Title
The biological activity of DNA dumbbells

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The Biological Activity of DNA Dumbbells

by

Carol S. Lim

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Chemistry

in the

GRADUATE DIVISION

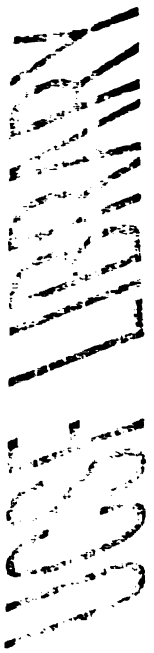
of the

UNIVERSITY OF CALIFORNIA

San Francisco

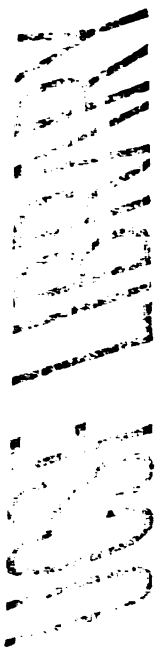
Date **University Librarian**

Degree Conferred: . . . . . .



DEDICATION

To my parents, Henry C. and Sun Boo Lim



ACKNOWLEDGMENTS

I would like to acknowledge Tony Hunt, my advisor, not only for scientific input and guidance, but for always having faith in me, for pep-talks and optimism in the face of adversity, for brainstorming about science and for talks having nothing to do with science. Tony encouraged creativity and independence, and helped shape me into the person I am today.

My husband, Tom Cheatham, deserves enormous credit for encouraging me to go beyond what I was at the time, for love and support, for help with science, and everything else.

My collaborators in the HHMI and in Immunogenetics helped make my experience at UCSF unique and scientifically rigorous. These people include B. Matija Peterlin, Marvin Garovoy, Nabila Jabrane-Ferrat, Joe Fontes, Hiroshi Okamoto, Tom Cujec, Bing Jiang, John Meyer, Helen Field, and Marianne Lantz. Without them this work would not have been possible. I would especially like to thank Nabila and Joe for their enthusiasm, guidance, time, patience, professional attitude, and sharing of their precious lab space. They both made molecular biology fun and easy to learn, and were pleasant to work with at all times. I looked forward to working with them at every opportunity I had.

I would like to also acknowledge my friends at UCSF, for giving me perspective, inspiration, chatting, diet coke breaks, and support-- they include Norris Turner, Jim Uchizono, Sherry LaPorte, Marci Schaner, Dallas Connor, Garrett Stanley, Justin Torpey, and Dennis Benjamin. Each of them, in their own way, made my experience at UCSF exciting, rewarding, and unforgettable. In particular, I would like to thank Norris for an incredible friendship over the years that words cannot describe; Jim for being like a nice big brother; Sherry for our instant camaraderie and sisterhood and for making science so exciting; and Marci for tons of emotional support, perspective, and escapes from the lab.

Past members of my lab, Murali Ramanathan, Renée Williard, Sarah Noonberg, Rod MacGregor should be acknowledged for their support and scientific inspiration. I give a special thanks to Murali for helping me when I first started graduate school, and for his continued mentoring over the years while he was here.

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Lastly, I would like to thank my parents who have shaped me into what I am today, and for their love and support over the years.

Other acknowledgments

Introduction

3-D model of DNA dumbbell (Figure 8) provided by Thomas E. Cheatham, III.

Chapter 1

Portions of the text of this thesis on pages 37-50 have been published in *Nucleosides and Nucleotides.* The co-author listed in this publication, C. A. Hunt, directed and supervised the research which forms the basis for the thesis. Also, melting temperature data kindly provided by Yoko S. Haga. Lourdes Nonato assisted greatly with the acquisition of gel scanning data.

Portions of the text of this thesis on pages 51-53 have been published in *Biotechniques.* The co-author listed in this publication, C. A. Hunt, directed and supervised the research which forms the basis for the thesis.

The Biological Activity of Dumbbell DNA

Carol S. Lim

ABSTRACT

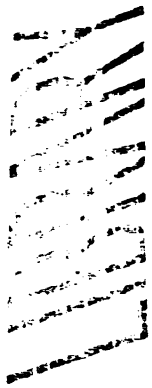
DNA dumbbells were tested for biological activity as transcription factor quenching agents (decoys). DNA dumbbells were ligated chemically or enzymatically from self-complementary phosphorylated oligonucleotides. DNA dumbbells had aberrant gel mobility and altered reactivity to DNA-substrate enzymes compared to their unligated counterparts.

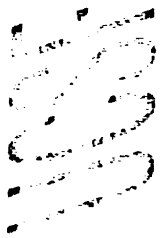
The X-box, a positive regulatory motif found in Major Histocompatibility Complex (MHC) class II DRA promoters, was chosen for the internal sequence of our DNA dumbbells. Several transcription factors target the X-box including the Regulatory Factor X (RFX) family. In electrophoretic mobility shift assays, all oligos containing the core X-box sequence could compete with wild-type for binding to X-box binding proteins. However, only ligated X-box dumbbells could directly bind to a protein identified as RFX1.

The first model system involved testing dumbbell DNA or other oligos for activity as measured by decreased DRA-driven reporter gene (chloramphenicol acetyl transferase, CAT) in Raji cells (which contain RFX). Ligated dumbbells were no more active than unligated, so unligated dumbbells (oligos) were used for the remainder of the studies. At 200 nM doses, both relevant and irrelevant sequence unligated dumbbells were able to decrease CAT activity, while other X-box oligos were inactive. Unligated dumbbells also reduced reporter gene readouts in control plasmid systems.

Due to these results, a simpler system was devised to elucidate whether dumbbells could block just RFX1 activity. A plasmid which creates a fusion protein of the DNA binding domain of RFX1 linked to the activation domain of VP16 was utilized

(pRFX1VP16). Co-transfection of pRFX1VP16 in Cos-7 cells with the reporter plasmid 4XBCAT results in synthesis of CAT protein. Fifty nanomolar doses of unligated dumbbells with the X-box sequence were able to block RFX1 activation. Ligated dumbbells were no more active than unligated as previously. However, double-stranded phosphorothioate oligos were the most potent, and hairpin and double-stranded X-box oligos also showed activity. Fifty nanomolar unligated control oligos were also active, and unrelated reporter gene systems were also "down-regulated" by unligated oligos. The most active oligos had no effect on transfection efficiency of plasmids based on a PCR assay. However, RNase protection assays indicated that active oligos decreased mRNA levels, suggesting an effect on polymerase II or its associated transcriptional machinery. The addition of stable segments of DNA such as dumbbells may act as competitors for transcriptional machinery and result in decreased polymerase II transcription.





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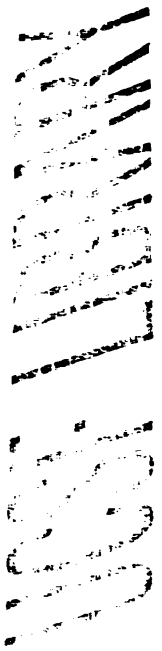

LIST OF FIGURES

Figure 12. Structural analysis of RFX1 (54). TAR = transactivating region; SP1, CTF, NF-1, GAL4, and GCN4 are all known transcription factors; Q, S/T, P, G, and E - one letter code for amino acids

Chapter 1

Figure 13. DNA dumbbell synthesis. Dumbbells may be synthesized from 2 intramolecularly annealing oligos with sticky ends (left) or from a single intramolecularly annealing oligo (right).

Figure 14A (chemical ligations) and Figure 14B (enzymatic ligations).

(1) unl ctrl 3; (2) ligated ctrl 3 dumbbell.

(3) unl hlyn; (4) ligated hlyn dumbbell.

(5) unl scr X; (6) ligated scr X dumbbell.

(7) unl X; (8) ligated X dumbbell.

(9) unl 5 T-X; (10) ligated 5 T-X dumbbell.

In all, upper band is unligated material; lower band is ligated material.

Note: Lanes 9 and 10 of the chemical ligation, Figure 14A, were run on a separate gel. Occasionally, oligos of this length contain shorter bands (Figure 14B, lane 5) which are either failed sequences or the full-length oligo folding back on itself. This is dependent on secondary structure formation of the newly synthesized oligo which sometimes is maintained despite highly denaturing conditions. Also, double bands may appear in the ligation product due to incomplete phosphorylation (Figure 14B, lane 10).

Figure 15. Klenow fragment (exonuclease) reactions. (1) no enzyme treatment; (2) 8 hour enzyme treatment; (3) 12 hour enzyme treatment. In (a), 5 T-X dumbbell and in (b) unl 5 T-X.

Figure 16. S1 nuclease reactions. (1) no S1 enzyme treatment; (2) 5 minute S1 treatment; (3) 20 minute S1 treatment. In (a), 5 T-X dumbbell and in (b) unl5 T-X .

Figure 17. *Mae* I cleavage. (1) *Mae* I digestion of 5 T-X dumbbell; (2) *Mae* I digest of unl 5 T-X; (3) 5 T-X dumbbell; (4) unl 5 T-X

Figure 18. Shrimp alkaline phosphatase (SAP) reactions. Odd-numbered lanes are SAP-treated. (1) unl 5 T-X + SAP; (2) phosphorylated unl 5 T-X; (3) phosphorylated unl 5 T-X + SAP; (4) 5 T-X dumbbell; (5) 5 T-X dumbbell + SAP.

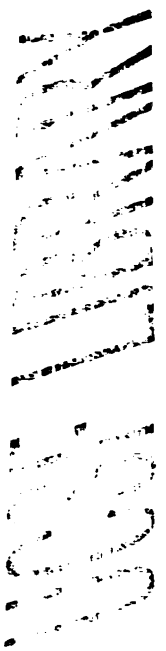

Figure 19. Melting temperature experiment. The unl X and X dumbbell sequences were subjected to heating/cooling (0.5°C/min.) from 20°C to 95°C to 20°C on a Cary 3E Spectrophotometer.

Figure 20. Scanning densitometer results for the chemical and enzymatic ligation reactions (unl X reactant, X dumbbell product). In this case, the yield for the chemical method was slightly higher (compare ligated AUCs).

Figure 21. A. Ethidium bromide stained gel. Lane 1, 42mer product (2.8 µg reaction mixture loaded). Lane 2, 42mer reactant (2 µg). **B.** Gel stained with methylene blue after ethidium bromide staining.

Oligonucleotide Sequences:

Lane 1. 42mer product: $_{T}{}^{T}{}^{T}$CCCCTAGCAACAGATG$^{T}{}_{T}{}_{T}$
$_{T}{}_{T}$GGGGATCGTTGTCTAC$_{T}{}^{T}$

Lane 2. 42mer reactant: 5'- CTAGGGGTTTTTCCCCTAGCAACAGATGTTTTTCATCTGTTG -3'

Figure 28. 1 μM oligos co-transfected with pDRASCAT in Raji cells. Toxicity seen with all oligos (except phosphorothioates, ds S-X and ds S-ctrl 3). Experiment performed in triplicate (n=3); error bars = ± 1 SD.

Figure 29. 0.1 μM oligos co-transfected with pDRASCAT in Raji cells. For the most part, all oligos were inactive (and non-toxic). Experiment performed in triplicate (n=3); error bars = ± 1 SD.

Figure 30. 0.2 μM oligos co-transfected with pDRASCAT in Raji cells. At this dose, most unligated dumbbells (unl X, unl CTTG-X, unl A-X, and unl ctrl 1) are active, yet non-toxic (unl rev X is toxic). Experiment performed in triplicate (n=3) except for linear QI and QII, where n=2; error bars = ± 1 SD.

Figure 31. Composite dose-response curves. In this and the following graph (Figure 32), response is measured by a decrease in CAT activity (response = 100 - %CAT).

Figure 32. Individual dose-response curves. Error bars (between experiment SD) are shown for experiments repeated 3 or more times (in duplicate or triplicate).

Figure 33. HCG assay. 1 μM oligos co-transfected with pDRASCAT and pACTHCG. % HCG = HCG activity compared to "no oligo;" activity measured by ^{125}I counts (not normalized). Experiment performed in triplicate (n=3) except for X dumbbell, where n=2; error bars = ± 1 SD.

Figure 34. Luciferase assay. 0.2 μM oligos co-transfected with pDRASCAT and pRSVluc. % luciferase = luciferase activity compared to "no oligo;" luciferase activity

p and ds S-X; lane 4, cells transfected with p and unl CTTG-X; lane 5, 2ng pRFX1VP16; lane 6, blank (water). Lane 7 contains molecular weight markers.

Figure 43. Total RNA accumulation assay. Total RNA levels are not decreased by the addition of oligos. CAT and live cells are included for comparison.

Figure 44. RNase protection. RNA harvested from cells transfected with: lane 1, plasmids pRFX1VP16 and p4XBCAT (p); lane 2, p + ds S X; lane 3, p + ds X; lane 4, p + unl X; lane 5, p + unl CTTG-X; all probed with the CAT probe (or the 18S RNA probe, below). Lane 6, RNA from cells transfected with p, probed with tRNA (control). Lane 7, 173 nt probe alone; lane 8, DNA molecular weight marker.

Figure 45. Distribution of RNA types in mammalian cells. Less than 5% of total RNA is mRNA.

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Chapter 4

ABBREVIATIONS

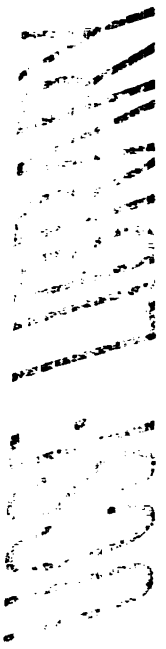
A, T, G, C	adenine, thymine guanine, cytosine
αRFX1	anti-RFX1 antibody
β-gal	beta galactosidase
bp	base-pair
BLS	Bare Lymphocyte Syndrome
BrCN	cyanogen bromide
CAT	chloramphenicol acetyltransferase
CID	class II-deficient combined immunodeficiency
CMV	cytomegalovirus
ctrl	control
CTTG	cytosine-thymidine-thymidine-guanosine loop
DTT	dithiothreitol
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
HCG	human chorionic gonadotropin
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
MHC	Major Histocompatibility Complex
nt	nucleotides
oligo	oligonucleotide (oligodeoxynucleotide)
p	plasmid(s)
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
RFX	Regulatory Factor X
rRNA	ribosomal RNA

RSV	Rous sarcoma virus
S	phosphorothioate backbone modified
SAP	shrimp alkaline phosphatase
SV	simian virus
TF	transcription factor
unl	unligated
wc	Watson-Crick base pairing
X	X-box sequence

I. Introduction

A. Evolution of this project

Although eukaryotic gene expression may be regulated at any of the steps from DNA transcription to RNA translation to protein, it is generally accepted that the primary control of gene expression is at the level of transcription. Many DNA- or oligonucleotide (oligo)-based strategies, e.g., antisense or triplex forming oligos, have exploited this fact. After joining the Hunt lab in the early 1990's, I became interested in developing alternative strategies to block gene expression. Studying the proteins that regulate gene expression, known as transcription factors, led me to the beginnings of this research project.



In order for transcription to occur, transcription factors must bind distinct regulatory sites or promoters on the gene. Once bound, transcription factors may interact with RNA polymerase or other factors to activate or repress transcription. With this in mind, a simple strategy was developed: use short segments of DNA with the same sequence as the regulatory sites or promoters on the gene to sequester or quench transcription factors, thus preventing their action on the gene.

The well-characterized Major Histocompatibility Complex (MHC) class II gene was selected as the target for gene regulation. Many of the transcription factors that regulate the MHC class II gene were also known and well characterized. In particular, the transcription factor RFX (Regulatory Factor X) became the target of this DNA based approach. The MHC proteins are involved in graft rejection; therefore, in transplantation, down-regulation of MHC II proteins on the donor organ may be of therapeutic use. MHC proteins are by far the preferred target antigen in T-cell mediated transplantation reactions. To conduct this research, an existing collaboration with Dr. Marvin Garovoy's group was utilized, and a new collaboration with Dr. B. Matija Peterlin's group was developed.

B. Oligonucleotide-based therapeutics and the central dogma of biology

Oligonucleotides, short stretches of single-stranded (or double-stranded, in some cases) DNA or RNA with or without chemical modification, can be designed to bind with very high specificity to a target gene, to RNA, or to a protein involved in regulating the gene. This binding can interrupt the cascade of biochemical events which would normally lead to the production of a protein (Figure 1). The specificity of these approaches is conferred by Watson-Crick base pair formation (for antisense), by Hoogsteen base-pairing (for triplexes) or by molecular recognition (for aptamers), assuming an appropriate target can be identified.

C. Oligonucleotide synthesis

The phosphoramidite method is the most popular technique currently employed to synthesize oligonucleotides (15, 29). Historically, Khorana and Gilham (1958) established the first approach to oligonucleotide synthesis called the phosphodiester method (52). The phosphotriester approach was developed in the mid-sixties to improve on this arduous synthesis. By 1976, the time-saving phosphite triester approach was in use. The current phosphoramidite method evolved in 1981 (Matteucci and Caruthers). This method consists of four basic steps: detritylation, condensation (or addition), capping, and oxidation (Figure 2). Synthesis occurs from the 3' to 5' direction. These steps are repeated until the desired sequence length has been achieved. The final product is cleaved from the support (Figure 3) and deprotected with strong ammonia (16, 24, 29). The oligonucleotides used in my experiments were synthesized using the latter approach and were obtained either from Keystone Laboratories or from Oligos, Etc.

D. DNA structures

The structures of DNA bases, sugar, and backbone are shown in Figure 4. Figure 5 shows the structure of a representative single-stranded DNA chain.

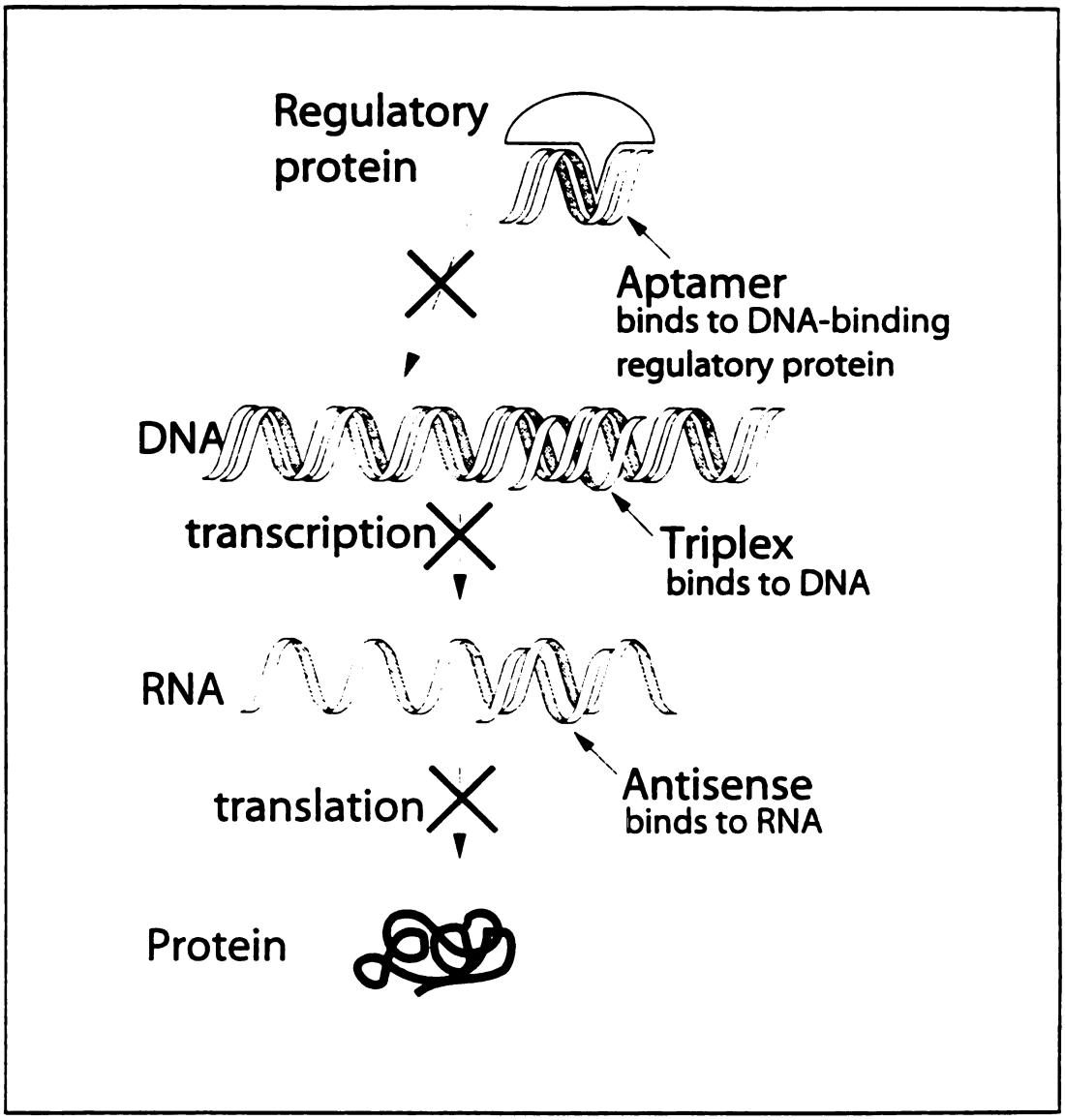


Figure 1. Nucleic acid (oligonucleotide)-based therapeutics [adapted from (38)]

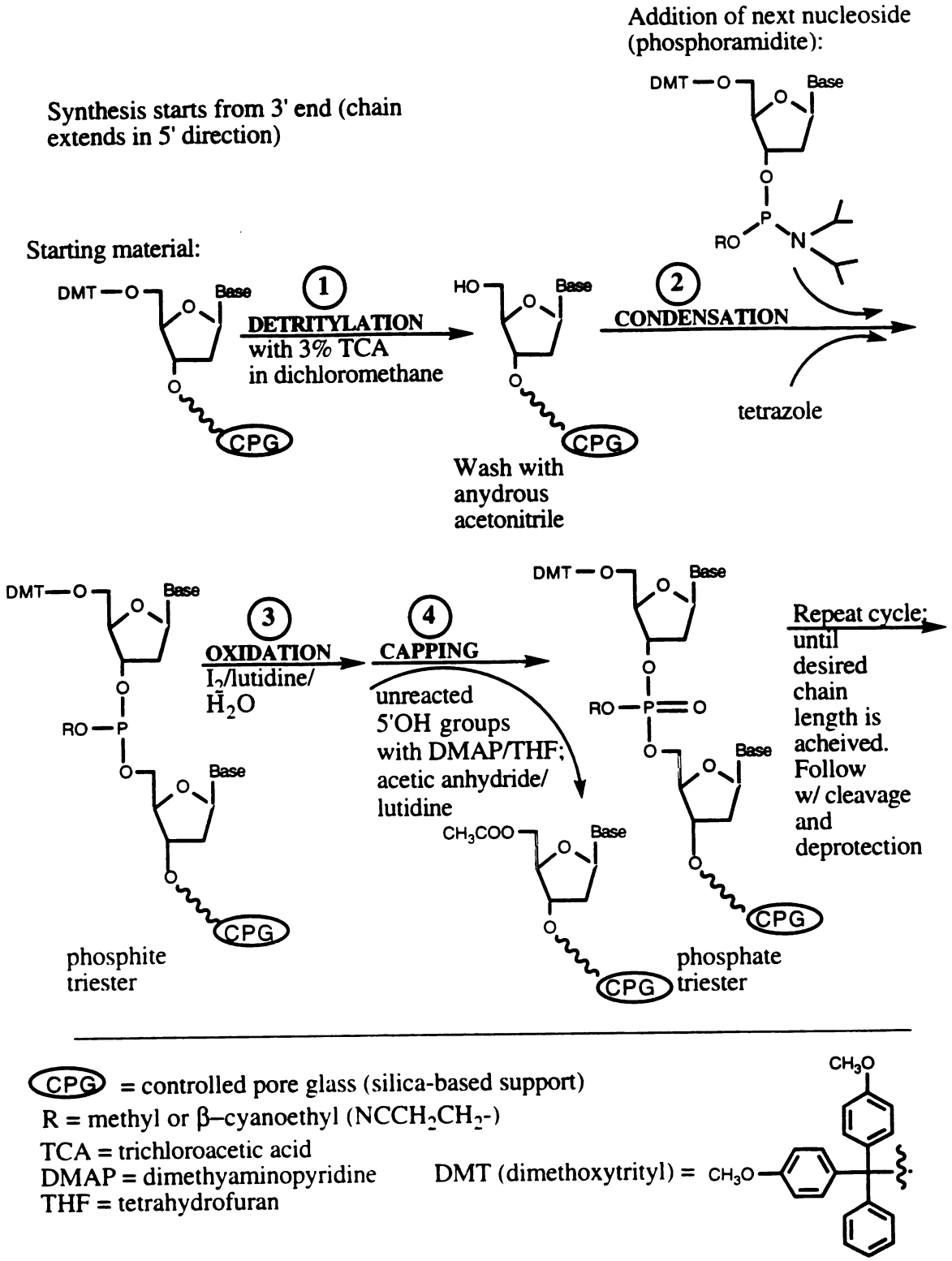


Figure 2. Synthesis of oligodeoxyribonucleotides by the phosphoramidite method

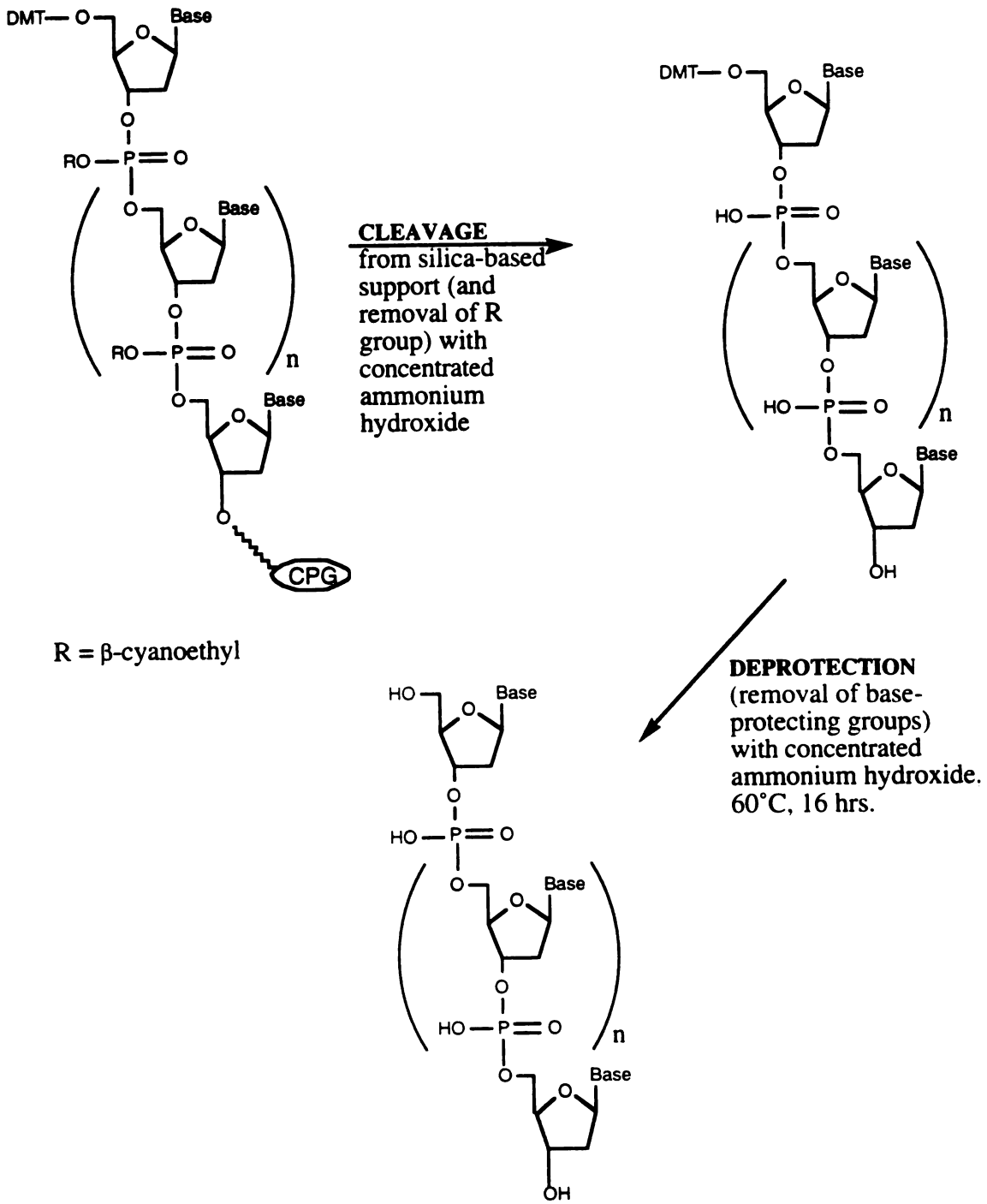
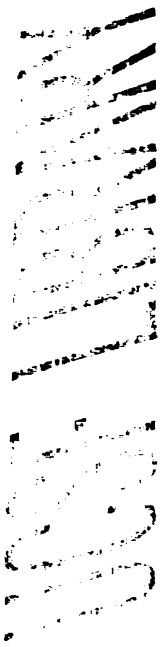


Figure 3. Cleavage and deprotection

E. Oligonucleotide-based therapeutics

1. Antisense

Antisense oligonucleotides (oligos) are short, single-stranded pieces of DNA or RNA targeted toward single-stranded DNA or RNA targets. Their specificity depends on Watson-Crick base-pairing (see Figure 6A) to their complementary target. Several mechanisms by which antisense oligos function are depicted in Figure 7. These mechanisms include inhibition of *transcription* via hybridization to locally open sections of DNA, or more commonly, of *translation* via blocking of binding of the initiation complex or by a RNase H mechanism (39). Antisense RNA occurs in nature as a gene repressor in prokaryotes (51, 58, 59). Since this discovery, many attempts at gene regulation using antisense modalities have been attempted. To date, the most successful strategies involve using backbone modified oligos such as phosphorothioates which are not only taken up by many cells, but are resistant to degradation by nucleases (26, 46, 61, 62). However, in recent years, phosphorothioate oligos have been plagued by numerous reports of non-specific effects (22, 72, 40, 53, 72) or binding to various proteins (35, 64). Variations on the antisense theme include derivatized oligos with reactive groups capable of either crosslinking to or cleaving the target sequence (4, 12, 36, 47, 68) including ribozymes.

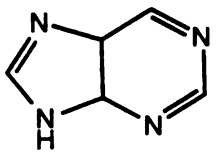


2. Ribozymes

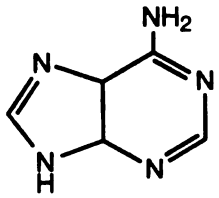
The first ribozyme or catalytic RNA activity was described by Cech (17) as a self-splicing intervening sequence of rRNA in *Tetrahymena thermophila*. Ribozymes are RNA molecules that cleave their target RNA. For cleavage to occur, ribozymes require a catalytic domain or core (conferred by secondary structure) and divalent cation (such as Mg^{2+}). Typical catalytic cores include the hammerhead motif (7, 25, 43) and the hairpin motif (8, 20). The major limitation in ribozyme therapeutics stems from the short half-life of RNA *in vivo* (39). However, Chartraud *et al.* described a catalytically active14-

BASES:

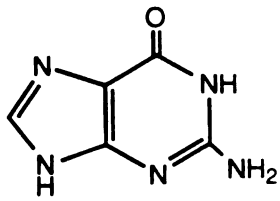
Purines:



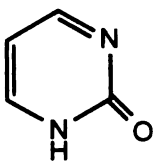
Adenine



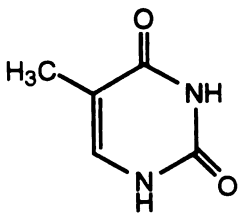
Guanine



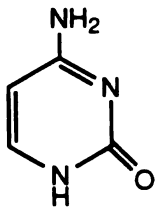
Pyrimidines:



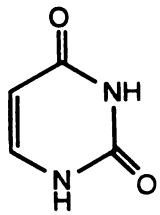
Thymidine



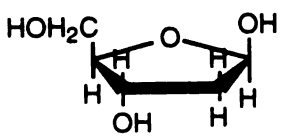
Cytosine



Uracil (RNA)

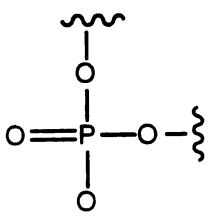


SUGAR:

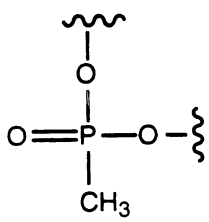


β-D-2 deoxyribose

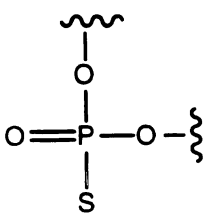
BACKBONE: Some common backbone modifications



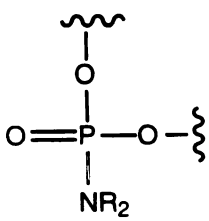
unmodified phosphodiester



methylphosphonate



phosphorothioate



phosphoramidate

Figure 4. DNA bases, sugar, and backbone

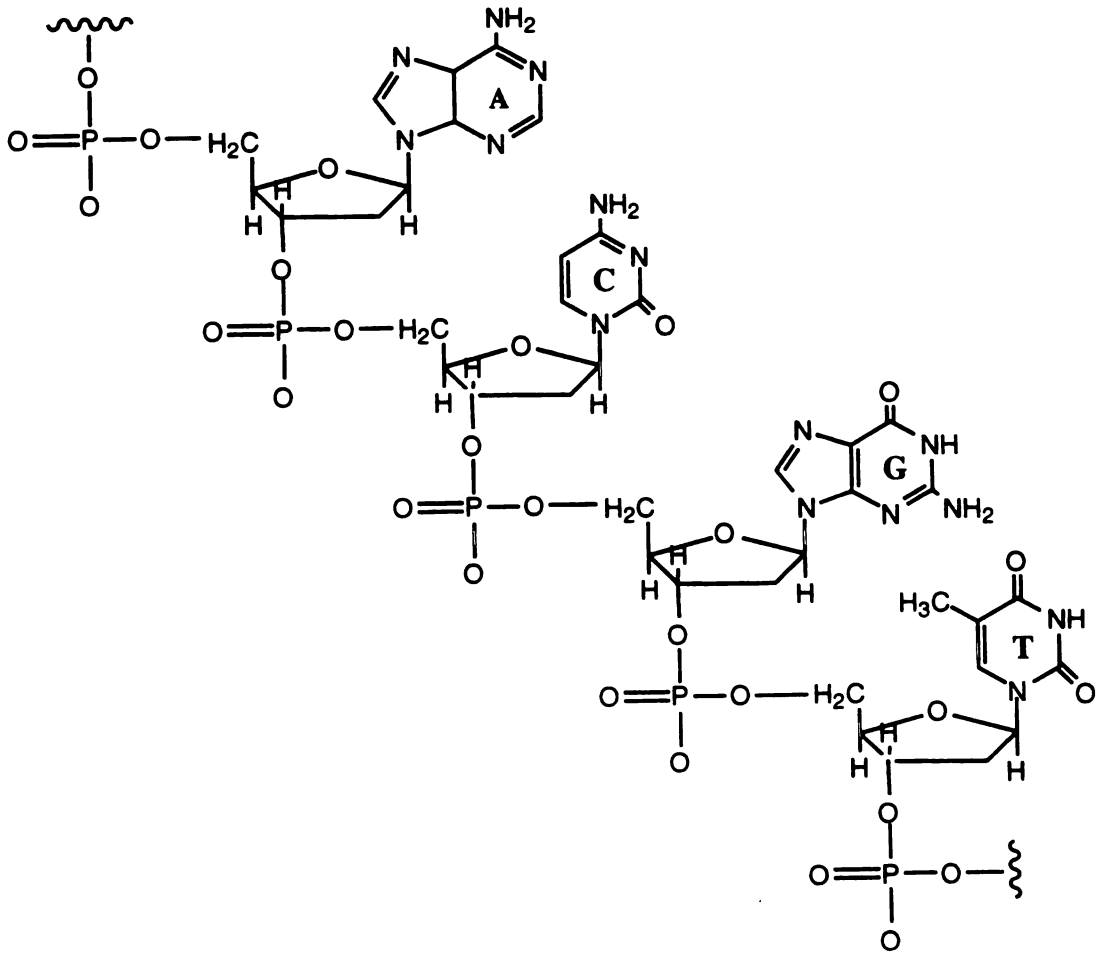
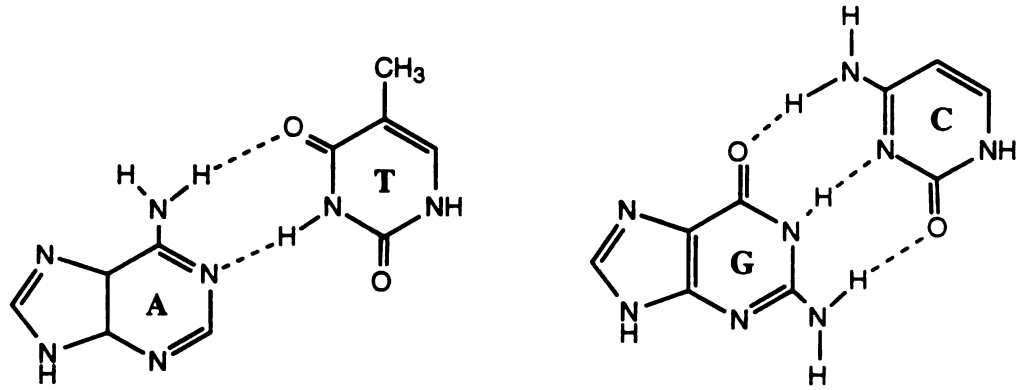
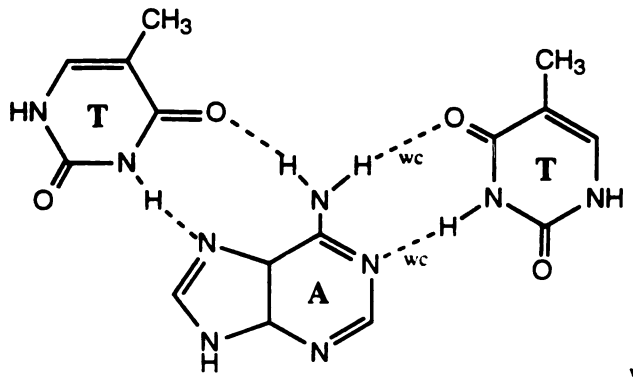


Figure 5. Representative DNA chain

A. Watson-Crick base pairing:



B. Hoogsteen base pairing:



wc denotes Watson-Crick base-pairing

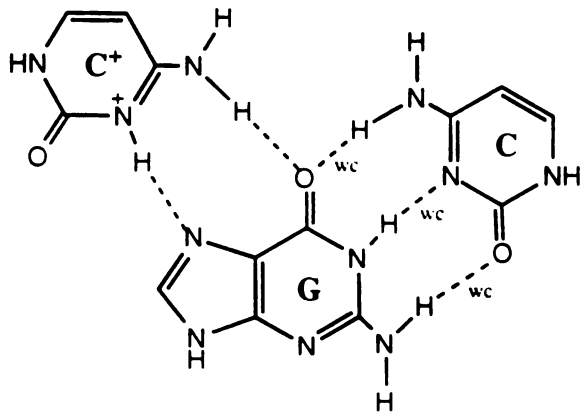


Figure 6. Watson-Crick (A:T and G:C) and Hoogsteen (T:A:T and C^+:G:C) base pairing.

