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# Antisense Inhibition of Virus Infections

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## I. Introduction

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Antiviral drugs and drug design have traditionally focused on inhibition of key proteins essential for successful viral replication. This approach is seen with the nucleoside and nucleotide analog inhibitors of the herpesvirus DNA polymerases, the human immunodeficiency virus (HIV) reverse transcriptase inhibitors, the HIV protease inhibitors, and the influenza hemagglutinin inhibitors (De Clercq, 1995; Kinchington and Redshaw, 1995; Haffey and Field, 1995; Field, 1994). It is apparent that this approach has resulted in a parade of clinically effective drugs, the best examples in the success story being acyclovir (ACV), which has been used both therapeutically and prophylactically to inhibit acute herpes simplex virus (HSV) lesion formation and duration, and zidovudine (AZT). But as successful as these approaches have been and as promising as the newer antiviral drugs may be, there are limitations to the present approaches.

These limitations are best illustrated by the concern for the emergence of clinically important drug-resistant virus mutants, a problem reviewed for the herpesviruses and for HIV (Field and Biron, 1994; De Clercq, 1995; Bowen *et al.*, 1995). For the herpesviruses, the problem has been most apparent in the immunocompromised host treated with suboptimal drug doses. For the HIV antivirals, no one drug treatment has resulted in total viral suppression, and the rapidity of resistance development has varied from drug to drug. As a result of the lack of potent and sustained virus suppression, attention has now turned to combination therapy in the clinic. Thus, the availability of a more potent and diverse group of inhibitors for any one infectious agent should provide a richer armamentarium from which to choose the most effective combinations.

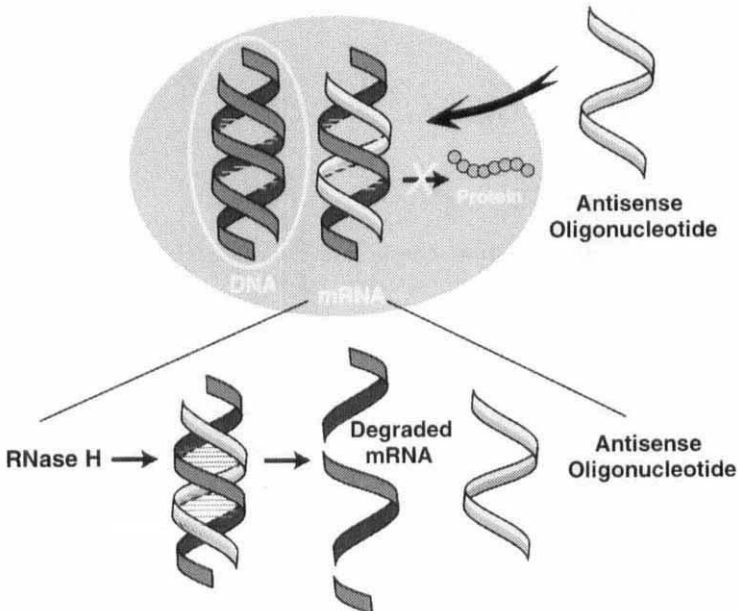
But how does one discover novel antiviral inhibitors? One approach has been the modification of a known inhibitor or an enzyme substrate in a set of structure–activity relationship (SAR) studies, to choose the inhibitor with the most attractive selectivity index (ratio of concentrations for antiviral efficacy compared to toxicity). This approach has produced the array of nucleoside and nucleotide inhibitors of HSV DNA polymerase and HIV reverse transcriptase, and the HIV protease substrate analog inhibitors. In the absence of a defined enzyme substrate, a traditional approach has employed a high-volume screen to evaluate thousands of compounds until a lead with some specificity of action can be identified and lead to the SAR refinement. As fruitful as these approaches have been, and as entrenched as these approaches are in the pharmaceutical *modus operandi*, they miss the great opportunity to design inhibitors against any of the gene targets provided by the virus. Herpes simplex virus has 71 open reading frames, for which the function of many of the resultant proteins is still poorly defined. Human immunodeficiency virus has nine open reading frames, yet only the reverse transcriptase and protease have been effectively addressed as antiviral targets by traditional approaches. Human cytomegalovirus (HCMV) more than 200 open reading frames, which should provide a rich hunting ground in the search for effective antivirals, if only we had the means to identify such inhibitors.

One new approach is just now being recognized as both versatile and practical for identifying and developing antiviral drugs. That approach is the use of antisense nucleic acids complementary to the viral RNAs to block mRNA translation or genome replication. It can take the form of antisense oligonucleotides applied to the infected cell, antisense RNA expressed within the cell, or as ribozymes capable of complementary binding to the target RNA and target cleavage via the inherent catalytic activity. In this chapter we summarize the opportunity to use these approaches to identify novel antiviral drug targets and to develop novel antiviral strategies. These are the approaches embodied in the emerging field of genetic pharmacology: the development of drugs and gene therapy to control gene expression (Field

and Goodchild, 1995; Yu *et al.*, 1994; Temsamani and Agrawal, 1996). We review genetic pharmacology as it relates to antiviral antisense research and drug development.

## II. Antisense Oligonucleotides as Potential Antiviral Agents

A major research emphasis has focused on the identification of oligonucleotides as antiviral agents. In principle, this suggests that if the sequence of a viral RNA (genome RNA or mRNA) is known, then one should be able to design a complementary oligonucleotide that will hybridize and inhibit its function as a replicating genome or mRNA (Fig. 1). This principle was first documented in the observations of Zamecnik and Stephenson, using a phosphodiester oligodeoxynucleotide to block Rous sarcoma virus replication (Zamecnik and Stephenson, 1978; Stephenson and Zamecnik, 1978). But the road from principle and initial observations in 1978 to practical antiviral utility is a long one, on which we have learned to choose preferred viral RNA target sequences for maximum antisense impact, to



**FIGURE 1** Mechanisms of antisense oligonucleotide inhibition. Antisense oligonucleotides can bind to target RNA in cells, preventing protein production directly by translation arrest. In addition, DNA-RNA hybrids formed by oligonucleotide binding to RNA are recognized by RNase H, which digests the RNA portion of the hybrid, destroying the mRNA and freeing the oligonucleotide for binding to another mRNA molecule.

chemically alter the antisense oligonucleotides to enhance stability and optimize activities in biological systems, and to recognize the array of biological actions of oligonucleotides that may contribute to antiviral activities and also to toxicities.

An antiviral antisense oligonucleotide should display a high degree of specificity, the reflection of the uniqueness of the nucleotide sequence provided by the genetic code, and the exact complementary base pairing with the target RNA. As a result, introducing mismatch nucleotides to interfere with hybridization of the oligonucleotide with the RNA target, or substituting inappropriate sequence by "scrambling" the order of the nucleotides or reversing the predicted antisense 5'-to-3' orientation, should reduce the antiviral activity. Furthermore, the antisense mechanism predicts selective inhibition of gene expression. Suggested guidelines for acceptance of an oligonucleotide activity as fitting these criteria were presented by Stein and Krieg (1994). Direct demonstration that an antisense oligonucleotide binds to the target RNA and violates its biological role within the cell has generally been difficult to obtain, although numerous indirect lines of evidence have suggested that this is so. For example, studies of antisense oligonucleotides against cytomegalovirus showed selective gene target inhibition resulting in a potent antiviral activity (Smith and Pari, 1995; Pari *et al.*, 1995). In addition, recent studies of antisense mechanism have been fruitful and have demonstrated the direct antisense activities *in situ* (Poltz *et al.*, 1995; Giles *et al.*, 1995a,b). The details of these observations as they relate to antiviral activities are described in Section III.

### A. Antiviral Target Selection

Antisense oligonucleotides should be highly selective compounds by virtue of their interaction with specific segments of RNA. For potential antivirals, identification of appropriate target RNA sequences for antisense oligonucleotides is performed at two levels: (1) the optimal gene within the virus, and (2) the optimal sequence within the RNA.

"Optimal" genes are those genes that are essential for virus replication and/or are essential for virulence. Thus, a wide variety of targets may be available, and through the antisense approach both validation of the target and discovery of an effective inhibitor can be attained simultaneously. This validation and discovery process are illustrated by studies on HCMV, which are discussed below. To be sure, oligonucleotides have been designed to target expression of structural genes of HIV (Lisziewicz *et al.*, 1993, 1994; Anazodo *et al.*, 1995) and hepatitis B virus (HBV) (Korba and Gerin, 1995), regulatory genes of HIV (Matsukura *et al.*, 1987), HCMV (Azad *et al.*, 1993; Pari *et al.*, 1995), Epstein-Barr virus (EBV) (Roth *et al.*, 1994; Daibata *et al.*, 1996), and human papillomavirus (HPV) (Cowsert *et al.*, 1993), and

a variety of functions in HBV (Wu and Wu, 1992; Offensperger *et al.*, 1993; Korba and Gerin, 1995).

But few studies have set out to compare viral targets for vulnerability to antisense inhibition, and fewer have done so while also controlling for nonantisense antiviral effects. For HIV, oligonucleotides targeted against a regulatory gene (*rev*), a viral enzyme (*pol*), and structural gene (*gag*) were compared in a single study (Kinchington *et al.*, 1992). All oligonucleotides were reported to be equally active in an acute infection assay. The *rev* targeted oligonucleotide was the only oligonucleotide active against chronically infected cells, suggesting that this was the "optimal" gene for antisense targeting. However, other sequences against these genes were not evaluated and the inhibition required a 30  $\mu$ M concentration, so it is not clear if the "optimal" sequences were actually chosen. For example, GEM 91, a 25-mer phosphorothioate oligonucleotide targeted at the *gag* gene, has been found to be active at submicromolar concentrations in acute infection assays and long-term model systems (Agrawal and Tang, 1992; Lisziewicz *et al.*, 1993). For HBV, RNA sequences have been identified that are essential for initiation of reverse transcription and packaging of viral RNA (Pollack and Ganem, 1994). Oligonucleotides against these regions were reported to be potent inhibitors of HBV replication (Korba and Gerin, 1995). For RNA viruses additional genetic targets may also be provided, since both genomic positive (picornaviruses), negative (influenza), and double-stranded (reoviruses), as well as individual transcripts, are available. For instance, we have targeted oligonucleotides to the genomic strand of respiratory syncytial virus to inhibit RNA replication, and it was previously shown that replication of both influenza virus and vesicular stomatitis virus was inhibited by oligonucleotides that were targeted to genomic RNA (Lemaitre *et al.*, 1987; Leiter *et al.*, 1990).

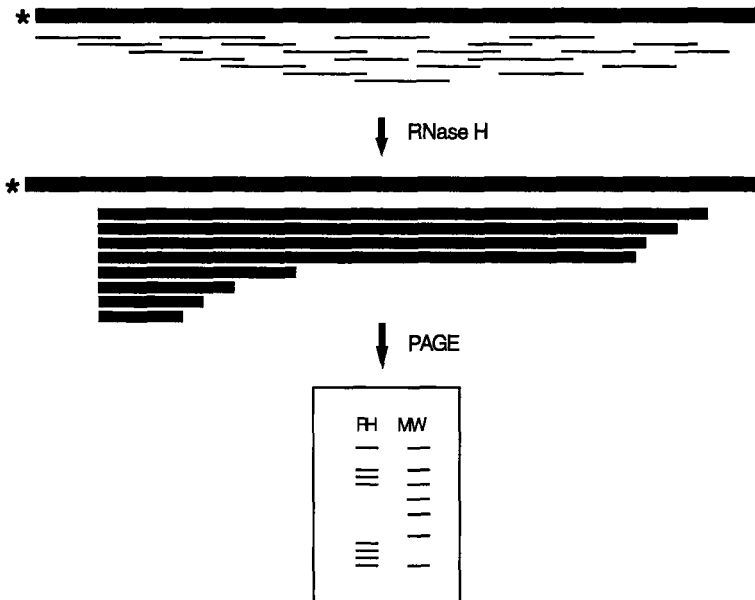
It is clear that there are no defined rules to identify the optimal antisense gene target for a virus, but the versatility of designing antisense inhibitors to heretofore unassailable targets provides great opportunity to eventually clarify those rules.

Much effort has also been made to identify the correct RNA target sequence within a gene. Again, there are no clear rules to easily predict which sequences are most accessible to oligonucleotides. Computer models of RNA secondary structure (Sczakiel *et al.*, 1993), as well as oligonucleotide hybridization efficiency (Stull *et al.*, 1992), and frequency (Han *et al.*, 1994) have been compared to antisense oligonucleotide activity. Antisense activity was shown to correlate well with hybridization strength; however, RNA secondary structure did not predict antisense activity. Often, *in vitro* translation inhibition has been used to screen a series of potential oligonucleotide inhibitors, and then the most active oligonucleotides were evaluated in cellular and/or antiviral assays (Chen *et al.*, 1996).

In our laboratories, oligonucleotide libraries have been screened for binding to target RNA *in vitro* by an assay described by Frank *et al.* (Frank and Goodchild, 1996; Frank *et al.*, 1993) and modified by Ho *et al.* (1996). Binding is measured by RNase H cleavage of RNA–oligonucleotide hybrids. The most sensitive sites are mapped and specific oligonucleotides synthesized and evaluated in cellular assays. An example of the technique is shown in Fig. 2. Correlation exists between accessible RNA regions *in vitro* and in cells, although one would expect more sites to be identified using this *in vitro* technique with the purified RNA target, than would be expected *in situ* where target RNA may carry numerous binding proteins.

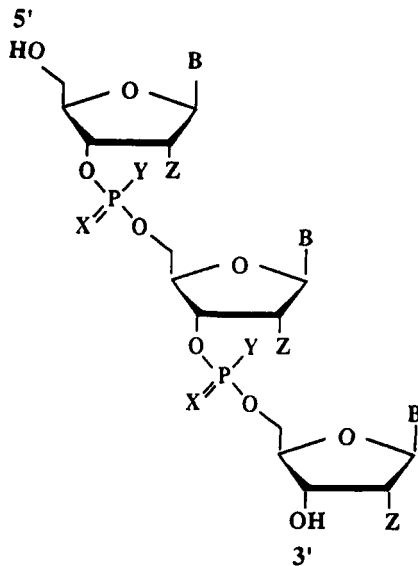
## B. Oligonucleotide Modifications

As previously mentioned, the fundamental mission of an antisense oligonucleotide is to hybridize to the viral RNA target and inhibit its function. This inhibition may be facilitated by cleaving the target RNA by RNase H, which recognizes the RNA–oligodeoxynucleotide complex, or by inhibiting RNA translation or splicing through hybrid arrest. The end result should



**FIGURE 2** Antisense oligonucleotide selection. Oligonucleotide libraries (thin lines) are incubated with 5' end-labeled RNA (thick line), then treated with RNase H. RNA regions with bound oligonucleotide are digested, producing families of shorter labeled RNAs, which are separated on polyacrylamide gels. Discrete families of RNAs are detected on gels (lane RH) and compared to molecular weight markers (lane MW) to identify regions of maximum oligonucleotide binding.

be either direct inhibition of the targeted RNA genome replication (for an RNA virus), or inhibition of translation of the targeted mRNA. In principle, unmodified oligodeoxynucleotides could satisfy this mission, but in practice chemical modifications of the oligonucleotide are necessary for robust antisense efficacy. Such modifications are designed to enhance stability, while retaining the capacity to hybridize to the target RNA and recruit RNase H. In addition, chemical modifications can be used to alter the hydrophobicity/hydrophilicity, thus altering the presentation of the oligonucleotide to the infected cell. Functional groups, such as phosphate and hydroxyl residues on natural nucleic acids, can be modified as indicated in Fig. 3. In general, those modifications that jeopardize the capacity of the oligonucleotide to participate in Watson–Crick base pairing are avoided, and most modifications have focused on the phosphodiester backbone and/or the sugar moiety.



**FIGURE 3** Structure of oligonucleotides. B, Any of the nitrogen heterocyclic bases found in DNA (A, C, T, G) or in RNA (where U replaces T). The terminal hydroxyl groups are distinguished as being at either the 5' or 3' ends.

	X	Y	Z
Deoxyoligonucleotides			H
Ribooligonucleotides			OH
2'-O-methyl derivatives			OMe
Unmodified	O	O	
Phosphorothioate	O	S	
Methyl phosphonate	O	CH <sub>3</sub>	
Phosphoramidate	O	NR <sup>1</sup> R <sup>2</sup>	



For instance, by replacement of the nonbridging oxygen of the phosphodiester backbone with sulfur, the resulting phosphorothioate (PS) has increased resistance to nuclease degradation. Although the duplex formed with the target RNA has a lower melting temperature ( $T_m$ ), it is a substrate for RNase H. A similar replacement of the nonester oxygen by a methyl group results in loss of negative charge and greater hydrophobicity, but at the cost of loss of RNase H activation. Alternatively, by replacing a hydrogen at the 2' position on the deoxyribose with a hydroxymethyl group, the sugar becomes a modified ribose, which hybridizes more strongly with the complementary mRNA ribonucleotide and provides greater stability to the duplex, indicated by an elevated  $T_m$ . These and other changes are demonstrated in Fig. 3, and allow one to synthesize tailored oligonucleotides with a balance of characteristics of hybridization affinity, hydrophobicity, and the capacity to recruit RNase H-mediated cleavage of the target RNA.

