Bispecific Oligonucleotides May Induce Interferon Expression in LNCaP Cells Enhancing Surface Antigen Expression: Effect of Intrastrand Base Pair Complementarity

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Abstract. *Antisense oligonucleotides (oligos) have been employed against in vivo and in vitro prostate cancer models. Most oligos consist of a single mRNA binding site, targeting a single gene product or others sharing sequence homology. However, our lab has developed bispecifics directed towards two (including unrelated) proteins. Previously we have shown that mono- and bispecific oligos targeting BCL-2 significantly inhibit LNCaP cell growth. Employing reverse transcriptase-polymerase chain reaction we found comparable suppression of expressed BCL-2. Computer models suggested that this activity could, in part, be enhanced by the formation of siRNA-like double-stranded regions, generated by intrastrand base pair complementarity. We hypothesize that these regions could be interferon inducers (like poly I:C) and enhance the expression of prostate specific cell surface antigens. The expression of cell surface prostate-specific membrane antigen (PSMA) and the secreted prostate-specific antigen (PSA) were candidates for evaluation. To test this theory, we evaluated the effects of mono- and bispecific oligos (with intrastrand complementarity), targeting BCL-2, upon the expression of non-targeted proteins PSMA, PSA and interferon-gamma (IFN-γ) in LNCaP cells. Levels of mRNA encoding PSMA were significaIntly elevated following treatment with the bispecific oligos (directed against both BCL-2 and the epidermal growth factor receptor) but not by the monospecific directed solely against BCL-2.*

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Furthermore, no differences were detected in mRNA levels encoding PSA following treatment with either mono- or bispecific forms. IFN-γ expression was also significantly increased by the bispecific and not by the monospecific oligos, supporting the hypothesis of interferon induction. This suggests that prostate cells (including LNCaP) retain an endogenous interferon-based antiviral defense mechanism (similar to that found in the testes) which is induced by double stranded oligos. Enhanced expression of cell surface differentiation antigens (such as PSMA) could increase targeting by cytotoxic T-cells and potentiate prostate cancer vaccines directed against tumor-associated cell surface antigens.

In 2010, the American Cancer Society (ACS) estimates that in spite of early detection, screening for prostate-specific antigen (PSA) and effective treatments for localized disease, in the United States there will be 32,050 deaths from prostate cancer with 217,230 newly diagnosed cases (1). For prostate cancer, there are two suggested etiologies: i) endogenous hormonal factors; and ii) oncogenic viruses.

The role of hormones in prostate growth and cancer control is well established. However, the involvement of oncogenic viruses, as either a primary cause or as a risk factor, has also been suggested. The ACS also believes that in the United States, infectious agents (viruses, bacteria and parasites) account for up to 10% of cancer causation (and up to 20% in developing countries) (2). The presence of viruses in the male urogenital tract is well documented and for the testes there is evidence of an endogenous (interferon-based) antiviral system (2). Double-stranded nucleic acids, as well as synthetic poly I:C, have already been shown to induce interferon when bound to toll-like receptor 3 (TLR3) (3). The induction of interferon by double- stranded nucleic acids (similar to viral replicative forms) in prostate cancer cells would suggest a similar defense.

