Title
Characterization and mechanism of action of interferon-gamma inhibitory oligodeoxynucleotides

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CHARACTERIZATION AND MECHANISM OF ACTION OF INTERFERON-GAMMA INHIBITORY OLIGODEOXYNUCLEOTIDES

by

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DISSERTATION

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This Section is more likely to remain incomplete than any other part of my thesis because I have so many people to thank. In any case, I hope not to miss the most important contributors..

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ABSTRACT

Interferon-γ (IFN-γ) is a pleiotropic cytokine that acts at various levels of the immune system to mediate graft rejection. Agents capable of blocking one or more physiological effects of IFN-γ may have useful therapeutic applications in transplantation.

In Section I, is a report on the characterization and mechanism of action of the oligodeoxynucleotide **I**, 5' GGG GTT GGT TGT GTT GGG TGT TGT GT—RNH_2, which inhibits the interferon-γ mediated enhancement of MHC Class I and ICAM-1 proteins in the K562 cell line. The inhibition is dose-dependent, with an EC_{50} value, 24 hours after dosing, of approximately 4 μM for 800 U/ml interferon-γ. The reverse complement **II**, 5' AC ACA ACA CCC AAC ACA ACC AAC CCC—RNH_2, is not inhibitory. Oligodeoxynucleotide **I** inhibits induction of MHC Class I by interferon-γ, but does not inhibit induction by either interferon-α or interferon-β. ICAM-1 enhancement by tumor necrosis factor-α (TNF-α), is also not inhibited by **I**. Oligo **I** belongs to a class of active oligodeoxynucleotides that inhibits interferon-γ induced MHC Class I and ICAM-1 in K562 cells. The activity and potency are sequence dependent, but remarkably different sequences can have comparable effects.

Oligonucleotide **I** acts as an aptamer to inhibit the binding of interferon-γ to cells. In binding studies carried out under conditions that minimize receptor endocytosis and recycling, **I** inhibits the association of radiolabeled interferon-γ to the cell surface. The inhibition of the association of interferon-γ with the cell surface is a novel site of action for oligos.

In Section II, a simple two compartment mathematical model (Hargrove, Hulsey et al. 1990; Hargrove and Schmidt 1989) is used to anticipate the effects of antisense oligodeoxyribonucleotide action within

single cells. The steady-state equations and dose-response relationships are derived for four mechanisms: 1) ribosome blockage, 2) mRNA cleavage by RNase H, 3) concurrent ribosome exclusion and RNase H action, and 4) decreased delivery of mature mRNA to the cytoplasm due to transcriptional blockage, interference with nucleocytoplasmic transport or splicing. Frequently translated mRNA producing stable proteins are the most attractive antisense targets because the protein levels are sensitive to the changes in the mRNA levels that can be effected using antisense oligonucleotides.

The nonsteady-state solutions show that both mRNA and protein half-life can determine the kinetics of antisense oligonucleotide action. A rapid onset of antisense effect will be observed in systems in which the mRNA is rapidly degraded and slowly translated and the translated protein is rapidly degraded. With a slowly degraded protein the kinetics of an antisense effect are limited by protein half-life. These results may be used to design experiments that discriminate among mechanisms of antisense action.

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dashed arrow represents the direction of information flow impacting material flow.

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Figure 4. Plot of effect vs. log f at different values of r for Case III. The fraction of the target mRNA that exists as a mRNA-oligo hybrid is f and $r = \frac{[k_{dm}]_{RNaseH}}{[k_{dm}]_{control}}$ is the ratio of the rate constants for oligo-induced RNase H degradation of target mRNA to its degradation rate constant under control conditions. Antisense activity in the system is assumed to be due to Case III, *both Ribosome exclusion and RNase H action* on hybrids.

Figure 5. In this Figure data from Figures 3 (Case II, solid lines) and 4 (Case III, dashed lines) are superimposed to highlight the differences between Cases II and III. If r is small, either due to low RNase H activity in a cell or rapid rate of target mRNA degradation, an antisense oligo targeted to include the

ribosome binding site may be more effective than an oligo targeted to some other coding portion of the mRNA.

Figure 6. Effect, as measured by the fractional inhibition of target protein synthesis, is plotted as a function of log $(\frac{[k_{dm}]_{RNaseH}}{[k_{dm}]_{control}})$, which is log of the ratio of the rate constant for RNase H activity to the mRNA degradation rate constant. The fraction of the total target mRNA that is in the form of mRNA-oligo hybrid is f. Two values of f are used: f = 0.5 when half the target mRNA exists as hybrid and f = 1 when all the target mRNA exists as a hybrid. The two S shaped curves with zero origins represent Case II, systems in which only the RNase H mechanism is operative. The uppermost curve represents a case when half the mRNA is in hybrid form (f = 0.5) and the ribosome exclusion mechanism plus the RNase H mechanism are operative (Case III).

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Characterization of the Oligodeoxynucleotide-Mediated Inhibition of Interferon-γ

ABSTRACT

Interferon-γ (IFN-γ) is a pleiotropic cytokine produced by lymphocytes and natural killer cells and has several modes of action. It is primarily an immunomodulatory agent that induces or enhances expression of major histocompatibility complex class I (MHC Class I), major histocompatibility complex class II and intercellular adhesion molecule-1 (ICAM-1) in a variety of cell types. At the cellular level, IFN-γ primes macrophages and stimulates natural killer cells. Clinically, the effects of IFN-γ at various levels of the immune system mediate graft rejection. Therefore, agents capable of blocking one or more physiological effects of IFN-γ may have useful therapeutic applications in transplantation.

We have observed that the effects of IFN-γ can be inhibited by several oligodeoxynucleotides (oligos) in K562 cells, a human myelogenous leukemia derived cell line which has low basal cell surface levels of MHC Class I that can be enhanced by IFN-γ. The observations were made serendipitously when we found a pattern of activity with oligos that were originally designed to provide experimental and control sequences for antisense and triple helix experiments.

We have shown that the oligodeoxynucleotide 5' GGG GTT GGT TGT GTT GGG TGT TGT GT—RNH_2, **I**, inhibits the interferon-γ mediated enhancement of MHC Class I and ICAM-1 proteins in the K562 cell line. The inhibition by oligodeoxynucleotide **I** 5' GGG GTT GGT TGT GTT GGG TGT TGT GT—RNH_2 is dose-dependent, with an EC_{50} 24 hours after dosing of approximately 4 μM for 800 U/ml interferon-γ. The reverse complement **II**, 5' AC ACA ACA CCC AAC ACA ACC AAC CCC—RNH_2, did not show activity. Oligodeoxynucleotide **I** inhibits induction of MHC Class I by interferon-γ, but does not inhibit induction by either interferon-α or interferon-β. ICAM-1

enhancement by tumor necrosis factor-α (TNF-α), is also not inhibited by **I**. Four other oligodeoxynucleotides were also evaluated, and three showed activity against interferon-γ at 25 μM. **I** belongs to a class of active oligodeoxynucleotides that similarly inhibit interferon-γ enhanced MHC Class I and ICAM-1 in K562 cells, but which differ in potency. The activity is sequence dependent, but remarkably different sequences can have comparable effects.

The activity of **I** in the HeLa S3 cell line inhibits the interferon-γ mediated enhancement of both ICAM-1 and MHC Class II DR, and the interferon-γ mediated reduction in transferrin receptor expression. Thus, **I** appears to specifically inhibit interferon-γ induced changes in protein expression in a variety of cell types, and this is consistent with **I** acting at an early step(s) in the induction process.

I have investigated the mechanism of action of **I** and report that it acts by inhibiting the binding of interferon-γ to cells. We also show that the dose response curves, the selectivity profile and the kinetics of **I** are consistent with this novel mechanism of action. The dose response curves for **I**, obtained using antibodies against MHC Class I heavy chain, β_2 microglobulin or ICAM-1 are almost superimposable at each observation time. Because MHC Class I induction by either interferon-α or interferon-β, or ICAM-1 enhancement by tumor necrosis factor-α (TNF-α), is not inhibited by **I**, the selectivity results show that a specific step involved in the interferon-γ induction process is inhibited. Furthermore, because the interferons share early signaling molecules, we interpret the data to suggest that the site of action is upstream of these common or shared elements. We also show that the synergistic induction of MHC Class I by mixtures of tumor necrosis factor-α and interferon-γ is inhibited. This result is consistent with a site of action

upstream of the step providing synergy and necessary for the induction of genes by interferon-γ.

In binding studies carried out under conditions that minimize receptor endocytosis and recycling, **I** inhibits the association of radiolabeled interferon-γ to the cell surface. Taken together, our results show that **I** exerts its effects by inhibiting the association of interferon-γ with the cell surface which is a novel site of action for oligos.

ABBREVIATIONS

D-PBS	Dulbeccos phosphate buffered saline
FBS	Fetal bovine serum heat inactivated at 56°C for 1 hour
FBSΔ	Fetal bovine serum heat inactivated at 65°C for 30 minutes
FITC	Fluorescein isothiocyanate
FL1	Fluorescence channel #1 in flow cytometer
FL2	Fluorescence channel #2 in flow cytometer
FSC	Forward scatter channel in flow cytometer
GAS	Gamma activated sequence
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MESF	Mean equivalent soluble fluorochromes
MHC	Major histocompatibility complex
oligo	Oligodeoxynucleotide
PBS	Phosphate buffered saline
PE	Phycoerythrin
PMT	Photomultiplier tube
SSC	Side scatter channel in flow cytometer
TNF-α	Tumor necrosis factor-α

INTRODUCTION

Research in immunotherapy has made possible life-saving clinical procedures, such as organ transplantation, and pharmaceutical products such as immunosuppressants, hematopoetic growth factors and vaccines. Successful pathogens such as the human immunodeficiency virus (HIV) and parasites such as *Leishmania* and *Listeria* however, continue to remain challenges. Much of current immunological research is directed toward understanding the cytokines and receptors that mediate interactions between the various cells types and the immune environment. The immune system offers targets for therapeutic intervention that are very rewarding but extremely challenging. The definition of the receptor ligand networks involved in the immune system has made possible the rational design of strategies for therapeutic intervention particularly for cancer, autoimmune diseases and allograft rejection. The strategies, targets and methodologies are reviewed by Waldmann (Waldmann 1992).

The primary target molecules for my research are the products of the Major Histocompatibility Complex (MHC) genes. The MHC proteins play a central role in the initiation of transplant rejection and the long-term goals of my project are to down-regulate the MHC proteins for applications in transplantation using nucleic acid based approaches.

Our laboratory has identified a class of oligodeoxynucleotides that specifically inhibits interferon-γ enhanced MHC expression in a variety of cell types. The specific aims of this thesis are to characterize these active oligodeoxynucleotides for therapeutic applications and to determine the mechanism and site of action.

The MHC genes encode a class of heterodimeric cell surface proteins that play a pivotal role in immune recognition by presenting antigenic

peptides to T cells and initiating the cascade of events that ultimately results in cellular and humoral immunity against viral, bacterial and parasitic pathogens. The MHC genes were first identified on the basis of skin graft experiments because grafts that are different at the MHC locus, termed allogeneic, are rejected, while grafts that do not differ at the MHC (or syngeneic grafts) have greatly improved survival. Even in the post-cyclosporin era, MHC compatibility is an important determinant of transplantation outcomes for a variety of solid organ and cellular transplants. In current transplantation practice, considerable effort is invested prior to transplantation to maximize MHC compatibility (Mickey 1987; Terasaki 1991) by identifying or 'typing' the MHC proteins on the donor and recipient by serological (Dyer and Martin 1991; Parham 1992) or molecular methods (Bidwell 1992; Charron 1993; Gyllensten and Allen 1991; Mach and Tiercy 1991; Wordsworth 1991). The currently available data suggests that decreased MHC expression will result in reduced graft immunogenicity and, for the transplant recipient, this translates to both a reduced likelihood of rejection and a lower immunosuppressant dosage. From a societal standpoint, reduced graft immunogenicity will improve both the cost and the logistics of transplantation because MHC typing can be minimized and transplants can be carried out with fewer MHC typing constraints.

In this Background section, I will discuss both the various oligodeoxynucleotide based strategies available and the regulation of the MHC by interferon-γ as these topics are most relevant to an understanding of my results. Antisense RNA is not discussed and the reader is referred to several comprehensive reviews (Green, Pines et al. 1986; Inouye 1988; Nellen and Lichtenstein 1993; Simons 1988; Simons and Kleckner 1988; van der Krol, Mol et al. 1988).

Oligonucleotide Based Therapeutic Strategies

The development of the phosphoramidite method for oligodeoxynucleotide synthesis has revolutionized the field of oligonucleotide biotechnology and, with the invention of the polymerase chain reaction, oligos have become indispensable reagents in the laboratory and in diagnostics. However, oligos are also being extensively investigated for several therapeutic applications because they offer the potential for a new generation of rationally designed drugs with high specificity and low toxicity. To overcome the problems associated with stability and cellular uptake, a variety oligonucleotide analogs have been synthesized and some these are shown in Figure 1. Several strategies have been used to translate the potential of oligos to practice and these are summarized in Table I.

Table I. The cellular targets for the various oligonucleotide based strategies.

STRATEGY OR APPROACH	CELLULAR TARGET OR RECEPTOR
Antisense	RNA
Antigene	Genomic DNA
Intercalator modified oligos	RNA or DNA
Reactive oligos	RNA or DNA
Ribozymes	RNA
DNA decoys	DNA binding proteins
Aptamers	Proteins

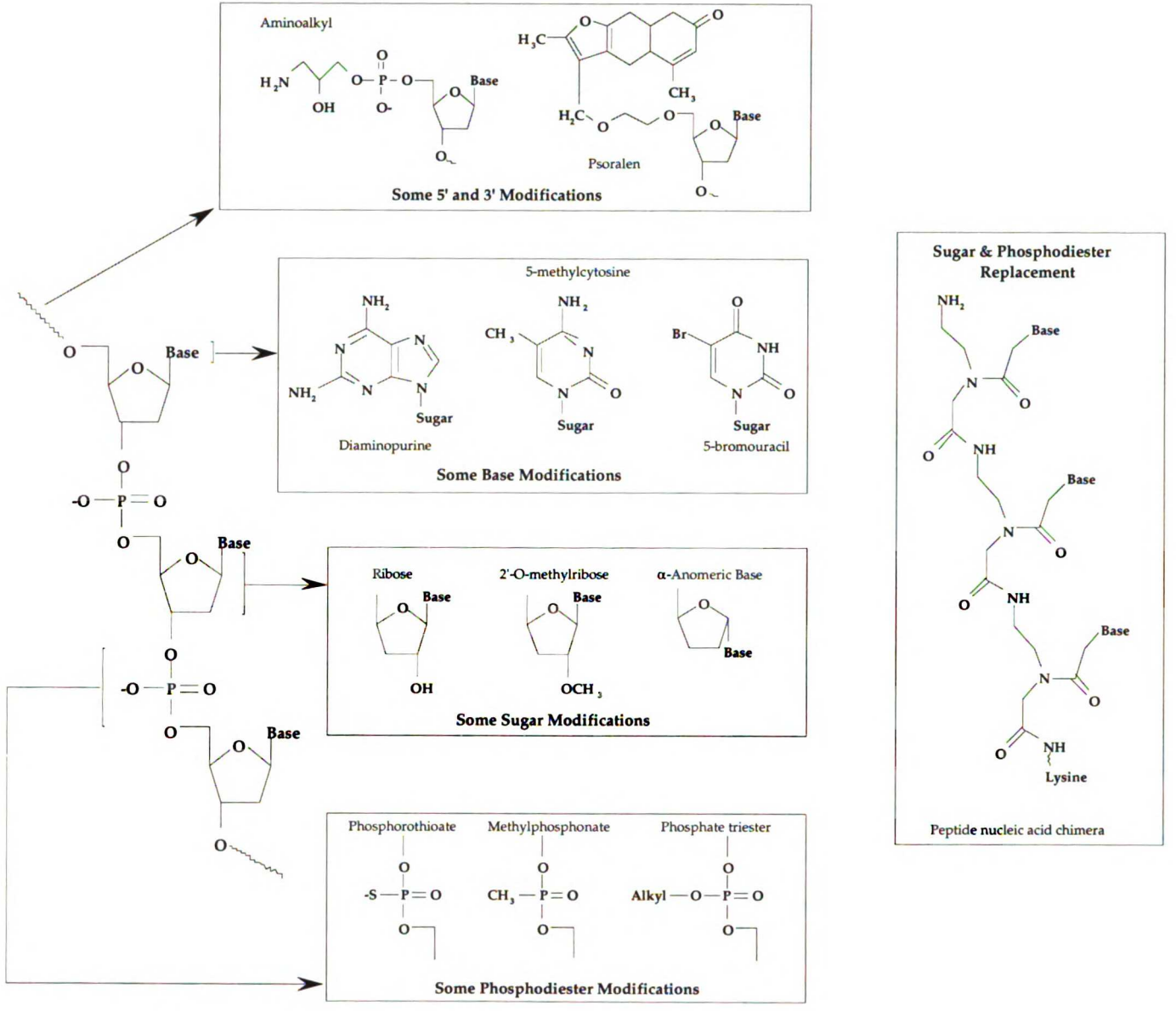


Figure 1. Some oligonucleotide analogs.

The Antisense Approach

The pharmacological receptor for an antisense oligo is RNA. The oligo is designed to be complementary (Miller, Braiterman et al. 1977; Stephenson and Zamecnik 1978; Zamecnik and Stephenson 1978) to the mRNA (or RNA) of interest and, on entry into the cell, binds the target RNA by forming Watson-Crick hydrogen bonds. The formation of a oligo-RNA hybrid ultimately results in reduced protein production via several effector mechanisms. These effector mechanisms include translational blockage, competition for the ribosomal assembly, and RNase H action. In the translational blockage mechanism, shown in Figure 2a, the formation of the oligo-mRNA hybrid hinders progress of the ribosome and results in a lowered rate of translation. In the competition for ribosomal assembly mechanism (Figure 2b), the oligo is usually designed to bind to the 5' untranslated region of a mRNA. This region of the mRNA contains sites that are important for the assembly of the ribosomal subunits and hybrid formation competes with ribosomal assembly resulting in a decreased rate of translational initiation. The principal effector in the RNase H mechanism is a ubiquitous enzyme, RNase H (Crouch and Dirksen 1985; Hostomsky, Hostomska et al. 1993), which recognizes and cleaves mRNA in mRNA-DNA hybrids. Thus, as shown in Figure 3a, when an antisense oligodeoxynucleotide forms a mRNA-oligo hybrid in the cell, RNase H (Boiziau, Larrouy et al. 1992; Walder and Walder 1988) specifically degrades the target mRNA. This reduces target mRNA levels and ultimately results in lower levels of the target protein. Post-transcriptional processes such as splicing and nucleocytoplasmic transport can also be inhibited using oligos. Methylphosphonate (Kulka, Smith et al. 1989; Miller, Agris et al. 1985; Smith, Aurelian et al. 1986) oligos directed against the splice sites of vesicular

stomatitis virus are quite effective suggesting that interference with splicing is a possible mechanism of action. Inhibition of nucleocytoplasmic transport is an effector for antisense RNA, and possibly antisense oligos, because high levels of RNA-RNA hybrids are detectable in the nuclear fraction (Kim and Wold 1985). These nuclear effector mechanisms are shown in Figure 3b.

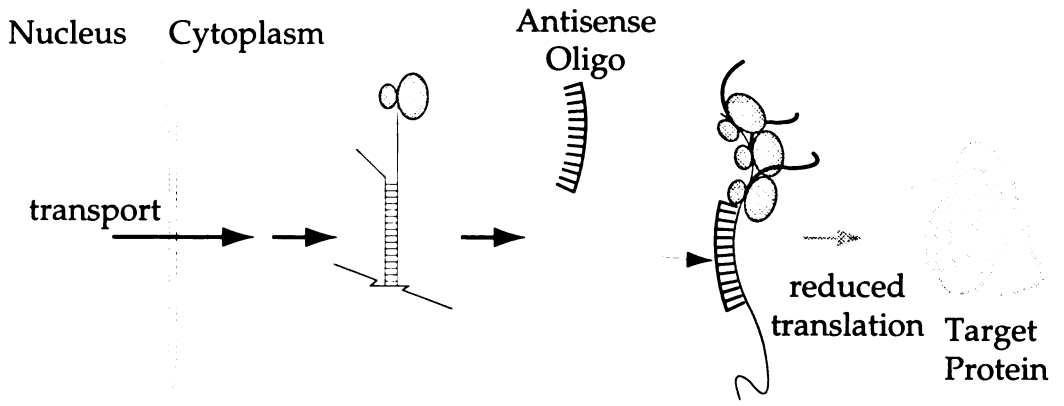


Figure 2a. Schematic representation of the translational blockage mechanism. Oligo binding to mRNA inhibits ribosomal progress.

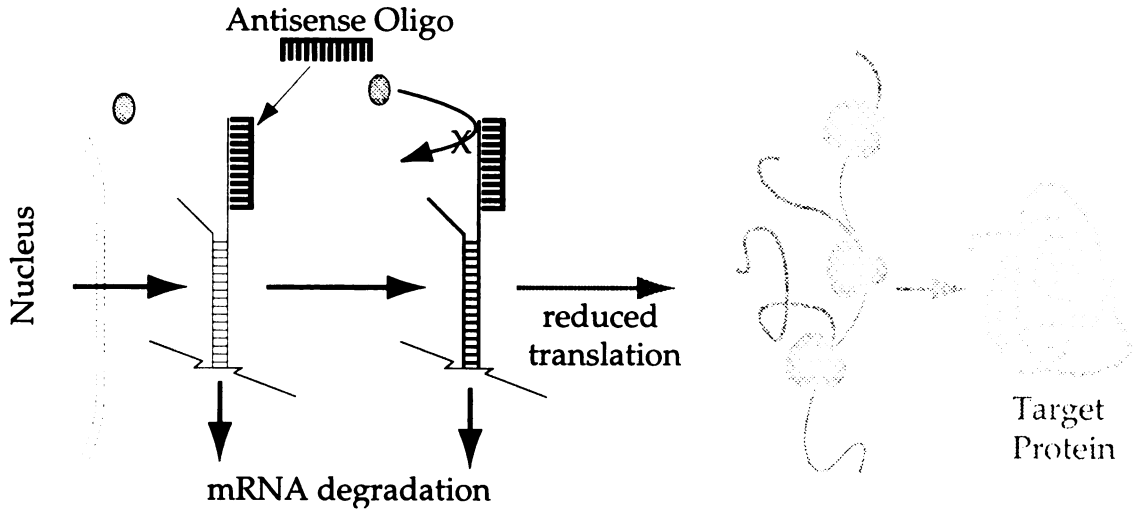


Figure 2b. Schematic representation of ribosomal competition mechanism for antisense oligo action. Oligo binding inhibits ribosomal assembly and the rate of translational initiation.

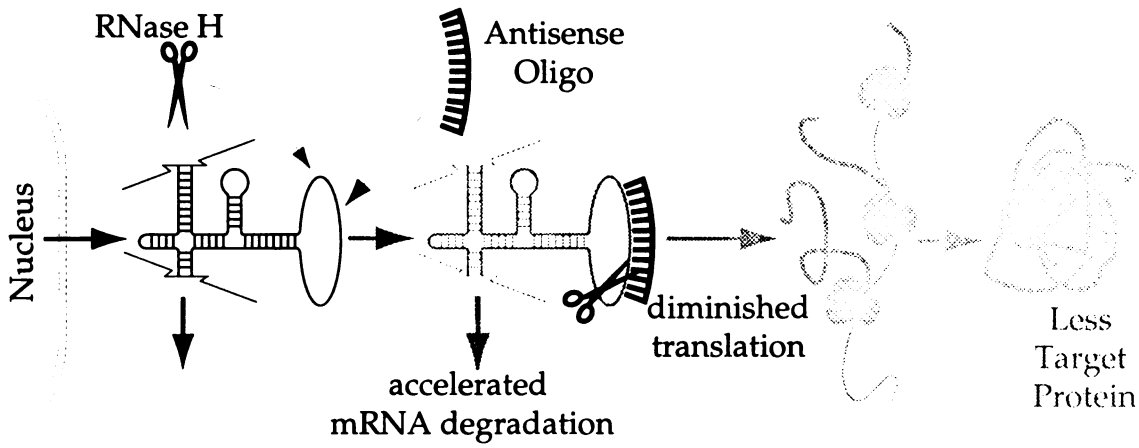


Figure 3a. Schematic representation of the RNase H mechanism for antisense oligo action. An enzyme, RNase H, cleaves mRNA in mRNA-oligo hybrids.

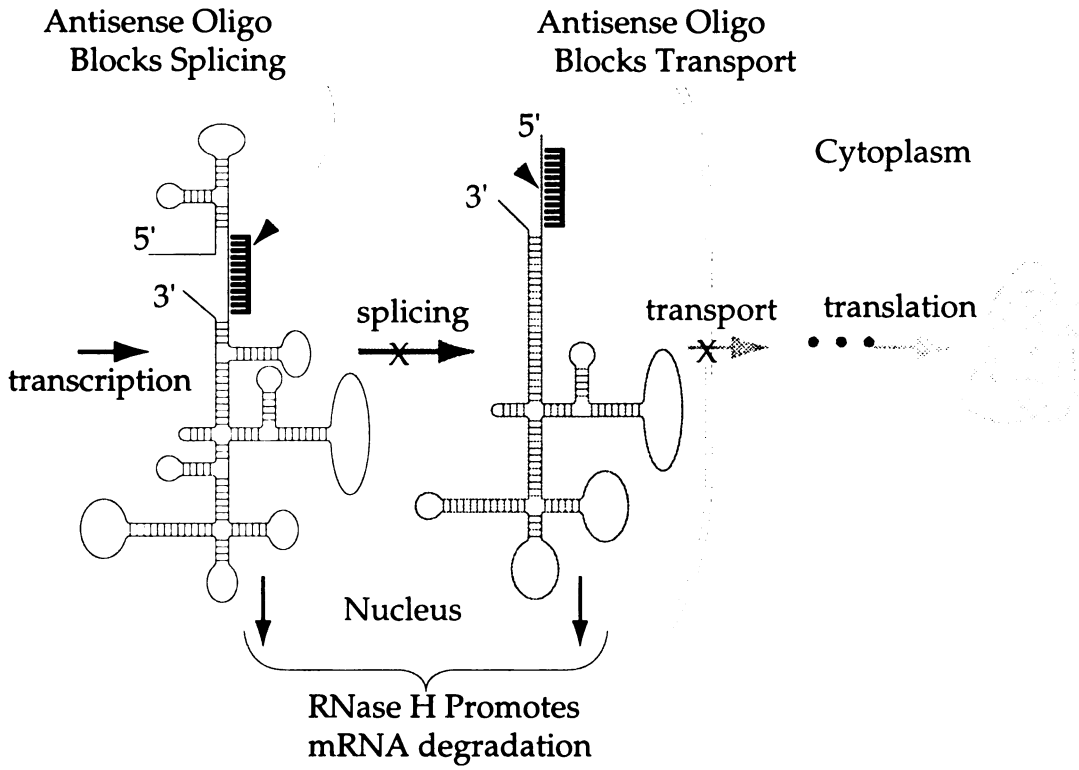


Figure 3b. Schematic representation of the antisense oligo effector mechanisms in the nucleus. Oligos inhibit splicing or nucleocytoplasmic transport of mRNA and this results in reduces steady-state levels of mRNA in the cytoplasm.

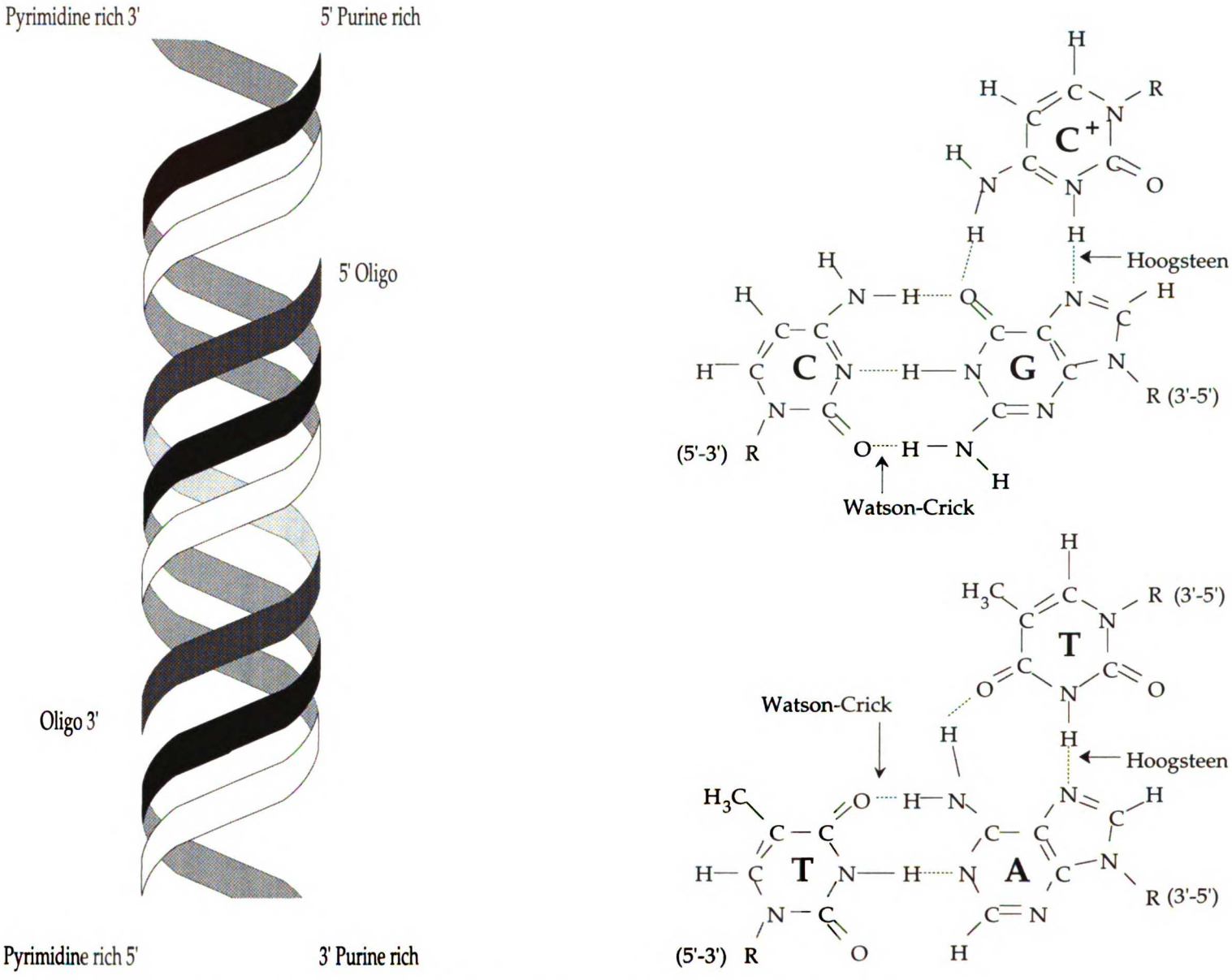


Figure 4. Schematic representation of triple helix formation via Hoogsteen bonds.

The Antigene Approach

The pharmacological receptor or target for the design of antigene oligos is genomic DNA. Because genomic DNA is double stranded, Watson-Crick complementarity cannot be used as a basis for oligo design and genes must be targeted using triple helix forming oligos that form Hoogsteen bonds. A schematic representation of triple helix formation and Hoogsteen bonding is shown in Figure 4.

Protonated cytosine and thymine containing oligos form triple helices on purine-pyrimidine tracts via C^+•G-C and T•A-T Hoogsteen bonds. The third strand in this motif is parallel to the purine rich strand of the Watson-Crick double helix, and is stabilized at low pH because of the requirement for cytosine protonation. However, the stability of C^+•G-C at physiological pH can be increased by using methyl cytosine (Hanvey, Williams et al. 1991; Lee, Woodsworth et al. 1984; Singleton and Dervan 1992; Xodo, Manzini et al. 1991), which has a higher pK_a, instead of cytosine.

The sequence motifs available for triple helix formation were extended by Hogan and coworkers who found that G•G-C, T•A-T triple helices are formed *in vitro* at physiological pH and inhibit transcription from a variety of promoters (Durland, Kessler et al. 1991; Postel 1992; Postel, Flint et al. 1991) containing purine rich tracts. The G•G-C, T•A-T helix is less sensitive to pH and is oriented antiparallel (Beal and Dervan 1991) to the purine rich strand. Triple helix formation reduces transcription by either binding to promoter sites (Durland, Kessler et al. 1991; Escude, Sun et al. 1992; Grigoriev, Praseuth et al. 1993b; Postel 1992), or by blocking RNA polymerase II initiation (Blume, Gee et al. 1992; Escude, Sun et al. 1992), or by inhibiting transcriptional elongation (Rando, DePaolis et al. 1994; Young, Krawczyk et al. 1991). An oligo with the same sequence as the purine rich strand has been reported to

interact with the interferon response element of the *6-16* gene via antiparallel triple helix formation (Roy 1993).

Triple helix formation has been achieved on single stranded mRNA by an ingenious technique referred to as the oligonucleotide clamp (Giovannangeli, Thuong et al. 1993) or a hairpin loop (D'Souza and Kool 1992; Wang, Booher et al. 1994). In this approach, the single stranded mRNA (or DNA) is targeted using an oligo designed to contain both the complementary Crick strand and the third Hoogsteen strand connected by a chemical linker (Giovannangeli, Thuong et al. 1993) or a pyrimidine loop (D'Souza and Kool 1992; Wang, Booher et al. 1994). The resulting triple helix has higher stability, presumably because the entropic benefits of third strand melting are significantly reduced.

The Intercalator Modified Oligo Approach

Oligos coupled to intercalating groups can be targeted against both genomic DNA and mRNA and have been used to the enhance effectiveness of the triple helix and the antisense approaches because intercalators stabilize the formation of oligo-target DNA complexes by stacking interactions with nearby bases. Intercalators such as acridine orange increase the affinities of oligos for their target double-stranded DNA, but these increases are sensitive to length, flexibility and composition (Orson, Kinsey et al. 1994). Acridine linked oligos have been shown to be effective against the SV40 virus (Birg, Praseuth et al. 1990) and against the NF-κB site in the IL-2 receptor α.

Benzo[e]pyridoindole derivatives that act as intercalators to specifically stabilize triplex formation have also been reported (Duval, Thuong et al. 1992b; Grigoriev, Praseuth et al. 1992; Pilch, Waring et al. 1993; Skoog and Maher 1993; Sun, Giovannangeli et al. 1991). These increases in affinity are

particularly valuable in the triple helix strategy because the third strand melting occurs at temperatures much lower than those for duplex formation. The use of intercalators is also desirable when the polypurine tracts required for triple helix formation are of limited length, have mismatches or do not permit high affinity triple helix formation. A potential disadvantage with the use of intercalating agents is toxicity caused by free intercalator released as result of oligo degradation.

The Reactive Oligo Approach

Oligos coupled to reactive groups are usually targeted to double stranded DNA. In this approach, chemically reactive groups are coupled to either the 5' or the 3' end of the oligos and these can either covalently link to the target DNA or cause chemical cleavage.

Alkylating agents, primarily nitrogen mustards, have been extensively investigated by Vlassov and his colleagues (Knorre, Vlassov et al. 1985a) in the antisense (Knorre and Vlassov 1985) strategy, antigene-triple helix (Vlassov, Gaidamakov et al. 1988) and sense (Knorre, Vlassov et al. 1985b) strategies. The primary site of reaction is the N7 of guanines. Inhibition of translation of viruses has been demonstrated (Knorre and Vlassov 1985), but these compounds tend also to react with proteins and lipids.

EDTA-Fe (Dreyer and Dervan 1985), phenanthroline-Cu (Francois, Saison et al. 1989a; Francois, Saison et al. 1989b; Francois, Saison et al. 1988a; Francois, Saison et al. 1988b; Francois, Saison et al. 1989; Sun, Francois et al. 1988; Sun, Francois et al. 1989), porphyrin-Fe (Le, Perrouault et al. 1986) have been investigated as oligo conjugates to effect sequence specific cleavage. The cleavage mechanism involves generation of an oxygen radical anion, $O_2^{\cdot-}$, by electron transfer from the metal center to an oxygen molecule. The radical

anion is converted to hydrogen peroxide, which in turn generates hydroxyl radicals by a Fenton reaction. The hydroxyl radicals cause cleavage of the backbone at the 1' and 4' positions of the deoxyribose.

Hélène and his coworkers showed that oligo-3' ellipticine conjugates bind double-stranded DNA to form triple helices (Perrouault, Asseline et al. 1990). Photoinduced cleavage of both strands of the double stranded DNA was achieved and the existence of a 5' ellipticine binding site at the triplex duplex junction was demonstrated. Phenanthroline modified oligos can cause double-strand DNA scission in the presence of reducing agents and the Cu^{2+} ion.