The importance of these oligonucleotide modifications in designing effective drugs is just now being evaluated, both in animal model systems and in the clinic. The first generation of widely used antisense oligonucleotides has been the PS compounds, and a body of data on biodistribution, pharmacokinetics, and metabolism in animals and in humans is now available. These studies were summarized by Agrawal and Tamsamani (1996), Field and Goodchild (1995), and Crooke and Bennett (1996). A second generation of antisense oligonucleotides is now emerging that includes combinations of nucleotide modifications within the oligonucleotide. For instance, *hybrid oligonucleotides* may be defined as substituted at the 3' and/or 5' ends with 2'-OCH<sub>3</sub> ribonucleosides, while maintaining the phosphorothioate backbone; *chimeric oligonucleotides* may be defined as substituted at the 3' and/or 5' ends with nonionic internucleotide linkages; and *self-stabilized oligonucleotides* may be defined as phosphorothioates or phosphodiesters that have two domains—a single-stranded antisense sequence and a hairpin loop at the 3' end. The pharmacological characteristics of these and other modifications are summarized in papers by Agrawal and Tamsamani (1996) and by Crooke *et al.* (1996). It is clear from the ongoing studies, that not only can one design an antisense inhibitor with potentially high selectivity in target inhibition, but one can now chemically modify that selected oligonucleotide to tailor the eventual drug for enhanced stability and perhaps tissue-specific uptake and metabolism (Crooke *et al.*, 1996). In addition, the first observation of oral uptake and tissue distribution of a hybrid oligonucleotide (Zhang *et al.*, 1995a) indicates that the opportunity to develop oligonucleotide therapeutics with oral bioavailability may be at hand.

To date, most of the antiviral cell culture studies and all of the animal efficacy and clinical studies have employed PS oligonucleotides. More recently, Hybridon (Cambridge, MA) has introduced the first advanced oligonucleotide, GEM 132, a hybrid oligonucleotide for clinical study (see Section III,B). These studies using PS oligonucleotides have generated useful informa-

tion concerning antisense activities, but it is apparent that PS compounds may also have additional nonantisense modes of antiviral activity. This can be readily demonstrated for HSV, EBV, and HIV (Gao *et al.*, 1990a; Yao *et al.*, 1993; Wyatt *et al.*, 1994; Ojwang *et al.*, 1994a, 1995; Buckheit *et al.*, 1994). The nonantisense mechanisms include inhibition of virus binding and internalization (Wyatt *et al.*, 1994; Buckheit *et al.*, 1994) and inhibition of virus-specific DNA polymerases (Yao *et al.*, 1993; Gao *et al.*, 1989, 1990a). These effects are related to the general polyanionic nature of oligonucleotides, but are more pronounced for specific sequences. For example, oligonucleotides containing four consecutive G residues formed tetrameric structures that potentially inhibited absorption of HIV or HSV (Buckheit *et al.*, 1994). Oligonucleotides containing only G and T residues inhibited absorption and integration of HIV (Ojwang *et al.*, 1994b); this is believed to be due to higher order structure of the oligonucleotides. Certain oligonucleotides containing CpG motifs may also stimulate B cell proliferation and have immunostimulatory characteristics (Krieg *et al.*, 1995). The nonantisense effects may contribute significantly to antiviral effects by oligonucleotides, enhancing their activity in cell culture. Unfortunately, these effects might in certain cases mask antisense effects and make rational selection of gene targets and sequence targets more difficult. These anomalies are more fully discussed below in the context of individual antiviral studies.

By far, most antiviral studies using oligonucleotides have been cell culture evaluations, some of which have neglected to apply a rigorous definition of antisense inhibition. However, as the field has matured and the criteria for an antisense antiviral oligonucleotide have become more precise, antisense mechanisms of antiviral activity have been repeatedly confirmed. In the studies reported below, we have emphasized those studies with a clear definition of the antisense mechanism of antiviral activity. Thus, the literature survey focuses on key studies that illustrate how the field has evolved, and the novelties of those key studies that have helped to build our understanding of the roles of oligonucleotides as antiviral agents.

### III. Oligonucleotides and Antiviral Activities \_\_\_\_\_

#### A. The Retroviruses

As was so correctly phrased by John Coffin, "No group of infectious agents has received as much attention from scientists in recent years as the retroviruses" (Coffin, 1996). This group has the capacity to replicate as a productive, cytolytic infection or become latent as genetic information inserted into the host genetic material; they have the capacity to capture and alter host genetic information in the form of oncogenes; they are readily mutable and thus can readily escape the antiviral effects of many initially

effective inhibitors; and they are associated with a wide variety of diseases including benign and malignant tumors and acquired immunodeficiency syndrome (AIDS). As a result, they have also been a favorite target for antiviral studies, including antisense studies.

### **1. Non-HIV Retroviruses**

The first detailed observations of inhibition of virus replication by oligonucleotides designed as antisense were published in 1978 (Zamecnik and Stephenson, 1978; Stephenson and Zamecnik, 1978). They used an unmodified 13-mer or a 13-mer blocked at both the 5' and 3' termini as the isourea derivatives. The oligonucleotide sequence was complementary to the reiterated 5'- and 3'-terminal repeats of the virion RNA, and blocked virus replication at about  $2 \mu\text{M}$ , as measured by appearance of Rous sarcoma virus reverse transcriptase. Furthermore, using an *in vitro* translation system, the authors demonstrated selectivity of inhibition of viral RNA translation, and sequence specificity of that inhibition. Since these early observations, an abundance of antisense papers concerning both animal and human retroviruses has appeared.

Bovine leukemia virus encodes a transactivating protein, Tax, which promotes viral transcription and activates cellular genes associated with tumorigenesis. Cantor and Palmer evaluated the capacity of unmodified 15-mers to inhibit Tax translation from Tax message in rabbit reticulocyte lysates (Cantor and Palmer, 1992). Apparent sequence-specific inhibition of translation was observed with oligonucleotides directed against the 5' portion of the Tax RNA, including the AUG. One oligonucleotide, containing a four-G sequence and targeted at a 3' sequence, was actually stimulatory to translation by an undefined mechanism. This research was extended by Kitajima *et al.*, who demonstrated, both in murine cells in culture and in implanted Tax-producing fibrosarcoma cells, that a 20-mer modified by the phosphorothioate substitution at the terminal three nucleotides on the 3' end inhibited Tax protein expression (Kitajima *et al.*, 1992a,b). Greater than 10-fold inhibition of Tax expression in mature tumors occurred with an intraperitoneal injection of  $40 \mu\text{g/g}$ . The similarly modified sense control oligonucleotide was ineffective. Curiously, in Tax-expressing cells the uptake of oligonucleotides was sevenfold higher than in non-Tax-expressing cells, and uptake appeared receptor mediated. The Tax protein causes transcriptional transactivation and is implicated in human T cell leukemia virus type I (HTLV-I)-mediated leukemogenesis, perhaps through activation and elevated expression of NF- $\kappa$ B. Kitajima also reported that antisense oligonucleotides, but not the complementary sense strand, targeted to NF $\kappa$ B mRNA translation start site inhibited Tax-transformed fibroblast growth and HTLV-I-transformed human lymphocyte growth. In mice, the antisense to NF- $\kappa$ B caused a rapid regression of the Tax-transformed tumors (Kitajima *et al.*, 1992a). The studies also suggested that although Tax is necessary to

transform HTLV-I-infected cells, it is the maintenance of high levels of NF- $\kappa$ B that is important to sustaining the malignant phenotype.

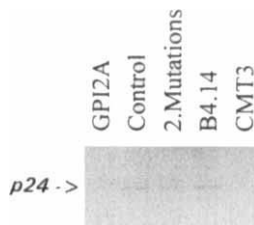
## 2. HIV

Since the identification and sequencing of HIV, there has been a strong interest in identifying a potent oligonucleotide inhibitor that would have the potential for development as a therapy for AIDS. Zamecnik *et al.* (1986) and Goodchild *et al.* (1988) described the inhibition of HIV replication by synthetic oligonucleotides, and the phosphorothioate oligonucleotide (GEM 91) designed by Agrawal and Tang to bind to the *gag* region of HIV RNA was identified for further therapeutic development in 1992 (Agrawal and Tang, 1992). GEM 91 was selected because of its potent antiviral effects, and was designed to bind to a well-conserved region of the viral genome of most clinical isolates. This illustrates one of the advantages of antiviral design through antisense—the potential to select among an array of molecular targets for inhibition (Liszewicz *et al.*, 1994). In these and other studies (Agrawal *et al.*, 1989), oligonucleotides were evaluated for their capacity to inhibit virus replication in long-term infected cell cultures, and selected antisense sequences were compared to unrelated or random sequence oligonucleotides. The results suggest that the selected antisense sequences have a considerable advantage of efficacy. Matsukura and colleagues also evaluated the inhibition of HIV replication by PS oligonucleotides, in this case targeted to the HIV *rev* RNA (Matsukura *et al.*, 1989). They reported sequence specificity of the antiviral effect (lack of activity of the sense, random, or homopolymeric sequences) and the expected effects on the HIV mRNA profile on treatment.

On the basis of the potent antiviral activity for GEM 91 in HIV cell culture infection studies and the well-conserved target sequence, phase I/II clinical evaluations for intravenous therapy of HIV has begun in both the United States and France (Martin and U.S. and French GEM 91 Collaborative Study Groups, 1995; Serini *et al.*, 1994). The US trial is a randomized, double-blind, placebo-controlled dose-escalating study using GEM 91 intravenous continuous infusion for 2 weeks. In the French study, GEM 91 is given as 2-hr infusion every other day for 28 days. Study entrants must have a viral burden of 25,000 copies of viral RNA/ml of plasma. To date, safety has been demonstrated for doses up to 4.4 mg/kg by continuous infusion and 3 mg/kg by repeated intermittent, 2-hr infusion. From the initial pharmacokinetic phase I single dose studies using <sup>35</sup>S-labeled GEM 91, plasma disappearance of the radioactivity associated with GEM 91 is the sum of two exponentials with mean half-lives of 0.18 and 26.71 hr. Both intact and degraded materials are found in the plasma. Elimination is mainly by urinary excretion of primarily lower molecular weight metabolites. Maximum tolerated doses have not yet been achieved (Zhang *et al.*, 1995b).

Anazodo *et al.* (1995) demonstrated that a partially phosphorothioated 20-mer targeted to a well-conserved coding region of the *gag* gene inhibited both expression of mRNA for the viral precursor protein p55 and p55 protein and its cleavage product, p24, in COS cells stably transfected with plasmids containing the *gag-pol* region (Fig. 4). In this system, the use of lipofectin with the oligonucleotide enhanced the activity. Inverse sequence and double mismatch control oligonucleotides were less effective. At 1  $\mu$ M the antisense 20-mer inhibited viral replication, as measured by reverse transcriptase levels, in a sequence-specific manner without inhibiting cell protein biosynthesis (large subunit of ribonucleotide reductase) or cell growth rate.

As with other oligonucleotide antiviral studies mentioned previously, anti-retroviral effects of oligonucleotides by nonantisense mechanisms have been amply demonstrated. Matsukura *et al.* have demonstrated that the phosphorothioate 28-mer homopolymer dC is a potent inhibitor of HIV infection (Matsukura *et al.*, 1988). Direct and potent ( $K_i$  values at 6–12 nM) inhibition of the reverse transcriptase (RT), RNase H, and primer extension functions has also been demonstrated for HIV (Hatta *et al.*, 1993; Bordier *et al.*, 1992; Austermann *et al.*, 1992) and avian myeloblastosis virus (Hatta *et al.*, 1993; Boiziau *et al.*, 1992). In addition to inhibition of RT, others have demonstrated the inhibition of HIV adsorption to cells (Zelphati *et al.*, 1994). Most recently an interesting phosphorothioate oligonucleotide sequence ( $T_2G_4T_2$ ) was identified that is a potent inhibitor of HIV viral envelope protein gp120 binding to the CD4 cell receptor. Specifically,  $T_2G_4T_2$  as well as other nonantisense oligonucleotide HIV inhibitors such as the phosphorothioate dC bind to the V3 loop of gp120 (Wyatt *et al.*, 1994; Stein *et al.*, 1993). The breadth of anti-HIV mechanisms of oligonucleotides was further expanded with the demonstration that a 17-mer (T3077),



**FIGURE 4** Inhibition of HIV p24 production. B4.14 cells, stably transfected to express HIV-1 p24, were treated with an antisense oligonucleotide (GPI2A) spanning bases 1189–1208 of HIV. Two control oligonucleotides containing either two mutations (2.mutations) or the inverse sequence (control), were also tested. p24 production, measured by immunoprecipitation using a rabbit polyclonal antibody, was decreased only in cells treated with the antisense oligonucleotide. Control and 2.mutation oligonucleotides did not alter p24 levels. Cells that were not transfected (CMT3) did not express p24 (Anazodo *et al.*, 1995).

composed of only deoxyguanosine and thymidine and with single phosphorothioate internucleotide linkages at the 5' and 3' ends, is a potent inhibitor of HIV integrase (Ojwang *et al.*, 1995). This same compound and related compounds (Ojwang *et al.*, 1994a) form tetramers owing in part to the motifs of G in the sequence, which appear to enhance the oligonucleotide capacity to block virus adsorption (Bishop *et al.*, 1996).

All of these observations emphasize the importance of defining mechanisms of action of a given oligonucleotide prior to identifying it as an antisense oligonucleotide. However, the fact that oligonucleotides may contribute numerous mechanisms toward the antiviral activity, in addition to the antisense mechanism, may in some cases be an asset in the pursuit of clinically useful antiviral drugs.

## **B. The Herpesviruses**

### ***1. Alphaherpesviruses: Herpes Simplex Viruses and Marek's Disease Virus***

Herpes simplex virus types 1 and 2 are responsible for acute infections (cold sores, herpes genitalis, herpes encephalitis) which may result in persistent latent infection of neurons in the sensory ganglia, and may be punctuated by acute recurrences (Roizman and Sears, 1996). Herpes simplex virus replication, like that of other herpesviruses, is the result of a coordinately regulated, sequentially ordered cascade of transcription and translation events that are broadly categorized as immediate early, early, and late events (Roizman and Sears, 1996). The immediate early genes are transcribed without the requirement for previous viral protein synthesis and include transactivators such as the  $\alpha$ -4 gene product, ICP 4. The early genes include many required for viral DNA synthesis, including the DNA polymerase, and are not transcribed until immediate early gene translation has been initiated. Finally, the late gene transcription is initiated following viral DNA synthesis. These include the structural proteins such as the virion capsid proteins from genes UL 13 and UL 48 (Vmw 65). Vmw 65 (also known as VP 16 and  $\alpha$ -TIF) has been identified as an essential structural element in the virion and also as a transactivator of immediate early gene expression (Roizman and Sears, 1996). Although this is a simplistic description of the events during the replication cycle, it serves to illustrate that multiple antiviral targets are potentially available to truncate replication at various stages.

Draper *et al.* reported the inhibition of transactivation of the ICP 4 promoter and virus replication by unmodified oligodeoxynucleotides targeted to the putative translation initiation site of Vmw 65 (Draper *et al.*, 1990). Sequence specificity of the activity was suggested by the relative activities of two different 18-mers, although rigorous studies to demonstrate that the specificity was an antisense effect were not performed. Further inhibition studies using Vmw 65 as a target were performed by Kmetz *et*

al. using the same 18-mer oligonucleotides, but as PS compounds (Kmetz *et al.*, 1991). The investigators found that a 25- $\mu\text{g/ml}$  (4.3  $\mu\text{M}$ ) concentration of oligonucleotide reduced replication of HSV-1 strain KOS by greater than 50%, and that the inhibition correlated well with the reduction of Vmw 65 protein. A random 18-mer served as control and had minimal inhibitory activity. Extension of the 18-mer by 5 nucleotides on the 3' end resulted in a more potent inhibitor. These studies suggested both a specific target effect (inhibition of Vmw 65 resulted in reduced infectivity), and a sequence specificity (the random 18-mer was less active) and thus potentially an antisense mechanism of action.

A series of studies by the Johns Hopkins group (Kulka and Aurelian, 1995; Kulka *et al.*, 1989, 1993, 1994; Kean *et al.*, 1995) have investigated antisense oligonucleotides targeted to the splice donor/acceptor sites of the immediate early pre-mRNAs of IE 4 (ICP 4 protein). For the earlier studies, the investigators used methyl phosphonate oligodeoxynucleotides, and reported a sequence specific inhibition of virus replication. A methyl phosphonate 12-mer targeted to the IE mRNA splice donor site was effective in reducing virus growth by 80% at 100  $\mu\text{M}$ , whereas the same oligonucleotide in which the central two residues were inverted was inactive. The same methyl phosphonate reduced virus yield by localized treatment in the HSV-1 mouse ear infection (Kulka *et al.*, 1993). Although these studies suggest specificity of activity, the effective concentrations make them impractical as potential therapeutic drugs. More recently this group has found that by using a 12-mer methyl phosphonate targeted to the intron/exon junction of the splice acceptor and by substituting 2'-OCH<sub>3</sub>U for dT, greater affinity for the RNA target and a fivefold reduction in 50% inhibitory concentration IC<sub>50</sub> (22 to 4  $\mu\text{M}$  versus HSV-1 with no inhibition against HSV-2) was achieved. The sequence specificity of the oligonucleotides was indicated by the relative lack of activity of mismatched oligonucleotides (Kean *et al.* 1995).

UL 13 is a late gene that encodes a protein kinase and appears to be a virion structural protein. A PS 21-mer oligonucleotide targeted to a translation initiation codon is a potent inhibitor of virus replication (IC<sub>50</sub> values of 0.4 to 1  $\mu\text{M}$ ) (Crooke *et al.*, 1992). This activity is quite potent, but demonstration of the characteristic sequence specificity and gene target selectivity must follow to label the antiviral activity as antisense.

Poddevin *et al.* investigated a series of phosphodiester oligonucleotides with 12-mer regions complementary to the target IE 4 pre-mRNA, and 3' noncomplementary flanking sequences that formed hairpin structures similar to the self-stabilized phosphorothioate oligonucleotides described previously. These studies, which were well controlled for non-sequence-dependent antiviral effects, demonstrated that by adding stability to degradation at the 3' terminus of an otherwise unaltered phosphodiester oligonucleotide, fairly potent inhibition (IC<sub>50</sub> value of 1.5  $\mu\text{M}$ ) could be achieved (Poddevin *et al.*, 1994).

