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NKX3.1 is a homeoprotein with prostate-specific expression in adults. Loss of NKX3.1 correlates with prostate cancer progression. NKX3.1 protein expression is reduced to varing degrees in virtually all primary prostate cancers. The NKX3.1 gene is affected by deletion and/or promoter hypermethylation in 90% of primary prostate cancers. NKX3.1 acts as a transcription factor by binding directly to DNA. NKX3.1 also complexes and coactivates other transcription factors such as serum response factor. We have now found that NKX3.1 complexes with the DNA unwinding enzyme toposiomerase I. NKX3.1 binds to toposiomerase I in a stoichiometric relationship and enhances scissile strand DNA cleavage by toposiomerase I. NKX3.1 does not affect religation of relaxed DNA by toposiomerase I. Toposiomerase I is involved in DNA replication, transcription, and repair and may be nder significant control of NKX3.1 in prostate epithelial cells.

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Introduction

NKX3.1 is an androgen-regulated NK-class homeobox gene with expression in both adult mice and humans restricted primarily in the prostate gland (1-5). The human *NKX3.1* gene has been mapped to chromosome 8p21 (6), a locus deleted in 85% of prostate cancer (7-9). Fine-structure mapping and sequencing of the minimally deleted region of 8p21 has placed *NKX3.1* in the center of the 8p21 deletion (10). However, the contralateral allele does not undergo somatic mutation in prostate cancer, suggesting that loss of a single allele may be important in prostate carcinogenesis (6). Consistent with this notion is the finding that heterozygosity for loss of *Nkx3.1* in mice confers prostatic dedifferentiation and hyperplasia suggesting that *NKX3.1* haploinsufficiency is dominant and explaining how *NKX3.1* may have a gatekeeper role in the 85% of prostate cancer cases in which the gene is deleted (4,10,11).

NKX3.1 has a complex function that includes binding directly to DNA via the homeodomain resulting, most commonly, in transcriptional suppression. NKX3.1 also binds to other transcription factors such as serum response factor, also via the NKX3.1 homeodomain. In this case NKX3.1 enhances transcription of SRF-responsive genes.

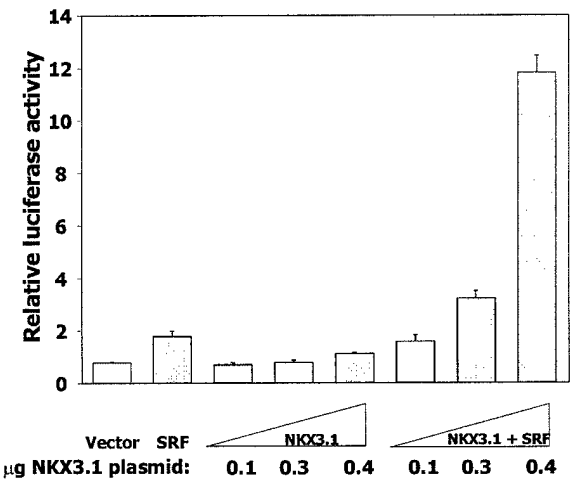
This project was aimed at two critical goals. The first was to identify a reporter that was sensitive to activation by NKX3.1. To this end we have reported and further clarify work with a fragment of the CMV early region promoter that is sensitive to NKX3.1. The second part of this project was to identify proteins that complexed with NKX3.1 and to explain the functional significance of the interaction between these proteins and both normal and polymorphic NKX3.1.

A. Construction and testing of NKX3.1 reporter genes with wild-type and mutant *NKX3.1* expression vectors. (Year 1)

1. Testing of human NKX3.1 effects on chicken SMGA reporter plasmid.

The effects of human NKX3.1 on activity of the chicken smooth muscle γ-actin (SMGA) promoter region are shown in Figure 1. Similar to findings of Carson et al, with murine Nkx3.1, we showed that human NKX3.1 can enhance the effect of serum response factor (SRF) on the serum-response element containing SMGA promoter (12). We also showed that the human SMGA promoter is equally responsive to SRF and NKX3.1 (Figure 2).

Fig 1
Effect of NKX3.1 on Chicken SMGA promoter



2. Construction of NKX3.1 and SRF expression vectors under control of HSVtk promoter.

As detailed in the year 1Progress Report these constructs could not be engineered and this element of the Statement of Work was abandoned.

3. Deletion analysis of CMV promoter

We have generated preliminary deletion fragments of the CMV early region promoter and placed them upstream from the *Renilla* luciferase reporter gene. These constructs are shown in Figure 3A. Interaction of these reporters with NKX3.1 and C-terminal truncated NKX3.1 is shown in Figure 3B. The effect of NKX3.1 was similar with all the CMV promoter constructs. However, the effect of the C-terminal truncated NKX3.1(Δ184-234) was markedly enhanced by deleting segments of the CMV promoter. It should be noted that the CMV promoter also has a TAAGTA hexanucleotide sequence that is an NKX3.1 recognition site (13). This site has been mutated without any affect on the reporter gene responses presented in Figure 3B.