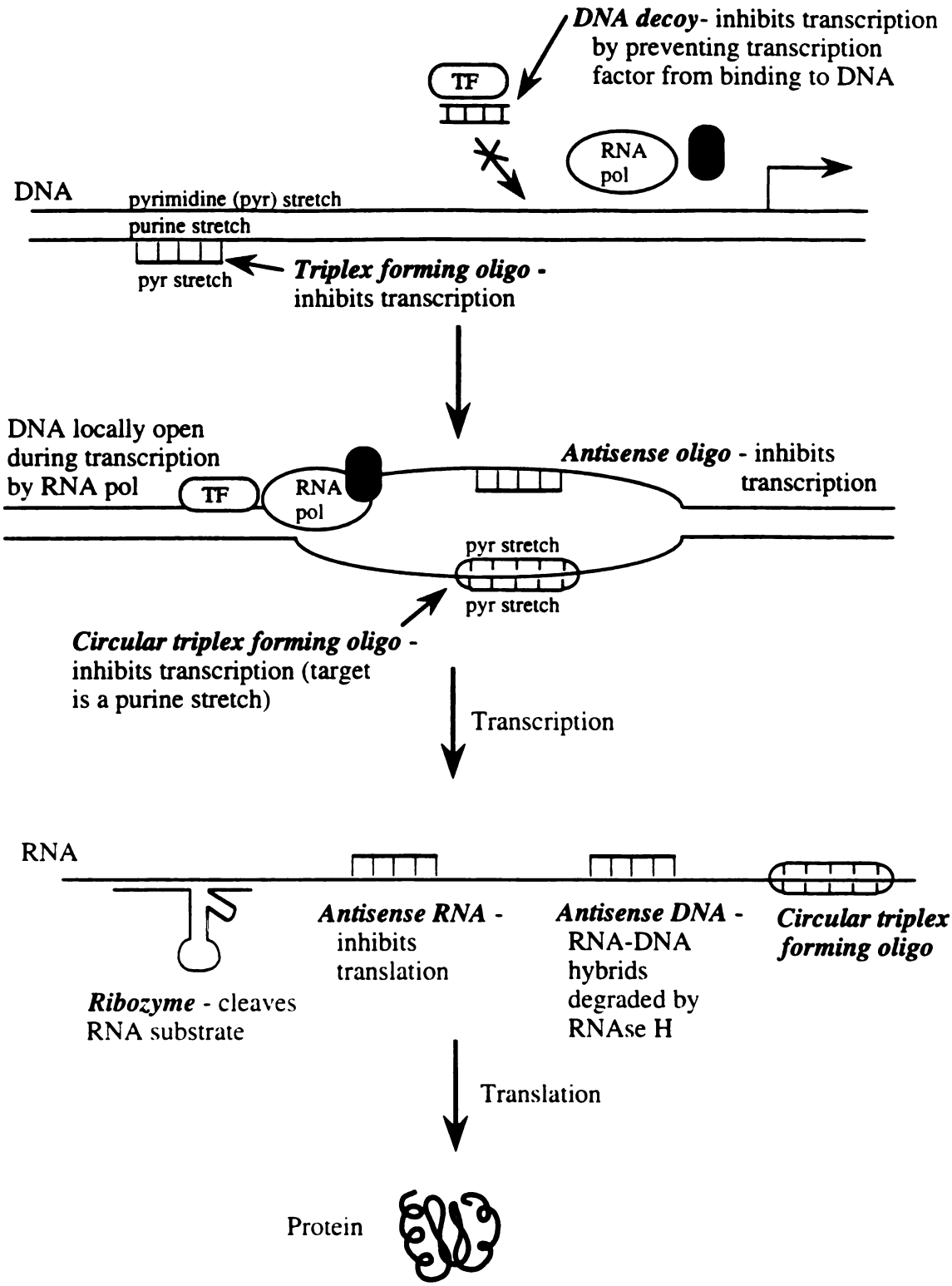
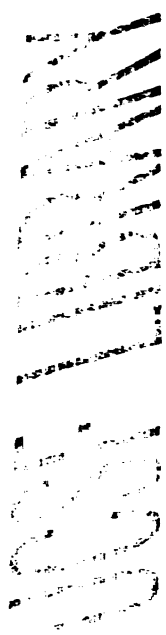


Figure 7. Targets for oligonucleotide-based therapeutics

mer oligodeoxynucleotide with a hammerhead motif—the first non-RNA ribozyme (19). Although this "all-DNA ribozyme" was less efficient in catalysis compared to the "all RNA ribozyme," a DNA analog with a single ribonucleotide in a critical position of the hammerhead was found to be a more efficient ribozyme than the "all RNA" version. In addition, chemically modified oligos may be substituted to prevent cellular degradation (49) of the ribozyme.

3. Antigene (triplex)

Antigene or triplex oligonucleotides are traditionally thought of as single-stranded homopyrimidine oligos targeted toward a double-stranded homopurine-homopyrimidine DNA target. The triplex forming oligo binds to the major groove of its DNA target, and recognition of the DNA target is conferred by Hoogsteen hydrogen bonding (Figure 6 B). Binding of the triplex forming oligo to DNA prevents transcription initiation or elongation. Like antisense oligos, triplex forming oligos may be backbone modified (71, 74). A new type of triplex former is a circular, non-self complementary oligo (44, 45, 70) targeted toward single-stranded DNA (locally open during transcription by RNA polymerase) or RNA (Figure 7). In the case of an RNA target, the mechanism of action is translation inhibition. Interestingly, recent studies have implicated DNA triple helix formation with *in vivo* mutagenesis. The triplex forming oligo may stall transcriptional machinery, which in turn may trigger error-prone spontaneous repair, and hence mutation (69).

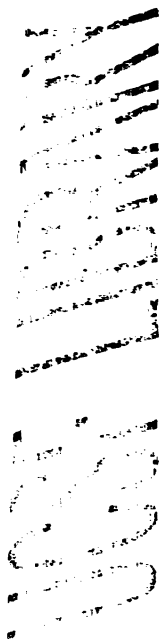


4. Aptamers, nucleic acid ligands, or decoys

Aptamers are single- or double-stranded nucleic acids which bind with high affinity to proteins or other factors (31, 37, 65). The specificity of aptamers, unlike antisense, ribozyme, or antigene oligos, relies on molecular recognition of the target protein to nucleic acid. Possible target proteins include transcription factors (6, 9, 41),

polymerases (65), growth factors (10), thrombin (11, 13), HIV integrase (1) and reverse transcriptase (57, 66), and even small non-protein molecules such as cyanocobalamin (48) and ATP (42). In the simplest case, if a target protein is known to bind to a specific DNA sequence, an aptamer can be made using that specific sequence. The classic example of a protein target is a DNA-binding protein or transcription factor (Figure 7). In the past 2 decades, DNA sequences have been mapped for hundreds of transcription factors, thus making them amenable to aptamer or decoy technology.

A more comprehensive approach to finding aptamers and other high affinity ligands involves screening large random libraries of nucleic acid sequences. This expansive type of approach includes SELEX (65) or *in vitro* selection (31, 37) and various other combinatorial methods (50). SELEX, systematic evolution of ligands by exponential enrichment, involves first chemically synthesizing a large random pool of DNA molecules of appropriate length(s) using an oligo synthesizing machine. This DNA pool is first amplified via PCR, then converted to RNA (when RNA aptamers are desired) using T7 RNA polymerase. After synthesizing an appropriate random pool of either DNA or RNA, the molecules are passed over an affinity column with the target molecule attached. The tightest binding DNA or RNA molecules are retained then eluted and amplified using PCR, which enriches the population of tightest binding molecules. This cycle is repeated, resulting in an exponential increase of molecules that bind the best. Using this method, DNA or RNA sequences other than the "wild type" or consensus sequence have been discovered (50, 65). Such results suggest that specific aptamers or decoys can be engineered for a variety of target molecules, even ones not known to normally bind DNA or RNA.

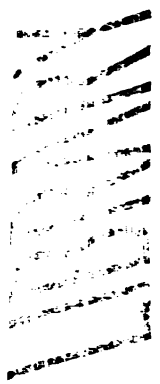


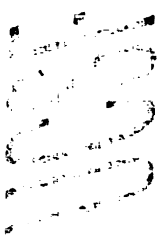
II. Background on DNA dumbbells

A. Utility of DNA dumbbells: physical chemistry

DNA dumbbells are short (typically 8-20 base pairs), double-stranded segments of

DNA with nucleotide "caps" on each end (Figure 8). In a number of cases, DNA dumbbells have historically been used as physical models of double-stranded DNA. In 1985, Wemmer and Benight used dumbbells to overcome problems of "end-fraying" of short pieces of duplex DNA in their studies of thermodynamic properties which govern base-pair stability (73). Dumbbells have been used as models for examining hairpins, cruciforms, and locally melted domains within naturally occurring DNA polymers (33). Antao *et al.* and others (2, 3) have suggested that DNA secondary structure, such as hairpin formation, may play a role in genetic regulation. Hence dumbbells have been used as a model for DNA hairpins. Dumbbells are ideal models since their formation is not concentration dependent, unlike hairpins. Dumbbells have also been used to overcome double-strand oligomer dissociation when investigating ethidium intercalation into DNA (56). Nicked dumbbells (gapped, unligated dumbbells) have been used as models for studying DNA conformations as substrates for repair enzymes (5).





B. Therapeutic application

In addition to their utility as physical models, DNA dumbbells may have biological relevance as aptamers or decoys for trapping proteins such as transcription factors (21, 23). Introduction of sequence-specific DNA decoys can selectively sequester transcription factors, making them unavailable to bind to their target DNA sequence within the gene. The final result is a blockade of transcriptional activation (41). Such a blockade is referred to in the literature as "the decoy approach." Several different forms of double-stranded DNA have been utilized as decoys for transcription factors (Table I) including unmodified oligonucleotide (oligo) duplexes (18), α·β-anomeric (chirally modified) oligos (63), phosphorothioate oligo duplexes (9) and dumbbell DNA (23). Use of phosphorothioate oligo duplexes may overcome the inherent disadvantage of nuclease susceptibility that is typical of unmodified oligos. However, recent studies have shown that double-stranded phosphorothioate oligos exhibit sequence independent effects due to

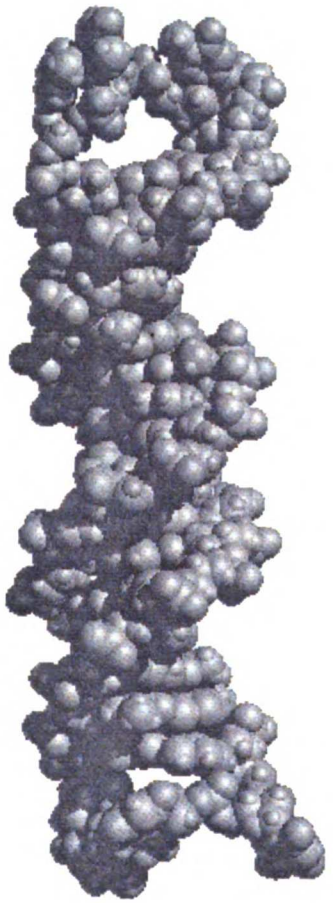


Figure 8. Three-dimensional representation of a DNA dumbbell (model is not energy minimized)

Table I. Summary of selected examples of transcription factor decoys

Author (reference)	Target(s)[1]	Type of Oligos	[Oligo]/cell #[2]	Activity[3]	Transfection[4]	Cell Line	Assay[5]
Cereghini, S. *et al.*. Genes and Dev., 2: 957-974, 1988	APF (HNF-1), seq/TF, albumin gene	unmodified ds (double-stranded)	n/a	~49-97%	n/a	(rat liver nuclear extract)	*in vitro* transcription
Berkowitz, L.. *et al.* Mol. Cell. Biol., 9: 4272-81, 1989	CRE, Fos protein, *c-fos* gene	unmodified ds	n/a	(relative comparison of stained cells)	microinjection	BALB/c 3T3	measurement of cAMP induced c-fos expression by flurorescence
Wu, H. *et al.* Gene, 89: 203-209, 1990	sp1 sequence, SP1 TF, adenovirus Eb1	phosphorothioate ds	0.17 to 4.9 μM/(n/a)	~50-80%	n/a	Molt4 human leukemic cells	*in vitro* transcription
Bielinska, A. *et al.* Science, 250(4983): 997-1000, 1990	κBseq, NF-κB, HIV enhancer	phosphorothioate ds	7.5 μM/10^5	~81%	DEAE-dextran; oligos added 4hrs post-transfection	EBV B-cell line clone 13	chloramphenicol acetyl transferase or CAT (pHIVCAT)
		unmodified ds	7.5 μM/10^5	~25%			
	Octamer seq/TF, IL-2 gene	phosphorothioate ds	7.5 μM/10^5	~92%		T-cell Jurkat leukemia	(pOCTCAT)
		unmodified ds	7.5 μM/10^5	~54%			
Holt, J. Antisense Res. and Dev., 1: 365-369, 1991	SRE seq, Fos protein, *c-fos* gene	unmodified ds	250-fold molar ratio/10^6	~100%	calcium phosphate coprecipitation with oligo	3T3 mouse fibroblasts	CAT (pFosCAT-FC4)
		unmodified ds, ss (single-stranded)	4 μM/10^6	~94% (ds) ~98% (ss)	passive adm. 24hrs post-transfection		

Table I. (continued)

Author (reference)	Target(s)[1]	Type of Oligos	[Oligo]/cell #[2]	Activity[3]	Transfection[4]	Cell Line	Assay[5]
Riabowol, K. *et al.* PNAS, 89: 157-161, 1992	AP-1 seq, Fos protein, *c-fos* gene	unmodified ds			microinjection	human Hs68 fibroblasts	thymidine incorporation
Clusel, C. *et al.* Nucl. Ac. Res., 21(15): 3405-3411, 1993	HNF-1 seq/ TF, liver-specific genes	ligated 5T dumbbell (unmodified)	2 and 10 nM/ $5x10^5$	~80%	calcium phosphate coprecipitation with oligo	C33 human epith tumor cells (pRSV-HNF1 added)	*in vitro* transcription and CAT (pΔE1 Alb-CAT)
		unligated 5T dumbbell (unmodified)	10 nM/$5x10^5$	~35%			
		unmodified ds	10 nM/$5x10^5$	~30%			
Tanaka, H. *et al.* Nucl.Ac. Res., 22(15): 3069-3074, 1994	κBseq, NF-κB (p50 subunit) TF, vimentin gene	α·β anomeric ds and unmodified ds	0.3 μM (100:1 oligo:plasmid)	~90%	calcium phosphate coprecipitation with oligo	HeLa cells	CAT (pVim241CAT)
			0.1 μM (30:1)	~40%			
Morishita, R. *et al.* PNAS, 92: 5855-5859, 1995	E2F seq/TF, c-myc, cdc2, PCNA	phosphorothioate ds	3 μM		hemagglutinating virus of Japan-liposome complex	VSMC (vascular smooth muscle cells)	cell count; c-myc mRNA and PCNA/ Cdc2 kinase protein levels
Yamakawa, H. *et al.* Nucleos. & Nucleot., 15(1-3): 519-529, 1996	rev HIV-1 mRNA seq, TF-N/A, HIV-1 gene	unligated 9T phosphorothioate loop dumbbell active	0.9 to 1.1 μM/ ml	50%	passive administration	MT-4 cells	protection against HIV-1 induced cytopathicity

[1]Target sequence(s), transcription factor (TF), gene(s)
[2]Concentration of oligo/number of cells per experiment
[3]Activity or percent inhibition
[4]Method of transfection
[5]Type of assay (plasmid used, if applicable)

non-specific protein binding (14). Chirally modified oligos are also nuclease resistant but require special synthesis techniques. Also, it is unclear which (if any) chiral form(s) will be recognized by a given transcription factor. As double-stranded targets, DNA dumbbells have the following advantages:

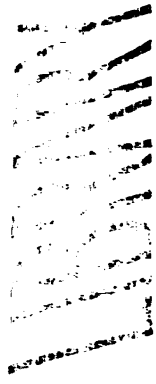
i. Dumbbells have increased stability to exonucleases (21), the major source of oligonucleotide degradation in many cell lines (34, 67).

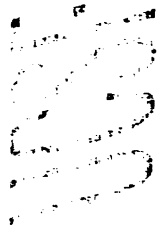
ii. Dumbbells are easily synthesized enzymatically or chemically.

iii. Dumbbells never dissociate (i.e., since they are covalently closed, strand dissociation in the classical sense does not occur).

iv. Duplex formation within the dumbbell is concentration independent (2).

v. The unmodified backbone of DNA dumbbells is non-toxic and physically identical to naturally occurring DNA.





III. Major Histocompatibility Complex (MHC)- target system

Down-regulating the molecules involved in transplantation rejection reactions was the focus of a collaborative effort between our lab and the Garovoy lab. Such molecules are the MHC molecules, by far the preferred target antigens for T-cell mediated transplantation reactions. Initially, DNA decoys were to be used in an attempt to modulate expression of MHC genes, with the ultimate goal of preventing graft rejection.

The MHC genes were discovered in the 1940's during transplantation studies in mice. In humans, the MHC is known as Human Leukocyte Antigen (HLA) and is located on chromosome 6 (Figure 9) and encodes MHC class I, II, and III molecules. The cell surface expressed-MHC molecules are the major determinants of graft rejection. Some key features of MHC I and II are listed in Table II. T-cells recognize MHC molecules bound to degraded peptide fragments. While the extracellular regions of the MHC class I and class II molecules are nearly superimposable, their structural organization differs

Major Histocompatibility Complex

Genes

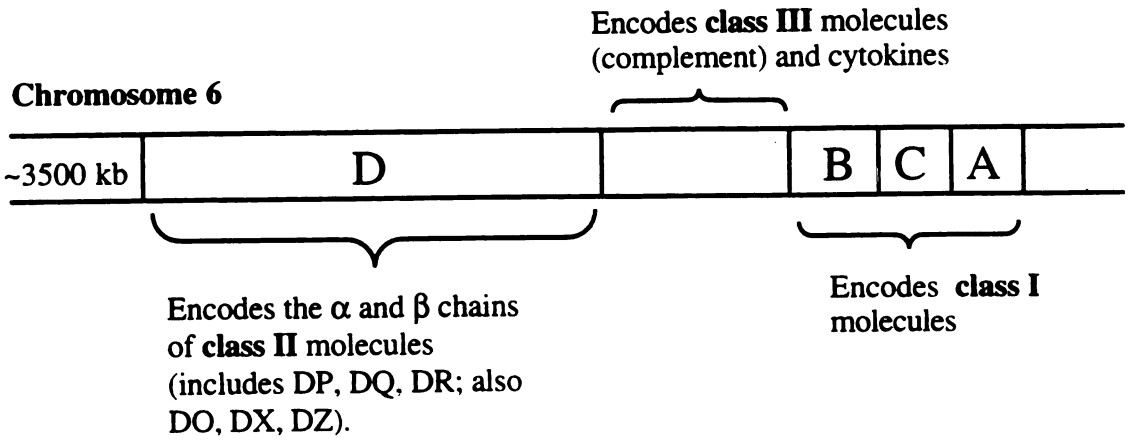


Figure 9. Chromosome 6 encodes the MHC molecules

(Figure 10). MHC class III—the complement system—complements and amplifies the action of antibodies and will not be discussed here.

Table II. Differences between human MHC class I and class II

	MHC class I	**MHC class II**
Cellular distribution	virtually all nucleated cells but excluding erythrocytes	B-lymphocytes, macrophages, activated T-cells, dendritic cells, monocytes, epithelial cells, other antigen presenting cells
Recognized by what type of T-cell?	cytotoxic T-cell	helper T-cell
Type of adhesion protein associated with on T-cells	CD8	CD4
Type of fragment bound to (antigen association)	endogenous peptides (degradation of proteins synthesized within the cell)	exogenous peptides (degradation of proteins synthesized outside the cell)
Structure	α (heavy) chain, ~44kD β_2 microglobulin (non-MHC encoded), ~12 kD	α chain, 32-34kD β chain, 29-32kD

A. MHC class II and the DRA promoter

The well-studied MHC class II gene was selected as a possible candidate for down-regulation. Class II molecules fall into distinct groups known as isotypes which, in humans, include DR, DQ, and DP. Due to the highly polymorphic nature of most MHC II genes, the essentially *monomorphic* DRA was selected as a potential target for regulation. The DRA gene contains a promoter region which is the target of many gene-regulatory DNA binding proteins. A schematic of the regions ("boxes") within the DRA

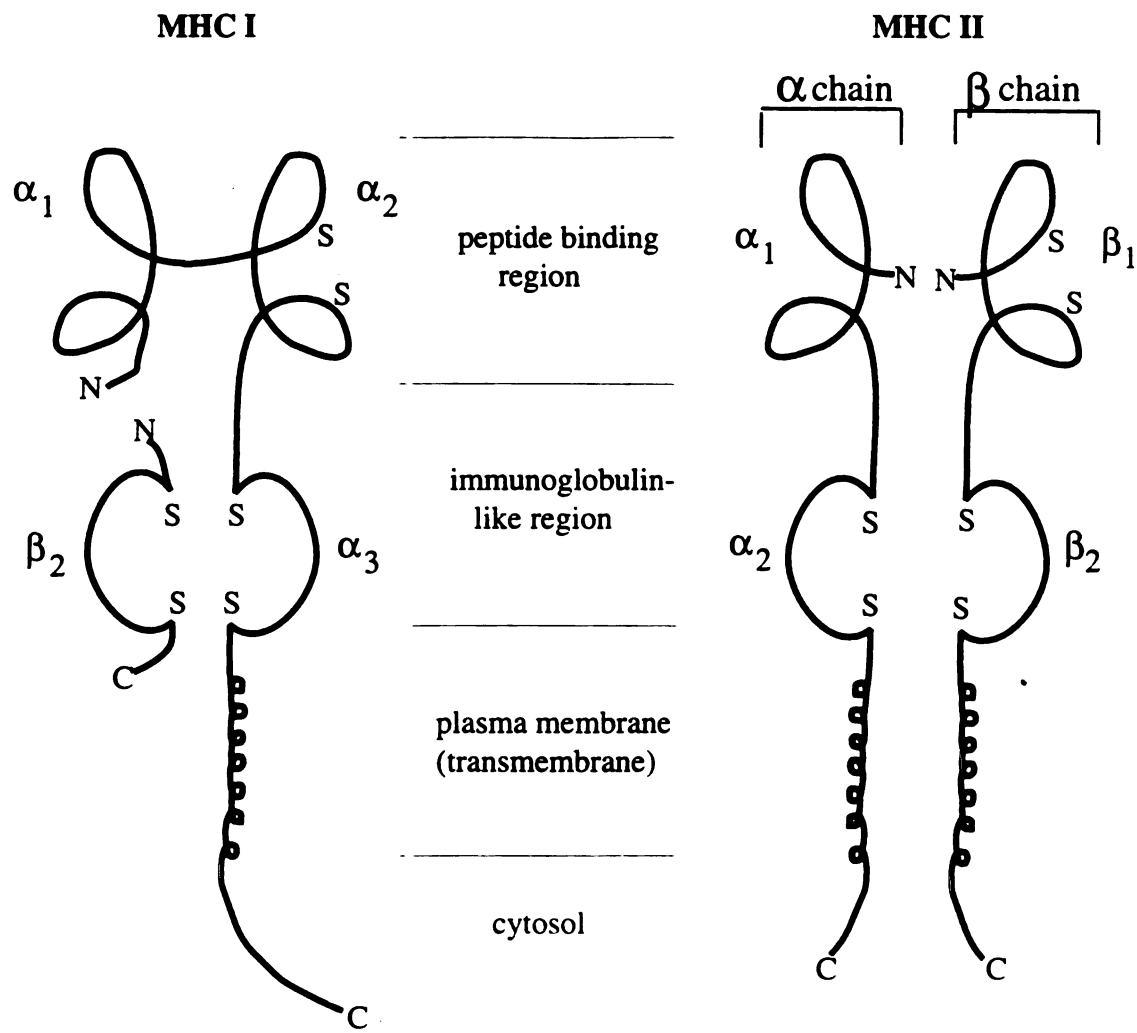


Figure 10. MHC I and II structures

promoter and the DNA binding proteins or transcription factors that bind to them are listed in Figure 11. The X-box region of the DRA promoter was selected as a potential DNA target to mimic because one of its transcription factors, RFX (Regulatory Factor X), was cloned and extensively characterized at that time. Since RFX was found to be essential for MHC class II expression, it made an attractive target for regulation of class

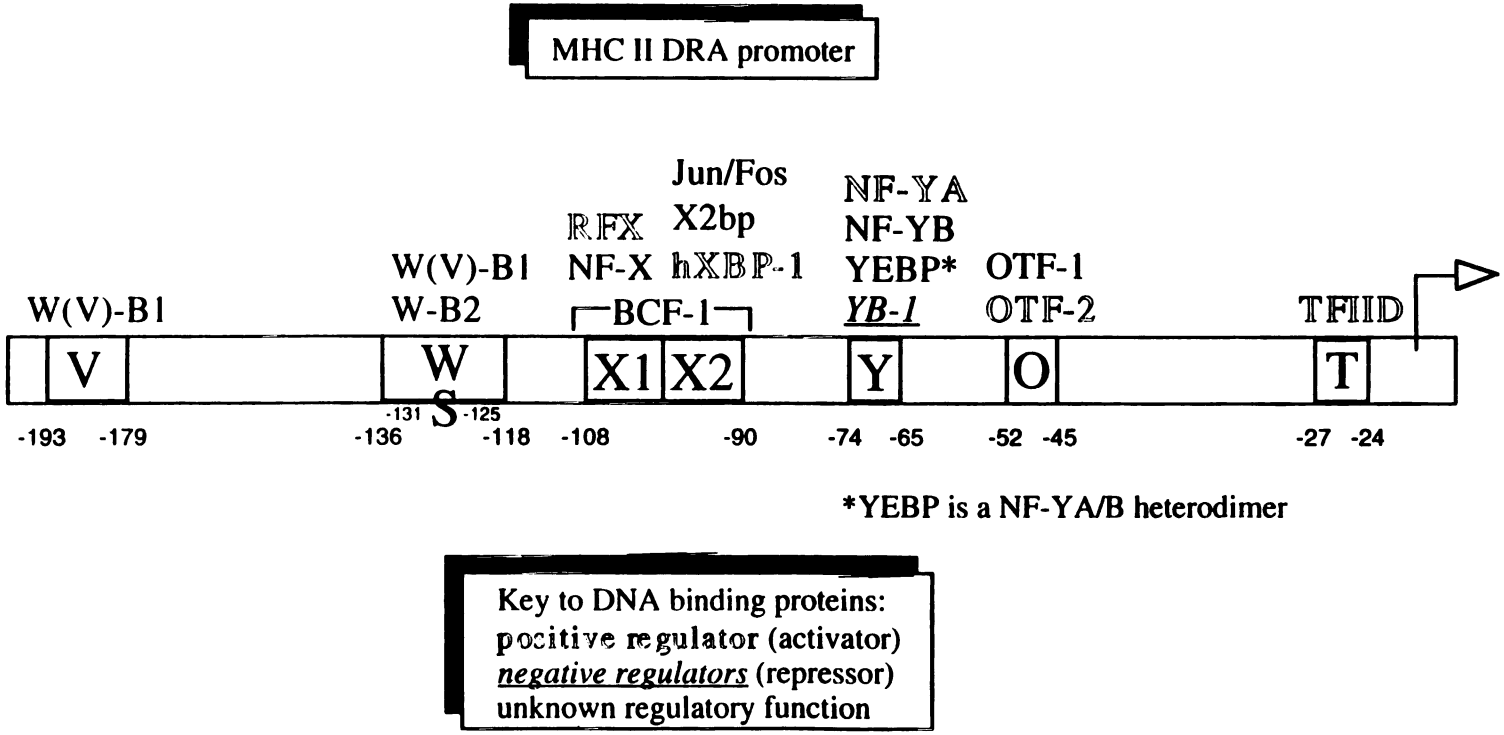


Figure 11. MHC class II DRA promoter. V, W (or Z), S (contained within W), X1, X2, Y, O and T are specific DNA sequences (or boxes) which interact with DNA binding proteins (listed above).

II expression. I therefore initiated a collaboration with the B. M. Peterlin group since his group was already studying RFX.

B. The RFX family of DNA binding proteins

RFX was originally identified (27, 28) by B. Mach's group, by virtue of its ability to bind to the X-box of the MHC II DRA promoter. RFX binding was thought to be defective in patients with class II-deficient combined immunodeficiency (CID, also known as Bare Lymphocyte Syndrome or BLS), a disease of class II gene regulation. Shortly thereafter, Mach's group cloned and characterized RFX which subsequently became known as RFX1 (54). RFX1 was found to be a 979 amino acid DNA binding protein which binds to DNA as a homodimer or monomer. RFX1 contains three separate domains—a dimerization domain, a DNA binding domain, and an activation domain (Figure 12). The dimerization and DNA binding domains are functionally independent of each other. The carboxy-terminus of RFX1 contains the dimerization domain, and a putative activation domain. The centrally located 91 amino acid DNA binding domain was found to be only distantly related to the helix-loop-helix DNA binding motif. It shares no extensive homology with any other known DNA binding motifs and therefore is believed to be a novel DNA binding motif. The amino terminal part of RFX1 contains 2 putative activation domains. The first activation domain consists of alternating glutamine-rich and serine/threonine-rich regions, whereas the second consists of a proline rich region.

Much controversy emerged in the years following the discovery of RFX. This controversy mainly arose as a consequence of identifying these proteins based on their specific DNA binding ability. Eventually it was determined that there is a family of RFX proteins which all share a common, 76 amino acid DNA binding motif (76-96% homologous). The controversy surrounding RFX1 stemmed from the fact that the cause of MHC class II deficiency was heterogeneous, despite an identical clinical phenotype.

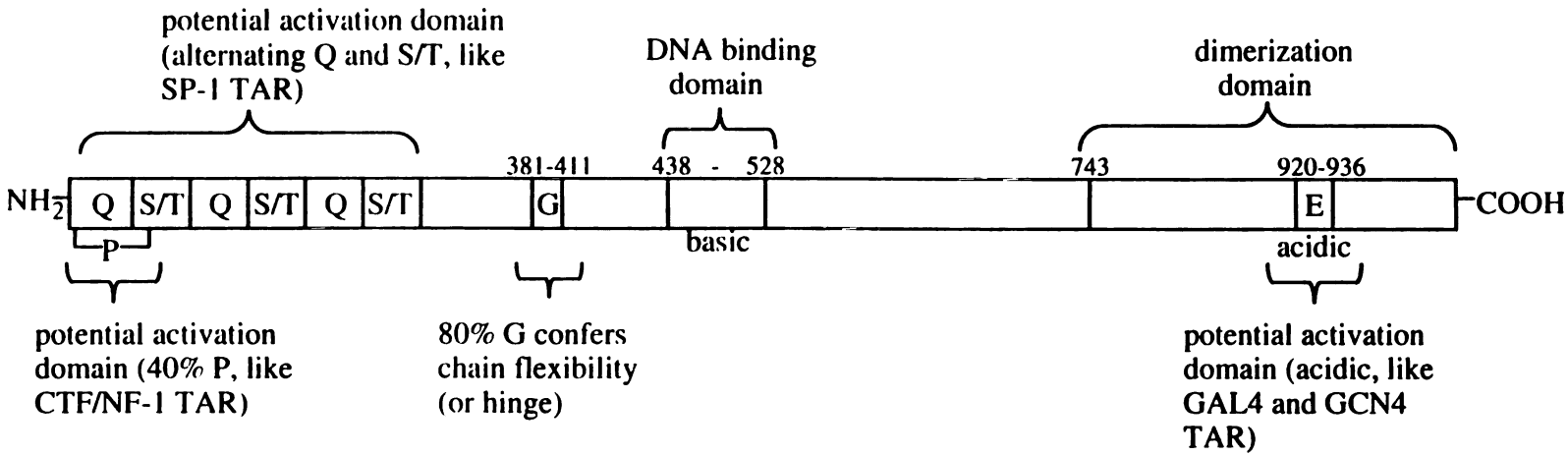


Figure 12. Structural analysis of RFX1 (54). TAR = transactivating region; SP1, CTF, NF-1, GAL4, and GCN4 are all known transcription factors; Q, S/T, P, G, and E - one letter code for amino acids.

There are now three known complementation groups (groups A, B, and C). In group A there is a defect in CIITA, a transactivator known to be essential for both constitutive and γ-IFN inducible expression of MHC II. In group B RFX-binding activity is defective. In group C addition of the 75-kD subunit of RFX known as RFX5 is enough to restore functional RFX binding in cells from patients with this defect. It is now known that there are at least 5 different members of the RFX family (Table III), along with RFX homologs found in yeast (*S. pombe* and *S. cerevisiae*) and nematodes (*C. elegans*) (32). The RFX proteins are now thought to be a family of DNA binding proteins conserved in the eukaryotic kingdom.

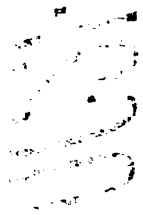
Table III. Regulatory Factor X family members

Name	**Year cloned**	**Cloned by** (first/last authors)	**Target site specificity**	**Suggested function**
RFX1	1990	Reith/Mach (54)	X-box sites (DRA > DPA, DQA); rpL30α and EF-C (MDBP) sites of rpL30 promoter and enhancers of HBV, polyomavirus, and CMV; methylated CpG MDBP sites	transactivator of HBV enhancer; other viral and cellular genes
RFX2	1994	Reith/Mach (55)	same as for RFX1	unknown; predominantly found in testis
RFX3	1994	Reith/Mach (55)	same as for RFX1	unknown; predominantly found in brain
RFX4	1992	Dotzlaw/ Murphy (30)	unknown (sequence of RFX4 found in aberrantly spliced estrogen receptor cDNAs from a human breast tumor)	unknown
RFX (RFX5)	1995	Steimle/ Reith (60)	RFX5 binds to the X-box only as part of the multimeric RFX complex; binds cooperatively with NF-Y (a Y-box binding protein)	essential for regulation of expression of MHC II genes

IV. Thesis objectives and organization of chapters

The overall goal of this project was to test the feasibility of using DNA dumbbells to block gene transcription (in particular, to block RFX transactivation of its target gene). There were three primary objectives: 1) synthesize and characterize DNA dumbbells; 2) utilize these DNA dumbbells and other double-stranded sequences for *in vitro* electrophoretic mobility shift assays (EMSAs or gel shift assays) with nuclear extracts containing DNA binding proteins (including RFX), and 3) test DNA dumbbells for functionality in a model system using a reporter gene assay. Upon reflection of the results obtained in pursuit of objective 3, a fourth objective was added: determine the mechanism by which DNA dumbbells decrease reporter gene activity.

Consistent with the above 4 goals the results have been divided into chapters. Chapter 1, Synthesis and Detection of DNA Dumbbells, is divided into 2 parts. Part 1 deals with the synthesis of DNA dumbbells with a focus on chemical vs. enzymatic ligation of self-complementary oligonucleotides. Part 2 describes sequential staining of short oligonucleotides in polyacrylamide gels with ethidium bromide and methylene blue



Chapter 2 describes RFX binding and competition with DNA dumbbells and other oligos using electrophoretic mobility shift assays (EMSAs). Chapters 3 and 4 are studies in model systems using reporter gene assays. Chapter 3, the results in Raji cells using pDRASCAT, shows that endogenous transcription of transiently transfected plasmids in Raji B-cells is blocked by "open" dumbbells but not by other double-stranded oligonucleotides. Finally Chapter 4 describes the results in Cos-7 cells using pRFX1VP16/P4XBCAT: sequence-independent inhibition of RNA transcription by DNA dumbbells and other decoys.

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Chapter 1. Synthesis and Detection of DNA Dumbbells

a. Synthesis of DNA Dumbbells: Chemical vs. Enzymatic Ligation of Self-Complementary Oligonucleotides

ABSTRACT

The chemical (cyanogen bromide) and enzymatic (T4 DNA ligase) ligation of five different self-complementary oligonucleotide sequences which form 14- or 16-base pair dumbbells are described and compared here. A review of both chemical and enzymatic methods is presented along with an improved enzymatic method. While both methods of ligation are effective, chemical ligation may be preferred because it is faster and less costly.

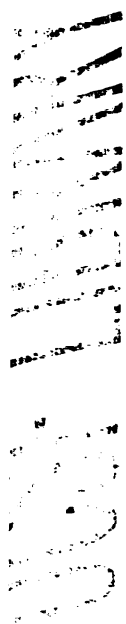
INTRODUCTION

DNA dumbbells have been used historically as physical models for analyzing local thermal stability in DNA (19), for examining hairpins, cruciforms, and locally melted domains within naturally occurring DNA polymers (9), for studying DNA conformations as substrates for various enzymes (2, 4) and for overcoming problems of double-strand oligomer dissociation when investigating nucleic acid drug targets (17). In addition to their utility as physical models, DNA dumbbells have biological relevance as aptamers or decoys for quenching or trapping proteins such as transcription factors (5, 6). Synthesis of DNA dumbbells has been achieved by enzymatically ligating a self-complementary (intramolecularly annealing) phosphorylated oligonucleotide using T4 DNA ligase (6, 9, 15, 19). Another method of ligation stems from the chemical "template-directed cyclization" using cyanogen bromide described by Kool (14). Template-directed polymerization of oligoadenylates on a poly (U) template was first described in 1986 by Kanaya and Yanagawa (13). No template is required for this ligation procedure because our oligos self-anneal (i.e., form their own template). This

chapter describes and compares an optimized enzymatic method of ligation and a chemical (BrCN) method of ligation, and demonstrates proof of ligation.