A further approach to avoid nonspecific effects and yet increase oligonucleotide stability was employed by Peyman *et al.* (1995). They screened an array of 20-mers that were phosphodiester oligonucleotides except for two phosphorothioate nucleotide residues at both the 5' and 3' ends. The antiviral efficacy in cell culture was evaluated by inhibition of virus-induced cytopathic effect (CPE). The most potent compound was targeted at the translation start site of IE 110 mRNA, and had an effective dose of 9  $\mu\text{M}$ . A 2-nucleotide shift in sequence reduced the efficacy by about ninefold, and mismatched oligonucleotides were inactive at 80  $\mu\text{M}$ . Thus, by definition of sequence specificity, the activity appears to be antisense mediated. However, no attempt was made to evaluate the selectivity of inhibition of expression of UL 110 compared to the coexpression of another immediate early gene, which would have provided additional evidence for the antisense mechanism of activity.

Numerous PS oligonucleotides, with no apparent antisense sequence specificity, can have an anti-HSV effect (A. K. Field, personal communication) This observation has been thoroughly investigated by Y.-C. Cheng and colleagues. The most potent antiviral compound was the phosphorothioate dC 28-mer, which had an  $\text{IC}_{90}$  against HSV-2 of 1  $\mu\text{M}$ , and probably inhibited virus replication by the capacity to block virus adsorption and penetration, and by the additional potent inhibition of viral DNA synthesis (Gao *et al.*, 1989, 1990a,b). The dC 28-mer competes as a template for the DNA polymerase and as a competitive inhibitor of viral DNA polymerase exonuclease activity. Most recently, Fennewald and colleagues described PS oligonucleotides that are potent HSV inhibitors ( $\text{IC}_{50}$  values of 0.02 to 0.2  $\mu\text{M}$ ) (Fennewald *et al.*, 1995). These oligonucleotides are composed entirely of dG and dT residues and effectively block virus adsorption and penetration, although these may not be the only antiviral effects.

Marek's disease virus (MDV) is an avian alphaherpesvirus that causes lymphoproliferative disease. Evidence suggests that maintenance of the tumorigenic state of MDV-derived lymphoblastoid cell lines is due to expression of a 1.8-kb gene family. Kawamura *et al.* evaluated a phosphodiester 18-mer oligonucleotide complementary to a splice donor sequence and demonstrated a sequence-specific inhibition of expression of the 1.8-kb mRNA and inhibition of colony growth in soft agar. The multiple copies of the MDV genome were maintained in the lymphoblastoid cells. Appropriate sense and unrelated oligonucleotides were inactive (Kawamura *et al.*, 1991). This relatively early (1991) and well-controlled study provided direct evidence that the transcription of the 1.8-kb region is required for tumorigenicity of MDV-transformed cells.

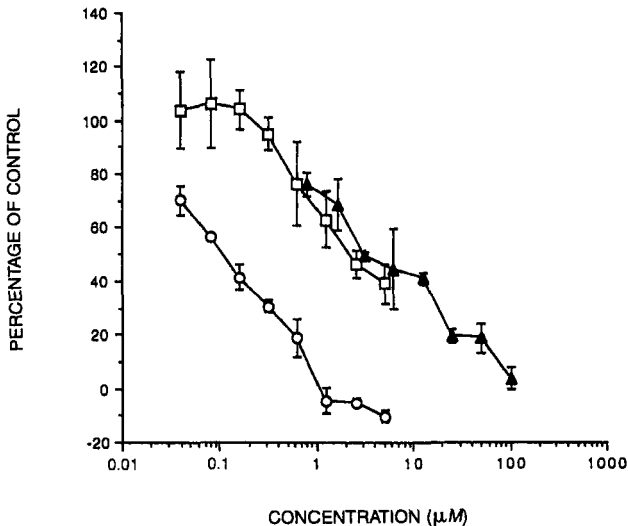
## **2. Betaherpesviruses: Human Cytomegalovirus**

Infections by HCMV are often silent and result in latency in a high proportion of the population. However, in immunocompromised individuals and in the newborn, HCMV infections may lead to a variety of disease



syndromes including retinitis, pharyngitis, esophagitis, systemic disease, and perhaps coronary artery disease (Speir *et al.*, 1994; Haffey and Field, 1995; Falloon and Masur, 1990). As with herpes simplex virus, the replicative cycle is a coordinately regulated cascade with immediate early, early, and late transcription events. However, whereas HSV has 71 open reading frames, HCMV has more than 200, suggesting a more complicated replication pattern and/or a more involved pathogenesis. However, the larger genome also potentially provides more opportunities for antiviral intervention by antisense oligonucleotides.

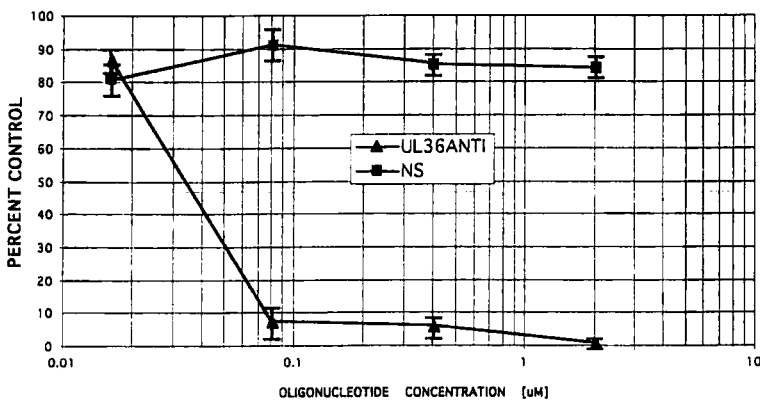
Two groups of researchers have provided extensive studies on oligonucleotide inhibition of HCMV. Azad *et al.* surveyed a series of oligonucleotides against translation start sites, coding regions, intron/exon regions, and 5' caps in a variety of genes including the DNA polymerase, and immediate early genes IE 1 and IE 2 (Azad *et al.*, 1993). They reported that the most potent oligonucleotide (ISIS 2922) was a PS 21-mer against the coding region of IE 2. According to the authors, ISIS 2922 reduced both IE 2 and HCMV replication proportionately, with an  $IC_{50}$  value of  $\sim 0.1 \mu M$  (Fig. 5). Although unrelated oligonucleotides were reported to be less active in both reduction of IE 2 and virus replication (suggesting a sequence-specific antiviral effect), mismatches in ISIS 2922 that substantially reduced hybridization did not alter the antiviral effects. The latter observations suggest that antiviral activity may be due in part to a nonantisense mechanism of



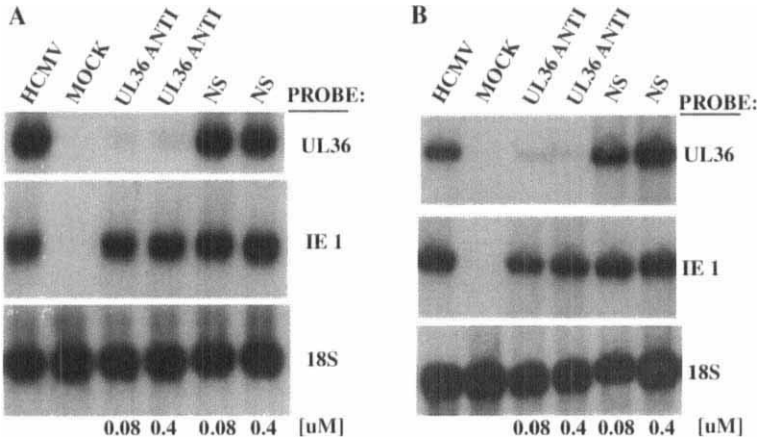
**FIGURE 5** Relative antiviral activities of phosphorothioate oligonucleotides and ganciclovir. ISIS 2922 (○, antisense oligonucleotide targeted to the HCMV major immediate early region) was a more potent inhibitor of HCMV antigen expression than ISIS 3383 (□, noncomplementary control oligonucleotide) or ganciclovir (▲) (Azad *et al.*, 1993).

action. When evaluated in combination with ganciclovir or foscarnet, ISIS 2922 was additive in antiviral activity versus HCMV; with AZT it was also mainly additive in inhibiting HIV (Azad *et al.*, 1995). Considering the relative lack of cytotoxicity on uninfected cells, ISIS 2922 was considered by the authors to be an attractive candidate for clinical evaluation. In further support, ISIS 2922 is cleared slowly from vitreous fluid, and intact oligonucleotide accumulates in the retina following intravitreal injection in rabbits. Subacute intravitreal dosing is also well tolerated in monkeys. In a phase I study of repeat intravitreal injections to AIDS patients with refractory HCMV retinitis, ISIS 2922 was well tolerated at doses up to 300  $\mu\text{g}$ —a calculated vitreal concentration of 8  $\mu\text{M}$ . Initial reports state that ISIS 2922 did inhibit the progression of HCMV retinitis in patients who had progressive disease during ganciclovir or foscarnet therapy and ISIS 2922 is now in expanded clinical efficacy studies (Anderson, 1994).

One virtue of the antisense approach to identifying novel antiviral drugs is the ability to target any of a large array of genes. Smith and Pari took this approach in identifying a potent 20-mer PS oligonucleotide (UL36 ANTI) complementary to the splice donor site of UL 36, an immediate early gene that was identified as essential for HCMV DNA origin of replication-dependent synthesis (Smith and Pari, 1995; Pari *et al.*, 1995). Sequence specificity of the antiviral activity, as measured by inhibition of viral DNA replication, was established by comparison of the efficacy of UL 36 ANTI [50% effective concentration ( $\text{EC}_{50}$ ) of 0.06  $\mu\text{M}$ ], with sense, reverse, and unrelated sequences ( $\text{EC}_{50}$  values in excess of 0.4  $\mu\text{M}$ ) (Fig. 6). Mismatches in the UL 36 sequence reduced activity. The gene target specificity was shown by Northern blots indicating the inhibition of UL 36 transcript, with



**FIGURE 6** UL36 ANTI ( $\blacktriangle$ , antisense oligonucleotide targeted to the UL 36 intron-exon boundary in unspliced RNA) inhibited production of HCMV UL 44 antigen as determined by ELISA of HCMV-infected human foreskin fibroblast cells. A nonspecific control oligonucleotide ( $\blacksquare$ ) did not inhibit antigen production (Pari *et al.*, 1995).



**FIGURE 7** UL36 ANTI selectively inhibits UL 36 mRNA. Northern analysis of RNA from oligonucleotide-treated cells shows that UL 36 ANTI decreased UL 36 RNA levels with no effect on either IE 1 RNA (A) or IE 2 RNA (B). Nonspecific control oligonucleotide did not inhibit any RNA production. Ribosomal RNA (18S) levels were constant throughout the experiment (Pari *et al.*, 1995).

no effect on the expression of either IE 1 or IE 2 (Fig. 7). UL 36 ANTI also reduced infectious virus yield by greater than 99% at 0.08  $\mu\text{M}$ . Thus, a novel antiviral target was identified using the antisense oligonucleotide approach, resulting in a potent and selective antiviral agent.

For future clinical evaluations, the UL 36 ANTI sequence has been investigated using chemical modifications described previously in this chapter. Preclinical evaluations are currently ongoing using a hybrid that is phosphorothioate at each nucleotide and that also contains 2'-OCH<sub>3</sub> substitutions at two nucleosides on the 5' end and at four nucleosides on the 3' end. This compound has been identified as GEM 132 (Fig. 8) and combines the potency embodied in the UL 36 ANTI oligonucleotide sequence with the enhanced stability of a hybrid, providing the potential for systemic and intravitreal use. Its initial entry into clinical studies as a systemic therapy occurred in August 1996. A phase I/II study to assess GEM 132 for intravitreal treatment of HCMV retinitis has been approved and studies are underway.

### 3. Gammaherpesviruses: Epstein-Barr Virus

Epstein-Barr virus is the causative agent for most cases of infectious mononucleosis, an acute infection associated with active virus replication

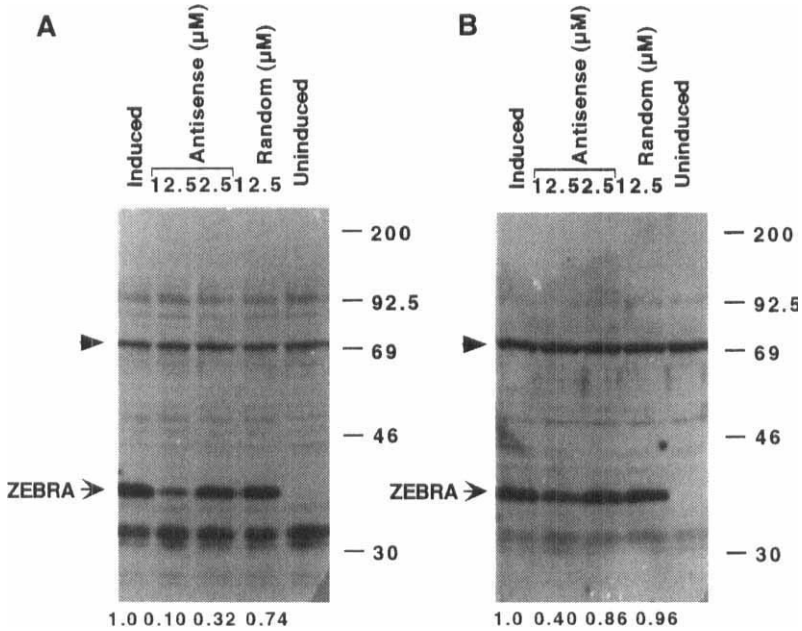


**FIGURE 8** GEM 132 chemical structure. The underlined bases are 2'-O-methyl-modified ribonucleotides; others are deoxyribonucleotides. All nucleotides are phosphorothioates.

(Pagano, 1995). Epstein–Barr virus infection can also result in latent infection, which can result in polyclonal B cell lymphoproliferative diseases in immunocompromised individuals and nasopharyngeal carcinoma, which is relatively common in southeast China (Huang, 1991). Among the genes expressed during latency and reactivation to productive infection, two have been the subject for design and evaluation of antisense oligonucleotides. These are the Epstein–Barr nuclear antigen EBNA 1 gene, which is required for maintenance of transformation of latently infected cells, and the BZLF gene, which is required to reactivate latently infected cells to virus-productive infection (Pagano, 1995).

Pagano *et al.* studied the potential for both unmodified and PS 18-mer antisense oligonucleotides as inhibitors of EBNA 1 (Pagano *et al.*, 1992). The oligonucleotides were targeted against the coding region just 3' of the AUG. Prolonged treatment of Raji cells, which carry 60 EBV episomes per cell, with 40  $\mu\text{M}$  unmodified antisense oligonucleotide was reported to result in a progressive reduction of EBNA 1 proteins determined by Western blots and in EBV copy number determined by Southern blots. Similar treatment with the sense oligonucleotide control was reported to be ineffective. Roth *et al.* (1994) also used unmodified oligonucleotides (40  $\mu\text{M}$ ) complementary to sequences in the EBNA 1 RNA and observed inhibition of EBNA 1 translation and inhibition of cell proliferation, with no effect on proliferation of EBV-negative cell growth. When PS oligonucleotides were used by Pagano in dose–response evaluations of growth inhibition of EBV-transformed cord blood B cells, control sense and scrambled sequences were reported to be partially effective as inhibitors of cell proliferation, while antisense oligonucleotides were reported to suppress growth totally at 20  $\mu\text{M}$ , and was partially effective at 5  $\mu\text{M}$  (Pagano *et al.*, 1992). Thus, as demonstrated in other virus infections, PS oligonucleotides may have a nonspecific inhibitory effect, and indeed this has been shown in the studies by Yao *et al.* (1993). They described potent inhibition ( $\text{EC}_{90}$  0.5  $\mu\text{M}$ ) of EBV yield from H1 cells, a chronically infected high-producer cell line, using PS 28-mer without sequence specificity for any EBV target. The mechanism of inhibition may be the result of blocking DNA synthesis, as was also described previously for the anti-HSV nonspecific oligonucleotide efficacy.

Sequence-specific and target-specific antisense inhibition of BZLF 1 expression was described by Daibata *et al.* (1996). Akata cells are latently infected with EBV and are inducible to the lytic viral cycle, a function that requires BZLF 1 expression of the Zebra protein. Both unmodified and PS 25-mer oligonucleotides complementary to the translation initiation codons inhibited the production of Zebra (as determined by Western blots) and replication of virus [shown by lack of production of the replicative linear DNA, viral early antigen (EA-D), and virus capsid antigen (VCA)]. Control sense, reverse sequence, and random oligonucleotides were less effective, and at the effective concentrations of antisense oligonucleotide no inhibition



**FIGURE 9** Immunoblot analysis of Zebra induction in antisense oligonucleotide-treated Akata cells. Antisense oligonucleotides targeted to the translation start site of Zebra inhibited Zebra production; a random mix of 20-mer oligonucleotides was much less potent. Inhibition was quantitated relative to induced Akata cells induced for EBV expression by stimulation with anti-IgG and normalized to a 72-kDa protein (see arrowhead). Phosphorothioate antisense oligonucleotides (A) and phosphodiester oligonucleotides (B) both inhibited Zebra induction. Reprinted from *Antiviral Res.* 29, Daibata, M., Enzinger, E. M., Monroe, J. E., Kilkuskie, R. E., Field, A. K., and Mulder, C., Antisense oligodeoxynucleotides against the BZLF1 transcript inhibit induction of productive Epstein-Barr virus replication, 243-260. Copyright 1996 with kind permission from Elsevier Science-NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands.

of cellular DNA synthesis or expression of CD19 (a B cell membrane protein) was apparent. Similar inhibition of virus production was observed in P3HR-1 cells, an EBV producer cell line, albeit after a prolonged antisense treatment. Thus, by the criteria of sequence and gene target specificity, these oligonucleotides designed to inhibit expression of the BZLF 1 gene and inhibit virus replication are probably functioning by an antisense mechanism, although nonspecific effects may also occur (Fig. 9 and Table I).