Psoralen causes double-stranded links when exposed to long wavelength 310-400 nm near-ultraviolet radiation. Psoralen-derivatized methylphosphonates have also been prepared and shown to crosslink to both single-stranded and double-stranded DNA (Bhan and Miller 1990). With single stranded DNA the psoralen stacks on the last base pair; with double-stranded DNA, it intercalates between the last two base pairs (Bhan and Miller 1990). Psoralen-derivatized methylphosphonates in an antisense approach were able to selectively discriminate between two forms of the *ras* oncogene that differed only by a single point mutation (Chang, Miller et al. 1991) and were inhibitory against the herpes simplex virus (Kulka, Wachsman et al. 1993). Hélène and his collaborators have demonstrated the effectiveness of psoralen-derivatized oligos in the triple helix strategy against the *bla* gene in the Tn3 transposon in E. coli (Duval, Thuong et al. 1992a), against HIV proviral DNA (Giovannangeli, Thuong et al. 1992; Giovannangeli, Thuong et al. 1993) , and against the NF-κB site on the IL-2 receptor α gene (Grigoriev, Praseuth et al. 1993a).

Pei et al (Pei, Corey et al. 1990) coupled a triple helix forming oligo to a nonspecific nuclease, staphyloccocal nuclease, and were able to demonstrate sequence specific cleavage of double-stranded DNA upon addition of calcium ions. The benzo[e]pyridoindole derivatives, that are triple helix specific ligands, have also been reported to form covalent adducts on exposure to near-ultraviolet radiation.

The Ribozyme Approach

Ribozymes are RNA molecules with catalytic activity (Brehm and Cech 1983; Cech, Zaug et al. 1981; Guerrier and Altman 1984; Guerrier, Gardiner et al. 1983; Kruger, Grabowski et al. 1982; Zaug, Grabowski et al. 1983). When recognition elements are added to a ribozyme, the result is an antisense ribozyme, a sophisticated antisense oligo that carries intrinsic effector activity and is capable of specifically inactivating target mRNA.

The most commonly investigated ribozyme for therapeutic applications is the hammerhead motif found in plant viroids (Forster, Davies et al. 1988a; Forster, Davies et al. 1988b; Forster and Symons 1987a; Forster and Symons 1987b). The active site of the hammerhead secondary structure contains the active site bases and 3' and 5' flanking sequences. Haseloff and Gerlach (Haseloff and Gerlach 1988) demonstrated that the flanking sequences of the ribozyme can be engineered to be complementary to mRNA containing a GUC, GUU or GUA triplet. The shortest ribozyme designed is a 13mer that catalyzes cleavage of a 41mer RNA substrate (Jeffries and Symons 1989). Koizumi et al. illustrated the exquisite specificity possible with ribozymes when they demonstrated specific cleavage of *Ha-ras* synthetic mRNA differing by a single point mutation GGU→GUU (Koizumi, Hayase et al. 1989a). They also demonstrated cleavage of rRNA transcribed *in vitro*

(Koizumi, Hayase et al. 1989b; Koizumi, Iwai et al. 1988). A hammerhead ribozyme derived from the sTobRV(+) virus has been reported to have RNA-RNA and RNA-DNA ligation activity and this activity may have potential applications in gene therapy (Tokumoto and Saigo 1992).

The usefulness of ribozymes containing RNA bases is limited because they have half-lives of less than a minute in cells. To improve the stability of ribozymes in biological media, chimeric ribozymes have been synthesized. The RNA bases in the active site cannot be dispensed with, but the other bases can be replaced with DNA (Chowrira, Berzal et al. 1993; Herschlag 1992; Herschlag, Eckstein et al. 1993; Tanaka, Hosaka et al. 1993). The replacement of flanking arms by DNA (Sawata, Shimayama et al. 1993) results in enhanced cleavage rates and improved substrate selectivity (Shimayama, Sawata et al. 1992) because the ribozyme turnover rate and selectivity is a balance between substrate binding and product dissociation (Herschlag 1991). Chimeric ribozymes containing a significant fraction of phosphorothioate modified DNA have been shown to be more than seven-fold more active (Shimayama, Nishikawa et al. 1993), and have a ten-fold longer half-life in human serum, than an all RNA hammerhead ribozyme with the same sequence.

The DNA Decoy Approach

DNA decoys, unlike the antisense and antigene approaches, target protein. For this reason, the approach is often is termed the 'sense' approach. DNA decoys are rationally designed competitive aptamers and usually contain sites for DNA binding protein. In theory, while any nucleic acid processing protein e.g. reverse transcriptase, mRNA binding factors or DNA polymerase, can be targeted using decoys, the approach has been most successfully used to target DNA binding proteins that are transcription factors

(Bielinska, Shivdasani et al. 1990). Upon entry into the cell, these oligos sequester transcription factors by mimicking the promoter or enhancer sites on DNA and can reduce occupancy of these sites by transcription factors. These effects result in a reduced transcription rate and, ultimately, in reduced protein expression. The decoy approach can also be used to enhance protein expression by targeting repressor proteins.

Double-stranded phosphorothioate oligos, designed to contain sites for the octamer transcription factor and NF-κB bound the target proteins *in vitro* and inhibited octamer driven IL-2 secretion and NF-κB driven activation of HIV in cell lines (Bielinska, Shivdasani et al. 1990).

A DNA dumbbell (Amaratunga, Snowden et al. 1992; Doktycz, Paner et al. 1993; Paner, Amaratunga et al. 1992; Paner, Gallo et al. 1993) is a double stranded oligo closed at both ends and can be prepared by chemical crosslinking (Chu and Orgel 1990a; Chu and Orgel 1990b) or enzymatic ligation (Germann, Schoenwaelder et al. 1985). Decoys have been synthesized as dumbbells because the absence of free 3' and 5' ends reduces the susceptibility of DNA to exonucleases and improves stability in biological fluids (Chu and Orgel 1992). Single stranded thymidine loops have been most commonly used to close the double stranded stem. For chemical crosslinking cis platinum diammine dichloride (cisPtII) , potassium platinous chloride (K_2PtCl_4) and trans platinum diammine dichloride have been used (Chu and Orgel 1990a; Chu and Orgel 1990b). The physical properties of the decoys are dependent on the loops size (Amaratunga, Snowden et al. 1992), e.g., for loops with more than three thymidines, the melting temperatures are inversely proportional to loop size. Specific binding of TRE and CRE containing phosphorothioate dumbbells to the transcription factors CREB and JUN has been demonstrated *in vitro*. A chloramphenicol acetyl transferase reporter

gene driven by hepatic nuclear factor-1 promoter has been inhibited by nanomolar concentrations of a phosphodiester dumbbell, while double stranded oligos were shown to be ineffective (Clusel, Ugarte et al. 1993).

Several laboratories have reported success using RNA decoys generated as antivirals. Inhibition of HIV expression has been achieved by using RNA decoys containing the TAT response element (TAR) (Sullenger, Gallardo et al. 1990; Sullenger, Gallardo et al. 1991). These decoys act by sequestering RNA binding proteins that activate the retroviral genome. In a variation of the RNA decoy approach, Sullenger and Cech (Sullenger and Cech 1993) attached a retroviral packaging signal to hammerhead ribozymes and showed that these colocalized with viral genome and markedly reduced viral titers. In this approach, the decoy packaging signal achieves compartmentalization and the ribozyme sequence is the effector mechanism for genome degradation. Lisziewicz et al. achieved resistance to HIV challenge infections when cell lines were previously infected with a recombinant retrovirus, containing a RNA construct with multiple TAR decoy sequences, and a hammerhead ribozyme targeted against the viral *gag* gene (Lisziewicz, Sun et al. 1993).

The Aptamer Approach

Aptamers, (Bock, Griffin et al. 1992; Griffin, Toole et al. 1993) are similar to DNA decoys in that the pharmacological target is protein. However, they differ from decoys in that binding and inhibition of protein function is not competitive. Aptamers for a given protein target can be efficiently isolated from a pool of random oligo sequences by affinity chromatography followed by repeated rounds of polymerase chain reaction selection. Aptamers that bind and inhibit thrombin have been extensively investigated. Bock et al. isolated 32 thrombin aptamers from a random pool of 60 base long oligo

sequences and identified a 14-17 base motif which was conserved (Bock, Griffin et al. 1992). These aptamers had association constants for thrombin in the 25-200 nM range and several inhibited thrombin mediated clot formation *in vitro* at nanomolar concentrations. One aptamer, GGTTGGTGTGGTTGG has been investigated *in vivo* in cynomolgus monkeys and in an extracorporeal hemofiltration circuit in sheep (Griffin, Tidmarsh et al. 1993). Infusion was used for delivery. Steady state effectiveness was reached in 10 minutes and the dose response relationship between oligo concentration and plasma prothrombin time was linear. The half-life of the aptamer was short, approximately 1.5-2 minutes, and the authors suggested that these aptamers may be useful as soft anticoagulants in acute situations and in extracorporeal devices. In an *ex vivo* whole arterial angioplasty model (Li, Kaplan et al. 1994), IC_{50} values of 70-80 nM were obtained and the aptamer specifically bound α-thrombin but not γ-thrombin (Paborsky, McCurdy et al. 1993). The aptamer adopts a compact secondary structure in solution (Macaya, Schultze et al. 1993; Schultze, Macaya et al. 1994; Wang, Krawczyk et al. 1993) that consists of two guanine quartets connected by two T-T loops at one end and a T-G-T loop at the other end. Macaya et al. found support for the formation of a quadruplex structure in solution and in the presence of thrombin. The thrombin binding site for aptamer has been biochemically characterized by hirudin competition experiments and by peptide mapping following fluorescein isothiocyanate labeling (Paborsky, McCurdy et al. 1993). The anion-binding exosite of thrombin is important for aptamer-thrombin interactions and the B chain Lys-21 and Lys-65, both in the anion-binding exosite, are protected from labeling in the presence of aptamer. The authors hypothesized that the aptamer binds to the positively charged anion-binding exosite and competes with the two physiological exosite substrates, fibrinogen

and platelet thrombin receptor. The three dimensional crystal structure of the aptamer-α-thrombin complex showed the aptamer bound between the fibrinogen recognition exosite and the putative heparin binding site of two symmetry-related thrombin molecules (Padmanabhan, Padmanabhan et al. 1993). Although both sites are positively charged, both hydrophobic and ionic interactions are involved in binding. There was no support for the existence of a quadruplex in the crystal structure.

Phosphodiester oligos have been shown to bind recombinant CD4 (Yakubov, Khaled et al. 1993), the cellular receptor for HIV, at two sites, with dissociation constants of approximately 0.1 and 1 μM. Phosphorothioate oligos also inhibit binding of recombinant CD4 with a monoclonal antibody that recognizes an epitope on the CDR3-like loop (D1 domain) of the CD4 protein, suggesting that this epitope contributes to part of the binding site.

Giver et al. have used the selection strategy to develop high affinity RNA aptamers for the HIV *rev* protein (Giver, Bartel et al. 1993a; Giver, Bartel et al. 1993b). Aptamers were identified with *rev* binding affinities 3-5 fold greater than the full length *rev*-response element. The sequences identified showed some similarity to a rev-binding element (RBE) localized within the *rev*-responsive element (RRE), but also contained novel sequence and structural motifs consisting of a short helical stem and bulged nucleotides CUC ... UYGAG not present in the wild-type element. Aptamers to a small molecule, vitamin B_{12} or cyanocobalamin, have also been identified (Lorsch and Szostak 1994) using a selection and amplification procedure.

The Molecules Involved In Graft Rejection

A phalanx of molecules mediate the antigen recognition process and initiate the process of rejection. Some the important receptor-ligand pairs on

the antigen presenting cell and on the T cell, which mediates the antigen specific response to the recognition process, are listed in Table II.

Table II. Receptor-ligand pairs involved in antigen recognition

ANTIGEN PRESENTING CELL	T CELL	ROLE
LFA-3	CD2	Adhesion
MHC	CD3	Signaling
MHC & Peptide	T cell Receptor	Antigen Recognition
MHC Class II	CD4	Helper T cell recognition
MHC Class I	CD8	Cytotoxic T cell recognition
ICAM-1	LFA-1	Adhesion

The Major Histocompatibility Complex in Transplant Rejection

The MHC genes were first identified on the basis of skin graft experiments. Grafts that are different at the MHC locus, termed allogeneic, are rejected, while grafts that do not differ at the MHC (syngeneic grafts) have greatly improved take. It was apparent in these pioneering studies that the physiological role of the MHC had little to do with artificially induced responses such as graft rejection. This realization ultimately led to the identification of the immunological role of the MHC.

The polymorphism of the MHC, coupled with their ability to present processed antigens, plays an important role in the initiation of an immune response against the graft. The high degree of polymorphism at the MHC loci results in an increased likelihood of immunologically significant differences between the host and the graft, and the recognition of the 'foreign' MHC derived peptides (Bradley, Mowat et al. 1992; Chandler and Passaro 1993; Sayegh, Watschinger et al. 1994; Watschinger, Gallon et al. 1994) may result in

immune attack directed against the graft. Furthermore, because of their role as antigenic peptide receptors, the MHC constantly present peptides including graft derived MHC peptides to the host immune system and thereby enhance the visibility of the graft to the host.

In a human donor-mouse recipient islet xenotransplant model, pretransplant treatment with $F_{(ab')2}$ antibodies to MHC Class I (Faustman and Coe 1991) improved survival as measured by C-peptide levels and histology. The $F_{(ab')2}$ antibodies to MHC Class I are also effective at prolonging survival in the mouse islet allograft model (Osorio, Ascher et al. 1994). Faustman and Coe (Faustman and Coe 1991) also reported improved survival of xenotranplanted human liver xenografts treated with $F_{(ab')2}$ antibodies to human MHC Class I. In studies using MHC Class I deficient β_2 microglobulin knockout mice, the absence of MHC Class I prolongs graft survival in kidney (Coffman, Geier et al. 1993) and islet allografts (Markmann, Bassiri et al. 1992; Markmann, Schachner et al. 1990; Osorio, Ascher et al. 1993).

The MHC Genes

The MHC genes are encoded on chromosome 6 in humans and are part of a complex known as the MHC, that contains over 70 genes and occupies 4 million base pairs (Trowsdale and Hanson 1993). Consistent with current usage, we use the term MHC to also refer to the human leukocyte antigen (HLA) genes and gene products. The gene complex is usually divided into three regions, with the MHC Class I genes at the telomeric end, Class III genes in the middle and the MHC Class II genes at the centromeric end (Trowsdale, Ragoussis et al. 1991).

The MHC Class I Genes

There are at least 17 genes in the MHC Class I region and these are labeled from the centromeric end as the loci B, C, E, A, H, G, F. Only the products of A, B, C loci are expressed. The exon-intron structure of the MHC Class I A, B, C genes parallels the domain structure of the expressed proteins in that separate exons are used to code for the signal sequence, the $\alpha 1$, $\alpha 2$, $\alpha 3$ domains, the connecting regions, the transmembrane region and the cytoplasmic tails of the protein.

The MHC Class I proteins found on the cell surface are heterodimers consisting of a MHC encoded heavy chain associated with β_2 microglobulin. The gene for β_2 microglobulin is not polymorphic in humans and is encoded on chromosome 15.

MHC Class I proteins are constitutively expressed on the surface of most somatic cells. MHC Class I A, B or C expression is absent on oocytes, spermatocytes, embryonic tissues and in certain cancerous cells.

The MHC Class II Genes

The Class II genes are coded in the D region of the MHC and span about 1 Mbp. This region contains functional genes as well as untranscribed pseudogenes and genes whose transcriptional status is currently unclear. The functionally important MHC Class II DR, DP and DQ proteins consist of α and β chains that are coded for by the R, P and Q subregions in the D region. A predominant fraction of MHC Class II heterodimers consist of α and β chains derived from the same subregion. The DRA gene locus which codes for the DRα chain is unusual in that it is not polymorphic and all individuals carry a single gene. The DRB subregion is also unique in the Class II subregion because individuals carry a variable number of β chain genes. The number of

DRB genes varies between one to four, but of these only one or two genes are expressed in an individual haplotype. In the DP and DQ subregions, the DPA1, DQA1 and DPB1, DQB1 genes are expressed, while the DPA2, DQA2 and DPB2, DQB2 genes are not. The RING4 gene is found centromeric of DP and encodes an ATP-dependent transporter implicated in peptide translocation to MHC Class I molecules in the endoplasmic reticulum. Several other novel genes have recently identified in this region of MHC Class II.

Unlike the MHC Class I genes, the MHC Class II genes have restricted tissue distribution and are constitutively expressed only on the surface of professional antigen presenting cells like B cells, activated T cells, macrophages, dendritic cells and the thymic epithelium.

The MHC Class III Genes

The Class III region occupies approximately 1.1 Mbp in the MHC and encodes a variety of genes differing in function. The genes coding for the C4, C2 and factor B proteins of the complement cascade are found in this region, as are the genes for the microsomal enzyme steroid 21-hydroxylase, the cytokines tumor necrosis factor α and β and the heat shock protein family HSP-70. The significance of the location of these disparate proteins in this region remains speculative.

Function of the MHC

The MHC proteins play a central role in antigen presentation and in the initiation of the cascade of events that allows the immune system to discriminate self from non-self. T cells recognize and respond to antigenic proteins only after the antigens have been processed by proteolysis to peptides

and presented in association with the appropriate MHC molecules (Rosenthal and Shevach 1973; Zinkernagel and Doherty 1974). The MHC proteins function as peptide receptors that present antigens to T cells for recognition and, in the course of normal development, the immune response is restricted only to antigenic peptides presented in the context of self MHC molecules. The requirement for MHC-peptide corecognition in T cell responses is termed MHC restriction.

Both helper (CD4 positive) and cytotoxic (CD8 positive) T cells use the same repertoire of T cell receptor proteins (Davis and Bjorkman 1988). MHC Class I and MHC Class II proteins in association with peptides differentially activate the cytotoxic and helper subsets of T cells, respectively. The MHC Class I proteins present antigens to cytotoxic T cells which express a marker called CD8, that recognizes a site on the $\alpha 3$ domain (Salter, Benjamin et al. 1990) of the MHC Class I heterodimer. Helper T cells express CD4 which binds MHC Class II at a site located on the β chain (König, Huang et al. 1992; Cammarota, Schierle et al. 1992), on a loop that is homologous to the CD8 binding site on the MHC Class I molecule.

The MHC Class I molecules present peptides derived from intracellular and cytoplasmic proteins to the immune system and this confers immunity to viral infection and tumors. The MHC Class II molecules bind peptides derived from extracellular proteins and are responsible for immunity against bacterial and parasitic infections.

The MHC proteins also play a critical role in the differentiation of T cells in the thymus. During development, only T lymphocytes that do not respond to self MHC, in association with autologous peptides, but bind self MHC are selected to undergo further differentiation

Structure of the MHC Proteins

A hallmark of the primary structure of the MHC proteins is the existence of polymorphism. MHC polymorphism is characterized by the existence of multiple alleles at each of the loci. Several alleles occur at significant frequencies in the general population and allelic differences often result in extensive changes in the size, hydrophobicity and charge at the MHC peptide binding site. For example, in the United States Caucasoid population there are 6 HLA-A and 5 HLA-B alleles with frequencies of over 5% (Imanishi, Akaza et al. 1991). The polymorphism of the MHC, the T cell receptor and the immunoglobulin genes together contribute to an immune repertoire that confers protection against diverse pathogens. However, allelic differences at the MHC loci are also critical determinants of the immune response to transplanted tissues and are associated with a variety of autoimmune disease states.

Structure and Polymorphism of MHC Proteins

The MHC proteins belong to the immunoglobulin superfamily and the Class I and Class II heterodimers have secondary structure characterized by four domains. The Class I heterodimer consists of the $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains of the heavy chain in association with β_2 microglobulin, which comprises the fourth domain. In MHC Class I proteins, the $\alpha 1$ and $\alpha 2$ domains are membrane distal and share sequence homology (Orr, Lancet et al. 1979; Orr, Lopez et al. 1979). These domains carry the peptide binding specificity and the polymorphism. The relatively constant $\alpha 3$ domain of MHC Class I is membrane proximal and is associated with β_2 microglobulin which is invariant in humans. The folding of the β_2 microglobulin and $\alpha 3$ domains are similar to that of the immunoglobulin constant regions, and each

contains two β pleated sheets which are connected by disulfide bonds (Bjorkman, Saper et al. 1987; Garrett, Saper et al. 1989). One β sheet has four β strands while the other is antiparallel and has three β strands. The α1 and α2 domains are structurally similar. Each consists of a four stranded antiparallel β sheet with a membrane distal α helix. The α1 and α2 domains have an axis of symmetry, because of which an open cleft 24 Å long and 10 Å wide, with a deep groove capable of binding a peptide 8-9 amino acids long is formed. The β sheets from the two domains form the floor, and the two long α helices form the distal boundary of this cavity. Many of the polymorphic residues and all the highly variable residues are located in this peptide binding groove. While the overall three dimensional structure of the various alleles is similar, the fine structure in the peptide binding site is quite different and this allows a diversity of peptides to be sequestered and presented.

In the Class II heterodimer, each subunit contributes two domains, and the α1 and α2 are derived from the α chain, while the β1 and β2 domains are derived from the β chain. The α1 and β1 domains contain the α helices and form the peptide-binding cleft.

The Role of Adhesion Molecules in Transplantation

The expression kinetics of cytokines (Martinez, Krams et al. 1992), and of accessory and adhesion molecules (Heemann, Tullius et al. 1994) has been extensively investigated to determine efficient predictors for acute rejection . Of the adhesion molecules, ICAM-1 has been most extensively investigated. In normal kidneys, ICAM-1 is expressed at low levels on endothelial cells and on the epithelia of glomeruli, but is absent on tubule derived cells. Several investigators have reported enhanced ICAM-1 expression on endothelial, tubular and infiltrating mononuclear cells in rejecting human kidneys (Faull

and Russ 1989; Moolenaar, Bruijn et al. 1991; Nocera, Cosimi et al. 1989). VCAM-1 and ELAM-1 are also up-regulated (Fuggle, Sanderson et al. 1993; Wuthrich, Jenkins et al. 1993) compared to pretransplant levels. The appearance of ICAM-1 on tubular cells, correlates with MHC Class II induction on endothelial cells and is associated with rejection (Faull and Russ 1989). In rejecting human cardiac allograft biopsies, *de novo* ICAM-1 expression on the intercalating disks (Rose, Page et al. 1991), and enhanced ICAM-1 and VCAM-1 (Briscoe, Schoen et al. 1991) expression on the endothelial cells, correlate with rejection. In rejecting rats heart allografts, ICAM-1 expression on myocardial cells and vascular endothelium is up-regulated. The endothelial cells have been implicated as antigen presenting cells in this model (Rose, Page et al. 1990). Increases in cell surface ICAM-1 (Adams, Hubscher et al. 1989) on bile ducts, endothelium, and perivenular hepatocytes and soluble ICAM-1 in bile (Adams, Mainolfi et al. 1993) are associated with rejection of liver allografts.

Monoclonal antibodies to ICAM-1 delay rejection of kidney allografts in humans and cardiac allografts in primates. Antibodies to LFA-1 alone have not shown activity in acutely rejecting human kidneys. In a mouse model, antibodies to both LFA-1 and ICAM-1 (Isobe, Yagita et al. 1992), when administered for 6 days post transplantation, facilitate indefinite survival. However, antibodies to either antigen alone were not effective. The treated mice also tolerate skin grafts from the donor but reject third party skin grafts. Monoclonal antibodies to the adhesion molecules VCAM-1, VLA-4 and LFA-1 have also been shown to prolong graft survival in rat cardiac transplant models.

Interferon-γ

The Molecular Effects of Interferon-γ

The expression of the MHC Class I and Class II proteins (Rosa and Fellous 1988; Wong, Clark et al. 1983) and ICAM-1 is regulated by interferon-γ (IFN-γ), a cytokine produced by cytotoxic T cells, by the T_H1 subset of T helper lymphocytes and by natural killer cells. Interferon-γ has immunomodulatory, antitumor (Aune and Pogue 1989; Takikawa, Habara et al. 1990; Taylor and Feng 1991) and antiviral activities in a variety of cell types. While it was originally identified (Wheelock 1965) on the basis of its antiviral activity, its primary function is in immunomodulation because it increases the cell surface levels of the major histocompatibility complex Class I and Class II proteins and of adhesion molecules such as intercellular adhesion molecule I (ICAM-1) (Dustin, Rothlein et al. 1986) and the F_c receptor. The increased expression of these proteins enhances antigen specific immune responses because the likelihood of antigen recognition is increased. This is a consequence of increased antigen presentation by the MHC molecules and the stabilization of T lymphocyte-antigen presenting cell interactions by the adhesion molecules (van Seventer, Shimizu et al. 1990).

IFN-γ also induces a variety of antigen nonspecific effector mechanisms that enhance its immunomodulatory role. It is the principal macrophage activating cytokine *in vivo*. It enhances levels of reactive oxygen and nitrogen compounds in macrophages and activates macrophage-mediated cytotoxicity against parasites and tumor cells. The IFN-γ- mediated increase in high affinity F_c receptor on monocytes and macrophages results in increased antibody dependent cellular cytotoxicity, and this is facilitated by the concomitant increases in the biosynthesis of a variety of complement proteins in macrophages and fibroblasts. In conjunction with second signals such as

lipopolysaccharide, interleukin-1 or TNF-α, IFN-γ triggers induction of an inducible isoform of nitric oxide synthase in macrophages.

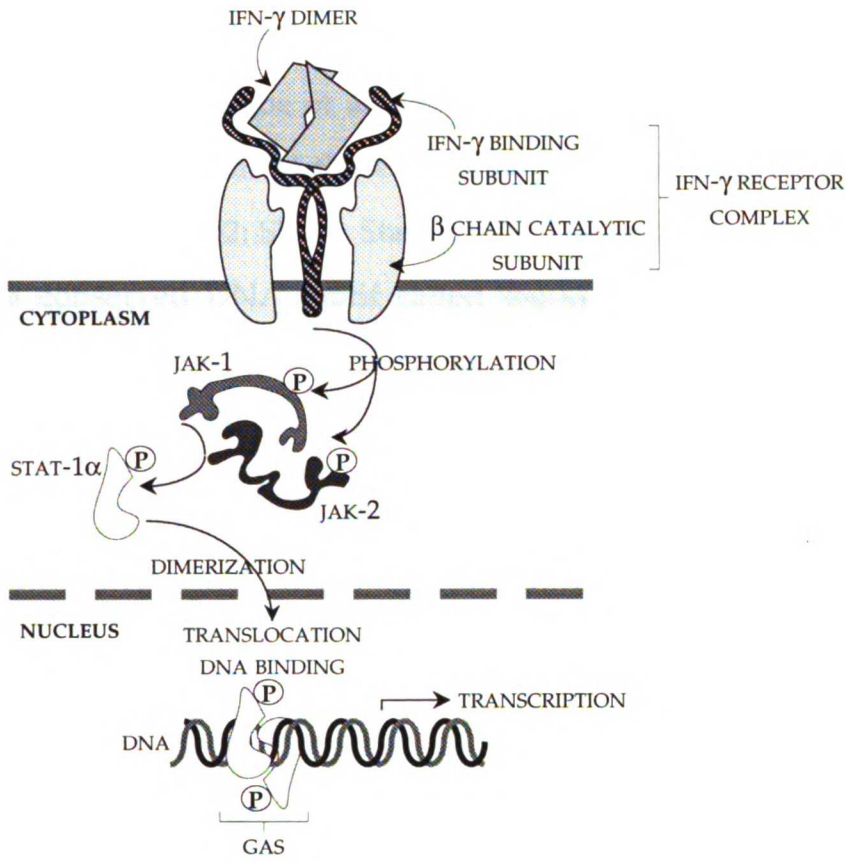


Figure 5. Schematic representation of the early events in IFN-γ signaling

Mechanism of MHC Induction by Interferon-γ

IFN-γ binds to its cell surface receptor complex with an affinity of 10^{-9}-10^{-10} M (Farrar and Schreiber 1993). This receptor complex consists of a 530 amino acid IFN-γ binding subunit and a second coreceptor referred to as AF-1 (Soh, Donnelly et al. 1994; Cook, Emanuel et al. 1994) or IFN-γR β (Hemmi, Bohni et al. 1994). The crystal structure of IFN-γ (Ealick, Cook et al. 1991) shows that the molecule adopts a predominantly (62%) α helical conformation with no β sheet. In the tertiary structure, IFN-γ is a

noncovalently associated antiparallel dimer with the amino terminus of one chain placed in close proximity to the carboxy terminus of the other chain. Each dimer may bind two receptor molecules. As depicted in Figure 5, upon binding to its receptor, IFN-γ induces rapid tyrosine phosphorylation of two proteins, Jak1 (Muller, Briscoe et al. 1993) and Jak2 (Watling, Guschin et al. 1993). The phosphorylation of these proteins results in the phosphorylation (Shuai, Schindler et al. 1992; Shuai, Stark et al. 1993) of Stat-1α (p91), a protein that binds a conserved DNA motif called the Gamma Activated Sequence (Kanno, Kozak et al. 1993; Decker, Lew et al. 1991; Lew, Decker et al. 1991; Pearse, Feinman et al. 1993) or GAS which confers susceptibility to IFN-γ.

The Cellular Effects of Interferon-γ

The molecular effects of IFN-γ have important consequences at the cellular level. IFN-γ activates macrophages and natural killer cells and plays a pivotal role in the development of T and B cell responses. It has antiproliferative effects on the T_H2 subset of T cells, and therefore promotes T_H1 responses.

It is the most significant monocyte/macrophage activating factor and regulates the differentiation and function of phagocytic cells. It plays an important role in the differentiation of myeloid precursors to mature monocytes. Lipopolysaccharide-treated macrophages produce higher levels of TNF-α in the presence of IFN-γ due to increased transcription and possibly by increasing TNF-α mRNA. IFN-γ induced nitric oxide production by macrophages is the principal effector mechanism for the resolution of parasitic infections such as *Leishmania* and *Listeria*. In B cells, it regulates isotype switching and inhibits IL-4 induced MHC Class II expression. Its antiproliferative effects on T_H2 cells results in reduced IL-10 production by T_H2

cells. Higher IL-10 levels favor antibody production in B cells and the presence of IFN-γ tends to facilitate cellular immune responses. The cross regulation of humoral and cellular responses by IL-10 and IFN-γ is an emerging paradigm in cellular immunology.

The Role of Interferon-γ in Clinical Situations

IFN-γ has been implicated in a variety of clinical and pathological situations, and these include graft rejection (Benvenuto, Bachetoni et al. 1991; Brok, Heidt et al. 1993; Bugeon, Cuturi et al. 1992; Dallman, Larsen et al. 1991; Merville, Pouteil et al. 1992; Merville, Pouteil et al. 1993; Nast, Zuo et al. 1994), inflammation (Halloran, Autenried et al. 1992), shock (Car, Eng et al. 1994) and autoimmunity.

Interferon-γ in Transplant Rejection

IFN-γ production is a central, possibly causative event in graft rejection (Halloran, Broski et al. 1993). Benvenuto et al. generated T cell clones from cells infiltrating rejected human kidney allografts and found that the $CD4^+$ and the $CD8^+$ clones had lectin dependent cytotoxicity and natural killer activity, and produced IFN-γ following phytohemagglutinin stimulation (Benvenuto, Bachetoni et al. 1991). These results implicate IFN-γ producing T cell clones in graft cytolysis. The authors suggest that the induction of MHC Class II by IFN-γ serves to amplify the host response. Noronha et al., using immunocytochemical methods, also found significantly elevated TNF-α, IFN-γ, and IL-2R expression on activated mononuclear cells infiltrating human kidney allograft undergoing acute cellular rejection (Noronha, Eberlein et al. 1992). Nast et al used reverse transcription-polymerase chain reaction (RT-PCR) on renal allograft fine-needle aspirates and concluded that

intragraft IFN-γ mRNA expression occurs in active acute rejection and precedes clinical acute rejection (Nast, Zuo et al. 1994). They suggested that IFN-γ mRNA detection be used as a diagnostic tool for the detection of acute cellular rejection. Skin grafts treated with antibodies to IFN-γ show prolonged survival, presumably because MHC Class II induction by IFN-γ is inhibited on keratinocytes and the antigen presenting cells (Rosenberg, Finbloom et al. 1990; Rosenberg and Singer 1992). The data of Bugeon et al., obtained in a rat cardiac allograft model, are complementary and show that IFN-γ and IL-2 mRNA accumulate to lower levels and with delayed kinetics in tolerated grafts when compared to rejected ones (Bugeon, Cuturi et al. 1992). This suggests that reduced IFN-γ and IL-2 are either involved in, or facilitate the induction of, graft tolerance. Transgenic mice which express IFN-γ under the control of an insulin promoter (Sarvetnick, Shizuru et al. 1990) develop diabetes because of the progressive destruction of pancreatic islets caused by the influx of inflammatory cells. While these results were obtained with histocompatible islets, the data demonstrate that IFN-γ is involved in the loss of tolerance *in vivo* and that its expression can cause activation of the cellular immune response.

Interferon-γ in Inflammation

A variety of cytokines cause and promote inflammatory responses. Cellular recruitment and the synergistic interaction between IFN-γ and TNF-α are both important mediators of inflammation. IFN-γ acts by increasing both the production and activity of TNF-α. In the presence of IFN-γ, both the production of TNF-α mRNA and protein are enhanced in lipopolysaccharide treated macrophages indicating transcriptional activation of the TNF-α gene. However, it is likely that some the effects of IFN-γ may be due to indirect

stabilization of TNF-α mRNA. IFN-γ also increases TNF-α receptor expression 3-5 fold in a variety of cell types. However, the relative contribution of this induction to the overall inflammatory response has not been established. The two cytokines enhance expression of a variety of adhesion molecules that facilitate cellular recruitment from the circulation. IFN-γ and TNF-α induce expression of ICAM-1, MHC Class I and ELAM-1 and these enhance the ability of the vascular endothelium to recruit circulating lymphocytes and monocytes.

Interferon-γ in Shock

TNF-α is the primary cytokine mediator in LPS induced models for shock. A commonly used animal model for shock is the Schwartzman reaction in which mice are treated with a low sensitizing dose of LPS followed by a larger intravenous dose of LPS 24 hours later. IFN-γ treatment of animals prior to LPS sensitization results in increased TNF-α production and causes increases in mortality. Antibodies that neutralize IFN-γ provide a measure of protection. Taken together, the data suggest that IFN-γ is a priming cytokine that can significantly enhance the toxicity associated with LPS induced TNF-α production.

Lessons from Knockout Mice

In the last few years, transgenic and knockout mice have significantly advanced our knowledge of the organismal effects of gene deletions and mutations and provided valuable insight into gene function *in vivo*. Most oligo based therapeutic approaches aim to reduce or eliminate target gene expression in a transient but specific manner and in the gene knockout animal this goal has already been completely accomplished. By reviewing the

results from knockout models, both the therapeutic value of targeting a specific gene and the likely spectrum of physiological side effects can be rationally assessed. Will elimination of gene expression have any impact on the physiological response of the target tissue? Will other genes compensate for loss of the targeted gene? Will reduced expression have any impact at the organismal level? These are all questions that can be addressed by studies in knockout animals before embarking on a gene down-regulation project. In the following, I review the literature (Raulet 1994) on mice with disruptions in the MHC Class I, MHC Class II, ICAM-1, IFN-γ, and IFN-γ receptor genes.

β_2 Microglobulin Deficient Knockout Mice

Mice homozygous for a disruption of the β_2 microglobulin gene are fertile and healthy and do not express MHC Class I at the cell surface (Zijlstra, Bix et al. 1990). The absence of cell surface MHC Class I confirms an important role for β_2 microglobulin in the stabilization of assembly of Class I heterodimers. T cell receptor α/β positive $CD4^-8^+$ T cells undergo positive selection on a framework containing MHC Class I in the thymus, and consistent with this model, these mice lack mature $CD4^-8^+$ T cells and are therefore, deficient in T cell cytotoxicity. The TCR γ/δ^+CD8^+, $CD4^+8^+$ and $CD4^+8^-$ T cell subpopulations were normal. The mice are sensitive to the cytoplasmic parasite Trypanasoma cruzi and show high parasitemia and no inflammatory response, despite higher IL-2 and IFN-γ production in response to mitogens (Tarleton, Koller et al. 1992). These results suggest an important role for either cell surface MHC Class I expression or $CD8^+$ cells in the resolution of T. cruzi infections.

Renal allografts from these β_2 microglobulin knockout mice had improved survival and function compared to allografts with MHC Class I

expression even though both graft types had mononuclear inflammatory infiltrates (Coffman, Geier et al. 1993). No differences were seen in the alloreactive proliferative and cytotoxic responses but the antibody responses differed. The primary antibody response produced by recipients of the MHC Class I deficient allografts was directed against donor MHC Class II antigens while recipients of allografts with MHC Class I produced antibodies directed against both donor MHC Class I and Class II antigens. The authors surmised that reduced MHC class I expression would be useful in overcoming some of the effects of MHC incompatibility in the kidney allograft model.