MATERIALS AND METHODS

Self-complementary oligonucleotides (oligos) were obtained from Keystone Laboratories or from Oligos, Etc. The sequences of the oligos used, their corresponding dumbbell structures, and a brief rationale for selection of sequences (expected activity in subsequent studies) are shown in Table IV. A more detailed explanation of the rationale behind sequence selection is discussed in subsequent chapters. Most oligos contain 4 T (thymidine) loops. The unl 5 T-X and 5 T-X dumbbell contain 5 thymidine loops.



Concentrations of reactants in all of the following reactions are listed in terms of final concentrations, unless noted otherwise. Oligos were phosphorylated as follows: 600 μM oligo, 3 units T4 polynucleotide kinase/nmol oligo, 2 mM ATP (Mg salt), 50 mM Tris HCl (pH 7.6), 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol were mixed together at 37°C. After 1 hour, the same amounts of ATP and T4 polynucleotide kinase were added, and the mixture was incubated for 24 hours. After phosphorylation, oligos were heated to 90-100°C for 10 minutes then cooled to room temperature over several hours to allow the oligos to anneal.

Chemical ligation was performed by incubating 50 μM phosphorylated oligo, 50 mM BrCN, 200 mM Imidazole (pH 7), 100 mM $NiCl_2 \cdot 6H_2O$ at 25°C for 24 hours (14). A two-fold excess of BrCN could be used without alteration in yield. For the enzymatic ligation 400 μM phosphorylated oligo, 3 units T4 DNA ligase/nmol oligo, 5% polyethylene glycol (PEG), 66 mM Tris HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol (DTT), and 66 μM ATP were incubated at 16°C for 48 hours.

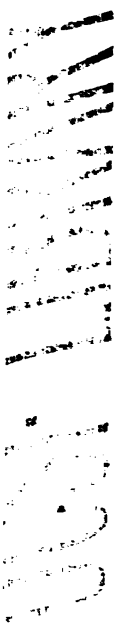
Ligation reactions contained 8 μg starting material for the chemical ligations and 5 μg for the enzymatic ligations. These reaction mixtures were mixed with formamide, boiled for 2 minutes, and quickly loaded onto 12% denaturing polyacrylamide gels with

Table IV. Oligonucleotide nomenclature, sequences, and brief rationale of sequence selection (expected activity). Underlined sequences are self-complementary; all dumbbells are self-complementary but are not underlined.

Oligo name and sequence	**Rationale**
unl (unligated) ctrl (control) 3:	control (inactive)
5'- TATACGGGTTTTCCCGTATACCACTCTGTTTTCAGAGTGG -3'	
ctrl 3 dumbbell:	control (inactive)
$_T{}^T$TCCCGTATACCACTCTG$^T{}_T$ $^T{}_T$GGGCATATGGTGAGAC$_T{}^T$	
unl hlyn:	active; see (21)
5'- TAACAACTTTTGTTGTTATAGTAACTTTTGTTACTA -3'	
hlyn dumbbell:	active; see (21)
$_T{}^T$GTTGTTATAGTAAC$^T{}_T$ $^T{}_T$CAACAATATCATTGT$_T{}^T$	
unl scrambled (scr) X:	scrambled control
5'- ATTTTTCCCTTTTGGGAAAAATTCCCCCCTTTTGGGGGGA -3'	(inactive)
scr X dumbbell:	scrambled control
$_T{}^T$GGGAAAAATTCCCCCCT_T $^T{}_T$CCCTTTTTAAGGGGGG$_T{}^T$	(inactive)
unl X:	wild-type (active)
5'- CTAGGGGTTTTCCCCTAGCAACAGATGTTTTCATCTGTTG -3'	
X dumbbell:	wild-type (active)
$_T{}^T$CCCCTAGCAACAGATGT_T $^T{}_T$GGGGATCGTTGTCTAC$_T{}^T$	
unl 5 T-X:	wild-type (active)
5'- CTAGGGGTTTTTCCCCTAGCAACAGATGTTTTTCATCTGTTG -3'	
5 T-X dumbbell:	wild-type (active)
$_T{}^T{}^T$CCCCTAGCAACAGATG$^T{}_T{}_T$ $^T{}_T{}_T$GGGGATCGTTGTCTAC$_T{}^T$	

ethidium bromide as described in (15). Ligation schematics are shown in Figure 13. The starting material in each ligation reaction was a single, intermolecularly annealing oligo (Figure 13, right).

All enzymatic reactions designed to show proof of synthesis were performed on the 5 T-X dumbbell product mixture at 37°C and were terminated by freezing to -20°C unless otherwise indicated. For Klenow exonuclease reactions, mixtures of 5 µg oligo, 6 units Klenow fragment, 66 mM Tris HCl pH 7.6, 6.6 mM $MgCl_2$, and 1 mM DTT in a reaction volume of 6 µl were incubated for either 8 or 12 hours (Figure 15). For S1 nuclease reactions, mixtures of 2.5 µg oligo, 2 units S1 nuclease, 250 mM NaCl, 30 mM sodium acetate pH 4.5, 1 mM $ZnSO_4$, and 5% glycerol in a total volume of 10 µl were incubated for either 5 or 20 minutes (Figure 16). *Mae* I reactions included 3 µg oligo, 10 units *Mae* I, 20 mM Tris HCl pH 8, 250 mM NaCl, 6 mM $MgCl_2$, and 7 mM mercaptoethanol in 25 µl total and were incubated for 24 hours (Figure 17). Lastly, shrimp alkaline phosphatase reactions containing 1.6 µg oligo, 100 mM Tris HCl pH 8, and 100 mM $MgCl_2$ in a total volume of 10 µl were incubated for 2 hours and terminated by heating to 65°C (Figure 18). For analysis, all enzyme reactions (on ligated dumbbell DNA or unligated starting material) were loaded with formamide onto 12% (19% for *Mae* I) denaturing polyacrylamide gels.



0.7 OD of gel purified unl X and X dumbbell were used for melting temperature experiments. Each sample was mixed with 7M urea and 1M Tris HCl, pH 7.5. Samples were boiled for 5 minutes and cooled before running on a Cary 3E Spectrophotometer. Samples were heated/cooled at a rate of 0.5°C/min. from 20°C to 95°C to 20°C (Figure 19).

For a quantitative comparison of the two reactions, the unl X and X dumbbell sequences were run on gels as previously, photographed, and scanned using the Bio-Rad GS 670 Imaging Densitometer. For analysis using Bio-Rad Molecular Analyst version 1.1..the gel images were inverted to obtain absorbance peaks. Gel bands were analyzed,

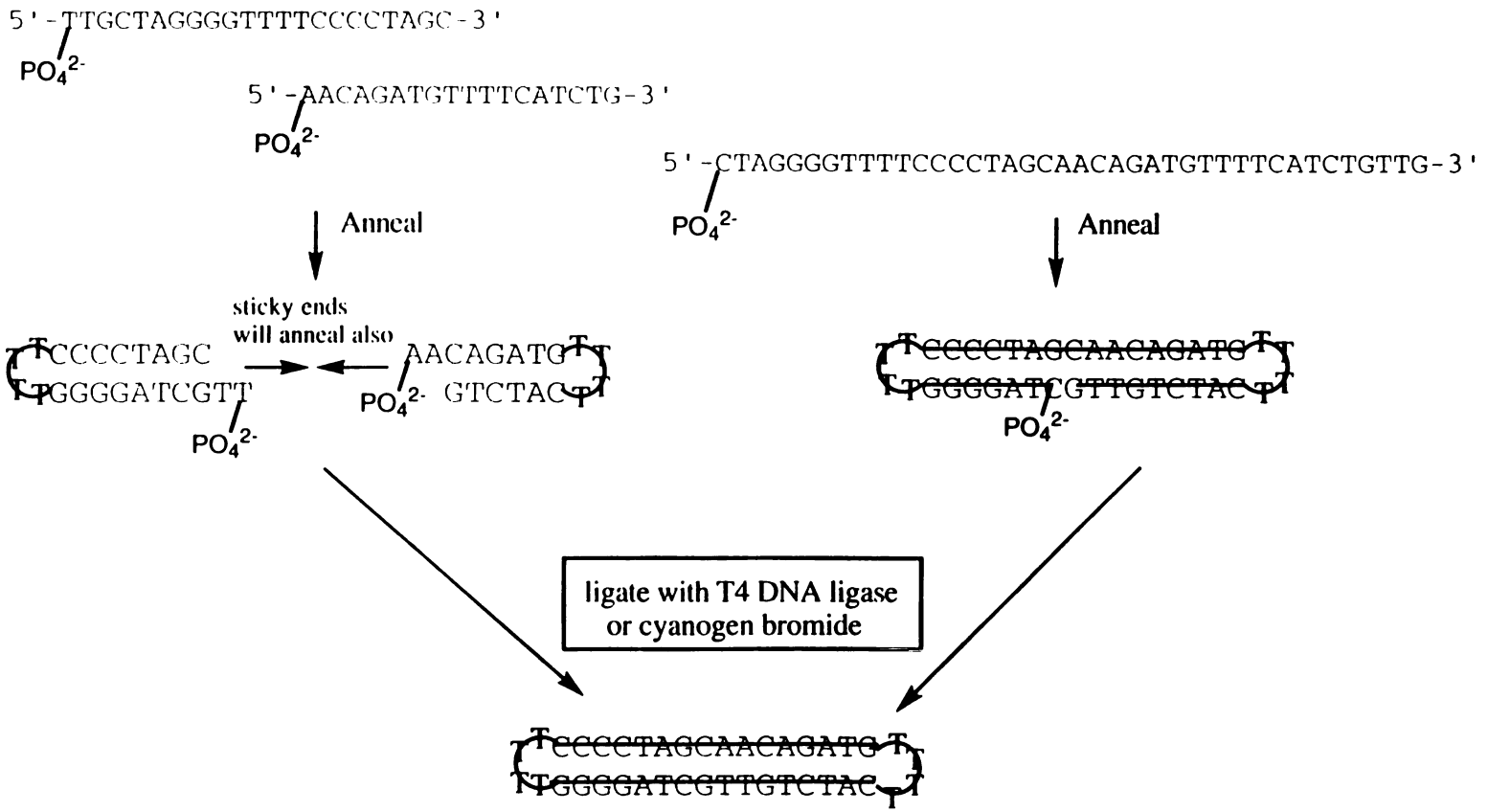


Figure 13. DNA dumbbell synthesis. Dumbbells may be synthesized from 2 intramolecularly annealing oligos with sticky ends (left) or from a single intramolecularly annealing oligo (right).

and absorbance vs. profile length (mm) was plotted to obtained a spectra with two major peaks (ligated and unligated, Figure 20). Area under the curve, AUC (obtained using Molecular Analyst), for each peak is indicated in Figure 20.

RESULTS AND DISCUSSION

For either the chemical or enzymatic method of ligation, proof of synthesis—covalent closure or formation of the dumbbell duplex—can be verified by altered electrophoretic mobility of product (1, 6, 15, 19) or by resistance of the product to degradation by various enzymes. These enzymes include exonucleases (9), single-strand specific nuclease (S1 nuclease) (6), alkaline phosphatase (9), or phosphodiesterase (6). Also, the existence of duplex formation within the dumbbell can be verified by sequence-specific cleavage by a restriction enzyme (1).

The chemically and enzymatically ligated dumbbells in Figures 14A and 14B were shown to migrate faster than their unligated counterparts for all sequences shown. Upon ligation, these oligonucleotides formed either 16 or 14 base-pair dumbbell structures. As shown in Figure 14, most reactions were incomplete, and reaction yields varied from sequence to sequence.

Further proof of ligation was demonstrated by resistance of the product to enzymatic degradation or by its susceptibility to restriction enzyme cleavage. An example is shown for the 5 T-X dumbbell. The 5 T-X dumbbell was found to be more resistant to the 5' to 3' exonuclease action of the Klenow fragment compared to its corresponding unligated sequence. After an 8-hour incubation with Klenow, the ligated sequence was unchanged whereas the unligated sequence was completely degraded (Figure 15, lanes 2a and 2b, respectively). After 12 hours, the ligated sequence showed signs of degradation as well (lane 3a).

After a 5 minute incubation, the 5 T-X dumbbell compared to the non-ligated sequence was degraded more slowly by S1 nuclease, a fairly single-strand specific

nuclease (Figure 16). After 20 minutes, S1 nuclease started degrading the loop ends of the dumbbell sequence (Figure 16, lane 3a).

The restriction enzyme *Mae* I cuts at "C↓TAG" and was used to show that the 5 T-X dumbbell DNA formed a recognizable, cleavable duplex. The 5 T-X dumbbell DNA should be cleaved into 2 segments, one a 15-mer and the other a 27-mer, by *Mae* I. Analysis of reaction products from *Mae* I treatment (Figure 17, lane 1) did indeed show 2 smaller fragments. The smallest fragment (Figure 17, lane 1, lower arrow) is very faint since the intensity of staining by ethidium bromide is proportional to the size of the DNA (20).

Figure 18 showed that shrimp alkaline phosphatase (SAP) cleaved the phosphate group from the phosphorylated unligated sequence, yet had no effect on the ligated dumbbell DNA. Two bands representing the phosphorylated and unphosphorylated forms (lane 2) were reduced to 1 band by SAP (lane 3). SAP had no effect on the dumbbell sequence (lanes 4 and 5). The results of the above reactions were not affected by shorter, failed sequence impurities which may appear in the purchased, non-HPLC purified starting oligo. These impurities appear as smears below main band in Figures 15-18.

Lastly, for further proof of ligation, a melting temperature experiment comparing purified unligated vs. ligated dumbbell was performed. As shown in Figure 19, the unligated and the ligated dumbbell structures (shown for unl X and corresponding dumbbell) clearly had different melting temperature profiles, suggesting a structural difference. The melting profile of the ligated dumbbell structure exhibited a cleaner transition and little or no hysteresis, compared to the unligated structure, as expected.

Chemical vs. enzymatic ligation yields were quantitated by scanning the gel photographs on an imaging densitometer as shown for unl X and X dumbbell in Figure 20. Due to the differential staining of double-stranded compared to single-stranded oligos (ethidium bromide stains double-stranded DNA more intensely (20)) only the

14 A) Chemical 1 2 3 4 5 6 7 8 9 10

14 B) Enzymatic 1 2 3 4 5 6 7 8 9 10

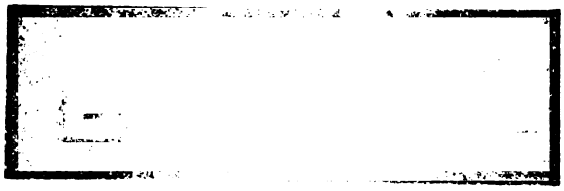


Figure 14A (chemical ligations) and **Figure 14B** (enzymatic ligations).

(1) unl ctrl 3; (2) ligated ctrl 3 dumbbell.

(3) unl hlyn; (4) ligated hlyn dumbbell.

(5) unl scr X; (6) ligated scr X dumbbell.

(7) unl X; (8) ligated X dumbbell.

(9) unl 5 T-X; (10) ligated 5 T-X dumbbell.

In all, upper band is unligated material; lower band is ligated material.

Note: Lanes 9 and 10 of the chemical ligation, Figure 14A, were run on a separate gel. Occasionally, oligos of this length contain shorter bands (Figure 14B, lane 5) which are either failed sequences or the full-length oligo folding back on itself. This is dependent on secondary structure formation of the newly synthesized oligo which sometimes is maintained despite highly denaturing conditions. Also, double bands may appear in the ligation product due to incomplete phosphorylation (Figure 14B, lane 10).

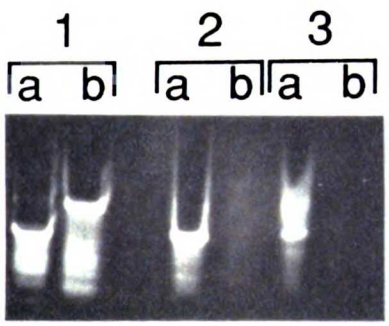


Figure 15. Klenow fragment (exonuclease) reactions. (1) no enzyme treatment; (2) 8 hour enzyme treatment; (3) 12 hour enzyme treatment. In (a), 5 T-X dumbbell and in (b) unl 5 T-X.

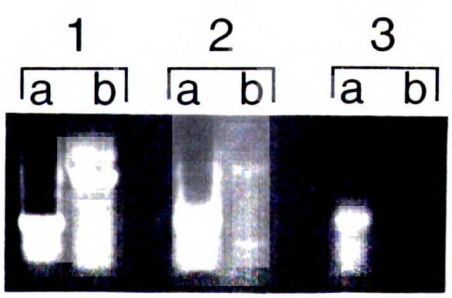


Figure 16. S1 nuclease reactions. (1) no S1 enzyme treatment; (2) 5 minute S1 treatment; (3) 20 minute S1 treatment. In (a), 5 T-X dumbbell and in (b) unl5 T-X .

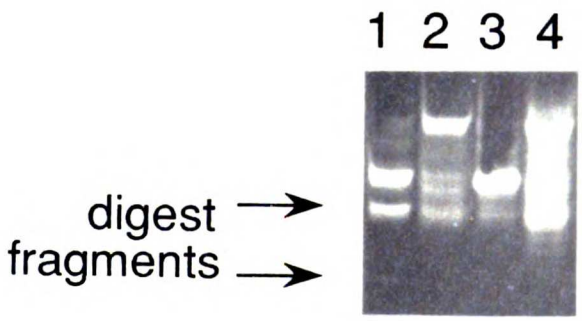


Figure 17. *Mae* I cleavage. (1) *Mae* I digestion of 5 T-X dumbbell; (2) *Mae* I digest of unl 5 T-X; (3) 5 T-X dumbbell; (4) unl 5 T-X

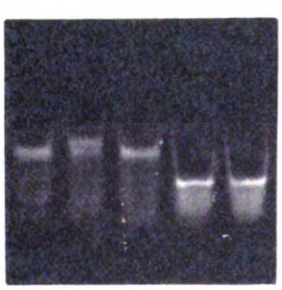


Figure 18. Shrimp alkaline phosphatase (SAP) reactions. Odd-numbered lanes were SAP-treated. (1) unl 5 T-X + SAP; (2) phosphorylated unl 5 T-X; (3) phosphorylated unl 5 T-X + SAP; (4) 5 T-X dumbbell; (5) 5 T-X dumbbell + SAP.

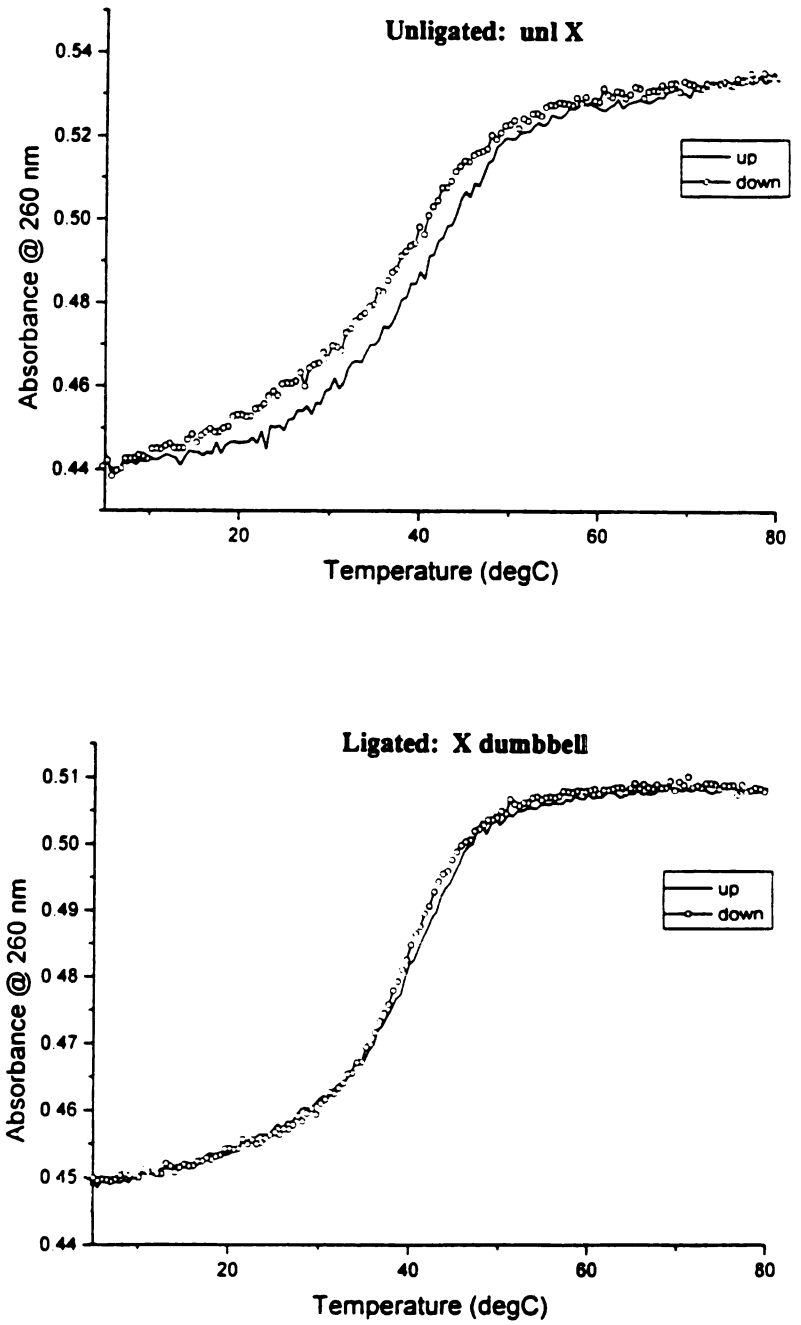


Figure 19. Melting temperature experiment. The unl X and X dumbbell sequences were subjected to heating/cooling (0.5°C/min.) from 20°C to 95°C to 20°C on a Cary 3E Spectrophotometer.

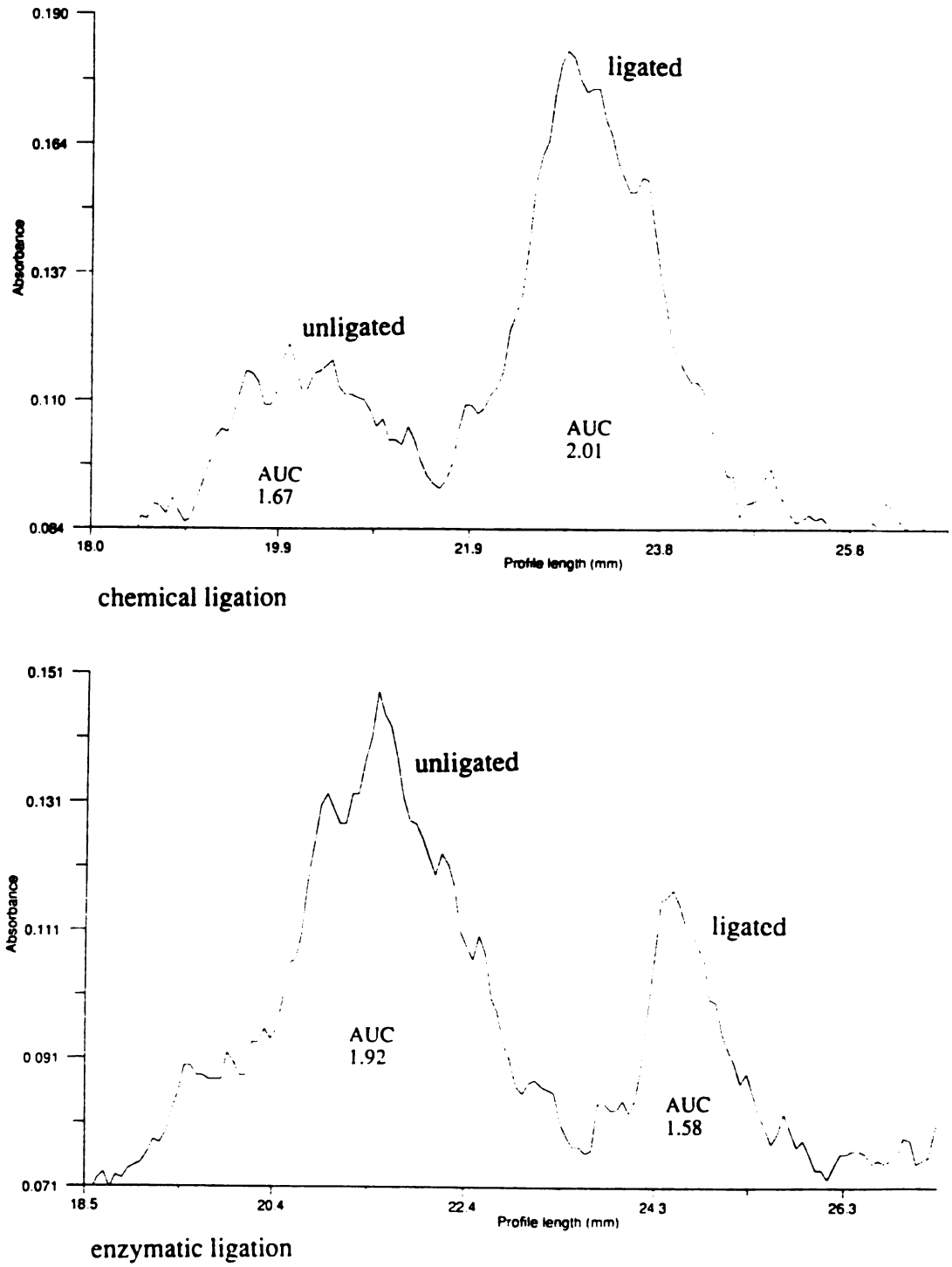


Figure 20. Scanning densitometer results for the chemical and enzymatic ligation reactions (unl X reactant, X dumbbell product). In this case, the yield for the chemical method was slightly higher (compare ligated AUCs).

relative yield of the ligated bands was compared. Apparently for this oligo sequence, the chemical ligation was more efficient (approximately 20% higher yield). Percent yield of these reactions may vary depending on the sequence of the oligos used as shown in Figures 14A and 14B.

The chemical method using cyanogen bromide described by Kool and others (7, 14) ligates *non*-self-complementary oligonucleotides by use of a complementary template oligo to position the 3' and 5 ' ends of the oligo in close proximity. The template is not incorporated into the final, circular oligo product. Since the self-complementary oligos described above form their own template, the 3'-OH and 5'-PO_4 ends are already near each other. This situation presents a simple, straightforward application of the cyanogen bromide method for DNA dumbbell synthesis. Recently, an improvement in the reaction rate of the cyanogen bromide method has been described (10), which uses N-morpholinoethanesulfonate instead of imidazole. In this case, the product is formed in minutes instead of 24 hours. An alternative chemical method uses 1-(3-dimethlyaminopropyl)-3-ethylcarbodiimide as the coupling agent (3), but requires longer reaction times (2-3 days). All of these methods require a phosphate on one end of the oligo. While we enzymatically phosphorylated our oligos, pre-phosphorylated oligos may also be purchased and used successfully for chemical or enzymatic ligations (data not shown).

While others have used an enzymatic method for ligating 5 T loop dumbbells (5, 6, 9) our enzymatic ligation method can successfully ligate 4 T loop dumbbells from single, self-complementary oligos. In a previous study (8, 9) failure to ligate a 4 T loop, 8 base-pair dumbbell was attributed to improper alignment of the oligo ends due to the distortion by the 4 T loop ends (8). However, Ashley and Kushlan attributed the failure to ligate this dumbbell on the substrate specificity of T4 DNA ligase (3) since they were able to ligate it chemically. Our enzymatic method, on the other hand, shows that 4 T loop dumbbells may be successfully ligated. This could reflect improved enzymatic

reaction conditions. Alternatively, it could reflect a possible substrate length requirement; i.e., a longer internal base-paired sequence may be required for proper recognition by T4 DNA ligase.

The concentrations of T4 DNA ligase used in published enzymatic ligation reactions varied widely from 1 U or less of enzyme per nmol oligo (6, 9) to 50 U or more of enzyme per nmol oligo (1, 19) which may be an important determinant of the successful outcome of ligation. Our enzymatic ligation method used a low amount of enzyme, 3 U/nmol, thus minimizing the cost of the reaction. Amaratunga *et al.* (1) also ligated 4 T loop dumbbells but used over 15 times more ligase. In general, yields of these ligation reactions may differ not only due to the method of ligation used but also due to the sequences, the loop size, and the composition of the starting oligonucleotide. Whereas the enzymatic method ligates the 5 T-X sequence better than the chemical method (Figures 14A and 14B, lanes 9 and 10), the chemical method ligates the scr X sequence better than the enzymatic method (Figures 14A and 14B, lanes 5 and 6). Another difference is that the enzymatic ligation reactions are performed on concentrated oligo solutions and may then be directly gel purified. In contrast, the chemical ligation reactions require larger volumes (lower oligo concentrations) and may require lyophilization prior to purification. Nevertheless, the chemical ligation method has advantageous overall. It is less time consuming and more cost effective. However, the potential toxicity of cyanogen bromide is a consideration.

b. Sequential Staining of Short Oligonucleotides in Polyacrylamide Gels With Ethidium Bromide and Methylene Blue

Oligonucleotides, commonly used as probes in molecular biology, are also utilized as antisense, triplex (11), and more recently, transcription factor quenching agents (12). Often these oligos are analyzed on polyacrylamide gels to determine their purity or to check for the presence of secondary structure (such as hairpins or dumbbells). Two non-radioactive stains are used to visualize such oligos in polyacrylamide gels: ethidium bromide and methylene blue. Methylene blue has been suggested as an inexpensive, non-toxic alternative to ethidium bromide (20) for staining both single- and double-stranded oligos. For most purposes methylene blue is adequate for staining short oligos. Ethidium bromide has an advantage when looking for oligo secondary structure because it preferentially stains double-stranded DNA. Due to its intercalative mechanism of interaction with DNA, ethidium bromide is inefficient for visualizing low concentrations of short single-stranded oligos. Methylene blue on the other hand interacts with DNA both by binding as an intercalator and by binding externally to DNA as a dimer (16). By using these dyes sequentially it is possible to both detect single- and double-stranded oligos at low concentrations and differentiate between them.

Table V illustrates the minimum amounts of single- and double-stranded oligos of variable sequences/lengths detectable using the two different dyes. Similar results were obtained using phosphorothioate and 3'-amino-modified oligos. Single-stranded oligos were loaded with formamide-containing sample buffer onto denaturing 12% polyacrylamide gels (4) for analysis. These gels consisted of 5 g urea, 4.69 ml 30% acrylamide solution, 1.88 ml 5 X TBE, 56 µl 10% ammonium persulfate, 3.5 µl TEMED, and 0.5 µl of 10mg/ml ethidium bromide solution. Double-stranded oligos were loaded with glycerol-containing sample buffer onto non-denaturing 20% gels (18), which consisted of 6.66 ml 30% acrylamide solution, 2 ml 5 X TBE, 1.27 ml water, 56 µl 10%

Table V. Approximate amounts (μg) of oligonucleotide detectable with methylene blue and ethidium bromide. A dilution series was performed to obtain this data.

	Approximate Amounts (μg) Detectable:	
Oligonucleotide	With Methylene Blue	With Ethidium Bromide
single-stranded 16mer 5'-CCCCTAGCAACAGATG-3'	0.05	>10
single-stranded 20mer 5'-GCCACGGAGCGAGACATCTC-3'	0.05	>1
single-stranded 40mer 5'-CTAGGGGTTTTCCCCTAGCAACAGATGTTTTCATCTGTTG-3'	0.05	0.01
double-stranded 16bp 5'-CCCCTAGCAACAGATG-3' 3'-GGGGATCGTTGTCTAC-5'	0.05	0.1
double-stranded 20bp 5'-GCCACGGAGCGAGACATCTC-3' 3'-CGGTGCCTCGCTCTGTAGAG-5'	0.05	0.05
double stranded 40mer decoy (16bp) T^{T}CCCCTAGCAACAGATG^{T}_{T} $^{T}_{T}$GGGGATCGTTGTCTAC_{T}^{T}	0.005-0.01	0.005-0.01

Note: oligos of the same length but different sequence may not stain comparably with ethidium bromide.

ammonium persulfate, 3.5 µl TEMED, and 0.5 µl of 10 mg/ml ethidium bromide solution. Both denaturing and non-denaturing gels were cast in 1 mm cassettes with 10-well combs (Novex, San Diego, CA) and electrophoresed using the Xcell Mini-cell system (Novex). Running buffer (1.25 X TBE) included 0.5 µg/ml ethidium bromide. After 1.5 hours of electrophoresis at 150 V (~15 mAmp), gels were removed from the cassette and photographed in the dark using a Polaroid camera and ultraviolet light source. Immediately after photography the gel was stained for approximately 5 minutes using 0.02% methylene blue. The gel was destained with 2-5 warm water washes until background was clear. Gels were then photographed on top of a fluorescent light box.

We have used this sequential staining technique to distinguish (on a 12% denaturing acrylamide gel) products from reactants in a ligation reaction (5) where the reactant is a 42 mer single-stranded, intramolecularly annealing oligo and the expected product is a closed, double-stranded, dumbbell-shaped oligo of the same length (Figure 21). Although the molecular weights of the product and reactant differ only by approximately 0.7% they migrate at significantly distinct rates due to their structural difference (5). This technique has also been successfully used to analyze ligation reactions involving oligos with different sequences and lengths. Ethidium bromide helps to distinguish between single-stranded reactants and double-stranded products while methylene blue allows quantitation of percent yield for the reaction. We are thus able to obtain more information from consecutively stained gels.

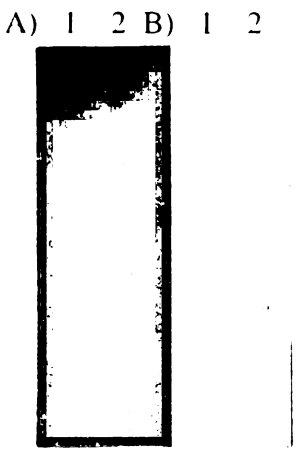


Figure 21. A. Ethidium bromide stained gel. Lane 1, 42mer product (2.8µg reaction mixture loaded). Lane 2, 42mer reactant (2µg). **B.** Gel stained with methylene blue after ethidium bromide staining.

Oligonucleotide Sequences:

Lane 1. 42mer product: $_{T}^{T}{}^{T}$CCCCTAGCAACAGATG$^{T}{}_{T}^{T}$
$_{T}^{T}$GGGGATCGTTGTCTAC$_{T}^{T}$

Lane 2. 42mer reactant: 5'- CTAGGGGTTTTTCCCCTAGCAACAGATGTTTTTCATCTGTTG -3'

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Chapter 2. RFX binding and competition with DNA dumbbells and other oligos: Electrophoretic mobility shift assays (EMSAs)

ABSTRACT

X-box dumbbells and other X-box oligos were evaluated in binding and competition studies with X-box binding proteins from Raji B-cell nuclear extracts. In our studies, any oligos with the "core X-box" sequence could compete with a longer double-stranded oligo containing the X-box for binding to X-box binding proteins. However, only the X-box dumbbell was able to directly bind to X-box binding proteins, including one identified as RFX1.

INTRODUCTION

The first step in evaluating possible transcription factor decoy targets is to identify protein-DNA interactions using electrophoretic mobility shift assays (also known as EMSAs or gel shift assays). Gel shift assays can be used as an initial screen for potential candidates for transcription factor-based therapeutics (2, 5). The principle of the EMSA is simple: isolate a source of the transcription factor(s) of interest which may be in a nuclear extract, and incubate the transcription factor with target DNA. After running this mixture on polyacrylamide gels, identify DNA-transcription factor complexes with altered mobility. In these studies, the transcription factors of interest were X-box binding proteins (which include the Regulatory Factor X or RFX family of transcription factors); the target DNA was the X-box. Raji nuclear extracts were utilized as a source of X-box binding proteins which contain RFX (9, 10, 14). Raji cells are Epstein-Barr virus- (EBV) positive, Burkitt's lymphoma human B-cells that constitutively express Major Histocompatibility Complex Class-II molecules (MHC II) on their cell surface. Since Raji cells constitutively express MHC II, they are a rich source of transcription factors that regulate MHC II gene expression. The DNA sequence known as the X-box is

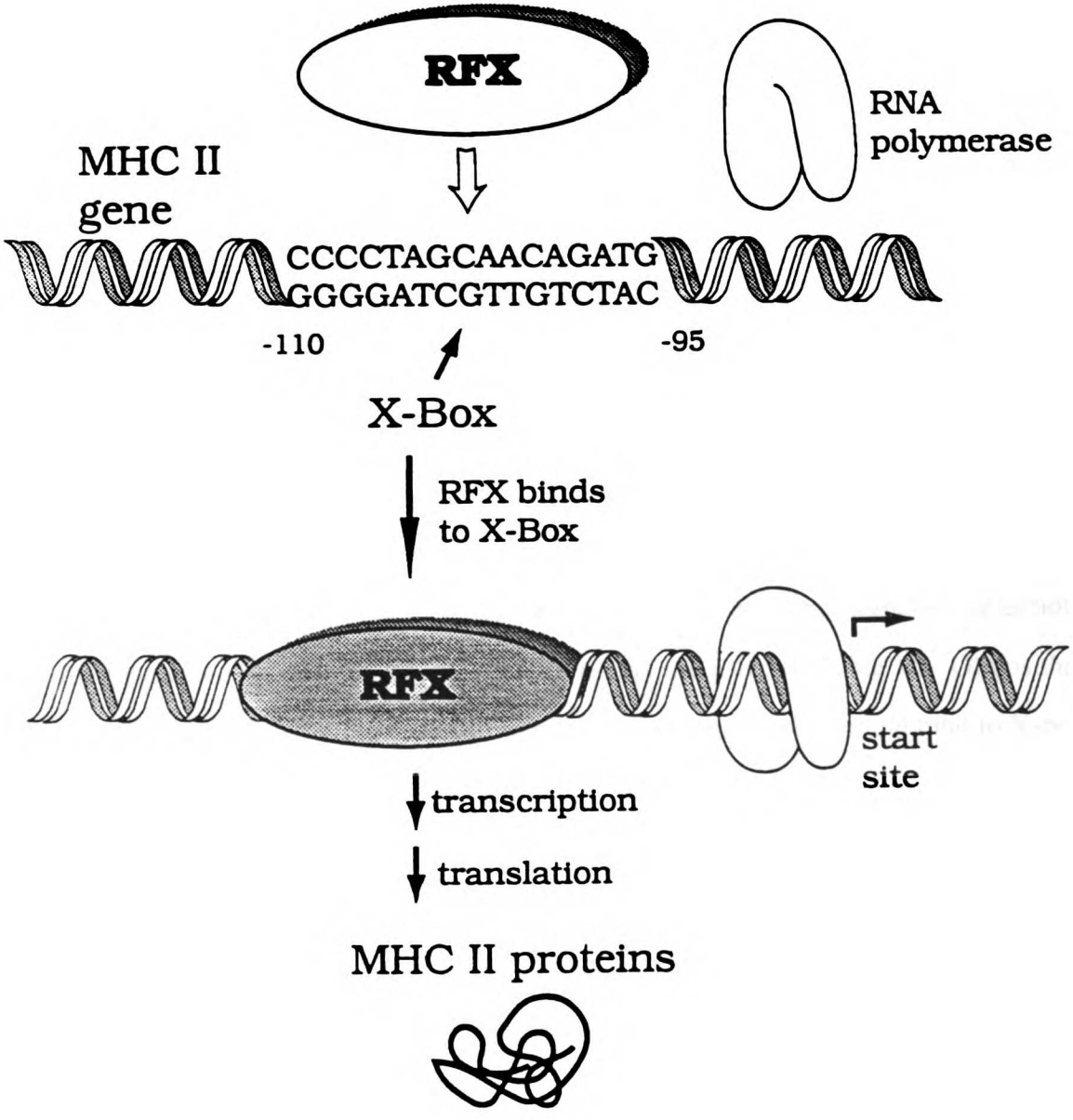

Figure 22. RFX binds to the X-box region of the MHC II gene to promote transcription.

located on the promoter region of the MHC II gene, and is vital for transcription of the MHC II gene (Figure 22). Mutations in the X-box region of the MHC II gene are not tolerated (9). The optimal conditions for RFX binding were modified from Hasegawa *et al.* 1991 (9).

