q24 harboring risk variants (Fig. 1A, all shaded regions; Ghoussaini et al. 2008; Al Olama et al. 2009). Of these prostate cancer-associated variants, rs6983267, is independently associated with colorectal cancer (Fig. 1A, green; Tomlinson et al. 2007), and a second prostate cancer-associated LD...
block harbors a distinct SNP (rs13281615) that shows association with breast cancer (Fig. 1A, pink; Easton et al. 2007). Although no well-annotated genes lie within this interval, the independent associated variants (or linked functional elements within the associated regions) may all be regulating the expression patterns of a single gene involved in cancer tumorigenesis and/or progression in various tissue types. The proto-oncogene MYC lies immediately downstream of this gene desert, raising the possibility that the associated regions of risk may harbor long-range cis-regulatory elements involved in the tissue-specific transcriptional regulation of MYC expression; under this hypothesis, each distinct association interval might harbor functional noncoding element involved in regulating MYC expression in the corresponding tissue type for each implicated cancer. A summary of the 8q24 gene desert and its numerous cancer loci is shown in Figure 1. Here, we have chosen to specifically focus on the multiple independent associations between this 8q24 gene desert and prostate cancer.

Encoding a well-known transcription factor essential to the regulation of cell proliferation and growth, MYC is up-regulated at both the mRNA and protein levels in aggressive prostate cancers (DeMarzo et al. 2003). In addition, copy-number analyses in prostate cancer specimens have identified the 8q24 region surrounding MYC as the most common recurrent region of chromosomal gain (Lapointe et al. 2007). These findings show that prostate cancers employ multiple mechanisms for achieving MYC overexpression, through transcriptional up-regulation or through amplification of gene copy number. We hypothesized that variation within MYC’s long-range cis-regulatory elements could disrupt the quantitative, temporal, or spatial expression patterns of MYC in the prostate, possibly underlying the GWAS signals identified in the 8q24 gene desert. In this study, we describe how an in vivo bacterial artificial chromosome (BAC) enhancer-trapping strategy efficiently scanned the 8q24 gene desert for cis-regulatory sequences, and report on the identification of both prostate and mammary gland enhancer activities within the assayed regions. We further refined the prostate enhancer interval, showing that it harbors the prostate cancer risk SNP rs6983267, and demonstrate that the two resultant allelic variants display functionally polymorphic prostate enhancer properties in vivo.

Results

Surveying the regulatory landscape of the 8q24 gene desert

To initially examine the 8q24 gene desert for regulatory elements, we surveyed the region using a broad-scale BAC scan approach
examination of large genomic regions for cis-regulatory elements, and can be readily applied to any locus of interest. We identified three overlapping human BACs encompassing the prostate cancer risk regions (Fig. 1A), which together span 480 kb of noncoding DNA. Each BAC carried the prostate cancer-associated risk haplotype and was tagged through a Tn7 transposon-mediated random insertion of a beta-galactosidase (lacZ) gene driven by a beta-globin minimal promoter (Spitz et al. 2003). The transposon-mediated insertion was performed using simple, commercially available kits (see Methods) and occurs in vitro; the protocol yields rapid results and can be easily scaled up for the simultaneous tagging of numerous BACs.

The lacZ cassette integration converts the BACs into enhancer-trapping systems, whereby any long-range enhancer(s) contained within each ~180-kb BAC can act upon the reporter gene to drive tissue- and temporal-specific beta-galactosidase expression. Any enhancers present within a given BAC are then simultaneously interrogated using a reporter assay system, allowing for the concurrent examination of large genomic regions for functional noncoding elements. The design of overlapping BACs aids in the efficiency of the system to narrow the critical region of interest, as expression profiles unique to only one BAC must be due to uniquely contained sequences; conversely, identical expression patterns present in overlapping BACs suggest that the functional element driving beta-galactosidase expression must be contained in the shared genomic region. Modified BACs were analyzed by PCR and pulsed-field gel electrophoresis to confirm the integration of the Tn7B-lacZ reporter cassette. To mitigate any possible effects of unknown insulator or silencer elements within the BAC sequence, we selected clones with at least two Tn7B-lacZ integration events. Each BAC was then injected into fertilized mouse oocytes to generate transgenic mice in accordance with IACUC regulatory standards. For each BAC, a minimum of two independent transgenic founders were obtained and studied; this is necessary to overcome potential position-dependent expression effects resulting from random integration of the transgene (BAC).