## C. Myxoviruses and Paramyxoviruses

### I. Influenza Viruses

Influenza viruses are enveloped virions with segmented single-stranded RNA genomes, and are the causative agents for epidemic acute respiratory

**TABLE 1** Inhibition of Productive Epstein–Barr Virus Replication by Anti-BZLF1 Antisense Oligonucleotides in Anti-IgG-Stimulated Akata Cells<sup>a</sup>

Oligonucleotide	Mean $\pm$ SE (% inhibition)			
	0.5 $\mu$ M	2.5 $\mu$ M	12.5 $\mu$ M	25 $\mu$ M
Phosphorothioate				
Antisense	35 $\pm$ 6 (3) <sup>b</sup>	48 $\pm$ 13 (3)	68 $\pm$ 9 (4)	69 $\pm$ 5 (4)
Random	5 $\pm$ 5 (3)	21 $\pm$ 10 (3)	24 $\pm$ 9 (4)	32 $\pm$ 10 (4)
Sense <sup>c</sup>			11 (1)	36 (1)
Reverse <sup>d</sup>			17 (1)	36 (1)
Phosphodiester				
Antisense	16 $\pm$ 13 (3)	25 $\pm$ 13 (3)	46 $\pm$ 10 (3)	56 $\pm$ 8 (3)
Random	14 $\pm$ 14 (3)	14 $\pm$ 7 (3)	21 $\pm$ 3 (3)	19 $\pm$ 6 (3)

<sup>a</sup> Reprinted from *Antiviral Res.* 29, Daibata, M., Enzinger, E. M., Monroe, J. E., Kilkuskie, R. E., Field, A. K., and Mulder, C., Antisense oligodeoxynucleotides against the BZLF1 transcript inhibit induction of productive Epstein–Barr virus replication, 243–260. Copyright 1996 with kind permission from Elsevier Science-NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands. Oligonucleotide-treated Akata cells were stimulated with anti-IgG for 24 hr. Inhibition was measured by the amounts of linear EBV DNA from gel analysis.

<sup>b</sup> The number of experiments (*n*) is listed in parentheses.

<sup>c</sup> Sense is the sequence complementary to the antisense.

<sup>d</sup> Reverse is the sequence with the identical nucleotide sequence as antisense but read in the opposite direction.

illness and severe disease. The negative-stranded RNA genome of influenza A and B viruses contains eight segments, and acts as a template for viral mRNA synthesis as well as synthesis of the antigenomic (positive-stranded) RNA intermediate (Lamb and Webster, 1996). The negative-stranded genome is packaged into virions along with the RNA-dependent RNA polymerase. Viral replication is a complex process; viral mRNA synthesis and replication occurs in the nucleus of infected cells. mRNA synthesis initiates with host cell-derived primers containing 5'-methylated capped RNA fragments from cellular RNA polymerase II transcripts. Four viral proteins are required for mRNA synthesis: NP, which is the major structural protein of the nucleocapsid, and the PB1, PB2, and PA proteins, which are polymerase proteins. Each of these proteins is encoded by a separate segment of the viral genome (Lamb and Webster, 1996), and each is an attractive target for antiviral inhibition.

Current antiviral therapies against influenza include amantidine and rimantidine, which act by blocking the ion channel formed by the M2 protein. This ion channel is pH activated and is required for uncoating of the viral nucleocapsid after virus infection. Amantidine and rimantidine are partially effective, especially when given as a prophylactic drug, but resistant virus variants can readily arise (Belshe *et al.*, 1988). More recently, specific inhibitors of the viral neuraminidase activity (required for virus binding

to cells) have been designed. Although this approach appears promising, additional therapies are required. Annual immunization has also been effective for high-risk populations; however, long-term immunization has not been possible due to antigenic variation from epidemic season to season.

Oligonucleotides can be effective anti-influenza agents in cell culture assays. Phosphorothioate oligonucleotides targeted against the viral RNA or mRNA of the PB1 gene inhibited the replication of influenza A and influenza C viruses (Leiter *et al.*, 1990). For influenza C virus, a PS oligonucleotide targeted against the viral RNA of the PB1 gene inhibited plaque formation by >90% at 20  $\mu\text{M}$  and caused a  $10^6$ -fold reduction in infectious virus at 80  $\mu\text{M}$ . A control oligonucleotide, containing one mismatch, was much less active, inhibiting plaque formation by about 50% at 20  $\mu\text{M}$ . Oligonucleotides containing three mismatches or lacking influenza sequence did not inhibit plaque formation at 20  $\mu\text{M}$ . Although this sequence-specific effect was observed, the active oligonucleotide contained a G quartet and mismatches eliminated this motif. It is not known whether the G quartet contributes to additional nonantisense mechanisms. Another study employed phosphodiester oligonucleotides modified at the 5' end with a hydrophobic group, *n*-undecanol (Kabanov *et al.*, 1990). A modified 10-mer targeted at the polymerase III gene was reported to inhibit influenza plaque formation at >10  $\mu\text{M}$ , whereas an unmodified 10-mer and a modified nonsense 10-mer were ineffective. The mechanism of inhibition, although apparently sequence specific, was not elaborated further. This is important since in addition to potential antisense inhibition, oligonucleotides have also been used as inhibitors of influenza virus RNA polymerase *in vitro* (Chung *et al.*, 1994). Short (<9-mer) oligonucleotides containing 5' cap structures (m7pppGm) bind *in vitro* to viral polymerase with high affinity and inhibit cap-dependent transcription. The antiviral effect of these oligonucleotides was not measured.

Clearly, sequence-specific inhibition of influenza virus replication is well documented, but whether this antiviral activity is the result solely of an antisense mechanism of action, or includes an unrelated inhibition of polymerase or adsorption/penetration or a combination of effects, is not entirely clear.

## 2. Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) causes severe lower respiratory tract disease in infants, young children, and immunocompromised adults (Collins *et al.*, 1996). Respiratory syncytial virus is an enveloped virus, containing an unsegmented, negative-stranded genome of approximately 15,000 nucleotides (Collins *et al.*, 1996). The genome encodes 10 viral proteins, which are translated from individual mRNAs. These RNAs are transcribed from the negative-stranded genome by the viral polymerase complex (N, P, and L proteins). In addition, this complex replicates the negative-stranded genome

from a positive-stranded intermediate for insertion into progeny virions. Replication occurs in the cytoplasm of the infected cells.

Currently, ribavirin is used therapeutically for RSV disease (Groothuis, 1994; Levin, 1994). Ribavirin is believed to inhibit RSV replication by several potential mechanisms: inhibition of viral polymerase, inhibition of 5' cap formation of mRNAs, and inhibition of IMP dehydrogenase, which decreases intracellular GTP levels (Gilbert and Knight, 1986). Which is the dominant mechanism of antiviral activity is uncertain. Unfortunately, clinical benefits from ribavirin are small and occur only in a portion of RSV-infected individuals (Levin, 1994). Clearly, there remains a need for development of effective therapeutics for RSV disease.

Oligodeoxyribonucleotides targeted against the genomic RNA inhibit RSV replication in cell culture by an apparent antisense mechanism. HEp-2 cells were infected with RSV strain A2 in the presence of oligonucleotides, with replication measured by enzyme immunosorbent assay (ELISA) or virus yield assay (Jairath *et al.*, 1997). Using ELISA,  $EC_{50}$  values were about 0.5–1  $\mu M$  for an antisense oligonucleotide targeted to the start of the NS2 gene. In all assays, the antisense oligonucleotide was more potent than (1) a control oligonucleotide containing the reverse sequence, (2) oligonucleotides targeted at RSV mRNA, (3) a random sequence oligonucleotide, and (4) ribavirin (Table II). Importantly, sequence-specific depletion of the genomic target following treatment of cells with the antisense oligonucleotide was demonstrated by reverse transcriptase-polymerase chain reaction (RT-PCR) (Fig. 10). Specific cleavage of the genomic target RNA has been detected at the antisense oligonucleotide-binding site, suggesting that cellular RNase H participates in the reaction. These observations provide *in situ*

**TABLE II** Respiratory Syncytial Virus Inhibition by Oligonucleotides<sup>a</sup>

Compound	$EC_{50} \pm SD$ ( $\mu M$ , F antigen)	$EC_{99} \pm SD$ ( $\mu M$ , infectious virus)
v590 (antisense)	0.64 $\pm$ 0.7 (12) <sup>b</sup>	6.6 $\pm$ 3.7 (6)
v590s (scrambled)	2.4 $\pm$ 2.1 (11) <sup>c</sup>	26 $\pm$ 8.9 (5) <sup>d</sup>
r20 (random mix of 20-mers)	17 $\pm$ 13 (10)	>30 (5)
Ribavirin	13 $\pm$ 5.7 (12) <sup>e</sup>	27 $\pm$ 6.2 (6) <sup>f</sup>

<sup>a</sup> Reprinted from *Antiviral Res.* 33, Jairath, S., Brown Vargas, P., Hamlin, H. A., Field, A. K., and Kilkuskie, R. E., Inhibition of respiratory syncytial virus replication by antisense oligodeoxyribonucleotides, 201–213. Copyright 1997 with kind permission from Elsevier Science-NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands.

<sup>b</sup> Number of experiments.

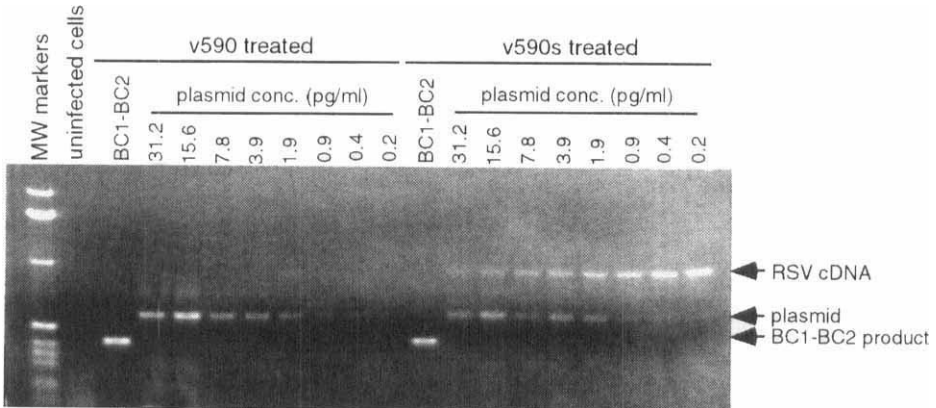
<sup>c</sup>  $p = 0.02$  vs v590.

<sup>d</sup>  $p = 0.006$  vs v590.

<sup>e</sup>  $p = 0.001$  vs v590.

<sup>f</sup>  $p = 0.0001$  vs v590.





**FIGURE 10** Quantitation of genomic RSV RNA. RSV cDNA was prepared by reverse transcription. The PCR was conducted using primers OD1 and BC6 to generate a 940-base pair product from the RSV cDNA. Serial dilutions of a competitive plasmid template that generated a 575-base pair product from the same primers were included with a constant amount of RSV cDNA to determine the concentration of RSV RNA present after oligonucleotide treatment. Cells treated with a scrambled control oligonucleotide (v590s) contained a measurable RSV cDNA PCR product. In cells treated with an antisense oligonucleotide (v590) no RSV cDNA PCR product was detected. Reprinted from *Antiviral Res.* 33, Jairath, S., Brown Vargas, P., Hamlin, H. A., Field, A. K., and Kilkuskie, R. E., Inhibition of respiratory syncytial virus replication by antisense oligodeoxyribonucleotides, 201–213. Copyright 1997 with kind permission from Elsevier Science-NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands.

evidence that the oligonucleotide, which was designed to bind to the target genome RNA, did trigger cleavage of the heteroduplex. This is precisely what is hoped for from an antisense mechanism of action.

However, the antisense oligonucleotide contains a 4-G quartet, and in some other systems four consecutive guanylic acid residues contribute to nonantisense, sequence-specific inhibition of viruses or other cellular functions (Wyatt *et al.*, 1994; Burgess *et al.*, 1995). The RSV antisense oligonucleotide was about fourfold more active than other oligonucleotides containing 4 Gs, including a control reversed sequence (Table II). This suggests that the four Gs may contribute significantly to the antiviral activity of the oligonucleotide, but are not the predominant antiviral factor. Thus, antisense oligonucleotides targeted against RSV genomic RNA can effectively inhibit RSV replication and may have therapeutic value.

#### D. Hepadnaviruses

Hepatitis B virus (HBV) is a member of the hepadnavirus family, which also includes hepatitis viruses of the Pekin duck, the heron, the woodchuck, and the ground squirrel. Worldwide, more than 400 million people are

infected with HBV, which causes acute (self-limiting) or chronic infection of the liver in the infected individual. Acute infections are characterized by the appearance of neutralizing antibodies, while chronically infected individuals continuously shed virus into the blood. Epidemiologic studies have linked chronic infection with hepatocellular carcinoma. While interferon  $\alpha$  has been used to treat chronic hepatitis, it has been only partially successful in suppressing virus shedding and in providing elimination of chronic infection (Locarnini and Cunningham, 1995). While other potential therapeutics such as lamivudine are in clinical evaluation (Dienstag *et al.*, 1995), the need for additional options in therapy has provided impetus to identify novel inhibitors for novel targets.

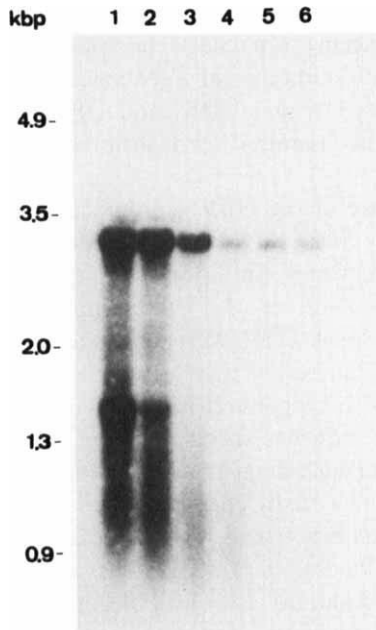
Hepatitis B virus is a compact DNA virus (3.2 kb) that contains four open reading frames encoding (1) three envelope or surface antigens, (2) two nucleocapsid (HBcAg and HBeAg) proteins, (3) the polymerase (P) gene product, and (4) the X gene product. The genome is transcribed to produce two predominant transcripts (3.5 and 2.1 kb) and two minor transcripts (2.5 and 0.7 kb). In addition to the open reading frames, HBV contains novel genetic targets for oligonucleotides. The encapsidation signal is a short (~90 base) RNA sequence that has well-defined secondary structure. This sequence is repeated at both ends of the pregenomic RNA and is recognized by viral proteins to package the viral RNA into nucleocapsids. Also, reverse transcription initiates at a sequence within this encapsidation signal. Other HBV RNA regions (DRI and DRII) are recognized by the HBV polymerase and are required for translocation of the enzyme during reverse transcription.

The complex nature of the HBV genome and its replication provides opportunity to identify antisense oligonucleotides with anti-HBV activity. Several groups have reported inhibition by targeting translation of HBV structural proteins (Goodarzi *et al.*, 1990; Blum *et al.*, 1991). A series of oligonucleotides against HBV were evaluated using a hepatocellular carcinoma-derived cell line stably transfected with HBV DNA (Hep2.2.15; Korba and Gerin, 1995). Oligonucleotides were reported to inhibit HBV virion production in a sequence-specific fashion. For oligonucleotides targeted against transcripts encoding structural proteins (S gene and C gene), HBV virion inhibition reportedly correlated well with inhibition of protein production. Antigen inhibition was also reported to be sequence specific; for example, oligonucleotides targeted against the S gene inhibited only HBsAg production, and had no effect on HBeAg or HBcAg levels. Interestingly, oligonucleotides targeted against the encapsidation sequence were reported to be among the most potent inhibitors identified in these experiments. These oligonucleotides inhibited virion production and intracellular HBV DNA replication, which is consistent with the requirement of this region in packaging viral RNA and viral DNA replication. None of the

oligonucleotides decreased the level of any HBV RNA species, suggesting that RNase H did not contribute to the antiviral activity in this assay system.

In a different study, a phosphorothioate oligonucleotide targeted against the duck hepatitis B virus pre-S gene inhibited virus replication *in vivo* (Offensperger *et al.*, 1993). This oligonucleotide was the most active of several oligonucleotides that were evaluated in cell culture. *In vivo*, virus replication was measured by the presence of viral DNA replicative intermediates in liver, as well as the presence of viral antigens (surface antigen and core antigen) in livers. *In vivo* inhibition was determined to be dose dependent and sequence specific. A dose of 20 mg/kg for 10 days caused a marked decrease in HBV DNA replication and a reduction in viral antigens in serum and liver, while 5 mg/kg had little effect on HBV DNA or antigen. Sense and random oligonucleotides did not inhibit virus production. These important results represent the first observation of antisense oligonucleotide inhibition of a virus infection *in vivo* (Fig. 11).

HBV X protein (HBx), which is a transactivator, was also reported to be targeted successfully by antisense oligonucleotides *in vivo* (Moriya *et al.*,



**FIGURE 11** Antisense oligonucleotide (AS2, targeted against HBV pre-S mRNA) inhibited DHBV replication *in vivo*. DNA was isolated from livers of DHBV-infected ducks and analyzed by Southern blot. Ducks were treated for 10 days with daily intravenous injections of AS2 at concentrations of 5  $\mu\text{g}$  (lane 2), 10  $\mu\text{g}$  (lane 3), or 20  $\mu\text{g}$  (lanes 4–6) per gram of body weight. Lane 1 is sample from an untreated control duck (Offensperger *et al.*, 1993).

1996). Transgenic mice expressing HBx developed liver lesions at the age of 2 months and liver tumors after 12 months. According to the authors, when treated with PS oligonucleotides targeted at the HBx translation start site intraperitoneally for 8 weeks, HBx production was inhibited as measured by RNA and protein production. In addition, hepatic lesions were reduced. The inhibition was reported to be sequence specific, in that a sense control did not inhibit; also, an oligonucleotide targeted within the HBx coding region was inactive. Subject to future positive studies, HBx therefore, appears potentially to be a useful target in HBV.