While dumbbell DNA has been extensively studied as a physical model for various DNA conformations (1, 8, 21) little is known about its biological relevance; i.e., will dumbbell DNA be even be recognized by its associated transcription factor? On a rudimentary level, if dumbbell DNA has a specified DNA recognition sequence, it should. However, compared to a long, typical duplex segment of DNA, dumbbell DNA might adopt a different, abiotic conformation. Additionally, the transcription factor itself may require flanking sequences or other factors for binding in addition to the specific DNA recognition sequence.

MATERIALS AND METHODS

Oligonucleotide sequences, a detailed rationale for sequence/structure selection, and their expected binding activity in gel shift assays are listed in Table VI. Oligos that are designated with a "+" were designed to be active sequences that should bind to X-box binding proteins. Those designated with a "-" were designed as inactive or control sequences.

To prepare ^{32}P phosphorylated oligos/dumbbells, 10 pmol oligo was incubated with 1 U T4 polynucleotide kinase (USB), 20 µCi ^{32}P γ-ATP (Amersham), 50 mM Tris HCl (pH 7.6), 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol in a 10 µl volume at 37°C for at least 1 hour. The DRAX probe was similarly phosphorylated but not ligated. Ligated refers to dumbbell products, and unligated (unl) refers to the starting reactant oligo. To ligate ^{32}P-labeled oligos, 5 pmol of ^{32}P-labeled unligated oligo was reacted in a 10 µl volume with 1 U T4 DNA ligase, 5% polyethylene glycol, 66 mM Tris HCl pH 7.6, 6.6 mM $MgCl_2$, 10 mM dithiothreitol (DTT), and 66 µM ATP at 16°C for 48 hours.

Table VI. Oligonucleotide nomenclature, sequences, and rationale for sequence/structure selection (underlined sequences are self-complementary; all dumbbells are self-complementary but are not underlined).

Oligo name, sequence, rationale	**Expected binding activity (+ = active; - = inactive)**
unl X (unligated 4 T loop X-box): 5'- CTAGGGGTTTTCCCCTAGCAACAGATGTTTTCATCTGTTG -3' Rationale: contains the entire X-box sequence, the target of RFX. The unligated dumbbell structure was tested for comparison to the ligated structure (X dumbbell); 4 T loop size.	+
X dumbbell (ligated 4 T X-box dumbbell): $_{T}^{T}$CCCCTAGCAACAGATG$^{T}_{T}$ $^{T}_{T}$GGGGATCGTTGTCTAC$_{T}^{T}$ Rationale: contains the entire 16 base-pair double-stranded X-box sequence, the target of RFX; 4 T loops on each end; ligated version of unl X.	+
unl 5 T-X (unligated 5 T-loop X-box oligo): 5'-CTAGGGGTTTTTCCCCTAGCAACAGATGTTTTTCATCTGTTG-3' Rationale: same as unl X except the loop size is 5 instead of 4 (does loop size makes any difference in transcription factor binding?).	+
5 T-X dumbbell (ligated 5 T-loop X-box dumbbell): $_{T}^{T}$ ^{T}CCCCTAGCAACAGATG^{T} $^{T}_{T}$ $^{T}_{T}$ TGGGGATCGTTGTCTAC$_{T}$ $_{T}^{T}$ Rationale: ligated dumbbell version of unl 5 T-X.	+
unl ctrl1 (unligated control oligo 1): 5'-TGAGTTGGTTTTCCAACTCACTTTCCGCTTTTGCGGAAAG-3' Rationale: contains an irrelevant unligated control sequence (this and all other controls should not be able to bind to RFX) taken from Bielinska *et al.* (3).	-

Table VI (continued)

Oligo name, sequence, rationale	**Expected binding activity (+ = active; - = inactive)**

ctrl dumbbell 1 (ligated control dumbbell 1): -

$_{T}{}^{T}$CCAACTCACTTTCCGC$^{T}{}_{T}$
$^{T}{}_{T}$GGTTGAGTGAAAGGCG$_{T}{}^{T}$

Rationale: ligated version of unl ctrl 1.

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ns 10 (non-specific 10-mer): -

5'-GAATTCGGCC-3'

Rationale: a standard sequence used by Peterlin's group, a non-specific single-stranded oligo used as a gel shift assay control.

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unl ctrl 3 (unligated control oligo 3): -

5'-TATACGGGTTTTCCCGTATACCACTCTGTTTTCAGAGTGG-3'

Rationale: contains the "m6 mutant" mutation of the X-box known to abolish RFX binding (19).

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ctrl 3 dumbbell(ligated control dumbbell 3): -

$_{T}{}^{T}$CCCGTATACCACTCTG$^{T}{}_{T}$
$^{T}{}_{T}$GGGCATATGGTGAGAC$_{T}{}^{T}$

Rationale: ligated version of unl ctrl 1.

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ds X (double-stranded phosphodiester X-box oligo) and ds S-X (double-stranded phosphorothioate X-box oligo): **+ for both**

5'-CCCCTAGCAACAGATG-3'
3'-GGGGATCGTTGTCTAC-5'

Rationale: both ds X and ds S-X are double-stranded oligos which contain the X-box sequence, the target of RFX.

Table VI (continued)

Oligo name, sequence, rationale	**Expected binding activity (+ = active; - = inactive)**
DRAX, double-stranded DRAX probe (-116 to -87): 5'- CTTCCCCTAGCAACAGATGCGTCATC-3' 3'-CCGGGAAGGGGATCGTTGTCTACGCAGTAGAGCT-5' Rationale: contains the X-box and flanking sequences (which may be necessary for RFX binding); this sequence was used by the Peterlin lab in gel shift assays involving RFX. This sequence was created by restriction enzyme cleavage (hence the overhanging ends) using *Apa* I and *Xho* of a plasmid known as "pDRASCAT" which contains the X-box.	+

Ligation and purification of non-radiolabeled dumbbells are described in Chapter 1.

Radiolabeled oligos/dumbbells were run on 12% denaturing (8.5 M urea) polyacrylamide gels (0.25 X TBE, Tris-borate-EDTA) at 10 mA and 150V, for purification. Gels were exposed briefly to Kodak X-OMat AR film (X-ray film) to determine the position of the radiolabeled product for excision. Gel slices containing radioactive product from these reactions were excised and eluted overnight with 100 µl TE (10 mM Tris Cl, 1 mM ETDA, pH 7.6) at room temperature. Typical counts of 1 µl of radiolabeled oligos/dumbbells were in the range of 10,000 to 40,000 as determined by scintillation counting; volumes were adjusted to give the same specific activity for each oligo/dumbbell for subsequent gel shift assays.

Raji cells (ATCC CCL 86) were used to obtain nuclear extracts. Raji cells are human EBV-positive, Burkitt's lymphoma B-cells that constitutively express MHC II, and were derived from a 11-year old black male in 1964; these cells were submitted to the ATCC after the 100th passage in 1967 (ATCC catalog). Generally, Raji cells used in these experiments were fed every 2-3 days in RPMI 1640 medium with 10% fetal bovine

serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (292 µg/ml) and split 1:10 (cells:media) once a week.

Nuclear extracts were prepared from Raji cells essentially as described previously (12); the protocol is as follows, all at 4°C: 5 x 10^8 Raji cells were centrifuged at 1000 rpm for 10 minutes to pellet. Cells were then washed with PBS (without Ca^{2+} or Mg^{2+}) and respun for 5 minutes. Cell pellets were resuspended in 5 volumes Buffer A, incubated for 10 minutes, and centrifuged for 10 minutes to pellet cells. Buffer A consists of 10 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) or HEPES pH 7.8, 15 mM KCl, 20 mM $MgCl_2$, 0.1 M EDTA, 1 mM DTT , 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM sodium metabisulfite. Cells were then resuspended in Buffer A and homogenized using a Dounce homogenizer (60 strokes) on ice to release the nuclei. The cells were then pelleted by centrifuging 10 minutes at 2500 rpm. The pellet was then resuspended in 1 ml Buffer A. To extract protein from the nuclei, enough 4 M $(NH_4)_2SO_4$ (pH 7.9) was added to make 0.3 M, and rocked gently for 30 minutes. Next, the cell mixture was centrifuged at 36.5K rpm in an ultracentrifuge for 1 hour. The supernatant was then collected (DNA pellet discarded), mixed with 0.2 g $(NH_4)_2SO_4$, incubated on ice for 30 minutes, and centrifuged at 36.5K rpm for 15 minutes. The nuclear extract-containing pellet was then resuspended in 300 µl Buffer B (50 mM HEPES pH 7.8, 50 mM KCl, 0.1 mM EDTA, 1 mM PMSF, 10% glycerol, and 1 mM DTT) and dialyzed for at least 3 hours against 500 ml Buffer B. Finally, the dialyzed supernatant was cleared by centrifugation at 10,000 rpm for 10 minutes, and the protein content (> 10 mg/ ml) measured (using the Bio-Rad Bradford protein assay reagent) prior to freezing in liquid nitrogen and storage at -70°C.

Polyclonal anti-RFX1 antibody (αRFX1) was prepared from rabbits as described previously (22) and recognizes the amino terminus of recombinant RFX1 (provided by N. Jabrane-Ferrat).

Electrophoretic mobility shift assays

Competition assays

Nuclear extracts containing RFX1 were incubated with radiolabeled DRAX and various unlabeled competitors. In one case (Figure 23), either X dumbbell, unl X, ctrl dumbbell 1, unl ctrl 1, ds X, ds S-X, or ns 10 was used as a competitor at 150-fold excess over radiolabeled DRAX. In another case (Figure 24), either ds X, X dumbbell, unl X, ctrl dumbbell 3, unl ctrl 3, or ns 10 was used as a competitor at 30-fold, 15-fold, or 7.5-fold excess over radiolabeled DRAX. Specifically, the binding assay at 0°C (on ice) included nuclear extract (10 μg protein); 10, 2, 1, or 0.5 pmol (for 150-, 30-, 15-, and 7.5-fold excess, respectively) unlabeled competitor in the binding buffer described by Reith (13) with the addition of 1-2 μg of poly(dI.dC)·poly(dI.dC). After a 10 minute incubation, ^{32}P-labeled DRAX probe (0.066 pmol or 12,000 cpm) was added and incubated for 20 additional minutes. For this and the following EMSA gels (the super shift and the direct binding assay), the entire reaction mixtures were individually loaded in separate lanes of a 5% native polyacrylamide gel and run at room temperature with recirculating 0.25 X TBE buffer for 2-3 hours at 10 mA and 150V. After drying, gels were exposed to X-ray film for 1-3 days at -70°C.

RFX1 super shift with antibody

Nuclear extract was incubated with either 2 μl of αRFX1 or 10 pmol unlabeled competitor oligo (either DRAX or X dumbbell) under the conditions described above, with the addition of 50 ng *E. coli* DNA (to compete out some of the non-specific protein binding (6)) and 0.1% Nonidet P-40 at the start of the incubation. After 10 minutes, radiolabeled DRAX was added, and the mixture was incubated for an additional 20 minutes.

Direct binding assay

Nuclear extracts were incubated with radiolabeled oligos/dumbbells (0.066 pmol or 15,000 cpm) for 20 minutes (without unlabeled competitor, except for lane 2) at 0°C in the binding buffer described by Reith (13). The following radiolabeled oligos were individually tested for binding to RFX: ds X, X dumbbell, unl X, ctrl dumbbell 1, unl ctrl 1, 5 T-X dumbbell, and unl 5 T-X.

RESULTS AND DISCUSSION

All oligonucleotides/dumbbells with the core X-box sequence were able to compete for binding to X-box binding proteins (RFX1)

In order to determine which oligos or dumbbells interact with X-box binding proteins, EMSAs were performed. B-cell nuclear extracts were first incubated with a radiolabeled 26 base-pair X-box probe (DRAX) with or without competitor oligos or dumbbells. RFX- DNA complexes typically appear as a "doublet of low electrophoretic mobility" in the position shown on these gels (6). The unlabeled competitors, X dumbbell, unl X, ds X, and the ds S-X (Figure 23, lanes 3, 4, 7, and 8, respectively) at 150-fold excess successfully competed with DRAX for binding to X-box binding proteins. Interestingly, the phosphorothioate (ds S-X) competed off other DNA binding proteins as well (Figure 23, lane 8), suggesting that it may non-specifically interact with other proteins. Oligos with irrelevant sequences were unable to compete for the formation of shifted complexes (Figure 23, lanes 5, 6, and 9).

Unl X was a better competitor than its corresponding ligated sequence, the X dumbbell

Different concentrations of competitors were used in another EMSA to determine which oligos and dumbbells were the best competitors. Unl X successfully competed at the lowest concentrations (Figure 24, lanes 7-9). Unl X was able to compete with the DRAX

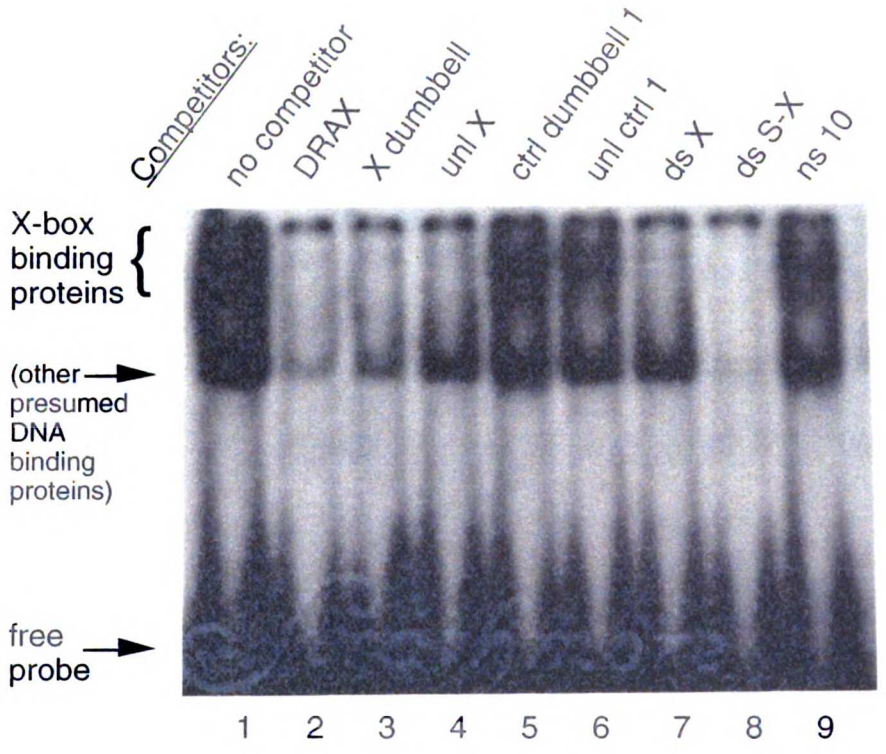

Figure 23. Competition gel. In all lanes, competitor oligos were added, at 150-fold excess, to ^{32}-P labeled DRAX probe incubated with nuclear extract. Other presumed DNA binding proteins are indicated on the gel (10, 22).

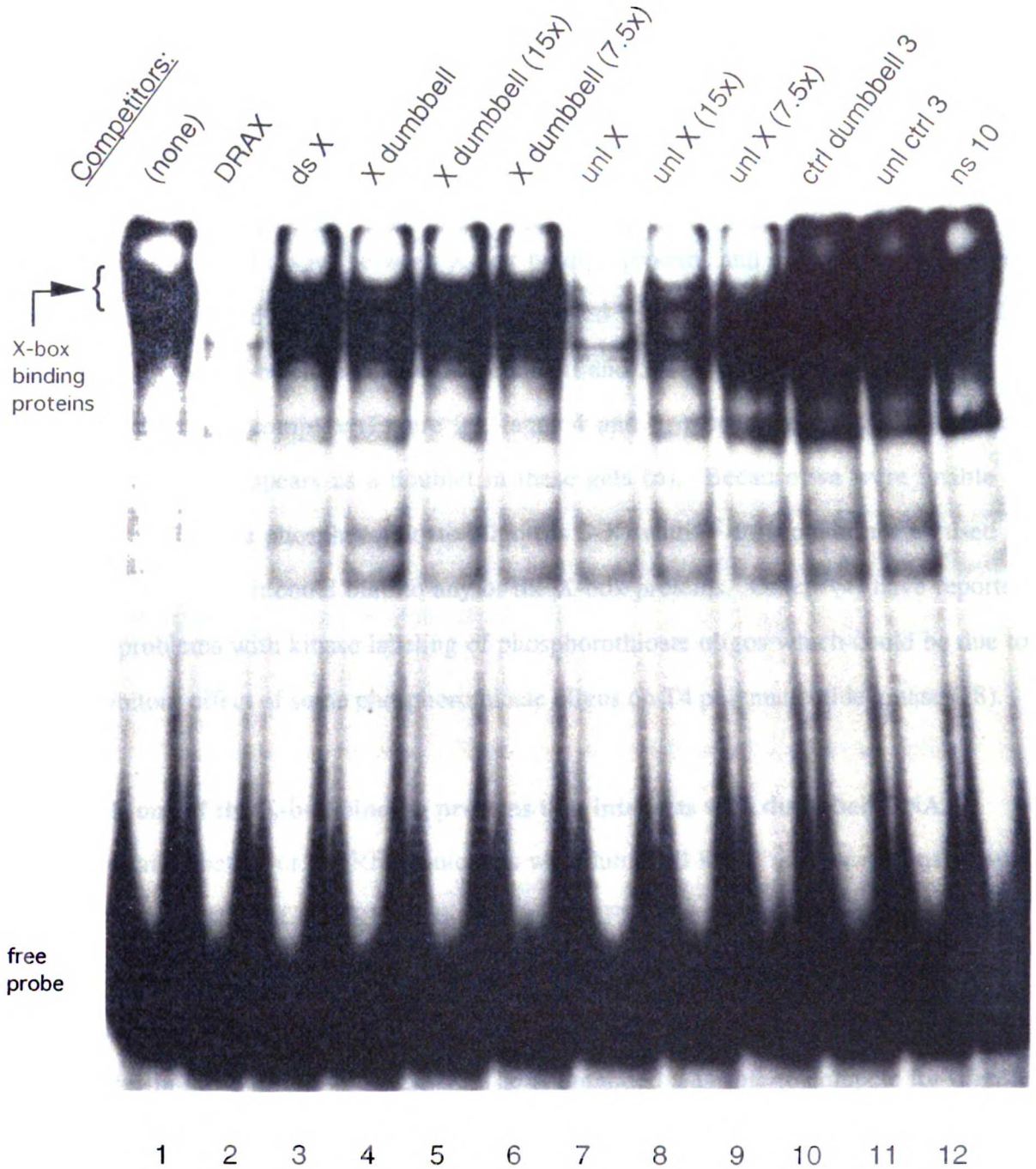

Figure 24. Competition gel with different competitor concentrations. Competitor concentrations are 30-fold over probe unless otherwise indicated in parenthesis. In all lanes, competitor oligos were added to ^{32}-P labeled DRAX probe incubated with nuclear extract.

probe at a 30:1 competitor:probe ratio for binding to X-box binding proteins (Figure 24, lane 7).

Only the ligated X-box dumbbells (X dumbbell and 5 T-X dumbbell) could bind to X-box binding proteins (RFX1)

Next, direct binding assays between X-box binding proteins and oligos/dumbbells were performed. B-cell nuclear extracts were incubated with radiolabeled oligos/dumbbells. Only the ligated X-box dumbbells, X dumbbell and 5 T-X dumbbell, were capable of forming a shifted complex (Figure 25, lanes 4 and 8, respectively). As mentioned previously, RFX appears as a doublet in these gels (6). Because we were unable to efficiently label the phosphorothioate oligo (ds S-X) with ^{32}-P, it could not be used to determine whether it could bind to any of the X-box proteins. Others (4) have reported similar problems with kinase labeling of phosphorothioate oligos which could be due to the inhibitory effect of some phosphorothioate oligos on T4 polynucleotide kinase (18).

RFX1 is one of the X-box binding proteins that interacts with dumbbell DNA

To ascertain whether or not RFX1 interacts with dumbbell DNA, a super shift assay was utilized. Inclusion of anti-RFX1 antibodies (αRFX1) in the EMSA binding reaction resulted in a decrease in the electrophoretic mobility of the complex, indicating that it contained RFX1. The larger complex (Figure 26, lane 1, a) was identified as RFX1 by super shifting by anti-RFX1 antibody. The X dumbbell was able to compete for (Figure 26, lane 4, a) and weakly bind to (Figure 25, lane 4) the RFX1 containing complex and to the lower unidentified complex as well (Figure 26, lane 4, b). This lower band is most likely another member of the RFX family (15, 16).

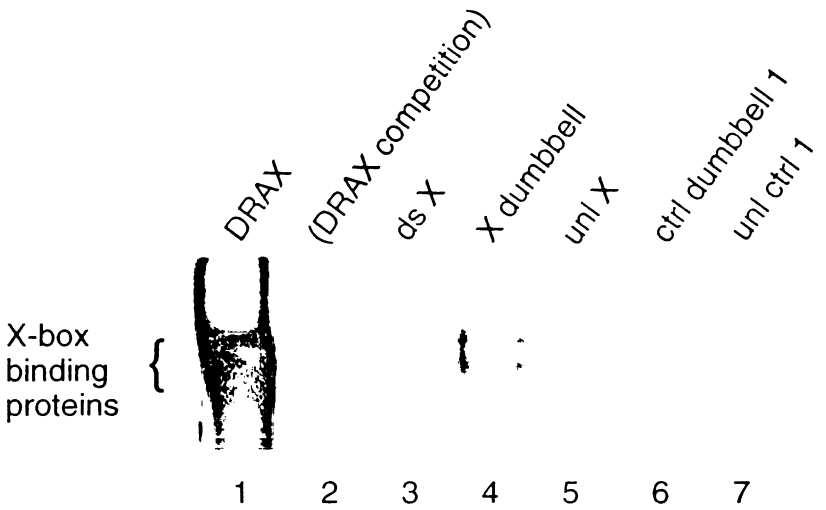

Figure 25. Direct binding gel. ^{32}-P labeled oligos (listed above the gel) were tested for their ability to directly bind to RFX. For lane 2, DRAX competition, unlabeled DRAX was added at a 150-fold excess as in Figure 23.

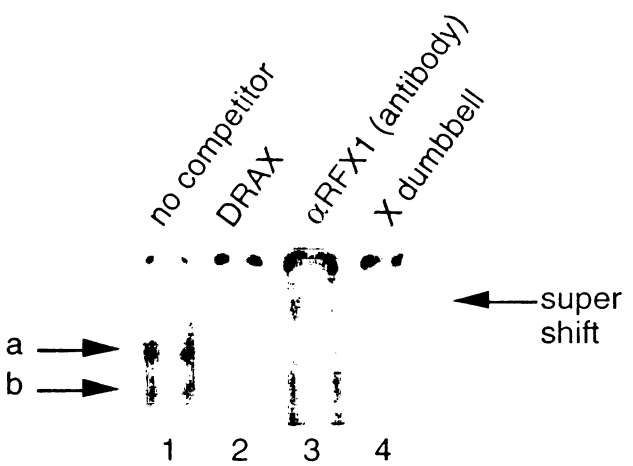

Figure 26. Super shift. All lanes contain ^{32}-P labeled DRAX, with various competitors (listed above gel). Lane 1, RFX binding to DRAX, indicated by bands a and b; lane 2, unlabeled DRAX at 150-fold excess over labeled DRAX; lane 3, αRFX1 (antibody) super shifting of band a; lane 4, X dumbbell at 150-fold excess competes off both bands a and b.

Multiple groups have suggested that transcription factors are an ideal target for therapeutics (2, 3, 5, 11). A simple, straightforward approach is to utilize information known about the interaction between a transcription factor and the precise sequence-specific DNA target. Given a particular transcription factor, and knowing its DNA target, one can create a specific decoy without much additional information. *In vitro* binding and competition studies can serve as the first step in evaluating the transcription factor-decoy interaction.

In this study we provide further evidence that dumbbell DNA can serve as decoys or mimics for transcription factors. Any oligo with the core X-box sequence could compete with the longer, "native" DRAX probe for binding to X-box binding proteins. These core X-box sequence oligos include ligated and unligated X-box dumbbells (X dumbbell and unl X, respectively); a double-stranded X-box (ds X), and a double-stranded phosphorothioate X-box (ds S-X). Apparently the thymidine loops on each end of the dumbbell do not interfere with RFX-DNA recognition. While the phosphorothioate ds S-X was also able to compete, it exhibited considerable non-specific affinity for other proteins, which has been demonstrated before (4). Unexpectedly, the unligated X-box dumbbell sequence, unl X, was a better competitor than its corresponding ligated sequence, the X dumbbell. The significance of this is not precisely known; however, one might speculate that unl X could be interacting directly with the double-stranded probe (DRAX) to prevent it from binding to RFX.

In direct binding studies, only ligated X-box dumbbells (X dumbbell and 5 T-X dumbbell) were able to bind directly to RFX proteins, including RFX1 (as identified by anti-RFX antibodies). In addition, unlike the ds X-box, X dumbbells can bind to X-box binding proteins (specifically RFX1), albeit weakly, which probably represents an increase in stability of DNA-protein interactions. Intriguingly, the 4 T loop X-box dumbbell bound the targeted protein better than the 5 T X-box dumbbells (under these

conditions). This differential affinity could possibly be due to a steric effect or to potential unfavorable interactions imposed by the larger 5 T-loops.

Although we have not precisely identified all of the X-box proteins that can bind to dumbbell DNA, one of these proteins is RFX1. Decoys for RFX1 may be of therapeutic interest since RFX1 has been implicated in γ-interferon induction of MHC II genes in monocytes (16) and may be a transactivator for a variety of viral and cellular genes (15).

DNA decoys come in several different forms, much like antisense oligonucleotides. Unmodified double-stranded oligonucleotides may be considered "first generation" DNA decoys. Second generation DNA decoys are an improvement due to decreased susceptibility to nucleases, via modified backbones (chiral modifications or phosphorothioates) or capped ends (DNA dumbbells). Along these same lines, third generation decoys may be non-DNA structures such as PNA (7), or small molecule mimics (17). The optimal type of decoy to use may vary from system to system, depending on the specific transcription factor-DNA interactions. Interactions between the decoy and the transcription factor may extend beyond simple base-pair recognition. Proteins may rely not only on base-specific hydrogen bonding for recognition of DNA, but also on overall structural features imposed by that particular sequence of DNA (i.e., groove geometry) (20). These sequence-specific determinants are more likely to be present in natural, unmodified DNA structures such as dumbbell DNA rather than decoys with modified backbones. The next step in the evaluation process is to test dumbbells for functional activity in a model system using a reporter gene assay (Chapter 3).

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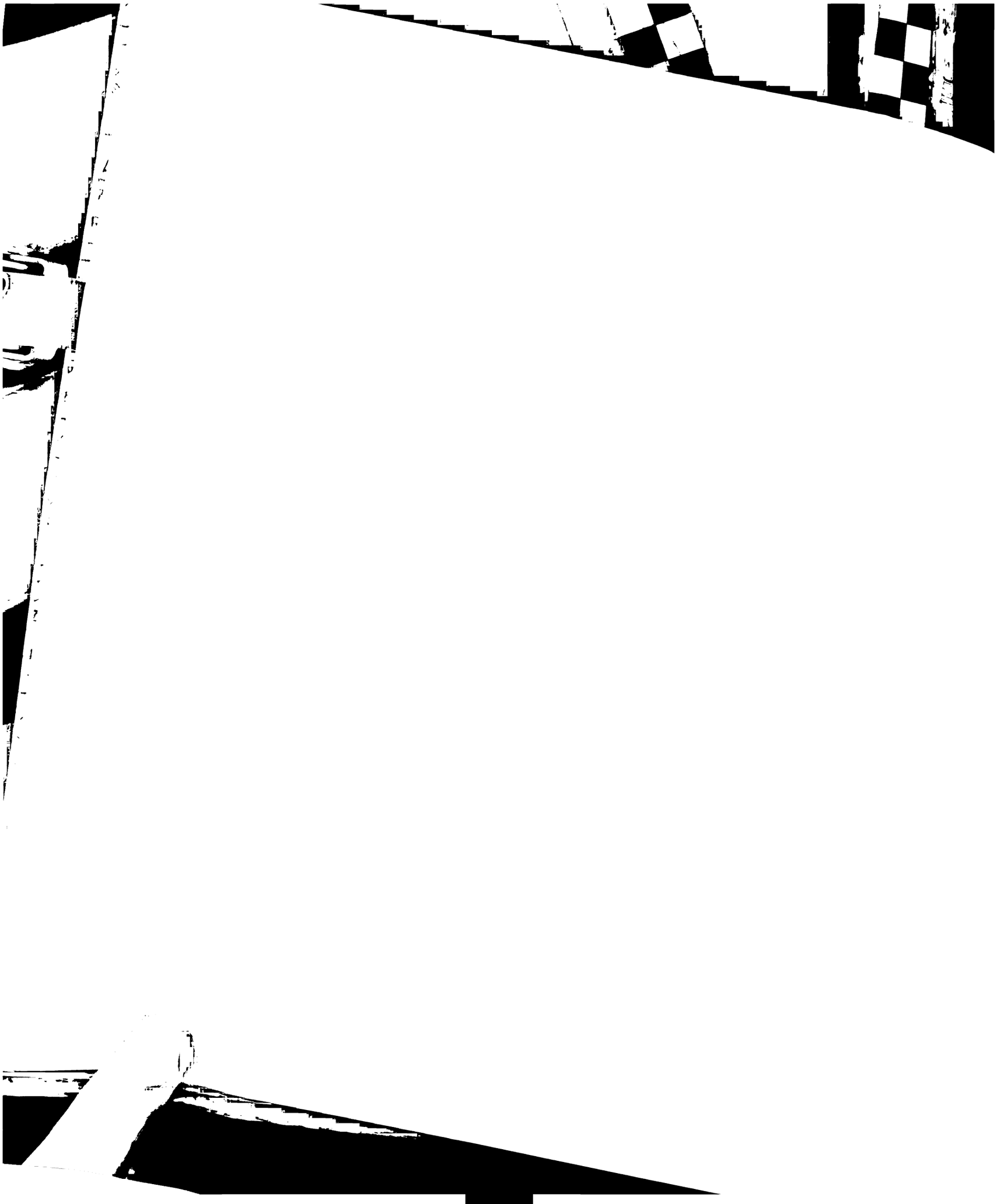

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Chapter 3. Results in Raji cells using pDRASCAT: Endogenous transcription of transiently transfected plasmids in Raji B-cells is blocked by dumbbells but not by other double-stranded oligonucleotides

ABSTRACT

DNA dumbbells were tested for biological activity in a reporter gene assay. Specifically, the ability of X-box dumbbells (X dumbbells) to down-regulate a plasmid containing the DRA promoter-driven CAT gene (pDRASCAT) was tested. The X-box sequence is a positive regulatory motif found within the DRA promoter of MHC class II genes. When pDRASCAT is transfected into RFX1-containing Raji cells, RFX1 binds to the X-box of the DRA promoter to drive CAT production. Results showed that ligated and unligated X dumbbells, as well as other irrelevant sequence dumbbells, were able to down-regulate transcription of the pDRASCAT gene. However, active oligos had no effect on levels of constitutively expressed MHC class II. In addition, dumbbells had an effect on 2 other unrelated reporter genes. Overall the results suggest a sequence-independent, but structurally specific effect of dumbbell DNA in this system.

INTRODUCTION

Transient transfection of a reporter gene followed by a subsequent analysis of gene product level is a commonly used procedure (5) for *in vivo* analysis of gene expression. Typically, the reporter gene has promoter and enhancer elements which drive the production of a reporter gene such as CAT (chloramphenicol acetyl transferase). Expression of such a reporter gene is governed by both general and specific transcription factors which interact with the gene. The reporter gene assay is ideal for testing the effect of oligos designed to specifically inhibit a transcription factor. An oligonucleotide (such as a DNA dumbbell) with the same sequence as a given promoter should be able to bind (decoy) the specific transcription factor and thus reduce its ability to interact with the

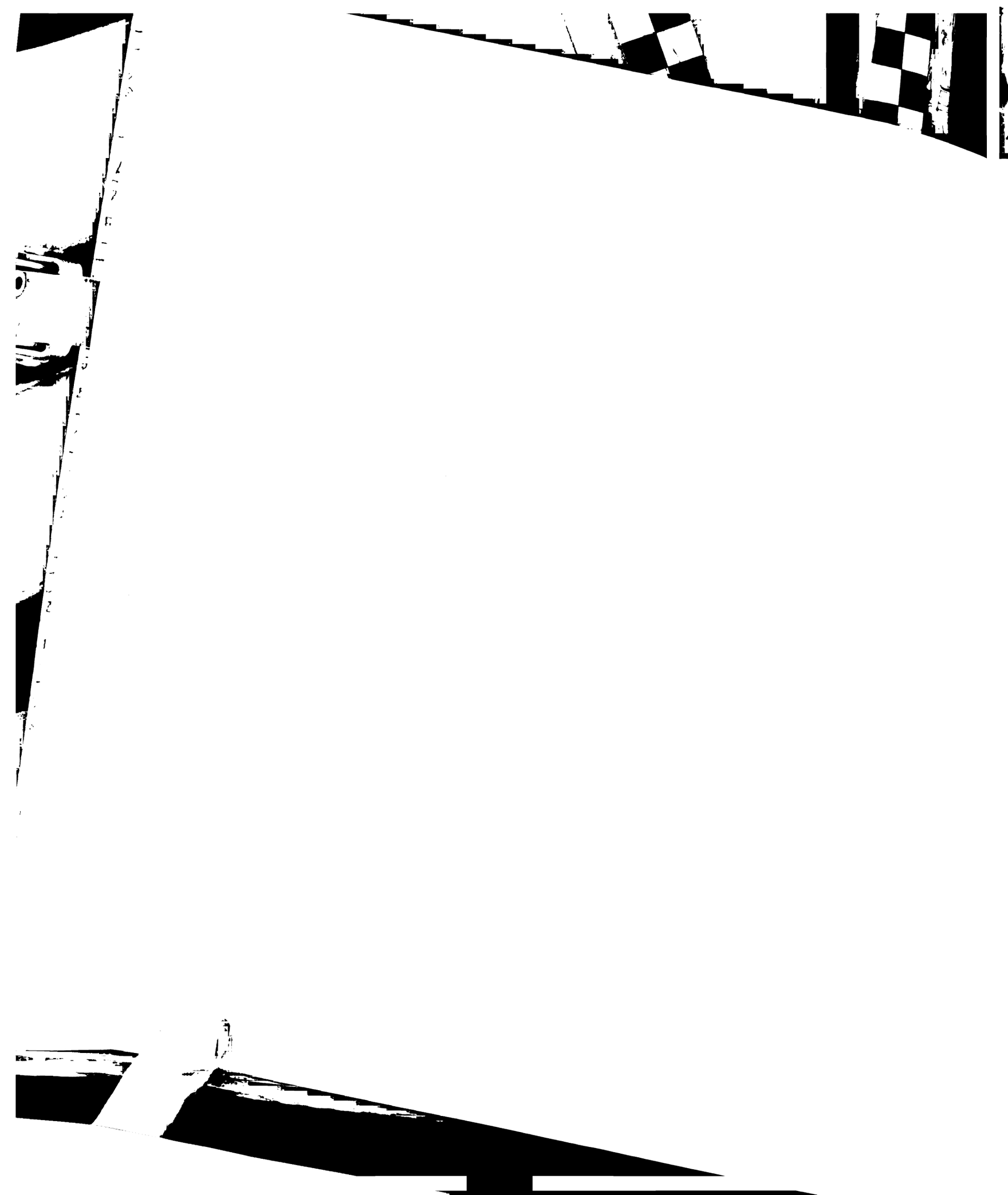
promoter on the reporter gene (Figure 27). Because the decoy-bound transcription factor can no longer interact with the reporter gene, the net result is a reduction in gene expression.

DNA dumbbells with loop composition of all thymidines (T's) with loop size of four nucleotides were chosen for the initial studies based on results from several DNA hairpin studies. Blommers *et al.* (3) showed that a particular DNA hairpin was maximally stable when the loop was comprised of four (or five) nucleotides. Additionally, Senior (9) found that for their specific hairpin sequence, homonucleotide loops allowed the formation of the most stable hairpin duplexes. Other hairpin experiments utilized A-loops and highly stable CTTG-loops (1).