Fig 2
Effect of NKX3.1 on SRF Activation of Human SMGA Promoter

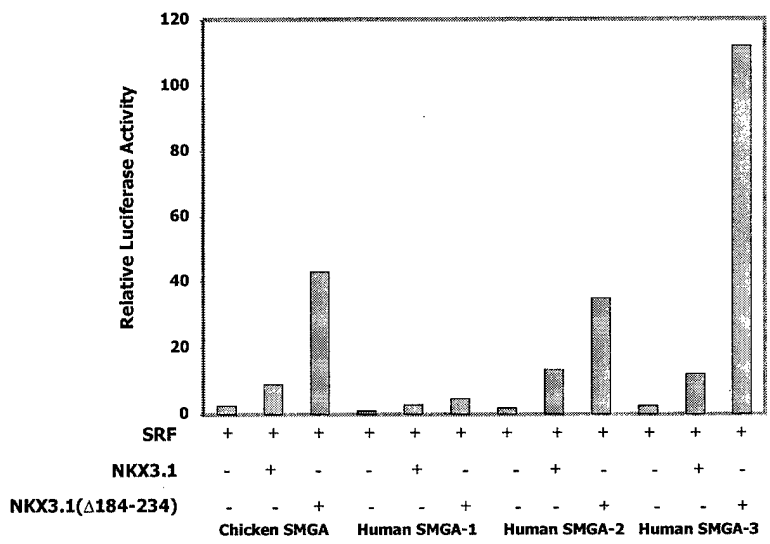


Fig 3A
Deletion Analysis of CMV Promoter

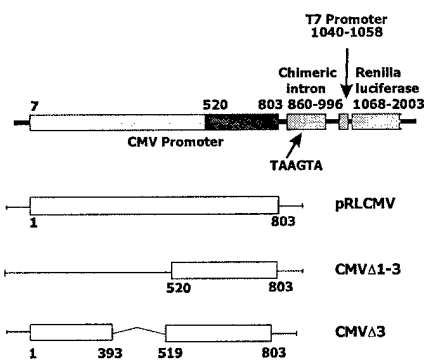
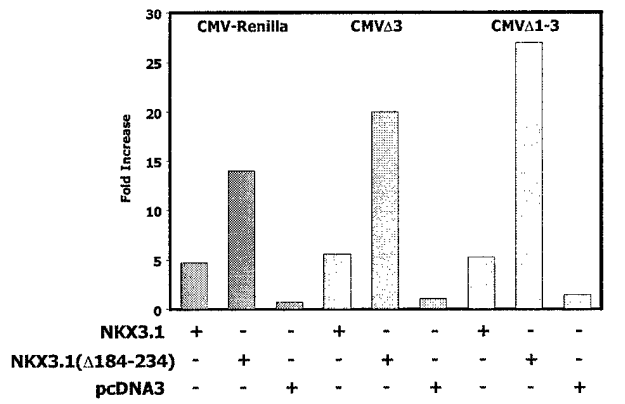


Fig 3B
Effect of NKX3.1 on CMV-Renilla Reporter Constructs



4. Analysis of NKX3.1 WT and mutant constructs driven by HSVtk on CMV and SMGA vectors

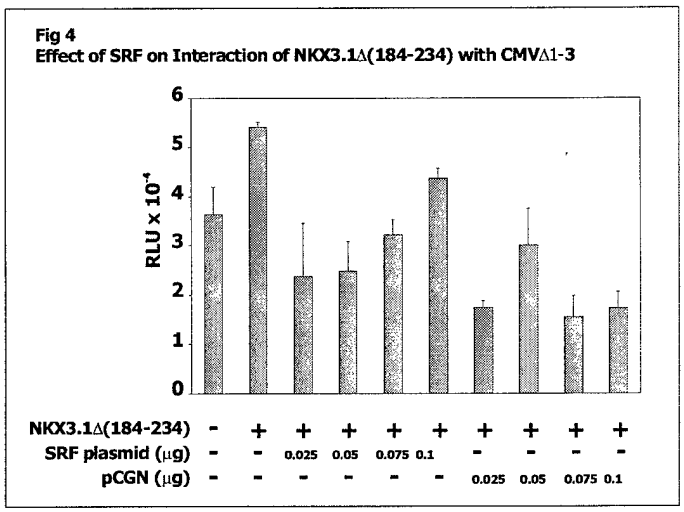
As described in A-2, these experiments cannot be completed and were deleted from the work plan.