We assayed lacZ expression at multiple points in prostate organogenesis and maturation; postnatal days 0 and 8 (P0 and P8) during prostate development, and P21, when prostate maturation is virtually complete (Sugimura et al. 1986). At each developmental stage, prostates were dissected and stained for beta-galactosidase expression using X-gal (Fig. 1B,C; Kothary et al. 1989).

These in vivo BAC transgenic reporter assays identified prostate enhancer activity contained within the 8q24 gene desert (Fig. 1C). While we did not observe beta-galactosidase prostate expression in BAC CTD-2533C10 transgenic mice (12 independent transgenics), animals harboring BACs CTD-2533C10 and RP11-124F15 displayed beta-galactosidase prostate expression at days P0 (data not shown), P8 (Fig. 1C), and P21 (data not shown). As illustrated in Figure 1C, the beta-galactosidase expression domain of both BAC RP11-124F15 and BAC CTD-2533C10 extends to other components of the urogenital system, including the coagulating glands, urethra, and the lining of the urinary bladder. While the seminal vesicles and ductus deferens also exhibit X-gal staining, we and others observed this expression pattern in both wild-type (Fig. 1B) and transgenic animals, reflecting the presence of endogenous beta-galactosidase in these structures (Wang et al. 2002; Krajnc-Franken et al. 2004). As 80% of the prostatic ducts are formed by day P15 in mice (Sugimura et al. 1986), our data indicate that the enhancer(s) contained within these two BACs are active both during and after prostate organogenesis and maturation.

Because some of the prostate cancer-associated regions also have been associated with breast and colorectal cancer (Fig. 1A), we chose to additionally assay the mammary glands, colon, and rectum of those animals transgenic for BACs containing the relevant regions (BAC RP11-124F15 for breast cancer, and both BACs RP11-124F15 and CTD-2533C10 for colorectal cancer). Mammary glands were examined at embryonic day 14.5 (E14.5), when the mammary buds have fully formed in female embryos, in 11- wk-old virgin females with mature branched glands, and in prelactating females 14 d after conception, when the mammary gland undergoes extensive hyperplasia and tissue remodeling (Hens and Wysolmerski 2005; Oakes et al. 2006; Ster Nicholson 2006).

We observed in vivo mammary gland enhancer activity in mice transgenic for BAC RP11-124F15 (Fig. 1E), which harbors associated intervals for not only prostate but also breast and colorectal cancer. Transgenic animals displayed beta-galactosidase expression in the epithelial compartment—ducts and alveoli (Hennighausen and Robinson 2005)—of the mammary glands of midgestational pregnant and 11-wk-old virgin females (Fig. 1E; data not shown). No enhancer activity was seen in E14.5 embryos. Of note, Jia et al. (2009) recently identified a noncoding element within this region capable of in vitro enhancer activity in breast cancer cell lines; this element should be viewed as a strong candidate for the mammary gland activity we see in vivo.

Characterizing the prostate enhancer

We next aimed to refine the location of the prostate enhancer(s) within the BACs driving prostate expression. Because of the highly similar reporter expression patterns obtained from BACs RP11-124F15 and CTD-2533C10, including prostate, coagulating gland, and urethral/bladder lining, we hypothesized that our BAC transgenic assays were identifying a single prostate enhancer within the 59-kb shared genomic segment of these two BACs. Interestingly, one of the most strongly associated prostate cancer risk SNPs, rs6983267, is contained within this 59-kb overlapping interval and disrupts an evolutionarily conserved sequence (Fig. 1A).