The internal duplex sequence of the DNA dumbbells was designed to mimic the X-box of the DRA promoter. This promoter is essential in regulating expression of the Major Histocompatibility Complex (MHC) Class II gene. The X dumbbell contains the full length 16 base-pair X-box (as found in the gene). The X-box is a positive regulatory motif conserved in all MHC II promoters. Several transcription factors appear to target that sequence, including the RFX family of transcription factors. For comparison, other sequences, such as a double-stranded X-box (ds X), a double-stranded phosphorothioate X-box (ds S-X), a single-stranded X-box (ss top X), a hairpin X box, and various control oligos were tested as well. Additionally, various oligos were tested for their ability to alter levels of constitutively expressed MHC class II as measured by fluorescence activated cell sorting.

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotide nomenclature, sequences, and rationale for sequence selection are described in Table VII.



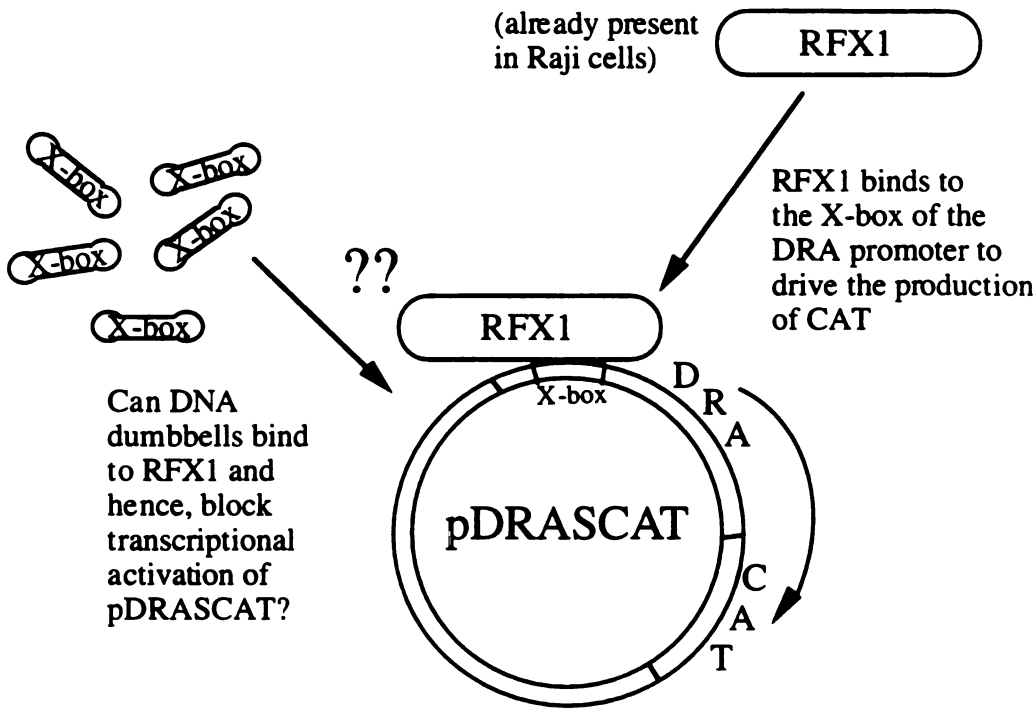


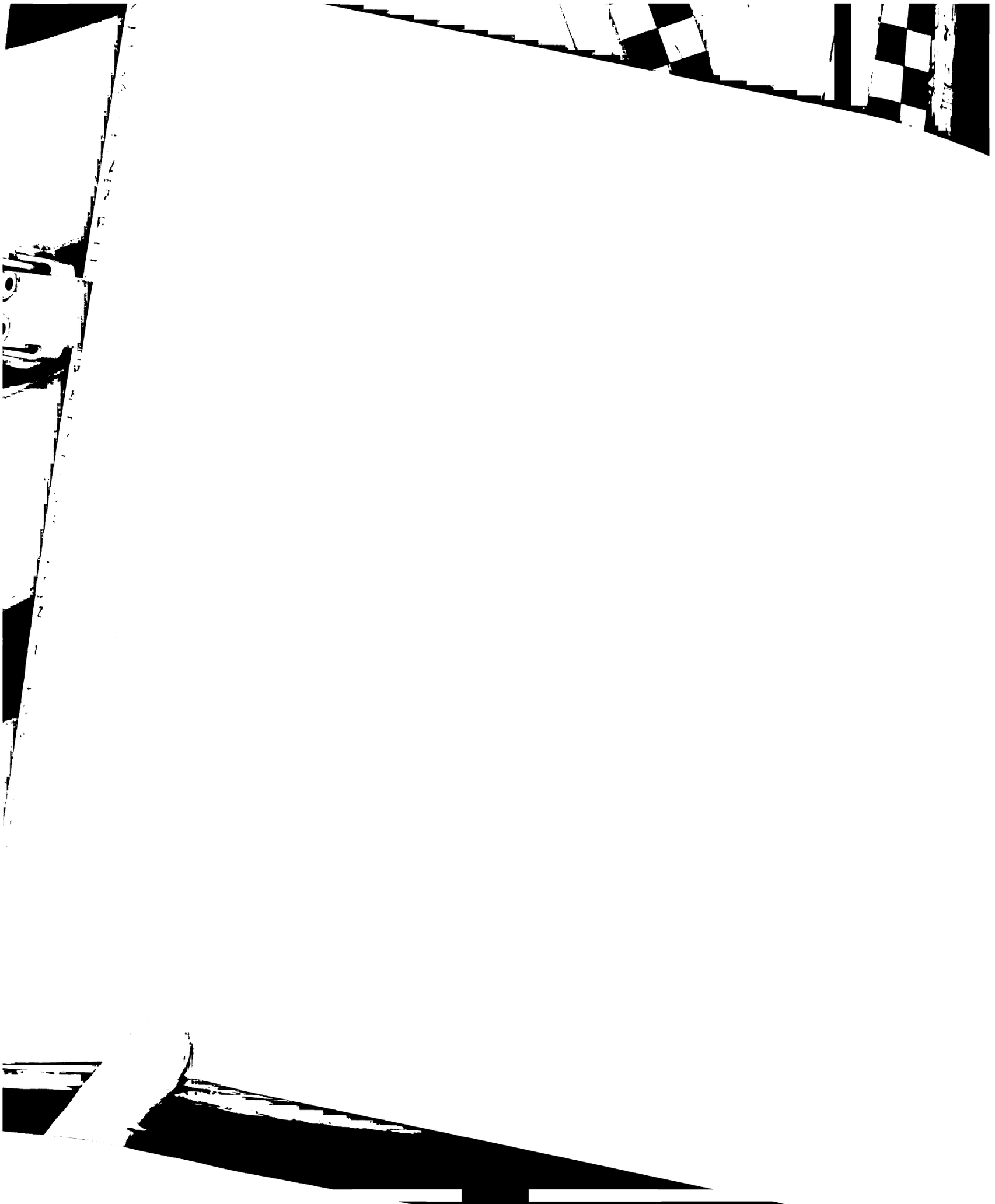
Figure 27. DNA dumbbells are designed to mimic a specific promoter sequence (e.g. act as decoys), the target of a particular transcription factor. In this figure, the specific promoter sequence is the X-box, and the transcription factor is RFX1.

Table VII. Oligonucleotide nomenclature, sequences, and rationale for sequence/structure selection (underlined sequences are self-complementary; all dumbbells and hairpins are self-complementary but are not underlined).

Oligo name, sequence, rationale	**Expected activity (+ = active; - = inactive)**
unl X (unligated 4 T loop X-box): 5'- CTAGGGGTTTTCCCCTAGCAACAGATGTTTTCATCTGTTG -3' Rationale: contains the entire X-box sequence, the target of RFX. The unligated dumbbell structure was tested for comparison to the ligated structure (X dumbbell); 4 T loop size.	+
X dumbbell (ligated 4 T X-box dumbbell): $_{T}^{T}$CCCCTAGCAACAGATG$^{T}_{T}$ $^{T}_{T}$GGGGATCGTTGTCTAC$_{T}^{T}$ Rationale: contains the entire 16 base-pair double-stranded X-box sequence, the target of RFX; 4 T loops on each end; ligated version of unl X.	+
unl CTTG-X (unligated CTTG-loop X-box): 5'-CTAGGGGCTTGCCCCTAGCAACAGATGCTTGCATCTGTTG-3' Rationale: same internal sequence as unl X; loops contain thermodynamically stable "CTTG" loops; more stable than T loops	+
unl A-X (unligated 4 A-loop X-box): 5'-CTAGGGGAAAACCCCTAGCAACAGATGAAAACATCTGTTG-3' Rationale: same internal sequence as unl X; A loops used (slightly less stable than T loops).	+
unl hlyn: 5'- TAACAACTTTTGTTGTTATAGTAACTTTTGTTACTA -3' Rationale: internal sequence should bind better to RFX than the wild-type X-box; see Ch. 1 (21)	+

Table VII (continued)

Oligo name, sequence, rationale	Expected activity (+ = active; - = inactive)
unl ctrl 1 (unligated control oligo 1): 5'-TGAGTTGGTTTTCCAACTCACTTTCCGCTTTTGCGGAAAG-3' Rationale: contains an irrelevant unligated control sequence (this and all other controls should not be able to bind to RFX) taken from Bielinska *et al.* (2).	-
unl ctrl 3 (unligated control oligo 3): 5'-TATACGGGTTTTCCCGTATACCACTCTGTTTTCAGAGTGG-3' Rationale: contains the "m6 mutant" mutation of the X-box known to abolish RFX binding (10).	-
unl rev X (unligated reverse sequence X-box): 5'-GTTGTCTACTTTTGTAGACAACGATCCCCTTTTGGGGATC-3' Rationale: designed as a control sequence (the X-box sequence in reverse).	-
ds X (double-stranded phosphodiester X-box oligo) and ds S-X (double-stranded phosphorothioate X-box oligo): 5'-CCCCTAGCAACAGATG-3' 3'-GGGGATCGTTGTCTAC-5' Rationale: both ds X and ds S-X are double-stranded oligos which contain the X-box sequence, the target of RFX. The ds S-X should not be degraded by cellular nucleases.	**+ for both**
ds S-ctrl 3: 5'-CCCGTATACCACTCTG-3' 3'-GGGCATATGGTGAGAC-5' Rationale: the phosphorothioate version of unl ctrl 3 (control sequence).	-
hairpin X: 5'-CCCCTAGCAACAGATG T T 3'-GGGGATCGTTGTCTAC T T	**+ (?)**



Rationale: contains the X-box sequence; an intermediate between a dumbbell and a double-stranded oligo.

Table VII (continued)

Oligo name, sequence, rationale	**Expected activity (+ = active; - = inactive)**
ss top X (single-stranded top strand of the X-box) 5'-CCCCTAGCAACAGATG -3' Rationale: contains the top strand of the X-box	-
ss B2 (an arbitrary control sequence) 5'-GCCACGGAGCGAGACATCTC-3' Rationale: contains a single-stranded control sequence	-
linear QI: 5'-AAAAGTCGACTGTGTAGGAATCCCAGCCAGATGCATCTCT-3' Rationale: a linear oligo 40-mer used to determine if activity is dependent merely on oligo size (length).	-
linear QII: 5'-AGAGATGCATCTGGCTGGGATTCCTACACAGTCGACTTTT-3' Rationale: another linear oligo 40-mer used to determine if activity is dependent merely on oligo size (length).	-

Cell culture

Class II expressing Raji cells (ATCC CCL 86, a human Burkitt's lymphoma B-cell line) were grown in RPMI 1640 medium with fetal bovine serum (10-20%), penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (292 µg/ml). Cells were fed with

fresh media every 2-3 days and split 1:10 (cells:media) once a week. Cells were split 1:2 the day before transfections. Raji cells can be grown continuously and typically were propagated for 6 months or longer for these experiments.

Plasmids

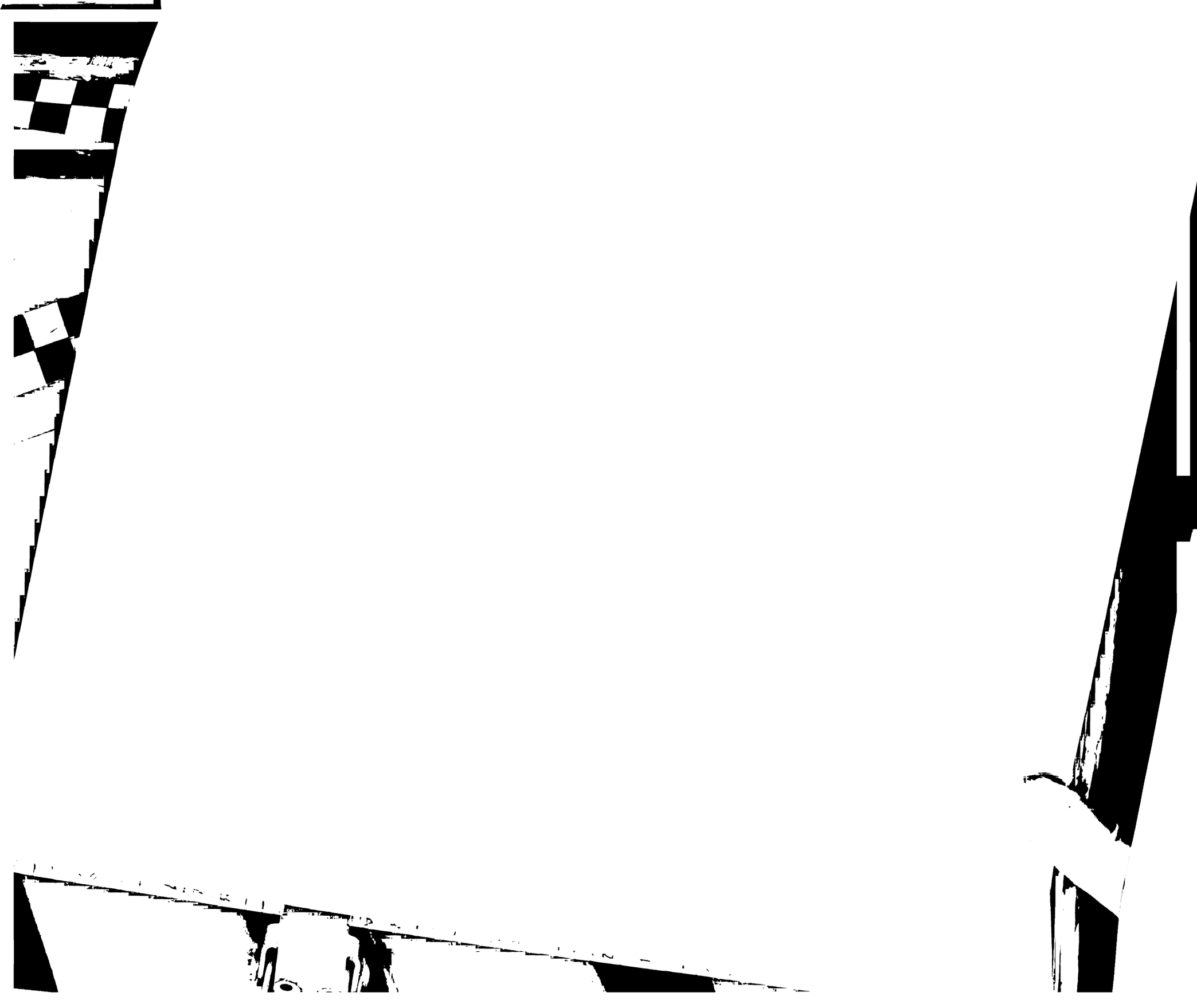
Construction of the plasmid pDRASCAT has been described previously (10, 11). It contains the DRA promoter from positions -150 to +31 linked to the CAT gene. In some cases either pRSVluc or pACTHCG plasmids were used as internal controls. The pRSVluc plasmid described by Garber *et al.* constitutively expresses the luciferase gene under the control of the Rous sarcoma virus enhancer (4); the actin promoter-driven pACTHCG described by Voliva *et al.* expresses secreted human chorionic gonadotropin (11).

Transfections

Between 1- 4 x 10^6 Raji cells (in 0.5 ml media or 0.4 ml serum-free media for luciferase assays) were used for electroporation. The same number of cells per electroporation were used within any given experiment. Cells were electroporated with 20 µg pDRASCAT and 20 µg pACTHCG or pRSVluc (1 x 10^7 cells used for luciferase), along with dumbbells or oligos using the Bio-Rad Gene Pulsar® set at 300 V (250 V for luciferase) and 960 µF. Cells were then transferred to 4.5 ml (9.5 ml for luciferase) complete media 10 minutes after electroporation and placed in a CO_2 incubator at 37°C for 48 hours prior to harvesting.

Reporter gene assays

Cells were harvested for reporter gene assays approximately 48 hours after electroporation. CAT assays were performed on cell lysates as described previously (7, 11). Briefly, cells were pelleted by centrifugation at 1500 rpm for 10 minutes at 4°C.



Supernatants were saved for HCG assays, described below. Pellets were then washed with 1.5 ml cold PBS (without Ca^{2+} or Mg^{2+}) and transferred to 1.5 ml microcentrifuge tubes, and spun at 14,000 rpm. Pellets were then mixed with 200 µl lysis buffer (0.25 M Tris HCl pH 7.5; 0.1% Triton-X 100), pipetted up and down, and vortexed to mix. After 5 minutes, samples were vortexed again, then centrifuged at 14,000 rpm for 5 minutes. Supernatants were saved for assaying (pellets discarded). Half of the supernatant (100 µl) was heated for 2 minutes in 65°C water bath (to eliminate endogenous acetyl CoA consuming activity) and cooled for 5 minutes. Ten microliters of this was saved for protein assays. The remaining 90 µl was transferred to a 5 ml plastic scintillation vial, 45 µl scintillation cocktail (25 µl chloramphenicol, 8.3 mg/5 ml; 0.2 µl ^3H Acetyl CoA, 0.5 µCi/ µl , 19.8µl H_2O) added, and 3 ml Econofluor II scintillation fluid added to each vial. Samples were then scintillation counted (^3H). Protein content of these lysates was estimated using the Bradford assay (using Bio-Rad Protein Assay Reagent) by measuring absorbance at 595nm. Protein content was used to normalize CAT activity. In some cases, cell counts using a hemocytometer were also taken to determine toxicity (using trypan blue exclusion).

Levels of HCG secreted into the cell media were assayed using the Tandem®-R HCG ImmunoRadioMetricAssay according to the manufacturer's directions (Hybritech, San Diego, CA) as follows: an Anti-HCG coated bead (containing monoclonal antibodies to the intact HCG molecule and the beta-subunit of HCG) was mixed with 100 µl sample and 100 µl ^{125}I-tracer antibody (to another epitope of the beta subunit of HCG) and incubated at 37°C for 1 hour. The bead was then washed twice with 2 ml wash solution (provided with the kit) and aspirated. Tracer antibody counts (^{125}I) were measured in a gamma counter.

Luciferase assays, normalized to cell counts, were performed on cell lysates using standard methods (5). First, live cell counts were taken on all samples (counted using trypan blue exclusion with a hemocytometer). Cells were then centrifuged for 7 minutes

at 1300 rpm. Cell pellets were then reconstituted to 1 x 10^6 cells/ml; 100 µl of this was mixed with 10 µl lysis buffer (50 µl luciferase buffer*, 10% Triton-X 100, 5 mM dithiothreitol) and incubated for 10 minutes at room temperature. Next, 100 µl assay buffer (800 µl luciferase buffer, 20 mM $MgCl_2$, 10 mM rATP) was added, followed by 100 µl 1 mM luciferin (Sigma); samples were immediately manually injected and read on a luminometer at 10 second intervals. (*Luciferase buffer consists of 220 mM K_2HPO_4, 30 mM KH_2PO_4, pH 7.8.)

Fluorescence activated cell sorting (FACS)

1.5 x 10^6 Raji cells were electroporated with oligo and transferred to 5 ml media, precisely in the same fashion as for the CAT assay. Treatments were performed and evaluated in quadruplicates. FACS readings were taken 24 and 48 hours after treatments using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) following the method of Ramanathan *et al.* (8). Twenty-four hours after electroporation, half of the cells from each treatment were removed and stained with antibodies for FACS analysis. The remainder of the cells were returned to the incubator and the procedure was repeated after an additional 24 hours (48 hours total). The antibodies used were anti-HLA-DR FITC and a control antibody, mouse IgG2a FITC (Becton Dickinson).

RESULTS

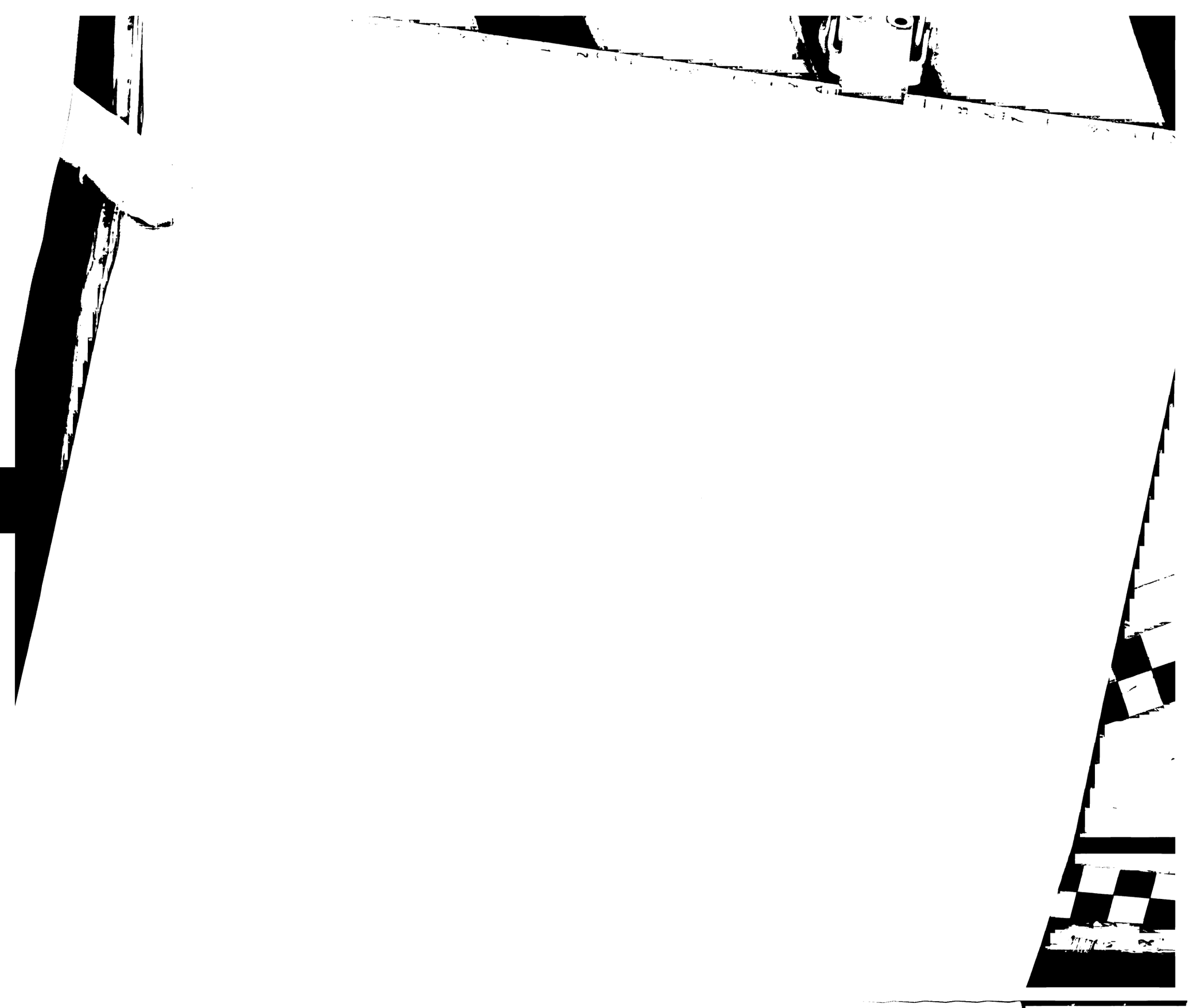
Optimizing oligo dose: 0.2 µM oligo concentrations

Initial studies were designed to determine the optimal oligonucleotide (decoy) concentrations for maintaining activity and minimizing toxicity. Since activity in these assays is measured by an oligo's ability to decrease CAT activity, the most active oligos exhibit the lowest CAT levels. A dose of 1 µM was toxic (as measured by cell counts) for several oligos tested (Figure 28), including unl CTTG-X, unl A-X, and hairpin X. At 0.5 µM, the X dumbbell and unl X decreased CAT levels to 4.65% and 11.32%,

respectively; however, cell counts were not taken. At this dose of 0.5 μM, since there was little difference in activity between unl X and the X dumbbell, further studies focused on unl X (and other unligated sequences). At 0.1 μM most oligos, including unl X, X dumbbell, unl CTTG-X, unl rev X, and hairpin X were non-toxic but fairly inactive (Figure 29). At 0.2 μM doses most unligated dumbbell oligos, including unl X, unl CTTG-X, unl A-X, and unl ctrl 1 were active and non-toxic (Figure 30). Unl rev X was the exception as this sequence exhibited toxicity. Two irrelevant sequence 40-mer oligos, linear QI and linear QII were tested to determine if activity correlated with oligo length. These oligos were inactive, indicating that some aspect of the structure—possibly in combination with some aspect of the sequence—of unligated dumbbells contributes to their activity. As shown by cell counts, most of these oligos were not toxic (except for unl rev X and hairpin X). In Figure 31, typical dose-response curves are shown. Note that response in this assay is defined as a measure of an oligo's ability to decrease CAT activity. Therefore response is defined as 100 - %CAT. Within the range of 0.1 to 1 μM, unl CTTG-X, X dumbbell, and unl X were the most potent. Hairpin, ds X, and ds S-X were essentially inactive. Figure 32 shows the dose-response curves for each oligo separately with error bars (if applicable).

Internal control reporter genes were also inhibited by unligated oligos

When either internal control pACTHCG or pRSVluc reporter genes were assayed they also were down-regulated by unligated oligos (Figures 33 and 34). The pACTHCG reporter gene was the first internal control tested and was only performed for 1 μM oligo concentrations. During the discovery that 1 μM oligos were potentially toxic, the HCG assay kit was discontinued by the manufacturer; therefore, in order to test the effects of lower dose oligos (e.g. 0.2 μM) on an internal control plasmid, another reporter gene (pRSVluc) was employed. Oligos had similar effects on the expression of these internal control reporter genes (Figures 33 and 34).



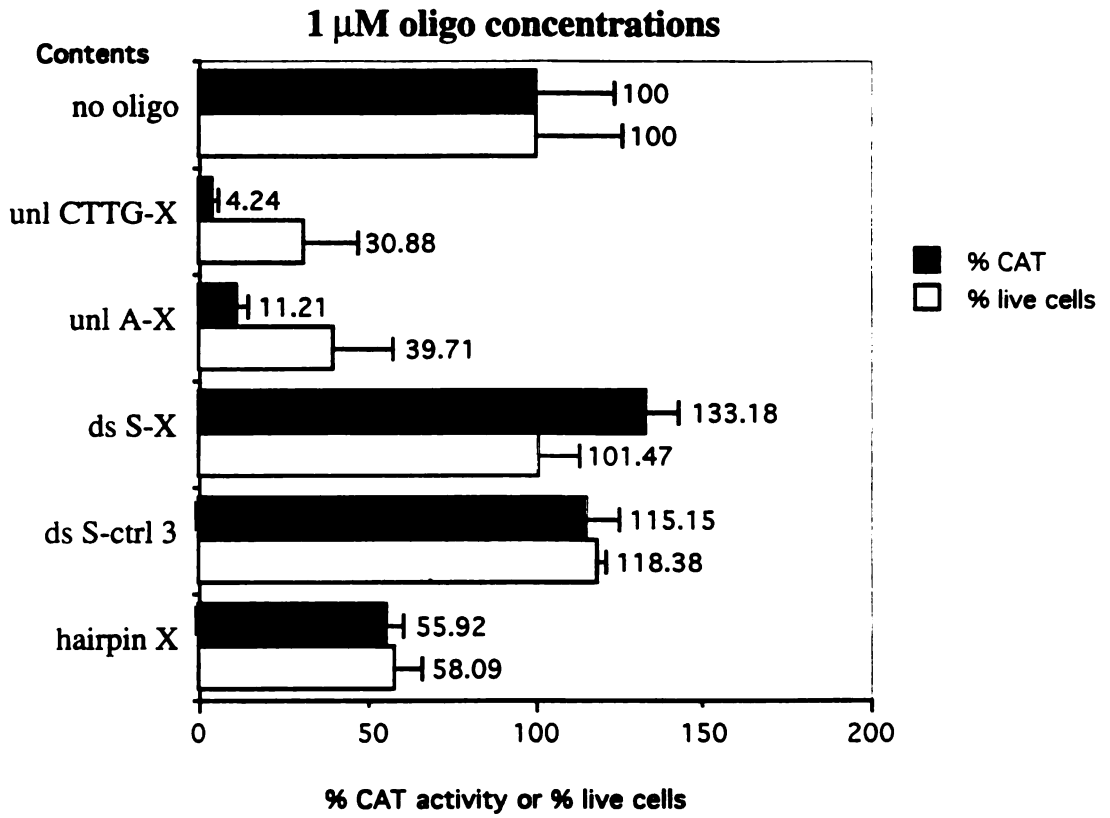


Figure 28. 1μM oligos co-transfected with pDRASCAT in Raji cells. Toxicity seen with all oligos (except phosphorothioates, ds S-X and ds S-ctrl 3). Experiment performed in triplicate (n=3); error bars = ± 1 SD.

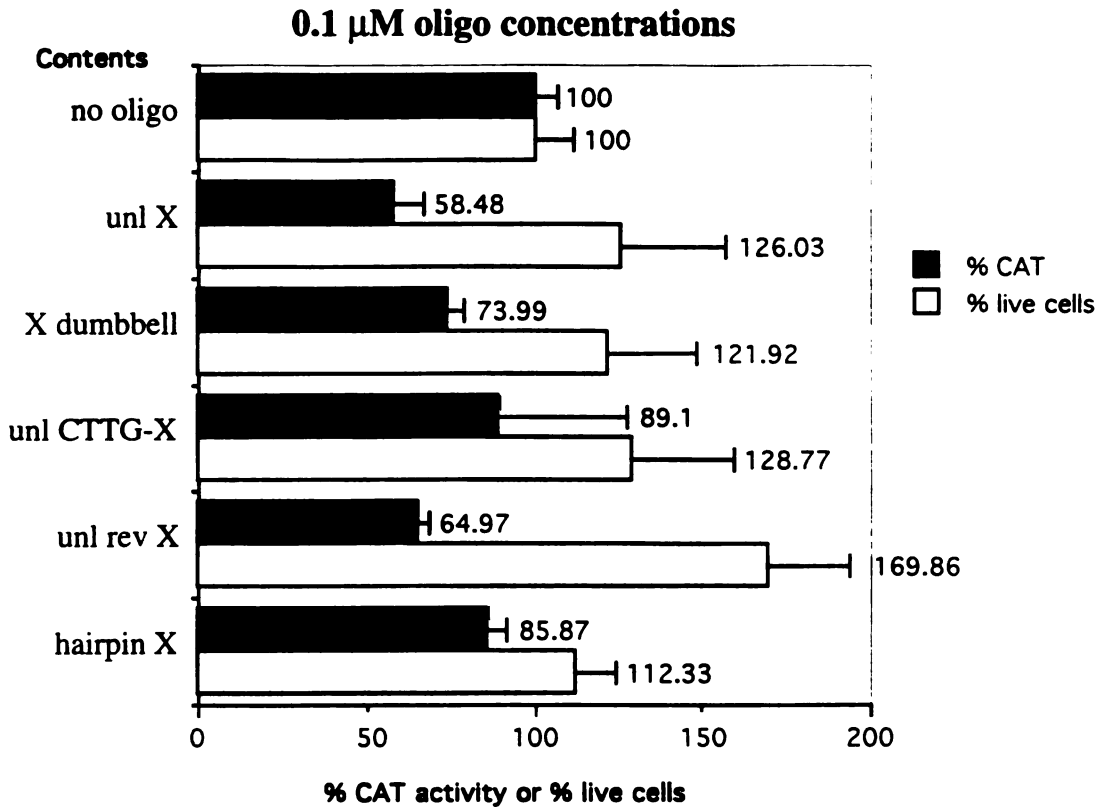


Figure 29. 0.1 µM oligos co-transfected with pDRASCAT in Raji cells. For the most part, all oligos were inactive (and non-toxic). Experiment performed in triplicate (n=3); error bars = ± 1 SD.