Previously, we reported bispecific oligos directed against BCL-2 and the epidermal growth factor receptor (EGFR) significantly inhibited the growth of LNCaP cells *in vitro* and similarly inhibited BCL-2 expression (4). In these experiments (4), employing reverse transcriptase-polymerase chain reaction (RT-PCR), we evaluated the effect of these oligos on the expression of two prostate-specific differentiation proteins, prostate specific membrane antigen (PSMA), PSA and an antiviral protein, interferon-gamma (IFN-γ). None of these proteins are directly targeted by the mRNA primers employed in RT-PCR, however, PSMA and PSA are surrogates for tumor progression, regression (in response to therapy) and recurrence (biochemical relapse). PSMA is a transmembrane-bound receptor with folate hydrolase activity. In contrast, PSA is a secreted kallikrien-related peptidase marker associated with prostate cancer recurrence and progression. It is often employed as a screening tool and has been evaluated as a target for directed therapy. PSMA, PSA and prostatic acid phosphatase (PAP) are targets for an activated immune system and are included in prostate cancer vaccines, such as ALPHAVAX ARV developed by the consortium of Cytogen and Progenics Pharmaceuticals employing PSMA, Prostavac-VF being developed by BN ImmunoTherapeutics employing PSA, and Provenge developed by Dendrion employing PAP. Since growth inhibition has been documented by these bispecifics, it was of interest to determine whether antigen expression is correlated to the percentage of growth inhibition or directly proportional to the number of cells present and therefore the amount of RNA extracted. An increase in antigen encoding mRNA could involve cytokine (IFN-γ) induction as a result of the unusual secondary structure of the bispecifics. Enhanced expression of HLA (5) and tumor necrosis factor receptors (6) following IFN-γ exposure has been reported and is thought to promote immune-mediated cytotoxicity. Greater expression of PSMA or PSA would presumably aid cytotoxic (CD8^{+}) T-cell targeting and increase antibody levels (anti-PSA) as reported by Lubaroff *et al.* (7).

Bispecific oligos, including those with intrastrand complementarity, are being evaluated because most types of cancer excessively express numerous proteins. Therefore, it is likely that for gene therapy to be effective at least several would need to be targeted. Bispecifics have been evaluated against genes involved in both a single autocrine loop (8), as well as those found in different growth regulatory paths (4, 9-12). While most experiments have evaluated the inhibition of *in vitro* cell growth or targeted gene expression, here we evaluate the expression of non-targeted genes. These non-specific effects (including those affecting PSMA expression) are important, and in complex pathways can lead to undesired compensatory responses. In a recently completed study (in press), we found that these same bispecifics, while directly targeting the apoptosis inhibitor BCL-2, had inadvertently inhibited the expression of caspase-3 (an apoptosis promoter), potentially allowing tumor variants to again be selected for apoptosis resistance (13). If gene therapy is to be successful, a full understanding of the unintended consequences of oligo therapy must be attained.

Materials and Methods

Oligonucleotides. Oligos (mono- and bispecific) were purchased from Eurofins MWG Operon (Huntsville, AL, USA). Each was phosphorothioated on three terminal bases at the 5' and 3' positions. Stock solutions were made to a final concentration of 625 µM in sterile Dulbecco phosphate-buffered saline (PBS).

Base sequences. Each oligo contained at least one CAT sequence and targeted the area adjacent to the mRNA AUG initiation codon for the respective targeted protein (EGFR or BCL-2).

MR$_4$ (monospecific targeting bcl-2) TCTCCCAGCGTGCGCCAT; MR$_{24}$ (bispecific targeting EGFR/BCL-2) GAGGGTCGCATCGC TGCTCTCTCCCAGCGTGCGCCAT; MR$_{42}$ (bispecific targeting BCL-2/EGFR) TCTCCCAGCGTGCGCCATGAGGGTCGCATCG CTGCTC.

Cell culture experiments.
Cell culture. LNCaP cells (Figure 1: Experiments 1 and 2) were grown in RPMI-1640 supplemented with 10% bovine serum, 1% L-glutamine and 1% penicillin/streptomycin in a 5% CO_2 incubator. Log- phase cells were harvested using EDTA/trypsin and equally distributed into 75 cm^2 flasks (Corning, NY, USA). At intervals, media were either supplemented or replaced with fresh.