To directly test the rs6983267-containing evolutionarily conserved element for regulatory potential in vivo, we cloned a 5-kb DNA fragment containing each allele of this SNP in a lacZ reporter cassette using Invitrogen’s Gateway cloning system (Kothary et al. 1989). Transgenic mice harboring either the risk or the non-risk variant of rs6983267 were generated and analyzed. We determined that the conserved sequence containing the prostate cancer GWAS SNP displayed allele-specific in vivo prostate enhancer properties (Fig. 2). Specifically, the risk allele, rs6983267-G, led to consistent, stronger beta-galactosidase expression in prostates and coagulating glands than the non-risk allele, rs6983267-T, in P0, P8, and P21 transgenic mice (Figs. 2A,B, 3B,C). The expression pattern driven by the rs6983267-G risk allele in three independent mouse transgenic lines closely resembled that observed in BACs RP11-124F15 and CTD-2533C10—both of which also harbor the risk allele. In contrast, the rs6983267-T non-risk allele led to weakened prostate and coagulating gland expression in three independent transgenic lines (Fig. 2B). For each allelic variant evaluated, those transgenic founders exhibiting enhancer activity showed highly concordant beta-galactosidase expression in the prostate, with a clear qualitative difference between the risk and non-risk variants.

To test whether this spatial reporter expression pattern of the rs6983267-containing enhancer correlates with endogenous MYC expression in prostate and other components of the urogenital larynx.
system, we performed whole mount in situ hybridizations using a full-length Myc probe in mouse prostates at P8 (Wilkinson and Nieto 1993). We observed Myc expression in the male genitourinary apparatus, including the prostate, in a pattern closely mimicking the reporter expression of the rs6983267-G enhancer and BACs CTD-2533C10 and RP11-124F15, both of which harbor the G risk allele as well (Fig. 2C).

This same prostate enhancer that we have characterized also has been shown to act as an allelic-specific long-range MYC enhancer in colorectal cancer cells (Jia et al. 2009; Pomerantz et al. 2009a; Tuupanen et al. 2009; Wright et al. 2010). Although we did not observe colorectal enhancer activity in our initial BAC screen of the region, we again assayed transgenic animals harboring either the risk or non-risk rs6983267-containing enhancer element for in vivo enhancer activity in the colorectal area at three developmental time points. We observed no beta-galactosidase expression in E14.5 intestines for either construct tested, and colorectal X-gal staining at P8 and P21 was indistinguishable between wild-type mice and transgenic animals harboring either enhancer variant (Supplemental material). Strong endogenous beta-galactosidase expression is observed in intestines of both wild-type and transgenic animals starting at E15.5, limiting our ability to identify in vivo colorectal enhancers in late embryogenesis and postnatally. These findings highlight the difficulty in assaying postnatal in vivo intestinal enhancers using lacZ reporter assays.

Investigations into the embryonic activity of the rs6983267-containing element demonstrated that while this enhancer has several spatial domains of expression, its allele-specific activity is restricted to the prostate and coagulating glands. Both the rs6983267-G and rs6983267-T enhancer elements drove expression in several spatial domains of E11.5 and E14.5 embryos, with no apparent allelic-specific enhancer activity (Fig. 3A). Transgenics harboring either haplotype variant showed similar X-gal staining in the limbs and tail at E11.5, consistent with previously reported patterns (data not shown; Tuupanen et al. 2009). We also observed enhancer activity in the developing urinary bladder, genital tubercle, and limbs in the E14.5 embryos. This pattern, which precedes prostate development, is also indistinguishable between the allelic variants of this enhancer (Fig. 3A).

Taken together, our data posit that the rs6983267-containing enhancer is part of MYC’s regulatory landscape, and that the variant within this enhancer may increase the risk of prostate cancer through its role in allelic-specific control of MYC expression in the prostate.

**Discussion**

The BAC enhancer-trapping strategy that we employed allowed us to rapidly interrogate the 440 kb of 8q24 prostate cancer-associated noncoding DNA for *cis*-regulatory elements. We effectively screened a half-megabase genomic interval in vivo using only three constructs, identifying the existence of mammary gland and prostate enhancers in the interval associated with each respective allele.
cancer type. We believe that this methodology provides a significant advance to current genomic techniques for following up on GWAS results in noncoding regions, as it can be easily adapted to examine loci in vivo on a megabase scale. As demonstrated by our results, this strategy can be used to concurrently identify spatially and temporally unique enhancers within a large sequence, and can be useful in refining the critical regions for enhancer mapping, while still permitting the use of a whole-systems, in vivo animal model.