Treatment of liver disease may be enhanced by targeting oligonucleotides to hepatocytes, the host cells for hepatitis virus replication. In two studies, oligonucleotides conjugated to asialoorosmucoid were reported to be taken up by cells containing the asialoglycoprotein receptor (Wu and Wu, 1992; Bunnell *et al.*, 1992). The conjugates reportedly attained ~5- to 10-fold greater cell association than did the unconjugated oligonucleotide, and this increase in uptake depended on the presence of the asialoglycoprotein receptor. In both studies, the reported increase in uptake resulted in antisense inhibition of the target gene. Antisense oligonucleotide complexes specifically decreased the production of HBV antigen in the medium of Hep2.2.15 cells by 80–90%; random oligonucleotide had no effect on virus production. In this report, uncomplexed antisense oligonucleotide reduced HBV antigen production by only 30–40%. This is in contrast to the observations of Korba and Gerin, who described uncomplexed phosphorothioates as effective inhibitors of HBV replication (Korba and Gerin, 1995). The differences in these results may be due to the oligonucleotide sequences used by the investigators. These results, especially the *in vivo* studies on duck hepatitis virus, show that antisense oligonucleotides can provide effective therapy against hepatitis B infection. Phosphorothioate oligonucleotides readily attain high concentrations in the liver (Crooke *et al.*, 1996; Agrawal and Tamsamani, 1996), and the opportunity to target hepatocytes with the antisense oligonucleotide may provide a unique advantage for therapy.

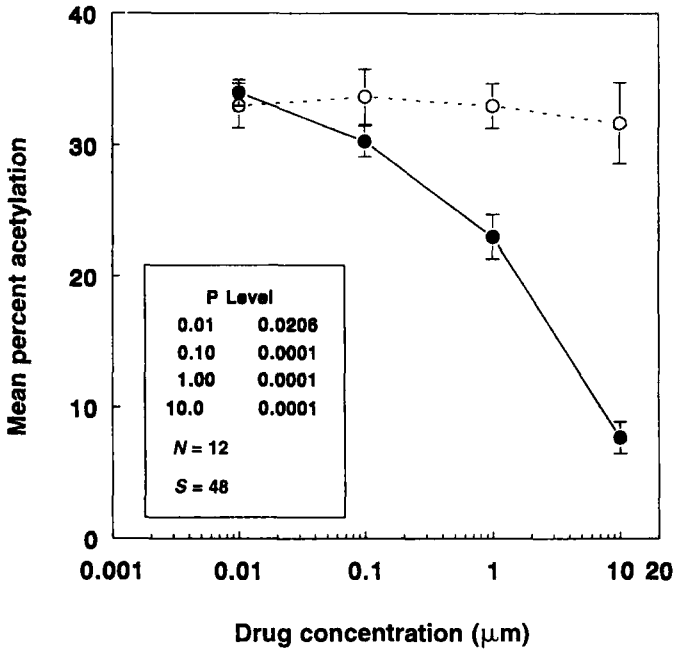
## E. Human Papillomaviruses

Human papillomaviruses (HPVs) include at least 65 types, based on DNA sequence diversity as measured by liquid hybridization. They infect epithelial cells at mucocutaneous surfaces, resulting in lesions from benign warts to cervical carcinoma, with each virus type having a specific anatomical site of replication. Several HPV types infect genital epithelia and represent the most prevalent etiologic agents of sexually transmitted viral disease. The genital HPV types can be further subdivided into “high-risk” types that are associated with the development of neoplasms (most commonly HPV-16 and HPV-18) and “low-risk” types that are rarely associated with malignancy (most commonly HPV-6 and HPV-11) (Shah and Howley, 1996).

The genome of all the HPV types is circular double-stranded DNA of approximately 7900 base pairs. The genome can be divided into three distinct functional domains; the upstream regulatory region, which contains the origin of viral DNA replication plus enhancers and promoters involved in transcription; the E region, which encodes genes required for vegetative functions; and the L region, which encodes the structural proteins L1 and L2. The polypeptides expressed from the E region have several functions: E1 is required for episomal DNA replication; E2 controls transcription and also may be involved in initiation of DNA replication; E4 interacts with cytokeratins; and E5, E6, and E7 are involved in cell transformation. The viral proteins are translated from families of alternatively spliced mRNAs (Howley, 1996).

Current therapies for treating HPV infections involve excision or destruction of infected tissue, rather than inhibition or reversal of the viral directed tumorigenesis. Even though viral replication and transformation are reasonably well understood, the use of that knowledge to identify selective new antiviral drugs has been lacking. This situation provides a rich opportunity for rational design through antisense, and antisense oligonucleotides designed to inhibit viral replication or inhibit cellular transformation have been evaluated. One compound, ISIS 2105, has been targeted at the HPV E2 mRNA translation initiation region of HPV-6 and HPV-11 (Cowsert *et al.*, 1993). They reported that cell culture studies using bovine papillomavirus (BPV) as a model system showed that oligonucleotides targeted at E2 could inhibit both E2-dependent transactivation in a transient assay and BPV-induced transformed cell focus formation, suggesting that the E2 gene is an appropriate target for antiviral therapy. Because HPV does not replicate in cell culture, further studies used a second model system in which E2-dependent transactivation of a reporter gene (chloramphenicol acetyltransferase) was measured. ISIS 2105 was reported to inhibit reporter expression, with an  $EC_{50}$  of approximately  $5 \mu M$  (Fig. 12). The inhibition was said to be sequence specific in that a control oligonucleotide targeted against the BPV E2 translation start site reportedly did not inhibit at concentrations up to  $10 \mu M$ . These studies led to Phase I and II clinical trials for treatment of genital warts with ISIS 2105; however, these trials were discontinued by the sponsor, ISIS Pharmaceuticals (Carlsbad, CA).

A combined effort of Hybridon (Cambridge, MA) and Roche Research Center (Welwyn-Garden City, UK) has resulted in the identification of sequence-specific and gene target-specific antisense oligonucleotides active in cell culture assays and in a mouse xenograft model of human papillomavirus replication (Roberts *et al.*, 1997; Lewis *et al.*, 1997). The antisense PS oligonucleotides targeted against HPV type 6 and 11 E1 gene selectively reduced E1 mRNA levels in cell culture. A modified version of the same sequence (containing 2'-OCH<sub>3</sub> nucleosides in addition to the PS backbone) significantly reduced growth of HPV-infected cysts in mice, while a mis-



**FIGURE 12** Inhibition of HPV-11 E2-dependent transactivation by an antisense oligonucleotide (ISIS 2105, ●). Chloramphenicol acetyltransferase (CAT) expression from a plasmid vector containing E2-dependent sequences was used to measure E2 transactivation. C127 cells were treated with oligonucleotide, then transfected with HPV-11 expression construct, HPV-11 E2-dependent CAT expression reporter. Analysis of variance on acetylated chloramphenicol was performed from four separate experiments. A control oligonucleotide (ISIS 2324, ○) did not inhibit CAT expression (Cowser *et al.*, 1993).

matched control oligonucleotide did not decrease cyst growth. Histologically, the antisense oligonucleotide-treated cysts carried fewer koilocytes, suggesting that antisense treatment decreased viral load. These findings will be further explored for the potential development of antisense oligonucleotide therapy for genital warts.

In several studies, the genes responsible for cellular transformation, E6 and E7, have been targeted with antisense oligonucleotides. Most studies have used cervical carcinoma-derived cell lines that contain HPV E6 and/or E7 and have measured growth inhibition to assess antisense oligonucleotide activity. In early experiments, plasmids expressing HPV antisense RNA against HPV18 E6 and/or E7 altered growth characteristics of HeLa cells (Steele *et al.*, 1992). Antisense expression was regulated by the inducible mouse mammary tumor virus (MMTV) promoter; induction resulted in a slowing of the growth rate, decrease in growth in soft agar, and an increase in serum requirement. Vero cells, which do not contain HPV sequences, were not effected by transfection with the antisense plasmids. Also, plasmids

expressing the sense constructs of E6 and E7 did not change growth characteristics of HeLa cells. All these characteristics suggested that expressed antisense RNA caused a decrease in E6 and/or E7 expression and an alteration of the transformed phenotype of the HeLa cells.

Similarly, HPV-specific PS oligonucleotides inhibited CaSki cell growth, which is dependent on HPV E6/E7 expression, by as much as 80–90% at concentrations between 10 and 20  $\mu\text{M}$  (Storey *et al.*, 1991). This effect was apparently sequence specific since only two oligonucleotides, targeted to the overlapping translation start sites for HPV E6 or HPV E7, were effective. Six HPV-specific oligonucleotides targeting other regions of the E6 or E7 genes, as well as a random sequence oligonucleotide, did not inhibit growth. However, there was no significant decrease in HPV E6 or E7 mRNA or protein, suggesting that the mechanism of inhibition, although selective, was not antisense.

Subsequent studies using PS oligonucleotides targeted at similar regions of E6 and E7 did show apparent antisense effects (Tan and Ting, 1995). In these experiments, oligonucleotides were delivered to cells with a cationic lipid. Cell growth and E7 expression were reportedly specifically inhibited by antisense targeted to E7 RNA. Interestingly, this report also suggested that E7 expression was also inhibited by an E6-specific antisense oligonucleotide, possibly due to the inhibition of the bicistronic E6–E7 transcript. The antisense oligonucleotides also were reported to inhibit tumor growth by SiHa cells in nude mice. In addition, a reported decrease in E7 expression was measured in the tumor, with no effect on a control gene (actin). A control oligonucleotide, containing randomized sequence, was not reported to inhibit tumor growth or E7 expression. These *in vivo* results suggest that E7 inhibition may be an effective treatment for HPV-induced tumors.

## F. Picornaviruses

Picornaviruses are nonenveloped viruses containing a single positive-stranded RNA genome. These viruses comprise a large family of human and agricultural pathogens, including poliovirus, hepatitis A virus, and foot and mouth disease virus (FMDV). Translation of picornavirus RNA occurs through a novel cap-independent mechanism involving initiation from internal sites on the viral RNA, specifically recognized by ribosomal and other cellular proteins. Several of these viruses have been targets for antisense oligonucleotide inhibition, and the studies demonstrate the versatility of the antisense approach.

Encephalomyocarditis virus (EMCV) has been used as a model system to study translation initiation by internal ribosomal entry (Jang *et al.*, 1988, 1989). Antisense phosphodiester oligonucleotides (13-mers), targeted at the 5' untranslated region and the translation start site of EMCV, inhibited cell-free translation in rabbit reticulocyte lysates (Sankar *et al.*, 1989). Specificity was demonstrated by showing that an oligonucleotide containing a

single mismatch did not inhibit translation. The importance of the 5' untranslated region was confirmed; oligonucleotides targeted at the coding region or the 3' end of the genome did not inhibit translation. Although viral replication assays were not performed, the results in the cell-free translation system suggest that inhibition of internal ribosomal entry by oligonucleotides provides a novel target for virus inhibition.

Antisense oligonucleotides inhibited replication of FMDV (Gutiérrez *et al.*, 1993). High concentrations (125–250  $\mu\text{M}$ ) of phosphodiester oligonucleotides were microinjected into uninfected cells, which were then infected with FMDV. A modest reduction (35–50%) in the percentage of cells expressing FMDV antigen was detected 5 hr after infection. Sequence specificity was observed, since a scrambled control oligonucleotide was ineffective. Studies following the fate of fluorescein-labeled oligonucleotide indicated localization in the nucleus after microinjection; high concentrations apparently saturated this transport process, and oligonucleotide was then detectable in the cytoplasm, the site of viral replication. Phosphorothioate versions of the same oligonucleotide sequences inhibited infectious virus yield by about 50% when used at high concentrations (50–100  $\mu\text{M}$ ). Again, the effect appeared to be sequence specific since the scrambled control was ineffective. However, the modest effects seen in this study using high oligonucleotide concentrations suggest that the utility of antisense oligonucleotides against FMDV is probably quite limited.

## G. Coronaviruses

Coronaviruses are large, enveloped virions containing a positive-stranded RNA genome, and the human strains are responsible for acute respiratory disease. Mouse hepatitis virus (MHV), which causes respiratory and gastrointestinal infections as well as hepatitis, has been used as a model system to study coronavirus replication. The viral RNA-dependent RNA polymerase synthesizes a full-length negative-sense copy of the genome prior to copying a series of subgenomic mRNAs from the negative strand. These subgenomic mRNAs contain at their 5' ends a common leader sequence that is derived from the 5' end of the genome. Subgenomic RNAs are derived from discontinuous RNA synthesis from this leader. An antisense oligonucleotide (14-mer) complementary to the leader sequence was reported to inhibit MHV plaque formation when the oligonucleotide was present during infection (Mizutani *et al.*, 1994). In addition, viral RNA was decreased. The effect was reported to be sequence specific and selective; a sense oligonucleotide did not inhibit virus replication, and cellular RNA was not effected by the antisense oligonucleotide.

## H. Flaviviruses

The Flaviviridae consist of three groups: flaviviruses, pestiviruses, and hepatitis C. A wide variety of arthropod-borne virus infections of humans



is caused by flaviviruses, resulting in severe disease (encephalitis, high fever, or hemorrhagic fever). The virions of all flaviviruses are enveloped and encapsidate a positive-stranded RNA genome that is translated into a single polyprotein. The polyprotein, which is processed by both cellular and viral proteases to produce mature proteins, contains similar functional organization, with structural proteins at the N terminus and nonstructural proteins at the C terminus (Rice, 1995).

The dengue virus group contains four distinct serotypes. Each can cause a debilitating, but nonfatal, fever. More severe disease, dengue hemorrhagic fever, results from reinfection with a heterologous serotype presumably caused by antibody-mediated enhancement of virus replication in monocytic cells. Because of this phenomenon, vaccine approaches have been unsuccessful, and it may be especially difficult to protect against all four serotypes (Monath, 1994).

Antisense oligonucleotides targeted against the translation initiation region and also to the 3' untranslated region were reported to be effective inhibitors of dengue virus type 2 replication (Raviprakash *et al.*, 1995). Specific modification of the C-5 position of uridine and cytosine with propynyl groups was required before sequence-specific inhibition of viral antigen production was observed at concentrations less than or equal to 1  $\mu$ M. Phosphodiester and PS oligonucleotides were reported not to inhibit in a sequence-specific fashion. Interestingly, inhibition was detected only after microinjection of oligonucleotides, suggesting that the effective presentation of the propynyl-modified compound to the intact cell may be problematic.

Hepatitis C virus (HCV) is a positive-stranded RNA virus that also infects hepatocytes and causes acute and chronic hepatitis. Hepatitis C virus is the major cause of non-A, non-B hepatitis, and like HBV is associated with hepatocellular carcinoma.

The organization of the 9.5-kb HCV genome is similar to that of pestiviruses and flaviviruses, with structural proteins at the 5' end and nonstructural proteins at the 3' end (Houghton, 1996). The virus encodes a single polyprotein that is processed by viral and cellular proteases. Hepatitis C virus also contains short 5' and 3' untranslated regions (UTRs), the 5' UTR representing the most highly conserved region of the virus (Bukh *et al.*, 1992). This 5' UTR region facilitates internal ribosomal entry, so that translation does not occur by ribosomal scanning from the 5' RNA cap. Instead, ribosomes bind to internal secondary structures formed by the 5' UTR (Wang *et al.*, 1994). In addition, separate experiments have shown that HCV 5' UTR sequences can control translation of downstream sequences (Yoo *et al.*, 1992). The conserved nature of the sequence and its requirement for translation suggest that the 5' UTR potentially provides an excellent target for an antisense oligonucleotide antiviral agent.

Hepatitis C virus was only recently suggested to replicate in cell culture (Yoo *et al.*, 1995). Virus replication in a hepatoma cell line was detected

only by the presence of both positive- and negative-strand RNA by RT-PCR. The presence of virus antigen was not reported, and although the infection model may have potential for the future it is not available for antiviral studies. However, oligonucleotides have been evaluated as inhibitors of HCV replication in a T lymphocyte cell line (Mizutani *et al.*, 1995). Sequence-specific inhibition was measured by the presence (or absence) of HCV RNA in the cells, although quantitation of RNA and direct evidence of RNA replication were not presented. These limited data do not provide confidence that virus replication was inhibited and that the cell-based assay systems are going to provide model HCV infections.

Because of the limitations of the virus replication systems, novel model systems for evaluation of antisense oligonucleotides have been developed. *In vitro* translation from RNA containing HCV 5' UTR sequences has been used to screen oligonucleotides (Wakita and Wands, 1994). Sequence-specific oligonucleotides have been identified that were reported to inhibit translation in this artificial system; oligonucleotides near the translation start site were active in all assays. Other domains in the 5' UTR that contained oligonucleotide-sensitive sequences were also identified. In other assay systems, oligonucleotides have been transfected into cells along with plasmids expressing the HCV 5' UTR fused to a reporter gene (luciferase) (Alt *et al.*, 1995). As a control, luciferase expression was compared to production of an unrelated gene (hepatitis B surface antigen) transfected on a separate plasmid. Again, sequence-specific inhibition was reported for oligonucleotides targeted near the translation start site and also in the 5' UTR. Several oligonucleotides targeted to the core protein-coding sequence also inhibited protein production (Alt *et al.*, 1995).

Hepatitis C virus gene expression was reported to be inhibited in stably transfected hepatocytes. The most active oligonucleotides were again targeted to sequences in the 5' untranslated region, reinforcing the importance of this region for regulating HCV expression (Hanecak *et al.*, 1996). Phosphorothioate oligonucleotides were reported to inhibit RNA and protein production in a sequence-specific, length-dependent fashion. Interestingly, oligonucleotides with all 2'-OCH<sub>3</sub> residues were reported to inhibit protein production without an effect on HCV RNA.