5. Effect of SRF on NKX3.1 interactions with CMV-derived reporter plasmids.

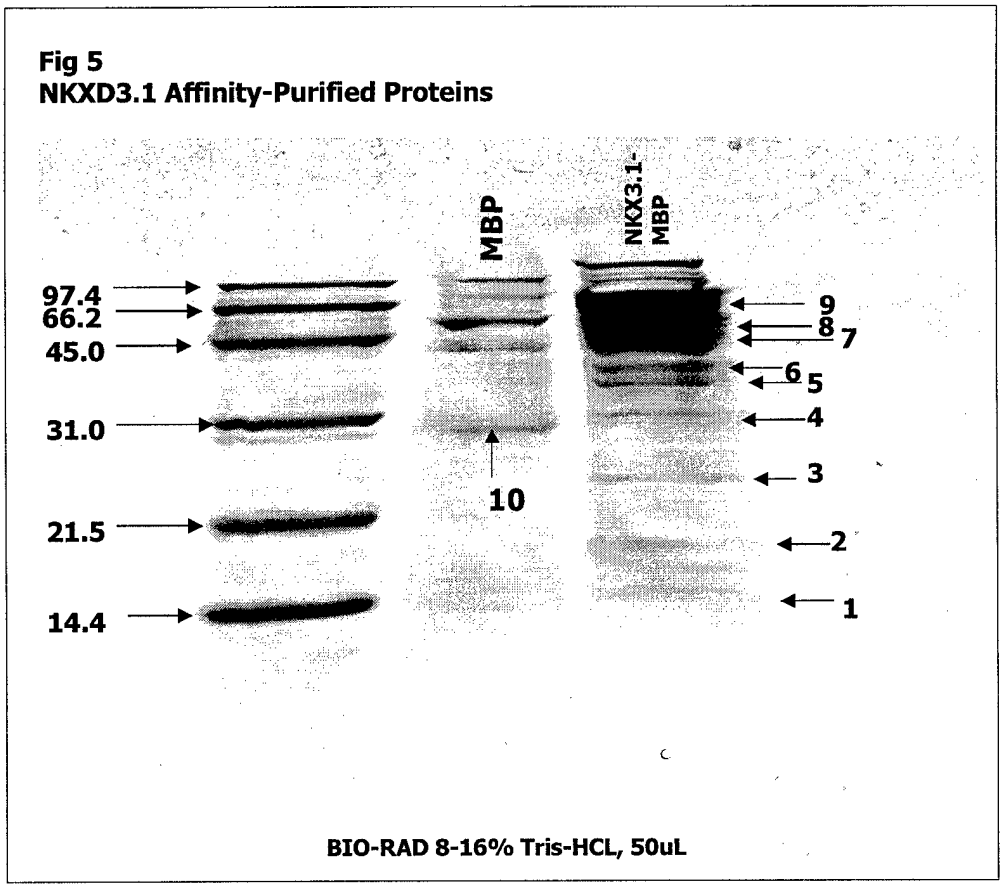
A suboptimal level of NKX3.1Δ(184-234) plasmid was used with the CMVΔ1-3 reporter in order to assess the effect, if any, of SRF on the interaction of NKX3.1 with the CMV promoter. We observed that at higher amounts of SRF plasmid there was about a 2-fold increase in NKX3.1 activation of the CMV(Δ1-3) reporter construct (Figure 4).

B. Yeast-two-hybrid cloning of NKX3.1 binding partners. (Year 1-2)

1. Isolation of Y2H clones that bind WT NKX3.1
2. Sequencing clones and confirmation by GST pull-down
3. Construction of NKX3.1 R52C DB plasmid and selection of clones that bind to NKX3.1 R52C
4. Back selection of NKX3.1 R52C-binding clones against WT NKX3.1
5. Analysis by sequencing and GST pull-down of any clones that bind NKX3.1 R52C but not WT NKX3.1



As described in the Year 1 Progress Report we had to abandon this element due to feasibility issues. The hybrid yeast two hybrid bait constructs – GAL4/NKX3.1 and VP-16/NKX3.1 were both biologically inactive presumably due to misfolding of the fusion proteins. Although we considered pursuing LEXA fusions that we used by C. Bieberich to identify PDEF as an NKX3.1 binding protein (14), but after discussions with C. Bieberich we decided not to pursue these experiments. Instead we have decided to exploit affinity chromatography to isolate proteins that complex with NKX3.1. We made an affinity column of NKX3.1/maltose-binding protein (MBP) fusion protein as an affinity reagent. PC-3 cell extract was passed through the column and through a control MBP column. Polyacrylamide gel electrophoresis of the affinity-purified peptides that adhered to the two columns is shown in Figure 5. Note that the NKX3.1/MBP fusion protein selected many more proteins than the control MBP



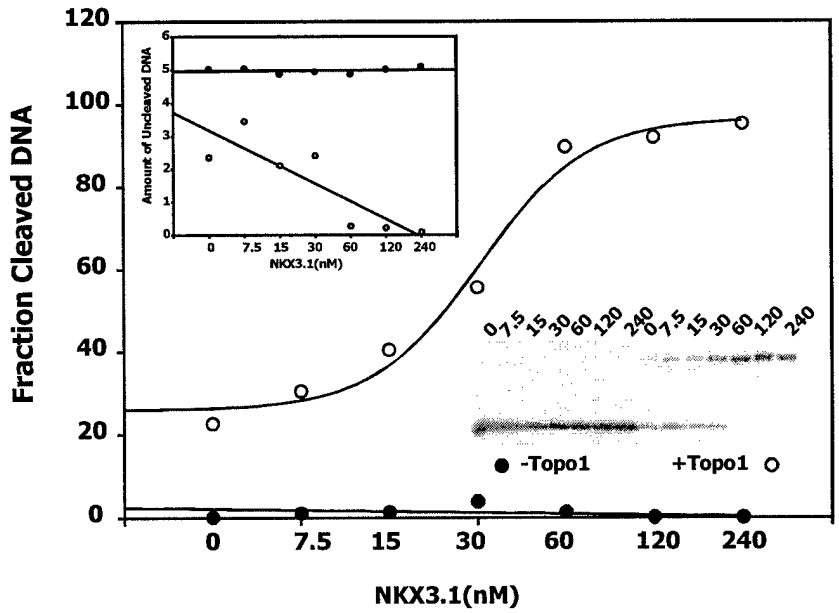
column.