Determination of growth. Four days prior to the addition of oligos (Figure 1: Experiments 1 and 2) 1×10^4 LNCaP cells were added, in a total 200 µl volume of media, to each depression of a 96-well plate and incubated at 37°C in a 5% CO_2 incubator. On the day of transfection, the following solutions were prepared: A) 1 µl of buffer containing either oligo or a diluent was added to 50 µl of OPTI-MEM and gently mixed. One dilution was made for each well; B) 1 µl of Lipofectin was diluted in 50 µl of OPTI-MEM and mixed gently for 5 min at room temperature; C) oligo dilutions were mixed with 50 µl of Lipofectin and gently mixed for 20 min at room temperature; D) 100 µl of the Lipofectin and oligo mixture were added to 100 µl of RPMI medium and mixed.

Cells were incubated for 24-48 h before solutions were aspirated and re-incubated for an additional 48 h in 200 µl of media. Cell counts were determined following the addition of WST-1 reagent to each well, and after 2 h the color intensity was measured by a microplate reader at a wavelength of 450 nm, using a reference of 650 nm. Values obtained were determined after the subtraction of paired blank samples from the experimental wells and were multiplied by a constant to give whole integers for analysis. Microsoft Excel software was utilized to calculate means and standard deviations and Student's *t*-tests were used to determine significance.

RT-PCR experiments.
Oligo treatment prior to PCR. Fours days prior to oligo addition, when cell density approached 75% confluence, 10 ml of fresh media were added. Cells were incubated for an additional 3 days before 5 ml of media were replaced with fresh the day before oligos were added; 100 µl of stock oligos were added to bring the final concentration to 6.25 µM. Incubation proceeded for an

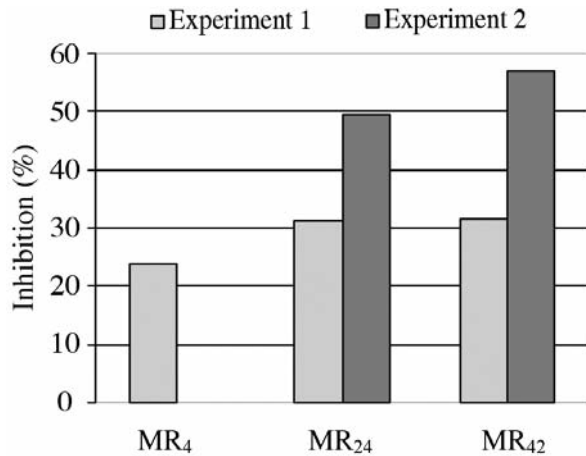Figure 1. *Inhibition of in vitro growth of LNCaP cells by mono- and bispecific oligos.*

Actin
353 base pair product

Figure 2. *Expression of actin in agarose gel.*

additional 24 h in the presence or absence of monospecific MR$_4$, or the MR$_{24}$ and MR$_{42}$ bispecifics.

RNA extraction. Following treatment, media were removed, 1 ml of cold (4˚C) RNAzol B was added to each 75 cm^2 culture flask and the monolayer lysed by repeated passage through a pipette. All procedures were performed at 4˚C. The lysate was removed, placed in a centrifuge tube to which 0.2 ml of chloroform was added, and shaken. The mixture stayed on ice for 5 min, was spun at 12,000×g for 15 min, and the upper aqueous volume removed and placed in a fresh tube. An equal volume of isopropanol was added, the tube shaken, and allowed to stay at 4˚C for 15 min before similar centrifugation to pellet the RNA. The supernatant was removed, the pellet washed in 1 ml of 75% ethanol, then spun for 8 min at 7500×g. The ethanol was pipetted off and the formed pellet air dried at –20˚C.

RNA quantitation. RNA was resuspended in 250 µl of diethylpyro-carbonate (DEPC)-treated H$_2$O, and quantitated using a Qubit flurometer and Quant-iT RNA assay kit (Invitrogen, Carlsbad, CA, USA). DEPC is an inhibitor of RNase activity.