The β_2 microglobulin knockout mice reject skin grafts normally across whole MHC and minor histocompatibility differences suggesting that the $CD4^-8^+$ T cell compartment is not essential for skin graft rejection (Zijlstra, Auchincloss et al. 1992). However, the ability to reject skin grafts across H-Y differences is reduced and natural killer mediated destruction of MHC Class I deficient skin grafts is not observed. The T cells from β_2 microglobulin deficient mice were targets of natural killer lysis, but spleen cells from these mice lacked the ability to lyse MHC Class I deficient targets, and normal natural killer cell tumor targets (Liao, Bix et al. 1991). These mice did not reject allogeneic bone marrow. The authors suggest that these results imply a role for MHC Class I molecules in the positive selection and tolerance of natural killer cells.

Considerable evidence suggests that the absence of MHC Class I on β_2 microglobulin knockout mice prolongs the survival of pancreatic islet allografts. Markmann et al. found that islet allografts from β_2 microglobulin deficient donors showed prolonged, even indefinite survival in allogeneic BALB/c recipients (Markmann, Bassiri et al. 1992; Markmann, Desai et al. 1994). However these transplanted pancreatic islet grafts did not show

improved survival in the autoimmune environment of non-obese diabetic mice. However, the absence of MHC Class I and $CD8^+$ T cells in a β_2 microglobulin deficient, non-obese diabetic model confers resistance to insulitis and diabetes (Wicker, Leiter et al. 1994). Osorio et al. also obtained improved islet survival when β_2 microglobulin deficient islets were used in allogeneic hosts (Osorio, Ascher et al. 1993). Desai et al. showed that when β_2 microglobulin mice are used as recipients, islet allografts had prolonged survival, but skin and xenografts did not (Desai, Bassiri et al. 1993). These studies provide evidence that reduction of MHC Class I expression may be useful in preventing pancreatic islet rejection.

Liver cells from β_2 microglobulin deficient mice, when transplanted into isogeneic, allogeneic, and xenogeneic recipients showed only slightly improved survival (Li and Faustman 1993). This suggests that host natural killer activity and reconstitution of graft MHC Class I by association with host soluble β_2 microglobulin are responsible for the disappointing allograft survival results.

β_2 Microglobulin and MHC Class II Knockout Mice

Mice deficient in both MHC Class I and Class II are healthy and fertile (Grusby, Auchincloss et al. 1993). The T cell compartment of these double knockouts lacks $CD4^+$ and $CD8^+$ T cells, and in the mixed lymphocyte reaction, spleen cells from MHC-deficient animals are poor stimulators and responders. The B-cell compartment and antibody responses on challenge with antigens are normal. However, these mice rapidly reject allogeneic skin grafts, and MHC deficient skin grafts are rapidly rejected by normal allogeneic recipients. The authors interpreted these results to imply that other,

undefined compensatory mechanisms were responsible for graft rejection in these immunocompromised double knockout mice.

ICAM-1 Knockout Mice

Mice deficient in ICAM-1 have higher neutrophil and lymphocyte counts but show normal T and B lymphocyte development (Xu, Gonzalo et al. 1994). The T cells respond normally in a mixed lymphocyte reaction but have reduced ability as stimulators. These mice are resistant to high dose endotoxin-induced shock despite normal production of TNF-α, IL-1 and IL-6. ICAM-1 deficient mice also show low mortality rates compared to wild type mice in a D-Gal sensitized exotoxin shock model. In this septic shock model, the effects are mediated by T cells. However ICAM-1 deficiency did not protect against the macrophage mediated shock induced by low dose endotoxin challenge following D-Gal sensitization. The knockout data suggest that reduced ICAM-1 expression may be useful in modulating T cell mediated responses in shock and elsewhere.

Interferon-γ Knockout Mice

Mice with a targeted disruption of the IFN-γ gene (Dalton, Pitts et al. 1993) develop normally and are healthy in the absence of pathogens. The splenocytes from IFN-γ deficient mice showed uncontrolled proliferation in response to mitogenic and alloantigenic stimuli and the T cell mediated cytotoxicity against allogeneic targets was increased following a mixed lymphocyte reaction. Macrophages from these mice express lower levels of MHC Class II, have lower antimicrobial activity and natural killer cell activity is reduced. The mice are sensitive to, and killed by a sublethal dose of intracellular pathogens such as Mycobacterium bovis (Cooper, Dalton et al.

1993; Flynn, Chan et al. 1993). This suggests an important role for IFN-γ in the function of several cell types in the immune system, particularly in the resolution of intracellular parasitic infections. Wang et al. showed that, in response to a Leishmania major challenge, the $CD4^+$ T cell compartments of these mice do not produce lymphotoxin (and of course IFN-γ) characteristic of differentiation along the T_H1 pathway but instead defaults to the T_H2 pathway as evidenced by IL-4, IL-5, IL-13 mRNA production and isotype switching (Wang, Reiner et al. 1994). This shows that in the absence of IFN-γ the T_H1 responses default to the T_H2 pathway.

Interferon-γ Receptor Knockout Mice

Mice lacking the IFN-γ receptor are healthy, show apparently normal immune system development and are normal in T cell proliferative and cytotoxic responses (Huang, Hendriks et al. 1993). Like the IFN-γ knockouts, these mice show high susceptibility to infection by Listeria monocytogenes and vaccinia virus. The mice are deficient in isotype switching and these results show that IFN-γ is necessary for a normal antigen-specific immunoglobulin G2a response.

Car et al. provide direct evidence for the critical role of IFN-γ in endotoxin or lipopolysaccharide [LPS]-induced shock because IFN-γ receptor knockout mice show increased resistance to LPS-induced toxicity and tolerated 100-10000 fold more LPS than control mice in a D-galactosamine-LPS model (Car, Eng et al. 1994). These mice express normal levels of the p55 and p75 TNFα receptors but have only 10% of normal serum TNFα level, and the lymphopenia, thrombocytopenia and weight loss characteristic of LPS induced shock are not as severe. The LPS binding to marrow and splenic macrophages is decreased due to reduced expression of the LPS receptor,

CD14. Additionally, the serum from mutant mice inhibits LPS binding to macrophages significantly via an unknown mechanism. The authors conclude that these secondary factors synergize with the absence of IFN-γ receptor mediated signaling to confer a phenotype with extremely high resistance to LPS induced shock.

These mice are very sensitive to the Bacillus Calmette-Guerin (BCG) strain of Mycobacterium bovis. BCG infection is not lethal for wild-type mice but IFN-γ receptor knockout mice died approximately 7-9 wk after inoculation. However, because of reduced TNF-α production, the IFN-γ receptor knockouts were less sensitive to the lethal effects of a LPS challenge following BCG inoculation. The authors conclude that the role of the IFN-γ receptor is critical to recovery from BCG inoculation and that it is an important mediator of the sensitivity to LPS following inoculation.

Kamijo et al. evaluated the effects of IFN-γ receptor gene disruption on macrophage activation (Kamijo, Shapiro et al. 1993). Nitric oxide is an important effector mechanism in macrophages and its induction in wild type mice macrophages requires two signals, usually TNF-α and LPS or IFN-γ. The IFN-γ receptor knockout mice are unresponsive to mixtures of TNF-α and IFN-γ but the nitric oxide induction from knockout mice is similar to wild type if mixtures of IFN-α or IFN-β and TNF-α or LPS are used. The knockout mice express normal levels of constitutive MHC Class II I-A, but the activated macrophages obtained after inoculation with Mycobacterium bovis had lower MHC Class II expression compared to control. In a systematic evaluation, the authors found that IL-4, granulocyte-macrophage colony-stimulating factor and IFN-α/β are less effective than optimal concentrations of IFN-γ in MHC Class II induction. The knockout mice are sensitive to Mycobacterium bovis

infection because other cytokines cannot compensate for the lack of IFN-γ signaling in macrophage activation.

The Evolution of this Project

This project was initiated in the Summer of 1992 as a collaboration between laboratories of Dr. C. Anthony Hunt and Dr. Marvin R. Garovoy. The goals of the project were to inhibit expression of the major histocompatibility complex on the surface of transplantable cells and tissues using nucleic acid based approaches. I joined the project in October 1992 and have been focusing on down-regulating the MHC Class I proteins.

I performed a theoretical analysis of nucleic acid based strategies to better understand the cellular factors that limit the efficacy of oligo based approaches. This work is summarized in the later part of this thesis.

Subsequently, I designed a variety of antisense and triple helix forming oligodeoxynucleotides against various mRNAs and genes in the Class I system and screened them for activity in a cell line that expressed MHC Class I constitutively. Unfortunately, these efforts were not successful. While troubleshooting our protocols we identified two problem areas in this constitutive model:

i. Activity may be difficult to detect because an active oligo has to act against a continuous flux of target mRNA
ii. Small changes in MHC antigen levels are measured against a relatively high background of constitutive expression

We realized that both these problems could be overcome if we identified a cell line that did not express high MHC Class I levels, but could be induced with cytokines. We found that the K562 cell line (ATCC CCL 243) met these criteria and confirmed that the line could be induced with IFN-γ.

We tested the oligos designed to inhibit the MHC in this inducible model and we identified active oligos that inhibited the cell surface induction of the MHC Class I and ICAM-1 by IFN-γ. We characterized the activity and found that the activity was directed against the effects of IFN-γ. We had serendipitously identified an antagonist for the effects of IFN-γ. The characterization and mechanism of action of these oligos is summarized in the first part of this thesis.

MATERIALS AND METHODS

Cell lines

All cell lines were obtained from the Cell Culture Facility at the University of California at San Francisco. The K562, HeLa S3, Raji and HUT78 cell lines were maintained in RPMI1640 containing 100 U/ml pennicillin, 100 μg/ml streptomycin and 10% Fetal Bovine Serum (FBS). The cells were passaged at 10^5 cells/ml once a week, usually on Fridays. Fresh growth medium was added to cultures twice weekly, usually on Mondays and Wednesdays, after carefully skimming approximately 50% of the spent growth medium. The cells were kept in a sterile Napco 5100 Controlled Environment Incubator (National Appliance Company, Tualatin, OR) set to maintain internal temperatures at 37°C and internal carbon dioxide concentrations at 5% v/v. Incubator temperatures and cylinder carbon dioxide pressures were checked every working day as part of the Immunogentics and Transplantation Laboratory Quality Assurance Protocol. Carbon dioxide concentrations were periodically checked and adjusted using a potassium hydroxide based FYRITE™ Orsat analyzer (Bacharach Instrument Company).

Cell culture medium was purchased from GIBCO Life Technologies (Grand Island, NY) and stored upon receipt at 4-8°C. Pennicillin-streptomycin was purchased as a single 100X stock solution from the Cell Culture Facility (University of California, San Francisco) and stored as 10 ml aliquots at -20°C. FBS was obtained from GIBCO Life Technologies (Grand Island, NY) and was heated to 56°C for 1 hour in a water bath to inactivate complement. The FBS was stored in 40 ml aliquots at -20°C.

Oligodeoxynucleotides

Oligodeoxynucleotides (oligos) were synthesized using standard phosphoramidite protocols by Keystone Laboratories (Menlo Park, CA). The oligos were purifed using high performance liquid chromatography by the manufacturer and supplied vacuum dried. The oligos were dissolved in twice autoclaved 0.1% diethyl pyrocarbonate treated water to give a stock solution with an estimated concentration of 1250 μM. Water was treated overnight with 0.1% v/v diethyl pyrocarbonate to inactivate nucleases. Oligo concentrations were quantitated spectrophotometrically using three 2 μl aliquots from the stock solution were diluted with 998 μl of Dulbecco's phosphate buffered saline. Five spectrophotometric measurements of absorbance at 260 nm were taken in a quartz cuvet on an Ultropec II spectrophotometer (Pharmacia LKB, Piscataway, NJ) and a conversion factor of 33 μg/unit of optical density was applied to convert from absorbance to concentration. The mean concentration obtained from these measurements was used as an operational estimate of the oligo concentration.

Oligodeoxynucleotide Sequences

The oligos used are shown below.

I 5' GGG GTT GGT TGT GTT GGG TGT TGT GT —RNH_2

II 5' AC ACA ACA CCC AAC ACA ACC AAC CCC —RNH_2

III 5' AGG GTT CGG GGC GCC ATG ACG GC —RNH_2

IV 5' CAG CCT TGA GGA TTC CCC AAC TCC G —RNH_2

V 5' GCC ACG GAG CGA GAC ATC TCC G —RNH_2

VI 5' CAT CTT CTG CCA TTC TGA AGC CGG —RNH_2

where R= —$CH_2CH(OH)CH_2$— (Glen Research, Sterling, VA).

Oligodeoxynucleotide Design

As indicated in the Abstract, these oligos were originally designed as experimental and control sequences for antisense and triple helix based antigene experiments. We serendipitously found anti-IFN-γ activities in the course of our experiments.

Oligo **I** was originally designed to form a G•GC, T•AT triple helix (Durland, Kessler et al. 1991) with a promoter region of the human MHC Class II DRA gene and oligo **II** is its reverse complement. The sequences of **III**, **V** and **VI** are antisense to the translation start regions of the MHC-I A locus consensus, β_2 microglobulin mRNA and nuclear factor NF-κB (DNA binding subunit) mRNA, respectively.

Interferons and Other Cytokines

Recombinant human IFN-γ was supplied by Collaborative Research Incorporated (Bedford, MA) as a 5 x 10^6 U/ml solution in sodium phosphate buffer containing sucrose and 2.7% human serum albumin; upon receipt it was diluted in Dulbecco's PBS (D-PBS) to a concentration of 50 U/ml. IFN-γ was stored at -70°C in aliquots to avoid multiple freeze-thaws and only thawed prior to use. Radiolabeled ^{125}I iodotyrosyl IFN-γ (Amersham, Arlington Heights, IL) was reconsitituted in 20 µl sterile water and stored in 5 µl aliquots at -20°C in accordance with the manufacturers instructions.

Recombinant human IFN-α (Chemicon, Temecula, CA) and IFN-β (Accurate Chemical Corporation, Westbury, NY) were diluted in D-PBS to 100 U/µl and 1000 U/µl respectively and stored at -20°C. Aliquots of recombinant TNF-α (Genzyme Corporation, Cambridge, MA) were stored at -70°C.

Antibodies for Flow Cytometry

Fluorescein isothiocyanate conjugated mouse monoclonal antibodies to human MHC Class I heavy chain (clone designation, UBI-HLA, A,B,C), β_2 microglobulin (clone designation UBI-β_2 microglobulin) and a mouse IgG_2b control were purchased from Olympus, Lake Success, NY. The MHC Class I heavy chain antibody is a mouse IgG_2b with kappa light chains and recognizes an SDS stable determinant on the MHC Class I heavy chain associated with β_2 microglobulin. The β_2 microglobulin anitbody is also mouse IgG_2b with kappa light chains, but recognizes human β_2 microglobulin both in its free monomeric form and in association with the MHC Class I heavy chain.

Additionally, fluorescein isothiocyanate conjugated mouse monoclonals to human ICAM-1 and an IgG_1 control were purchased from AMAC (Westbrook, ME). Fluorescein isothiocyanate conjugated mouse monoclonals to human MHC Class II DR and transferrin receptor were purchased from Becton Dickinson (San Jose, CA).

All antibodies were stored at 4-8°C in the dark.

Oligonucleotide treatment of cells

Protocol A: Triplicate samples of 0.5 x 10^6 K562 cells were placed in Falcon 2051 tubes in 0.25 ml of either a) serum free medium b) serum free medium and 800 Units/ml IFN-γ or c) serum free medium, 800 Units/ml IFN-γ and oligo. For the specificity experiments IFN-α (6400 U/ml), IFN-β (6400 U/ml) or TNF-α (800 U/ml) were used in place of IFN-γ. The cytokine doses were selected to give cell surface MHC Class I enhancements comparable to 800 U/ml IFN-γ. After 1 hour at 37°C, medium containing FBS that was heat inactivated at 65°C for 30 minutes (FBSΔ) was added to a final concentration of 10% in 1 ml. Stated concentrations of IFN-γ and oligo are for

those in the first hour. Samples were drawn from each tube at 24, 48 or 72 hours and stained for flow cytometry using fluorescein isothiocyanate conjugated mouse monoclonal antibodies to human MHC Class I heavy chain, β_2 microglobulin, ICAM-1 and a mouse IgG_2b control.

Protocol B: Protocol A was modified to eliminate the 1 hour preincubation of cells in serum-free medium. The volume of medium was 1 ml and contained 0.5 x 10^6 K562 cells, 10% FBSΔ, and the indicated concentrations of cytokines and/or oligo.

Northern Analysis

Cytoplasmic RNA was extracted from 3 x 10^6 cells harvested 15 hours after treatment. Cells were lysed in a buffer containing 150 mM sodium chloride, 10 mM Tris (pH 8), 2 mM magnesium chloride, 0.5% NP-40 and 10 mM vanadyl ribonucleoside. Nuclei were removed by centrifugation, and the supernatants were denatured in a buffer containing 7 M urea, 450 mM sodium chloride, 10 mM EDTA 1% sodium dodecyl sulfate and 10 mM Tris (pH 7.4). The RNA was extracted with two 650 µl phenol-chloroform-isoamyl alcohol (24:24:1 v/v) extractions. Three volumes of 100% ethanol containing 10% v/v of 3M sodium acetate, pH 6.0 were added to the extracts. The ethanol precipitation of RNA was completed by overnight cooling at -70°C. The precipitated RNA was dried in a SpeedVac (Savant Instruments, Farmingdale, NY) without heating and then resuspended in 25 µl of diethyl pyrocarbonate treated water. Absorbances at 260 and 280 nm were measured and the RNA concentration was estimated using a conversion factor of 40 µg RNA per unit of OD at 260 nm. The concentration estimates were verified by electrophoresing 1 µg of RNA from each sample on a 1% agarose in TAE gel

containing 1 μg/ml of ethidium bromide. The ribosomal RNA bands were visualized using UV transillumination to confirm that the RNA was intact and that the spectrophotometric concentration estimates gave uniform loading. Aliquots containing 20 μg of RNA were removed and diluted to 200 μl with diethyl pyrocarbonate treated water. After the addition of 20 μl of 3M sodium acetate (pH 6.0) and 600 μl of 100% ethanol, the RNA was reprecipitated by overnight incubation at -70°C. The precipitated RNA was washed with 1 ml of cold 70% ethanol at -20°C and dried prior to transfer into the gel loading buffer.

The RNA (20 μg/lane) was separated on a 1% agarose gel containing 2% formaldehyde. The gel was vacuum blotted with a LKB 2016 VacuGene™ system (Pharmacia LKB, Bromma, Sweden) to a nylon membrane (0.2 μm, ICN Biochemicals, Irvine, CA) and stored at -70°C. The membrane was sequentially probed with 5' end-labeled (labeled using adenosine 5'-[γ-^{32}P] triphosphate and polynucleotide kinase) antisense oligonucleotides to β-actin (5' GAC GAC GAG CGC GGC GAT ATC ATC ATC), ICAM-1 (probe cocktail from R & D Systems, Minneapolis, MN) and MHC-I A, B (5' CCA ATA CTC CGG CCC CTC C). Prehybridization for 4 hours and overnight hybridization were carried out at 45°C in 5X SSC, 10X Denhardt's solution, 50 mM sodium phosphate (pH 7.2), 7% SDS. The membrane was washed twice at room temperature with 2X SSC, 0.1% SDS and again with 0.5X SSC, 0.1% SDS at 50°C and then autoradiographed. Bound label was dehybridized for 2 hours with a buffer containing 1 mM Tris, 1 mM EDTA and 0.1X Denhardt's solution to prepare the membrane for the next probe.

Kinetics Experiment

For the kinetics of inhibition experiment, K562 cells were treated with 200 U/ml IFN-γ according to Protocol B. At 0, 2, 4, 8, 12 and 24 hours, **I** was added to one set of cells to give a final concentration of 6.25 μM. Additional sets of cells were washed twice with 5 ml of serum-free medium at 0, 2, 4, 8, 12 or 24 hours to remove external IFN-γ and served as controls. Aliquots were drawn from each tube at 24 and 48 hours for flow cytometry.

Competition Studies with Denatured IFN-γ

IFN-γ was heat denatured by incubation at 65°C for 1 hour. K562 cells were treated according to Protocol B with 50 U/ml active IFN-γ containing a 100 fold excess of denatured IFN-γ and the **I** concentrations indicated in Figure 20. The control cells were not treated with denatured IFN-γ but were otherwise processed identically. Cell surface expression of MHC Class I and ICAM-1 were measured 24 and 48 hours after treatment.

*Testing the Hypothesis that **I** Acts By Irreversibly Modifying IFN-γ*

To challenge the hypothesis that **I** acts by irreversibly modifying external IFN-γ we modified Protocol B by incorporating the following treatments: i) control ii) 200 U/ml IFN-γ iii) 6.25 μM **I** alone, or iv) a mixture of IFN-γ and **I**. After 15 hours , the tube contents were pooled and 1 ml aliquots of the supernatant were either treated with medium, IFN-γ, **I**, or **II**. Supenatants from cells that had already received a given reagent in the earlier treatment were not treated with the same reagent again. The IFN-γ activity remaining in the media was assayed by measuring ICAM-1 induction produced after 24 hours in a fresh lot of K562 cells. To recover and quantitate

the bioactivity in the IFN-γ and **I** supernatant, we used 6.25 μM **II**, which we knew to be an antagonist for **I**.

Preincubation Experiment

In order to study the effect of preincubation with **I** on inhibition, K562 cells were treated with 6.25 μM **I** for 0, 2, 4, 8, or 12 hours according to Protocol B. The cells were washed twice to remove externally accessible **I** and 200 U/ml IFN-γ was added. Aliquots were analyzed for ICAM-1 expression by flow cytometry 12 and 36 hours after IFN-γ addition.

Binding Studies

Binding studies were carried out to evaluate the effect of **I** and **II** on the binding of IFN-γ spiked with ^{125}I labeled IFN-γ. In these experiments each tube contained 2 x 10^6 K562 cells treated with appropriate concentration of labelled IFN-γ. The assay medium was RPMI 1640 containing 10% FBSΔ. Aliquots of the labeled IFN-γ were analyzed in a Cobra AutoGamma (Downers Grove, IL) gamma counter and the specific activity calculated. Immediately after IFN-γ addition, 10 μM **I** or **II** was added to the experimental tubes. The tubes were incubated on ice for 1 hour and then centrifuged to pellet the cells. Following centrifugation aliquots of the supernatant were removed for determining the IFN-γ concentration. The cell pellet was washed three times with cold PBS containing 1% FBSΔ and the radioactivity was quantitated in the gamma counter. One set of experiments was carried out with a 100 fold excess of cold IFN-γ to ensure that the radiolabeled IFN-γ competed with cold IFN-γ for cell surface binding, and to provide assurance that the behavior of the labeled cytokine did not differ significantly from that of the unlabeled material.

Binding Studies with Immobilized Receptor and with Anti-IFN-γ Antibody

Binding studies were carried out to evaluate the effect of **I** and **II** on the binding of ^{125}I labeled IFN-γ to the purified soluble extracellular domain of the IFN-γ binding subunit of the human IFN-γ receptor complex. The purified partial receptor was obtained as a gift from Dr. Robert Schreiber (Washington University, St Louis, MO) and will be referred to simply as receptor. To accomplish receptor immobilization, 100 μL/well of a 1.5 μg/ml solution of the receptor in carbonate buffer (pH 9.6) was added to Removawell® (Dynatech Laboratories, Chantilly, VA) strips. The control strips received only the buffer. The strips were incubated overnight at 4-8°C to allow receptor binding and then treated with D-PBS containing 1% bovine serum albumin at 4-8°C to minimize nonspecific binding.

The immobilized receptor and control strips were treated with various concentrations of radiolabeled IFN-γ in the presence and absence of oligos. The labeled IFN-γ was allowed to bind to the strips for 1 hour on ice. The strips were then washed 4 times with D-PBS containing 0.05% Tween and the bound label was quantitated using the Cobra gamma counter.

The protocol was used to immobilize the anti-IFN-γ antibody was identical to that used to immobilize the soluble extracellular domain of the IFN-γ receptor, except that the antibody was used instead of receptor. The antibody used was a neutralizing antibody to IFN-γ (Clone H21, Catalog code: 1598-00, Genzyme Corporation, Cambridge, MA). Control strips were treated with OKT3 antibody (Ortho, Raritan, NJ).

To better identify the site of action, we also determined the effect of oligo treatment on the binding of two anti-IFN-γ receptor antibodies. In these studies, immobilized receptor strips and control strips were treated with 0, 2.5, 5, 10, 20 or 40.5 μM of either **I** or **II** in 100 μl of D-PBS containing 1% FCSΔ and

incubated at room temperature for 1 hour. The strips were then washed 4 times with D-PBS containing 0.05% Tween to remove unbound oligo. One μg of either a blocking mouse anti-human IFN-γ receptor antibody (Clone GIR-208, Catalog code: 1223-01, Genzyme Corporation, Cambridge, MA) or a non-blocking mouse anti-human IFN-γ receptor antibody (Clone GIR-94.5.92, Catalog code: 1224-00, Genzyme Corporation, Cambridge, MA) was added to the strips in 100 μl of PBS containing 1% FCSΔ. After incubation at room temperature for 1 hour, the strips were treated with 100 μl of horseradish peroxidase-coupled goat anti-mouse antibody and incubated again at room temperature for 1 hour. The strips were washed 4 times with D-PBS containing 0.05% Tween to remove unbound goat anti-mouse antibody. The amount of antibody bound was quantitated by adding the horseradish peroxidase substrate, o-phenylenediamine followed by absorbance measurements at 490 nm on a microplate reader (Bio-Rad, Hercules, CA). The background absorbance, due to nonspecific binding of of horseradish peroxidase-coupled goat anti-mouse antibody, was obtained on control and receptor wells not treated with anti-IFN-γ receptor antibodies.

Staining Protocol for Flow Cytometry

Usually, 0.075-0.1 x 10^6 cells were stained with each antibody for flow cytometry. The cells were first washed with 1 ml of D-PBS containing 2% FBS and 0.1% sodium azide. The cells were resuspended in 50 μl of D-PBS containing 2% FBS and 0.1% sodium azide, and 1 μg of the appropriate fluorescently labeled antibody was then added. This mixture was incubated for 30 minutes in the dark, washed twice with D-PBS containing 0.1% sodium azide to remove unbound antibody, and then resuspended in 100 μl of D-PBS containing 0.1% sodium azide. The samples were treated with 50 μl of 50

μg/ml propidium iodide in D-PBS prior to analysis so that nonviable cells could be excluded during data acquisition on the basis of the higher fluorescence in the bandpass 585/42 nm channel.

A 50 μl aliquot of the Quantum Simply Cellular ® bead suspension (Flow Cytometry Standards Corp., Research Triangle Park, NC) was also stained with each antibody. The Quantum Simply Cellular® Beads kit is a suspension of five (four plus one blank) different populations of uniform size polystyrene microbeads, each having a different number of binding sites for mouse antibodies. After the last wash, the beads were resuspended in D-PBS containing 0.1% azide and 0.5% bovine serum albumin and 10000-30000 events were acquired and stored using the same cyotmeter FL1, FL2 and compensation settings on the cytometer as used for the cells. During acquisition the FSC vs SSC plot was gated appropriately to ensure that only singlet events are acquired. After acquisition, the data was analyzed in three steps. In the first step, a smoothed FL1 histogram for analysis was generated. This step improves the estimate of central tendency and renders it less sensitive to random fluctuations. Five markers were set on the smoothed histogram so as to include the peaks corresponding to the five populations that comprise the Quantum Simply Cellular® Beads mixture. In the second step, the peak channel value or mode on each of these subpopulations was computed. The mode was preferred over the mean or median because it provides a measure of central tendency that is independent of the location of markers. In the final step, the mean number of antibody binding sites, obtained from the manufacturer's data sheet for each lot of beads, was plotted against the peak channel value. The data were fitted using a least squares routine in CricketGraph III to an equation of the form:

$$\text{Number of antibody binding sites} = A(10^{(B \times \text{Channel Number})})$$

The coefficients of correlation were usually greater than 0.99. The parameters A and B computed by the program for each antibody were transferred to an Excel (Microsoft, Redmond, WA) spreadsheet and the mean number of specific antibody sites on cells after each treatment was computed.

Calibration and Quality Assurance of the Flow Cytometer

A FACScan® flow cytometer (Becton Dickinson, San Jose, CA) was used throughout this study. To ensure accuracy and reproduciblity of the flow cytometry results, the following calibration procedures were carried out at the start of each day according to the manufacturers recommended protocols (Becton Dickinson 1991; Flow Cytometry Standards Corp. 1992).

- AutoCOMP™ test (Becton Dickinson, San Jose, CA)
- QuickCal™ test (Flow Cytometry Standards Corp., Research Triangle Park, NC)

The AutoCOMP™ procedures performs detector gain adjustment, automatic fluorescence compensation and sensitivity testing; the purpose of the QuickCal™ protocol is to correct the flow cytometer settings for variations due to instrument performance. Together, these corrections allow data obtained on different days to be compared and improve both the accuracy and the reproducibility of longitudinal studies. Additionally, each antibody was calibrated for each experiment using Quantum Simply Cellular™ beads because the AutoCOMP™ and QuickCal™ procedures do not i) compensate for variations in the specific fluorescence intensity of antibodies; ii) allow the number of antibody binding sites on cells to be quantitated; iii) allow for small variations in staining protocol; iv) allow settings other than QuickCal™ settings to be used.

RESULTS

In this Section, results from characterization and mechanistic studies on the active IFN-γ inhibitory oligos identified in our laboratory are presented. The characterization experiments were designed to evaluate the suitability of these oligos for therapeutic applications and the mechanistic studies were directed towards identifying the site of action.

As a first step towards establishing the therapeutic usefulness of these oligos, we determined whether a wide range of IFN-γ mediated effects could be inhibited by these active oligos. The potency, dose dependence, and time course of the IFN-γ inhibitory effects of **I** were measured using flow cytometric assays for IFN-γ enhanced cell surface MHC Class I, β_2 microglobulin and ICAM-1. We also confirmed that these oligos did not act via nonspecific inhibition of cellular processes and that constitutive ICAM-1 expression was not inhibited. In experiments designed to challenge the specificity of **I**, we found that IFN-α or IFN-β induced MHC Class I, and TNF-α enhanced ICAM-1 were not inhibited by **I**. Furthermore, **I** was also active in a synergistic mixture of TNF-α and IFN-γ. These results established **I** as a specific, selective, and potent inhibitor for the effects of IFN-γ and suggested that it was a promising lead candidate for therapeutic applications in transplantation and septic shock.

The characterization studies suggested that **I** exerts its effects at an early step in the IFN-γ induction process and that it does not act via antisense and antigene effector mechanisms. In ligand binding studies on K562 cells, we found that **I** inhibited the cellular association of labeled IFN-γ. Inhibition of labeled IFN-γ binding was also observed in *in vitro* binding assays employing either the purified extracellular domain of the IFN-γ binding subunit of the human IFN-γ receptor or a neutralizing anti-IFN-γ

antibody. In summary, the results show that **I** acts by inhibiting the binding of IFN-γ to its receptor complex.

Evidence for Dose Dependent Down-Regulation

To assess the relative potency of **I** for inhibiting the IFN-γ mediated enhancement of MHC Class I and ICAM-1, we constructed dose response curves using Protocol A. The induction of MHC Class I and β_2 microglobulin in K562 cells remained approximately constant at 24 and 48 hours and declined at 72 hours while the induction of cell surface ICAM-1 was highest at 24 hours and declined thereafter. Increases in the constitutive background were also observed at 72 hours for these markers. The dose response curves for **I** were fitted to a Hill equation. In the fitting procedure, the ED_{50} and Hill n values were calculated with minimum and maximum effect values constrained to the constitutive and the IFN-γ induced expression levels, respectively. Oligo **II** was inactive and was not fitted using this procedure. Figure 6 shows that **I** inhibits IFN-γ mediated induction of cell surface MHC Class I heavy chain proteins at 24, 48 and 72 hours. Inhibition is dose-dependent, and Figure 6 gives ED_{50} values of approximately 4 μM and 8 μM for 800 U/ml IFN-γ at 24 and 48 hours, respectively.

The human MHC Class I protein is heterodimeric and consists of a 45 kD polymorphic heavy chain closely associated with β_2 microglobulin, a nonpolymorphic, 12 kD, light chain. If expression of the heavy chain is reduced, then a quantitatively identical inhibition of β_2 microglobulin is to be expected. We verified this prediction and show in Figure 7 that **I** concomitantly inhibits the expression of β_2 microglobulin. Taken together, the data suggest that IFN-γ induced expression of intact MHC

Class I heterodimers can be completely blocked in a dose-dependent fashion by **I**. If **I** is acting at a step(s) unique to the generation of cell surface MHC Class I (e.g. transcription or translation) then it is unlikely that the up-regulation of other proteins by IFN-γ will be inhibited. However, the data in Figure 8 demonstrate that **I** also inhibits IFN-γ mediated enhancement of ICAM-1. Representative flow cytometric histograms obtained using the MHC Class I heavy chain antibody for treatment with 10 μM oligo **I** and IFN-γ are shown in Figure 9.

The data in Figures 6-8 allow a direct comparison of the results obtained with the three markers. It is evident that the dose response relationships, at a given time, for all markers are essentially the same. Oligo **II** is inactive at 24 and 72 hours, but an unusual dose dependent up-regulation of IFN-γ enhanced MHC Class I and ICAM-1 is evident at 48 hours.

Evidence that the Activity is Not Nonspecific Inhibition

Figure 10 shows data from an experiment designed to test the possibility that nonspecific inhibition of genes by **I** is the basis for the observed activity.

K562 cells were treated with 25 μM oligo **I** or **II** in the presence of IFN-γ and analyzed by flow cytometry for transferrin receptor expression at 24 and 48 hours. Transferrin receptor expression is readily assayed using flow cytometry and is not enhanced or repressed by IFN-γ in K562 cells. K562 cells treated with IFN-γ and **I** did not show reduced cell surface transferrin receptor expression when compared to cells treated with IFN-γ and **II**. The results show that **I** does not cause nonspecific inhibition of cell surface transferrin receptor expression.

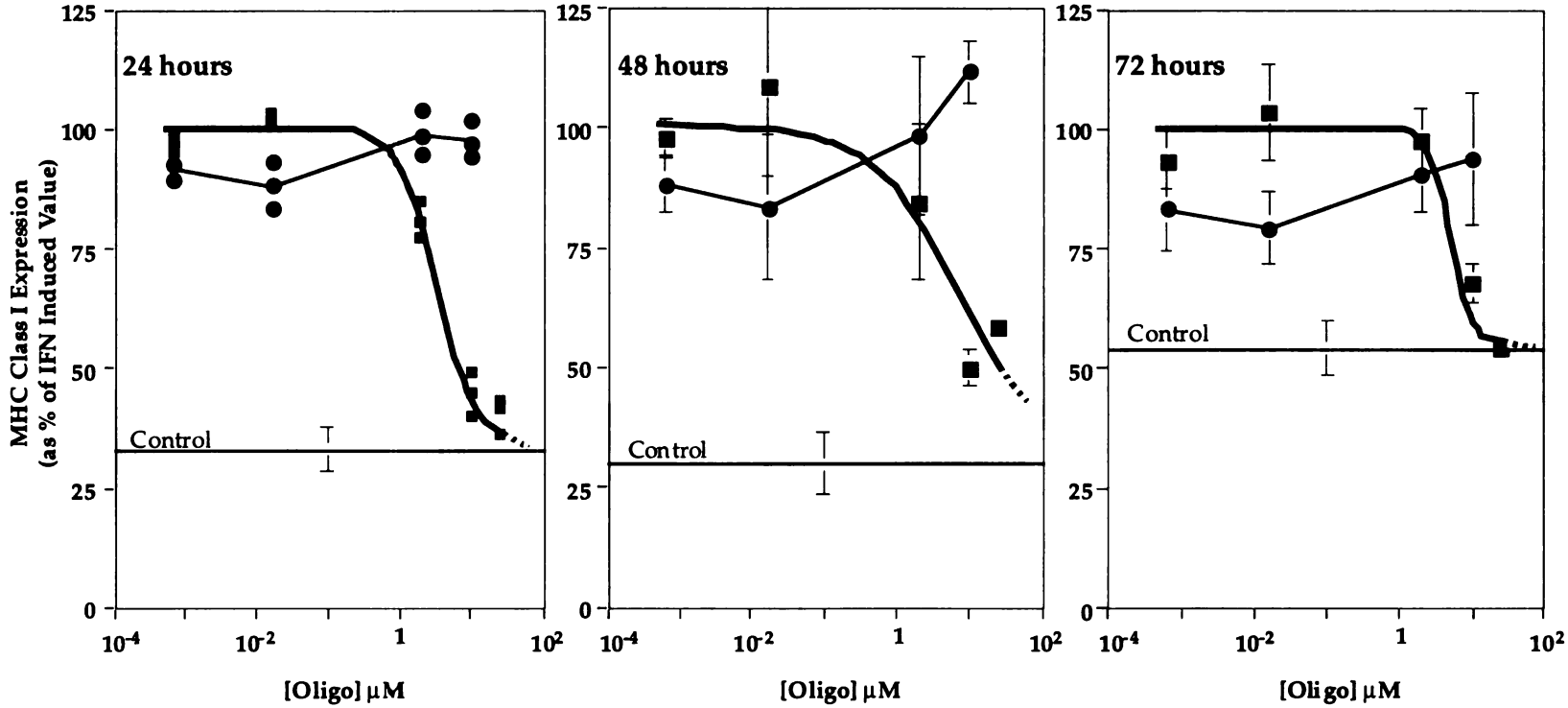


Figure 6. K562 cells were treated with either —■— oligo I or —●— oligo II and stained for cell surface MHC Class I expression 24, 48 and 72 hours later. Individual data points are represented on the 24 hour graph while the 48 and 72 hour data are represented as mean values with error bars (Protocol A; n = 3; error bars: ±1 SD). The data from flow cytometry are expressed as a percentage of the level obtained with IFN-γ in the absence of oligo.