The ten enumerated bands were subjected to in-gel digestion procedure with capillary column LC-microelectrospray MS analysis to generate and sequence a number of peptides. Proteins were identified by internal sequencing experiments performed by tandem mass spectrometry in the Mass Spectrometry Center in the Analytical Chemistry Lab. The LC-MS system is based on Finnigan LCQ-Deca XP ion trap mass spectrometers with Agilent 1100 microcapillary LC systems. Extracts were injected and the peptides are eluted from a 10 cm x 75 μm id Phenomenex Jupiter C18 reversed-phase capillary column with acetonitrile/0.1% formic acid gradient at a flow rate of 0.5 μL/min. The LC effluent was directly analyzed by coupling it to the mass spectrometer using a Protana nanoelectrospray ion source. The peptides were analyzed using the data-dependent multitask capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights (MS) and product ion spectra (MS/MS) to determine amino acid sequence in successive instrument scans. This mode of analysis is completely automated. The data are analyzed interpreting the CID spectra of the ions to produce the tabulated results for each digest. The interpretation process is performed by searches of the SwissProt and NCBI protein sequence databases using the search program SEQUEST.

Band #7 was thus identified as representing human toposiomerase I, a type I toposiomerase that is ubiquitous expressed in cells. Toposiomerase I (Toposiomerase I) is a member of the type IB subfamily of toposiomerases I. Toposiomerase I can relax positively or negatively supercoiled DNA by binding and cleaving a single strand via a covlaent linkage between a tyrosine and a 3' end of DNA. The binding and unwinding is cofactor-independent and does not require Mg^{++} (15,16). Toposiomerase I is assayed using a pUC12 plasmid with a 50 nucleotide insert derived from the tetrahymena ribosomal gene repeat (pHOT1) (17). Toposiomerase I nicks DNA preferntially, but not exclusively, as a combination of nucleotides that extends from positions -4 to –1 on the scissile strand that read 5'-(A/T)(G/C)(A/T)T-3'. The enzyme attaches covalently to the –1 T residue.

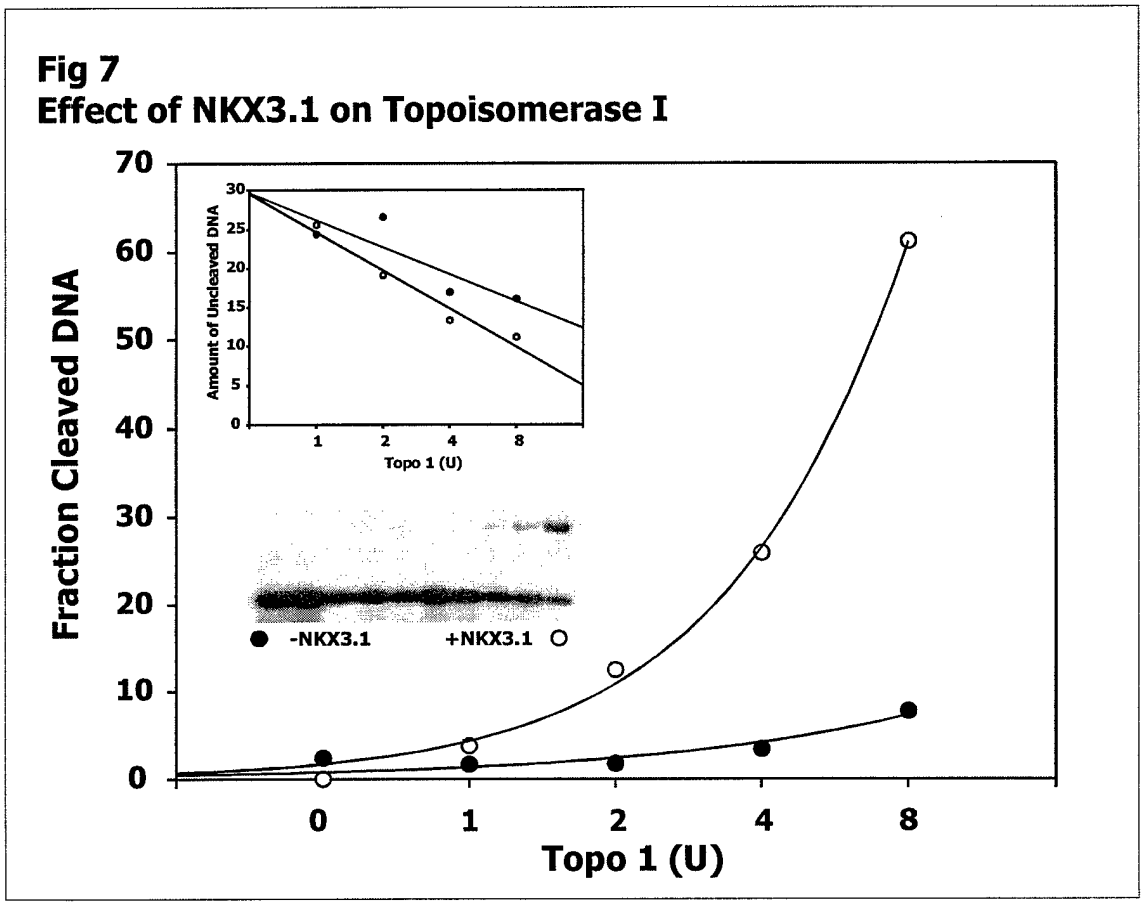
We first demonstrated the effect of NKX3.1 on toposiomerase I cleavage of pHOT1 super coiled DNA. The result is seen in Figure 6 where we see that NKX3.1 alone had not effect on unwinding of pHOT1 DNA, but had a marked effect on the toposiomerase activity. The reaction