These relatively straightforward BAC transgenic reporter assays also provide a way to more closely approximate the genomic context of relevant enhancers. By testing ~200 kb of sequence simultaneously, enhancers are assayed in a context much closer to their true genomic environment, one where they are subjected to (largely unknown) modifications by neighboring repressors, insulators, chromatin changes, and/or various other interactions with nearby cis sequences. In traditional plasmid-based reporter assays, this important genomic context is lost. We conducted our clone selection strategy so as to minimize the potential negative effects of such insulators or repressors; tagged BACs containing at least two copies of the Tn7b-lacZ reporter cassette—integrated near each end of the BAC sequence—were selected for experimental use. We hypothesized that this would diminish false-negative results caused by repressive elements in a single-copy integration clone. When compared with BACs tagged with just a single Tn7b-lacZ cassette, we observed more reproducible results in mice transgenic for BACs harboring two Tn7b-lacZ integrations (M.A.N, unpubl.).

Because we observed the same urogenital system spatial pattern of expression in both of the overlapping BACs tested, we deduced that the enhancer was within the small interval shared between those BACs. However, it is possible that other prostate enhancers also exist within the BACs we tested. To formally exclude this possibility, other approaches could have been used, including the analysis of additional enhancer-trapping BACs with complementary overlapping patterns. Alternatively, BAC recombinant could have been employed to specifically delete our known enhancer from the BACs assayed. Both approaches are logical follow-ups to the in vivo BAC transgenic reporter assays, and would maintain the analytical strengths of assaying enhancers in their genomic environments.

Recent studies have reported on the colorectal and prostate enhancer activities of the rs6983267-containing sequence we describe here (Jia et al. 2009; Pomerantz et al. 2009a; Tuupanen et al. 2009; Sotelo et al. 2010; Wright et al. 2010). Using a combination of genome-wide in vitro assays, this sequence has been highlighted as possessing attributes of an enhancer, including specific chromatin modifications and binding of transcription factors. Several groups have demonstrated that in colorectal cancer cell lines, TCF712 (TCF4) binds preferentially to the risk allele (rs6983267-G) of this enhancer (Pomerantz et al. 2009a; Tuupanen et al. 2009; Wright et al. 2010). Reports regarding the enhancer properties of this sequence in prostate cancer cell lines have been mixed, however. When tested in LNCaP and PC3 prostate cancer cell lines, this sequence displayed enhancer properties only in the former, possibly due to the PC-3 line’s lack of androgen receptor expression (Jia et al. 2009). In a second study, this rs6983267-containing enhancer was unable to drive luciferase expression above promoter-only levels in LNCaP or PC-3 cells, unless cells were cotransfected with Tc4 and beta-catenin expression vectors (Sotelo et al. 2010). Under those conditions, the rs6983267-containing element demonstrated allelic-specific enhancer activity in LNCaP cells, but with the non-risk rs6983267-T variant driving stronger expression than the risk rs6983267-G allele.

Our in vivo results—showing the cancer risk allele demonstrating stronger enhancer potential than the non-risk allele—corroborate those reported in colorectal cancer cell lines (Pomerantz et al. 2009a; Tuupanen et al. 2009; Wright et al. 2010), and are concordant with MYC’s known role as a proto-oncogene. Our whole-animal experimental strategy obviated the experimental variation added by cell lines to clearly show that this element is a functional prostate enhancer in vivo, while also adding the ability to investigate enhancer activity throughout organogenesis. We believe that this broad spatial and temporal characterization of regulatory potential is ideally afforded by in vivo experimentation, and propose this as the standard in the follow-up to GWAS risk variants implicated in human disease.