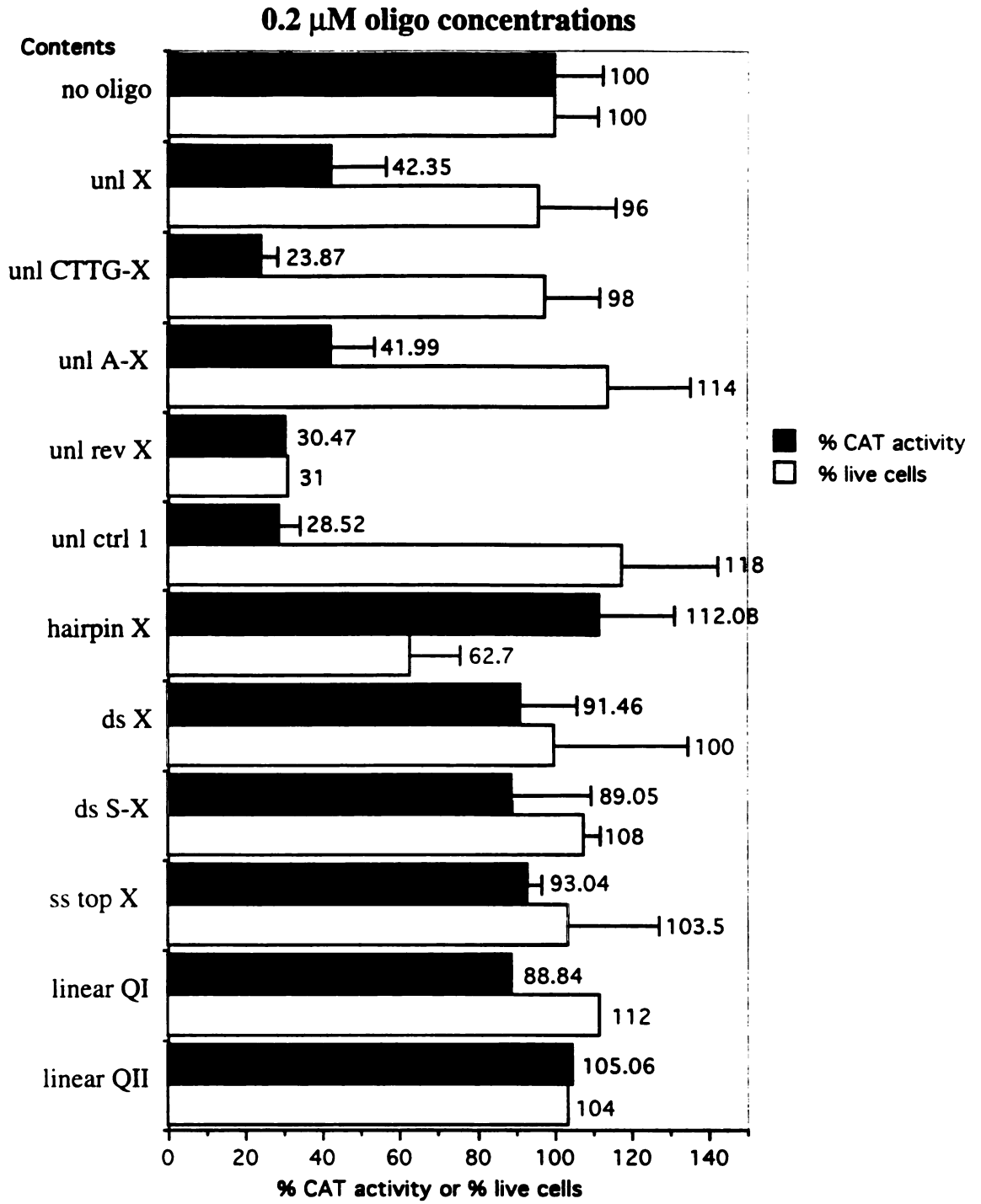
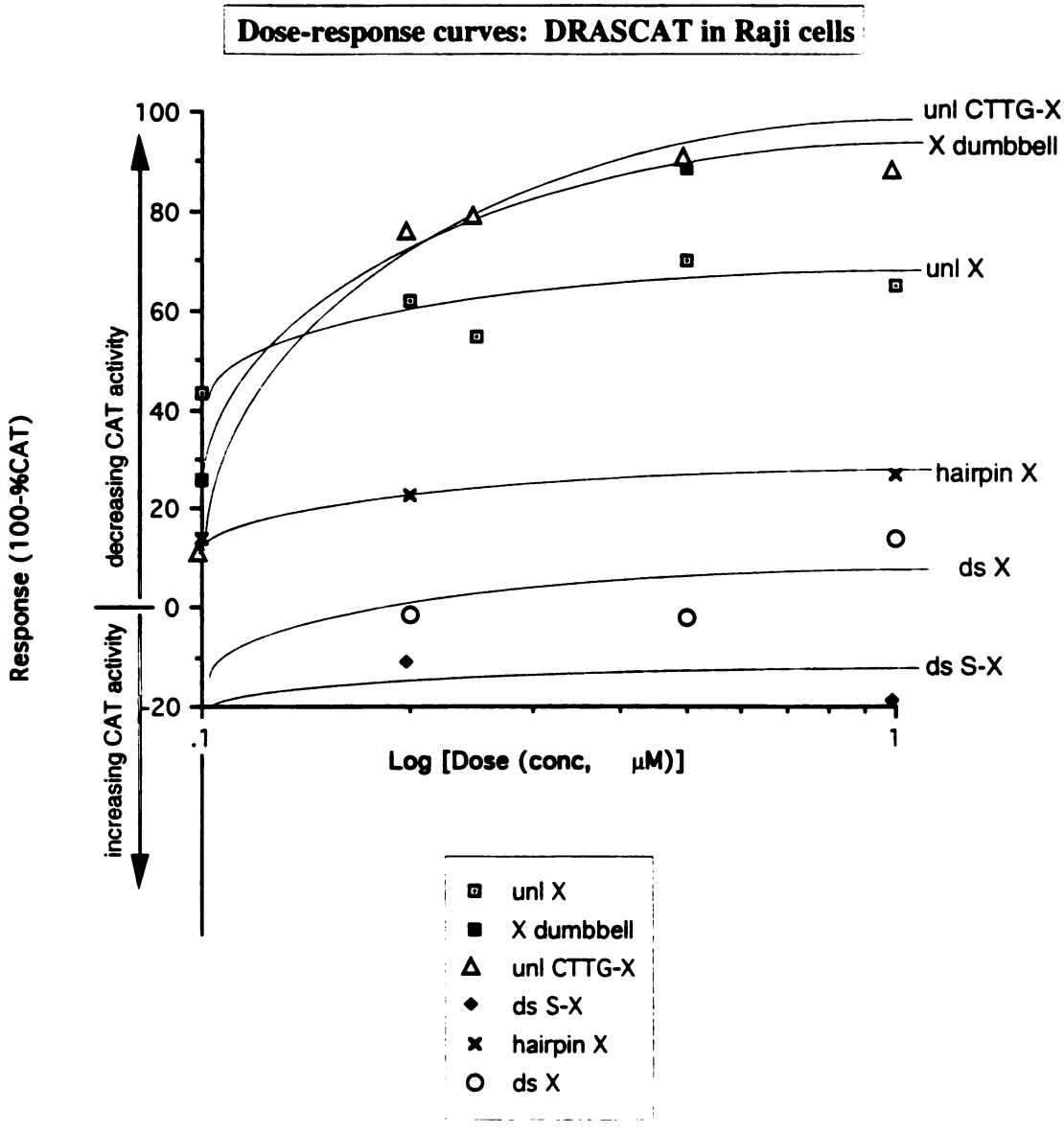
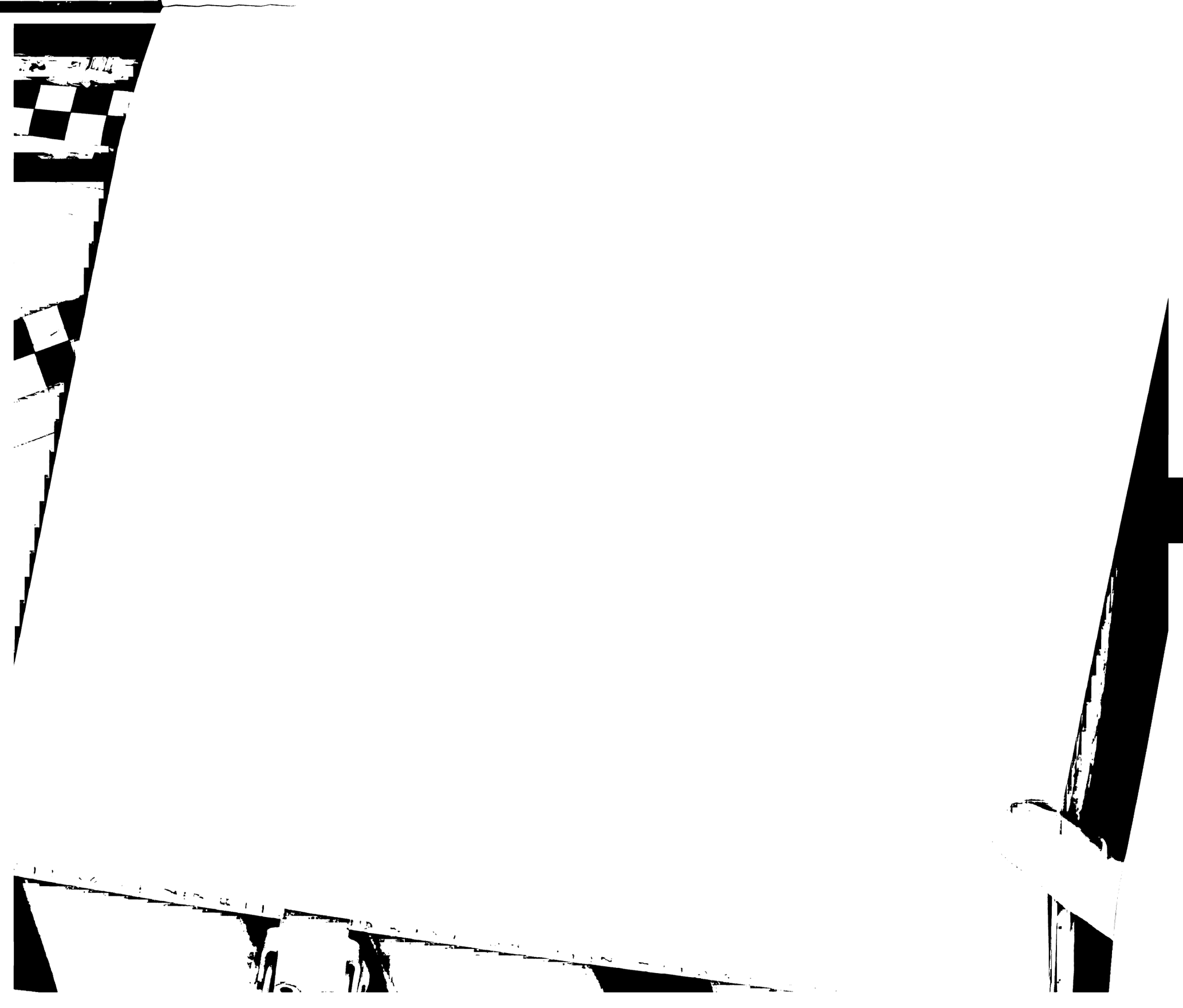


Figure 30. 0.2 µM oligos co-transfected with pDRASCAT in Raji cells. At this dose, most unligated dumbbells (unl X, unl CTTG-X, unl A-X, and unl ctrl 1) are active, yet non-toxic (unl rev X is toxic). Experiment performed in triplicate (n=3) except for linear QI and QII, where n=2; error bars = ± 1 SD.



(Curves represent apparent trend lines)

Figure 31. Composite dose-response curves. In this and the following graph (Figure 32), response is measured by a decrease in CAT activity (response = 100 - %CAT).



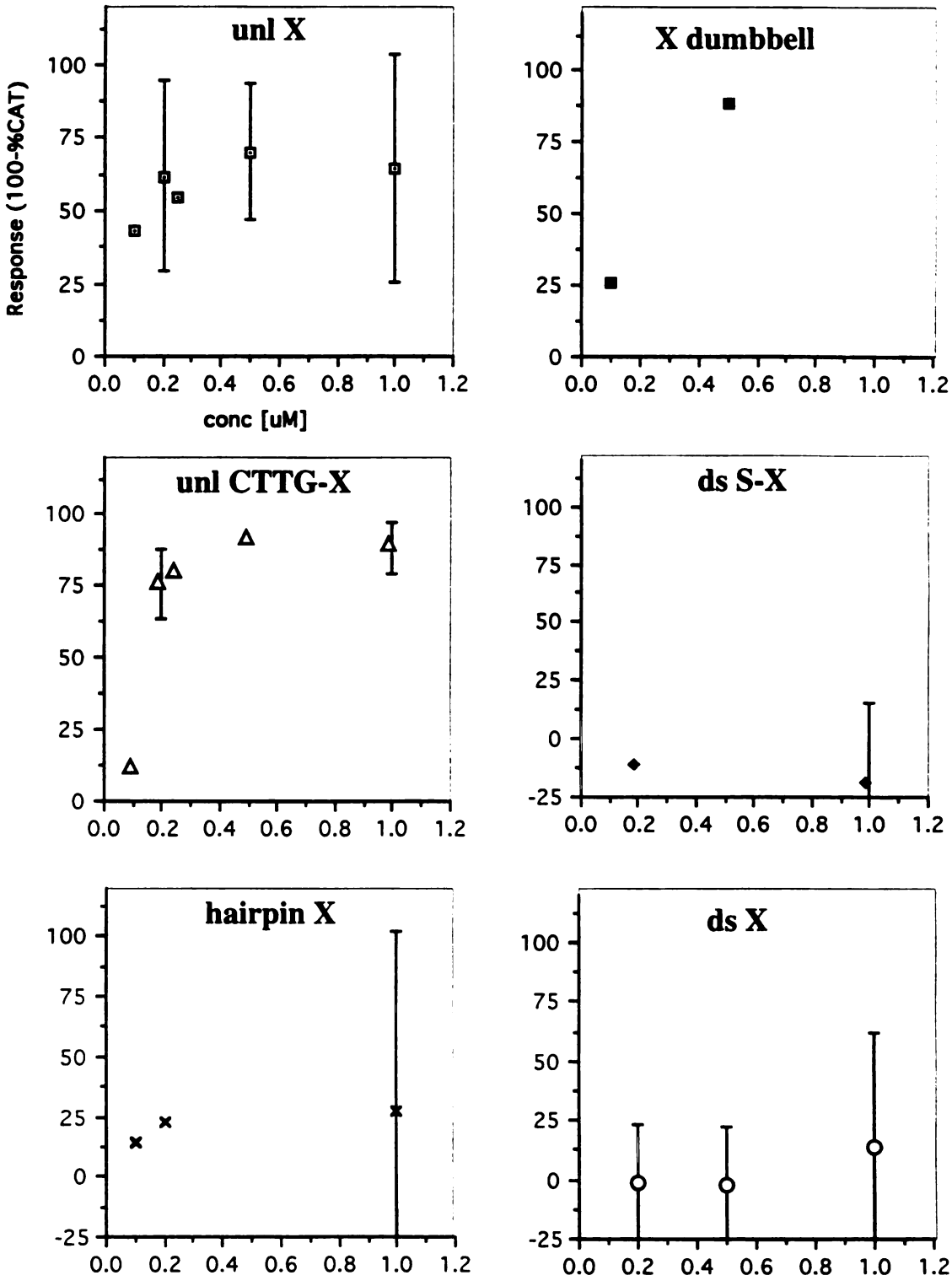


Figure 32. Individual dose-response curves. Error bars (between experiment SD) are shown for experiments repeated 3 or more times (in duplicate or triplicate).

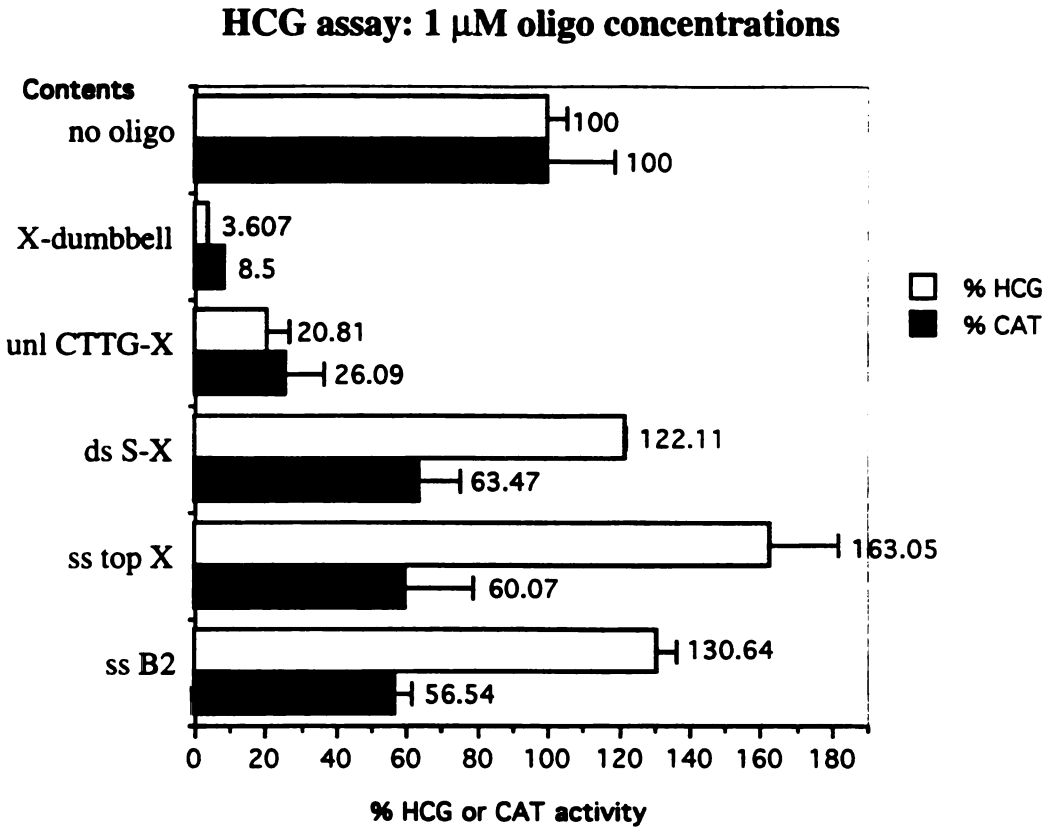
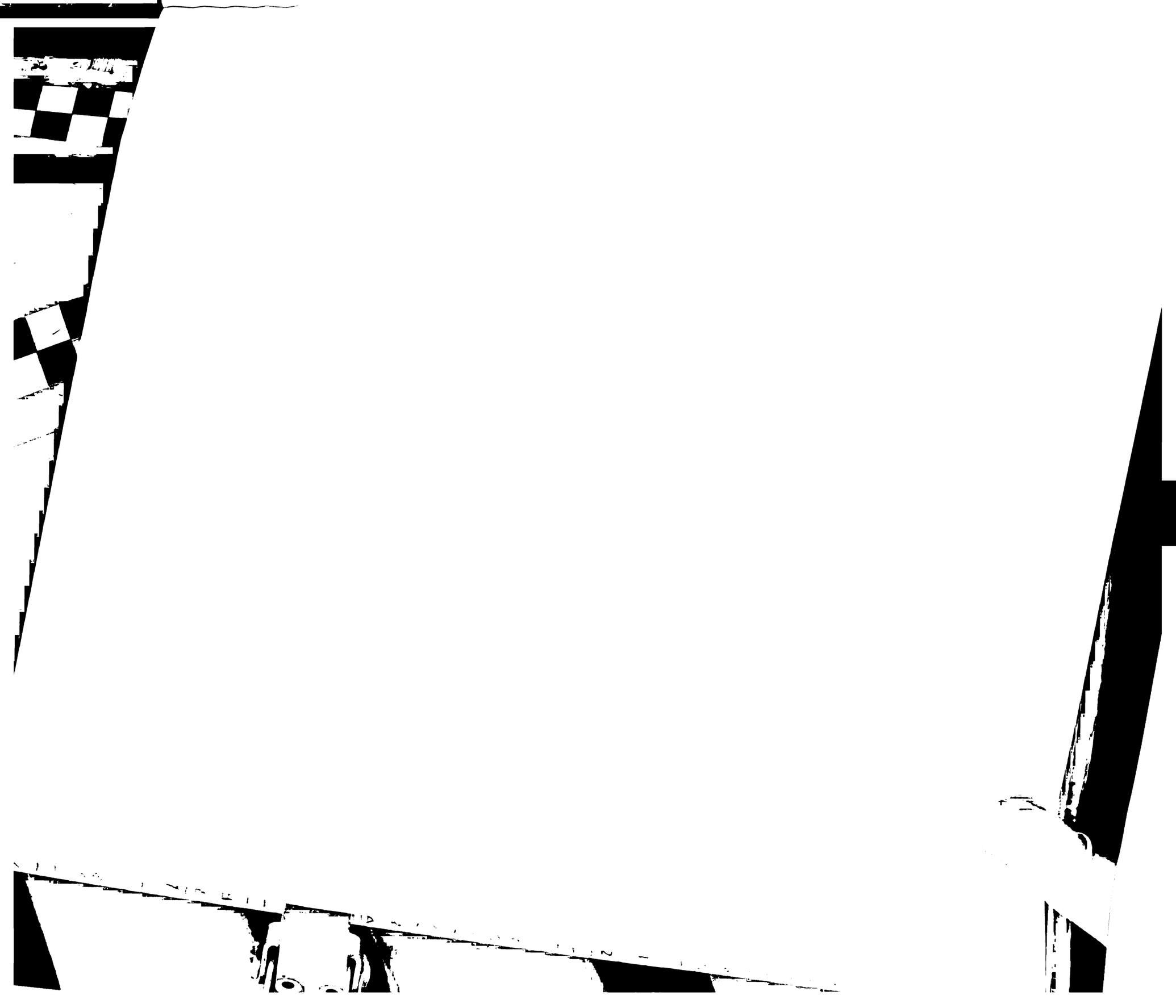


Figure 33. HCG assay. 1 µM oligos co-transfected with pDRASCAT and pACTHCG. % HCG = HCG activity compared to "no oligo;" activity measured by ^{125}I counts (not normalized). Experiment performed in triplicate (n=3) except for X dumbbell, where n=2; error bars = ± 1 SD.



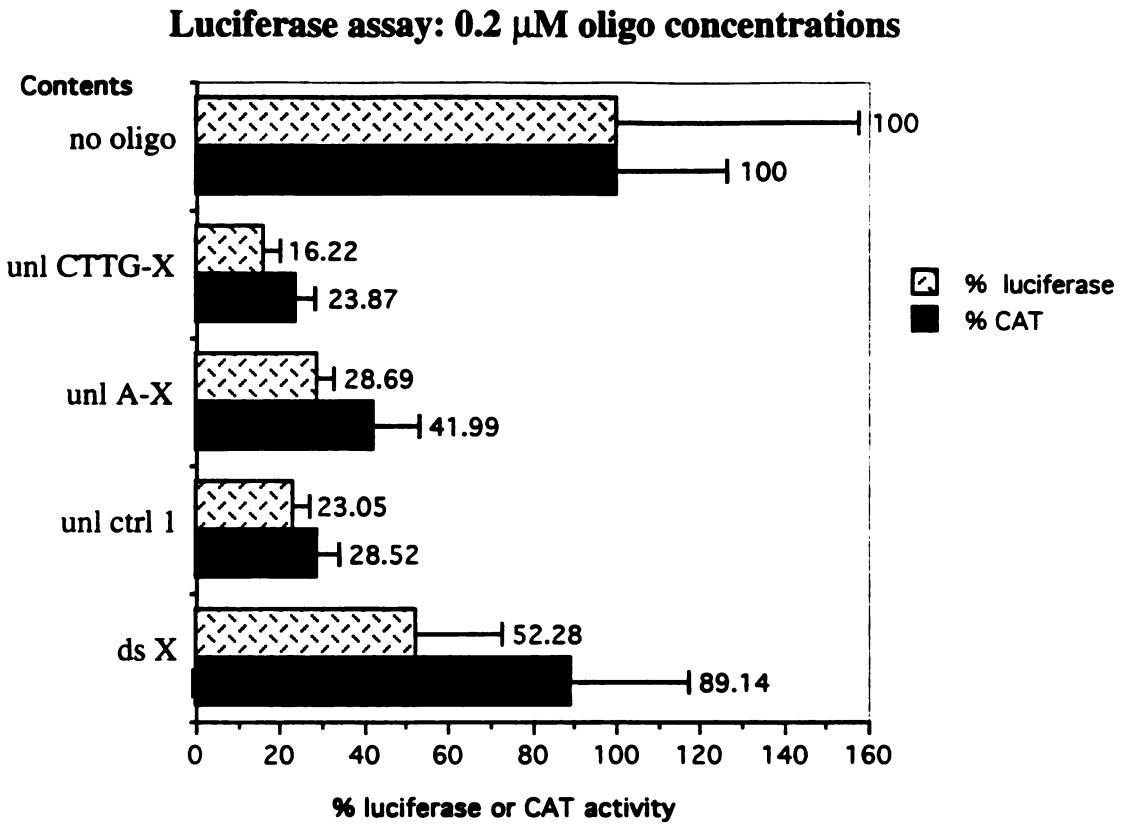


Figure 34. Luciferase assay. 0.2 μM oligos co-transfected with pDRASCAT and pRSVluc. % luciferase = luciferase activity compared to "no oligo;" luciferase activity (light units) measured by a luminometer, normalized to cell number. Experiment performed in triplicate (n=3); error bars = ± 1 SD.

Fluorescence activated cell sorting: constitutively expressed MHC II levels were not altered by active oligos

Lastly, to determine if active oligos had an effect on constitutive expression of MHC class II on Raji cells, fluorescence activated cell sorting of treated cells was performed. As shown in Figure 35, none of the 1 μM oligo treatments had an effect on cell-surface expressed MHC (HLA) DRA levels at 24 or 48 hours. IgG levels should not be altered by oligo treatment and therefore were measured as an internal control.

DISCUSSION

In this study, unligated oligos (unl CTTG-X and unl A-X) at 1 μM doses were found to be toxic. When doses were lowered to 0.1 μM, unligated oligos (unl X, unl CTTG-X, unl rev X) were no longer toxic, but were only marginally active in down-regulating CAT activity. The X dumbbell at this dose was also only weakly active. Finally, at 0.2 μM, unligated dumbbells of any sequence (unl X, unl CTTG-X, unl A-X, and unl ctrl 1) were active, as determined by their ability to down-regulate pDRASCAT. These sequences were also found to be non-toxic at this dose. Double-stranded oligos, hairpins, and linear oligos, on the other hand, were inactive at 0.2 μM. These oligos are all highly susceptible to cellular exonuclease activity which could account for their inactivity. In other words, oligos degraded rapidly by exonucleases would not be active. One might assume that improving resistance to exonucléases by substituting a phosphorothioate modified backbone could enhance activity. This was not the case, though, as the phosphorothioate oligo (ds S-X) tested was also inactive in this system. Phosphorothioates are known to have decreased duplex stability compared to the unmodified phosphodiester parent compound (6). This duplex instability may contribute to phosphorothioate inactivity in this system. These results suggests that the specific, stable structure (but not the sequence) of unligated oligos may confer activity.

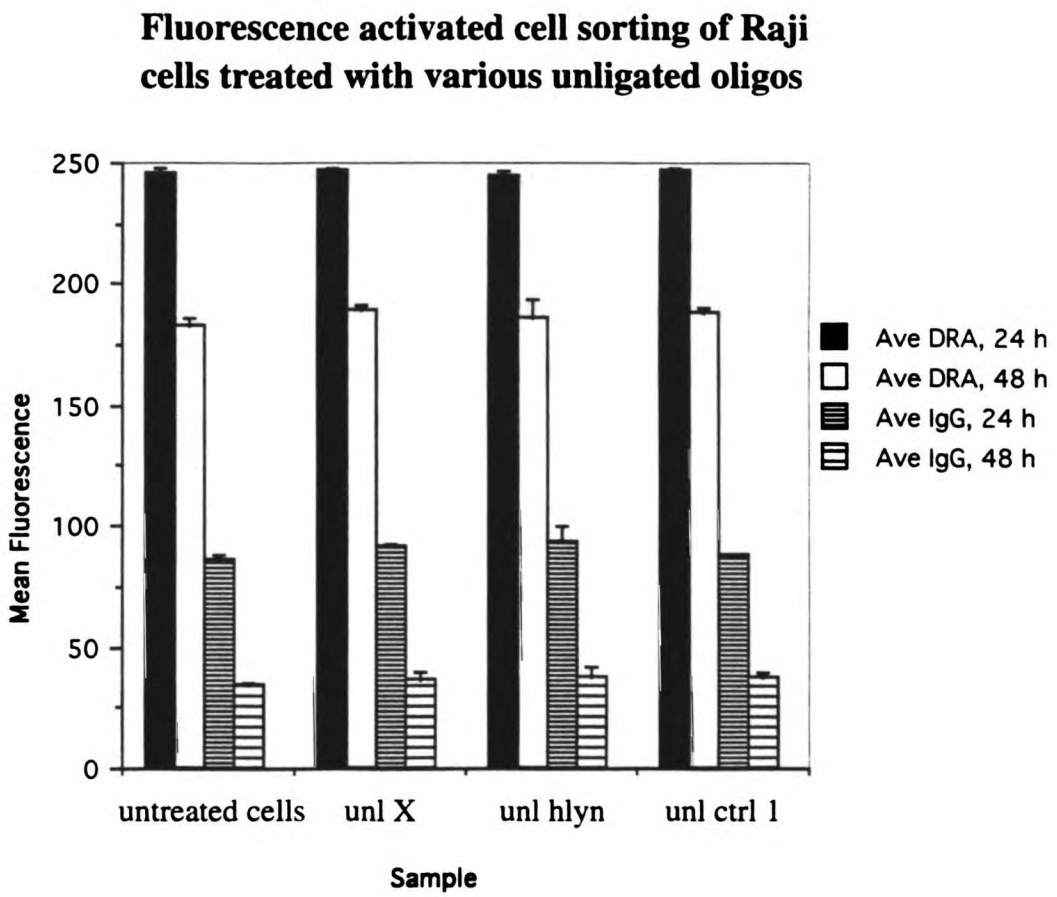


Figure 35. Fluorescence activated cell sorting of MHC II levels of Raji cells with various oligo treatments at 24 and 48 hours. Oligos have no effect on MHC II DRA levels. Experiment performed in quadruplicate (n=4); error bars = ± 1 SD.

Combining the evidence showing that unligated oligos are active in unrelated "control" reporter gene assays with their inability to down-regulate constitutively expressed MHC class II suggests that unligated oligos act by some mechanism other than interference with transcription of MHC class II genes. This mechanism is likely to be fairly general (such as interference with a general transcription factor), although it could be cell line- or system-dependent. In order to address this issue more thoroughly, an alternative system is described in the following chapter (Chapter 4).

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Chapter 4. Results in Cos-7 cells using pRFX1VP16 and p4XBCAT: Sequence-independent inhibition of RNA transcription by DNA dumbbells

ABSTRACT

An alternative reporter gene assay in Cos-7 cells was utilized to determine if activity of DNA dumbbells was cell line- or system-dependent. In this model system, dumbbells were tested for their ability to block RFX1VP16 activation of a plasmid containing multiple repeats of the X-box linked to the CAT gene. While it appeared that dumbbells could block this activation, the effect was not dependent on the sequence of the dumbbells. A PCR-based assay showed that transfection efficiency of plasmid DNA was not altered by dumbbells, nor were total RNA levels (using a RNA accumulation assay). Finally, a RNase protection assay suggested an inhibition of transcription by dumbbells, most likely via an interaction with general transcriptional machinery.

INTRODUCTION

The X-box dumbbell was found to interact with at least one of the RFX family members, RFX1 (Chapter 2) as shown by *in vitro* binding assays. The first *in vivo* model system utilized the pDRASCAT plasmid, which contained the CAT gene driven by the DRA promoter. Results showed that unligated dumbbells were able to block expression of this and 2 other reporter plasmids in a manner not dependent on their specific sequence (Chapter 3). To test if these results were cell line- or system-dependent, an entirely different test system was designed with different reporter gene assay, cell line, and mode of transfection. A reporter gene assay was devised to elucidate whether dumbbells could block transcriptional activation of exogenously added RFX1 in Cos-7 cells. A plasmid containing the cDNA of RFX1 fused to the transcriptional activation domain of adenovirus VP16 was constructed, and is referred to as pRFX1VP16. The product of this plasmid can bind to and activate p4XBCAT which contains 4 repeats of the X box linked

to the CAT gene (Figure 36). The cell line used in the first system was the B-cell line Raji, transformed by the Epstein-Barr virus. The cell line selected for this protocol was the Simian virus-transformed adherent monkey kidney line Cos-7. For Raji cells, plasmids were transfected by electroporation, whereas Cos-7 cells were transfected using lipofectin. Besides using a different cell line, the main difference between this system and the previous pDRASCAT system is the fact that transcription of the reporter gene here (p4XBCAT) is completely dependent on the formation of exogenously added RFX1VP16 fusion protein. A comparison of these two systems is presented in Table VIII. The initial results of this assay showed that oligos with the core X-box sequence were able to block RFX1 activation. Ligated dumbbells were no more active than unligated oligos, as in the previous system. However, control oligos were also active, and transcription of 2 other unrelated reporter gene systems was reduced. To determine at what level the oligos were affecting this assay, additional experiments were conducted. Oligos had no effect on transfection efficiency of plasmids based on a PCR assay. However, an RNase protection assay indicated that active oligos decreased mRNA levels without altering total RNA levels, suggesting an effect on RNA polymerase II (pol II) or its associated transcriptional machinery.

Table VIII. Comparison of assay systems

Assay system/ plasmids	**Cell line and characteristics**	**Type of cell line**	**Method of transfection**	**RFX1 present in cells?**
DRA- driven CAT gene/pDRASCAT	Raji B-cell line, EBV-transformed	suspension	electroporation	yes
RFX1VP16 activation of X-box-driven CAT gene/pRFX1VP16, p4XBCAT	Cos-7 monkey kidney cells, SV40-transformed	adherent	cationic lipid (lipofectin)	no

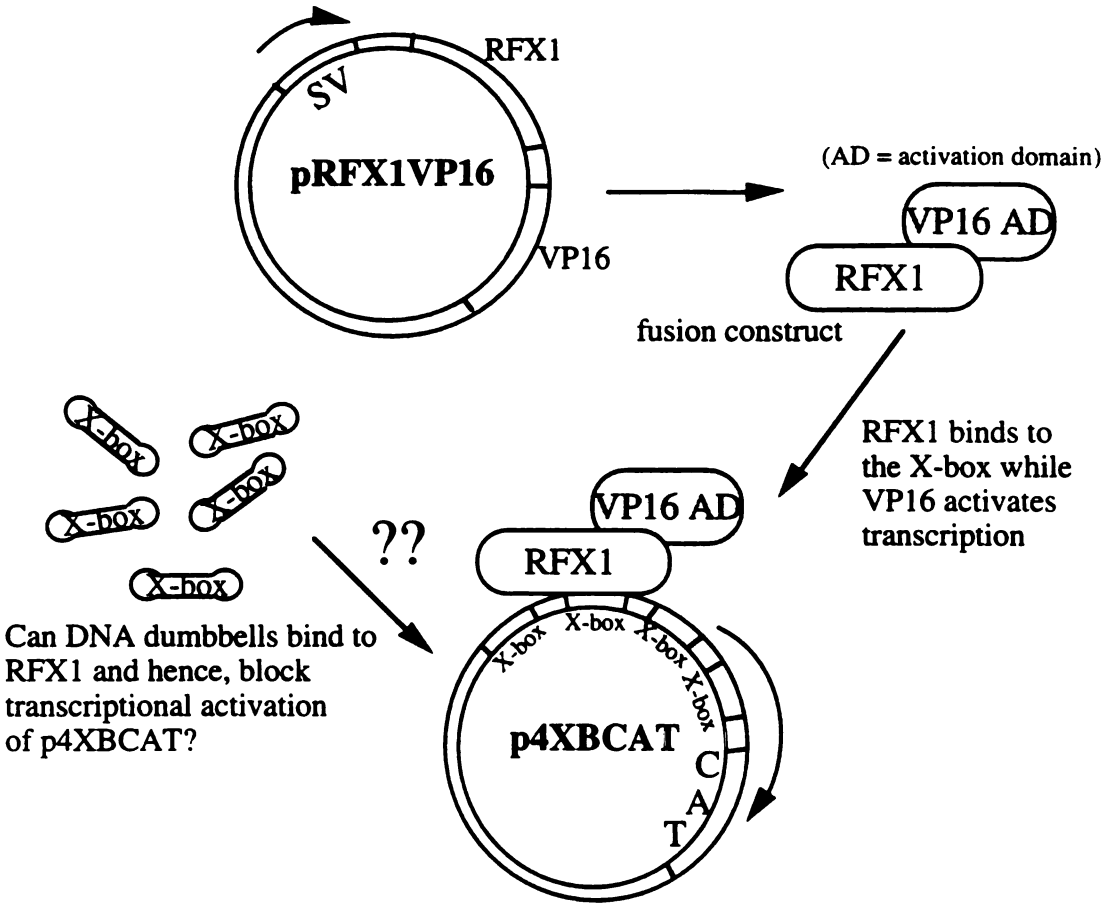

Figure 36. Co-transfection of plasmids pRFX1VP16 and p4XBCAT. Without pRFX1VP16, p4XBCAT is not transcribed, and CAT is not produced.

MATERIALS AND METHODS

Oligonucleotide sequences

Oligonucleotide sequences, nomenclature, and rationale for sequence selection are in Table IX. Core X-box oligos refer to all oligos with the X-box internal sequence and include all oligos in Table IX except for the "ctrl" oligos and ns 10. Synthesis, purification, and proof of ligation have been described in Chapter 1.

Table IX. Oligonucleotide nomenclature, sequences, and rationale for sequence/structure selection (complementary sequences are underlined where it is not apparent).

Oligo name, sequence, rationale	**Expected activity (+ = active; - = inactive)**
unl X (unligated 4 T loop X-box): 5'- CTAGGGGTTTTCCCCTAGCAACAGATGTTTTCATCTGTTG -3' Rationale: contains the entire X-box sequence, the target of RFX. The unligated dumbbell structure was tested for comparison to the ligated structure (X dumbbell); 4 T loop size.	+
X dumbbell (ligated 4 T X-box dumbbell): $_{T}^{T}$CCCCTAGCAACAGATG$^{T}_{T}$ $^{T}_{T}$GGGGATCGTTGTCTAC$_{T}^{T}$ Rationale: contains the entire 16 base-pair double-stranded X-box sequence, the target of RFX; 4 T loops on each end; ligated version of unl X.	+
unl CTTG-X (unligated CTTG-loop X-box): 5'-CTAGGGGCTTGCCCCTAGCAACAGATGCTTGCATCTGTTG-3' Rationale: same internal sequence as unl X; loops contain thermodynamically stable "CTTG" loops (3, 4); more stable than T loops	+
unl A-X (unligated 4 A-loop X-box): 5'-CTAGGGGAAAACCCCTAGCAACAGATGAAAACATCTGTTG-3' Rationale: same internal sequence as unl X; A loops used (slightly less stable than T loops).	+
unl ctrl 1 (unligated control oligo 1): 5'-TGAGTTGGTTTTCCAACTCACTTTCCGCTTTTGCGGAAAG-3' Rationale: contains an irrelevant unligated control sequence (this and all other controls should not be able to bind to RFX) taken from Bielinska *et al.* (6).	-

Table IX (continued)

Oligo name, sequence, rationale	Expected activity (+ = active; - = inactive)

ctrl dumbbell 1 (ligated T-loop control dumbbell 1): -

$_{T}{}^{T}$CCAACTCACTTTCCGC$^{T}{}_{T}$
$^{T}{}_{T}$GGTTGAGTGAAAGGCG$_{T}{}^{T}$

Rationale: ligated version of unl ctrl 1.

---

unl ctrl 2 (unligated control oligo 2): -

5'-TCCCTTGGTTTTCCAAGGGACTTTCCGCTTTTGCGGAAAG-3'

Rationale: contains the "κB" sequence used by Bielinska *et al.* (6) which is the target of the transcription factor NF-κB. This was chosen as a control sequence in this system.

---

unl ctrl 3 (unligated control oligo 3): -

5'-TATACGGGTTTTCCCGTATACCACTCTGTTTTCAGAGTGG-3'

Rationale: contains the "m6 mutant" mutation of the X-box known to abolish RFX binding (24).

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ds X (double-stranded phosphodiester X-box oligo) and ds S-X (double-stranded phosphorothioate X-box oligo): **+ for both**

5'-CCCCTAGCAACAGATG-3'
3'-GGGGATCGTTGTCTAC-5'

Rationale: both ds X and ds S-X are double-stranded oligos which contain the X-box sequence, the target of RFX.

---

hairpin X: **+ (?)**

5'-CCCCTAGCAACAGATG$^{T}{}_{T}$
3'-GGGGATCGTTGTCTAC$_{T}{}^{T}$

Rationale: contains the X-box sequence; an intermediate between a dumbbell and a double-stranded oligo.

Cell culture

Cos-7 cells (ATCC CRL-1651) were grown in Dulbecco's modified Eagle's medium (with 4.5 g/L glucose), 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (292 µg/ml). Adherent Cos-7 cells are fibroblast-like, SV40-transformed, African green monkey kidney cells derived from CV-1 cells. Cos-7 cells were split 1:20 (cells:media) at least once a week. Every 3 days, media was aspirated off cells and fresh media added.

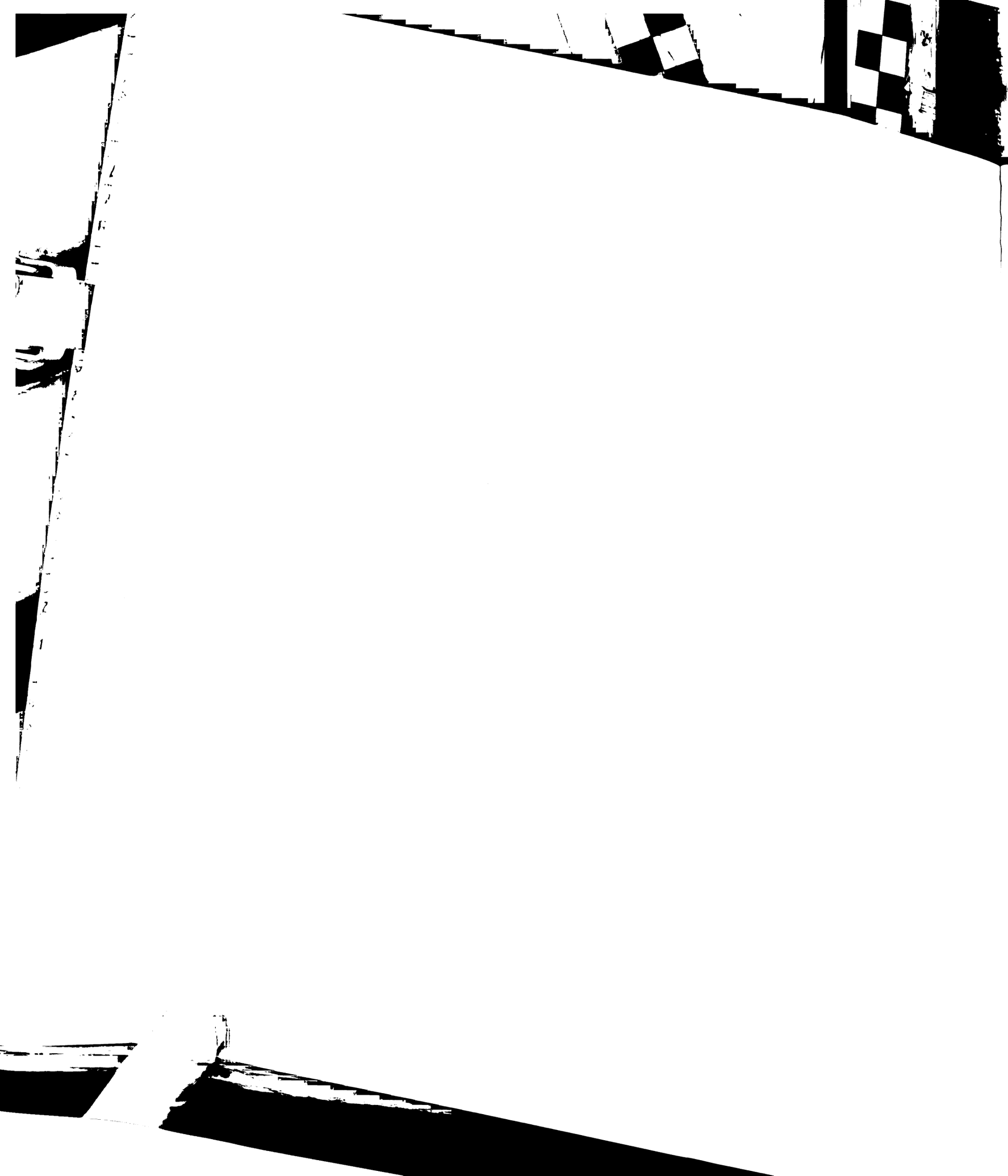
Transfections and reporter gene assays

RFX1 activation of 4XBCAT

1 x 10^5 Cos-7 cells were plated in 6-well plates the day before transfection. The plasmids pRFX1VP16 (2 µg) and p4XBCAT (1 µg) plus oligo were co-transfected with lipofectin (Gibco BRL, Gaithersberg, MD) using the manufacturer's suggested protocol. All experiments were performed at least twice, and within each experiment, independent transfections were performed in triplicate. For RNase protection assays large-scale transfections of 5 x 10^5 Cos-7 cells in 100 mm plates (2.4 µg pRFX1VP16 and 1.6 µg p4XBCAT, 50 nM oligos) were performed.