RT-PCR. Extracted RNA was diluted in DEPC treated water to 40 µg/µl then 1-4 µl of this RNA was added to 1 µl of both sense and antisense primers (forward and reverse sequences) for PSMA, PSA, IFN-γ or human actin (used as a control). From a kit purchased from Invitrogen the following reactants were added for RT-PCR: 25 µl of 2×reaction mixture, 2 µl SuperScript III RT/platinum *Taq* mix, tracking dye, and 3 µl MgSO$_4$ (of a 5 mM stock concentration). DEPC-treated water was added to yield a final volume of 50 µl. As a control for RT-PCR products, human actin expression was tested in RNA extracted from HeLa cells which was provided in a kit purchased from Invitrogen. RT-PCR was performed for 2×25 cycles using the F54 program in a Sprint PCR Thermocycler.

Primers. Actin: Antisense primer sequence: 5' CAA ACA TGA TCT GGG TCA TCT TCT C 3', sense primer sequence: 5' GCT CGT CGT CGA CAA CGG CTC; PCR product was 353 base pairs in length. *PSMA [human folate hydrolase 1 (FOLH1)]:* Antisense primer sequence: 5' CCT CTG CCC ACT CAG TAG AA 3', sense primer sequence: 5' GAG GAG CTT TGG AAC ACT GA 3'; PCR product was 113 base pairs in length. *PSA [human kallidrein-related*
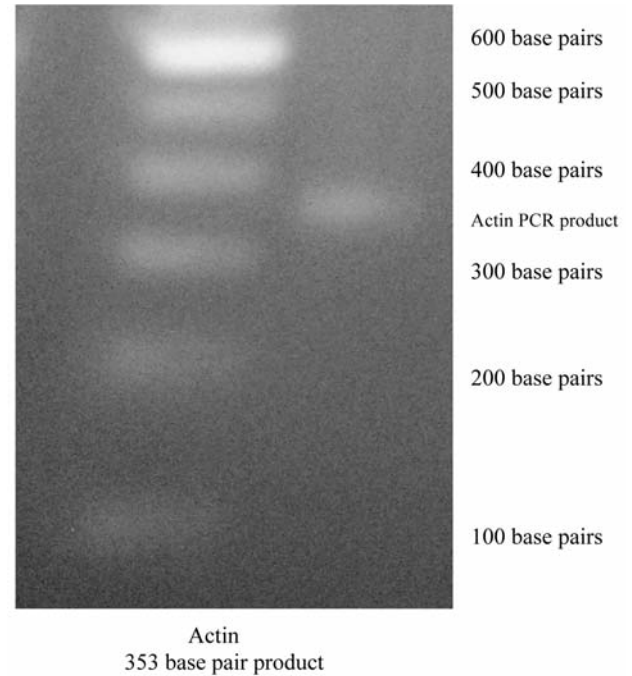
peptidase 3 (KLK3)]: Antisense primer sequence: 5' CTG AGG GTT GTC TGG AGG AC 3', sense primer sequence: 5' CCA GAC ACT CAC AGC AAG GA 3'; PCR product was 204 base pairs in length. *IFN-γ [human interferon, gamma (IFNG)]:* Antisense primer sequence: 5' AAG CAC CAG GCA TGA AAT CT 3'; sense primer sequence: 5' TCC CAT GGG TTG TGT GTT TA 3'; PCR product was 198 base pairs in length.

Detection and quantitation of product.
Agarose gel electrophoresis. 1.5% Agarose gels were prepared in a 50 ml volume of TBE buffer (1×solution: 0.089 M Tris borate and 0.002M EDTA, pH 8.3), containing 3 µl of ethidium bromide in a Fisher Biotest electrophoresis system (Pittsburg, PA, USA). Samples were run for 2 h at a constant voltage of 70 using a BioRad 1000/500 power supply source. To locate the amplified PCR product, 3 µl of a molecular marker (Invitrogen) which contained a sequence of bases in 100 base pair increments (Invitrogen), as well as 2 µl of a sucrose based bromphenol blue tracking dye were run in each gel. For actin product localization, the tracking dye was included in each sample run; for the BCL-2 product the tracking dye was run separately.