Fig 6
Effect of NKS3.1 on Topoisomerase I



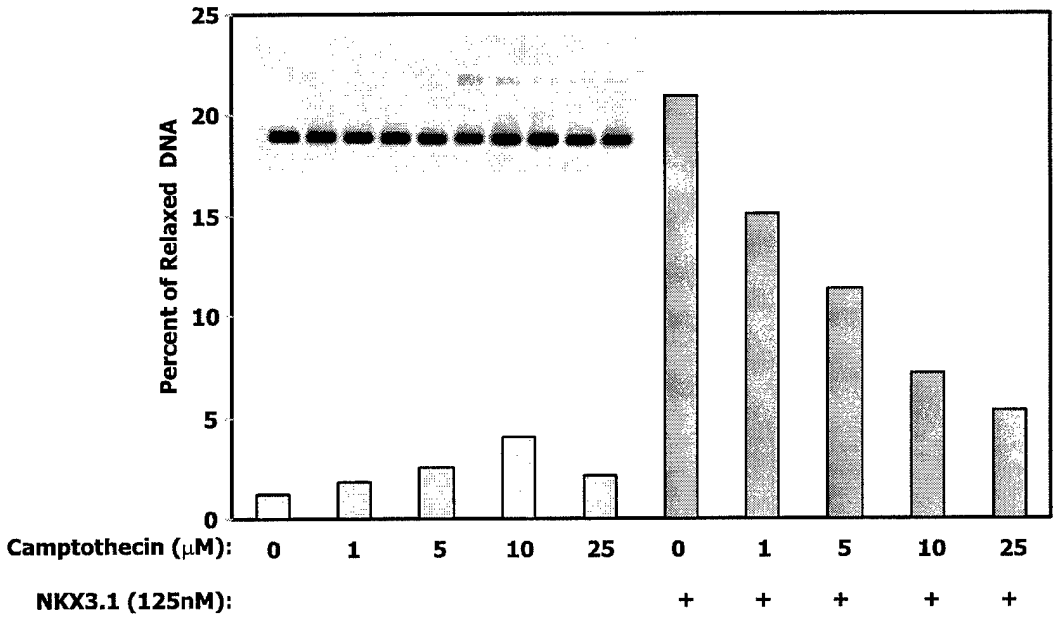
was performed according to a protocol provided by Topogen, Inc. (Columbus, OH). Briefly, 0.4U (approximately10ng to a final concentration of 5.5nM) of toposiomerase 1 was incubated with indicated amount of recombinant NKX3.1, 2 μl of 10x buffer containing 100mM Tris-Cl, pH7.5, 1M NaCl or KCl, 10mM PMSF, 10mM β-mercaptoethanol, and 250ng (7.1nM) of pHOT1 plasmid in a final reaction volume of 20μl at room temperature. The reaction was stopped by the addition of 5μl of 50mM EDTA, 0.5% SDS, 0.1% bromophenol blue and 50% (v/w) sucrose) in reaction mixture. DNA was electrophoresed in 1% agarose gels at 15V (2V/cm) for 18 hours. Gels were stained with ethidium bromide and photographed under UV light.

We also titrated toposiomerase I in the presence of a fixed amount of NKX3.1 and demonstrated that NKX3.1 changed the rate at which toposiomerase I effected DNA cleavage. This result is shown in Figure 7.



We next examined the interaction of NKX3.1 and toposiomerase I in the presence of camptothecin, a chemotherapeutic agent and known inhibitor of toposiomerase I. Camptothecin intercalates into DNA at the site of toposiomerase I binding to DNA and prevents religation, thus stabilizing the covalent complex of toposiomerase I and cleaved DNA and promoting cell death (18,19). The assay was performed as described for the DNA relaxation assay with the exception of adding the indicated amount of camptothecin (18,20). Figure 8 shows the expected effect of camptothecin on the cleavage of pHOT1 DNA by toposiomerase I. Substantially lower concentrations of NKX3.1 have a much greater effect on toposiomerase cleavage activity that is antagonized by increasing concentrations of camptothecin. The data suggest that the physiologic interaction of NKX3.1 and toposiomerase is disrupted by the binding of camptothecin to DNA and competitive obstruction of the religation and release of toposiomerase I from the covalent enzyme/DNA complex.

Fig 8
Effect of NKX3.1 on Camptothecin and Topoisomerase I



Toposiomerase I cleaves one of the two strands of double-stranded DNA (the scissile strand) and forms a covalent bond between a tyrosine and a thymidine residue. The DNA duplex then unwinding through at least one turn and the enzyme religates DNA and releases. The two functions, DNA cleavage and religation can be analyzed separated by the use of specially designed oligonucleotide reagents as shown below (21).