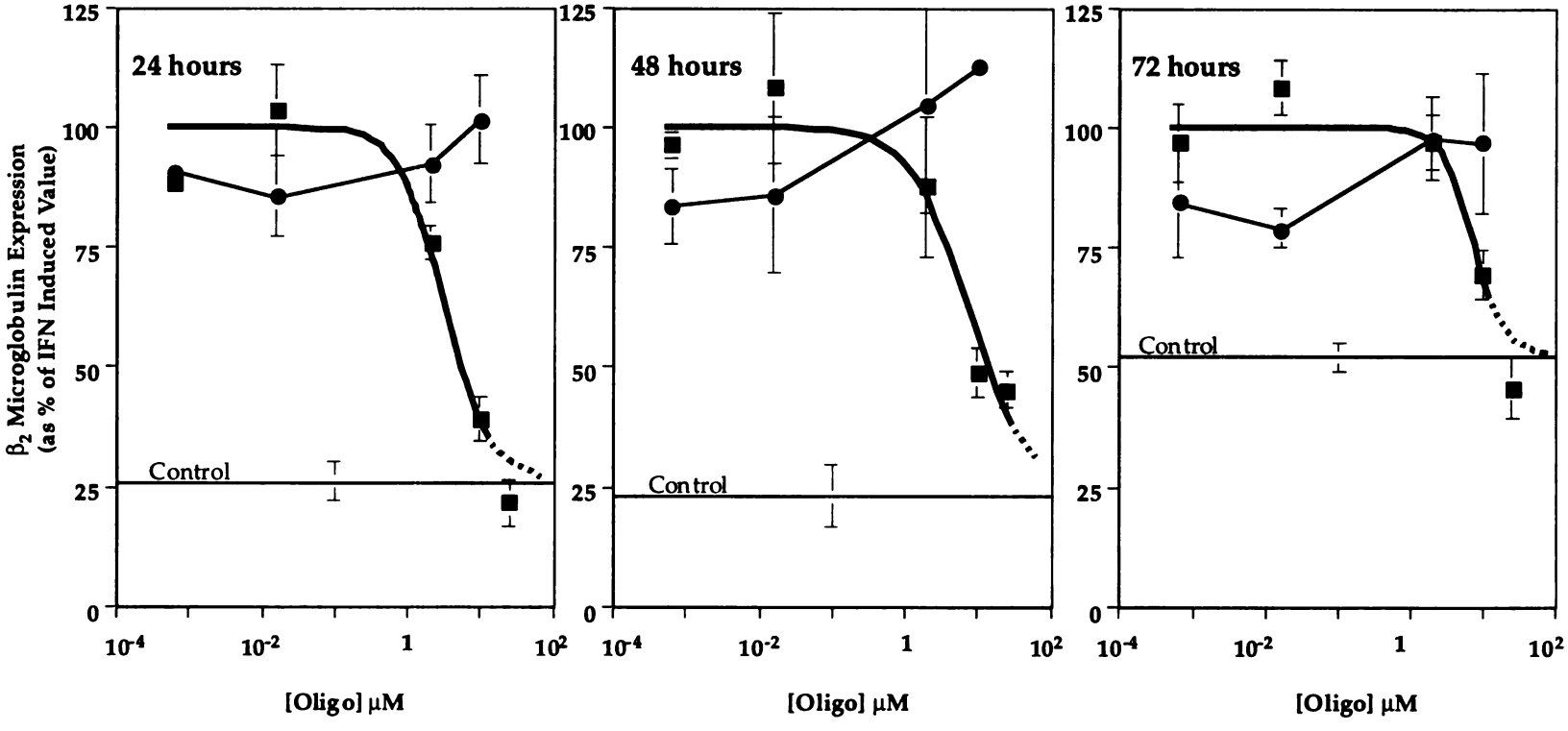


Figure 7. K562 cells were treated with either —■— oligo I or —●— oligo II and stained for cell surface β_2 microglobulin expression 24, 48 and 72 hours later (Protocol A; n = 3; error bars: ±1 SD). The data from flow cytometry are expressed as a percentage of the level obtained with IFN-γ in the absence of oligo. Control cells were not treated with IFN-γ or oligo.

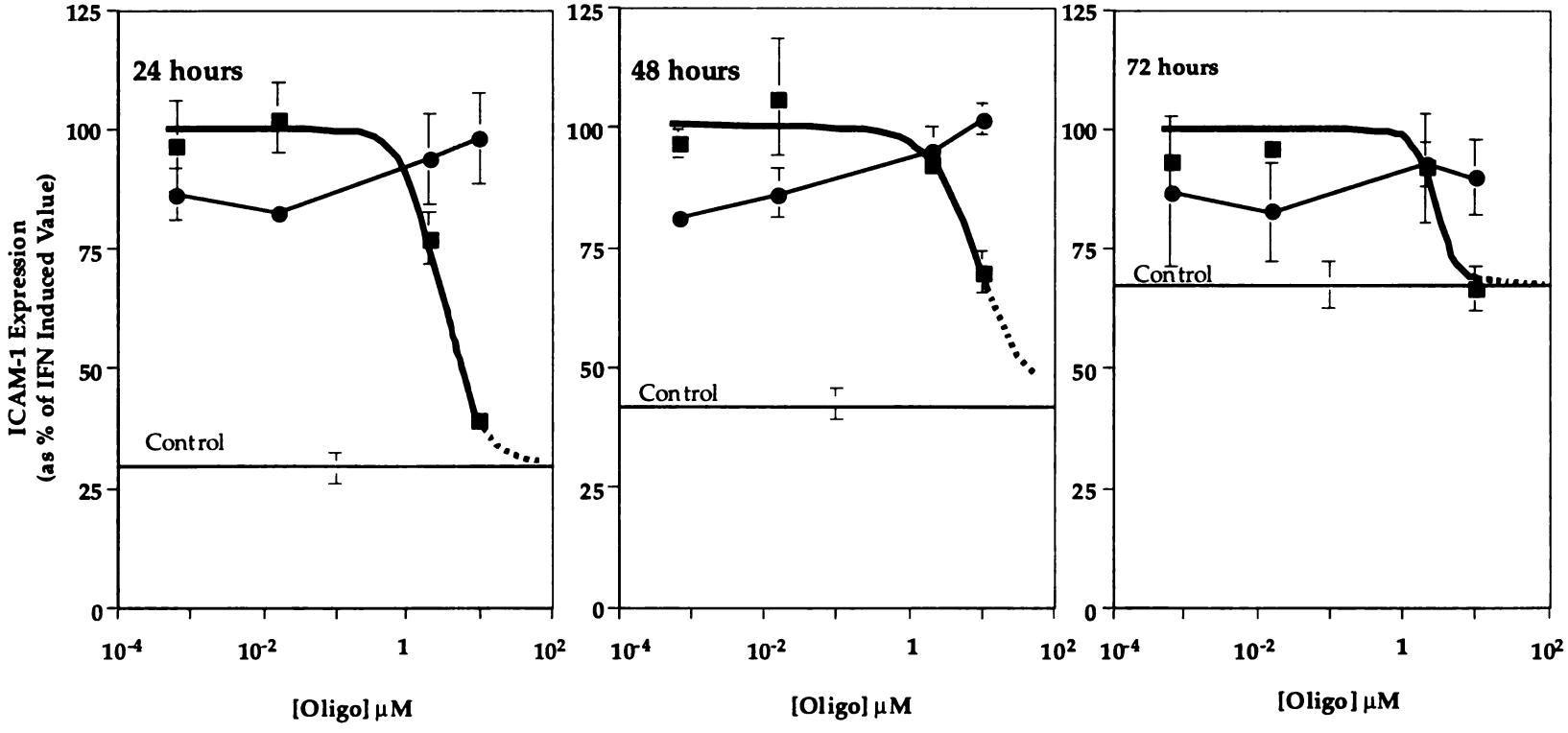


Figure 8. K562 cells were treated with either —■— oligo I or —●— oligo II and stained for cell surface ICAM-1 expression 24, 48 and 72 hours later (Protocol A; n = 3; error bars: ±1 SD). The data from flow cytometry are expressed as a percentage of the level obtained with IFN-γ in the absence of oligo. Control cells were not treated with IFN-γ or oligo.

Evidence that I does not Modulate Constitutive ICAM-1

An antisense mechanism of action is expected to reduce constitutive expression as well as inducible expression. We decided to evaluate the effect of **I** on constitutive ICAM-1 expression in K562 cells in order to test whether **I** acted via an antisense mechanism. Constitutive ICAM-1 is unaffected by treatment with **I** or **II**. The data in Figure 11 were obtained at 24 and 48 hours using Protocol B and K562 cells treated with 3.125, 6.25 or 12.5 μM Oligo **I** in the absence of IFN-γ. Furthermore, 25 μM of either **I** or **II** in the absence of IFN-γ, (Protocol A) does not decrease ICAM-1 expression in K562 cells (data not shown). The data show that **I** does not act at a step required for the basal expression of ICAM-1 and that it does not exert its effects via an antisense mechanism. Because antisense effector mechanisms act on mRNA, these results strongly suggest that an early step in the IFN-γ pathway, upstream of mRNA degradation and translation is inhibited by **I**.

Evidence that the Activity of I is Enhanced by the 3' Amino Modification

The presence of the 3' amino modification on **I** is not sufficient to cause activity because **II**, which has the same modification, is not active. To obtain more information on the role of the 3' amino modification in determining activity, we compared the activity of **I** to that of unmodified **I**. The latter had the same sequence as **I** but did not have the 3' (3-amino-2-propanol) modification.

Equivalent concentrations of both oligos were tested using Protocol B. As shown in Figure 12, unmodified **I** is able to inhibit the IFN-γ mediated enhancement of cell surface MHC Class I and ICAM-1, but is less active than **I** confirming that the activity of **I** is not simply due to the 3-amino-2-

propanol modification. At 48 hours (data not shown), unmodified **I** is inactive while **I** still retains activity. This difference in activity between **I** and unmodified **I** is postulated to be a consequence of the increased stability of **I** in the medium (Dagle, Weeks et al. 1991; Wickstrom 1986).

Evidence that a Family of Oligos has Activity

To determine whether **I** was unique in its ability to inhibit the effects of IFN-γ, we compared 25 μM **I, III, IV, V** and **VI** at 48 hours using Protocol A. Figure 13 shows that activity is sequence dependent, yet broadly similar for all three markers. An analysis of variance (ANOVA) showed a treatment effect at $p<0.001$. A post hoc comparison of mean values was also carried out using the Fisher test at a significance level of 95%. Treatment with IFN-γ and oligos **I** or **III** was not different from control treatment but differed significantly from treatment with IFN-γ alone. However, treatment with IFN-γ and **VI** was ineffective and did not differ significantly from treatment with IFN-γ alone. Using similar criteria, oligos **IV** and **V** had intermediate activity.

The ability of each oligo to inhibit both MHC Class I and ICAM-1 induction is consistent with the earlier observations. Taken together, the data suggest that a family or class of oligos exists that inhibits MHC Class I and ICAM-1 induction by IFN-γ in K562 cells and that **I, III, IV** and **V** are members of this family.

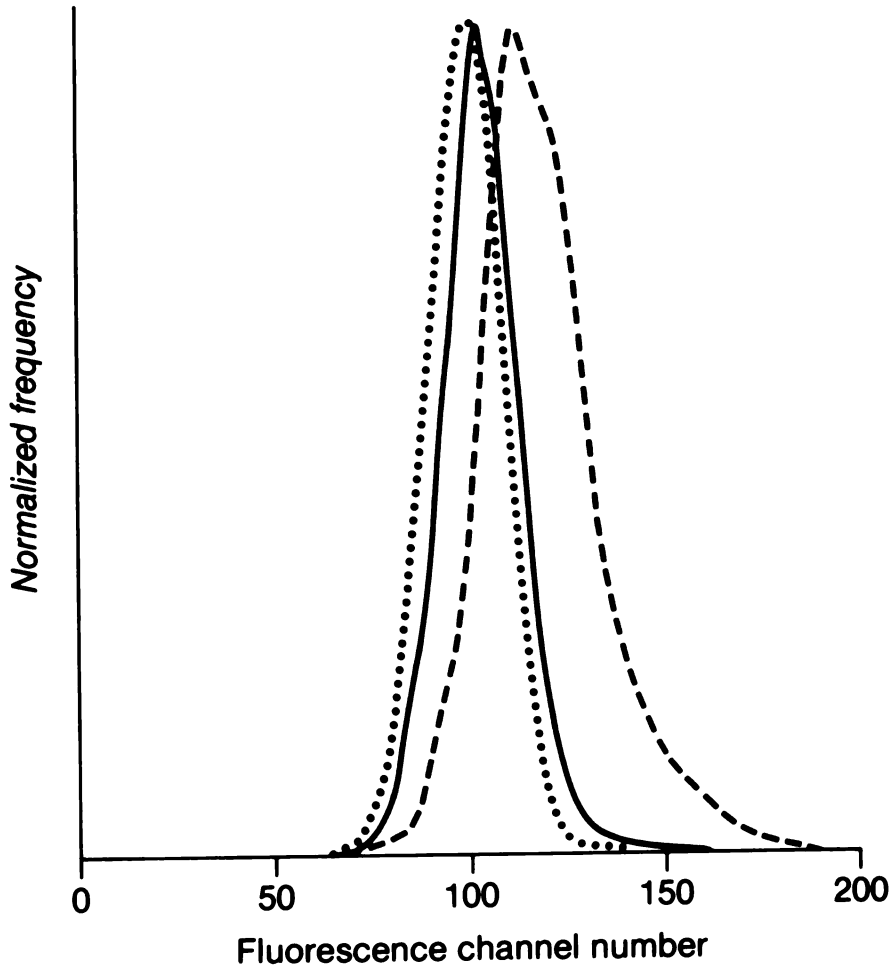


Figure 9. Representative flow cytometric histograms obtained using the antibody directed to the MHC Class I heavy chain. K562 cells were treated using Protocol A with IFN-γ in the absence (- - - - - - -) and presence (———) of 10 μM oligo I. Control cells are shown in (.....).

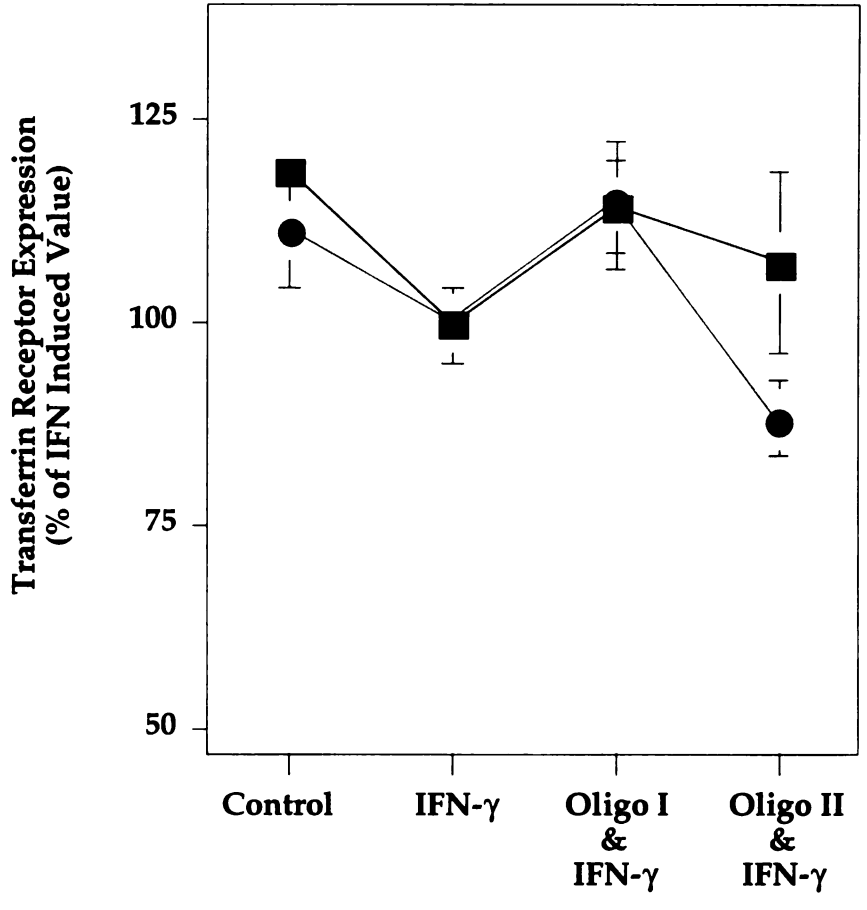


Figure 10. K562 cells were treated (n=3 replicate tubes; error bars= ±1 SD) with IFN-γ and 25 μM of either **I** or **II** and stained for transferrin receptor expression at 24 hours (—■—) and at 48 hours (—●—). This figure shows that treatment with **I** does not result in a decrease in transferrin receptor expression, suggesting that it does not act via a nonspecific decrease in transcription or translation.

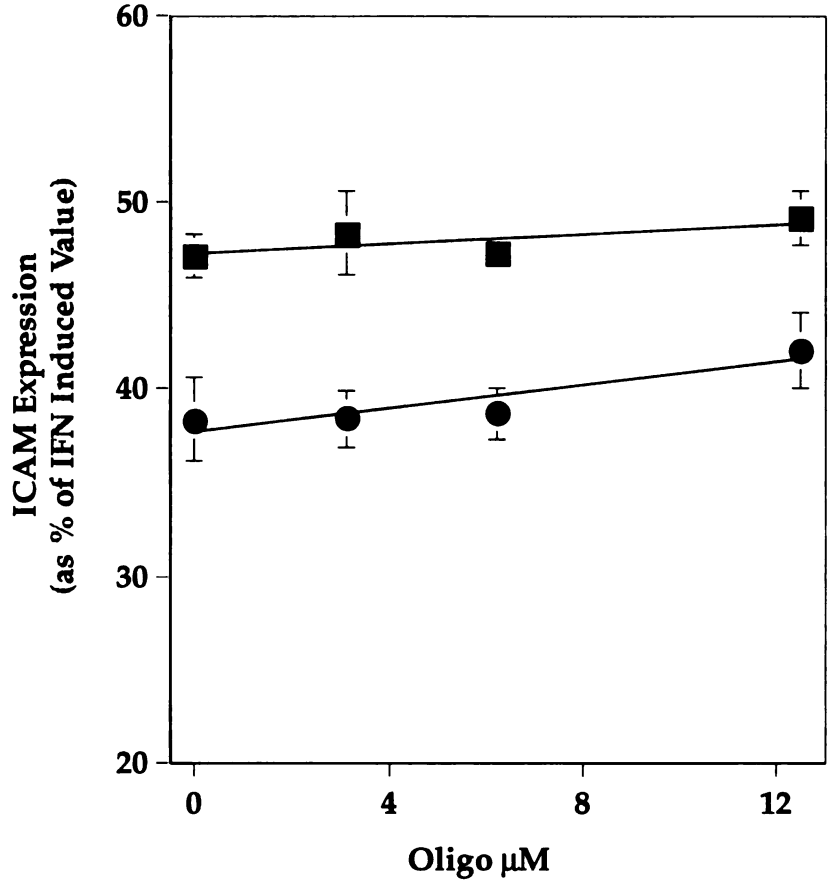


Figure 11. K562 cells were analyzed for cell surface ICAM-1 expression 24 hours (—■—) or 48 hours (—●—) after treatment with the indicated concentrations of oligo **I** alone (Protocol B; n = 2; error bars: range). The data from flow cytometry are expressed as a percentage of the level obtained with IFN-γ in the absence of oligo.

Evidence for Specificity

To challenge the hypothesis that **I** exerts its effects at a step(s) shared by several induction pathways, we turned to the cytokines IFN-α, IFN-β (Lindahl, Gresser et al. 1976) and TNF-α (Collins, Lapierre et al. 1986), which also induce MHC Class I.

The data in Figure 14, obtained using Protocol A, show that **I** selectively inhibits IFN-γ induced MHC Class I but has little or no effect on MHC Class I induction by 6400 U/ml of either IFN-α or IFN-β. Flow cytometry results with the anti-β_2 microglobulin monoclonal antibody confirmed these results (data not shown).

IFN-α and IFN-β do not induce cell surface ICAM-1 in K562 cells. Therefore, we examined the effect of **I** on the TNF-α mediated enhancement of ICAM-1 (Rothlein, Dustin et al. 1986). The results (Figure 15) demonstrate that **I** is inactive in blocking the enhancement of ICAM-1 by TNF-α. Both studies support the hypothesis that the mechanism(s) leading to blockage of cell surface MHC Class I and ICAM-1 in K562 cells by **I** is specific for the IFN-γ pathway.

Evidence that I Blocks the Synergy Between TNF-α and IFN-γ

MHC Class I is synergisitically induced by mixtures containing IFN-γ and TNF-α. To adduce additional evidence for the selectivity of **I**, and to characterize its effect on the synergy between IFN-γ and TNF-α, we evaluated the effect of 0 or 6.25 μM oligo **I** on K562 cells treated with 0, 5, 50, or 500 U/ml TNF-α with or without 50 or 200 U/ml IFN-γ. The results were also used to determine whether the site of action of **I** was upstream or downstream of the step TNF-α activated step providing synergy.

The 24 hour data for β_2 microglobulin and ICAM-1 are summarized in Figure 16. Over a 100 fold range of TNF-α, in the absence of IFN-γ, the addition of 6.25 μM **I** had no effect on the enhancement of ICAM-1 expression, confirming that **I** is specific for IFN-γ. TNF-α alone did not significantly induce β_2 microglobulin, but mixtures of TNF-α and 50 U/ml IFN-γ synergistically increased β_2 microglobulin levels. When **I** was added to mixtures containing TNF-α and 50 U/ml IFN-γ both the IFN-γ mediated induction of β_2 microglobulin and the synergy were inhibited and β_2 microglobulin expression returned to levels comparable to background. Interestingly, ICAM-1 levels observed after the addition of **I** to mixtures of TNF-α and 50 U/ml IFN-γ were always higher than those observed after treatment with TNF-α alone, suggesting that the IFN-γ enhanced component and the synergy component contributed by IFN-γ were inhibited, but that the TNF-α mediated enhancement of ICAM-1 was independent of **I**.

The synergy observed with TNF-α/200 U/ml IFN-γ mixtures was higher than that observed with TNF-α/50 U/ml IFN-γ. The addition of **I** gave similar results for both proteins: both the synergy and the IFN-γ induced components were inhibited. ICAM-1 expression in the presence of **I** was higher than the levels with TNF-α alone.

These results suggest that **I** selectively inhibits the effects of IFN-γ alone and when mixed with TNF-α. Because the inhibition of the synergistic component is not overcome with a high concentration of TNF-α, **I** must act upstream of the TNF-α activated step providing synergy.

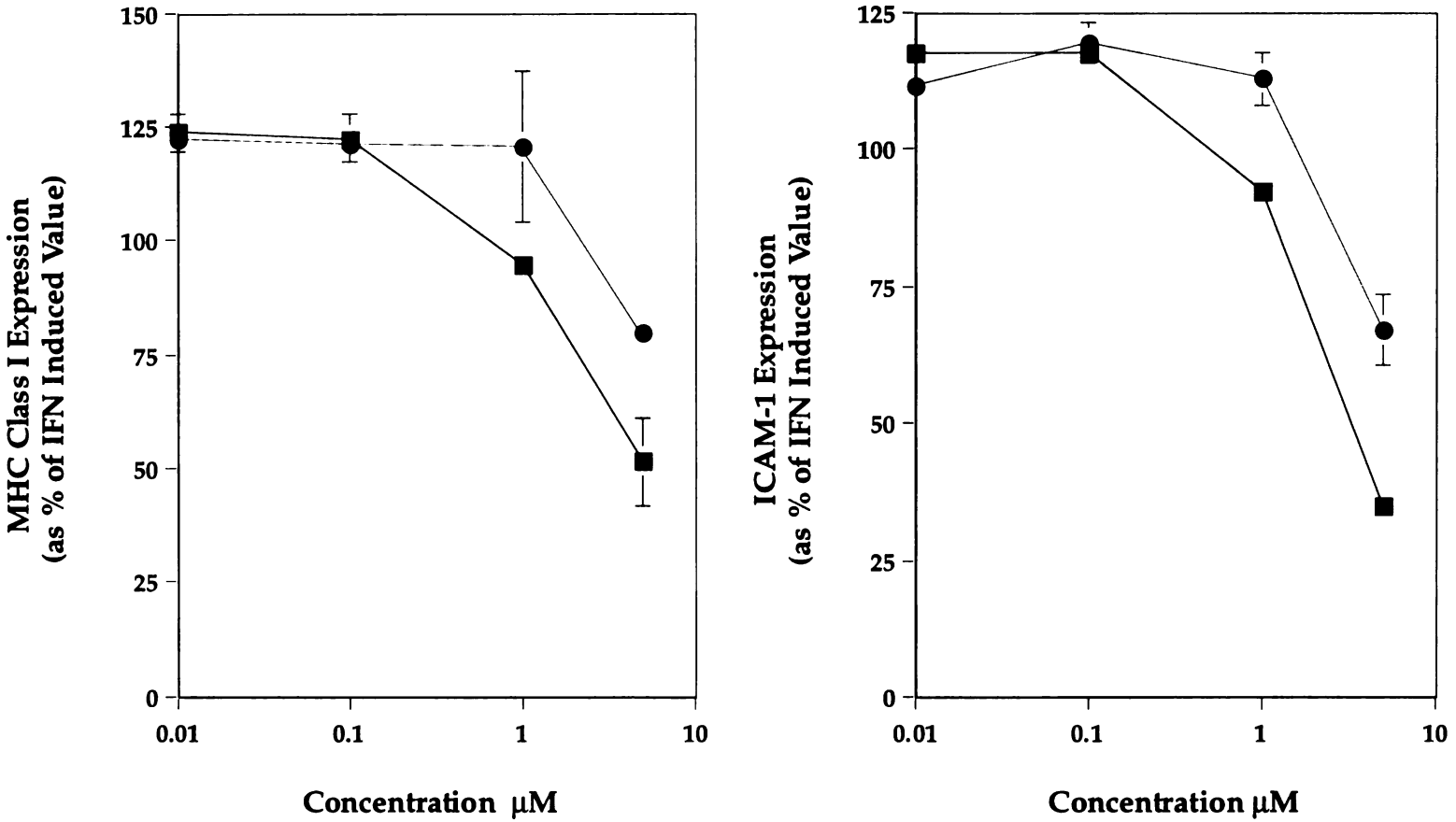


Figure 12. K562 cells were analyzed for cell surface MHC Class I and ICAM-1 expression 24 hours after treatment with the indicated doses of **I** (—■—) or unmodified oligo **I** (—●—) (Protocol B; n = 2; error bars: range). The data from flow cytometry are expressed as a percentage of the level obtained with IFN-γ in the absence of oligo.

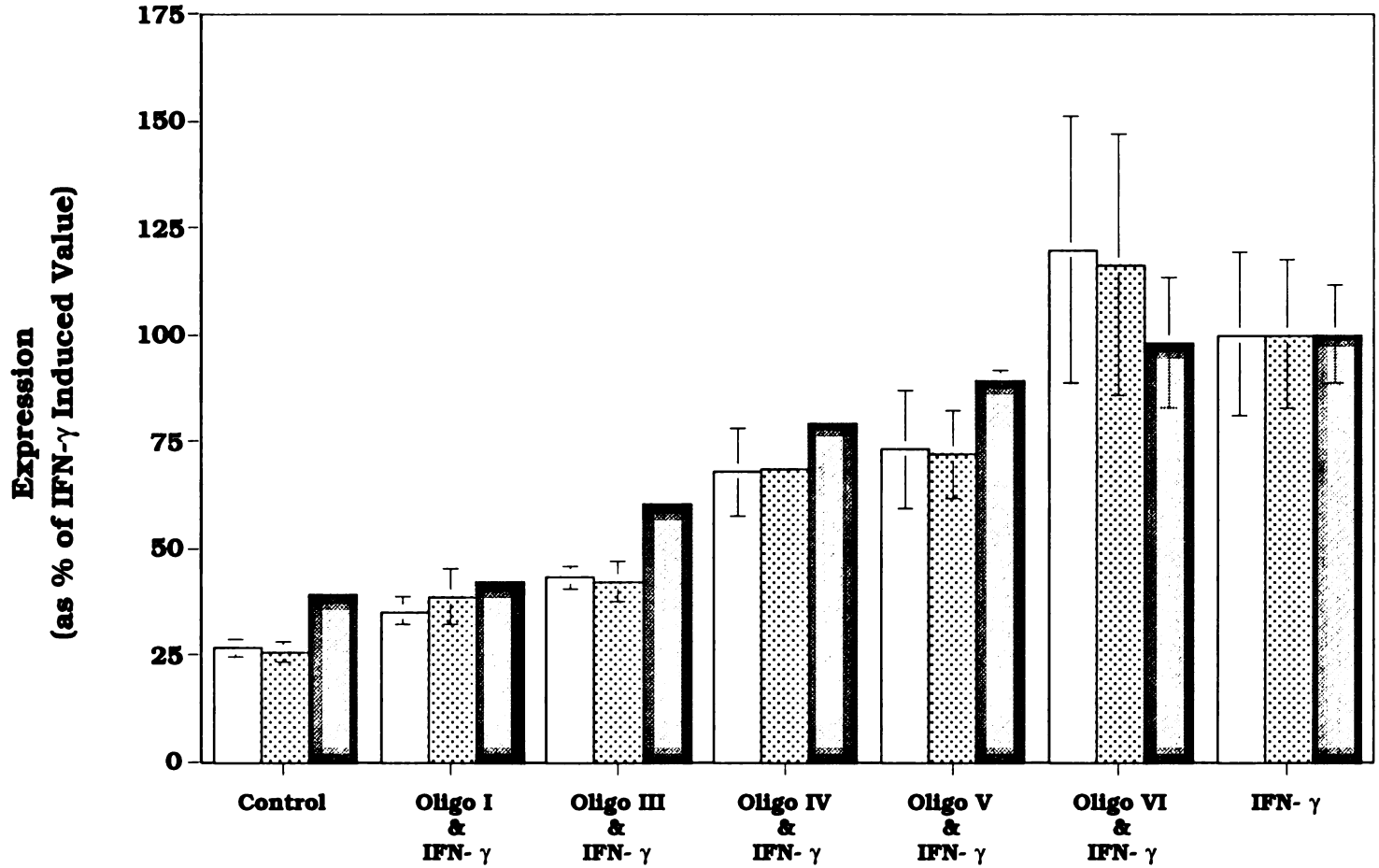


Figure 13. K562 cells were treated with either oligo **I, III, IV, V** or **VI** and IFN-γ using Protocol A, and stained for cell surface (□) MHC Class I, (▨) β_2 microglobulin and (■) ICAM-1 expression 48 hours later (n = 3; error bars: ±1 SD). The data from flow cytometry are expressed as a percentage of the level obtained with IFN-γ in the absence of oligo.

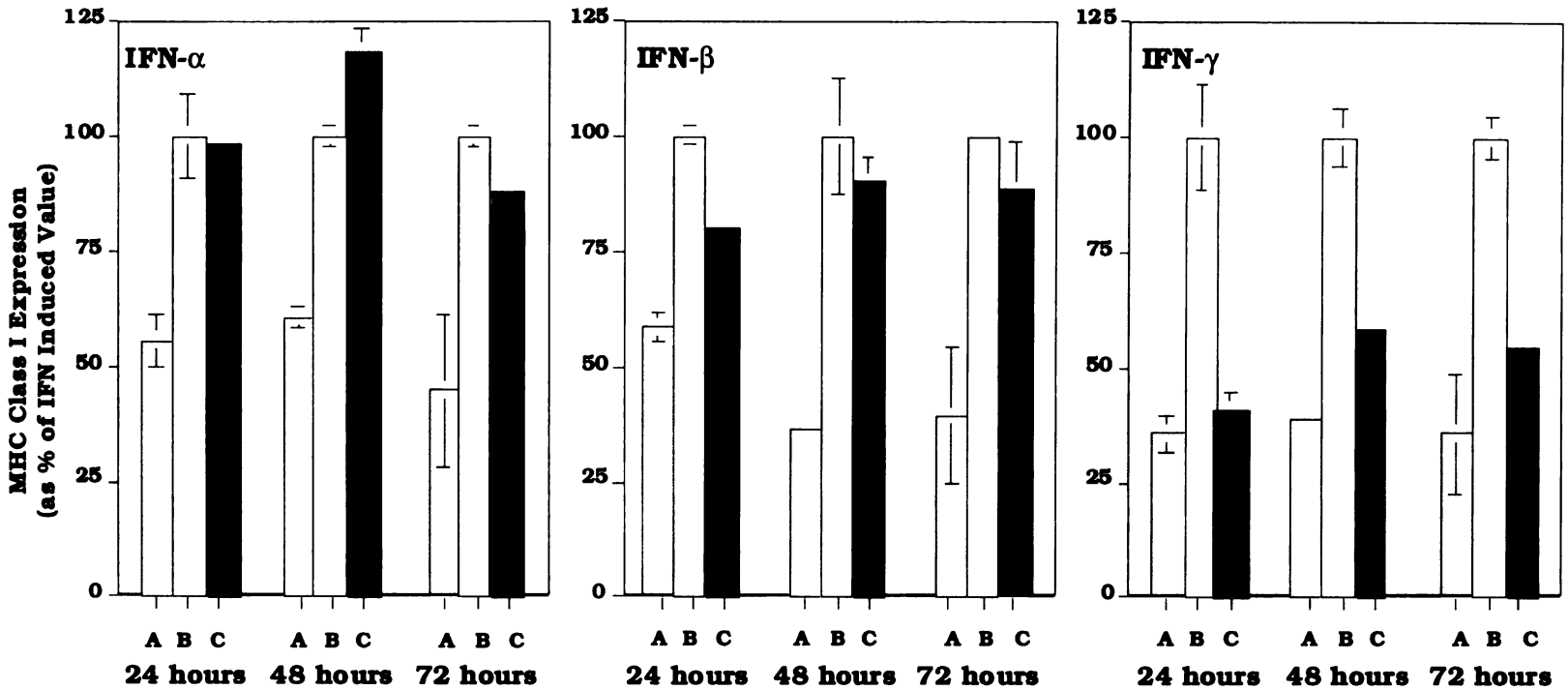


Figure 14. K562 cells (Protocol A; n = 3; error bars: ±1 SD) were treated with 25 μM oligo **I** and either IFN-γ (800 U/ml), IFN-α (6400 U/ml) or IFN-β (6400 U/ml). Cell surface MHC Class I levels, 24, 48 and 72 hours later are expressed as a percentage of the appropriate interferon induced value for (**A**) control cells (**B**) cells treated only with the indicated interferon, and (**C**) cells treated with the indicated interferon plus 25 μM oligo **I**.

Evidence for Activity in other Cell Lines

In order to exclude the possibility that the anti-IFN-γ activity of **I** was due to cell line specific factors, we extended these studies to Burkitts lymphoma-derived Raji cells, to uterine carcinoma derived HeLa S3 cells, and to T cell lymphoma derived HUT78 cells. These cell lines are reasonable *in vitro* models for B cells, epithelial cells and mature T cells, respectively. Cells were treated with 25 μM oligo **I** according to Protocol A modified to also stain for MHC Class II DR and transferrin receptor protein expression at 48 hours.