Control transfections

In separate transfections, 0.5 µg of the CMV-promoter driven plasmid pCR™3-CAT (Invitrogen, San Diego, CA) was used as an expression control plasmid. These control transfections were performed at least twice in duplicate. Another control utilized 2 µg of pSVβgal (Promega, Madison, WI) transfected into 2 x 10^5 cells on 60 mm plates, and these were performed twice in triplicate. Both control transfections were performed on Cos-7 cells.



Plasmid constructions

The plasmid pRFX1VP16 was constructed by Joseph Fontes by first amplifying the RFX1 cDNA (a gift of B. Mach) with primers that introduced a *Hind* III site after the final amino acid codon. This cDNA was subcloned into pSVSPORT1 (Life Technologies, Grand Island, NY). The cDNA coding for the activation domain of VP16 from herpes simplex virus (amino acids 400-479) was ligated into the introduced *Hind* III and *Sal* I sites of the plasmid. The plasmid p4XBCAT contains four X-box repeats and has been described previously (26). Plasmids were restriction enzyme digested and run on agarose gels to verify their construction using standard methods (19).

CAT assays

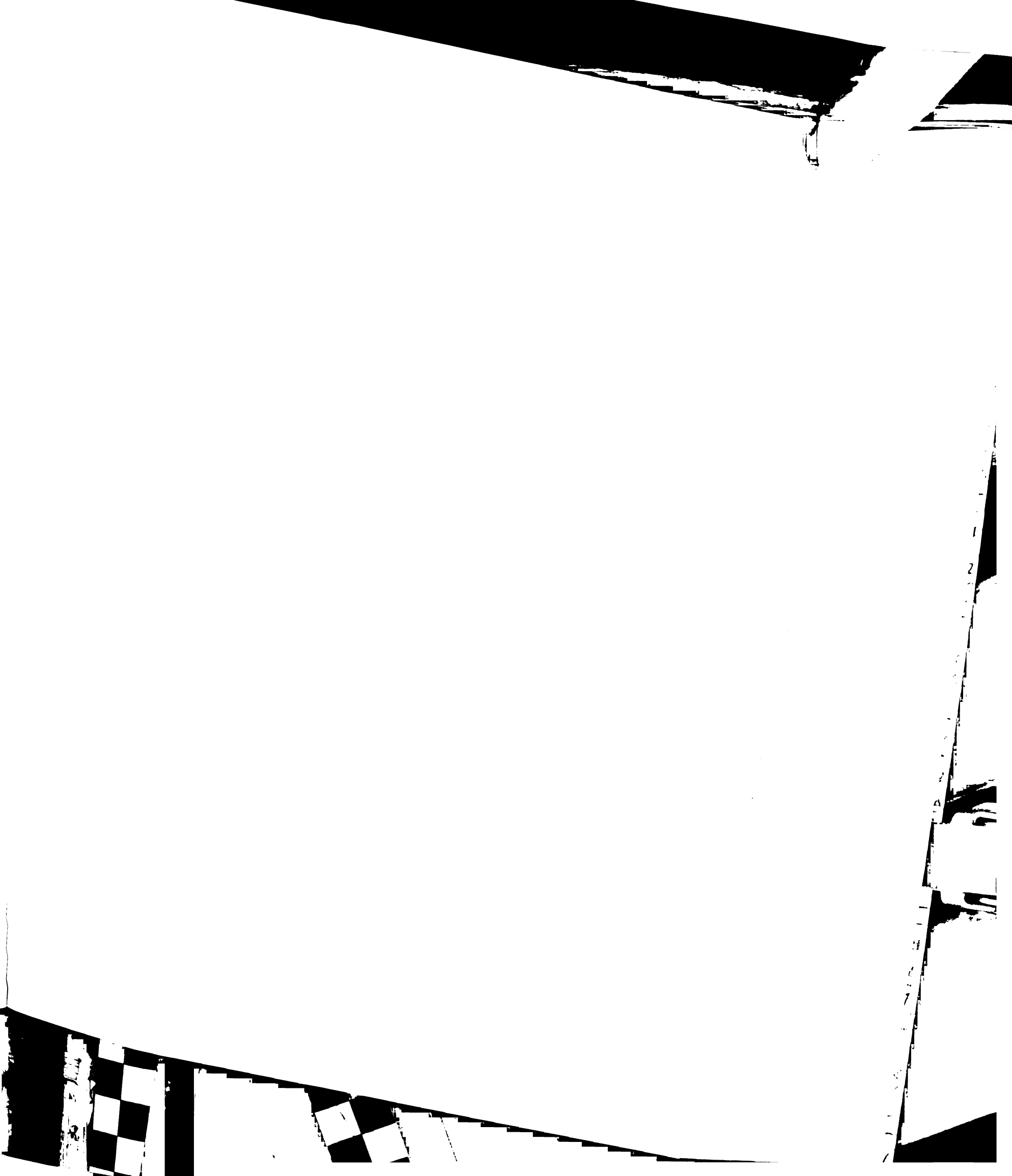
Cells were briefly trypsinized and harvested for CAT assays approximately 48 hours after transfection. CAT assays were performed on cell lysates and normalized to protein content as described previously (Chapter 3, methods). In some cases, cell counts (using a hemocytometer) were also taken to determine viability (by trypan blue exclusion).

β-galactosidase assays

Cells were stained *in situ* for β-galactosidase activity. Activity was confirmed visually upon detection of blue stained cells. A qualitative result was obtained by counting the number of cells stained blue in a fixed area of cells of approximately 90% confluency, or by removing the cells from the plates with trypsin and counting cells stained blue on a hemocytometer using a microscope.

PCR of transfected DNA

The first PCR experiment was performed on 2 known concentrations of plasmid (pSV40βgal) as a standard (Figure 41). Each PCR reaction in a total volume of 100 µl contained 10 µl template (plasmid DNA, either 2.25 ng or 4.5 ng), 0.2 mM dNTPs, 100

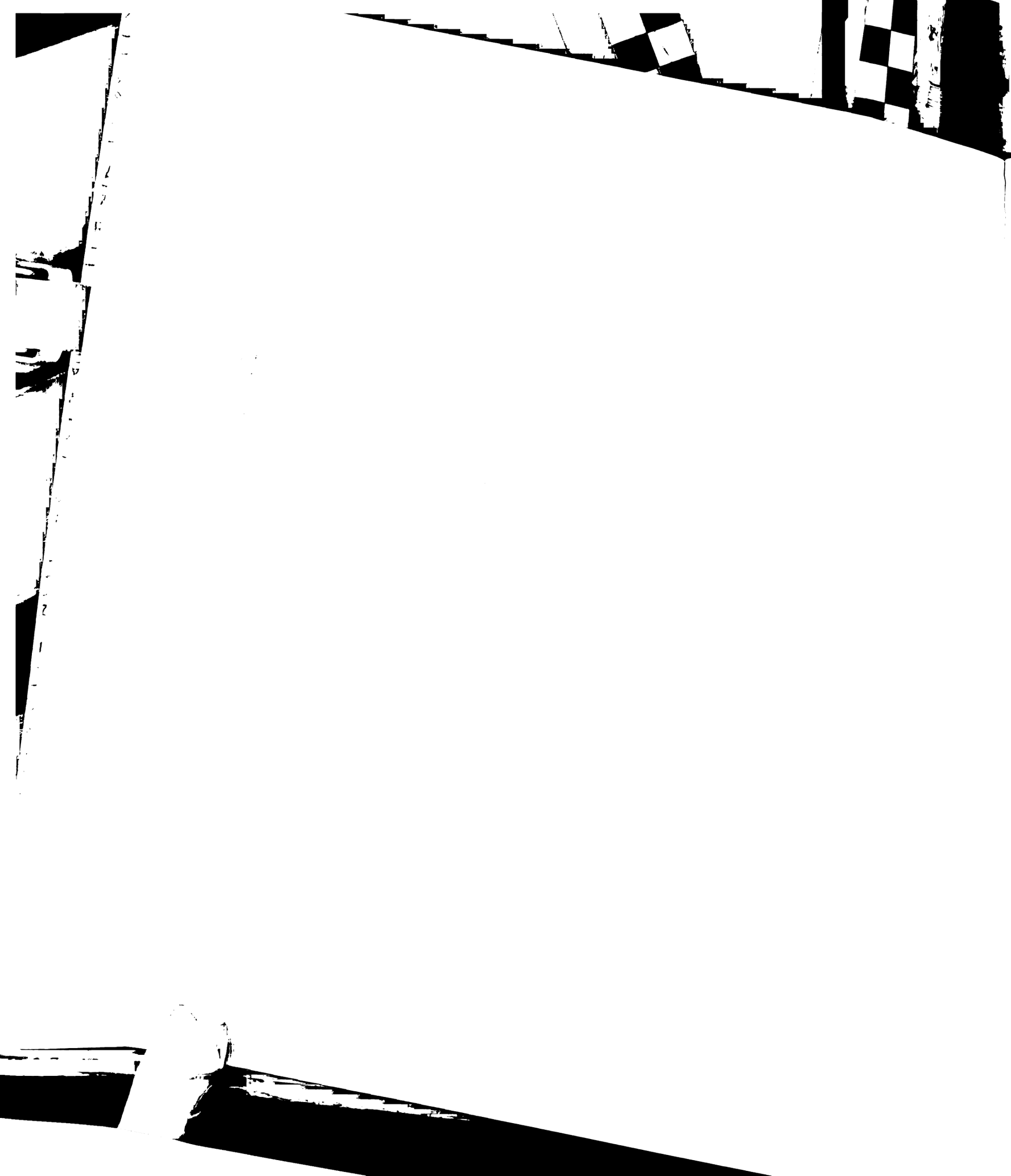


pmol each of primers gal1/gal2 which amplify pSV40βgal (gal1: CTA.GGG.AAC.CCA.CTG.CTT.AAG.CCT.C; gal2: CCG.GAT.CCG.TCA.GAA.AGC. AGA.CC), 1 unit Taq DNA polymerase (Boehringer Mannheim), 10 mM Tris HCl, 50 mM KCl (pH 8.3), and 2.5 mM $MgCl_2$. Both samples were subjected to 25 cycles of amplification (60 seconds denaturing at 94°C, 60 seconds annealing at 60°C, 3 minutes extension at 74°C) and were loaded onto a 1.5% agarose gel with ethidium bromide and photographed under UV illumination for analysis.

For the second PCR experiment, DNA was extracted from 1 x 10^5 cells transfected (as described above) with both 2 µg pRFX1VP16 and 1 µg p4XBCAT plasmids with (100 nM ds S-X or unl CTTG-X) or without oligo, using a modified Hirt assay (23). Incubation time and temperature for this extraction were one hour and 50°C, respectively. PCR was performed on these DNA-extracted samples as above using primers V1/V2 which amplify pRFXVP16 (V1: GCC.ACC.ATG.GCA.TCG.ACG.GCC.CCC.CCG.ACC.GAT; V2: GTC.GAC.CCC. ACC.GTC.CTC.GTC.AAT.TCC). All samples (including controls) were subjected to 25 cycles of amplification (30 seconds denaturing at 94°C, 30 seconds annealing at 60°C, 60 seconds extension at 74°C) and were loaded onto an agarose gel and visualized as above.

Total RNA Accumulation Assay

To detect alterations in the levels of newly synthesized RNA, a ^{3}H-uridine pulse-chase method (15) was used. Cells were transfected with plasmids and oligos (in triplicate) in the normal fashion, but with 100 nM active oligos to dramatically lower CAT readouts. One replicate of each transfection was used for the CAT assay and for determining cell viability; 2 replicates were used for the RNA assay. Cell media were removed 48 hours after transfection, replaced with 1 ml Opti-MEM I (Gibco BRL) and 40 µCi 5, 6 ^{3}H-uridine (Amersham), and placed in a CO_2 incubator at 37°C. After 2 hours, the cells were washed twice with PBS (without Ca^{2+} or Mg^{+}). Total RNA was isolated using the



RNeasy kit (Qiagen). Aliquots of ^{3}H-labeled RNA were counted for radioactivity in a scintillation counter. RNA assays were performed twice in duplicate.

RNase protection assay

Cells from large-scale transfections were harvested as pooled duplicates 48 hours post-transfection. Cytoplasmic RNA was isolated from these cells using the method of Kao *et al.* (14) for use in RNase protection assays. The RNA probe was prepared by linearizing pCR™3-CAT with *Pvu* II. This created 3 fragments, one of which was *in vitro* transcribed by T7 polymerase using the MAXIscript™ system (Ambion, Austin, TX) to generate a ^{32}P-labeled fragment of 173 nucleotides. This probe was complementary to a 111 nucleotide region of the CAT transcript. In addition, a ^{32}P-labeled-18S ribosomal RNA internal standard probe (116 nucleotide transcript, 80 nucleotides of which are complementary with ribosomal RNA from all vertebrates) was prepared using linearized pT7 RNA 18S (Ambion). Protection assays were performed using the Guardian RNase protection assay kit (Clontech, Palo Alto, CA) with hybridization at 42°C overnight. CAT RNA and 18S RNA were probed separately. Samples were heated at 95°C for 5 minutes before loading onto a 6% denaturing (8 M urea) acrylamide gel and electrophoresed in TBE buffer for 1.5 to 2 hours at 500V. After drying, gels were exposed to X-ray film with an intensifying screen overnight at -70°C.

RESULTS

Ligated/unligated dumbbells of *any* sequence and the phosphorothioate oligo (ds S-X) were able to block RFX1VP16 activation of p4XBCAT

Since core X-box oligos and dumbbells were able to interact with RFX1 *in vitro*, the next step was to determine if these oligos and dumbbells could specifically block transcriptional activation by RFX1 *in vivo*. The plasmid pRFX1VP16 expresses a protein consisting of RFX1 fused to the strong transcriptional activating domain of VP16. This

fusion protein is a potent transcriptional activator dependent only RFX1 binding to its target sequence. The p4XBCAT plasmid contains multiple repeats of the X-box, the target for RFX1, thus resulting in strong transactivation. Dumbbells, unligated oligos, double-stranded oligonucleotides, and double-stranded phosphorothioate oligos were tested for their ability to block RFX1VP16 activation of p4XBCAT. Initial studies indicated that optimal (e.g., maximal effect with no toxicity) oligo concentrations in Cos-7 cells were in the range of 50-100 nM (data not shown).

Because the ligated dumbbell (X dumbbell) was the only oligo capable of directly binding to RFX1 in EMSAs, it was anticipated that it would be the most potent inhibitor of RFX1VP16 activation of 4XBCAT. Clusel *et al.* showed that ligated, but not unligated, dumbbells specific for the transcription factor HNF-1 were able to block HNF-1 activation of a CAT reporter plasmid (10). In our system, however, ligated dumbbells were no more active than their unligated counterparts in blocking RFX1VP16 activation of p4XBCAT (see Figure 39, top 2 panels). Even under highly denaturing conditions (7 M urea, 10 mM Tris HCl), the unligated X-box oligo had a melting temperature of 42.5°C as determined by a Cary 3E Spectrophotometer, demonstrating a highly stable structure. Under physiologic conditions, unligated dumbbells may indeed exist in a folded (double-stranded) state (see also Figure 14 A, lanes 7-8); certain loop ends such as the ones used here (T loops, CTTG loops) additionally contribute to the overall stability of this type of molecule. In other words, the stability and hence apparent activity of these oligos in this system is not dependent on a *ligated* structure. Therefore we chose to focus on unligated oligos in further studies.

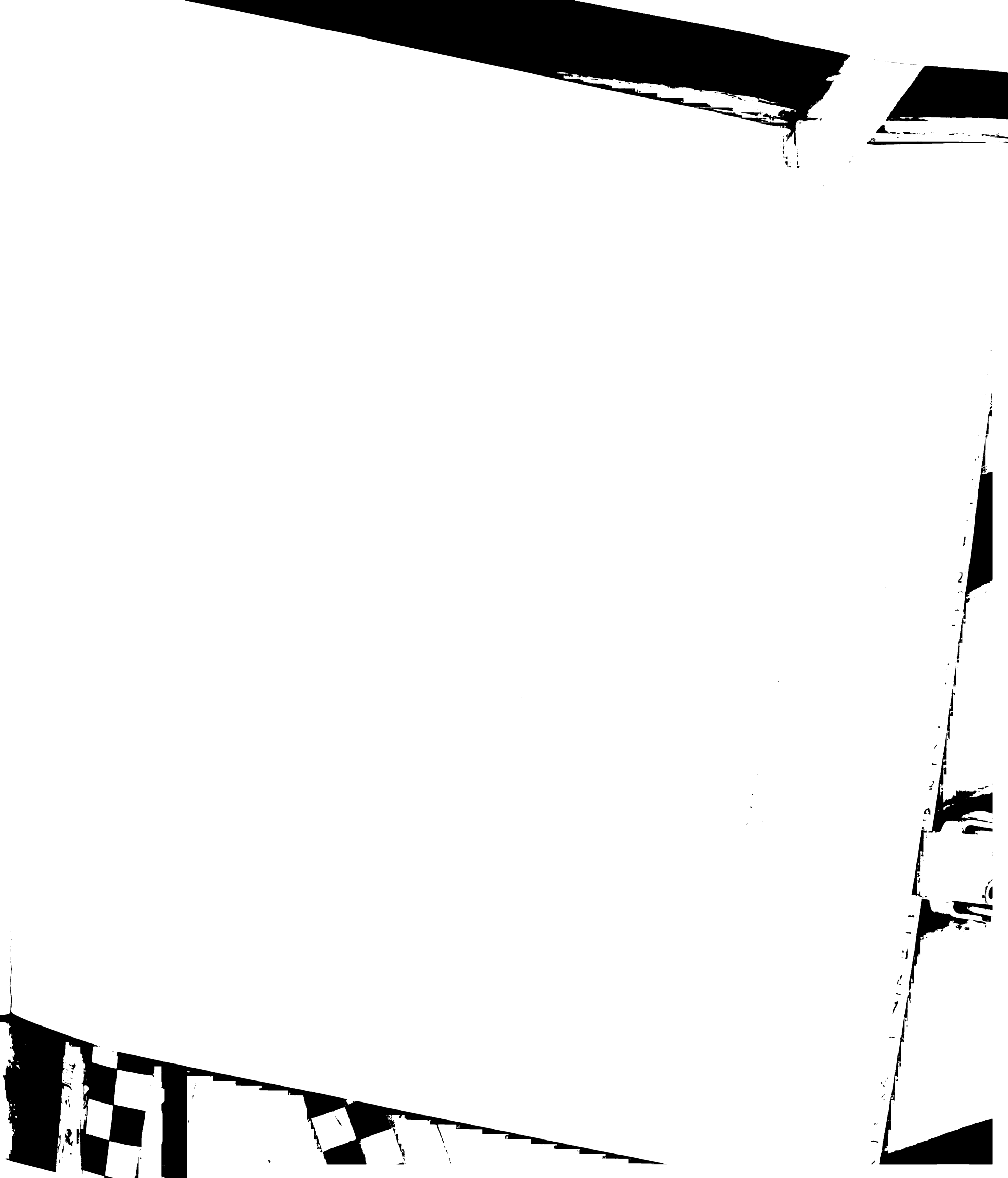
To further investigate what effect the loop structure of these unligated oligos had on activity, unligated X-box oligos with different loop sequences were tested. The first contains CTTG loops (unl CTTG-X-box); the second contains A-loops (unl A-X), both in place of T loops. Unl CTTG-X contains highly thermodynamically stable G(CTTG)C

loop structures (3, 4) on each end instead. Unl A-X should be the least stable of these 3 based on DNA hairpin studies (20).

As seen in Figure 37, all core X-box oligos, including unl X, unl CTTG-X, unl A-X, and ds S-X, were effective in down-regulating CAT activity. The ds X was much less active, presumably due to degradation by exonucleases. A hairpin X box with loop on one end only was of intermediate activity. Cell viabilities were relatively stable among all samples. Significantly, the control or irrelevant sequence unligated oligos unl ctrl 2 and unl ctrl 3 inhibited RFX1VP16 activation of 4XBCAT. The activity of these irrelevant oligos suggested a non-specific effect of unligated oligos in general on the function of RFX1VP16. Another double-stranded phosphorothioate having the same internal sequence as unl ctrl 3 was also able to decrease RFX1VP16 activation of 4XBCAT to the same extent as ds S-X (data not shown; done at 100 nM doses). A summary of the activity of several different oligos (unl X, X dumbbell, unl CTTG-X, ds S-X, hairpin X and ds X) is presented as dose-response curves in Figure 38. Figure 39 depicts individual dose-response plots, including between experiment standard deviations, when applicable.

Active oligos and dumbbells decreased reporter gene readouts in 2 other systems

As control experiments, some of the core X-box oligos were tested to determine their effect on other reporter gene systems, including CMVCAT and SVβgal, neither of which were expected to be specifically regulated by any of the core X box oligos. CMVCAT transfection results are shown in Figure 40. Reduction of reporter gene readout by various oligos in this system followed the same trend as seen with the RFXVP16/4XBCAT system. A possible explanation is that active oligos could possibly interact with the CAT gene itself or some aspect of the assay. Therefore, an entirely different reporter gene was tested. The pSVβgal reporter gene consists of SV40 promoter/enhancer sequences which drive the lac Z gene, encoding the beta galactosidase



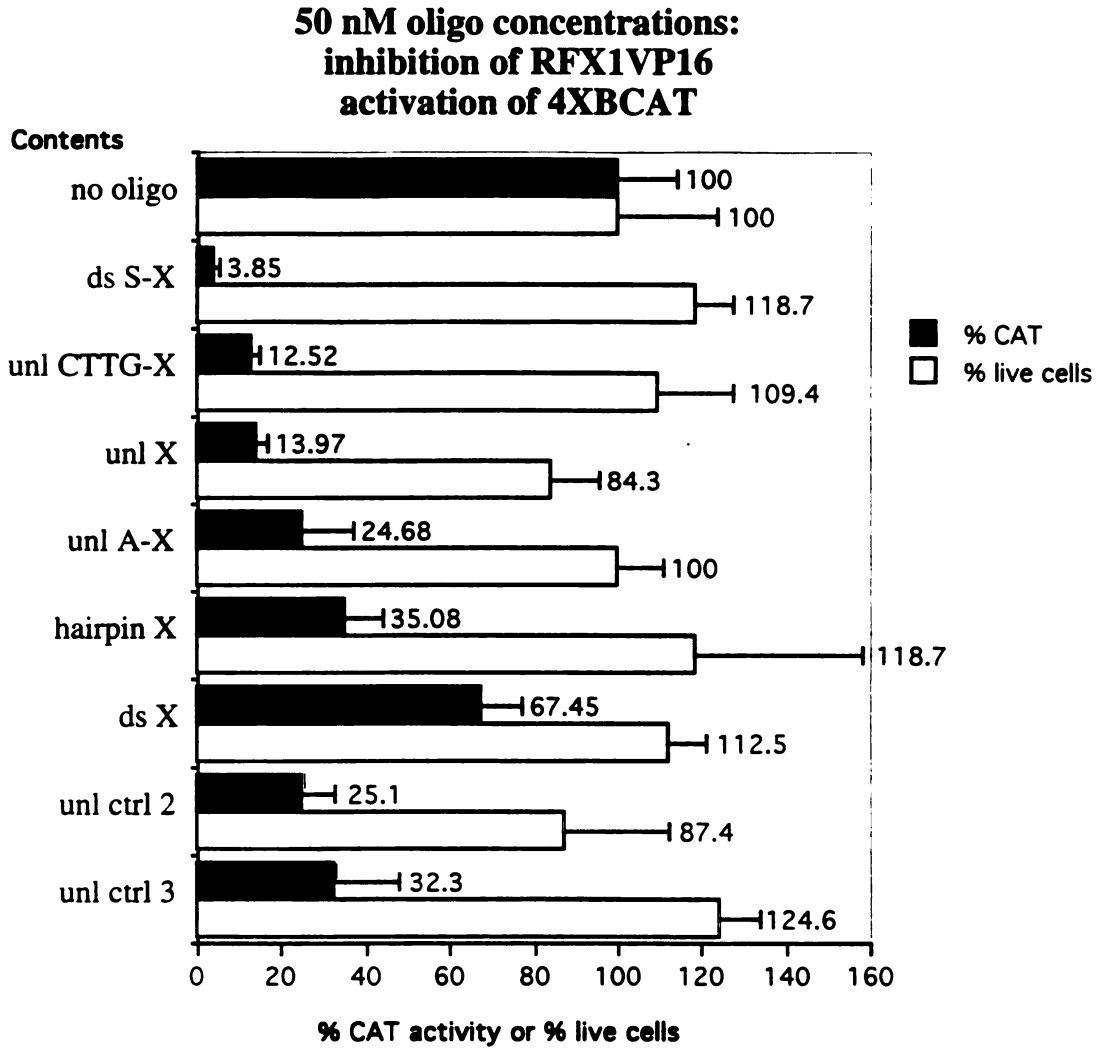
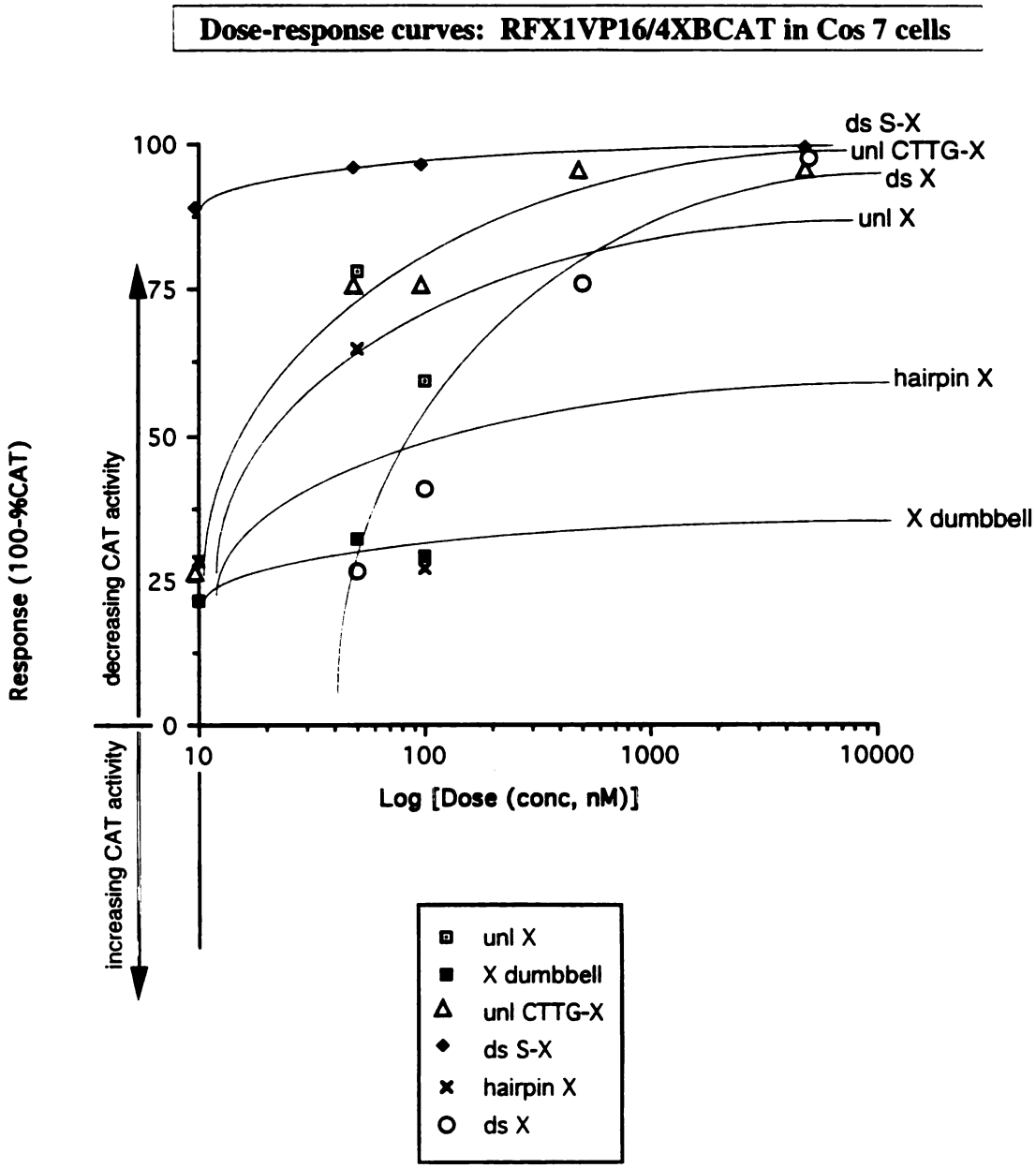


Figure 37. Oligo inhibition of RFX1VP16 activation of 4XBCAT. Fifty nanomolar oligo concentrations used. Experiment performed in triplicate (n=3); error bars = ± 1 SD.



(Curves represent apparent trend lines)

Figure 38. Composite dose-response curves. In this and the following graph (Figure 39), response is measured by a decrease in CAT activity (response = 100 - %CAT).

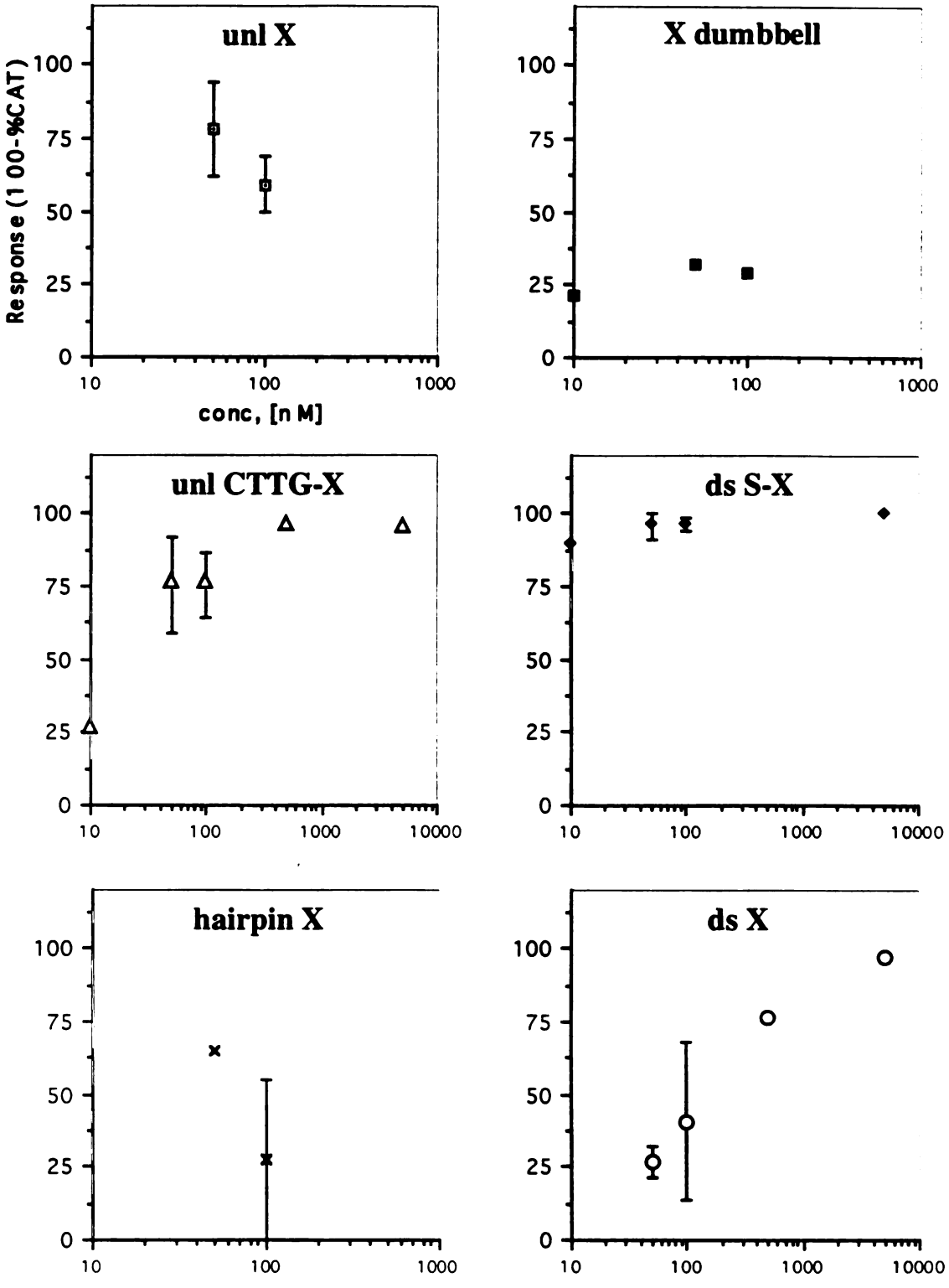
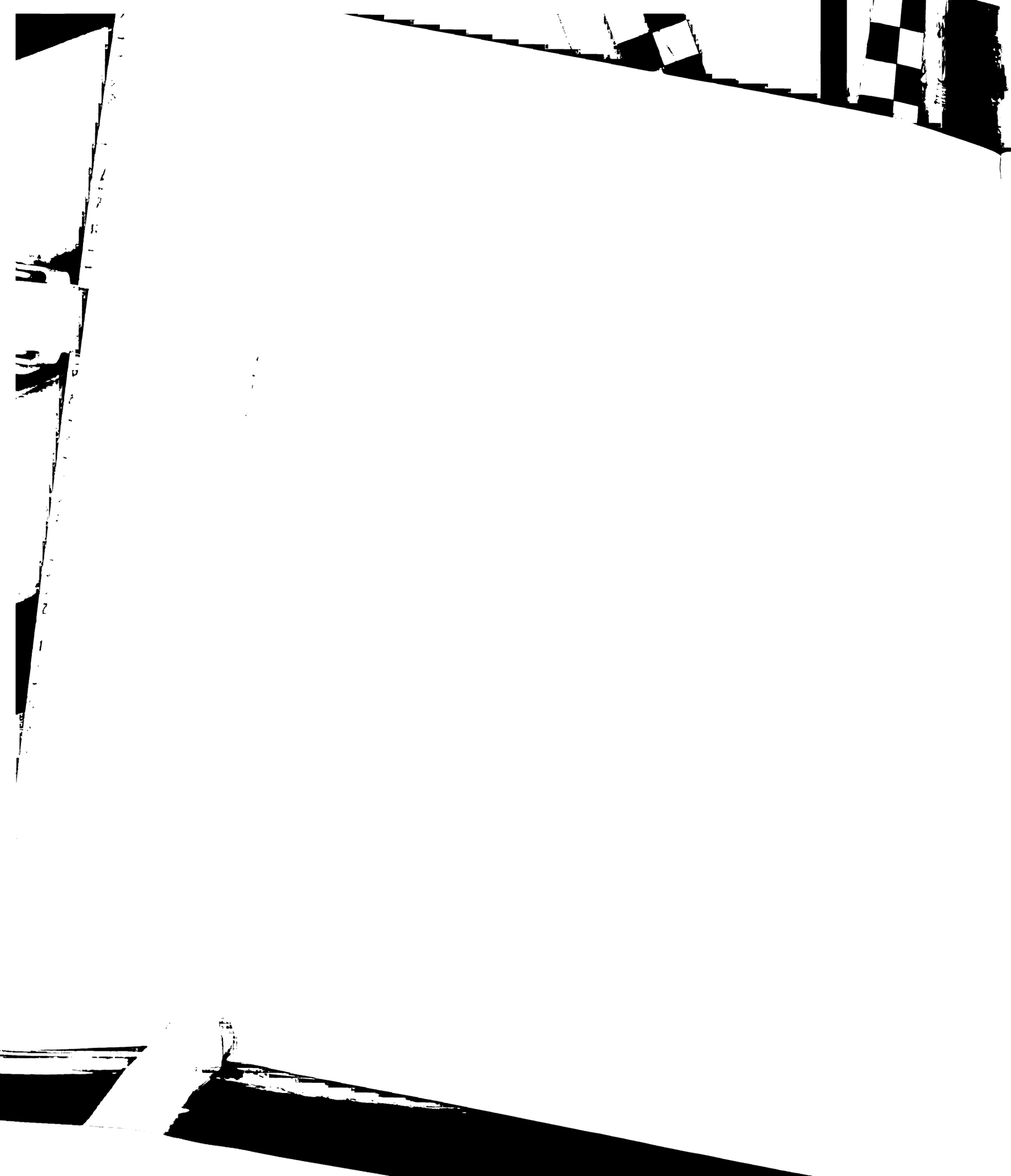


Figure 39. Individual dose-response curves. Error bars (between experiment SD) are shown for experiments repeated 3 or more times (in duplicate or triplicate).