Reagents:

14 mer 5'-*GAAAAAAG**ACTT**AG- 3'
25 mer 3'- C TTT TTT CTGAATCTTTTTAAAAAT-5'

11 mer acceptor 5'-AGAAAAATTTT-3'

Toposiomerase I cleaves the partial duplex:

↓ ↓
5'-*GAAAAAAG**ACTT**AG- 3'
3'- C TTT TTT CTGAATCTTTTTAAAAAT-5'

↓ Top I

5'-*GAAAAAAG**ACTT**- 3'
3'- C TTT TTT CTGAATCTTTTTAAAAAT-5'

11-mer acceptor now can form a duplex with the cleaved partial duplex.

5'-AGAAAAATTTT-3' acceptor

↓

5'-*GAAAAAAG**ACTT**AGAAAAATTTT-3'
3'- C TTT TTT CTGAAT CTTT TTAAAAAT-5'

A 14mer oligonucleotide was labeled at the 5' terminus in a 25μl reaction containing 10pmol of 14mer oligonuleotide, 1.5μl 6000Ci/mmol of γ[^{32}P]-ATP, and 10U T4 polynucleotide kinase in polynucleotide kinase reaction buffer (NEB Inc. Beverly, MA) at 37°C for 20min. T4 polynucleotide kinase was inactivated by heating at 65°C for 20min. Labeled DNA was purified with a QIAquick Nucleotide Removal Kit (QIAGEN Sciences, Valencia, CA). 14/25mer partial duplex was generated by annealing the labeled 14mer oligonucleotide with 100-fold molar excess 25mer to ensure maximal annealing of the 14mer into a partial duplex. The annealing reaction of 100μl included 87μl [^{32}P]-14mer, 3.5μl 300μM 25mer oligonucleotide, and 10μl of 10X annealing buffer containing 100nM Tris-HCl (pH7.5), 1M NaCl and 10mM EDTA. The annealing mixture was heated at 95°C for 3-5min and then cooled to room temperature for 1 to 2 hours prior to usage. The DNA cleavage reaction was performed in 20μl with 1μl (0.1pmol) of suicide substrate and 4μl (1.2 – 2.2U) of Toposiomerase I (TopoGEN, Inc, Columbus, OH) in 20mM Tris-HCl (pH7.5), 100mM KCl, 1mM EDTA and 1mM of DTT incubated for 30min at 37°C. Reactions that included recombinant NKX3.1 were preincubated with Toposiomerase I in water at room temperature for 60min prior to the addition of suicide substrate. The reaction was terminated by addition of SDS at 37°C to a final concentration of 1%. The cleaved DNA substrate was purification using a QIAquick Nucleotide Removal Kit. 30μl of eluted cleaved DNA substrate was incubated at 37°C for 30min with 3μl 1mg/ml of proteinase K at a final concentration of 0.1μg/μl. 10μl of reaction was removed and added to 30μl loading dye (96% formamide, 20mM EDTA, pH 8.0, 0.03 % xylene cyanol, 0.03 % bromphenol blue), denatured at 90°C for 5min. and electrophoresed on a 16% polyacrylamide gel with 7M urea. The gel was autoradiographed after drying and quantitated with Scion Imaging software (Scion Corporation, Frederick, MD).

For the religation assay the 14/25mer suicide substrate was incubated with human toposiomerase I as described in suicide cleavage assay in the presence of a 1000-fold excess 11mer acceptor oligonucleotide in a 20μl reaction. NKX3.1 and 11mer acceptor were added at the initiation of reaction and incubated at 37°C for 30min followed by terminating reaction by 1% SDS and then treated with 1mg/ml proteinase K at 37°C for 30min. To discriminate cleavage and religation reactions 0.5MNaCl was added to the reaction mixture to terminate the cleavage reaction. NKX3.1 was applied either prior to or after the addition of 0.5NaCl to determine either its influence on cleavage of DNA duplex, or to determine its effects on DNA religation by Toposiomerase I. Following the addition of the 11mer acceptor, reaction mixtures were incubated at 37°C for 60min and terminated by the addition of 1% SDS followed by and proteolysis at 37°C for 30 min. Ten μl of sample was dissolved in 30μl of formamide loading buffer. Samples were heated at 90°C for 30min and resolved on a 16% denatured polyacrylamide gel with 7M urea. The gel was autoradiographed after drying and quantitated with Scion Imaging software (Scion Corporation, Frederick, MD).

To assay spontaneous cleavage and religation [^{32}P]-14mer/25mer partial duplex was incubated with toposiomerase I in the presence of excess amount of 11mer acceptor oligonucleotide in a reaction mixture with or without NKX3.1 for 30min at 37°C. The reaction was terminated with 1% SDS (22). The data showed that NKX3.1 significantly increased DNA cleaved products and slightly increased religated products (Figure 9 left panel). As a result, roughly all the cleaved products were religated. To clarify if the elevated level of religated product was due to stimulation of toposiomerase I religation activity by NKX3.1, or to the supply of cleaved DNA products augmented by NKX3.1, single turnover religation assays were performed by assaying the addition of a 5'OH-terminated 11mer to the cleaved 12mer oligonucleotide product to form an end-product 23mer. The first step of the reaction was carried out by incubating the suicide cleavage substrate 14/25mer with toposiomerase I in the presence, or absence of NKX3.1 to generate corresponding covalent intermediate complex. The religation reaction was initiated by the addition of 0.5M NaCl and the 11mer oligonucleotide. 0.5M NaCl promoted dissociation of the toposiomerase I after strand closure and prevent recleavage of the product (21,23,24) (Figure 9 center and right panels).