Raji cells constitutively express high levels of MHC Class I, MHC Class II DR and transferrin receptor. IFN-γ did not significantly change these levels, and **I** does not appear to down-regulate any of the markers examined (data not shown). In HUT78 cells the IFN-γ induced expression of ICAM-1 was inhibited by **I**. However, neither IFN-γ nor **I** altered levels of MHC Class I, β_2 microglobulin, MHC Class II DR or transferrin receptor in HUT78 cells (data not shown). Thus, in Raji and HUT78 cells, as in K562 cells, **I** can inhibit the up-regulation of proteins by IFN-γ, but does not alter constitutive levels of cell surface proteins.

IFN-γ induces MHC Class I heavy chain, ICAM-1 and MHC Class II DR in HeLa S3 (Figure 17). Oligo **I** inhibits MHC Class DR and ICAM-1, induction but in contrast to K562 cells, has little or no effect on MHC Class I or β_2 microglobulin (data not shown) expression. Unlike the other markers examined, the transferrin receptor is down-regulated by IFN-γ in HeLa S3 cells and 25 μM **I** also inhibits this down-regulation. Thus, **I** is capable of inhibiting both the up-regulation and the down-regulation of some IFN-γ regulated genes.

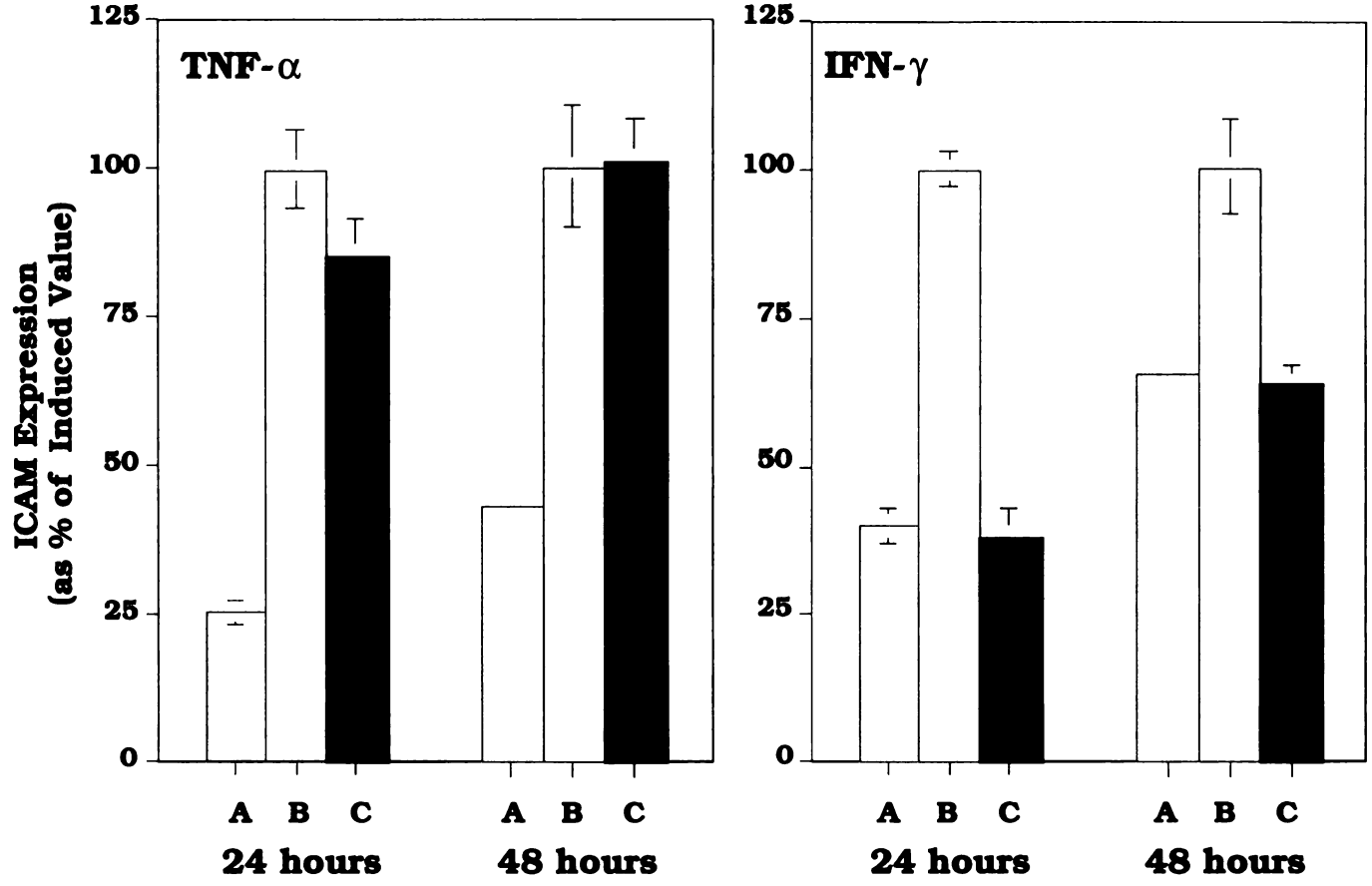


Figure 15. K562 cells (Protocol A; n = 3; error bars: ±1 SD) were treated with 25 μM oligo **I** and either IFN-γ (800 U/ml) or TNF-α (800 U/ml). Cell surface ICAM-1 levels 48 hours later are expressed as a percentage of the appropriate induced value for (**A**) Control cells (**B**) Cells treated with the indicated cytokine, and (**C**) Cells treated with the indicated cytokine and 25 μM oligo **I**.

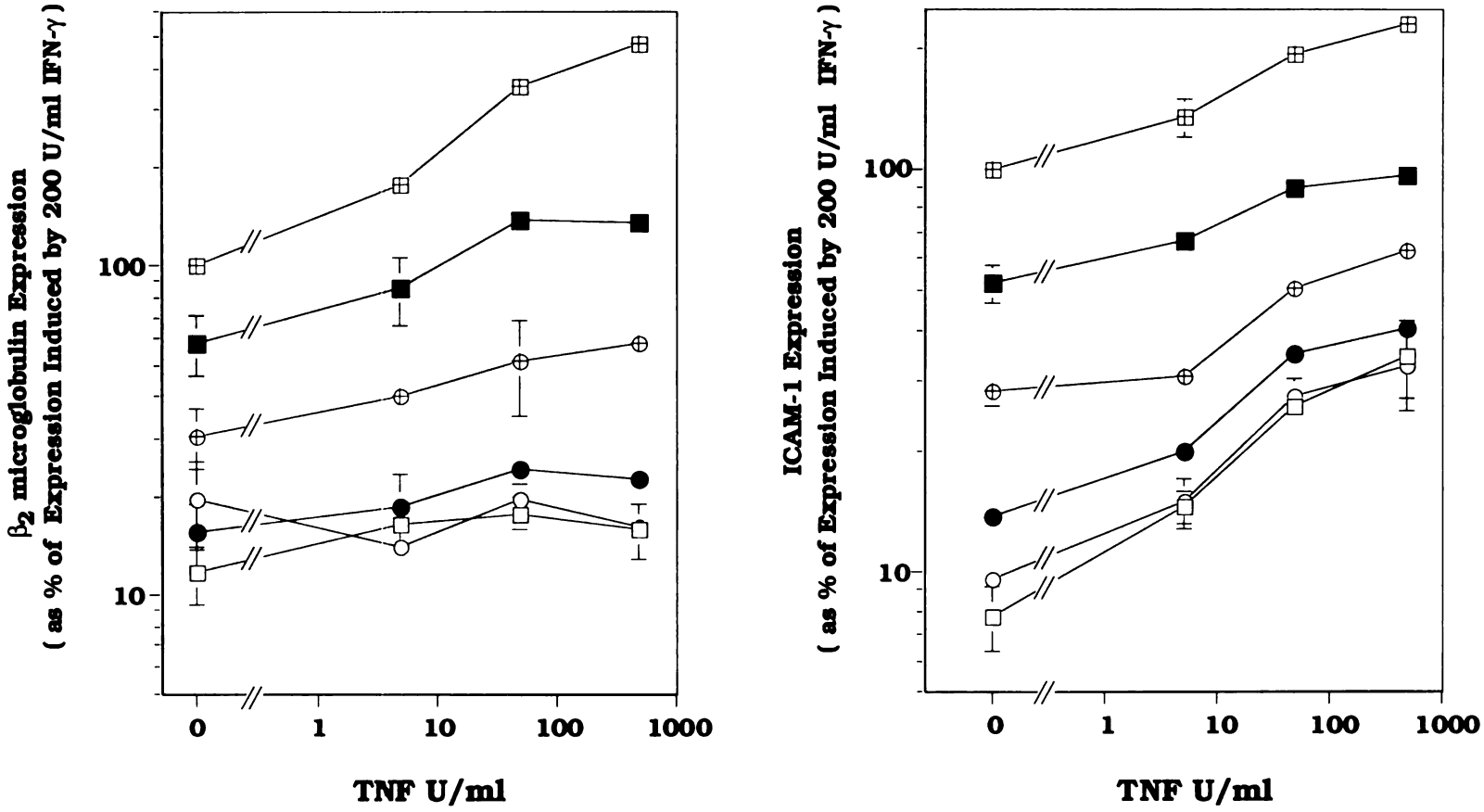


Figure 16. K562 cells were treated (Protocol B; n = 2; error bars: range) with either (—□—) TNF-α alone; (—○—) TNF-α and 6.25 μM oligo I; (—■—) 50 U/ml IFN-γ and TNF-α; (—●—) 50 U/ml IFN-γ, TNF-α and 6.25 μM oligo I; (—⊞—) 200 U/ml IFN-γ and TNF-α; or (—⊕—) 200 U/ml IFN-γ, TNF-α and 6.25 μM oligo I. Cell surface MHC Class I and ICAM-1 levels at 24 hours are expressed as a percentage of the expression observed with 200 U/ml IFN-γ alone. Both axes are log scales.

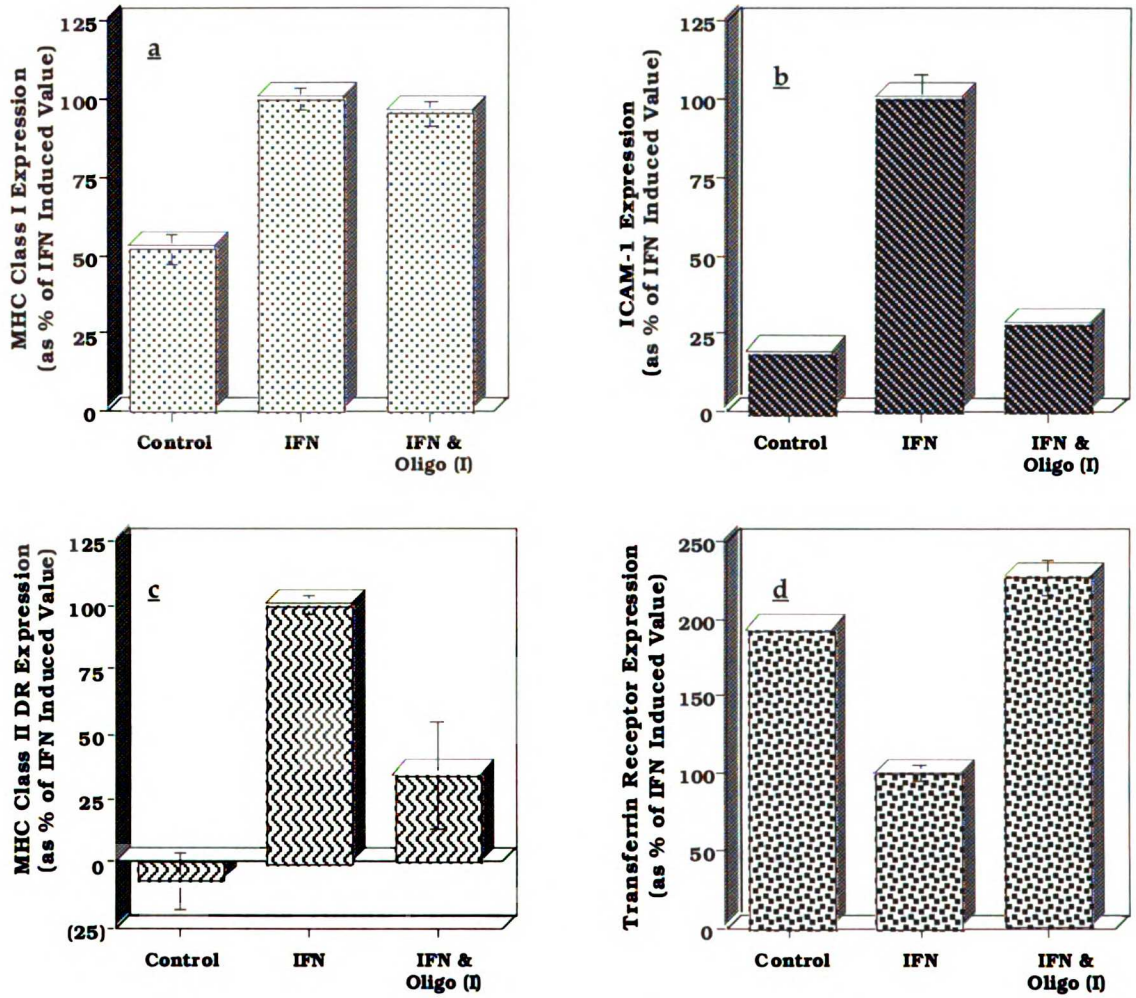


Figure 17. HeLa S3 cells were either control treated, treated with IFN-γ (800 U/ml) or treated with IFN-γ (800 U/ml) and 25 μM oligo **I** (Protocol A; n = 2; error bars: range). Cells were stained 48 hours later for (**a**) MHC Class I (**b**) ICAM-1 (**c**) MHC Class II DR (**d**) transferrin receptor and the results are expressed as a percentage of the corresponding IFN-γ induced value.

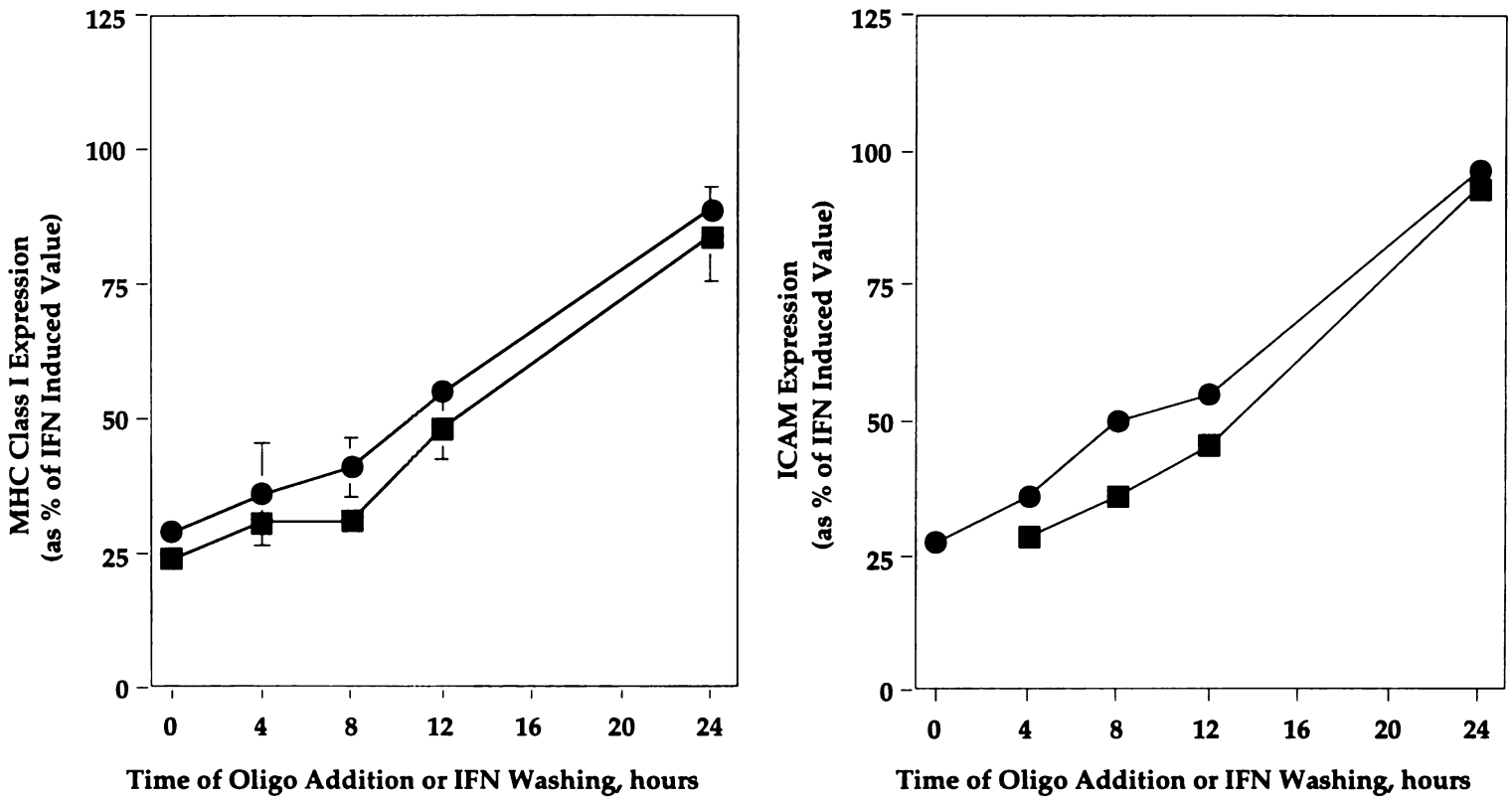


Figure 18. K562 cells were treated (Protocol B; n = 2; error bars: range) with 200 U/ml IFN-γ at time t = 0 and either treated with 6.25 μM oligo I (—●—) or washed twice to remove external free IFN-γ (—■—)at 0, 2, 4, 8, 12 or 24 hours. Cell surface MHC Class I and ICAM-1 levels at 24 hours are expressed as a percentage of the IFN-γ induced value of cells neither washed nor treated with I.

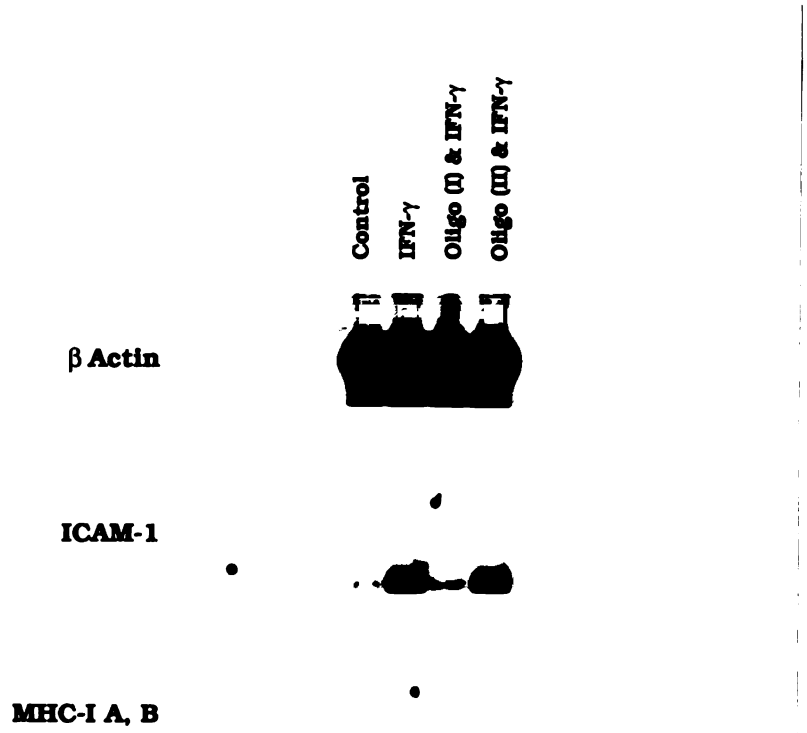


Figure 19. This Figure shows the results of a Northern analysis of mRNA extracted from K562 cells after 15 hours of the treatments indicated on the lanes. Oligos I and II were used at 25 μM. The blot was probed with ^{32}P labeled antisense oligodeoxynucleotide probes for β-actin, MHC-I heavy chain and ICAM-1 mRNAs.

Kinetics of Inhibition

This experiment was designed to define the kinetic properties of the oligo inhibition of IFN-γ and to compare the effectiveness of **I** to the removal of IFN-γ. We determined the effectiveness of **I** on K562 cells that were previously exposed to IFN-γ for varying lengths of time prior to the addition of **I** to study the oligos' ability to overcome prior IFN-γ treatment. Controls were not treated with **I**, but instead were washed to remove extracellular IFN-γ; these experiments allowed us to determine the effect that time of exposure to IFN-γ had on the inhibitory activity of **I**. These controls provide a reasonable basis for comparing the inhibitory activity of **I** because the repeated washing removes external free IFN-γ. Cells were stained for flow cytometry 24 hours after the addition of IFN-γ, and the results for both MHC Class I and ICAM-1 are shown in Figure 18.

Decreasing the duration of exposure to IFN-γ causes a linear decrease in the cell surface expression of both MHC Class I and ICAM-1. Similarly, the addition of **I** at progressively earlier times also results in a linear decrease in MHC Class I and ICAM-1 expression. Notably, the treatment with **I** is slightly less effective than the physical removal of extracellular IFN-γ. The data clearly show that **I** exerts inhibitory activity even when added to cells previously exposed to IFN-γ, and suggests that the underlying mechanism(s) of action of **I** in K562 cells has effective kinetic properties equivalent to the removal of IFN-γ by washing.

Evidence from Northern Analysis of mRNAs

The Northern analysis of mRNA was carried out to determine whether the site of action of **I** was downstream to IFN-γ induced mRNA accumulation. If the site of action of **I** were post-mRNA accumulation or

post-translational, the ICAM-1 and MHC Class I mRNA levels in cells treated with IFN-γ and **I** would be similar to those in cells treated with IFN-γ alone. Northern analysis was also used to determine whether **I** was specific or nonspecific because mRNAs for both IFN-γ induced proteins (MHC-I and ICAM-1) and a protein (β-actin) not modulated by IFN-γ were probed.

Figure 19 shows results of a Northern analysis of mRNA from K562 cells with the following treatments i) control ii) IFN-γ iii) 25 μM oligo **I** and IFN-γ and iv) 25 μM oligo **II** and IFN-γ. The blot was probed for the β-actin, MHC-I heavy chain and ICAM-1 mRNAs. However, the results show that treatment with **I** inhibits the IFN-γ mediated increases in mRNA levels for both ICAM-1 and MHC-I heavy chain, but does not alter the β-actin mRNA level. Oligo **II** does not inhibit the effects of IFN-γ on mRNA levels. These data are consistent with the flow cytometry results and show that **I** does not act at a step downstream of mRNA accumulation.

*Oligo **I** Does Not Act by Binding to the Primary Structure of IFN-γ*

To test whether **I** exerts its effects on IFN-γ treated cells by binding to the primary structure of IFN-γ, we treated cells with a mixture of active IFN-γ containing a 100-fold excess heat-treated IFN-γ. We confirmed that the heat treatment inactivated IFN-γ, and reasoned that if the primary structure of IFN-γ was sufficient to provide the putative receptor for **I**, then **I** would be sequestered by the large excess of inactive IFN-γ and would be unavailable to act on active IFN-γ. Figure 20 shows the MHC Class I and ICAM-1 dose response curves for **I** obtained in the presence and absence of a 100 fold excess of inactive IFN-γ. The curves in the presence and absence of inactive IFN-γ are similar and this shows that **I** does not exert its effects by binding to the primary structure of IFN-γ.

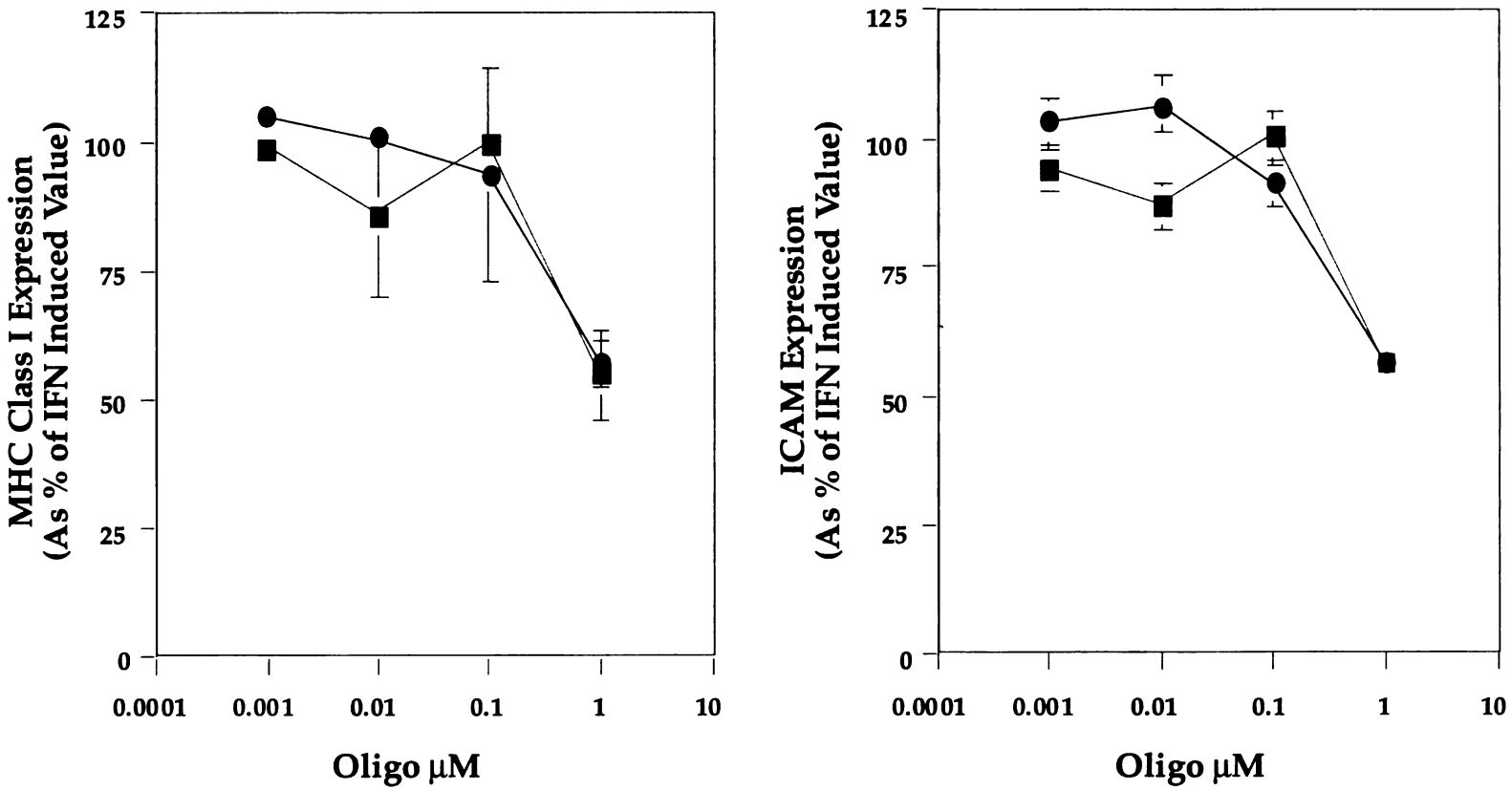


Figure 20. In an experiment designed to evaluate whether denatured IFN-γ was sufficent to provide the putative receptor for **I**, K562 cells were treated (Protocol B; n = 2; error bars: range) with 50 U/ml IFN-γ and the indicated concentrations of **I** either in the presence (—■—) or in the absence (—●—) of a 100 fold excess of heat denatured IFN-γ. Cell surface MHC Class I and ICAM-1 levels at 24 hours are expressed as a percentage of the IFN-γ induced value.

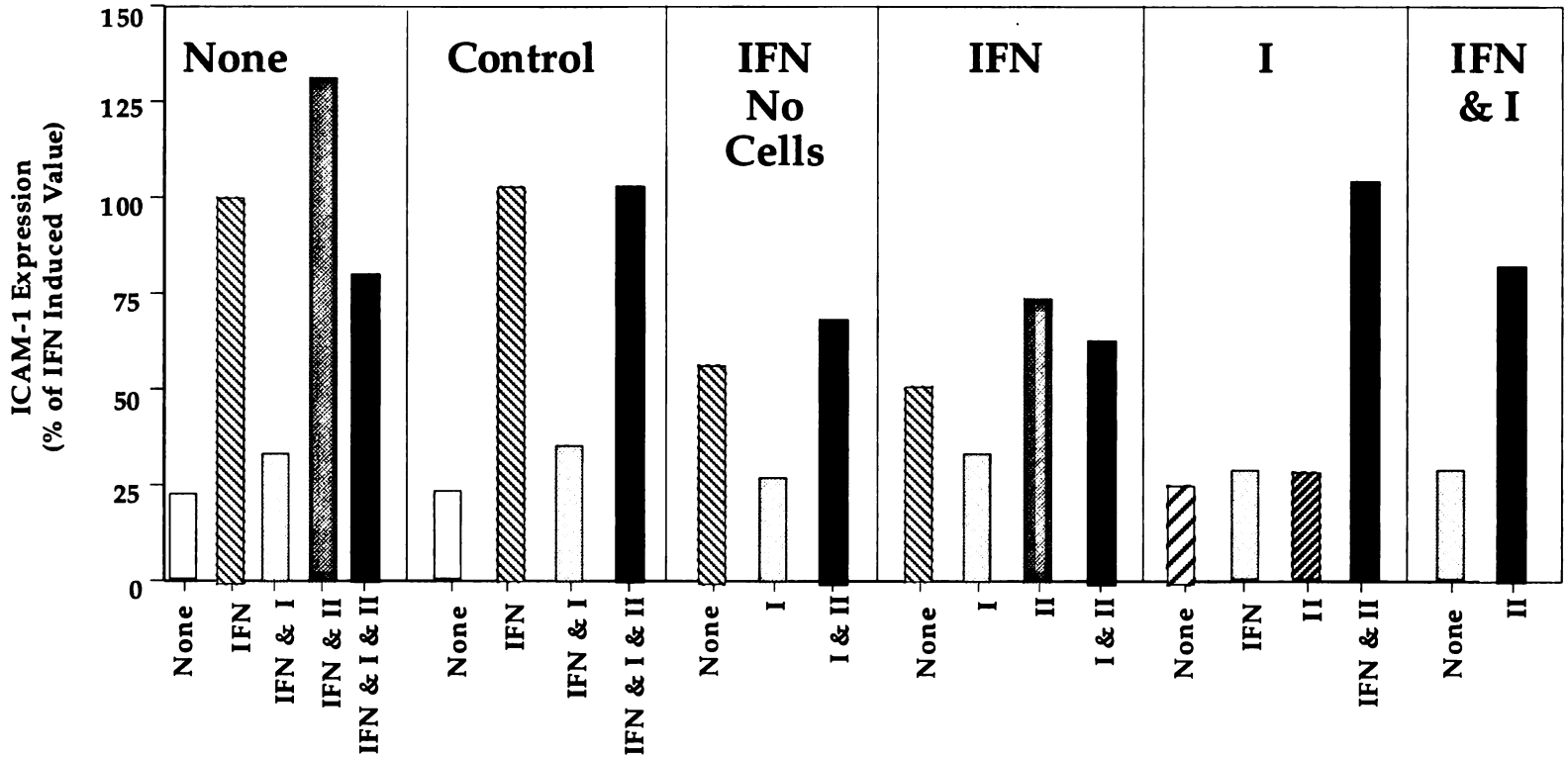


Figure 21. To test whether **I** exerted its effects by irreversibly modifying IFN-γ, K562 cells were treated in four replicate tubes with either i) media ii) 200 U/ml IFN-γ iii) 6.25 μM **I** or iv) 6.25 μM **I** and IFN-γ. After 15 hours, cell free supernatant from the four replicate pooled and aliquots treated as indicated along the category axis. The IFN-γ activity in these aliquots was bioassayed by measuring ICAM-1 expression at 24 hours in K562 cells. The samples that received the same total additions are shown shaded with the same pattern.

Oligo I Does Not Act by Irreversibly Modifying IFN-γ

The results of an experiment designed to exclude irreversible binding of **I** to IFN-γ as a candidate mechanism are shown in Figure 21. The question itself is not trivial because IFN-γ acts on its receptor as a homodimer and has been shown to be sensitive to pH and temperature.

Supernatants were removed after 12 hours from K562 cells treated with either IFN-γ or a mixture of IFN-γ and **I**. After removal, **II**, the reverse complement of **I**, was added to the supernatant from IFN-γ and **I** treated cells and both **II** and **I** were added to the supernatant from cells treated with IFN-γ alone. The addition of **II**, which is an antagonist for the IFN-γ inhibitory activity of **I**, allowed us to bioassay for IFN-γ. The results show that equivalent amounts of IFN-γ activity are recovered from cells treated with **I** suggesting that irreversible chemical modification of IFN-γ is not responsible for the inhibitory activity of **I**.

Effect of Preincubation with Oligo

The preincubation experiment was designed to determine whether any **I** that is taken up by cells was sufficient to provide inhibitory activity and to determine whether the site of action was accessible for the removal of **I** by cell washing. The data in Figure 22 show that washing abrogates the inhibitory activity of **I**.

Evidence that Oligo I Inhibits the Cellular Association of IFN-γ

To determine whether or not **I** acts prior to the cell membrane, the effects of 10 μM **I** and 10 μM **II** on the binding of radiolabeled IFN-γ to K562 cells was studied. The experiments were carried out on ice in order to minimize receptor endocytosis and recycling.

In the presence of **I**, significantly less IFN-γ associated with cells (Figure 23a). At the 10 μM level, **II** was less effective than **I** at inhibiting the cellular association of IFN-γ. In the presence of a 100-fold excess of unlabeled IFN-γ, labeled IFN-γ binding was significantly reduced. This confirmed that the labeled compound was competing effectively with unlabeled, native IFN-γ and that the results are not artifacts of the labeling process.

A Scatchard analysis of the data was carried out (Figure 23b) and also showed that labelled IFN-γ binding in the presence of **I** was greatly reduced compared to binding in the absence of oligo or in the presence of 10 μM **II**. These results strongly suggest that the effects of **I** are a result of reduced IFN-γ binding in the presence of **I**. Because the binding studies were carried out at 2-4°C, the role of receptor recycling was minimal. Therefore, we can conclude that **I** acts to reduce labeled IFN-γ binding at the cell surface.

The doses of IFN-γ shown in Figure 23a were selected to be representative of those at which physiological effects such as MHC Class I, MHC Class II and ICAM-1 induction occur. Similar results were obtained when higher IFN-γ doses were examined.

*Evidence that Oligo **I** Inhibition of the Cellular Association of IFN-γ is Dose Dependent*

This experiment was designed to determine the effect of varying the dose of **I** and **II** on the binding of labeled IFN-γ. In this experiment, oligo concentrations of 0, 0.01, 0.1, 1 and 10 μM were used with the IFN-γ dose kept constant at 200 U/ml. Figure 24 shows that **I** is more potent than **II** and inhibits the cellular association of labeled IFN-γ at lower doses than **II**.

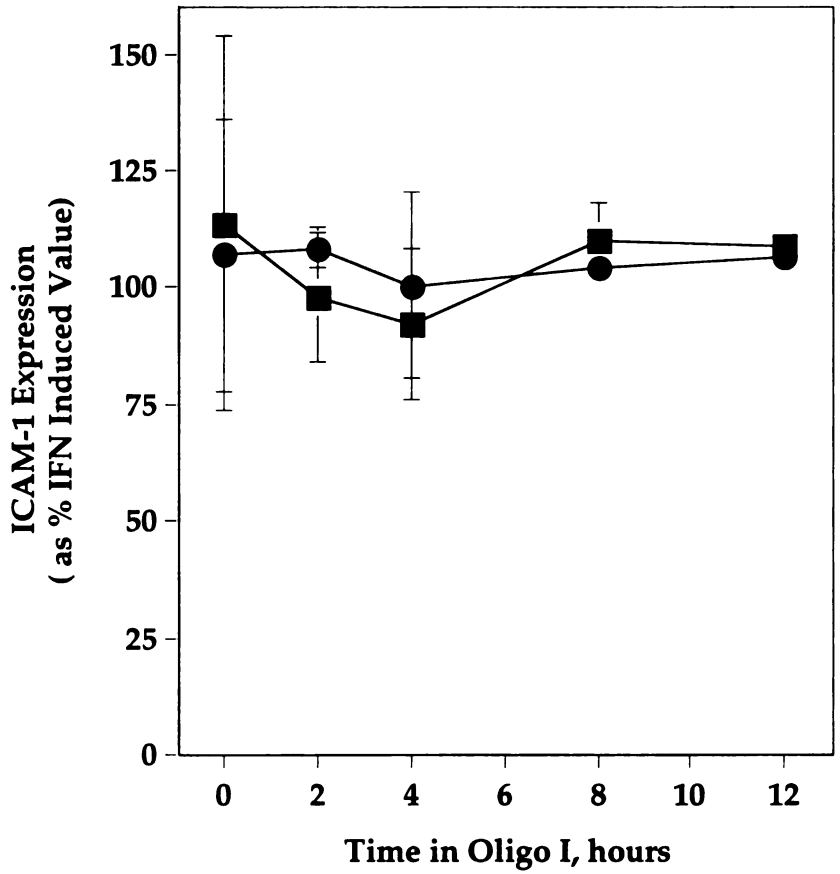


Figure 22. K562 cells were treated (Protocol B; n = 2; error bars: range) with treated with 6.25 μM oligo **I** for 0, 1, 2, 4, 8 or 12 hours and then washed twice to remove external free oligo **I**. The cells were then exposed to 200 U/ml IFN-γ and analyzed for cell surface ICAM-1 expression 12 hours (—■—) and 36 hours (—●—) later. The results are expressed as a percentage of the IFN-γ induced ICAM-1 level on cells not treated with **I**.