In a similar study, sequence-specific interaction of oligonucleotides with the HCV 5' UTR was reported in cells stably transfected with HCV bases 52–1417 (P. Brown Vargas, H. A. Hamlin, B. Frank, D. Walther, A. K. Field, and R. E. Kilkuskie, unpublished observations). These cells expressed HCV C and E1 proteins; translation was controlled by the 5' UTR. When treated with individual oligonucleotides in the presence of cationic lipids, HCV RNA cleavage was detected using a ribonuclease protection assay. Cleavage was mapped to the oligonucleotide-binding site. Cleavage required RNase H, since treatment with oligonucleotides containing all 2'-OCH<sub>3</sub> residues did not result in RNA cleavage. Importantly, these results suggest

that PS oligonucleotides acted by an antisense mechanism through specific interaction with their target ribonucleotide sequence.

### I. Rhabdoviruses

Rhabdoviruses are a widespread family that includes rabies virus. Vesicular stomatitis virus (VSV) is a well-studied member of the rhabdovirus family, and causes flulike symptoms in infected humans. Vesicular stomatitis virus is an enveloped virus containing five proteins and a negative-stranded genome. Five individual messenger RNAs are transcribed from the genome. Novel modifications of oligonucleotides have been evaluated as anti-VSV agents. Psoralen-modified methyl phosphonate oligonucleotides were reported to bind specifically to VSV mRNAs *in vitro* (Levis and Miller, 1994). Specific cross-linking occurred for 16-mer oligonucleotides targeted to the N or M mRNAs. A shorter (12-mer) oligonucleotide designed to bind specifically to M mRNA was also reported to bind to N mRNA. The oligonucleotide was partially complementary (9 of 12 residues) to N mRNA; and N mRNA was the predominant RNA species in the mixture. The high degree of homology of different mRNAs in VSV makes specific oligonucleotide targeting more difficult. However, the ability to target more than one RNA with the same oligonucleotide could potentially improve potency of an antiviral compound. Antiviral activity of these psoralen-modified oligonucleotides was not reported.

In a separate study, a 15-mer oligonucleotide, conjugated at the 3' end to poly-L-lysine, reportedly inhibited VSV antigen synthesis and virus yield (Lemaitre *et al.*, 1987). The conjugated oligonucleotide, complementary to the 5' end of VSV N mRNA, reportedly inhibited virus production by >95% at 400 nM. Specificity was suggested by the lack of effect on the replication of a control virus, encephalomyocarditis virus, under the same conditions. Also, a second conjugated oligonucleotide, a 13-mer targeted to the coding sequence of N mRNA, had no effect on virus yield. Unconjugated oligonucleotides were not evaluated, so the effect of polylysine on delivery could not be addressed directly.

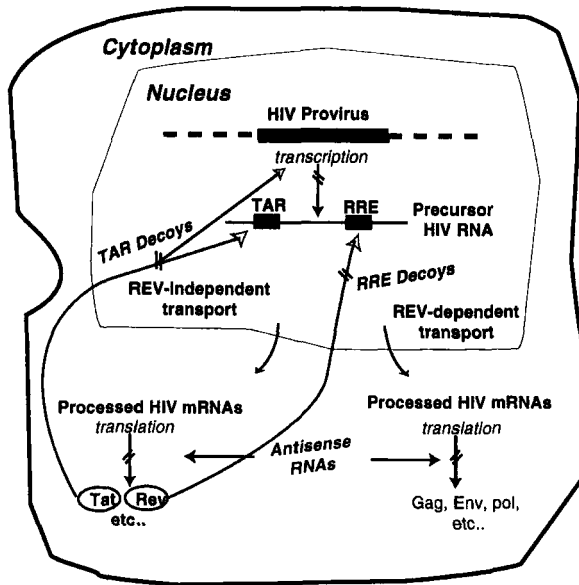
### IV. Antisense RNA, RNA Decoys, and Ribozymes \_\_\_\_\_

A second approach to developing an effective antisense antiviral agent is *in situ* transcription of antisense RNA as a selective blockade of virus replication. Antisense RNA has been used to define the activity of a gene product by observing the phenotypic effects of selective inhibition of translation. This approach was used to help define the roles of the human papillomavirus-encoded E6 and E7 transcripts in cervical carcinoma cells. Von Knebel Doeberitz and Gissmann demonstrated that expression of anti-

sense RNA following transfection into HPV-transformed cells reduced the number of surviving cells capable of colony formation (von Knebel Doeberitz and Gissmann, 1987). Similar transfection of the vector expressing the sense RNA did not alter colony formation, and transfection of the antisense RNA vector into control cells that did not carry HPV had no effect on cell growth.

The expression of antisense RNA has been successful for inhibition of mouse hepatitis virus (Mizutani *et al.*, 1994; Thieringer *et al.*, 1995), parvovirus (Ramirez *et al.*, 1995), polyoma virus (Liu *et al.*, 1994), simian immunodeficiency virus (Tung, 1994), human T lymphotropic virus type I (Fujita and Shiku, 1993), and measles virus (Koschel *et al.*, 1995). But not all antisense RNA studies have resulted in virus inhibition, or in readily interpretable results. Leiter *et al.* demonstrated that even with successful inhibition of influenza replication in cells expressing virus-specific RNA but not the vector, the inhibition probably resulted from the induction of interferon following the formation of double-stranded RNAs hybridized from the sense and antisense strands (Leiter *et al.*, 1989). Double-stranded RNA has been repeatedly confirmed as a potent inducer of interferon  $\alpha$ , and is a primary mechanism by which interferon is induced during virus infections (Marcus, 1994). In most studies the induction of interferons has not been checked, leaving suspect the direct role of the antisense mechanism in the antiviral activity resulting from vector-expressed antisense RNA; thus further emphasizes the need for extensive controls in such studies.

For HIV, several approaches (Fig. 13) have been taken to inhibit replication by RNA expression (Yu *et al.*, 1994). Transfection of cells to transcribe antisense RNA to the transactivation response (TAR) element (Chuah *et al.*, 1994), the *gag* region (Sczakiel and Pawlita, 1991), the *tat* and *rev* regions (Junker *et al.*, 1994; Sczakiel *et al.*, 1992), and the 5' leader region of HIV (Sun *et al.*, 1995a) conferred various degrees of protection to HIV challenge infection in cells in culture. An additional nonantisense approach has been the overexpression of TAR or the Rev response element (RRE) as decoys. This provides a means to inactivate the Tat and Rev proteins by alternate binding to the antiviral decoy. This approach has had some success as demonstrated by Bevec *et al.* (1994) and by Lori *et al.* (1994). In a single vector, Chang *et al.* combined the use of a TAR decoy and vector-expressed antisense RNA to the Tat mRNA both to block the activity of the Tat protein and block its translation (Chang *et al.*, 1994). Compared to the vector controls, the combined decoy and antisense approach inhibited 94 to 98% of the Tat activity. A combination of RNA decoy and antisense RNA was taken a step further by the construction of a vector that expresses both the polymeric TAR decoy and the antisense to *tat* (referred to as *antitit*). Lisiewicz *et al.* demonstrated that AIDS patient peripheral blood mononuclear cells transfected with the retroviral vectors carrying the *antitit* gene resisted HIV replication and expanded in culture, providing a possible



**FIGURE 13** HIV inhibition by antisense RNAs and decoys. Antisense RNAs prevent HIV mRNA translation. Alternatively, RNA decoys of TAR and RRE can bind and inactivate the Tat and Rev proteins.

avenue for *ex vivo* gene therapy to provide a competent and expanding pool of CD4<sup>+</sup> cells (Liszewicz *et al.*, 1995).

Ribozymes, which are catalytic RNAs, theoretically combine the specificity of an antisense molecule in binding to the complementary RNA target, with a catalytic core. This combination should provide a potent and selective antiviral agent, and expressed ribozymes are undergoing evaluation and refinement in many laboratories. Sarver *et al.* first demonstrated in 1990 the use of an expressed hammerhead ribozyme targeted to the *gag* sequence of HIV to increase the resistance of HeLa CD4<sup>+</sup> cells to HIV infection (Sarver *et al.*, 1990). Since then numerous publications using expressed hammerhead or hairpin ribozymes have appeared and the first clinical trials in AIDS patients should soon be at hand (Leavitt *et al.*, 1996). For a review of the antiviral ribozyme literature see Kijima *et al.* (1995). However, for the purpose of this chapter we highlight only certain key studies. Wong-Staal and colleagues have concentrated on developing potently active hairpin ribozymes that can inhibit HIV replication in peripheral blood lymphocytes and mononuclear cells derived from CD34<sup>+</sup> hematopoietic stem cells (Leavitt *et al.*, 1994; Yamada *et al.*, 1994a,b; Yu *et al.*, 1995). They have targeted the ribozymes at either the 5' leader sequence of HIV-1 or a conserved region of the HIV-1 *pol* gene, and expressed it from a retroviral vector. Hammerhead ribozymes have been utilized by numerous investigators (Ho-

mann *et al.*, 1993; Crisell *et al.*, 1993; Sun *et al.*, 1995b; Lo *et al.*, 1992) to target the *tat* gene or the LTR/*gag* region. Both specificity and the requirement of the catalytic activity to obtain optimal anti-HIV activity suggest that the ribozymes are functioning in a catalytic manner, and not merely by an antisense mechanism of action.

## V. Future Directions

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The aim of this chapter has been to increase awareness of both the opportunities and advances of antisense/gene therapy technology as a means to identify novel antiviral gene targets and to identify molecules with attractive therapeutic potential. Through the use of antisense oligonucleotides and antisense RNA, novel antiviral targets have been reported for HCMV, HIV, the hepatitis viruses and others; in many cases providing the proof of principle needed to launch a concerted effort for serious antiviral drug discovery and development. But in addition, the technology has already yielded antiviral drug candidates, with antisense clinical trials ongoing against HIV and HCMV and others in the preclinical stages of development. In parallel, the antisense, decoy, and ribozyme gene therapy approaches are also gaining credibility as potentially attractive means to enhance cellular resistance to HIV infection.

But where will all this lead? It is our belief that the success of one or more of the antisense therapeutic approaches that are now undergoing evaluation in the clinic will result in accelerated momentum toward developing an array of effective antisense therapeutic oligonucleotides. Viruses that have heretofore been unassailable by conventional therapy should be vulnerable to the antisense approach. And like any evolving field, both specific antisense mechanisms of action and nonspecific activities will be more precisely defined and appreciated for their contribution to the overall antiviral efficacy. In parallel, the identification of novel chemical modifications of the oligonucleotides will provide attractive pharmacological properties as the next generation of oligonucleotide drugs emerges. The end result should be the identification of antisense oligonucleotides that fulfill the promise of antisense—potently active antiviral agents that demonstrate high selectivity for the viral target by virtue of the sequence complementarity of antisense hybridization.

For antisense RNA, RNA decoys, and expressed ribozymes the problems are different. Here the issues concern controlled expression in the appropriate cell. Consequently, emphasis on inducible vector constructs, cell targeting, and RNA stability will be key to practical application.

There should be little doubt that regardless of the outcome of present efforts to show clinical efficacy, the field of genetic pharmacology has scored impressive gains and progress in the field is gaining momentum. This momen-

tum is introducing a new paradigm for antiviral target identification and antiviral drug discovery.

## References

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- Agrawal, S., and Tang, J. Y. (1992). GEM 91—an antisense oligonucleotide phosphorothioate as a therapeutic agent for AIDS. *Antisense Res. Dev.* **2**, 261–266.
- Agrawal, S., and Tamsamani, J. (1996). In “Molecular Medicine: Antisense Therapeutics” (S. Agrawal, ed.), pp. 247–270. Humana Press, Totowa, New Jersey.
- Agrawal, S., Ikeuchi, T., Sun, D., Sarin, P. S., Konopka, A., Maizel, J., and Zamecnik, P. C. (1989). Inhibition of human immunodeficiency virus in early infected and chronically infected cells by antisense oligodeoxynucleotides and their phosphorothioate analogues. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7790–7794.
- Alt, M., Renz, R., Hofschneider, P. H., Paumgartner, G., and Caselmann, W. H. (1995). Specific inhibition of hepatitis C viral gene expression by antisense phosphorothioate oligodeoxynucleotides. *Hepatology* **22**, 707–717.
- Anazodo, M. I., Wainberg, M. A., Friesen, A. D., and Wright, J. A. (1995). Sequence-specific inhibition of gene expression by a novel antisense oligodeoxynucleotide phosphorothioate directed against a nonregulatory region of the human immunodeficiency virus type 1 genome. *J. Virol.* **69**, 1794–1801.
- Anderson, K. P. (1994). Pre-clinical and clinical antiviral activity of an antisense oligonucleotide complementary to cytomegalovirus immediate early RNA. *Antivirals, Second Annual IBC Conference* (Abstr.).
- Austermann, S., Kruhoffer, M., and Grosse, F. (1992). Inhibition of human immunodeficiency virus type 1 reverse transcriptase by 3'-blocked oligonucleotide primers. *Biochem. Pharmacol.* **43**, 2581–2589.
- Azad, R. F., Driver, V. B., Tanaka, K., Crooke, R. M., and Anderson, K. P. (1993). Antiviral activity of a phosphorothioate oligonucleotide complementary to RNA of the human cytomegalovirus major immediate-early region. *Antimicrob. Agents Chemother.* **37**, 1945–1954.
- Azad, R. F., Brown-Driver, V., Buckheit, R. W., Jr., and Anderson, K. P. (1995). Antiviral activity of a phosphorothioate oligonucleotide complementary to human cytomegalovirus RNA when used in combination with antiviral nucleoside analogs. *Antiviral Res.* **28**, 101–111.
- Belshe, R. B., Smith, M. H., Hall, C. B., Betts, R., and Hay, A. J. (1988). Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. *J. Virol.* **62**, 1508–1512.
- Bevec, D., Volc-Platzer, B., Zimmermann, K., Dobrovnik, M., Hauber, J., Veres, G., and Böhnlein, E. (1994). Constitutive expression of chimeric *neo*-Rev response element transcripts suppresses HIV-1 replication in human CD4<sup>+</sup> T lymphocytes. *Hum. Gene Ther.* **5**, 193–201.
- Bishop, J. S., Guy-Caffey, J. K., Ojwang, J. O., Smith, S. R., Hogan, M. E., Cossum, P. A., Rando, R. F., and Chaudhary, N. (1996). Intramolecular G-quartet motifs confer nuclease resistance to a potent anti-HIV oligonucleotide. *J. Biol. Chem.* **271**, 5698–5703.
- Blum, H. E., Galun, E., Weizsacker, F., and Wands, J. R. (1991). Inhibition of hepatitis B virus by antisense oligodeoxynucleotides (letter). *Lancet* **337**, 1230.
- Boiziau, C., Thuong, N. T., and Toulmé, J.-J. (1992). Mechanisms of the inhibition of reverse transcription by antisense oligonucleotides. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 768–772.
- Bordier, B., Hélène, C., Barr, P. J., Litvak, S., and Sarih-Cottin, L. (1992). *In vitro* effect of antisense oligonucleotides on human immunodeficiency virus type 1 reverse transcription. *Nucleic Acids Res.* **20**, 5999–6006.

- Bowen, E. F., Atkins, M., Weller, I. V. D., and Johnson, M. A. (1995). In "Antiviral Chemotherapy" (D. J. Jeffries and E. De Clercq, eds.), pp. 65–80. John Wiley & Sons, New York.
- Buckheit, R. W., Jr., Roberson, J. L., Lackman-Smith, C., Wyatt, J. R., Vickers, T. A., and Ecker, D. J. (1994). Potent and specific inhibition of HIV envelope-mediated cell fusion and virus binding by G quarter-forming oligonucleotide (ISIS 5320). *AIDS Res. Hum. Retroviruses* 10, 1497–1506.
- Bukh, J., Purcell, R. H., and Miller, R. H. (1992). Sequence analysis of the 5' noncoding region of hepatitis C virus. *Proc. Natl. Acad. Sci. U.S.A.* 89, 4942–4946.
- Bunnell, B. A., Askari, F. K., and Wilson, J. M. (1992). Targeted delivery of antisense oligonucleotides by molecular conjugates. *Somatic. Cell Mol. Genet.* 18, 559–569.
- Burgess, T. L., Fisher, E. F., Ross, S. L., Bready, J. V., Qian, Y., Bayewitch, L. A., Cohen, A. M., Herrera, C. J., Hu, S. S.-F., Kramer, T. B., Lott, F. D., Martin, F. H., Pierce, G. F., Simonet, L., and Farrell, C. L. (1995). The antiproliferative activity of *c-myb* and *c-myc* antisense oligonucleotides in smooth muscle cells is caused by a nonantisense mechanism. *Proc. Natl. Acad. Sci. U.S.A.* 92, 4051–4055.
- Cantor, G. H., and Palmer, G. H. (1992). Antisense oligonucleotide inhibition of bovine leukemia virus tax expression in a cell-free system. *Antisense Res. Dev.* 2, 147–152.
- Chang, H.-K., Gendelman, R., Lisziewicz, J., Gallo, R. C., and Ensoli, B. (1994). Block of HIV-1 infection by a combination of antisense *tat* RNA and TAR decoys: A strategy for control of HIV-1. *Gene Ther.* 1, 208–216.
- Chen, T. Z., Lin, S. B., Wu, J. C., Choo, K. B., and Au, L. C. (1996). A method for screening antisense oligodeoxyribonucleotides effective for mRNA translation-arrest. *J. Biochem. (Tokyo)* 119, 252–255.
- Chuah, M. K. L., Vandendriessche, T., Chang, H.-K., Ensoli, B., and Morgan, R. A. (1994). Inhibition of human immunodeficiency virus type-1 by retroviral vectors expressing antisense-TAR. *Hum. Gene Ther.* 5, 1467–1475.
- Chung, T. D. Y., Cianci, C., Hagen, M., Terry, B., Matthews, J. T., Krystal, M., and Colonno, R. J. (1994). Biochemical studies on capped RNA primers identify a class of oligonucleotide inhibitors of the influenza virus RNA polymerase. *Proc. Natl. Acad. Sci. U.S.A.* 91, 2372–2376.
- Coffin, J. (1996). In "Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, eds.), 3rd Ed., pp. 1767–1847. Lippincott-Raven, Philadelphia.
- Collins, P. L., McIntosh, K., and Chanock, R. M. (1996). In "Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, eds.), 3rd Ed., pp. 1313–1351. Lippincott-Raven, Philadelphia.
- Cowsert, L. M., Fox, M. C., Zon, G., and Mirabelli, C. K. (1993). *In vitro* evaluation of phosphorothioate oligonucleotides targeted to the E2 mRNA of papillomavirus: Potential treatment for genital warts. *Antimicrob. Agents Chemother.* 37, 171–177.
- Crisell, P., Thompson, S., and James, W. (1993). Inhibition of HIV-1 replication by ribozymes that show poor activity *in vitro*. *Nucleic Acids Res.* 21, 5251–5255.
- Crooke, R. M., Hoke, G. D., and Shoemaker, J. E. (1992). *In vitro* toxicological evaluation of ISIS 1082, a phosphorothioate oligonucleotide inhibitor of herpes simplex virus. *Antimicrob. Agents Chemother.* 36, 527–532.
- Crooke, S. T., and Bennett, C. F. (1996). Progress in antisense oligonucleotide therapeutic. *Annu. Rev. Pharmacol. Toxicol.* 36, 107–129.
- Crooke, S. T., Graham, M. J., Zuckerman, J. E., Brooks, D., Conklin, B. S., Cummins, L. L., Greig, M. J., Guinasso, C. J., Kornblust, D., Manoharan, M., Sasmor, H., Schleich, T., Tivel, K. L., and Griffey, R. H. (1996). Pharmacokinetic properties of several novel oligonucleotide analogs in mice. *J. Pharm. Exp. Ther.* 277, 923–937.
- Daibata, M., Enzinger, E. M., Monroe, J. E., Kilkuskie, R. E., Field, A. K., and Mulder, C. (1996). Antisense oligodeoxynucleotides against the BZLF1 transcript inhibit induction of productive Epstein-Barr virus replication. *Antiviral Res.* 29, 243–260.
- De Clercq, E. (1995). Antiviral therapy for human immunodeficiency virus infections. *Clin. Microbiol. Rev.* 8, 200–239.