In the single turnover religation reaction with the presence of NKX3.1 in the cleavage step, we saw more cleaved product as expected and somewhat more relegated product. In the single turnover reaction with the addition of NKX3.1 at the initiation of the religation step following the addition of 0.5M NaCl and 11mer oligonucleotide, there was no obvious enhancement of religated product stimulated by NKX3.1 beyond the amount expected from the level of cleavage product observed. This suggested that NKX3.1 could facilitate DNA access by toposiomerase I despite the dissociative effects of 0.5M NaCl.

In summary, based on our DNA religation data, we conclude NKX3.1 did not separately affect the enzymatic religation activity of toposiomerase I. NKX3.1 appears to affect the access of toposiomerase I to DNA and enhanced cleavage as seen by the cleavage and relaxation assays.

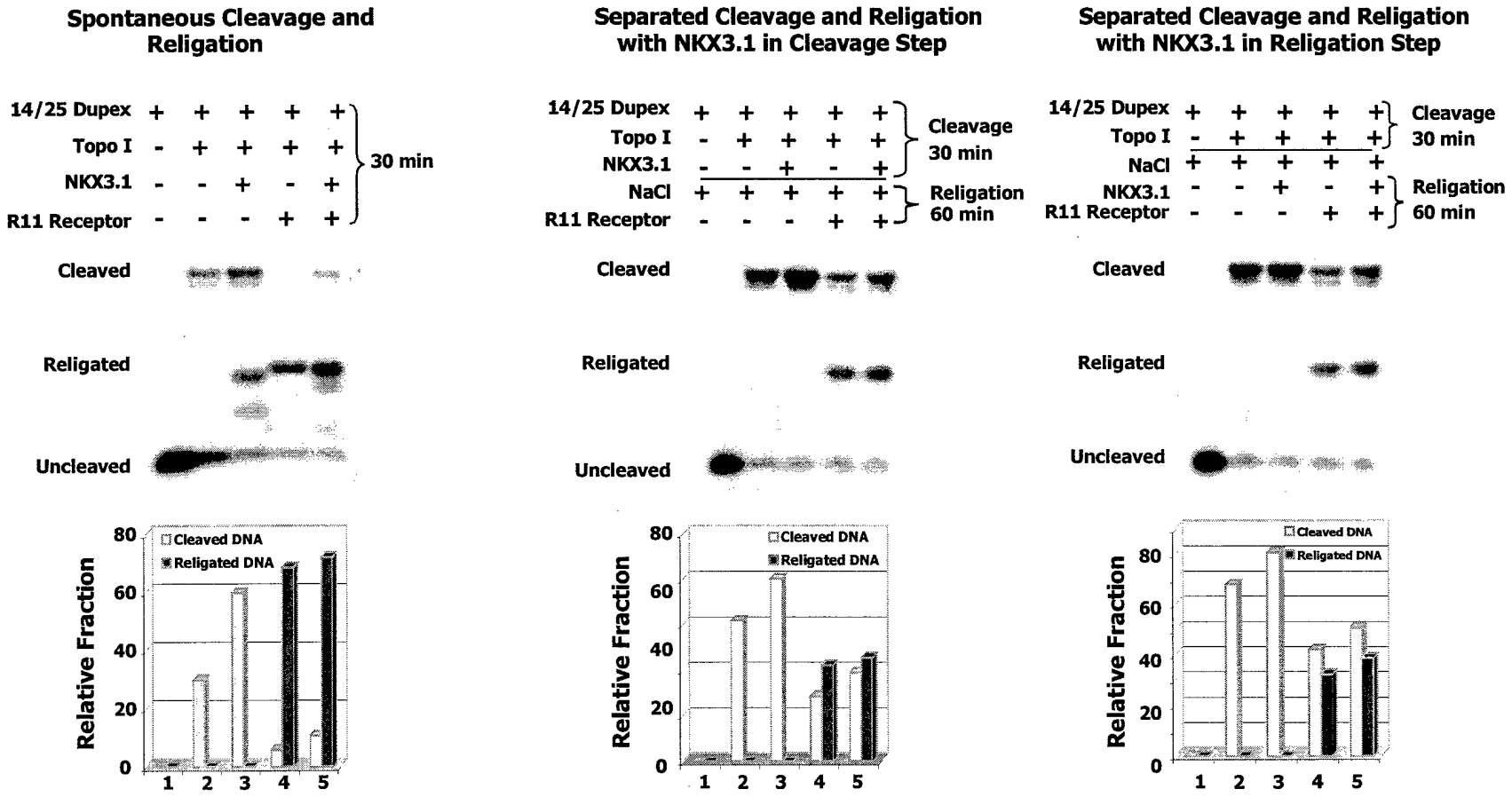
In summary we have shown that NKX3.1 binds to topoisomerase I and affects the cleavage of DNA. The remainder of the project will focus on the interaction of NKX3.1 and the variant NKX3.1(R52C) protein and identify the region of NKX3.1 that binds to topoisomerase I.