Quantitation. Gels were visualized under UV light and photographed using a Canon 800 digital camera. Photos were converted to black and white format and bands quantitated using Mipav software provided by the National Institute of Health (NIH).

Results

Cell culture experiments. LNCaP cells were incubated with MR$_4$, MR$_{24}$ and MR$_{42}$ and compared to lipofectin-containing controls (Figure 1).
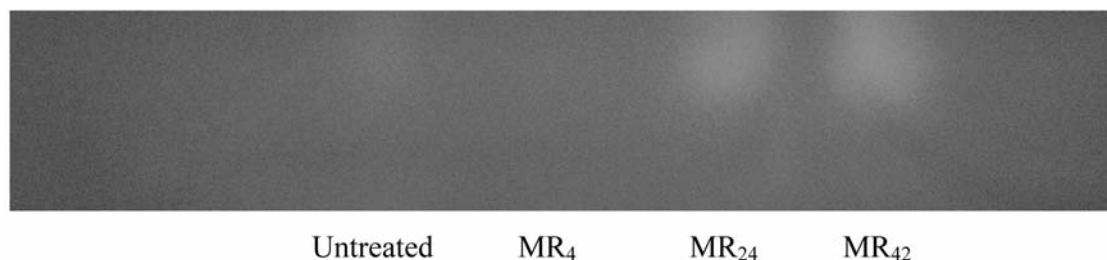
Figure 3. *PSMA expression in LNCaP cells treated with mono- and bispecific oligos directed against BCL-2 and EGFR.*
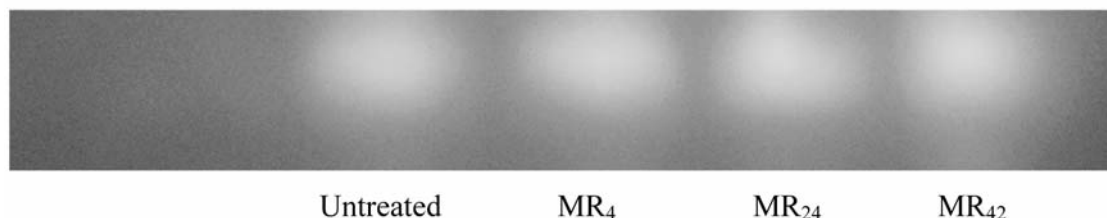

Figure 4. *PSA expression in LNCaP cells treated with mono- and bispecfic oligos directed against BCL-2 and EGFR.*

Figure 5. *IFN-γ expression in LNCaP cells treated with mono- and bispecific oligos directed against BCL-2 and EGFR.*

In Experiment 1, each oligo significantly inhibited the growth of LNCaP cells: MR_4 by 23.8% ($p=0.0004$); MR_{24} by 31.2% ($p<0.001$); and MR_{42} by 31.7% ($p<0.001$).

In Experiment 2, LNCaP cells were similarly incubated with bispecific oligos, alone, and compared to lipofectin-containing controls. MR_{24} and MR_{42} respectively produced significant inhibitions of 49.5% ($p<0.001$) and 56.8% ($p<0.001$).

We conclude that bispecific oligos are at least as effective as the mono, specific MR_4 directed only towards BCL-2 in the inhibition of *in vitro* cell growth.

RT-PCR experiments. Comparable amounts of RNA from LNCaP cells treated with mono- or bispecific oligos directed against BCL-2 (and EGFR in bispecifics) was evaluated by RT-PCR using primers for actin, PSMA, PSA and IFN-γ. Products were run on agarose gels and digitally photographed. The identified product bands were cropped and scanned with Mipav software. Figure 2 provides an example of one such band (the actin control) suitable for scanning and quantitation. The molecular weight markers shown in the left column are (in 100 base pair increments) 600 and 100 base pairs (from top to bottom). The band visualized between molecular markers of 300 and 400 base pairs is the expected 353 base pair human actin PCR product. Representative bands for PSMA, PSA and IFN-γ are presented in Figures 3, 4 and 5 respectively.