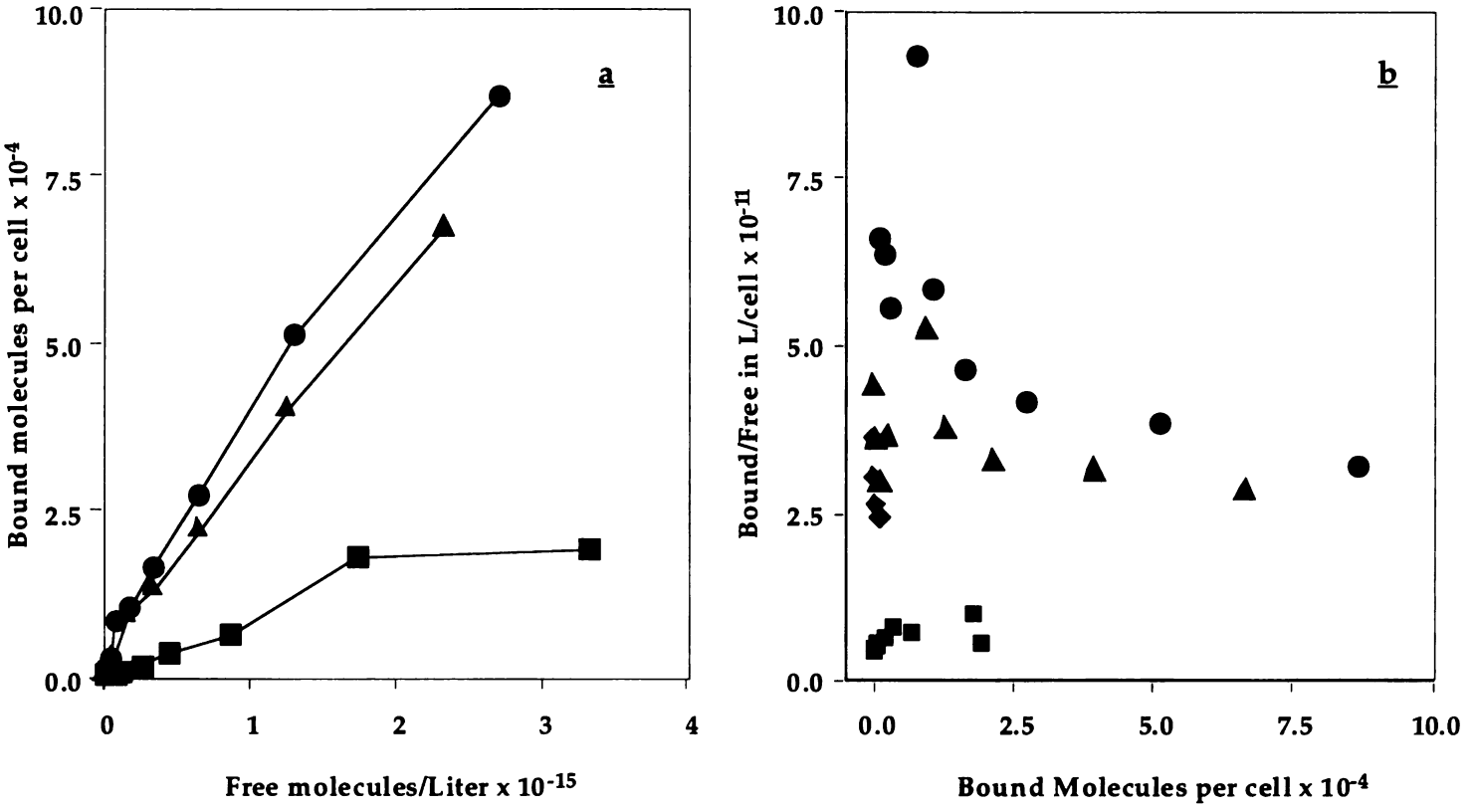


Figure 23a. The binding of ^{125}I labeled IFN-γ to K562 cells is shown. 2 x 10^6 cells were treated on ice with various concentrations of labeled IFN-γ and either media(—●—), 100 fold excess of cold IFN-γ (—◆—), 10 μM I (—■—), or 10 μM II (—▲—). After 1 hour, radioactivity in the cell free supernatant and in the cell pellet were measured in a gamma counter. Figure 23b is a Scatchard plot of the data from Figure 23a.

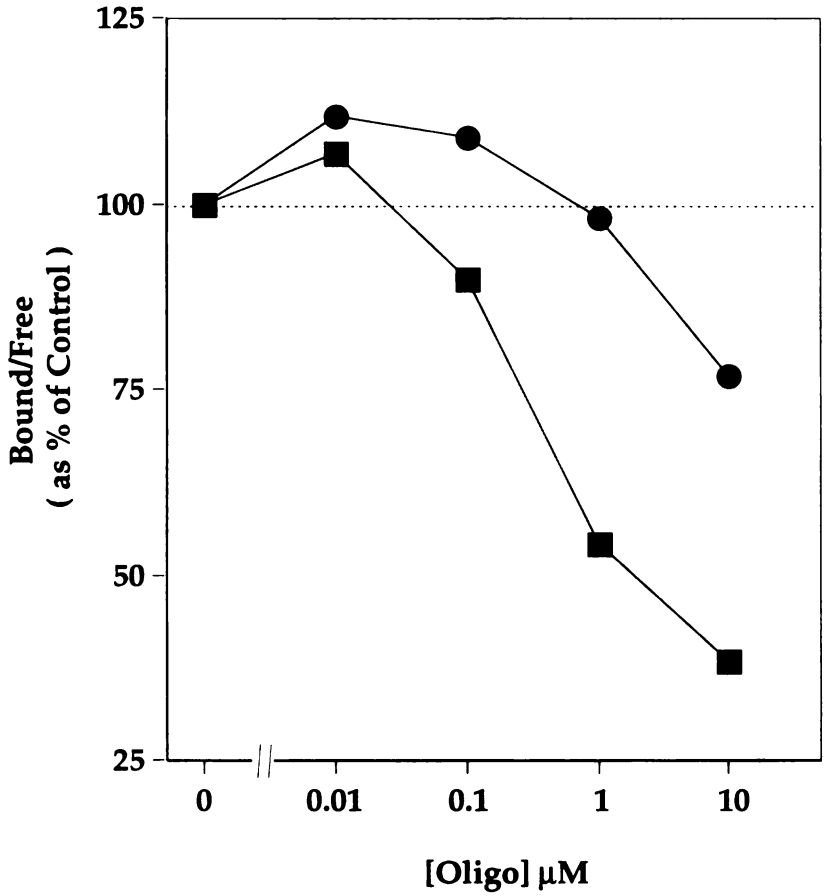


Figure 24. The dose dependence of the binding of ^{125}I labeled IFN-γ to K562 cells in the presence of oligo **I** and **II** is shown. 2 x 10^6 K562 cells were treated on ice with 200 U/ml of labeled IFN-γ and the **I** (—■—) or **II** (—●—) concentrations shown. After 1 hour, radioactivity in the cell free supernatant and in the cell pellet were measured in a gamma counter. The data are expressed as a percentage of the bound radioactivity to free radioactivity in cells not treated with either oligo.

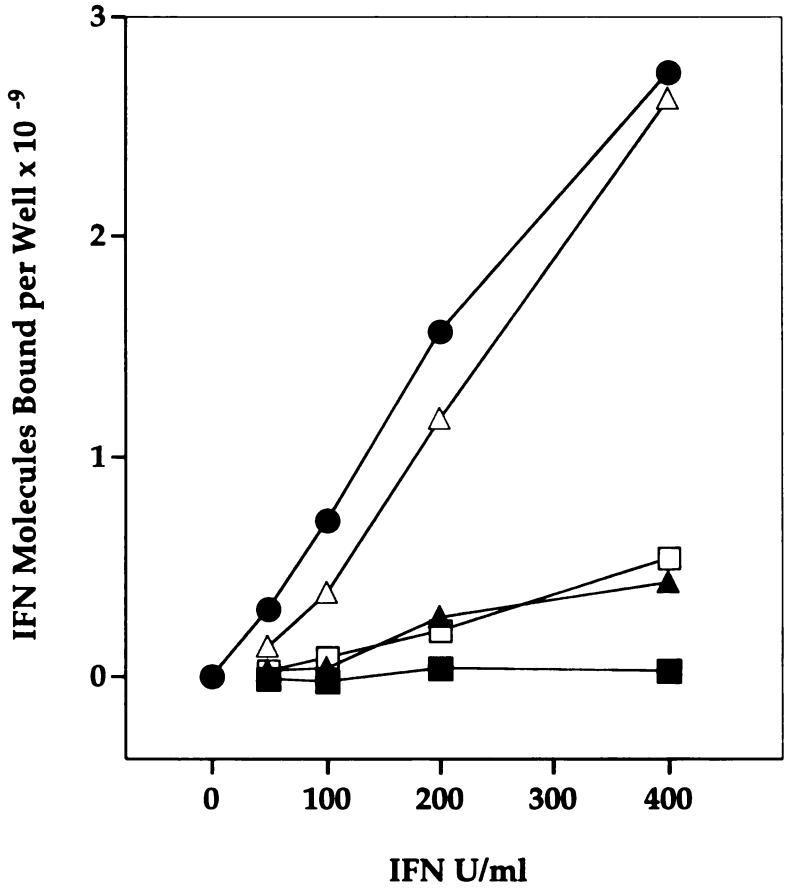


Figure 25. The binding of ^{125}I labeled IFN-γ to the extracellular domain of the human IFN-γ receptor. The receptor was immobilized on plastic wells and treated with 50, 100, 200 or 400 U/ml of labeled IFN-γ in the absence (—●—) of oligos or in the presence of 10 μM **I** (—■—), 1 μM **I** (—□—), 10 μM **II** (—▲—) or 1 μM **II** (—△—). After 1 hour on ice, the radioactivity in bound to the wells was measured in a gamma counter.

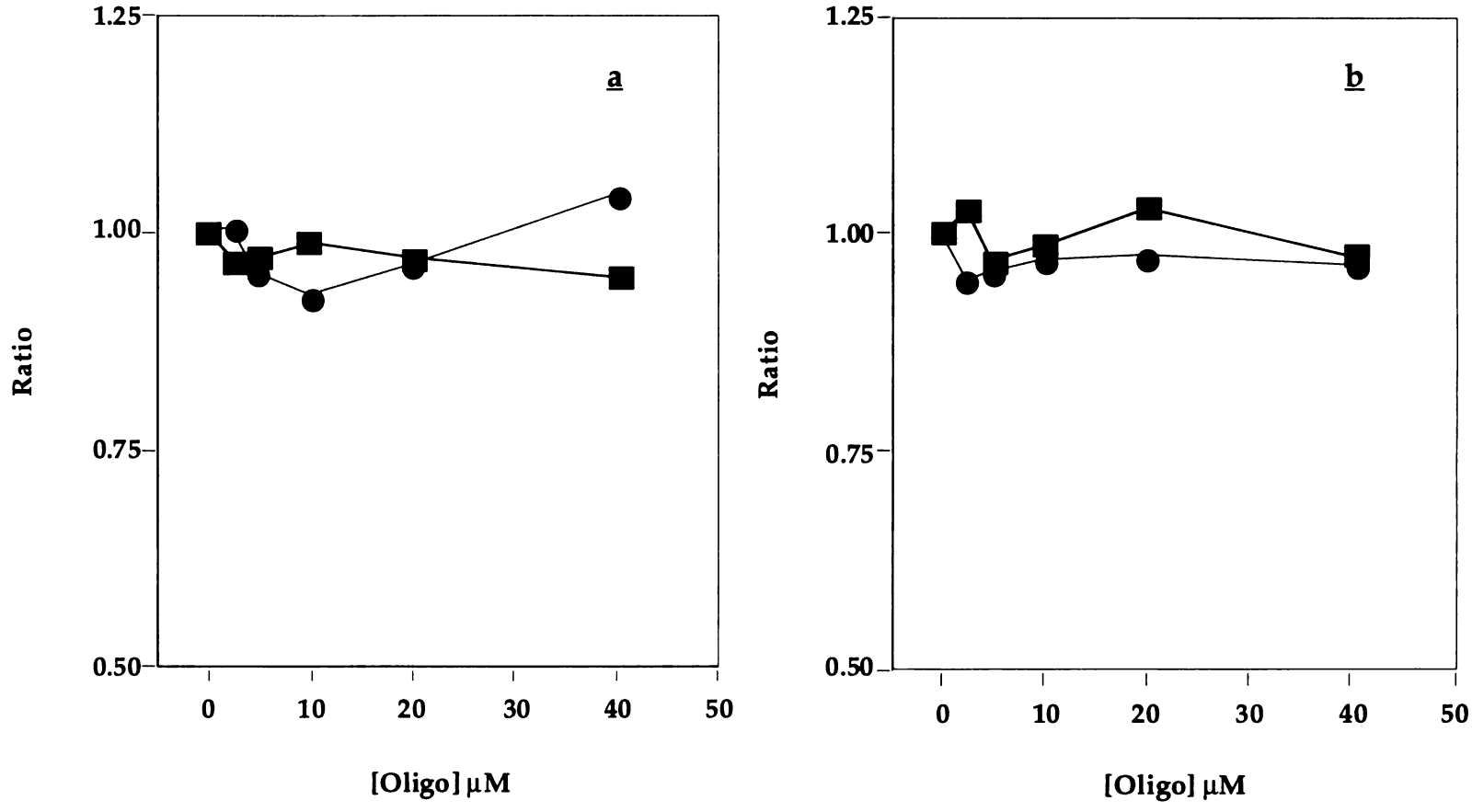


Figure 26. The effect of oligo treatment of the extracellular domain of the human IFN-γ receptor on subsequent binding of either a blocking (Figure 26a) or a nonblocking antibody (Figure 26b) to the human IFN-γ receptor. The extracellular domain was treated with either **I** (—■—) or **II** (—●—) and probed after washing with a blocking or a nonblocking antibody. The data are normalized to the absorbance observed in the absence of oligo.

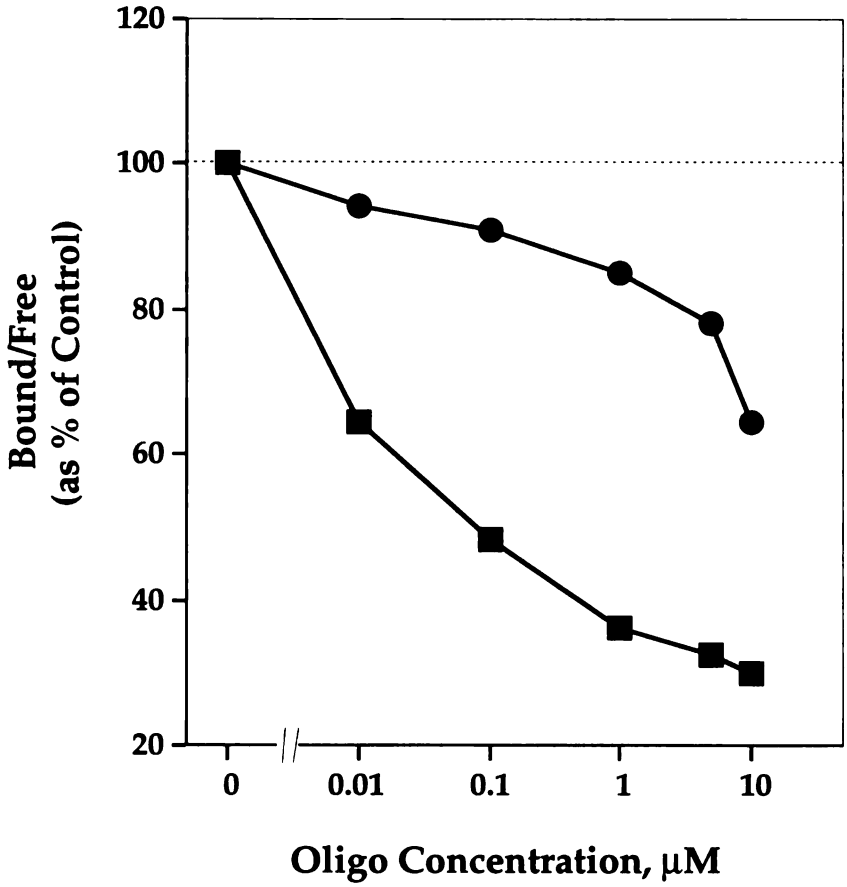


Figure 27. The dose dependence of the binding of ^{125}I labeled IFN-γ to immobilized anti-IFN-γ antibody in the presence of oligo **I** (—■—) and **II** (—●—) is shown. The data are expressed as a percentage of the bound radioactivity to free radioactivity in wells not treated with either oligo.

Oligo I Inhibits Binding of Labeled IFN-γ to the Purified IFN-γ Binding Subunit of the IFN-γ Receptor Protein

We then designed experiments to determine whether **I** was capable of inhibiting the binding of IFN-γ to the purified extracellular domain of the IFN-γ binding subunit of the human IFN-γ receptor *in vitro*. The extracellular domain lacks the hydrophobic transmembrane domain but retains excellent IFN-γ binding ability (Fountoulakis, Lahm et al. 1991). Unlike the native receptor which can aggregate in solution due to its hydrophobicity, the purified extracellular domain of the IFN-γ binding subunit is soluble in aqueous buffers. The results were used to determine whether the presence of the extracellular domain of the IFN-γ receptor alone was sufficient to confer susceptibilty to inhibition by **I**.

We studied the binding of labeled IFN-γ to its purified receptor which was immobilized on plastic. We found (Figure 25) that 10 μM of both **I** and **II** inhibited binding of labelled IFN-γ. At lower doses **I** was more potent than **II** at inhibiting the IFN-γ binding. This finding suggests that the reduced binding of IFN-γ to cells in presence of **I** is possibly a result of interference with binding to the receptor.

Oligo I does not Inhibit Binding of Antibodies to the Purified IFN-γ Binding Subunit of the IFN-γ Receptor Protein

Our earlier exeriments had allowed us to narrow the primary site of action of **I** to one of two proteins – IFN-γ or the extracellular domain of the IFN-γ receptor. To better identify the site of action of **I**, we immobilized purified extracellular domain of the IFN-γ binding subunit of the human IFN-γ receptor protein on plates and treated it with various concentrations of **I**. This was followed by treatment with one of two mouse antibodies

against the human IFN-γ receptor. The amount of antibody binding to the immobilized receptor was quantitated by using a goat anti-mouse IgG antibody conjugated to horse radish peroxidase. We used both a blocking antibody and a non-blocking antibody because we reasoned that this would allow us to narrow the site of **I** action to the neighborhood of the epitopes recognized by these antibodies. The results with **I** and **II** are summarized in Figure 26. The data are expressed as a fraction of the value obtained in the absence of any oligo. If the binding of **I** competed with or inhibited antibody binding to the immobilized IFN-γ receptor, then the epitope could be considered a likely candidate for the site of **I** action. However, over the concentration range examined we found that **I** did not inhibit binding of either antibody to the epitopes. This result suggests that **I** does not interact with these epitopes with sufficient affinity to compete with the antibodies. This experiment also confirms that the results obtained with the immobilized receptor are not artifacts caused by the **I** mediated detachment of the immobilized receptor from the plates.

*Oligo **I** Inhibits Binding of IFN-γ to an Anti-IFN-γ Antibody*

To determine whether **I** could inhibit binding of labeled IFN-γ to a ligand other than the IFN-γ receptor, we studied the effect of **I** on the interaction between labeled IFN-γ and an anti-IFN-γ antibody. As shown in Figure 26, **I** inhibits the binding of IFN-γ to the antibody in a dose-dependent manner. Oligo **II** is inactive except at the 10 μM concentration. Taken together with the results in Figures 21 and 22, this suggests that **I** acts by interacting with IFN-γ.

DISCUSSION

In this thesis, I have identified a class of oligodeoxynucleotides that specifically inhibits the IFN-γ induced up and down-regulation of cell surface proteins. In the Introduction, I outlined my research objectives which were: a] the characterization of the activity of IFN-γ-inhibitory oligos for therapeutic applications and, b] the determination of the mechanism and site of action.

Characterization Of The IFN-γ Inhibitory Activity

The dose response curves, the potency, the specificity and selectivity profiles and the kinetics results on the IFN-γ-inhibitory oligos were obtained to address the first of the two research goals. Oligo **I** is effective against a wide range of genes modulated by IFN-γ, and the induction of MHC Class I, ICAM-1, transferrin receptor, MHC Class II DR (Tam, Huey et al. 1994; Tam, Li et al. 1994) and the F_c receptor (Fedoseyeva, Li et al. 1994) proteins are inhibited. The results in this thesis clearly show that the activity is directed only against the effects of IFN-γ because: a] the levels of constitutively expressed proteins and, b] the expression of proteins induced by other cytokines (IFN-α, IFN-β or TNF-α) is not inhibited. The inhibitory effects of **I** are both specific (Figures 14, 15 and 16) and selective (Figure 16) for IFN-γ.

The activity of **I** is not unique because several of the oligos tested show IFN-γ inhibitory activity (Figure 13). This lack of uniqueness is not surprising because **I** acts as an aptamer, and the aptamers reported for thrombin (Bock, Griffin 1992) are also not unique. The thrombin aptamers contain a conserved 14-17 base pair consensus sequence. However, the only structural characteristics separating the most active from the less active, or inactive, IFN-γ inhibitory oligos is the high fraction of guanosine bases in **I** and **III**. Alignment of **I-VI** using a dynamic programming algorithm did not reveal

significant sequence similarity. To date, I have been unable to identify structure-activity relationships based on the potential for hairpins and inverted repeats that may facilitate the interaction of DNA with binding proteins. When the primate database in GenBank was searched with sequences **I-VI** as probes, I did not discover any common targets. As a result, the sequence-function, structure-function relationships and consensus sequence motif for the IFN-γ-inhibitory activity are not yet known.

The inability to identify a consensus motif for IFN-γ-inhibitory oligos may have been limited by the small number of active sequences for which information is available. Bock et al. (Bock, Griffin 1992) inferred the thrombin aptamer consensus motif, GGtTGGNNNGGtTGG, by sequencing over 32 aptamers. The thrombin aptamer motif contains several highly conserved bases, but they are separated by a number of highly variable bases. By analogy, the IFN-γ-inhibitory motif may be structurally similar, but this makes identifying a consensus motif from a limited number of sequences difficult. The site of action needs to be delineated before the screening and amplification approach used for the thrombin aptamers can be successfully applied for identifying the IFN-γ-inhibitory oligo consensus sequence.

An alternative hypothesis is that a conserved consensus sequence does not exist because the binding of IFN-γ-inhibitory oligos to the target site is driven entirely by the negative charge on the oligos via electrostatic interactions. This is a possibility because my mechanistic studies indicate that the **I** acts directly on IFN-γ, and IFN-γ is a basic, positively charged protein with a pI of 8.6-8.7 (Yip, Pang et al. 1981). Several basic residues in the carboxy-terminal end have been shown to be necessary for activity (Griggs, Jarpe et al. 1992). However, the likelihood that no consensus motif exists is low because oligos **II** and **VI** have much lower activity compared to oligos **I** and **III**, and

because thrombin, which is also basic, has aptamers that have a well defined consensus motif. The electrostatic interactions may be necessary but they are not sufficient for high affinity binding. From the practical standpoint, however, this 'non-uniqueness' is an advantage because it may allow the identification of more potent IFN-γ-inhibitory oligos with clinically useful therapeutic profiles.

The results of Figure 12, provide insight into the structural requirements for activity and show that the nucleic acid part of **I**, in the absence of the 3' amino modification, has IFN-γ inhibitory activity. However, the potency with unmodified **I** is greatly reduced, probably because the 3' modification increases the half-life in biological media as previously reported (Dagle, Weeks et al. 1991; Tidd and Warenius 1989; Wickstrom 1986). An interesting possibility, not explored in this thesis, is that both oligos have the same intrinsic inhibitory activity and that the differences in potency are entirely due to differences in oligo stability.

The most active of the oligos studied are **I** and **III** which have ED_{50} values in K562 cells in the micromolar range. All the oligos tested showed a correlation between the inhibition of MHC Class I and the inhibition of ICAM-1. I will argue later in this Discussion, that this is indicative of a common mechanism of action. The micromolar ED_{50} values for **I** compare favorably with the oligos and peptide IFN-γ antagonists reported by others. The oligo AS5 (Siegrist, Mach et al. 1993), reduces IFN-γ induction of MHC Class II DR expression in THP-1 cells to 23% of control levels at 10 μM, but not does not affect constitutive expression. The phosphorothioate oligos ISIS 1570 and ISIS 1939 (Chiang, Chan 1991), when used at 1 μM, reduce IFN-γ induction of ICAM-1 expression in A549 lung carcinoma cells to 38.9% and 18.3% of control levels, respectively. A peptide comprising the amino

terminal amino acids 1-39 of mouse IFN-γ inhibits the binding and antiviral activity of human IFN-γ with an ED_{50} of 500 μM (Jarpe, Johnson 1993). Synthetic peptide mimics (Seelig, Prosise et al. 1994) that juxtapose residues 22-29 and 12-19 from the amino terminus and residues 131-139 from the carboxy terminus of IFN-γ show improved potency and inhibit IFN-γ binding with ED_{50} values in the 15-50 μM range.

The results obtained with HeLa S3 cells are instructive. Oligo **I** inhibits MHC Class II DR and ICAM-1 induction, as well as the IFN-γ mediated down-regulation of the transferrin receptor. This is consistent with action of **I** as an IFN-γ antagonist because it demonstrates that **I** is capable of inhibiting not only the up-regulation, but also the down-regulation of specific IFN-γ regulated genes. However, given the effectiveness of **I** against IFN-γ induced MHC Class I in K562 cells, the absence of significant inhibitory effect on IFN-γ induced MHC Class I or β_2 microglobulin in HeLa S3 cells is unexpected. Because the IFN-γ doses used in both cell lines are the same, this result suggests that some of the effects of **I** are cell line dependent. Additionally, because inhibition of some but not all IFN-γ regulated genes is observed, all IFN- induced genes in HeLa S3 cells are not equally sensitive to inhibition by **I**. A plausible mechanism for the low responsiveness of MHC Class I to inhibition by **I** is that this marker is highly sensitive to IFN-γ. Because of this sensitivity, the few IFN-γ receptors that are occupied in the presence of **I** are capable of 100% MHC Class I induction. This hypothesis can be tested by obtaining dose-response curves for MHC Class over a range of IFN-γ and **I** doses. A low IFN-γ ED_{50} value is indicative of high IFN-γ sensitivity. An alternative hypothesis, clearly not parsimonious, is that the mechanism of action **I** in HeLa S3 cells is different from that in K562 cells.

Mechanistic Studies

Figure 28 is a working model with possible sites of action for **I**. In the remainder of this discussion, I will refer frequently to Figure 28 to interpret the data from a mechanistic perspective. The experimental results will be systematically evaluated in terms of the constraints each result imposes on the mechanism and site of action.

In Figures 6-8, the dose-response curves for three proteins (MHC Class I heavy chain, β_2 microglobulin and ICAM-1) in K562 cells at 3 times (24, 48 and 72 hr) show striking similarities. The simplest explanation is that a step common to, or necessary for, the induction of all three proteins is inhibited. This hypothesis accounts for the similarities in the dose response curves and the absence of gene selectivity. However, the absence of gene selectivity can be explained for, albeit less plausibly, by an alternative hypothesis. In the alternative hypothesis, the activity profile of **I** is the result of a specific effect on one gene, say MHC Class I, and a combination of nonspecific and fortuitous effects on the others. Such effects have been observed with antisense oligos (Herschlag 1991; Woolf, Melton et al. 1992). Specific effects differ from nonspecific effects in the mechanism or site of action, but fortuitous effects result from the same mechanisms as specific effects; e.g., with antisense oligos, fortuitous side effects can be a consequence of the short hybridization length required for RNAse H cleavage (Woolf, Melton et al. 1992). However, it is highly unusual for nonspecific and fortuitous effects to show the same dose dependence and kinetics as specific effects. Because the kinetics and dose response curves for MHC Class I and ICAM-1 are similar, I conclude that these results are consistent with the parsimonious working hypothesis: A common specific mechanism of action is involved.

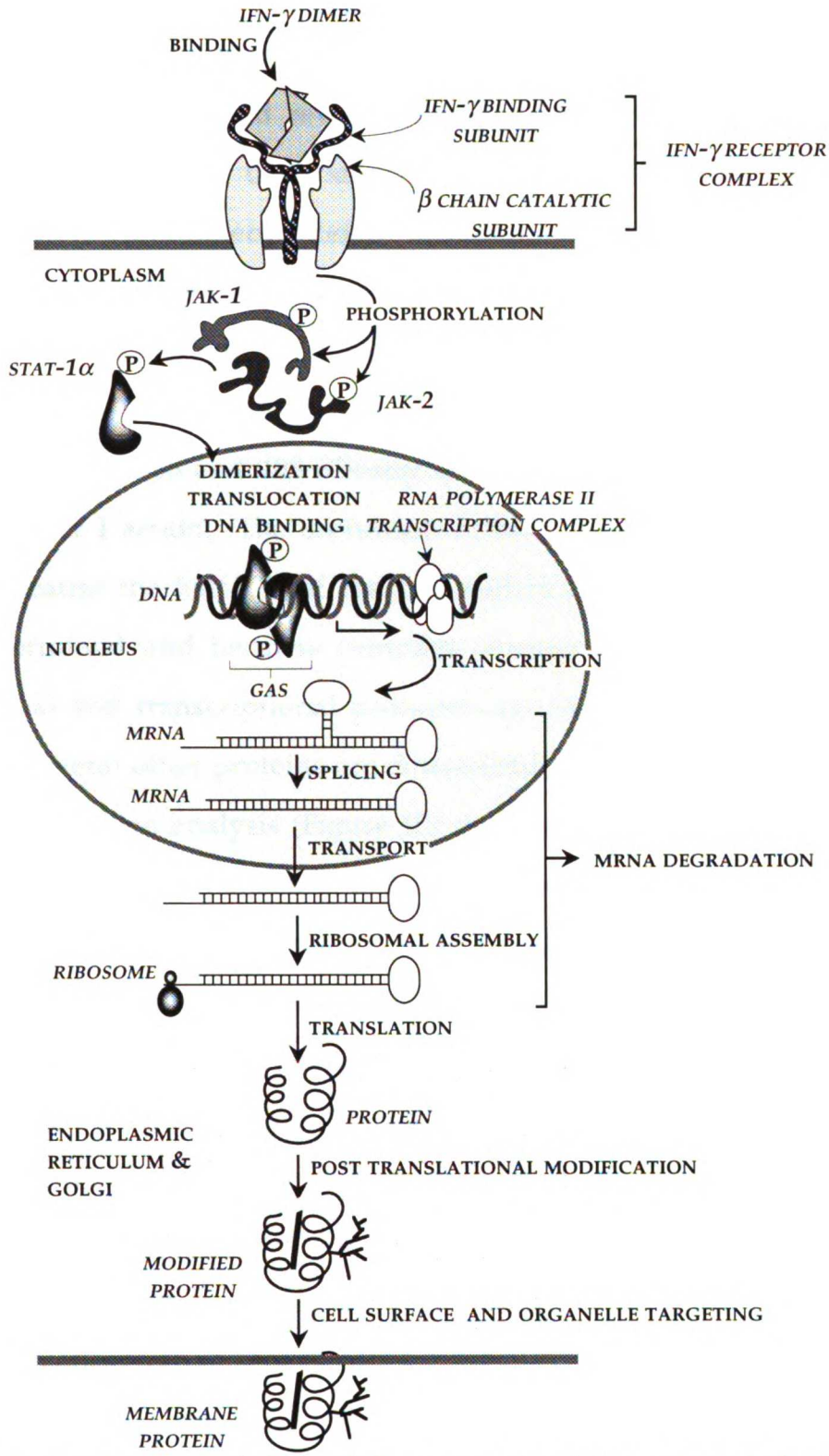


Figure 28. A schematic of the cellular processes that are initiated when IFN-γ binds to its cell surface receptor.

Oligos have been shown to have inhibitory effects on a variety of nucleic acid processing enzymes such as human DNA polymerase, RNAse H (Gao, Han et al. 1992) and reverse transcriptase (Stein, Cheng 1993). The results in Figure 10 show that **I** does not nonspecifically inhibit the enzymes, proteins and ribonucleoproteins involved in transcription, splicing, nucleocytoplasmic transport and translation. Post-translational processes in the endoplasmic reticulum and Golgi apparatus that are shared by all proteins are also not inhibited. In Figure 28, only RNA polymerase II transcriptional complex, and the 60S and 40S ribosomal subunits are depicted and these are not targets for **I** action. The usefulness of the data in Figure 10 is however, limited because the half life of the cell surface transferrin receptor has not been determined and because complete absence of nonspecific effects on translational and transcriptional processes can be established only after the effects on several other proteins are determined.

The Northern analysis (Figure 19) was carried out to develop a refined hypothesis for the mechanism and the site of action. This analysis provides information on whether **I** acts prior to, or upstream of IFN-γ induced mRNA accumulation and is necessary because an active oligo, ISIS 1570 (Chiang, Chan et al. 1991), targeted against the AUG codon of ICAM-1 mRNA inhibits IFN-γ induced ICAM-1 protein expression without inhibiting ICAM-1 mRNA accumulation. Chiang et al. concluded that ISIS 1570 acts post-transcriptionally at a step downstream of mRNA accumulation, possibly by translational arrest or at a post translational step.

At a concentration of 6.25 μM, **I** but not **II**, inhibits the enhancement of ICAM-1 and MHC Class I mRNA levels by IFN-γ and this pattern in Figure 19 is qualitatively consistent with the pattern observed in Figures 6-9 for cell surface protein expression. Therefore, **I** acts at a step at or prior to IFN-γ

induced mRNA accumulation. The ribosomal assembly, translation, post-translational modification and cell surface and organelle targeting steps in Figure 28 are now excluded as candidate sites of action. The observation that the mRNA accumulation of actin, a gene not induced by IFN-γ, is not inhibited by either **I** or **II**, provides additional support for the absence of nonspecific effects upstream of, and including mRNA accumulation.

The mRNA accumulation results do not exclude antisense or antigene effects as candidates because both mechanisms reduce mRNA levels (Uhlmann, Peymann 1990; Hélène, Toulmé 1990; Stein, Cheng 1993). However, these mechanisms provide other unique experimental signatures against which my results can be compared. The important experimental signatures of these mechanisms are summarized in Table III.

Table III. Experimental signatures of the antisense and antigene mechanisms.

EXPERIMENTAL SIGNATURE[1]	ANTISENSE	ANTIGENE
Reduction in mRNA levels?	Yes	Yes
Specific for targeted gene?	Yes	Yes
Decrease constitutive expression?	Yes	Yes[2]
Independent of inducing cytokine?[3]	Yes	No[4]

[1](Uhlmann, Peymann 1990; Hélène, Toulmé 1990; Stein, Cheng 1993).

[2]Oligo must be targeted to a site downstream of the constitutive promoter.

[3](Chiang, Chan 1991).

[4]Inhibition depends on relative locations of the target *vis-a-vis* the cytokine promoters.

Oligo **I** inhibits several genes, e.g. IFN-γ induced MHC Class I, MHC Class II, ICAM-1, F_c receptor, and transferrin receptor, and does not demonstrate the gene specificity that is characteristic of antigene and antisense effector mechanisms. Figure 11 is additional evidence that reduces the likelihood of the antisense and antigene mechanisms as a potential

mechanisms because **I** does not inhibit constitutive ICAM-1 expression in K562 cells over the dose range examined. The last criterion in Table III is addressed by the data in Figures 14 and 15 which show that **I** does not inhibit MHC Class I and ICAM-1 induction by cytokines other than IFN-γ. Again, **I** does exhibit the response characteristics expected of an antisense effector mechanism.