- Dienstag, J. L., Perillo, R. P., Schiff, E. R., Bartholomew, M., Vicary, C., and Rubin, M. (1995). A preliminary trial of lamivudine for chronic hepatitis B infection. *N. Engl. J. Med.* **333**, 1657-1661.
- Draper, K. G., Ceruzzi, M., Kmetz, M. E., and Sturzenbercker, L. J. (1990). Complementary oligonucleotide sequence inhibits both Vmw65 gene expression and replication of herpes simplex virus. *Antiviral Res.* **13**, 151-164.
- Falloon, J., and Masur, H. (1990). In "Antiviral Agents and Viral Diseases of Man" (G. J. Galasso, R. J. Whitley, and T. C. Merigan, eds.), 3rd Ed., pp. 669-690. Raven Press, New York.
- Fennewald, S. M., Mustain, S., Ojwang, J., and Rando, R. F. (1995). Inhibition of herpes simplex virus in culture by oligonucleotides composed entirely of deoxyguanosine and thymidine. *Antiviral Res.* **26**, 37-54.
- Field, A. K. (1994). In "Encyclopedia of Virology" (R. G. Webster and A. Granoff, eds.), pp. 42-49. Academic Press, London.
- Field, A. K., and Biron, K. K. (1994). "The end of innocence" revisited: Resistance of herpesviruses to antiviral drugs. *Clin. Microbiol. Rev.* **7**, 1-13.
- Field, A. K., and Goodchild, J. (1995). Antisense oligonucleotides: Rational drug design for genetic pharmacology. *Exp. Opin. Invest. Drugs* **4**, 799-821.
- Frank, B. L., and Goodchild, J. (1997). In "Ribozyme Protocols" (P. C. Turner, ed.), pp. 1-7. Human Press, Totowa, New Jersey.
- Frank, B. L., Trainor, B. L., Cocuzza, A. J., Hobbs, F. W., and Chidester, D. R. (1993). Selection of mRNA sequences for targeting with antisense reagents. *International Conference on Nucleic Acid Medical Applications* 4-14 (Abstr.).
- Fujita, M., and Shiku, H. (1993). A human T lymphotropic virus type I (HTLV-I) long terminal repeat-directed antisense *c-myc* construct with an Epstein-Barr virus replicon vector inhibits cell growth in a HTLV-I-transformed human T cell line. *FEBS Lett.* **322**, 15-20.
- Gao, W., Stein, C. A., Cohen, J. S., Dutschman, G. E., and Cheng, Y.-C. (1989). Effect of phosphorothioate homo-oligodeoxynucleotides on herpes simplex virus type 2-induced DNA polymerase. *J. Biol. Chem.* **264**, 11521-11526.
- Gao, W., Jaroszewski, J. W., Cohen, J. S., and Cheng, Y.-C. (1990a). Mechanisms of inhibition of herpes virus type 2 growth by 28-mer phosphorothioate oligodeoxycytidine. *J. Biol. Chem.* **265**, 20172-20178.
- Gao, W.-Y., Hanes, R. N., Vazquez-Padua, M. A., Stein, C. A., Cohen, J. S., and Cheng, Y.-C. (1990b). Inhibition of herpes simplex virus type 2 growth by phosphorothioate oligodeoxynucleotides. *Antimicrob. Agents Chemother.* **34**, 808-812.
- Gilbert, B. E., and Knight, V. (1986). Biochemistry and clinical applications of ribavirin. *Antimicrob. Agents Chemother.* **30**, 201-205.
- Giles, R. V., Ruddell, C. J., Spiller, D. G., Green, J. A., and Tidd, D. M. (1995a). Single base discrimination for ribonuclease H-dependent antisense effects within intact human leukaemia cells. *Nucleic Acids Res.* **23**, 954-961.
- Giles, R. V., Spiller, D. G., and Tidd, D. M. (1995b). Detection of ribonuclease H-generated mRNA fragments in human leukemia cells following reversible membrane permeabilization in the presence of antisense oligodeoxynucleotides. *Antisense Res. Dev.* **5**, 23-31.
- Goodarzi, G., Gross, S. C., Tewari, A., and Watabe, K. (1990). Antisense oligodeoxyribonucleotides inhibit the expression of the gene for hepatitis B virus surface antigen. *J. Gen. Virol.* **71**, 3021-3025.
- Goodchild, J., Agrawal, S., Civiera, M. P., Sarin, P. S., Sun, D., and Zamecnik, P. C. (1988). Inhibition of human immunodeficiency virus replication by antisense oligonucleotides. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5507-5511.
- Groothuis, J. R. (1994). Role of antibody and use of respiratory syncytial virus (RSV) immune globulin to prevent severe RSV disease in high-risk children. *J. Pediatr.* **124**(Suppl.), S28-S32.

- Gutiérrez, A., Rodríguez, A., Pintado, B., and Sobrino, F. (1993). Transient inhibition of foot-and-mouth disease virus infection of BHK-21 cells by antisense oligonucleotides directed against the second functional initiator AUG. *Antiviral Res.* **22**, 1–13.
- Haffey, M. L., and Field, A. K. (1995). In "Antiviral Chemotherapy" (D. J. Jeffries and E. De Clercq, eds.), pp. 83–126. John Wiley & Sons, New York.
- Han, J., Zhu, Z., Hsu, C., and Finley, W. H. (1994). Selection of antisense oligonucleotides on the basis of genomic frequency of the target sequence. *Antisense Res. Dev.* **4**, 53–65.
- Hanecak, R., Brown-Driver, V., Fox, M. C., Azad, R. F., Furusako, S., Nozaki, C., Ford, C., Sasmor, H., and Anderson, K. P. (1996). Antisense oligonucleotide inhibition of hepatitis C virus gene expression in transformed hepatocytes. *J. Virol.* **70**, 5203–5212.
- Hatta, T., Kim, S.-G., Nakashima, H., Yamamoto, N., Sakamoto, K., Yokoyama, S., and Takaku, H. (1993). Mechanisms of the inhibition of reverse transcription by unmodified and modified antisense oligonucleotides. *FEBS Lett.* **330**, 161–164.
- Ho, S. P., Britton, D. H. O., Stone, B. A., Behrens, D. L., Leffet, L. M., Hobbs, F. W., Miller, J. A., and Trainor, G. L. (1996). Potent antisense oligonucleotides to the human multidrug resistance-1 mRNA are rationally selected by mapping RNA-accessible sites with oligonucleotide libraries. *Nucleic Acids Res.* **24**, 1901–1907.
- Homann, M., Tzortzakaki, S., Rittner, K., Sczakiel, G., and Tabler, M. (1993). Incorporation of the catalytic domain of a hammerhead ribozyme into antisense RNA enhances its inhibitory effect on the replication of human immunodeficiency virus type 1. *Nucleic Acids Res.* **21**, 2809–2814.
- Houghton, M. (1996). In "Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, eds.), 3rd Ed., pp. 1035–1058. Lippincott-Raven, Philadelphia.
- Howley, P. M. (1996). In "Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, eds.), 3rd Ed., pp. 2045–2076. Lippincott Raven, Philadelphia.
- Huang, D. P. (1991). In "Nasopharyngeal Carcinoma" (C. A. van Hasselt and A. G. Gibb, eds.), pp. 23–36. The Chinese University Press, Hong Kong.
- Jairath, S., Brown Vargas, P., Hamlin, H. A., Field, A. K., and Kilkuskie, R. E. (1997). Inhibition of respiratory syncytial virus replication by antisense oligodeoxyribonucleotides. *Antiviral Res.* **33**, 201–213.
- Jang, S. K., Krausslich, H. G., Nicklin, M. J. H., Duke, G. M., Palmenberg, A. C., and Wimmer, E. (1988). A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during *in vitro* translation. *J. Virol.* **62**, 2636–2643.
- Jang, S. K., Davies, M. V., Kaufman, R. J., and Wimmer, E. (1989). Initiation of protein synthesis by internal entry of ribosomes into the 5' nontranslated region of encephalomyocarditis virus RNA *in vivo*. *J. Virol.* **63**, 1651–1660.
- Junker, U., Rittner, K., Homann, M., Bevec, D., Bohnlein, E., and Sczakiel, G. (1994). Reduction in replication of the human immunodeficiency virus type 1 in human T cell lines by polymerase III-driven transcription of chimeric tRNA-antisense RNA genes. *Antisense Res. Dev.* **4**, 165–172.
- Kabanov, A. V., Vinogradov, S. V., Ovcharenko, A. V., Krivonos, A. V., Melik-Nubarov, N. S., Kiselev, V. I., and Severin, E. S. (1990). A new class of antivirals: Antisense oligonucleotides combined with a hydrophobic substituent effectively inhibit influenza virus reproduction and synthesis of virus-specific proteins in MDCK cells. *FEBS Lett.* **259**, 327–330.
- Kawamura, M., Hayashi, M., Furuichi, T., Nonoyama, M., Isogai, E., and Namioka, S. (1991). The inhibitory effects of oligonucleotides, complementary to Marek's disease virus mRNA transcribed from the BamHI-H region, on the proliferation of transformed lymphoblastoid cells, MDCC-MSB1. *J. Gen. Virol.* **72**, 1105–1111.
- Kean, J. M., Kipp, S. A., Miller, P. S., Kulka, M., and Aurelian, L. (1995). Inhibition of herpes simplex virus replication by antisense oligo-2'-O-methylribonucleoside methylphosphonates. *Biochemistry* **34**, 14617–14620.
- Kijima, H., Ishida, H., Ohkawa, T., Kashani-Sabet, M., and Scanlon, K. J. (1995). Therapeutic applications of ribozymes. *Pharmacol. Ther.* **68**, 247–267.

- Kinchington, D., and Redshaw, S. (1995). In "Antiviral Chemotherapy" (D. J. Jeffries and E. De Clercq, eds.), pp. 3–40. John Wiley & Sons, New York.
- Kinchington, D., Galpin, S., Jaroszewski, J. W., Ghosh, K., Subasinghe, C., and Cohen, J. S. (1992). A comparison of *gag*, *pol*, and *rev* antisense oligodeoxynucleotides as inhibitors of HIV-1. *Antiviral Res.* 17, 53–62.
- Kitajima, I., Shinohara, T., Bilakovics, J., Brown, D. A., Xu, X., and Nerenberg, M. (1992a). Ablation of transplanted HTLV-1 tax-transformed tumors in mice by antisense inhibition of NF- $\kappa$ B. *Science* 258, 1792–1795.
- Kitajima, I., Shinohara, T., Minor, T., Bibbs, L., Bilakovics, J., and Nerenberg, M. (1992b). Human T-cell leukemia virus type 1 tax transformation is associated with increased uptake of oligodeoxynucleotides *in vitro* and *in vivo*. *J. Biol. Chem.* 267, 25881–25888.
- Kmetz, M. E., Ceruzzi, M., and Schwartz, J. (1991). Vmw65 phosphorothioate oligonucleotides inhibit HSV KOS replication and Vmw65 protein synthesis. *Antiviral Res.* 16, 173–184.
- Korba, B. E., and Gerin, J. L. (1995). Antisense oligonucleotides are effective inhibitors of hepatitis B virus replication *in vitro*. *Antiviral Res.* 28, 225–242.
- Koschel, K., Brinckmann, U., and Hoyningen-Huene, V. V. (1995). Measles virus antisense sequences specifically cure cells persistently infected with measles virus. *Virology* 207, 168–178.
- Krieg, A. M., Yi, A.-E., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A., and Klinman, D. M. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature (London)* 374, 546–549.
- Kulka, M., and Aurelian, L. (1995). Antiviral activity of and oligo(nucleoside methylphosphonate) that targets HSV-1 immediate-early pre-mRNA 4,5 is augmented by cotreatment with replication-defective adenovirus. *Antisense Res. Dev.* 5, 243–249.
- Kulka, M., Smith, C. C., Aurelian, L., Fischelevich, R., Meade, K., Miller, P., and Ts'o, P. O. P. (1989). Site specificity of the inhibitory effects of oligo(nucleoside methylphosphonate)s complementary to the acceptor splice junction of herpes simplex virus type 1 immediate early mRNA 4. *Proc. Natl. Acad. Sci. U.S.A.* 86, 6868–6872.
- Kulka, M., Wachsman, M., Miura, S., Fischelevich, R., Miller, P. S., Ts'o, P. O. P., and Aurelian, L. (1993). Antiviral effect of oligo(nucleoside methylphosphonate)s complementary to the herpes simplex virus type 1 immediate early mRNAs 4 and 5. *Antiviral Res.* 20, 115–130.
- Kulka, M., Smith, C. C., Levis, J., Fischelevich, R., Hunter, J. C. R., Cushman, C. D., Miller, P. S., Ts'o, P. O. P., and Aurelian, L. (1994). Synergistic antiviral activities of oligonucleoside methylphosphonates complementary to herpes simplex virus type 1 immediate-early mRNAs 4, 5, and 1. *Antimicrob. Agents Chemother.* 38, 675–680.
- Lamb, R. A., and Webster, R. G. (1996). In "Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, eds.), 3rd Ed., pp. 1353–1396. Lippincott Raven, Philadelphia.
- Leavitt, M. C., Yu, M., Yamada, O., Kraus, G., Looney, D., Poeschla, E., and Wong-Staal, F. (1994). Transfer of an anti-HIV-1 ribozyme gene into primary human lymphocytes. *Hum. Gene Ther.* 5, 1115–1120.
- Leavitt, M. C., Yu, M., Wong-Staal, F., and Looney, D. J. (1996). *Ex vivo* transduction and expansion of CD4+ lymphocytes from HIV+ donors: Prelude to a ribozyme gene therapy trial. *Gene Ther.* 3, 599–606.
- Leiter, J. M., Krystal, M., and Palese, P. (1989). Expression of antisense RNA fails to inhibit influenza virus replication. *Virus Res.* 14, 141–159.
- Leiter, J. M. E., Agrawal, S., Palese, P., and Zamecnik, P. C. (1990). Inhibition of influenza virus replication by phosphorothioate oligonucleotides. *Proc. Natl. Acad. Sci. U.S.A.* 87, 3430–3434.
- Lemaitre, M., Bayard, B., and Lebleu, B. (1987). Specific antiviral activity of a poly(L-lysine)-conjugated oligodeoxyribonucleotide sequence complementary to vesicular stomatitis virus N protein mRNA initiation site. *Proc. Natl. Acad. Sci. U.S.A.* 84, 648–652.
- Levin, M. J. (1994). Treatment and prevention options for respiratory syncytial virus infections. *J. Pediatr.* 124(Suppl.), S22–S27.