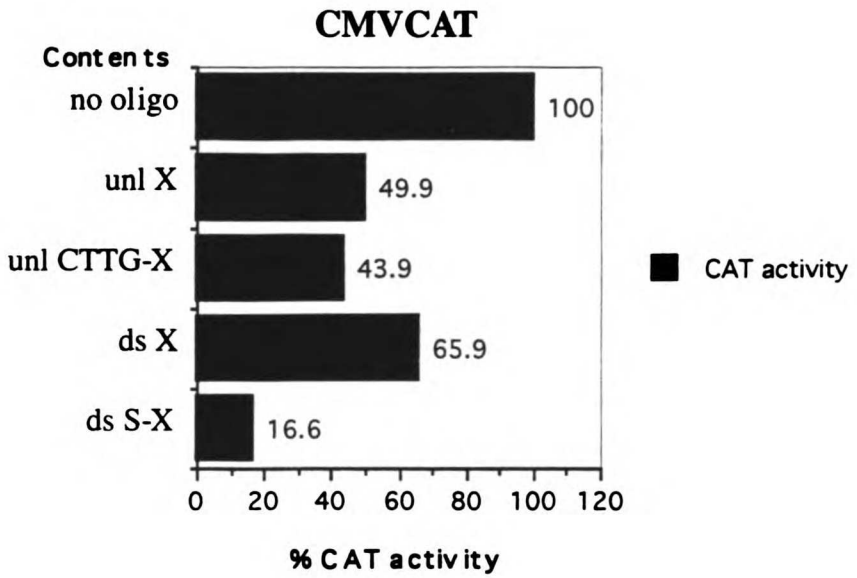


Figure 40. CMVCAT control. Unligated dumbbells and ds S-X are active in this system.

enzyme. Production of βgal can be detected by the appearance of a blue color when mixed with substrate (1). For SVβgal assays, only the 2 most potent oligos were tested (ds S-X and unl CTTG-X). Compared to SVβgal alone, ds S-X treated cells expressed only 2-3% blue cells while unl CTTG-X treated cells expressed ~20% blue cells. Thus, the same effect was seen with these 2 reporter genes as well.

Active oligos and dumbbells did not alter transfection efficiency of reporter plasmids

The ability of active oligos and dumbbells to decrease reporter gene readouts may have been due to their ability to reduce transfection efficiency. To address this issue, Cos-7 cells transfected with plasmids in the presence or absence of oligo were subject to PCR amplification. Treated cells were harvested and lysed to extract their DNA which contains transfected plasmid DNA. This DNA was then subjected to PCR amplification.

The amount of DNA in each sample is proportional to the ethidium bromide-stained band intensity when run on a gel if low cycle number (<25 cycles) PCR is used (17), i.e., the amount of DNA remains in the linear amplification range. To verify that this protocol can be used to detect at least a 2-fold difference in the amount of plasmid present, a standard was performed: two different concentrations of plasmids, 2.25 ng and 4.5 ng, were amplified using this method. The results in Figure 41 show that there was a discernible difference between the band intensities with a 2-fold difference in plasmid concentrations..

The second PCR experiment was performed on DNA extracted from cells transfected with plasmid and oligo. Two of the most active oligos, ds S X-box and unl CTTG-X-box, were tested. PCR analysis of transfected samples showed that the amounts of amplification product from all transfections were similar (Figure 42, lanes 2-4), indicating that the oligos had no effect on transfection efficiency. Negative controls included a DNA sample from mock-transfected cells (Figure 42, lane 1) and a water blank (Figure 42, lane 6). In addition to transfected pRFX1VP16, another positive control included 2 ng pRFX1VP16 as template (Figure 42, lane 5).

Total RNA levels were not altered by active oligos or dumbbells

Total RNA levels were checked to determine if the oligos were inhibiting transcription in general. The results of this assay (Figure 43) showed that despite the dramatic decrease in CAT activity upon addition of 100 nM oligo, there was no distinguishable decrease in overall transcription at the time point selected. However, if only polymerase II, which transcribes mRNA, was being affected, this would not be discernible since the majority of the total RNA is ribosomal RNA. Less than 5% of total cytoplasmic RNA is mRNA. Therefore, to detect a possible difference in message, the RNase protection assay was performed.

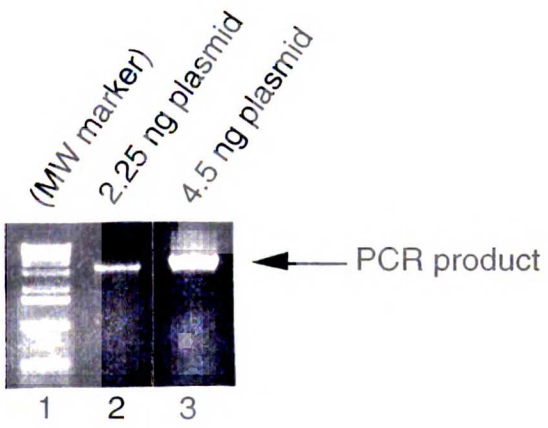


Figure 41. PCR amplification of 2 concentrations of plasmids, 2.25 ng and 4.5 ng.

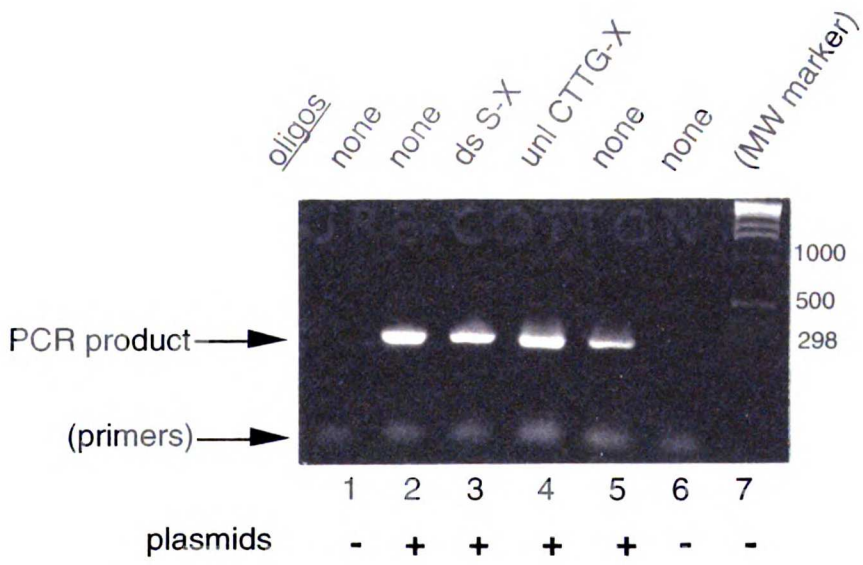
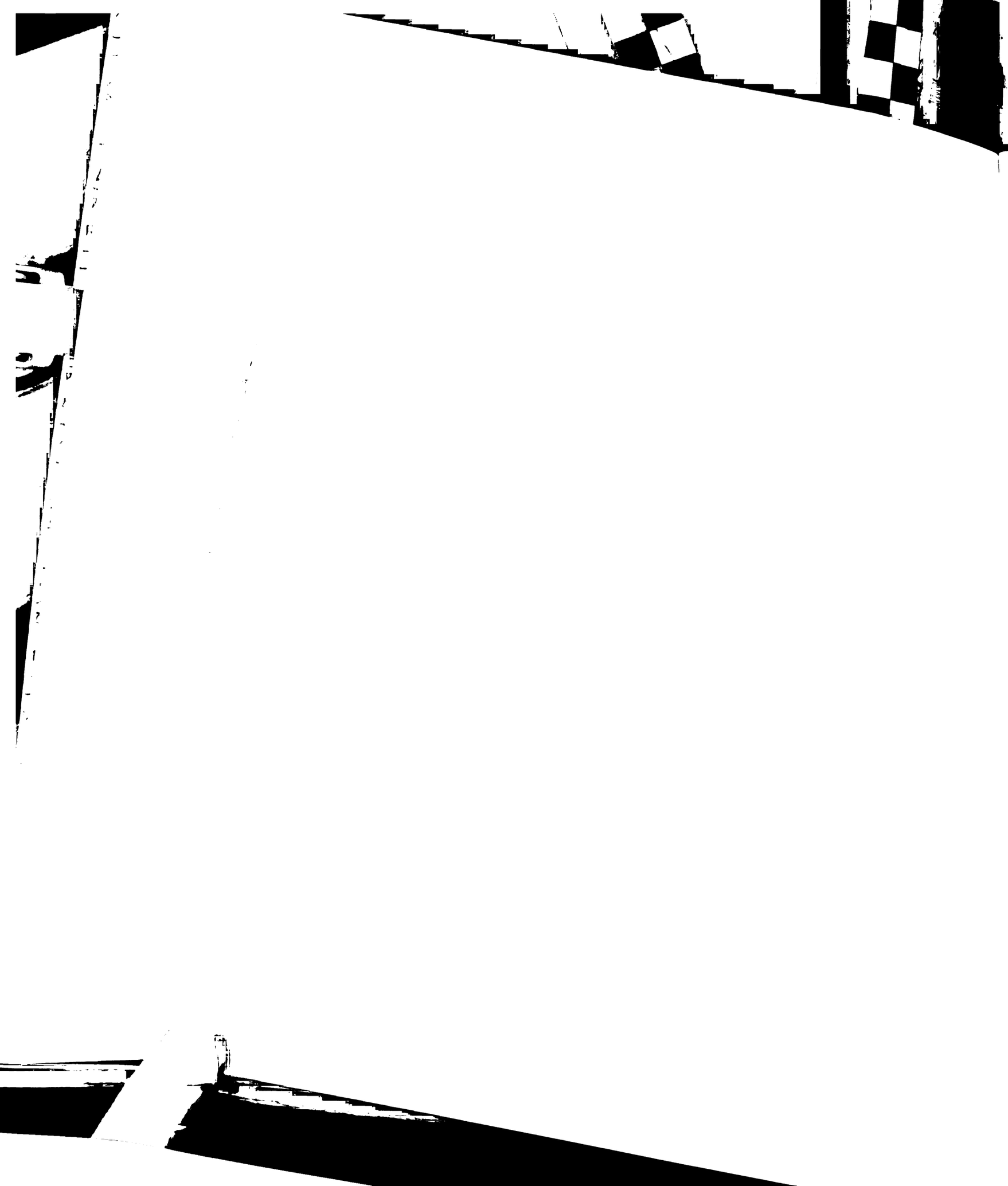


Figure 42. PCR-based assay for transfection efficiency. Lanes contain PCR-amplified DNA from: lane 1, cells alone (mock transfection); lane 2, cells transfected with plasmids pRFX1VP16 and p4XBCAT (p = both plasmids) ; lane 3, cells transfected with p and ds S-X; lane 4, cells transfected with p and unl CTTG-X; lane 5, 2ng pRFX1VP16; lane 6, blank (water). Lane 7 contains molecular weight markers.



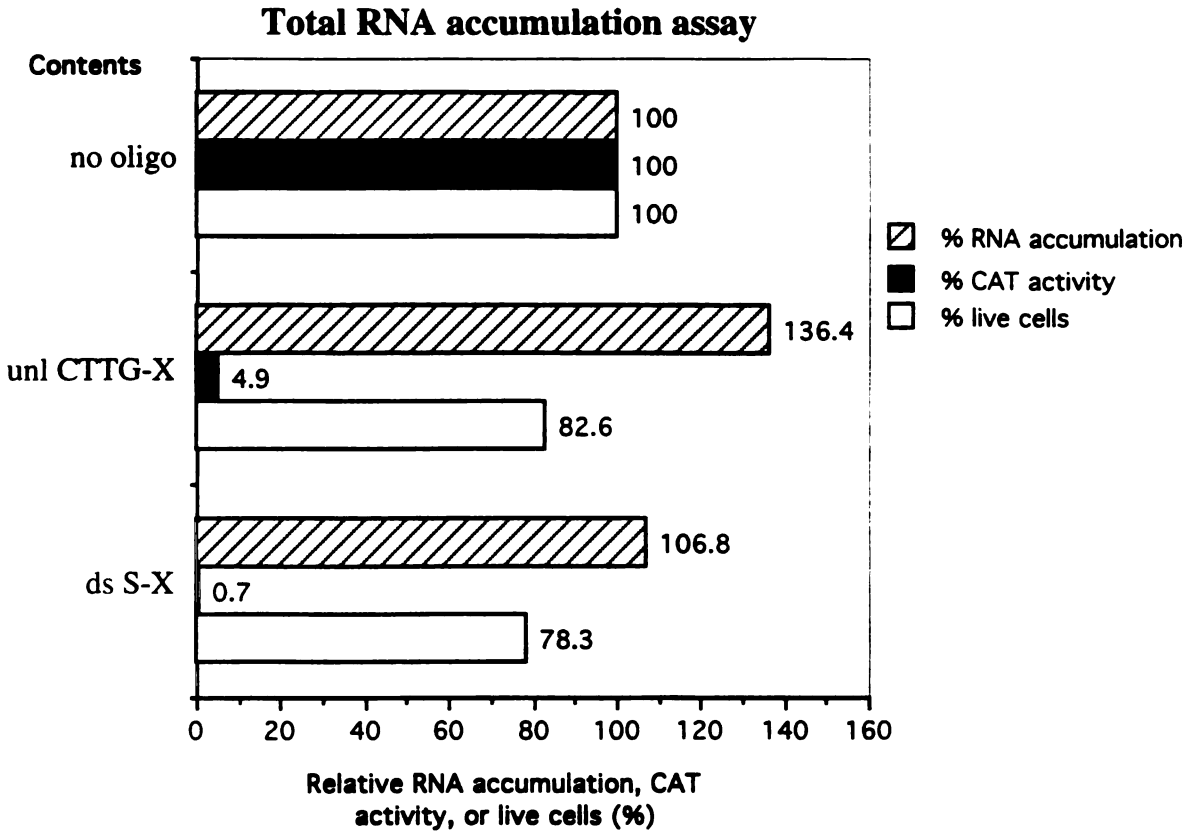


Figure 43. Total RNA accumulation assay. Total RNA levels are not decreased by the addition of oligos. CAT and live cells are included for comparison.

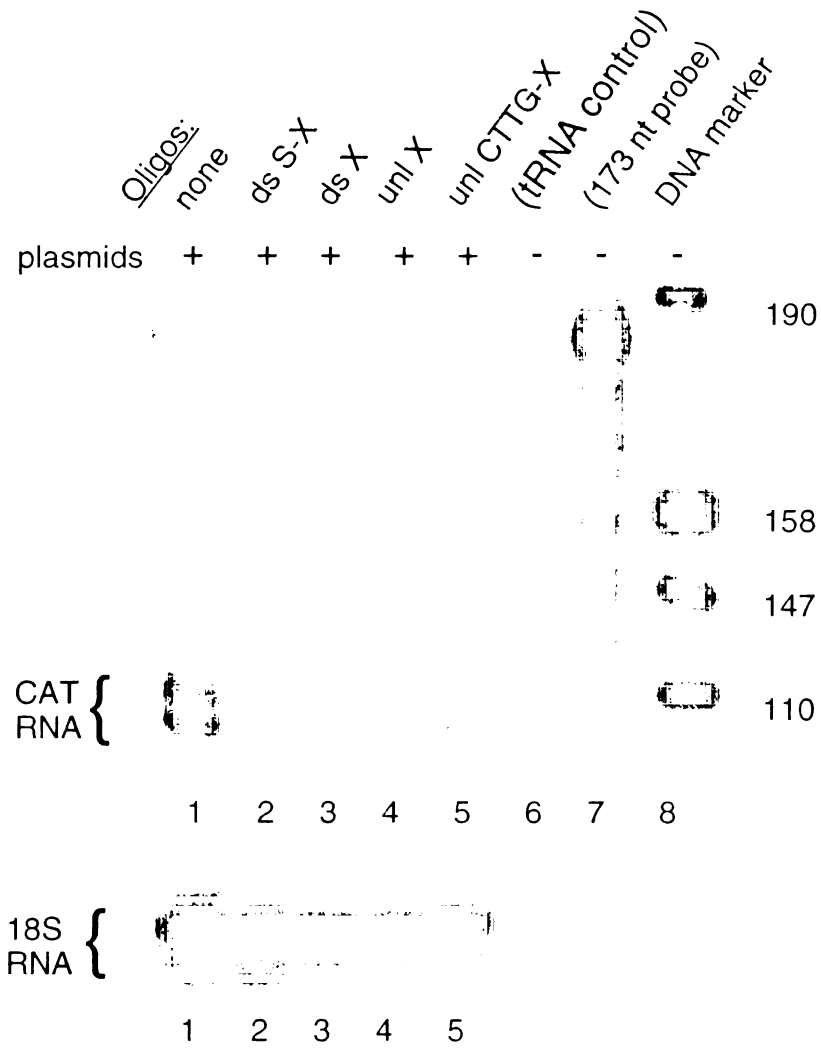


Figure 44. RNase protection. RNA harvested from cells transfected with: lane 1, plasmids pRFX1VP16 and p4XBCAT (p); lane 2, p + ds S X; lane 3, p + ds X; lane 4, p + unl X; lane 5, p + unl CTTG-X; all probed with the CAT probe (or the 18S RNA probe, below). Lane 6, RNA from cells transfected with p, probed with tRNA (control). Lane 7, 173 nt probe alone; lane 8, DNA molecular weight marker.

Note: the presence of 2 bands in this gel is most likely due to RNA:DNA hybrid formation, which is also resistant to RNase. This often will arise when RNA from transient transfections are used (hence contaminating DNA is present) (17). The protected 80bp fragment of 18S RNA should normally appear as 2 closely migrating bands "due to a portion of the antisense probe rehybridizing to its complementary sequence" according to the manufacturer (Ambion).

Levels of mRNA were decreased by the addition of active oligos and dumbbells

To determine if active oligos and dumbbells were effecting general transcription of a reporter plasmid, mRNA transcripts were measured using an RNase protection assay. RNase assays revealed a decrease in CAT mRNA, which is transcribed by pol II, upon the addition of 50 nM core X-box oligos, (Figure 44; compare lane 1, no oligo added, to lanes 2-5). The ds S-X oligo (Figure 44, lane 2) yielded the largest decrease in mRNA levels. The protected band(s) are consistent with the predicted size (111 base pairs). In contrast, levels of 18S RNA (transcribed by polymerase I) were not altered by the addition of oligos (Figure 44, lanes 2-5; CAT RNA and 18S RNA were probed separately).

DISCUSSION AND FINAL SUMMARY

Correlation between *in vitro* and *in vivo* data

In EMSAs using Raji nuclear extracts and a labeled X-box oligo probe, competitor oligos with the core X-box sequence were able to compete efficiently for complex formation. However, in direct binding assays, only the X dumbbell was able to form a complex with an X-box protein identified as RFX1 (Chapter 2). In the first *in vivo* model system tested, unligated dumbbells had a non-specific effects on reporter gene assays (Chapter 3). Unligated irrelevant sequence oligos did not compete *in vitro* (Chapter 2), but in the system described in this last chapter, had activity in 3 different *in vivo* reporter gene assays. This suggested that the oligos inhibited transcription in a manner not predicted, i.e., not by competition with the reporter plasmid target for RFX1 binding. The PCR-based assay indicated that the oligos were not directly interfering with plasmid transfection, nor were they inhibiting all transcription (based on total RNA levels). However, RNase protection assay analysis indicated that the oligos were inhibiting RNA polymerase II transcription. In retrospect, the *in vitro* gel shift assays were not a good predictor of oligo activity since many of these oligos had unanticipated effects.

Inhibition of RNA polymerase II: the mechanism of action of DNA dumbbells

In Chapter 4, it was shown that active oligos and dumbbells did not decrease levels of total RNA (Figure 43). This suggested that RNA polymerases (Table X) in general were not being inhibited. The next step was to determine if a subset of these polymerases were

Table X. Eukaryotic RNA polymerases.

RNA polymerase	Transcribes:
RNA pol I	large rRNA (ribosomal RNA) including 18S RNA
RNA pol II	genes whose RNAs (mRNAs) will be transcribed into proteins (messenger RNA); snRNPs (small nuclear ribonucleoproteins)
RNA pol III	small stable RNAs including 5S and tRNA (transfer RNA)

being altered by active oligos and dumbbells. The most likely candidate was RNA pol II, which transcribes DNA into messenger RNA. Since only 5% of total RNA is mRNA (Figure 45), alterations in mRNA levels would not be detectable in a total RNA assay. The RNase protection assay (Figure 44) did indeed show that mRNA levels were diminished upon the addition of active oligos or dumbbells. It remains unclear how these active oligos or dumbbells interact with RNA pol II to decrease mRNA levels. However, there are several mechanisms which can explain decreased mRNA levels. First, active oligos or dumbbells could interact directly with RNA pol II, to form inactive, non-productive complexes with RNA pol II. Second, active oligos or dumbbells could interact with RNA pol II-associated general transcription factors, which include general initiation factors TFIIB, TFIID, TFIIE, TFIIF, and TFIIH; as well as general elongation

factors SII, SIII (Elongin), TFIIF, ELL, and P-TEFb (5). The protein RAP30, part of the TFIIF complex, prevents RNA pol II from associating with non-specific DNA. If active oligos or dumbbells were capable of interacting with RAP30, they could cause RNA pol II to bind to non-productively to non-specific DNA which would result in decreased transcription. Third, active oligos or dumbbells could prematurely terminate transcription. An example of a compound that interrupts RNA pol II transcription by premature termination is the chemical inhibitor 5,6-dichlorobenzimidazole (DRB).

There is a precedent for oligonucleotide inhibition of RNA pol II in the literature. Golub *et al.* describe a specific triple-helix forming oligo which prevented *in vitro* transcription by RNA pol II (13) but only in the presence of RecA protein. RecA protein is involved in genetic recombination in *E. coli* and in this study, was thought to enhance the ability of the triple-helix forming oligo in finding its double-stranded target. There are several other examples in the literature of triple-helix inhibition of bacterial T7 RNA polymerase (2, 16, 18) as well.

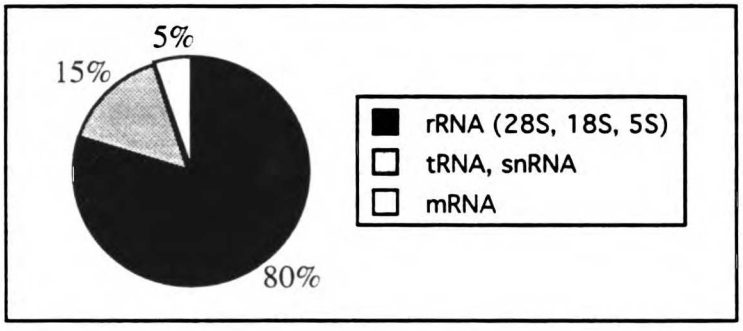


Figure 45. Distribution of RNA types in mammalian cells. Less than 5% of total RNA is mRNA.

Comparison of the 2 systems tested: Cos-7/RFX1VP16 vs. Raji/DRASCAT

Interestingly, double-stranded phosphorothioates were the most active oligos in the

Cos-7/RFX1VP16 system; in the Raji/DRASCAT system, they were the least active (Figure 31 in Chapter 3 vs. Figure 38). Phosphorothioate duplexes were initially chosen as positive controls for these studies. The ds S-X duplex was expected to be active both *in vitro* and in reporter gene assays. However, the use of phosphorothioate oligos warrants careful considerations. In recent years, it has been shown that phosphorothioates exhibit a wide range of non-sequence specific activities (21). These non-sequence specific activities include inhibition of cell growth and adhesion (8), inhibition of DNA polymerases (12), inhibition of T4 polynucleotide kinase (22), and binding to proteins (7) such as C23/nucleolin (27). This list is by no means exhaustive. It merely serves to reinforce that it is critical to recognize that phosphorothioate oligonucleotides exhibit a multitude of non-sequence specific effects.

Overall, the Cos-7 system was more sensitive to oligonucleotide dose. In general, oligos and dumbbells that were active in the Raji system (200 nM) required 4 times the dose used in the Cos-7 system (50 nM). This of course, could have been due to the method of transfection used, the cell type, cell number, reporter genes used, etc. as well as the sensitivity of the cells to the oligo treatment. Comparison of Figures 31 and 38 show other differences in oligo activity; notably, X dumbbells were active in the Raji system, and relatively inactive in the Cos-7 system. Perhaps, in the Cos-7 system, the fully closed, ligated dumbbell could not adopt the conformation required for inhibition of RNA pol II-associated transcription.

Toxicity considerations of oligonucleotide therapeutics

Toxicity or cell viability is an important factor that should be considered when interpreting results. In the typical reporter gene assays used for assessing oligo activity, cell death can inadvertently contribute to "activity" since activity is commonly measured by decreases in reporter gene protein. While others have reported the sequence-specific blockage of transcription factors with oligo doses ranging from 10 nM to 7.5 μM, no

mention of cell viability/toxicity is made in several of the studies (6, 10). Some of our earlier studies indicated that certain oligos were more toxic than others, usually when in the micromolar range (data not shown). There have been several reports of toxicity of phosphorothioates (8, 11); however, in our studies, phosphorothioates were never toxic (as measured by cell viability) at the concentrations we tested (up to 5 μM). Toxicity of phosphorothioates is thought to be length dependent (11), so perhaps our phosphorothioate duplexes were non-toxic due to their relatively short length (16 mers).

Correlation between activity and overall oligonucleotide stability

Factoring out toxicity, in our reporter gene assays, all core X-box oligos were able to block RFX1VP16 activation of 4XBCAT. Interestingly, activity correlated with predicted oligo stability (i.e., the more stable the oligo, the more potent it was). Phosphorothioates would be expected to be the most stable due to their nuclease resistance in cells (25). Chu (9) reported the following order of stability (in human sera): dumbbells > hairpins > double strands > single strands, due to active single-strand endonucleases and less rapidly acting double-strand exonucleases. Thermodynamically, among the unligated dumbbells, the expected order of stability is: CTTG loop > T loop > A loop, based on DNA hairpin studies (3). Therefore, in terms of resistance to exonucleases and thermodynamic stability, the expected order of overall stability (and coincidentally, the order of activity) for our oligos in the Cos-7 system was: ds S-X > unl CTTG-X > unl X > unl A-X > hairpin X > ds X. The melting temperature of unl X under highly denaturing conditions was 42.5°C; therefore under physiologic conditions unligated dumbbells would be expected to exist as stable duplex molecules. Even with a nick in the dumbbell (i.e., the unligated state), endonuclease degradation in this cell line could be minimal, allowing the oligos ample time to exert their effects. The stability of unligated dumbbells (and phosphorothioate oligos) may contribute to their apparent activity.

DNA dumbbells: a new class of biologically active compounds

In summary, factors that may influence the success of DNA decoys or dumbbells include: the dose and toxicity profile of the oligo, the cell line, the affinity and specificity of the transcription factor for the decoy *in vivo*, the affinity of the oligo toward transcriptional machinery and other non-specific effects. The study of DNA dumbbells presents a unique opportunity to test the effects of the addition of short, unmodified or "naturally occurring" phosphodiester oligonucleotide duplexes to cell systems. These dumbbells may have non-sequence specific effects on the intracellular milieu, including inhibition of RNA pol II transcription. Hence dumbbells may prove to be useful tools for studies involving pol II transcription and gene expression. Interestingly, unligated dumbbells (referred to as "nicked" dumbbells) with phosphorothioate substitutions in the loops were recently found to have anti-HIV activity in a human T-cell line (28). Unligated (nicked) dumbbells represent a new class of biologically active oligonucleotides with activities yet to be discovered.

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APPENDICES

Appendix I. All oligonucleotide sequences, structures, and rationale for sequence selection

Oligo name, sequence, rationale	**Expected activity (+ = active; - = inactive)**

unl X (unligated 4 T loop X-box): +

5'- CTAGGGGTTTTCCCCTAGCAACAGATGTTTTCATCTGTTG -3'

Rationale: contains the entire X-box sequence, the target of RFX. The unligated dumbbell structure was tested for comparison to the ligated structure (X dumbbell); 4 T loop size.

X dumbbell (ligated 4 T X-box dumbbell): +

$_{T}{}^{T}$CCCCTAGCAACAGATG$^{T}{}_{T}$
$^{T}{}_{T}$GGGGATCGTTGTCTAC$_{T}{}^{T}$

Rationale: contains the entire 16 base-pair double-stranded X-box sequence, the target of RFX; 4 T loops on each end; ligated version of unl X.

---

unl 5 T-X (unligated 5 T-loop X-box oligo): +

5'-CTAGGGGTTTTTCCCCTAGCAACAGATGTTTTTCATCTGTTG-3'

Rationale: same as unl X except the loop size is 5 instead of 4 (does loop size makes any difference in transcription factor binding?).

---

5 T-X dumbbell (ligated 5 T-loop X-box dumbbell): +

$_{T}{}^{T}{}_{T}$CCCCTAGCAACAGATG$^{T}{}_{T}$
$^{T}{}_{T}$GGGGATCGTTGTCTAC$_{T}{}_{T}{}^{T}$

Rationale: ligated dumbbell version of unl 5 T-X.

---

unl CTTG-X (unligated CTTG-loop X-box): +

5'-CTAGGGGCTTGCCCCTAGCAACAGATGCTTGCATCTGTTG-3'

Rationale: same internal sequence as unl X; loops contain thermodynamically stable "CTTG" loops; more stable than T loops

---

Oligo name, sequence, rationale	Expected activity (+ = active; - = inactive)

unl A-X (unligated 4 A-loop X-box): +

5'-CTAGGGGAAAACCCCTAGCAACAGATGAAAACATCTGTTG-3'

Rationale: same internal sequence as unl X; A loops used (slightly less stable than T loops).

---

unl hlyn: +

5'- TAACAACTTTTGTTGTTATAGTAACTTTTGTTACTA -3'

Rationale: internal sequence should bind better to RFX than the wild-type X-box; see Ch. 1 (21)

---

hlyn dumbbell: +

$_{T}^{T}$GTTGTTATAGTAAC$^{T}_{T}$
$^{T}_{T}$CAACAATATCATTG$_{T}^{T}$

Rationale: internal sequence should bind better to RFX than the wild-type X-box; see Ch. 1 (21); ligated version of unl hlyn

---

unl scrambled (scr) X: -

5'- ATTTTTCCCTTTTGGGAAAAATTCCCCCCTTTTGGGGGGA -3'

Rationale: scrambled control sequence

---

scr X dumbbell: -

$_{T}^{T}$GGGAAAAATTCCCCCC$^{T}_{T}$
$^{T}_{T}$CCCTTTTTAAGGGGGG$_{T}^{T}$

Rationale: scrambled control sequence; ligated version of unl scr X

---

unl ctrl1 (unligated control oligo 1): -

5'-TGAGTTGGTTTTCCAACTCACTTTCCGCTTTTGCGGAAAG-3'

Rationale: contains an irrelevant unligated control sequence (this and all other controls should not be able to bind to RFX) taken from Bielinska *et al.* (3).

Oligo name, sequence, rationale	Expected activity (+ = active; - = inactive)

ctrl dumbbell 1 (ligated control dumbbell 1): -

$_{T}{}^{T}$CCAACTCACTTTCCGC$^{T}{}_{T}$
$^{T}{}_{T}$GGTTGAGTGAAAGGCG$_{T}{}^{T}$

Rationale: ligated version of unl ctrl 1.

---

unl ctrl 2 (unligated control oligo 2): -

5'-TCCCTTGGTTTTCCAAGGGACTTTCCGCTTTTGCGGAAAG-3'

Rationale: contains the "κB" sequence used by Bielinska *et al.* (6) which is the target of the transcription factor NF-κB. This was chosen as a control sequence in this system.

---

unl ctrl 3 (unligated control oligo 3): -

5'-TATACGGGTTTTCCCGTATACCACTCTGTTTTCAGAGTGG-3'

Rationale: contains the "m6 mutant" mutation of the X-box known to abolish RFX binding (19).

---

ctrl 3 dumbbell(ligated control dumbbell 3): -

$_{T}{}^{T}$CCCGTATACCACTCTG$^{T}{}_{T}$
$^{T}{}_{T}$GGGCATATGGTGAGAC$_{T}{}^{T}$

Rationale: ligated version of unl ctrl 1.

---

unl rev X (unligated reverse sequence X-box): -

5'-GTTGTCTACTTTTGTAGACAACGATCCCCTTTTGGGGATC-3'

Rationale: designed as a control sequence (the X-box sequence in reverse).

---

ds X (double-stranded phosphodiester X-box oligo) and ds S-X (double-stranded phosphorothioate X-box oligo): **+ for both**

5'-CCCCTAGCAACAGATG-3'
3'-GGGGATCGTTGTCTAC-5'

Rationale: both ds X and ds S-X are double-stranded oligos which contain the X-box sequence, the target of RFX. The ds S-X should not be degraded by cellular nucleases.

Oligo name, sequence, rationale	Expected activity (+ = active; - = inactive)

DRAX, double-stranded DRAX probe (-116 to -87): +

```
5'-     CTTCCCCTAGCAACAGATGCGTCATC-3'
3'-CCGGGAAGGGGATCGTTGTCTACGCAGTAGAGCT-5'
```

Rationale: contains the X-box and flanking sequences (which may be necessary for RFX binding); this sequence was used by the Peterlin lab in gel shift assays involving RFX. This sequence was created by restriction enzyme cleavage (hence the overhanging ends) using *Apa* I and *Xho* of a plasmid known as "pDRASCAT" which contains the X-box.

---

ds S-ctrl 3: -

```
5'-CCCGTATACCACTCTG-3'
3'-GGGCATATGGTGAGAC-5'
```

Rationale: the phosphorothioate version of unl ctrl 3 (control sequence).

---

hairpin X: + (?)

```
5'-CCCCTAGCAACAGATG T T
3'-GGGGATCGTTGTCTAC T T
```

Rationale: contains the X-box sequence; an intermediate between a dumbbell and a double-stranded oligo.

---

ss top X (single-stranded top strand of the X-box) -

```
5'-CCCCTAGCAACAGATG -3'
```

Rationale: contains the top strand of the X-box

---

ss B2 (an arbitrary control sequence) -

```
5'-GCCACGGAGCGAGACATCTC-3'
```

Rationale: contains a single-stranded control sequence

---

ns 10 (non-specific 10-mer): -

```
5'-GAATTCGGCC-3'
```

Rationale: a standard sequence used by Peterlin's group, a non-specific single-stranded oligo used as a gel shift assay control.

Oligo name, sequence, rationale	Expected activity (+ = active; - = inactive)
linear QI:	-
5'-AAAAGTCGACTGTGTAGGAATCCCAGCCAGATGCATCTCT-3'	
Rationale: a linear oligo 40-mer used to determine if activity is dependent merely on oligo size (length).	
linear QII:	-
5'-AGAGATGCATCTGGCTGGGATTCCTACACAGTCGACTTTT-3'	
Rationale: another linear oligo 40-mer used to determine if activity is dependent merely on oligo size (length).	

Appendix II. Activity of other oligos in DRASCAT/Raji system:

Oligo	Dose (μM)	% CAT activity	N. (STDEV)	n. (stdev)	activity
unl 5T-X	1	118.34	2	2	I
unl X-PO_4	1	102.6	3 (5.87)	2,2x3 (8.71, 21.78)	I
unl A-X	1	16.02	2	2,3 (2.91)	A++
	0.2	38.9	1	3 (10.33)	A+
unl hlyn	1	16.72	3 (26.43)	2x2,3 (5.55)	A++
	0.2	58.78	1	2	A
unl ctrl 1	1	88	7 (48.23)	1,6x2	I
	0.5	31.3	1	2	A+
	0.25	44.6	1	2	A+
	0.2	35.29	2	2,3 (5.03)	A+
	0.1	76.68	1	3 (2.98)	I
unl ctrl 2	1	27.2	2	2,3 (36.94)	A+
	0.5	8.1	1	2	A++
	0.2	50.85	1	2	A
unl ctrl 3	1	70.49	6 (34.3)	5x2,3 (59.32)	A
	0.5	30	1	2	A+
	0.25	37.65	1	2	A+
	0.2	50.23	1	2	A
unl rev X	0.2	41.53	1	2	A+
	0.1	64.97	1	3 (3.86)	A
unl scr1	1	26.53	1	2	A+
unl scr2	0.5	8.09	1	2	A++
	0.2	33.95	1	2	A+
scr2 X dumbbell	0.5	0.655	1	2	A++
ds S-ctrl 3	1	134.69	2	2,3 (9.88)	I
ss top X	1	64.65	2	3 (25.78, 15.09)	A
	0.2	93.1	1	3 (3.87)	I

ss B2	1	56.5	1	3 (6.18)	A
linear QI	0.2	76.29	1	2	I
linear QII	0.2	85.73	1	2	I

N = number of times experiment was performed

STDEV = between experiment standard deviation (if N=3 or more)

n = number of replicates per experiment (n= 1, 2, or 3)

stdev = within experiment standard deviation (if n=3)

activity ranges from I (inactive) to A (active)++:

I >75% CAT activity

50% < A <75%

25% < A+ <50%

A ++ <25%

Appendix III. Activity of other oligos in RFX1VP16/Cos 7 system:

Oligo	Dose (nM)	% CAT activity	N, (STDEV)	n, (stdev)	activity
unl A-X	50	24.68	1	3 (12.42)	A++
	100	14.05	1	1	A++
unl hlyn	100	39.82	1	1	A+
unl ctrl 2	50	36.45	2	2,3 (7.24)	A+
	100	68.31	1	1	A
unl ctrl 1	100	13.14	1	1	A++
unl ctrl 3	50	46.36	2	2,3 (15.23)	A+
	100	57.44	2	1,2	A
unl rev X	100	29.63	1	1	A+
unl scr2	100	21.84	1	1	A++
ds S-ctrl 3	100	3.19	2	2x3 (0.5, 0.67)	A++
linear QI	100	8.68	1	1	A++
linear QII	50	32	1	1	A+
	100	8.24	1	1	A++
	500	7.14	1	1	A++

N = number of times experiment was performed

STDEV = between experiment standard deviation (if N=3 or more)

n = number of replicates per experiment (n= 1, 2, or 3)

stdev = within experiment standard deviation (if n=3)

activity ranges from I (inactive) to A (active)++:

I >75% CAT activity

50% < A <75%

25% < A+ <50%

A ++ <25%