The specificity data in Figures 14 and 15, however, represent only single points on the dose-response surface. It does not eliminate possibility that **I** may be inhibitory at higher oligo to cytokine ratios. The experimental results in Figure 16 address this possibility. They strengthen the specificity profile argument and show that **I** does not inhibit TNF-α induced ICAM-1 expression over a 100-fold range of TNF-α concentration.

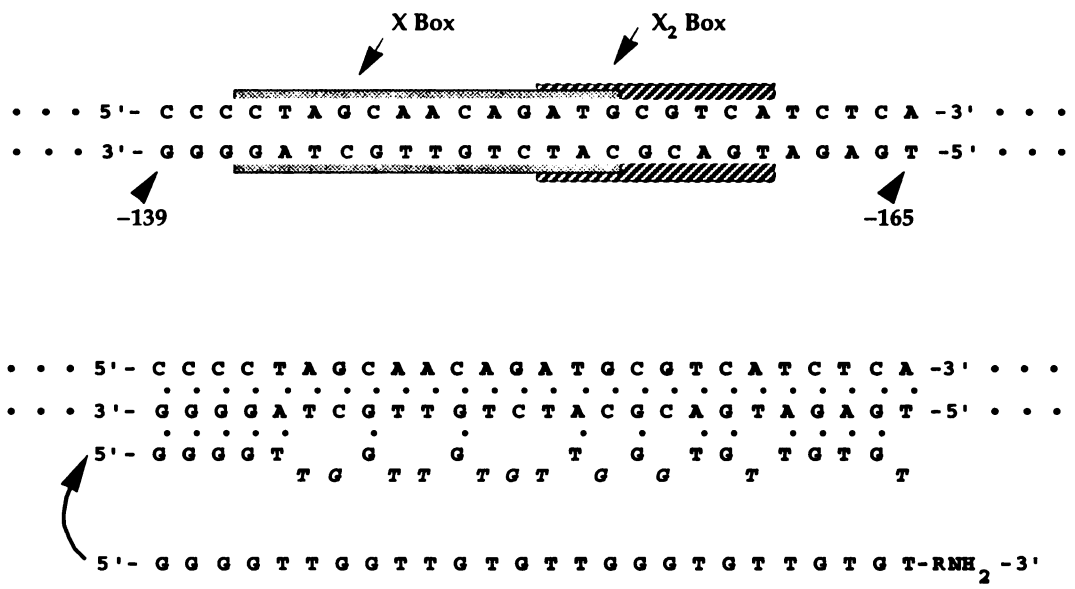
A possibility that has not been excluded is that **I** exerts its effects by antigene triple helix formation on DNA sequence elements common to all these genes. The Gamma Activated Sequence (GAS) consensus sequence TTNCNNNAA, is not particularly A or G rich, and in most genes (Table IV) only 4 or 5 out of nine bases can form a G•GC, T•AT triple helix. As an example, Figure 29 shows that **I** forms only 11 (out of a possible 26) Hoogsteen bonds with the X-X_2 box of the MHC Class II DRα. The longest triplex run is 5. Prakash and Kool (Prakash, Kool 1992) report on the effect of helix length on the triplex T_m and show that: a] an 8mer third strand yields a melting point of approximately 12°C and, b] a 13-15mer third strand is required for a T_m of 37°C. Thus, the formation of a triple helix is not likely to be sustained at 37°C over the immediate vicinity of the GAS by a 5-mer third strand. To cause gene inhibition in cells, a triple helix forming oligo must not only bind DNA but also compete successfully with DNA binding proteins for the promoter site. From a detailed thermodynamic study of triple helix formation, Roberts and

Crothers (Roberts, Crothers 1992) estimate that a RNA third strand 12-16 base long is required for obtaining the stability normally associated with protein-DNA complexes. Because DNA•(DNA-DNA) triple helices are about 11 kcal/mole less stable than the RNA•(DNA-RNA) triple helices and about 7 kcal/mole less stable than RNA•(DNA-DNA), the length requirements for DNA third strands is expected to be longer. I conclude that **I** does not meet the currently accepted criteria for triplex formation and for protein displacement at the GAS. Thus, in Figure 28, I have excluded all the steps downstream of (and including) Stat1α binding to the GAS as candidates for the mechanism of action.

In Figure 16, **I** not only inhibits IFN-γ induced MHC Class I, but also blocks the synergistic induction of MHC Class I by mixtures of IFN-γ and TNF-α. This shows that **I** acts at a step upstream of the TNF-α activated step that results in synergy. If **I** action occurred at a step downstream of the synergy junction, then the inhibition of IFN-γ by **I** could be overcome by high TNF-α concentrations.

Table IV. Sequences of the Gamma Activated Sites of various genes (from James E. Darnell Jr, Ian M. Kerr, George R. Stark, Science, 264, 1415-1421).

GENE	GAMMA ACTIVATED SITE
Guanylate binding protein	TTACTCTAA
Ly-6E	TTCCTGTAA
FcγR1	TTCCCAGAA
MIG	TTACTATAA
ICSBP	TTCCGAGAA
MHC Class II DRB1 J Element	CTTCATACAG
Consensus	TTNCNNNAA



Oligo I

Figure 29. The alignment of oligo **I** with its original design target, X-X_2 box of the MHC Class II DRα promoter.

The data in Figure 14 shows that **I** specifically inhibits MHC Class I induced by IFN-γ but has no effect on MHC Class I induced by IFN-α and IFN-β. Considerable evidence shows that IFN-γ shares signaling proteins (Schindler, Fu et al. 1992; Schindler, Shuai et al. 1992; Shuai, Schindler et al. 1992; Shuai, Stark et al. 1993) with IFN-α and IFN-β. The shared signaling proteins include Jak1, Stat-1α and Stat-1β. Figure 30 and Table V (adapted from Darnell, Kerr et al. 1994) demonstrate the extent of the overlap between the two pathways. Because **I** inhibits IFN-γ, but not IFN-α induced MHC Class I, the mechanism of action of **I** involves a site upstream of these common signaling elements. Thus, in Figure 28, the Jak1 and Stat-1α phosphorylation steps can be excluded as potential sites of action.

Figure 18 shows that the addition of **I** is kinetically similar to the rapid removal of external IFN-γ. Such data are consistent with, but not proof for, a

reduction in the receptor occupancy, which in turn can be the consequence of IFN-γ sequestration, receptor blockage or the displacement of receptor bound IFN-γ. The data in Figure 22 are also consistent with a site of action that is located at, or upstream of, the extracellular face of the cell membrane. The site of action is accessible to washing and the removal of the free and "washable" oligo results in the loss of inhibitory activity. If there are inhibitory amounts of internalized or membrane associated oligo after washing, then it either rapidly equilibrates with the extracellular oligo or it is inaccessible to the site of action. The latter is more likely given that the $t_{1/2}$ for loss of cell associated oligo (Stein, Tonkinson et al. 1993) is 3.7 hours. These results provide the rationale for hypothesizing that **I** inhibits binding of IFN-γ to its cell surface receptor.

Table V. Signaling proteins shared by the IFN-α, IFN-β and IFN-γ pathways (From James E. Darnell Jr, Ian M. Kerr, George R. Stark, Science, 264, 1415-1421). The complementation groups U1-U6 are defective in IFN-α responses and the complementation groups γ1 and γ2 are defective in IFN-γ responses.

COMPLEMENTATION GROUP	RESPONSE TO LIGAND			COMPLEMENTING PROTEIN
	IFN-α	IFN-β	IFN-γ	
U1	–	Partial	+	Tyk2
U2	±[1]	±	±	p48
U3	–	–	–	Stat1 (p91, p84)[2]
U4	–	–	–	Jak1
U5	–	–	+	?
U6	–	–	+	Stat2 (p113)
γ1	+	+	–	Jak2
γ2	+	+	–	?

[1]± indicates that some genes respond, but other genes do not.

[2]The older nomenclature is shown in parentheses.

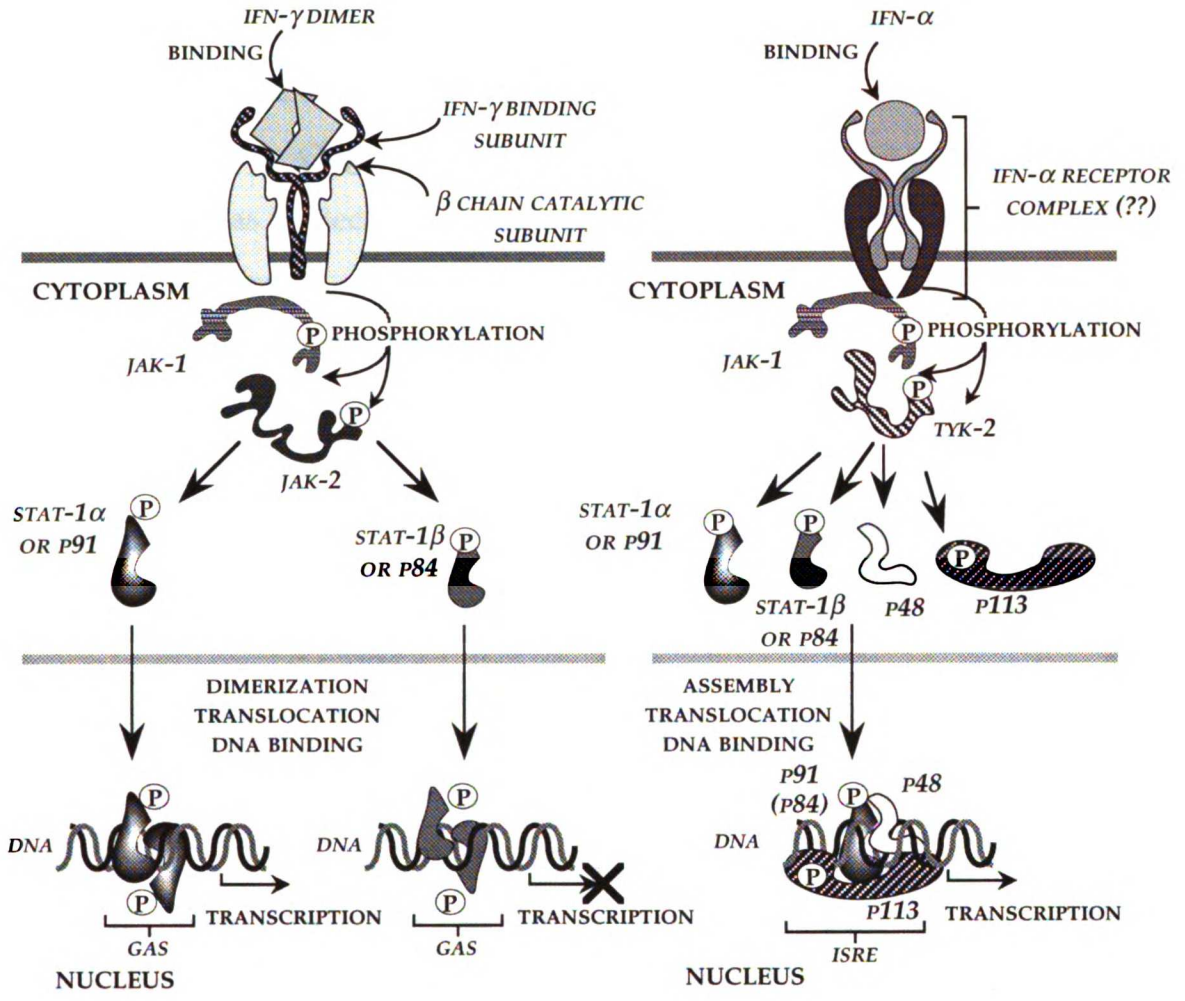


Figure 30. The signaling pathways for IFN-α and IFN-γ are depicted (from James E. Darnell Jr, Ian M. Kerr, George R. Stark, Science, 264, 1415-1421). The two pathways share several key proteins involved in signaling and transcriptional activation.

The binding experiments (Figure 23 and 24) were carried out on ice because over 95% of bound murine (Celada, Schreiber 1987) and human IFN-γ (Finbloom 1988) can be recovered from the plasma membrane under these conditions implying that ligand internalization, receptor recycling and IFN-γ degradation are minimized. Since the labeled IFN-γ is not irreversibly associated with the cells, the data can be interpreted in the using a simple reversible receptor-ligand binding model. The data show that I is an effective

inhibitor of cell surface IFN-γ binding. The ED_{50} values from the bioassay (Figure 6) and the ED_{50} value from Figure 24 are comparable at 4 μM and 2 μM, respectively. Oligo **II** also has inhibitory activity but is only about 0.03 fold as potent as **I**. Because **I** inhibits the cellular association of labeled IFN-γ, the site of action is either extracellular or at the extracellular face of the plasma membrane. The nonlinear nature of the Scatchard plot (Figure 23b) obtained in the absence of oligo shows that there are high affinity and low affinity sites for IFN-γ on the K562 cells. The low affinity sites may represent nonspecific binding. In Figure 23, the labeled IFN-γ binding to cells in the presence of 10 μM **I** is less than the labeled IFN-γ binding in the presence of a 100 fold excess cold IFN-γ. This decreased binding implies that **I** reduces specific and nonspecific IFN-γ binding because labeled IFN-γ binding in the presence of excess cold IFN-γ is a measure of nonspecific binding (Celada, Gray et al. 1984; Finbloom, Hoover et al. 1985).

If significant binding interactions do occur between the oligo or IFN-γ and the proteins in the media, the net effect is a reduction in the chemical potential of the individual interacting species i.e. a reduction in the free fraction of the binding species. The binding experiments were carried out in RPMI media supplemented with 10% FBSΔ in order to approximate the conditions used to obtain Figures 6-9. Serum or albumin supplemented media is used in all the IFN-γ binding studies I have been able to identify in the literature (Branca, Braglioni 1981; Celada, Gray et al. 1984; Anderson, Yip et al. 1983; Finbloom, Hoover et al. 1985; Celada, Schreiber 1987; Finbloom 1988). In our binding assays, only the cell bound IFN-γ is measured, and the measured "free concentration" really reflects the total concentration of serum bound, oligo associated and truly free IFN-γ. Because, K_d = [Free IFN-γ][Free Receptor]/[Receptor bound IFN-γ], this can result in an overestimate for the

true dissociation constant between IFN-γ and its receptor. The same argument is true for oligos. I did not measure either IFN-γ or oligo binding to media proteins. However, the dissociation constants for phosphorothioate binding to albumin and α_2-macroglobulin are approximately 400 μM and 5-10 μM, respectively and the binding constants for phosphodiester oligonucleotides is significantly lower (P. A. Cossum, personal communication). In my experiments, the true effectiveness of **I** is underestimated because the association of oligos with cell surface (Stein, Tonkinson et al. 1993) and serum proteins reduces the free concentration of oligo. Therefore, protein association does not qualitatively change the conclusions — **I** inhibits the binding of IFN-γ and acts at or prior to the extracellular face of the plasma membrane.

A working hypothesis consistent with the cell binding data is that **I** inhibits the interaction of IFN-γ with the IFN-γ binding subunit of the IFN-γ receptor complex. Empirical support for this hypothesis is presented in Figure 25 which shows that **I** inhibits the binding of labeled IFN-γ to the purified extracellular domain of the IFN-γ binding subunit of the human IFN-γ receptor complex. The binding characteristics are similar to those obtained using K562 cells, but there are differences. Notably, IFN-γ binding is abrogated in the presence of 10 μM **II** and the shapes of binding curves of **I** and **II** are virtually indistinguishable. However, at 1 μM, **I** is more active than **II**. The exact reasons for the differences in binding behavior however, are still unclear but may be a result of altered receptor configuration, mobility or nonspecific binding. The receptor configuration is relevant because the probable stoichiometry between IFN-γ dimers and the IFN-γ binding subunit of the human IFN-γ receptor is 1:2 (Fountoulakis, Zulauf 1993). The soluble receptor subunit may be unable to bind IFN-γ with the same affinity

(Fountoulakis, Juranville et al. 1990) and stoichiometry as the native receptor because of differences in disulfide linkages between the cysteines in the protein and due to destabilization of the dimer. Further, the immobilized soluble receptor subunit may expose receptor surfaces that are not normally exposed on cells to the labeled IFN-γ, and binding sites normally available in cells may be obscured by adsorption of the receptor to the plastic wells. Therefore, in the adsorbed receptor subunit, the relative ratio of high affinity binding sites to low affinity sites is likely to be decreased relative to the native receptor. The apparent increase in the effectiveness of **II** in the soluble receptor experiments may be a result of inhibitory effects on the binding of IFN-γ to the physiologically irrelevant low affinity sites. Because **I** inhibits the binding of IFN-γ to the immobilized receptor *in vitro*, the site of action (see Figure 28) is either on IFN-γ or at the extracellular domain of the IFN-γ binding subunit of the IFN-γ receptor complex.

The results of Figure 26 and 27, taken together, provide preliminary support to the hypothesis that **I** acts by interacting with, or binding to IFN-γ. Because **I** inhibits the binding of labeled IFN-γ to both the receptor and an anti-IFN-γ blocking antibody, **I** either binds to both proteins or acts on IFN-γ. The former appears less probable because the kinetic results of Figure 22 show that external free oligo is required for activity, and that cell associated oligo alone is insufficient. My results therefore, provide preliminary support for a site of action located on IFN-γ. Figure 26 is consistent with the absence of a high affinity interaction between **I** and the soluble subunit of the IFN-γ receptor. However, this result should be cautiously interpreted because the approach is indirect, using antibodies to detect binding. While a positive result in this assay is meaningful, this negative result is of significantly less value because bound **I** can dissociate during the washing steps, it can be

displaced by antibodies with higher affinity, and the antibodies may not recognize epitopes masked by **I**.

The competition experiments with denatured, inactive IFN-γ (Figure 20) provide evidence that the primary amino acid sequence of IFN-γ alone does not contain the requisite structural information to provide the putative binding site for **I**. This result is inconsistent with a simple electrostatic interaction between **I** and IFN-γ because the putative charge-charge interactions should occur even with denatured IFN-γ. The electrostatic interaction possibly occurs between the negatively charged **I** and the positively charged carboxy terminus of human IFN-γ. This interaction can plausibly occur in denatured IFN-γ because the crystal structure of IFN-γ (Ealick, Cook et al. 1991) shows weak electron density in this region that is indicative of high mobility and flexibility. However, the possibility that the primary structure was rendered inaccessible during heat denaturation by processes such as IFN-γ aggregation and entrapment in carrier protein cannot be ruled out. The results of Figure 21 show that the interaction between **I** and its putative receptor is reversible. This excludes irreversible or covalent modification of IFN-γ as a potential mechanism of action. Therefore, **I** does not act via mechanisms that involve denaturation or suicide inhibition of IFN-γ.

While the class of oligos I have discussed modulates the expression of immunologically important proteins through an effect(s) on IFN-γ binding, other workers report down-regulating these proteins using oligos that function by a different mechanism. Antisense oligos targeted to MHC Class I, β_2 microglobulin or ICAM-1 mRNA down-regulate their targets in the absence of IFN-γ (Chiang, Chan et al. 1991; Kanbe, Arita et al. 1992; Lichtenstein, Fady et al. 1992). Only Chiang et al. (Chiang, Chan et al. 1991) determine the mechanism of action. Both the active oligos, ISIS 1570 and ISIS

1939, act post-transcriptionally, but by distinct mechanisms. The activity of ISIS 1939 is RNAse H-mediated, but the site of action of ISIS 1570 is downstream of mRNA accumulation. An oligo, AS5, has been reported by Siegrist et al. (Siegrist, Mach et al. 1993) to have activities that closely resemble those observed with **I**. AS5 is antisense to RFX-1 and blocks IFN-γ inducible MHC Class II but not IFN-γ inducible MHC Class I or constitutive MHC Class II in THP-1 cells. Constitutive MHC Class II expression in B cells is also not inhibited. Siegrist et al. discuss plausible mechanisms by which AS5 could alter IFN-γ induced, but not constitutive MHC Class II expression and conclude that an antisense effector mechanism is responsible.

The biological effect of **I** arises from a mechanism that is clearly distinct from any of the above oligos. Oligo **I** acts as an aptamer to inhibit IFN-γ binding. Interestingly, Chiang et al. (Chiang, Chan et al. 1991) report that IFN-γ induced ICAM-1 mRNA is more sensitive to down-regulation by a phosphorothiate oligo than either IL-1β or TNF-α induced ICAM-1 mRNA. This result allows one to speculate that the oligos used by Chiang et al. may have some aptameric anti-IFN-γ activity as well as antisense activity. Both oligos were shown to act via a post-transcriptional inhibitory mechanisms and the activity was shown to be independent of the cytokine used for induction.

Oligos are uniquely interesting because both the antisense and the aptamer approaches can be used to regulate the IFN-γ induced expression of the MHC and ICAM-1 proteins. They provide strategies for modulating both the constitutive expression of immunologically important proteins and their up-regulation by cytokines such as IFN-γ. The simultaneous use of multiple oligonucleotide strategies may be uniquely beneficial in a variety of clinical situations.

In conclusion, we have identified oligodeoxynucleotides that specifically inhibit the effects of IFN-γ in a variety of cell lines by acting as aptamers at an unexpected step. The mechanism of action of **I** involves inhibition of the IFN-γ binding but the exact site of action is yet undetermined. The results are most consistent with a site of action located on IFN-γ but the possibility that **I** acts by binding the IFN-γ receptor is not formally eliminated. The activities of oligos **I** plus **III-V** can have potential applications in transplantation, and in the treatment of inflammation, autoimmune disease and other situations where IFN-γ contributes to the underlying pathology.

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SECTION II

Predictions of Effect for Intracellular Antisense Oligodeoxyribonucleotides

ABSTRACT

We have analyzed the implications of a simple two compartment mathematical model (Hargrove, Hulsey et al. 1990; Hargrove and Schmidt 1989) to anticipate the effects of antisense oligodeoxyribonucleotide action within single cells. Steady-state and nonsteady-state solutions for mRNA and protein levels are obtained for constant levels of antisense oligonucleotide.

The steady-state equations are derived for four special cases representing the following mechanisms: 1) ribosome blockage, 2) mRNA cleavage by RNase H, 3) concurrent ribosome exclusion and RNase H action, and 4) decreased delivery of mature mRNA to the cytoplasm due to transcriptional blockage, interference with nucleocytoplasmic transport or splicing. Our results indicate that frequently translated mRNA producing stable proteins are the most attractive antisense targets because the protein levels are sensitive to the changes in the mRNA levels that can be effected using antisense oligonucleotides. Dose-response relationships have been derived for the four mechanisms action under ideal conditions.

The nonsteady-state solutions show that both mRNA and protein half-life can determine the kinetics of antisense oligonucleotide action. A rapid onset of antisense effect will be observed in systems in which the mRNA is rapidly degraded and slowly translated and the translated protein is rapidly degraded. With a slowly degraded protein the kinetics of an antisense effect are limited by protein half-life. When the translational rate constant is large compared to the absolute difference between the mRNA and protein degradation rate constants, the kinetics of antisense action is determined by both degradation rate constants but is limited by the slower of the two degradative processes.

We also show that the steady state and nonsteady-state solutions may be used to design experiments that discriminate among mechanisms of antisense action.

INTRODUCTION

Considerable experimental data is now available describing antisense oligonucleotide (oligo) activity in models of viral infection (Ts'o 1992) and cancer (Calabretta 1991; Wickstrom 1992). Theoretical approaches to antisense oligo therapeutics have been largely restricted to identifying optimal sites on target mRNA using RNA secondary structure prediction algorithms. These approaches compute mRNA secondary structure from sequence and base pairing free energy data and identify regions that are more likely to be single stranded and therefore more accessible to oligos. The identification of single stranded regions provides a framework for the rational design of antisense oligos (Stull, Taylor et al. 1992; Wickstrom, Bacon et al. 1988; Wickstrom, Wickstrom et al. 1987). The computations use base pairing free energies estimated from melting point (T_m) experiments with oligonucleotides and cannot take into account the intracellular environment in which the mRNA exists.

In this paper, we examine factors within the intracellular milieu that can impact oligo action. We employ a mathematical model of the protein synthesis response in cells treated with antisense oligos. The approach is based on interpretations of a dual compartment model presented by Hargrove and Schmidt (Hargrove and Schmidt 1989) for mRNA-protein kinetics. We intentionally use parameters that can be experimentally measured.

Several questions are addressed using this approach. Are some target mRNAs more suited to the antisense treatment than others? Do functional signatures exist that discriminate among the various mechanisms proposed for oligo action? The results demonstrate that suitable mRNA targets can be identified using the Hargrove and Schmidt model. Further, we show that the model can be used to design experiments capable of discriminating among

various proposed mechanisms for oligo action; e.g. between the RNase H and translational blockade mechanisms. A preliminary assessment of feasibility, dose and response time can also be made. Such a model, despite its simplisitic representation of a complex physiological process, can be expected to complement a target identification algorithm by taking intracellular environmental factors into account.

MATERIALS AND METHODS

The Model

The model used by Hargrove and Schmidt (Hargrove and Schmidt 1989) is shown in Figure 1. It is a two compartment model in which a single mRNA compartment is translated to protein. The mRNA and protein are assumed to degrade independently with first order rate constants k_{dm} and k_{dp}. The model does not explicitly incorporate the presence of an oligo molecule; however, the equations are valid at constant oligo levels.

The pertinent intracellular processes and the biological basis for the mathematical treatment are shown in Figure 2.

The differential equations describing this system are

$$\frac{dM}{dt} = T_0 - k_{dm} M \tag{1}$$

$$\frac{dP}{dt} = k_{tm} M - k_{dp} P \tag{2}$$

The steady-state mRNA and protein levels, M_{ss} and P_{ss} respectively, are:

$$M_{ss} = \frac{T_0}{k_{dm}} \tag{3}$$

$$P_{ss} = \frac{k_{tm} M_{ss}}{k_{dp}} = \frac{k_{tm} T_0}{k_{dp} k_{dm}} \tag{4}$$

The degradation rate constants are inversely proportional to the half lives of mRNA ($t_{\frac{1}{2}\,dm}$) and protein ($t_{\frac{1}{2}\,dp}$) and:

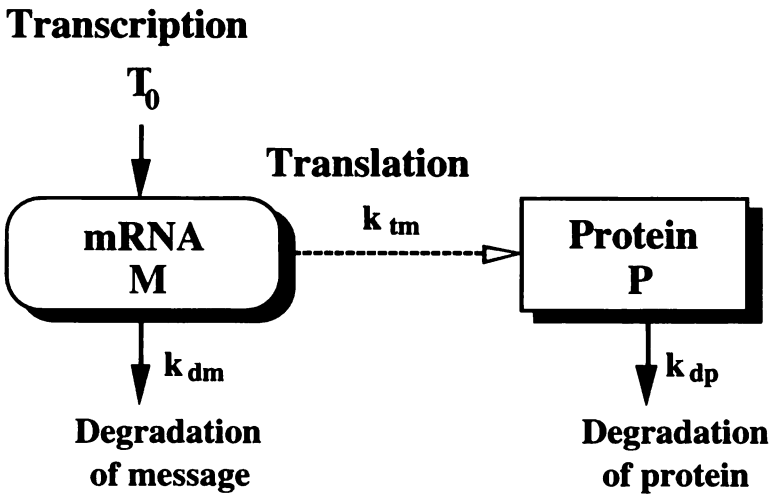

Figure 1. A schematic representation of the model (Hargrove, Hulsey et al. 1990; Hargrove and Schmidt 1989) used for analyzing the effect of steady oligo levels on cells. The two well mixed compartments are the pool of target protein (**P**) and its messenger RNA (**mRNA**), the chosen antisense target. Symbols are explained in Nomenclature. The apparent first order rate constants for each of the processes are shown. The solid arrows represent the direction of actual material flows from the compartment of origin. The dashed arrow represents the direction of information flow impacting material flow.

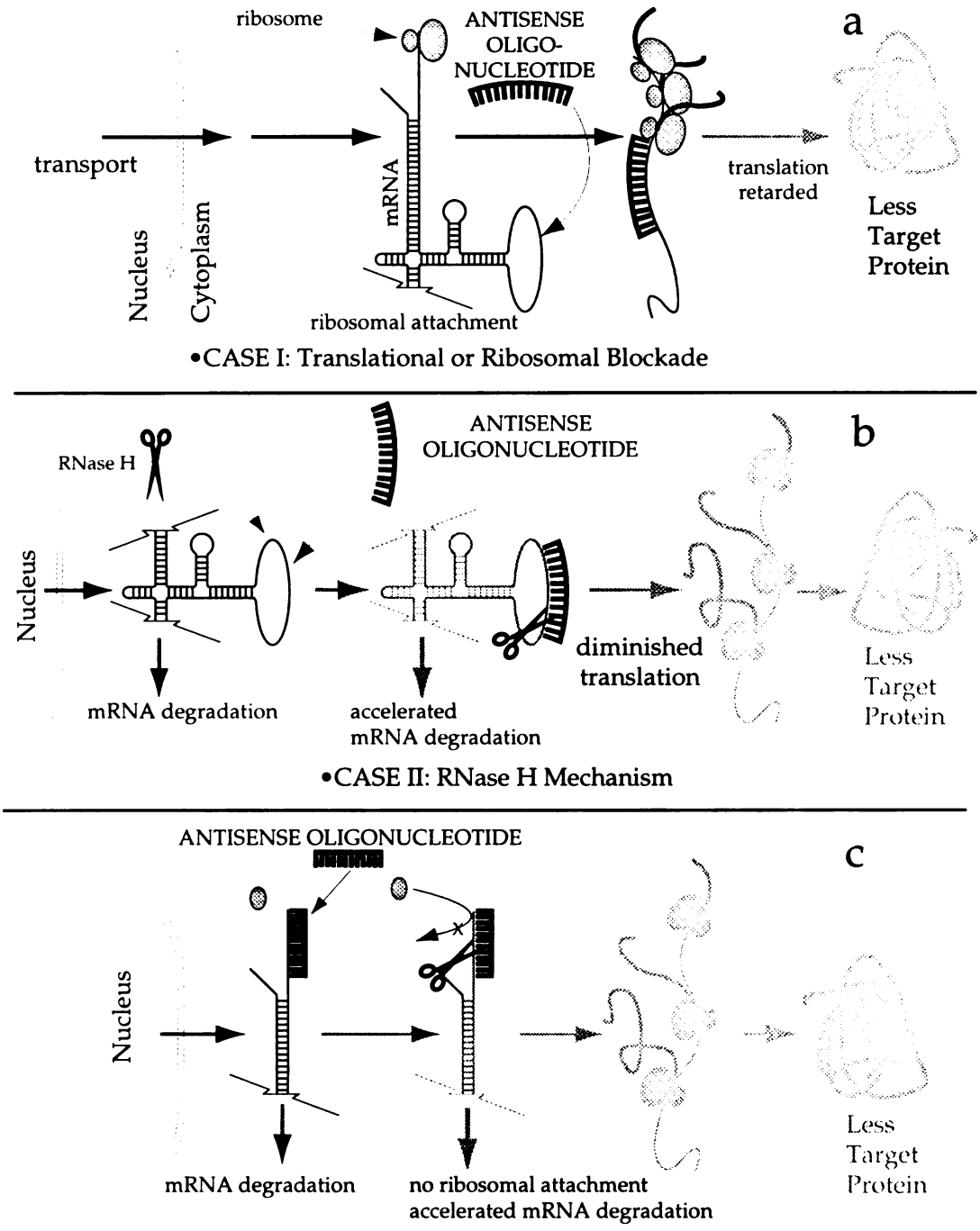

Figure 2. A schematic showing mechanisms considered important for antisense oligodeoxyribonucleotide action. **a**) The translational blockage mechanism, Case I. **b**) The RNase H mechanism, Case II. **c**) The RNase H and ribosomal competition mechanism, Case III.

$$k_{dm} = \frac{\ln 2}{t_{\frac{1}{2}\,dm}} \text{ and } k_{dp} = \frac{\ln 2}{t_{\frac{1}{2}\,dp}}$$

These half-lives can be measured experimentally and used to determine the rate constants k_{dm} and k_{dp}. Equations 3 and 4 are rewritten in terms of these experimentally measurable parameters as:

$$M_{ss} = \frac{T_0\, t_{\frac{1}{2}\,dm}}{\ln 2} \tag{5}$$

$$P_{ss} = \frac{k_{tm}\, T_0\, t_{\frac{1}{2}\,dm}\, t_{\frac{1}{2}\,dp}}{(\ln 2)^2} \tag{6}$$

The ratio $\frac{P_{ss}}{M_{ss}}$ is defined as the steady-state amplification (A_{ss}) and can be derived from Equations 3 and 4.

$$A_{ss} = \frac{P_{ss}}{M_{ss}} = \frac{k_{tm}\, t_{\frac{1}{2}\,dp}}{\ln 2} \tag{7}$$

A_{ss} is a measure of the mRNA to protein conversion efficiency and represents the average number of protein molecules translated per mRNA. For antisense effectiveness, a high value of A_{ss} is desirable; i.e. when A_{ss} is high, the protein will be sensitive to a modest reduction in its mRNA.

The nonsteady-state solutions for Equations 1 and 2 when T_0 is a constant are:

$$M(t) = M_0\, e^{-k_{dm}t} + M_{ss}\,(1 - e^{-k_{dm}t}) \tag{8}$$

$$P(t) = P_{ss} + (P_0 - P_{ss})\, e^{-k_{dp}t} + \frac{k_{tm}}{(k_{dm} - k_{dp})} \cdot (M_0 - M_{ss})\,(e^{-k_{dp}t} - e^{-k_{dm}t}) \qquad (9a)$$

When the rate constants of degradation are equal and, $k_{dm} = k_{dp} = k$ the nonsteady-state solution is:

$$P(t) = P_{ss} + (P_0 - P_{ss})\, e^{-kt} + k_{tm}\,(M_0 - M_{ss})\,(t\, e^{-kt}) \qquad (9b)$$

RESULTS

The Steady State Solutions

The parameters of the two compartment Hargrove and Schmidt model are responsive to the mechanism of antisense oligo action. In translational arrest or ribosome blockage, the mRNA-oligo hybrid slows the progress of mRNA translation on the ribosome resulting in decreased k_{tm}. The enzyme Ribonuclease H (RNase H), on the other hand, contributes to oligo activity by degrading the RNA in mRNA-oligo hybrids, thus causing an increase in k_{dm}. Antigene approaches and oligos directed toward regulatory and transcription factors will impact protein production by changing T_0, the rate of delivery of mature mRNA to the cytoplasm. Approaches that impede nucleocytoplasmic transport or accelerate nuclear degradation of transcripts also reduce the effective value of T_0.

Equation 6 shows that P_{ss} and $[k_{tm}\, T_0\, t_{\frac{1}{2}\,dm}\, t_{\frac{1}{2}\,dp}]$ are equivalent measures of the 'size' of a system. Protein downregulation to a given absolute steady-state level is likely to require a higher steady-state level of oligonucleotide in a system with a large initial value of $[k_{tm}\, T_0\, t_{\frac{1}{2}\,dm}\, t_{\frac{1}{2}\,dp}]$. The derivation of Equation 8 indicates that complete elimination of the protein is possible by blocking either transcription ($T_0 \rightarrow 0$) or translation ($k_{tm} \rightarrow 0$), or by instantaneous degradation of either the mRNA ($t_{\frac{1}{2}\,dm} \rightarrow 0$, $k_{dm} \rightarrow \infty$) or protein ($t_{\frac{1}{2}\,dp} \rightarrow 0$, $k_{dp} \rightarrow \infty$).

How does one select between protein targets in a given therapeutic situation? Because the effect of antisense oligos is usually mediated through mRNA, amplification can be used to select between target proteins. Amplification is equivalent to measuring the slope of a 'dose response' curve. The potential impact of an oligo on a given target can be evaluated because proteins that are sensitive to their respective mRNA levels will have

high values of A_{ss}. Proteins with high values of A_{ss} are more attractive as antisense targets because they are more sensitive to changes in mRNA levels.

Effect of Antisense Oligonucleotide

Equations 5, 6 and 7 can be compared with and without oligo treatment and the ratios shown in Equations 10a- c can be calculated.

$$\frac{[M_{ss}]_{treatment}}{[M_{ss}]_{control}} = \frac{[T_0\ t_{\frac{1}{2}dm}]_{treatment}}{[T_0\ t_{\frac{1}{2}dm}]_{control}} \qquad (10a)$$

$$\frac{[P_{ss}]_{treatment}}{[P_{ss}]_{control}} = \frac{[k_{tm}\ T_0\ t_{\frac{1}{2}dm}]_{treatment}}{[k_{tm}\ T_0\ t_{\frac{1}{2}dm}]_{control}} \qquad (10b)$$

$$\frac{[A_{ss}]_{treatment}}{[A_{ss}]_{control}} = \frac{[k_{tm}]_{treatment}}{[k_{tm}]_{control}} \qquad (10c)$$

Equations 10a-c follow directly from Equations 5-7 if we assume that $t_{\frac{1}{2}dp}$ is the same with and without oligo. This is a reasonable assumption because the known protein stability determining pathways are not sensitive to oligo levels.