Analysis of protein binding by NKX3.1 WT and mutant proteins. (Year 2-3)

1. Y2H assay with NKX3.1 WT, R52C and S48A in GAL4-DB constructs with SRF and other proteins in GAL4-AD constructs.
2. GST pull down assays using NKX3.1 WT, R52C and S48A unphosphorylated and phosphorylated in vitro to bind to SRF and other binding partners.
3. Reporter gene assays using CMV promoter-luciferase and SMGA-luciferase reporters to assay activation by NKX3.1 WT, R52C and S48A with SRF and other binding proteins. Assay will be done in the presence and absence of 100nM TPA.

This element of the Statement of Work was premised on the assumption that the NKX3.1 binding proteins would modify the effects of NKX3.1. However, we have now shown that the enzymatic activity of topoisomerase I is affected by NKX3.1. Therefore we will analyze the effects of different mutant forms of NKX3.1 to affect topoisomerase I activity. We will also analyze binding of these mutant proteins and compare them to wild type protein in a gel retardation assay in which topoisomerase I is bound to an oligonucleotide recognition sequence that is not itself recognized by NKX3.1. Preliminary development of such a gel retardation assay is shown in Figure 10. Different conditions were tested to optimize the binding of topoisomerase I to the DNA recognition sequence. The mobility shift assay was performed using either the 14/25mer partial duplex oligonuleotide or the 25mer full-length duplex oligonucleotide, labeled at the 5'end as described in DNA cleavage assay. In 20μl volume of reaction, 110nM of topoisomerase I was

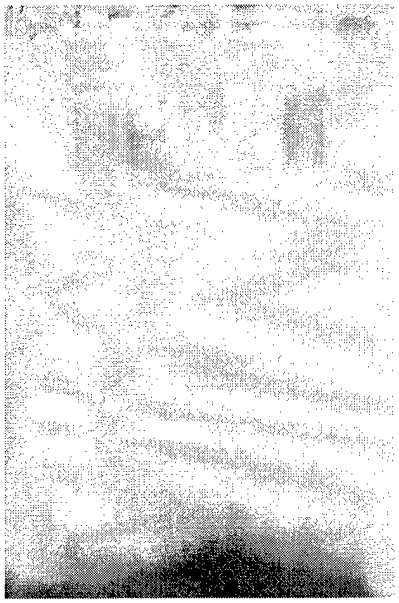
Fig 9
Effect of NKX3.1 on Topoisomerase Cleavage and Religation



incubated in either binding buffer containing 10mM Tris-HCl (pH 7.5), 50mM NaCl, 1mM $MgCl_2$, 0.5mM EDTA, 0.5mM DTT, and 0.05mg/ml poly(dI-dT), or 10mM Tris-HCl (pH7.5) in the presence of one of the reagents in the binding buffer at room temperature for 15min, and then incubated with 0.1pmol (5nM) of DNA at room temperature for another 15min. 5μl of 25% glycerol was added in reaction mixture and the total sample was run on 8% native polyacrylamide gel for 2-3 h at 200V (25,26).

Fig 10
Preliminary Human Topoisomerase I Gel Shift

Topo	-	+	+	+	+	+	+
Tris	+	-	+	+	+	+	+
Added:	-	bb*	-	NaCl	$MgCl_2$	DTT	p(dI-dT)



***bb = binding buffer**

C. Structural analysis of NKX3.1 – regions necessary for protein/protein interactions. (Year 3)

1. Construct GAL-4-DB vectors with *NKX3.1* deletion constructs.
2. Y2H analysis of deletion mutants binding to GAL4-AD-SRF and other protein binding partners.
3. GST pull down assay using deletion mutants of NKX3.1-GST fusion protein and SRF or other cloned proteins
4. SMGA reporter gene analysis of transcriptional activation by *NKX3.1* deletion mutants and SRF. CMV reporter gene analysis of *NKX3.1* deletion mutants cotransfected with clones isolated in aim 2.

To address this element of the Statement of Work we will use out panel of recombinant NKX3.1 proteins that have the following deletions: Δ1-44, Δ1-64, Δ1-117, Δ183-234, and homeodomain only (123-182). This proteins will be tested in vitro for the ability to complex with toposiomerase I and enhance the cleavage of pHOT1 super coiled DNA substrate.

Key Research Accomplishments

1. Identification topoisomerase I as a target for NKX3.1 binding.
2. Modulation of topoisomerase enzymatic activity by NKX3.1.

Reportable Outcomes

Same as key accomplishments. An abstract describing this work was submitted to the 2004 Annual Meeting of the American Association for Cancer Research.

Conclusions

1. NKX3.1 binds topoisomerase I in the absence of DNA.
2. NKX3.1 enhances topoisomerase I cleavage of DNA either by facilitating DNA binding or by accelerating DNA cleavage.
3. NKX3.1 appears to have minimal effects on DNA religation by topoisomerase I.

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