The rs6983267-containing element physically interacts with MYC’s promoter in both colorectal cancer and prostate cancer cell lines, providing evidence that this enhancer is involved in regulating MYC expression in these two tissue types (Pomerantz et al. 2009a; Sotelo et al. 2010; Wright et al. 2010). Despite these compelling findings and the fact that altered MYC expression has been implicated repeatedly in the pathogenesis of prostate cancers (Williams et al. 2005), no association has been seen between rs6983267 genotype and MYC mRNA levels in normal prostate cells or prostate tumors (Pomerantz et al. 2009b). This lack of genotype–phenotype correlation implies that steady-state MYC mRNA levels in adult prostate tissue may not be the correct biological entity underlying risk. Our findings demonstrate that the rs6983267-containing enhancer exhibits differential in vivo activity throughout prostate organogenesis, and raise the possibility that this variant asserts its influence on prostate cancer risk long before tumorigenesis occurs. With widely varying risk allele frequencies in different populations—from 49% in American Caucasians to 81% in African Americans (HapMap, merged Phase 1, 2, and 3 frequencies)—this SNP may also have an effect on the population prevalence of both prostate cancer and colorectal cancer (Jemal et al. 2009).

We have described how a noncoding SNP strongly associated with disease can in fact alter the in vivo activity of its encompassing cis-regulatory element, suggesting a possible impact on cancer risk before tumorigenesis actually occurs. Although further studies are warranted, our in vivo temporal data hint at an underlying molecular explanation for this nongenic SNP’s contribution to prostate cancer risk. These findings emphasize the notion that thorough investigations into the regulatory impact of polymorphisms are an indispensable component to the functional follow-up of GWAS scans, and stress the importance of conducting these experiments using in vivo systems.

Methods

Transposon-mediated BAC modification

BACs CTD-2506D10, RP11-124F15, and CTD-2533C10 were modified by in vitro random transposition of Tn7b-lacZ (Spitz et al. 2003). BAC DNA was extracted by using the Nucleobond AX Kit (Macherey-Nagel). Twenty nanograms of Tn7b-lacZ vector was mixed with 20–40 ng of BAC DNA, G3P buffer, and TnABC transposase (New England Biolabs), followed by incubation for 10 min at 37°C. Start solution was added and the reaction was extended for 1 h. After heat inactivation for 10 min at 75°C and a 1-h dialysis, electrocompetent DH10B cells were transformed with 2 µL of the transposition reaction. Cells were plated on LB agar containing...
20 μg/mL kanamycin and 20 μg/mL chloramphenicol. Positive colonies were first identified by polymerase chain reaction (PCR) using beta-globin and lacZ primers (Tn7-taglacZ beta-globin F: AGCA TCTATTGCTTACATTTGC; Tn7-taglacZ R: ATAGGTTACGTTGG TGTAAGTGG TGTA). Modified BAC colonies were then digested with NotI and separated by pulsed-field gel electrophoresis overnight on a 1% agarose gel to determine the number of copies and the position(s) of the integrated Tn7-taglacZ cassette. Clones with two copies of the cassette were chosen for further analysis to minimize the possible influence of silencer or insulator elements with the BACs.

lacZ plasmid generation
The 5 kb of sequence surrounding the rs6983267-containing conserved element was PCR amplified from human genomic DNA heterozygous for the rs6983267 SNP (rs6983267 F: TCTTGACCCTG ATTGCTGAAAAAT; rs6983267 R: TCTGGGGGTAGTTAAATGA TAA). The fragment was then purified using the QIAquick PCR Purification Kit (Qiagen) and cloned into the pDONR 221 Gateway entry vector (Invitrogen). Colonies were selected by restriction enzyme analysis for successful fragment insertion, and positive clones were sequenced to determine the allelic status of SNP rs6983267 (rs6983267-seq F: TAGACACCAAGGGAGGTATCA; rs6983267-seq R: CCAGGTAAAGGAACACTG). Clones containing sequence harboring both the risk (G) and non-risk (T) rs6983267 allele were transferred to a Gateway-HSP68-lacZ reporter vector using the LR recombination reaction (Invitrogen) (Poulin et al. 2005). All plasmids were again verified by restriction analysis and direct sequencing prior to pronuclear mouse injections.