PSMA: When the background intensity was subtracted, the amount of PSMA product (from control, MR_4, MR_{24} and MR_{42}) was significantly enhanced by the bispecific MR_{24} and MR_{42}. The intensity of the bands and significance from the control were respectively 19.3±10.5, 20.9±4.7 (NS), 33.7±11.4 ($p=0.03$) and 33.0±10.8 ($p=0.02$). These results were pooled from both duplicate PCR runs and gels. In contrast, no significant difference was seen in cells treated with the MR_4 monospecific oligo. A representative band can be seen in Figure 3.

When results are normalized for extracted RNA, in spite of significant growth inhibition produced by mono- and bispecific oligos, PSMA expression is indeed enhanced in cells by the bispecifics and not affected by the lower cell numbers in the treated groups.

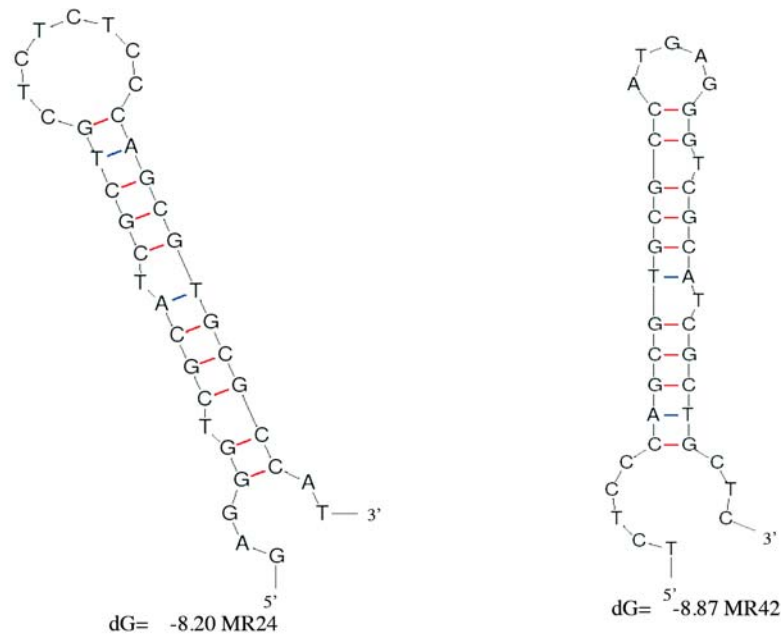*PSA:* When the background intensity was subtracted, treated cells were not significantly different from the

Figure 6. *Suggested complementary binding conformation of MR$_{24}$ and MR$_{42}$.*

untreated controls. The intensity of bands containing PSA product were 60.9±11.2, 60.4±8.1, 69.6±4.6 and 66.1±7.3 respectively for the untreated cells and those treated with monospecific MR$_4$ and bispecifics MR$_{24}$ and MR$_{42}$. A representative band can be seen in Figure 4.

Although LNCaP cell growth is inhibited by both mono and bispecific oligos, it appears that when results are normalized for extracted RNA, PSA expression remains constant in cells and is similarly not reduced by the diminished number of cells present in the treated groups.

IFN-γ: When the background intensity was subtracted, cells treated with the bispecifics had significantly greater IFN-γ expression (*p*<0.05) than the untreated controls and bands were visually more intense than those seen in the mono-specific treated group. A representative band can be seen in Figure 5. The intensity of bands containing IFN-γ product, when compared to controls, were increased 3.12%±10.6 (NS), 28.5%±23.1 (*p*=0.025) and 19.2%±15.9 (*p*<0.05) respectively for those treated with mono specific MR$_4$ and bispecific MR$_{24}$ and MR$_{42}$. This supports the hypothesis that double-stranded oligos can act as an interferon inducer.