We have derived steady-state equations for four special cases that represent the mechanisms of: 1) ribosome blockage, 2) mRNA cleavage by RNase H, 3) concurrent ribosome exclusion and RNase H cleavage, and 4) transcriptional blockage or interference with nucleocytoplasmic transport or splicing.

Case I: When the mechanism of oligo action is predominantly ribosomal blockade, as illustrated in Figure 2a, only k_{tm} will be affected.

$$\frac{[M_{ss}]_{treatment}}{[M_{ss}]_{control}} = 1 \qquad (11a)$$

$$\frac{[P_{ss}]_{treatment}}{[P_{ss}]_{control}} = \frac{[A_{ss}]_{treatment}}{[A_{ss}]_{control}} = \frac{[k_{tm}]_{treatment}}{[k_{tm}]_{control}} \qquad (11b)$$

Case II: When oligo action is mediated predominantly through the RNase H mechanism, as depicted in Figure 2b, the only parameter that changes is $t_{\frac{1}{2}\,dm}$, and:

$$\frac{[M_{ss}]_{treatment}}{[M_{ss}]_{control}} = \frac{[P_{ss}]_{treatment}}{[P_{ss}]_{control}} = \frac{[t_{\frac{1}{2}}\,dm]_{treatment}}{[t_{\frac{1}{2}}\,dm]_{control}} \qquad (12a)$$

$$\frac{[A_{ss}]_{treatment}}{[A_{ss}]_{control}} = 1 \qquad (12b)$$

Case III: A predominantly RNase H mechanism can alter both $t_{\frac{1}{2}\,dm}$ and k_{tm} when an antisense molecule is directed to the 5' untranslated region of a mRNA so that it can compete with ribosomal binding, as portrayed in Figure 2c. In this case T_0 and $t_{\frac{1}{2}\,dp}$ are assumed to be constant and the effect of a constant level of antisense oligo is given by:

$$\frac{[M_{ss}]_{treatment}}{[M_{ss}]_{control}} = \frac{[t_{\frac{1}{2}}\,dm]_{treatment}}{[t_{\frac{1}{2}}\,dm]_{control}} \qquad (13a)$$

$$\frac{[P_{ss}]_{treatment}}{[P_{ss}]_{control}} = \frac{[k_{tm}\,t_{\frac{1}{2}}\,dm]_{treatment}}{[k_{tm}\,t_{\frac{1}{2}}\,dm]_{control}} \qquad (13b)$$

$$\frac{[A_{ss}]_{treatment}}{[A_{ss}]_{control}} = \frac{[k_{tm}]_{treatment}}{[k_{tm}]_{control}} \qquad (13c)$$

Case IV: The primary effect of an oligo acting in the nucleus at the level of transcription or nucleocytoplasmic transport or splicing, is to reduce the value of T_0.

$$\frac{[M_{ss}]_{treatment}}{[M_{ss}]_{control}} = \frac{[P_{ss}]_{treatment}}{[P_{ss}]_{control}} = \frac{[T_0]_{treatment}}{[T_0]_{control}} \quad (14a)$$

$$\frac{[A_{ss}]_{treatment}}{[A_{ss}]_{control}} = 1 \quad (14b)$$

These four cases are summarized in the upper part of Table 1. Note that a system acting only through the RNase H mechanism (Case II) will have the same amplification ratio with and without oligo. Thus, the $\frac{[A_{ss}]_{treatment}}{[A_{ss}]_{control}}$ ratio can be used to discriminate between the ribosome blockade mechanism and a RNase H mechanism. In the former case, this ratio will, in general, be a function of oligo concentration, while in the latter case it will be approximately unity at all oligo concentrations. The $\frac{[A_{ss}]_{treatment}}{[A_{ss}]_{control}}$ ratio cannot, however, discriminate between a RNase H-based mechanism and the transcription blockade mechanism. A parameter to discriminate between these two mechanisms is discussed in Results under *The Nonsteady-State Solutions*. The ribosome blockage mechanism leaves the mRNA ratios unchanged and $\frac{[M_{ss}]_{treatment}}{[M_{ss}]_{control}}$ is unity.

Towards an Idealized Dose Response Curve

A series of restrictive assumptions allows us to derive idealized expressions for the dose response curve for each mechanism. In the following sections, dose is expressed in terms of f, the fraction of the mRNA

involved in hybrid formation. At steady-state this fraction is expected to be related to the extracellular oligo concentration. The fractional inhibition of protein synthesis ($1 - \frac{[P_{ss}]_{treatment}}{[P_{ss}]_{control}}$) can be used as a measure of therapeutic effect or response to antisense oligo administration. The exact relationship between f and the intracellular oligo concentration will depend on variables such as the free energy of hybrid formation and temperature. Other variables such as the T_m, the likelihood of the binding site being involved in secondary structure, the ionic environment and subcellular oligo distribution also contribute to f.

For Case I, where the mechanism is primarily one of blocking ribosomal progression at a site unlikely to influence ribosome assembly on mRNA. Such mRNA-oligo hybrids may slow the progress of translation and thus the fraction of mRNA with bound oligo has a decreased k_{tm}. If the translational rate constant for mRNA-oligo hybrids is $[k_{tm}]_{hybrid}$, then:

$$[k_{tm}]_{treatment} = [k_{tm}]_{control}(1 - f) + f[k_{tm}]_{hybrid}$$
$$= [k_{tm}]_{control}(1 - f[1 - \rho]) \quad (15a)$$

where $\rho = \frac{[k_{tm}]_{hybrid}}{[k_{tm}]_{control}}$ is expected to be positive and less than 1.

$$\frac{[M_{ss}]_{treatment}}{[M_{ss}]_{control}} = 1 \quad (15b)$$

$$\frac{[P_{ss}]_{treatment}}{[P_{ss}]_{control}} = \frac{[A_{ss}]_{treatment}}{[A_{ss}]_{control}} = (1 - f[1 - \rho]) \quad (15c)$$

Therefore, for Case I Effect = $f[1 - \rho]$ (15d)

For Case II, we assume that antisense oligos act via accelerated mRNA degradation in systems with high RNase H activity. Further, we assume that

the changes in mRNA degradation rate are linear in hybrid concentration, [H]. If oligos do not affect the cellular nucleases responsible for mRNA degradation, then:

$$\frac{dM}{dt} = T_0 - [k_{dm}]_{control}\, M - [k_{dm}]_{RNaseH}\, H$$

$$= T_0 - [k_{dm}]_{control}\, M\,(1 + rf) \qquad (16a)$$

$$[k_{dm}]_{treatment} = [k_{dm}]_{control}\,(1 + r\,f) \qquad (16b)$$

where $r = \frac{[k_{dm}]_{RNaseH}}{[k_{dm}]_{control}}$ and in general $r > 0$. The fraction $\frac{H}{M}$ is approximated by f, the fraction of the mRNA that is in a mRNA-oligo hybrid. In deriving Equations 16a and 16b we have assumed that RNase H and the cellular nucleases act independently, i.e., do not act on the same mRNA, in order to arrive at an upper limit for antisense effect. Thus:

$$\frac{[M_{ss}]_{treatment}}{[M_{ss}]_{control}} = \frac{[P_{ss}]_{treatment}}{[P_{ss}]_{control}} = \frac{1}{(1 + rf)} \qquad (16c)$$

$$\frac{[A_{ss}]_{treatment}}{[A_{ss}]_{control}} = 1 \qquad (16d)$$

$$\text{So, for Case II effect} = \frac{r\,f}{1 + rf} \qquad (16e)$$

Effect for Case II is plotted against log f in Figure 3 at three levels of r. The maximum amount of hybrid that can form is $[M_{ss}]_{control}$ for which f = 1. The upper limit for the antisense response is then

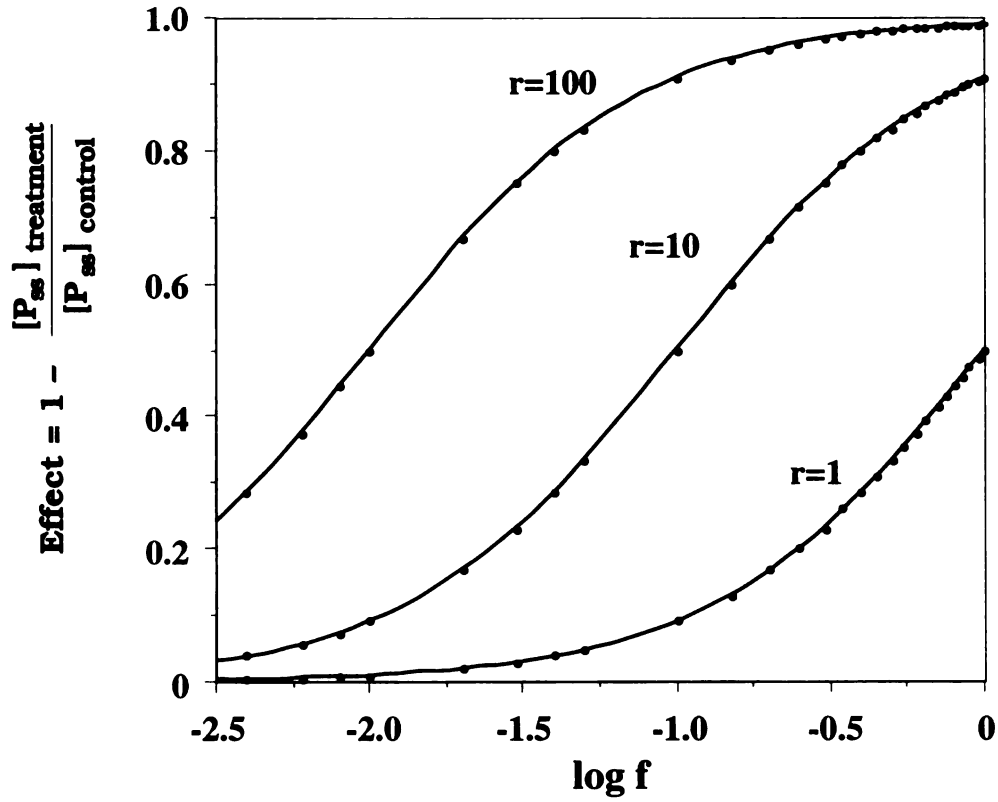

Figure 3. Plot of effect vs. log f at different values of r for Case II. The fraction of the target mRNA that exists as a mRNA-oligo hybrid is f and r = $\frac{[k_{dm}]_{RNaseH}}{[k_{dm}]_{control}}$ is the ratio of the rate constants for oligo-induced RNase H degradation of target mRNA to its degradation rate constant under control conditions. Antisense activity in the system is assumed to be due only to Case II, *RNase H action* on hybrids.

$$\left(\frac{[M_{ss}]_{treatment}}{[M_{ss}]_{control}}\right)_{max} = \left(\frac{[P_{ss}]_{treatment}}{[P_{ss}]_{control}}\right)_{max} = \frac{1}{1+r} \qquad (16f)$$

$$\text{So, for Case II } \quad effect_{max} = \frac{r}{1+r} \qquad (16g)$$

Equation 16g clearly indicates that a significant steady-state antisense effect may not be observed if $r << 1$, even when the oligo reaches and binds to its target effectively. Such low activity can be expected under conditions where RNase H activity is low, or when the mRNA is relatively unstable (i.e. when $[k_{dm}]_{control}$ is large). Equations 16a - 16g show that r, the RNase H activity *relative* to the mRNA degradation rate constant, is a more effective predictor of steady-state oligo activity than is absolute RNase H activity. Thus, a single cell type with a given RNase H activity can exhibit different levels of inhibition (by differently targeted antisense oligos) and these differences may be attributable to differences in target mRNA half-life. In general, if r is similar for two systems, then the steady-state outcomes are expected to be similar for similar doses, all other factors being equal.

In Case III an oligo molecule targeted to a region of the mRNA that is needed for ribosome binding reduces the amount of mRNA available for translation *and* initiates RNase H mediated mRNA degradation. If ribosome and oligo binding to mRNA are mutually exclusive, and oligo is bound tightly (ideally with $\rho \cong 0$) then:

$$[k_{tm}]_{treatment} = [k_{tm}]_{control}\ (1 - f) \qquad (17a)$$

$$[k_{dm}]_{treatment} = [k_{dm}]_{control}\ (1 + rf) \qquad (17b)$$

$$\frac{[M_{ss}]_{treatment}}{[M_{ss}]_{control}} = \frac{1}{(1 + rf)} \quad (17c)$$

$$\frac{[P_{ss}]_{treatment}}{[P_{ss}]_{control}} = \frac{1 - f}{(1 + rf)} \quad (17d)$$

$$\frac{[A_{ss}]_{treatment}}{[A_{ss}]_{control}} = 1 - f \quad (17e)$$

$$\text{So, for Case III effect} = \frac{f(1 + r)}{(1 + rf)} \quad (17f)$$

Effect for Case III is plotted vs. log f in Figure 4 for three values of r. Cases II and III are contrasted in Figures 5 and 6 . We see that, at low values of r, a much greater effect will be seen for Case III. Figure 6 shows that the differences between Case III and Case II diminish as r increases and at r = 19 the effect in Case II is 95% that of Case III. When r is large enough, the effect becomes less sensitive to f, the fraction of the mRNA bound to antisense oligo. Figure 6 also provides an estimate for the RNase H activity required for antisense effect under ideal conditions.

For Case IV, the nucleocytoplasmic delivery of target mRNA is altered by the oligo either directly or indirectly. Equation 10 indicates that protein syntesis will be decreased in proportion to the decrease in mRNA delivered, and prevented completely when T_0 is zero. Clearly, Class IV offers therapeutically attractive targets. Effect for this case is a simple linear function of $[T_0]_{treatment}$, and this obviates the need for a graphical representation of the results.

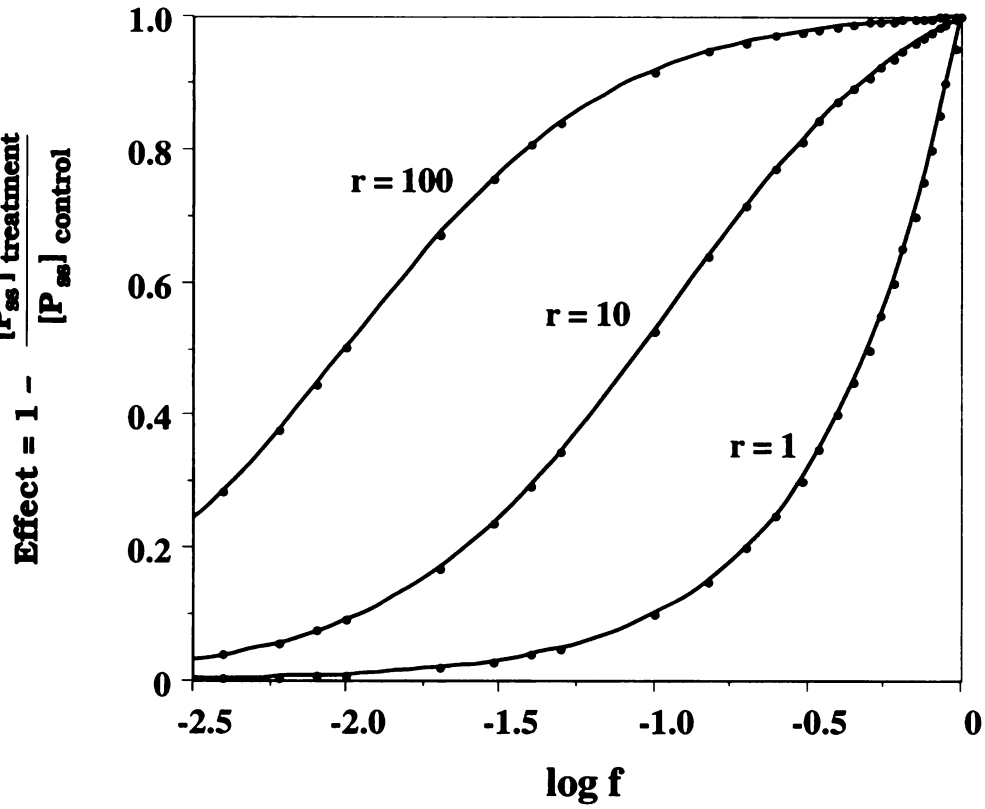

Figure 4. Plot of effect vs. log f at different values of r for Case III. The fraction of the target mRNA that exists as a mRNA-oligo hybrid is f and r = $\frac{[k_{dm}]_{RNaseH}}{[k_{dm}]_{control}}$ is the ratio of the rate constants for oligo-induced RNase H degradation of target mRNA to its degradation rate constant under control conditions. Antisense activity in the system is assumed to be due to Case III, *both Ribosome exclusion and RNase H action* on hybrids.

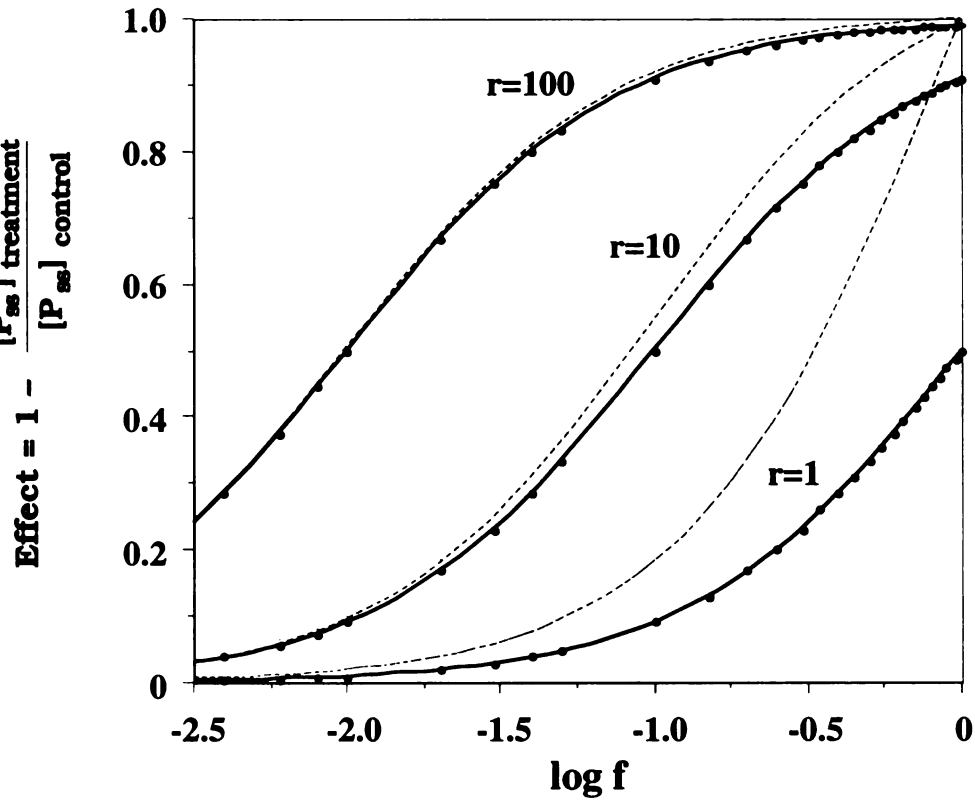

Figure 5. In this Figure data from Figures 3 (Case II, solid lines) and 4 (Case III, dashed lines) are superimposed to highlight the differences between Cases II and III. If r is small, either due to low RNase H activity in a cell or rapid rate of target mRNA degradation, an antisense oligo targeted to include the ribosome binding site may be more effective than an oligo targeted to some other coding portion of the mRNA.

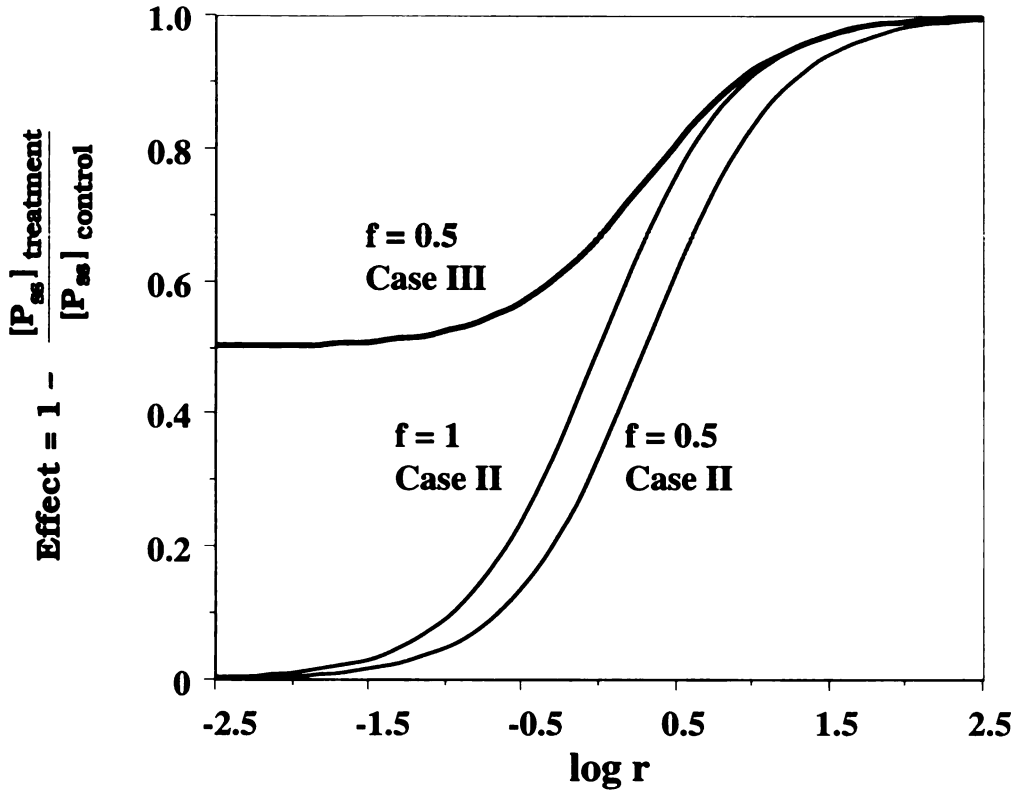

Figure 6. Effect, as measured by the fractional inhibition of target protein synthesis, is plotted as a function of log $(\frac{[k_{dm}]_{RNaseH}}{[k_{dm}]_{control}})$, which is log of the ratio of the rate constant for RNase H activity to the mRNA degradation rate constant. The fraction of the total target mRNA that is in the form of mRNA-oligo hybrid is f. Two values of f are used: f = 0.5 when half the target mRNA exists as hybrid and f = 1 when all the target mRNA exists as a hybrid. The two S shaped curves with zero origins represent Case II, systems in which only the RNase H mechanism is operative. The uppermost curve represents a case when half the mRNA is in hybrid form (f = 0.5) and the ribosome exclusion mechanism plus the RNase H mechanism are operative (Case III).

Table 1. A summary of parameters that can be used to discriminate among the mechanisms in Cases I - IV.

	STEADY STATE			
	Ribosome Block only	**RNase H only**	**Ribosome binding site & RNase H**	**Transcription blockage**
$\frac{[M_{ss}]_{treatment}}{[M_{ss}]_{control}}$	1	$\frac{[t_{\frac{1}{2}}\ dm]_{treatment}}{[t_{\frac{1}{2}}\ dm]_{control}}$	$\frac{[t_{\frac{1}{2}}\ dm]_{treatment}}{[t_{\frac{1}{2}}\ dm]_{control}}$	$\frac{[T_0]_{treatment}}{[T_0]_{control}}$
$\frac{[P_{ss}]_{treatment}}{[P_{ss}]_{control}}$	$\frac{[k_{tm}]_{treatment}}{[k_{tm}]_{control}}$	$\frac{[t_{\frac{1}{2}}\ dm]_{treatment}}{[t_{\frac{1}{2}}\ dm]_{control}}$	$\frac{[k_{tm}t_{\frac{1}{2}}\ dm]_{treatment}}{[k_{tm}t_{\frac{1}{2}}\ dm]_{control}}$	$\frac{[T_0]_{treatment}}{[T_0]_{control}}$
$\frac{[A_{ss}]_{treatment}}{[A_{ss}]_{control}}$	$\frac{[k_{tm}]_{treatment}}{[k_{tm}]_{control}}$	1	$\frac{[k_{tm}]_{treatment}}{[k_{tm}]_{control}}$	1

	TRANSIENT RESPONSE			
Coefficients	Changed	Changed	Changed	Changed
Time constant	Unchanged	Changed	Changed	Unchanged

The Nonsteady-State Solutions

The nonsteady-state Equations 5 and 6 can be rewritten in the following form.

$$\frac{M - M_{ss}}{M_0 - M_{ss}} = e^{-k_{dm}t} \tag{18a}$$

$$\frac{P - P_{ss}}{P_0 - P_{ss}} = e^{-k_{dp}t} + \frac{k_{tm}}{k_{dm}-k_{dp}} \cdot \frac{M_0 - M_{ss}}{P_0 - P_{ss}} \left(e^{-k_{dp}t} - e^{-k_{dm}t} \right) \tag{18b}$$

Equation 18 can also be written in terms of amplification A_{ss} and protein, mRNA half lives.

$$\frac{P - P_{ss}}{P_0 - P_{ss}} = e^{-k_{dp}t} + \frac{A_{ss}}{\left(\frac{t_{\frac{1}{2}\,dp}}{t_{\frac{1}{2}\,dm}} - 1 \right)} \cdot \frac{M_0 - M_{ss}}{P_0 - P_{ss}} \left(e^{-k_{dp}t} - e^{-k_{dm}t} \right) \tag{18c}$$

When $k_{dm} = k_{dp} = k$ Equation 19 must be used.

$$\frac{P - P_{ss}}{P_0 - P_{ss}} = e^{-kt} + k_{tm} \cdot \frac{M_0 - M_{ss}}{P_0 - P_{ss}} \cdot te^{-kt} \tag{19}$$

Can the transient response shed light on the mechanism? Experimental determination of k_{dp} and k_{dm} through $t_{\frac{1}{2}}$ measurements allows discrimination between the RNase H mechanism and the transcriptional block mechanisms. In either the pure RNase H based mechanism (Case II) or in the mixed Ribosome block-RNase H based mechanism (Case III) k_{dm} is altered. However, an oligo acting in the nucleus at the level of transcription, splicing, nucleocytoplasmic transport or through nuclear RNase H mediated degradation (Case IV) cannot modify k_{dp} or k_{dm}. Only the term T_0, which implicitly occurs in the coefficients of Equation 18 is altered. Translational blockage influences k_{tm} and it is noteworthy that this term occurs only as a

coefficient in Equation 18 rather than in the exponential terms. Analysis of the transient behavior of the system can thus be used to discriminate between an cytoplasmic RNase H dominated system and a system with decreased delivery of mature mRNA to the cytoplasm. These results are summarized in the lower part of Table 1.

Equations 18b and c consist of two terms both of which are positive provided $\frac{M_0 - M_{ss}}{P_0 - P_{ss}}$ is positive, a condition that is valid whenever down-regulation is occurring. The first term represents degradation of the previously synthesized protein, and would be the only term present if the oligo functioned ideally *and* f = 1. Such an ideal situation could either cause an instantaneous block of translation ($k_{tm} \rightarrow 0$) or a near instantaneous degradation of the target mRNA ($k_{dm} \rightarrow \infty$). The second term can, therefore, be viewed as arising from non-ideal behavior in an actual situation. What is role of mRNA and protein stability in antisense experiments? The second term shows that both mRNA and the protein stability contribute significantly to the kinetics of antisense action when the ratio $\frac{k_{tm}}{k_{dm}-k_{dp}}$ is large. Under conditions in which protein stability is low, (k_{dp} is large, $e^{-k_{dp}t} \rightarrow 0$) the kinetics of protein loss are limited by k_{dm}, the rate constant for mRNA breakdown. This rate constant can be enhanced by antisense oligos that exploit RNase H, assuming sufficient levels of the enzyme exists in the cell type being studied. High protein stability (i.e. $e^{-k_{dp}t} \rightarrow 1$), on the other hand, can only result in a slow, prolonged loss of protein because k_{dp} is not influenced by any of the known mechanisms of antisense action. Equation 18b provides insight into this situation. Consider the idealized case where an oligo either instantaneously causes the steady-state value of M_{ss} to be reached or instantaneously blocks all translation of a target mRNA. Such a state will

be approximated when oligo treatment causes $k_{dm} >> k_{dp}$. Even under such extreme conditions, the kinetics of protein level will depend solely on k_{dp}, and so the observed onset of oligo effect is limited to the normal rate of loss of the protein present at the time of treatment. As a specific numerical example of this limiting situation, consider the effect of a large dose of an ideal oligo, one that causes both instantaneous degradation of the target mRNA pool and blocks transcription. When $k_{dp} = 0.23$ day^{-1}, which is characteristic of alanine aminotransferase (Hargrove, Hulsey et al. 1990) one estimates a time to half-effect of 3 days. This example illustrates that antisense techniques may not be useful for rapidly downregulating relatively stable proteins. Antisense oligos act at the level of information supply (mRNA) but an effect is desired at the level of the protein. Thus, if a large pool of relatively stable protein is already present in a cell, a significant antisense effect at the level of protein may not be observed, even after a large fraction of the mRNA pool has been degraded, which may require several cell cycles.

In contrast, the preceding analysis shows that antisense oligos can generate a rapid onset of effects when the target protein is being rapidly turned over and the mRNA is neither stable nor frequently translated.

DISCUSSION

The equations developed in this paper provide estimates of the ideal activity that can be expected in a given system when oligo uptake and stability limitations are overcome. The models used were kept simple so that the few parameters needed to provide useful predictions could be determined experimentally. Some interesting results and implications of this work are discussed below, as are several limiting assumptions and weaknesses.

The derivation of the dose response curves for Cases II and III assumes that the presence of the antisense oligo does not significantly inhibit cellular nucleases since this ties up oligo in a process that detracts from activity. For maximal activity the mRNA-oligo hybrids must serve as substrate for RNase H and remain substrates for other cellular nucleases. An additional assumption is that mRNAs degraded by either cellular nucleases *or* by RNase H are incapable of synthesizing active protein. If the presence of oligos does inhibit or saturate cellular nucleases, then an overall decrease in mRNA degradation rate is expected. As a consequence, one may observe a nonspecific increase in the levels of some proteins concomitant with down-regulation of the targeted protein. The case in which oligo treatment causes induction of additional nucleases is not considered, nor have we considered the effect of feedback.

In deriving the steady-state dose-response relationships we have treated each mechanism in isolation so as to highlight the effect on protein synthesis. In reality, however, more than one mechanism may contribute, and appropriate dose-response relationships must be derived from Equations 10a-10c.

The assumption of linearity in the rate processes may not hold if the actual range of values in the systems studied is large. However, over small

ranges, the assumption of linearity seems reasonable because nonlinear continuous functions may be reasonably estimated using linear approximations.

We have not incorporated the effect of intracellular compartmentalization of oligo (Chin, Green et al. 1990; Leonetti, Mechti et al. 1991) in our model. Such a consideration may become important in anticipating effect for some antigene approaches, particularly those where the oligo has been designed to bind to double helical DNA (G. Felsenfeld et al. 1957; (Beal and Dervan 1991; Durland, Kessler et al. 1991). However, oligo activity in the nucleus only alters transcript flow T_0 into the cytoplasm in the model presented here. We assume that because the nucleus is isolated from the translational apparatus, and because only cytoplasmic mRNA is available to ribosomes, that neither nuclear RNase H activity nor interference with mature mRNA delivery (covered by Case IV) is likely to influence the time constants of the system. In contrast, cytoplasmic RNase H activity can modify the system's time constants. The nuclear RNase H activity required to produce a given level of steady-state antisense effect is greater than that for cytoplasmic RNase H activity because such nuclear RNase H activity must be at least comparable to the sum of the nuclear mRNA degradation rate and nucleocytoplasmic mRNA transport rate to be significantly felt. This is a more demanding requirement than that derived here for cytoplasmic RNase H activity which has to be comparable to the rate of mRNA degradation for significant effect.

More complex kinetic forms, e.g., inclusion of Michaelis Menten kinetics or enzyme induction, can be introduced into Equations 1 and 2, but will probably require numerical solutions. We have used a zero-order

transcription rate simply because the analytical solutions were expected to yield general principles.

This model-based approach does have limitations. A major assumption is that oligo concentrations in the cytoplasm are maintained constant or are at steady-state. The steady-state assumption is reasonable when oligo transport is rapid *relative* to the other processes such as mRNA degradation and protein degradation. However, when oligonucleotide uptake is slow *relative* to the other processes, a model that accounts for the oligo degradation and transport kinetics is required. This situation and others are appropriate topics for a subsequent manuscript.

There are fundamental differences, which the equations developed here help us examine, between antisense oligonucleotides and conventional drugs. The therapeutic effect of a conventional drug is often measurable soon after drug target binding. Following absorption and distribution of the drug to its target protein or enzyme, a fundamental rate-limiting step is often the kinetics of drug binding to its target enzyme or protein. Oligos directed against the same target enzyme or protein, on the other hand, are expected to cause a therapeutic effect primarily by decreasing the amount of enzyme or protein. Target protein degradation can therefore be a fundamental rate-limiting event and may cause a significant delay between the oligo reaching target mRNA and a detectable pharmacological effect.

An interesting situation may arise when oligo-mRNA hybrid formation precludes degradation of that mRNA by the cellular nucleases. The rate constant of mRNA degradation for the case when exclusion of nucleases is perfect can be expressed as $[k_{dm}]_{treatment} = [k_{dm}]_{control} (1 - f + r f)$ and the effect or fractional inhibition of protein synthesis is $f(r - 1)/(1 - f + rf)$. This relationship suggests that for r values less than unity one may observe an

actual up regulation of the target protein because hybrid formation causes a net increase in the mRNA half-life. On the other hand, for r values larger than unity, target protein down regulation is predicted. Thus, one should not be surprised to observe up regulation with $0 < r < 1$, especially if the targeted protein has a short half-life. We point out this theoretical outcome to emphasize the unusual behaviors predicted by the model simply by manipulating mRNA degradation.

Biological activities have been observed with sense, nonsense and scrambled oligonucleotides and the question of *appropriate* controls has been discussed in the literature (Hawkins 1991). While binding to the target sequences may be partly responsible for observed activity, our analysis suggests that simple competition of the oligo for cellular nucleases and stability determining proteins can also contribute to such activity. Thus, the presence of oligonucleotides within cells, as opposed to binding of oligonucleotides to unintended nucleic acid sequences, can result in unexpected activities.

What are the effects of oligos binding to unintended mRNA? Systems that have large values of r are attractive as antisense targets because they are less sensitive to f, the fraction of the mRNA bound to the oligo. However, they are also likely to exhibit *side effects* if a large fraction of the unintended mRNA is bound or if the unintended mRNA has a long half-life.

With appropriate modifications many of the findings presented here are applicable to other antisense approaches including those that employ plasmids producing antisense RNA. Mechanisms other than RNase H may be operative with antisense RNA, and as they are identified, they could be accommodated within a similar theoretical framework.

Using a simple two compartment kinetic model we have presented unanticipated predictions, helped explain observations already in the literature and provided a tool that may prove helpful in designing experiments and explaining their outcome.

NOMENCLATURE

M	Total cytoplasmic level of mRNA of interest, in moles.
P	Level of protein of interest, in moles.
A	Amplification ratio. Non-dimensional.
H	mRNA-oligo hybrid level in moles.
$\frac{dM}{dt}$	Rate of change of mRNA levels in moles·time^{-1}.
$\frac{dP}{dt}$	Rate of change of protein levels in moles·time^{-1}.
T_0	Rate of delivery of mature target mRNA to the cytoplasm in moles·time^{-1}.
f	Fraction of the mRNA that is in a DNA-RNA hybrid. Non-dimensional.
k_{dm}	Effective mRNA degradation rate constant in time^{-1}.
k_{dp}	Effective protein degradation rate constant in time^{-1}.
k_{tm}	Effective mRNA translation rate constant in time^{-1}.
Effect	$\text{Effect} = 1 - \frac{[P_{ss}]_{treatment}}{[P_{ss}]_{control}}$ = Fractional inhibition of protein synthesis by the oligo. It is used as a measure of therapeutic effect. Non-dimensional.
ρ	$\rho = \frac{[k_{tm}]_{hybrid}}{[k_{tm}]_{control}}$ and is the ratio of hybrid to control mRNA translational rate constants.
r	$r = \frac{[k_{dm}]_{RNaseH}}{[k_{dm}]_{control}}$ and is the ratio of the oligo induced RNase H mRNA degradation to control mRNA degradation rate constants.

Subscripts

0	Initial state.
ss	Steady state.
treatment	Results after treatment with antisense oligonucleotide.
control	Results after appropriate control treatment.
hybrid	Results due to mRNA-oligo hybrid formation.
RNase H	Results due to Ribonuclease H activity.

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