Production of transgenic mice
Tn7-taglacZ tagged BAC DNA was purified using the Nucleobond BAC 100 Kit (Macherey-Nagel), rehydrated in injection buffer (10 mM Tris at pH 7.5; 0.1 mM EDTA), and diluted to a concentration of 2 ng/μL. BAC DNA was injected in its circular form.

Plasmid DNA was purified using the Plasmid Maxi Kit (Qiagen), and 50 μg of each plasmid was digested with Sall to excise the vector backbone. Following a gel purification step using the QIAquick Gel Extraction Kit (Qiagen), the DNA to be injected was further purified using a standard ethanol precipitation. The purified DNA was dialyzed for 24 h against injection buffer (10 mM Tris at pH 7.5; 0.1 mM EDTA), and its concentration was determined fluorometrically and by agarose gel electrophoresis. The DNA was diluted to a concentration of 2 ng/μL. Purified BAC and plasmid DNA were then used for pronuclear injections of CD1 mouse embryos in accordance with standard protocols approved by the University of Chicago.

For the Tn7-taglacZ tagged BACs, multiple stable transgenic lines were generated for each construct, and F1 animals were analyzed for each line at multiple postnatal developmental time points. BAC CTD-2506D10 DNA injections yielded 12 independent lines (0/12 positive for prostate beta-galactosidase expression); injections of RP11-124F15 and CTD-2533C10 both resulted in two independent beta-galactosidase-expressing lines.

For the rs6983267-containing enhancer plasmid, a total of two beta-galactosidase-expressing independent transgenics was obtained for rs6983267-G; three beta-galactosidase-expressing independent transgenic animals/lines were also obtained for rs6983267-T. For several of these independent lines, the F1 animals themselves were analyzed at P8; this excluded any analysis of the line at other time points. For the risk allele, rs6983267-G, we obtained two F0 transgenic animals in the prostate. The third independent rs6983267-G transgenic was maintained as a stable line. For the non-risk allele, rs6983267-T, one F0 transgenic animal was obtained; the remaining two independent transgenics were maintained as stable lines.

Mouse in vivo transgenic reporter assay
Prostates and mammary glands were harvested from mice at P0, P8, and P21 and dissected into cold 10 mM phosphate buffer (PBS) (pH 7.3), followed by 30–45 min of incubation with 4% paraformaldehyde at 4°C. E14.5 embryos were incubated in 4% paraformaldehyde for 2 h. Tissues were then washed twice for 20 min with wash buffer (2 mM MgCl2; 0.01% deoxycholate; 0.02% NP-40; 100 mM phosphate buffer at pH 7.3), and stained for 18 h at room temperature with freshly made staining solution (0.8 mg/mL X-gal; 4 mM potassium ferrocyanide; 4 mM potassium ferricyanide; 20 mM Tris at pH 7.5 in wash buffer). After staining, samples were rinsed five times for 20 min in PBS and post-fixed in 4% paraformaldehyde. For each animal analyzed, tail samples were taken at the time of dissection and DNA was isolated through the addition of lysis buffer (100 mM Tris-HCl at pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 1 mg/mL proteinase K) and incubation overnight at 55°C. Genotyping was performed by PCR with primers within the reporter cassette/vector (using beta-globin and lacZ primers for the Tn7-taglacZ tagged BACs, rs6983267-seq primers for the plasmids).

Imaging
All photographs were taken using a Leica MZ16 F stereomicroscope and QCapture Pro software. Settings (lighting, exposure time) were kept constant between structure- and aged-matched samples. Images displayed in the paper were generated using an image processing software package (CombineZM) that allows for the creation of extended depth of field images. Multiple pictures of each structure were taken at varying depth of fields and then computationally integrated; the focus areas are blended to create a composite high-resolution image with an extended depth of field. This allowed for the production of images where all the multiple plains of the urogenital apparatus appear well focused and defined.

In situ hybridization
In situ hybridization analysis on whole P8 prostates using digoxigenin-labeled Myc antisense and sense riboprobes was performed according to standard protocols (Wilkinson and Nieto 1993). The probes were generated from a full-length mouse Myc cDNA clone (IMAGE ID 3962047). Staining was performed for 48 h, and the stained prostates were then transferred to 10% buffered formalin phosphate prior to imaging.

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