Discussion

Antisense oligos have been employed against prostate cancer models in both *in vivo* and *in vitro* systems. Genes targeted include protein growth factors, androgens, receptors for each of these stimulating factors, inhibitors of apoptosis (BCL-2 and clusterin) and oncogenes. Most reports of oligo activity

quantitate results indirectly by inhibition of *in vitro* growth, however, more direct methods can use PCR to measure suppression of protein-specific mRNA.

In this study, we evaluated the effect of oligo-mediated growth suppression on seemingly unrelated markers of tumor progression, PSMA, PSA and IFN-γ. Although LNCaP cell growth is inhibited by bispecific oligos, it appears that when results are normalized for extracted RNA, PSA expression remains constant. In contrast, the expression of PSMA, under similar conditions, is enhanced by the bispecifics (targeting EGFR and BCL-2) while remaining unchanged from controls when treated with the mono-specific oligo targeting only BCL-2.

Previously, we have suggested that cell growth inhibition by some bispecifics could be attributed to the extended region of complementary base pairing seen in Figure 6. This low energy conformation resembles siRNA in its double stranded form. No other bispecific oligos we have evaluated have this configuration. How this accounts for transcriptional inhibition is not known, but could involve nuclease or dicer-like protein hydrolysis, resulting in release of a single reactive strand.

Double-stranded nucleic acids are also known inducers of interferon, similar to poly I:C. Interferon promotes the expression of tumor cell HLA antigens (5) as well as the surface receptors for tumor necrosis factor (6). Both HLA and tumor necrosis factor receptors are targets for tumor-directed cytotoxic responses. If induced, interferon could similarly enhance PSMA expression on prostate tumor cells, enhancing

their recognition as targets for cytotoxic (CD8+) T-cells. Therefore, in addition to their potential for growth inhibition, the effect of bispecific oligos on enhanced antigenic expression could also be clinically (immunologically) significant, particularly when administered with prostate cancer vaccines which enhance presentation of PSMA or PSA.

We suggest the presence of an IFN-based antiviral defense system in the prostate similar to that found in the testes, and there is ample documentation of prostatic infection.

DNA from oncogenic human papillomavirus (HPV) has been identified in integrated or episomal forms in tissue samples of prostatic carcinoma (14). However, the majority of reports identify the presence of members of the herpes family. Cytomegalovirus (CMV) infection has been demonstrated by the identification of viral antigens (15) and specific antibodies (16), and its DNA has been found in both normal and cancerous tissue (17). CMV capable of *in vitro* transformation of primary cells has also been isolated from normal tissue (16). CMV has also been isolated from a patient with prostatitis (18). In perhaps the strongest (and relatively recent) report, Epstein-Barr virus (EBV) was identified in seven out of nineteen prostate carcinomas, with EBV immune reactivity present in 5-30% of the neoplastic nuclei. The seven positive cases represented tissue derived from all Gleason grades of cancer and three included EBV reactivity in focal areas of benign glandular hyperplasia. In one case with high-grade prostatic intraepithelial neoplasia, convincing EBV staining was found in both basal and luminal cells. Ten normal prostate samples were negative. In at least one case, positive staining of an architecturally and cytologically distorted carcinoma was found following androgen-deprivation therapy (19).

Finally, several studies identify human herpesvirus-8 in both hyper- and neoplastic prostate glands, as well as within seminal fluid (20).

Endogenous retroviruses in animal prostates have also been identified in several (not all) strains of inbred rats (21). Expression of these C-type particles was restricted to epithelial cells and appeared to be under androgen control (22); viral particles were expressed in the ventral prostates of hormonally intact rats, diminished in the same tissue following castration, and expressed again following subcutaneous testosterone administration. This is in contrast to the EBV staining cited above which appeared only after androgen deprivation (19).

As antisense oligos enter the clinical setting, it is important to choose those which not only specifically suppress the desired target, but also do not initiate compensatory responses negating the original effect. In addition, if oligos are designed with multiple specificities and through intrastrand complementarity potentiate an immune response through interferon induction, this would be an additional benefit, particularly if given with prostate cancer vaccines, based on antigen presentation.

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