- Levis, J. T., and Miller, P. S. (1994). Interactions of psoralen-derivatized oligodeoxyribonucleoside methylphosphonates with vesicular stomatitis virus messenger RNA. *Antisense Res. Dev.* **4**, 223–230.
- Lewis, E. J., Szymkowski, D. E., Greenfield, I. M., Kreider, J. W., Roberts, P. C., Frank, B. L., Wolfe, J. L., Kilkuskie, R. E., and Mills, J. S. (1997). Identification of an antisense oligonucleotide with *in vivo* activity against human papillomavirus. 2. Efficacy against benign human genital condyloma in a mouse xenograft model. *Antiviral Res.* (in press).
- Lisziewicz, J., Sun, D., Metelev, V., Zamecnik, P., Gallo, R. C., and Agrawal, S. (1993). Long-term treatment of human immunodeficiency virus-infected cells with antisense oligonucleotide phosphorothioates. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3860–3864.
- Lisziewicz, J., Sun, D., Weichold, F. F., Thierry, A. R., Lusso, P., Tang, J., Gallo, R. C., and Agrawal, S. (1994). Antisense oligodeoxynucleotide phosphorothioate complementary to Gag mRNA blocks replication of human immunodeficiency virus type 1 in human peripheral blood cells. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 7942–7946.
- Lisziewicz, J., Sun, D., Lisiewicz, A., and Gallo, R. C. (1995). Antitumor gene therapy: A candidate for late-stage AIDS patients. *Gene Ther.* **2**, 218–222.
- Liu, Z., Batt, D. B., and Carmichael, G. G. (1994). Targeted nuclear antisense RNA mimics natural antisense-induced degradation of polyoma virus early RNA. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4258–4262.
- Lo, K. M. S., Biasolo, M. A., Dehni, G., Palú, G., and Haseltine, W. A. (1992). Inhibition of replication of HIV-1 by retroviral vectors expressing tat-antisense and anti-tat ribozyme RNA. *Virology* **190**, 176–183.
- Locarnini, S. A., and Cunningham, A. L. (1995). In “Antiviral Chemotherapy” (D. J. Jeffries and E. De Clercq, eds.), pp. 441–530. John Wiley & Sons, New York.
- Lori, F., Lisiewicz, J., Smythe, J., Cara, A., Bunnag, T. A., Curiel, D., and Gallo, R. C. (1994). Rapid protection against human immunodeficiency virus type 1 (HIV-1) replication mediated by high efficiency non-retroviral delivery of genes interfering with HIV-1 tat and gag. *Gene Ther.* **1**, 27–31.
- Marcus, P. I. (1994). In “Encyclopedia of Virology” (R. G. Webster and A. Granoff, eds.), pp. 733–739. Academic Press, London.
- Martin, R. R., and U.S. and French GEM® 91 Collaborative Study Groups (1995). Antisense therapy with GEM 91: Phase III clinical trials to evaluate antiretroviral activity of continuous and intermittent IV regimens. *CHI Second Annual HIV Clinical Trials Meeting* (Abstr.).
- Matsukura, M., Zon, G., Shinozuka, K., Mitsuya, H., Reitz, M., Cohen, J. S., and Broder, S. (1987). Phosphorothioate analogs of oligodeoxynucleotides: Inhibitors of replication and cytopathic effects of human immunodeficiency virus. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7706–7710.
- Matsukura, M., Zon, G., Shinozuka, K., Stein, C. A., Mitsuya, H., Cohen, J. S., and Broder, S. (1988). Synthesis of phosphorothioate analogues of oligodeoxynucleotides and their antiviral activity against human immunodeficiency virus (HIV). *Gene* **72**, 343–347.
- Matsukura, M., Zon, G., Shinozuka, K., Robert-Guroff, M., Shimada, T., Stein, C. A., Mitsuya, H., Wong-Staal, F., Cohen, J. S., and Broder, S. (1989). Regulation of viral expression of human immunodeficiency virus *in vitro* by an antisense phosphorothioate oligodeoxynucleotide against rev (art/trs) in chronically infected cells. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4244–4248.
- Mizutani, T., Hayashi, M., Maeda, A., Sasaki, N., Yamashita, T., Kasai, N., and Namioka, S. (1994). In “Coronaviruses” (H. Laude and J. F. Vautherot, eds.), pp. 129–135. Plenum Press, New York.
- Mizutani, T., Kato, N., Hirota, M., Sugiyama, K., Murakami, A., and Shimotohno, K. (1995). Inhibition of hepatitis C virus replication by antisense oligonucleotide in culture cells. *Biochem. Biophys. Res. Commun.* **212**, 906–911.

- Monath, T. P. (1994). Dengue: The risk to developed and developing countries. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2395–2400.
- Moriya, K., Matsukura, M., Kurokawa, K., and Koike, K. (1996). *In vivo* inhibition of hepatitis B virus gene expression by antisense phosphorothioate oligonucleotides. *Biochem. Biophys. Res. Commun.* **218**, 217–223.
- Offensperger, W. B., Offensperger, S., Walter, E., Teubner, K., Igloi, G., Blum, H. E., and Gerok, W. (1993). *In vivo* inhibition of duck hepatitis B virus replication and gene expression by phosphorothioate modified antisense oligodeoxynucleotides. *EMBO J.* **12**, 1257–1262.
- Ojwang, J., Elbaggari, A., Marshall, H. B., Jayaraman, K., McGrath, M. S., and Rando, R. F. (1994a). Inhibition of human immunodeficiency virus type 1 activity *in vitro* by oligonucleotides composed entirely of guanosine and thymidine. *J. Acquir. Immune Defic. Syndr.* **7**, 560–570.
- Ojwang, J., Elbaggari, A., Marshall, H. B., Jayaraman, K., McGrath, M. S., and Rando, R. F. (1994b). Inhibition of human immunodeficiency virus type 1 activity *in vitro* by oligonucleotides composed entirely of guanosine and thymidine. *J. Acquir. Immune Defic. Syndr.* **7**, 560–570.
- Ojwang, J. O., Buckheit, R. W., Pommier, Y., Mazumder, A., De Vreese, K., Esté, J. A., Reymen, D., Pallansch, L. A., Lackman-Smith, C., Wallace, T. L., De Clercq, E., McGrath, M. S., and Rando, R. F. (1995). T30177, an oligonucleotide stabilized by an intramolecular guanosine octet, is a potent inhibitor of laboratory strains and clinical isolates of human immunodeficiency virus type 1. *Antimicrob. Agents Chemother.* **39**, 2426–2435.
- Pagano, J. S. (1995). In "Antiviral Chemotherapy" (D. J. Jeffries and E. De Clercq, eds.), pp. 155–195. John Wiley & Sons, New York.
- Pagano, J. S., Jimenez, G., Sung, N. S., Raab-Traub, N., and Lin, J.-C. (1992). Epstein-Barr viral latency and cell immortalization as targets for antisense oligomers. *Ann. N.Y. Acad. Sci.* **660**, 107–116.
- Pari, G. S., Field, A. K., and Smith, J. A. (1995). Potent antiviral activity of an antisense oligonucleotide complementary to the intron–exon boundary of human cytomegalovirus genes UL36 and UL37. *Antimicrob. Agents Chemother.* **39**, 1157–1161.
- Peyman, A., Helsing, M., Kretzschmar, G., Mag, M., Grabley, S., and Uhlmann, E. (1995). Inhibition of viral growth by antisense oligonucleotides directed against the IE110 and the UL30 mRNA of herpes simplex virus type-1. *Biol. Chem. Hoppe-Seyler* **376**, 195–198.
- Poddevin, B., Meguenni, S., Elias, I., Vasseur, M., and Blumenfeld, M. (1994). Improved anti-herpes simplex virus type 1 activity of a phosphodiester antisense oligonucleotide containing a 3'-terminal hairpin-like structure. *Antisense Res. Dev.* **4**, 147–154.
- Politz, J. C., Taneja, K. L., and Singer, R. H. (1995). Characterization of hybridization between synthetic oligodeoxynucleotides and RNA in living cells. *Nucleic Acids Res.* **23**, 4946–4953.
- Pollack, J. R., and Ganem, D. (1994). Site-specific RNA binding by a hepatitis B virus reverse transcriptase initiates two distinct reactions: RNA packaging and DNA synthesis. *J. Virol.* **68**, 5579–5587.
- Ramirez, J. C., Santaren, J. F., and Almendral, J. M. (1995). Transcriptional inhibition of the parvovirus minute virus of mice by constitutive expression of an antisense RNA targeted against the NS-1 transactivator protein. *Virology* **206**, 57–68.
- Raviprakash, K., Liu, K., Matteucci, M., Wagner, R., Riffenburgh, R., and Carl, M. (1995). Inhibition of dengue virus by novel, modified antisense oligonucleotides. *J. Virol.* **69**, 69–74.
- Rice, C. M. (1995). In "Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, eds.), 3rd Ed., pp. 931–960. Lippincott Raven, Philadelphia.
- Roberts, P. C., Frank, B. L., Boldt, S. E., Walther, D. M., Wolfe, J. L., Kilkuskie, R. E., Szymkowski, D. D., Greenfield, I. M., Sullivan, V., and Mills, J. S. (1997). Identification

- of an antisense oligonucleotide with *in vivo* activity against human papillomavirus. 1. *In vitro* evaluation of oligonucleotides. *Antiviral Res.* (in press).
- Roizman, B., and Sears, A. E. (1996). In "Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, eds.), 3rd Ed., p. 2231. Lippincott-Raven, Philadelphia.
- Roth, G., Curiel, T., and Lacy, J. (1994). Epstein-Barr viral nuclear antigen 1 antisense oligodeoxynucleotide inhibits proliferation of Epstein-Barr virus-immortalized B cells. *Blood* **84**, 582-587.
- Sankar, S., Cheah, K. C., and Porter, A. G. (1989). Antisense oligonucleotide inhibition of encephalomyocarditis virus RNA translation. *Eur. J. Biochem.* **184**, 39-45.
- Sarver, N., Cantin, E. M., Chang, P. S., Zaia, J. A., Ladne, P. A., Stephens, D. A., and Rossi, J. J. (1990). Ribozymes as potential anti-HIV-1 therapeutic agents. *Science* **247**, 1222-1225.
- Sczakiel, G., and Pawlita, M. (1991). Inhibition of human immunodeficiency virus type 1 replication in human T cells stably expressing antisense RNA. *J. Virol.* **65**, 468-472.
- Sczakiel, G., Oppenlander, M., Rittner, K., and Pawlita, M. (1992). Tat- and rev-directed antisense RNA expression inhibits and abolishes replication of human immunodeficiency virus type 1: A temporal analysis. *J. Virol.* **66**, 5576-5581.
- Sczakiel, G., Homann, M., and Rittner, K. (1993). Computer-aided search for effective antisense RNA target sequences of the human immunodeficiency virus type 1. *Antisense Res. Dev.* **3**, 45-52.
- Serini, D., Katlama, C., Gouyette, A., Re, M., Lascoux, C., Tubiana, R., and Tournier, C. (1994). An open-label safety and pharmacokinetic study of single intravenous or subcutaneous ascending doses of GEM 91 in untreated, adult, HIV positive, asymptomatic human volunteer patients. *34th Intersci. Conf. on Antimicrob. Agents and Chemotherapy* Abst. M10. (Abstr.).
- Shah, K. V., and Howley, P. M. (1996). In "Virology" (B. N. Fields, D. M. Knipe, and P. M. Howley, eds.), 3rd Ed., pp. 2077-2110. Lippincott-Raven, Philadelphia.
- Smith, J. A., and Pari, G. S. (1995). Expression of human cytomegalovirus UL36 and UL37 genes is required for viral DNA replication. *J. Virol.* **69**, 1925-1931.
- Speir, E., Modali, R., Huang, E.-S., Leon, M. B., Shawl, F., Finkel, T., and Epstein, S. E. (1994). Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. *Science* **265**, 391-394.
- Steele, C., Sacks, P. G., Adler-Storthz, K., and Shillitoe, E. J. (1992). Effect on cancer cells of plasmids that express antisense RNA of human papillomavirus type 18. *Cancer Res.* **52**, 4706-4711.
- Stein, C. A., and Krieg, A. M. (1994). Problems in interpretation of data derived from *in vitro* and *in vivo* use of antisense oligodeoxynucleotides. *Antisense Res. Dev.* **4**, 67-69.
- Stein, C. A., Cleary, A. M., Yakubov, L., and Lederman, S. (1993). Phosphorothioate oligodeoxynucleotides bind to the third variable loop domain (V3) of human immunodeficiency virus type 1 gp120. *Antisense Res. Dev.* **3**, 19-31.
- Stephenson, M. L., and Zamecnik, P. C. (1978). Inhibition of Rous sarcoma virus viral RNA translation by a specific oligodeoxyribonucleotide. *Proc. Natl. Acad. Sci. U.S.A.* **75**, 285-288.
- Storey, A., Oates, D., Banks, L., Crawford, L., and Crook, T. (1991). Anti-sense phosphorothioate oligonucleotides have both specific and non-specific effects on cells containing human papillomavirus type 16. *Nucleic Acids Res.* **19**, 4109-4114.
- Stull, R. A., Taylor, L. A., and Szoka, F. C. (1992). Predicting antisense oligonucleotide inhibitory efficacy: A computational approach using histograms and thermodynamic indices. *Nucleic Acids Res.* **20**, 3501-3508.
- Sun, L. Q., Pyati, J., Smythe, J., Wang, L., Macpherson, J., Gerlach, W., and Symonds, G. (1995a). Resistance to human immunodeficiency virus type 1 infection conferred by transduction of human peripheral blood lymphocytes with ribozyme, antisense, or poly-

- meric trans-activation response element constructs. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7272–7276.
- Sun, L. Q., Wang, L., Gerlach, W. L., and Symonds, G. (1995b). Target sequence-specific inhibition of HIV-1 replication by ribozymes directed to tat RNA. *Nucleic Acids Res.* **23**, 2909–2913.
- Tan, T. M. C., and Ting, R. C. Y. (1995). *In vitro* and *in vivo* inhibition of human papillomavirus type 16 E6 and E7 genes. *Cancer Res.* **55**, 4599–4605.
- Temsamani, J., and Agrawal, S. (1996). Antisense oligonucleotides as antiviral agents. *Adv. Antiviral Drug Design* **2**, 1–39.
- Thieringer, H. A., Takayama, K. M., Kang, C., and Inouye, M. (1995). Antisense RNA-mediated inhibition of mouse hepatitis virus replication in L2 cells. *Antisense Res. Dev.* **5**, 289–294.
- Tung, F. Y. T. (1994). Suppression of simian immunodeficiency virus replication in primary peripheral mononuclear cells by antisense RNA. *J. Med. Virol.* **42**, 255–258.
- von Knebel Doeberitz, M., and Gissmann, L. (1987). Analysis of the biological role of human papilloma virus (HPV)-encoded transcripts in cervical carcinoma cells by antisense RNA. *Hematologie Bluttransfusion* **31**, 377–379.
- Wakita, T., and Wands, J. R. (1994). Specific inhibition of hepatitis C virus expression by antisense oligodeoxynucleotides. *In vitro* model for selection of target sequence. *J. Biol. Chem.* **269**, 14205–14210.
- Wang, C., Sarnow, P., and Siddiqui, A. (1994). A conserved helical element is essential for internal initiation of translation of hepatitis C virus RNA. *J. Virol.* **68**, 7301–7307.
- Wu, G. Y., and Wu, C. H. (1992). Specific inhibition of hepatitis B viral gene expression *in vitro* by targeted antisense oligonucleotides. *J. Biol. Chem.* **267**, 12436–12439.
- Wyatt, J. R., Vickers, T. A., Roberson, J. L., Buckheit, R. W., Jr., Klimkait, T., DeBaets, E., Davis, P. W., Rayner, B., Imbach, J. L., and Ecker, D. J. (1994). Combinatorially selected guanosine-quartet structure is a potent inhibitor of human immunodeficiency virus envelope-mediated cell fusion. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 1356–1360.
- Yamada, O., Kraus, G., Leavitt, M. C., Yu, M., and Wong-Staal, F. (1994a). Activity and cleavage site specificity of an anti-HIV-1 hairpin ribozyme in human T cells. *Virology* **205**, 121–126.
- Yamada, O., Yu, M., Yee, J.-K., Kraus, G., Looney, D., and Wong-Staal, F. (1994b). Intracellular immunization of human T cells with a hairpin ribozyme against human immunodeficiency virus type 1. *Gene Ther.* **1**, 38–45.
- Yao, G.-Q., Grill, S., Egan, W., and Cheng, Y.-C. (1993). Potent inhibition of Epstein-Barr virus by phosphorothioate oligodeoxynucleotides without sequence specification. *Antimicrob. Agents Chemother.* **37**, 1420–1425.
- Yoo, B. J., Spaete, R. R., Geballe, A. P., Selby, M., Houghton, M., and Han, J. H. (1992). 5' End-dependent translation initiation of hepatitis C viral RNA and the presence of putative positive and negative translational control elements within the 5' untranslated region. *Virology* **191**, 889–899.
- Yoo, B. J., Selby, M. J., Choe, J., Suh, B. S., Choi, S. H., Joh, J. S., Nuovo, G. J., Lee, H.-S., Houghton, M., and Han, J. H. (1995). Transfection of a differentiated human hepatoma cell line (Huh7) with *in vitro*-transcribed hepatitis C virus (HCV) RNA and establishment of a long-term culture persistently infected with HCV. *J. Virol.* **69**, 32–38.
- Yu, M., Poeschla, E., and Wong-Staal, F. (1994). Progress towards gene therapy for HIV infection. *Gene Ther.* **1**, 13–26.
- Yu, M., Poeschla, E., Yamada, O., Degrandis, P., Leavitt, M. C., Heusch, M., Yees, J.-K., Wong-Staal, F., and Hampel, A. (1995). *In vitro* and *in vivo* characterization of a second functional hairpin ribozyme against HIV-1. *Virology* **206**, 381–386.
- Zamecnik, P. C., and Stephenson, M. L. (1978). Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc. Natl. Acad. Sci. U.S.A.* **75**, 280–284.

- Zamecnik, P. C., Goodchild, J., Taguchi, Y., and Sarin, P. S. (1986). Inhibition of replication and expression of human T-cell lymphotropic virus type III in cultured cells by exogenous synthetic oligonucleotides complementary to viral RNA. *Proc. Natl. Acad. Sci. U.S.A.* 83, 4143–4146.
- Zelphati, O., Imbach, J.-L., Signoret, N., Zon, G., Rayner, B., and Leserman, L. (1994). Antisense oligonucleotides in solution or encapsulated in immunoliposomes inhibit replication of HIV-1 by several different mechanisms. *Nucleic Acids Res.* 22, 4307–4314.
- Zhang, R., Lu, Z., Zhao, H., Zhang, X., Diasio, R. B., Habus, I., Jiang, Z., Iyer, R. P., Yu, D., and Agrawal, S. (1995a). *In vivo* stability, disposition and metabolism of a “hybrid” oligonucleotide phosphorothioate in rats. *Biochem. Pharmacol.* 50, 545–556.
- Zhang, R., Yan, J., Shahinian, H., Amin, G., Lu, Z., Saag, M., Jiang, Z., Tamsamani, J., Martin, R. R., Schechter, P. J., Agrawal, S., and Diasio, R. B. (1995b). Pharmacokinetics of an anti-HIV antisense oligonucleotide phosphorothioate (GEM 91) in HIV-infected subjects. *Clin. Pharmacol. Therapeut.* 58, 44–53.