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Antisense Oligonucleotides as Therapeutic Agents—Is the Bullet Really Magical?

C. A. Stein and Y.-C. Cheng

Because of the specificity of Watson-Crick base pairing, attempts are now being made to use oligodeoxynucleotides (oligos) in the therapy of human disease. However, for a successful outcome, the oligo must meet at least six criteria: (i) the oligos can be synthesized easily and in bulk; (ii) the oligos must be stable in vivo; (iii) the oligos must be able to enter the target cell; (iv) the oligos must be retained by the target cell; (v) the oligos must be able to interact with their cellular targets; and (vi) the oligos should not interact in a non–sequence-specific manner with other macromolecules. Phosphorothioate oligos are examples of oligos that are being considered for clinical therapeutic trials and meet some, but not all, of these criteria. The potential use of phosphorothioate oligos as inhibitors of viral replication is highlighted.

It is the idea of specificity that provides the underlying allure of oligodeoxynucleotide technology (Fig. 1). Standard cytotoxic chemotherapy for conditions such as neoplastic disease is fraught with systemic toxicity. The ratio of the toxic dose to the therapeutic dose is relatively low, which reflects the large number of cellular targets affected by the chemotherapeutic agent and the agent's inability to distinguish between normal and diseased cells. In theory, this problem can be solved by taking advantage of the specificity conferred by Watson-Crick base pair formation, if an appropriate target can be identified. For example, an oligo of more than 15 to 17 nucleotides in length would have a unique sequence relative to the entire human genome. In principle, a suitable oligo should be able to interfere, in a sequence-specific manner, with processes such as the translation of mRNA into protein (*1*). If the synthesis of that protein is a requirement for cell growth or, alternatively, for intracellular viral replication, then these processes would be slowed by the antisense agent. Furthermore, the property of complementarity may also be used to inhibit other physiologic processes in addition to mRNA translation; for example, oligos targeted to donor-acceptor sites for splicing pre-mRNA inhibit human immunodeficiency virus–type 1 (HIV-1) replication (*2*). In addition, oligos complementary to genomic DNA can interact with it, by means of Hoogsteen base pairing in the major groove, to form a triple-helical structure. Investigators have inhibited transcription in tissue culture by inducing triple helix formation, and several examples are given below.

Phase I clinical trials of oligos, designed to evaluate the toxicity of these compounds in cancer patients, have already commenced. A phosphorothioate (PS) oligo complementary to the p53 mRNA has been administered to a patient with chemotherapy-refractory acute myelogenous leukemia (*3*). Another trial that has been proposed [for a methylphosphonate (MP) oligo] is a phase I-II trial in patients with chronic myelogenous leukemia. The trial design calls for patients to receive transplants of autologous oligo-purged marrow (*4*) after they receive standard chemotherapy and radiotherapy.

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Requirements for the Therapeutic Use of Oligos

The use of antisense oligos as therapeutic agents presupposes that six criteria can be satisfied.

The oligos can be synthesized easily and in bulk. The development of phosphoramidite chemistry by Caruthers and co-workers and its elaboration into an automated technology (*5*) have greatly enhanced the ease with which oligos are synthesized and consequently their availability. Methods for large-scale oligo synthesis are being commercially pursued (*6*). Although the cost has been dramatically reduced, the final cost to the consumer of a "treatment" with oligo has not been determined.

The oligos must be stable in vivo. This precludes the use of phosphodiester (PO) oligos as therapeutics because serum and intracellular nucleases (both endo- and exonucleases) will degrade them (*7*). For the past decade, significant effort has been expended by synthetic chemists to develop nuclease-resistant oligos. Perhaps the greatest successes in doing so have been achieved with the PS (*8*) and MP oligos (*9*), which can be synthesized with relative ease.

The oligos must be able to enter the target cell. The ability of oligos to penetrate the cell membrane and the mechanism of entrance are critical considerations in devel-

oping these agents as therapeutics. Unlike many other antineoplastic agents, which are of low molecular weight and hydrophilic, oligos (with the exception of MP types) are polyanionic and cannot passively diffuse across cell membranes. Despite this, PO oligos are internalized within cells, and many examples of sequence-specific inhibition of mRNA translation exist, some of which are shown in Table 1 and Fig. 2.

As mentioned above, it has been demonstrated that the binding of a PO oligo in a triple helix (*12*) to a promoter region of the genomic DNA inhibits the in vivo transcription of the interleukin-2α (IL-2α) receptor (*10*) and c-*myc* (*11*). When microinjected into the cytoplasm, rhodamine-labeled PO oligos (*13*) will rapidly accumulate in the nucleus; however, the physiologic mechanisms of oligo internalization are not well understood. Lymphoid and other cells can bind DNA on their membranes (*14*). Recently (*15, 16*), an ~80-kD protein (p80) was isolated from the membranes of CHO fibroblasts, HL60 cells, and other cell types (*17*). This protein binds PO [in a calcium-dependent manner (*18, 19*)] and PS oligos but not MP oligos, mononucleotide phosphates, D-ribose 5-phosphate, or chondroitin sulfate. The binding of oligos to p80, however, is competitive with respect to other polyanions, including dextran sulfate and pentosan polysulfate. The polyanion-binding behavior of p80 resembles that of at least three other PS and PO oligo-

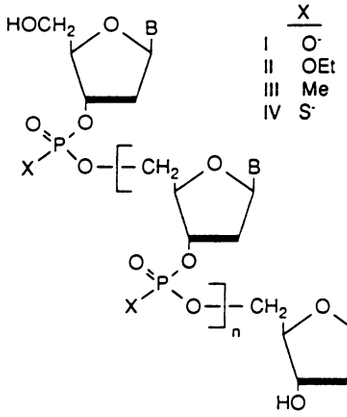

Fig. 1. The structure of an oligo. The nucleotide bases adenine, cytosine, guanine, and thymine are represented by the letter "B." Four classes (I through IV) of oligo are shown in the upper right: O, phosphodiester, or normal DNA; OEt (ethyl), phosphotriester; Me (methyl), methylphosphonate; and S, phosphorothioate. Phosphorothioate and methylphosphonate oligos either will soon be tested or are being tested in clinical trials; phosphotriester oligos are not currently being intensively studied. [Reprinted with permission from (*117*), American Association for Cancer Research, Inc.]

binding proteins, including recombinant soluble CD4 (*20*), the HIV-1 envelope glycoprotein gp120 [which will bind PS oligos at the v3 loop (*21*)], and protein kinase C β1 isoform (PKC β1) (*18*).

Data obtained at pH 7.0 by titrating HL60 cells at 4°C with a 5′ fluorescein-labeled 15-nucleotide (15-mer) homopolymer of thymidine (FOdT15) suggest that PO oligos bind to only a single site on the cells. However, at pH 4.0 to 4.5, at least one other oligo surface binding site exists (*19, 22*), which has a molecular mass of 30 kD. The Michaelis constant K_m (or apparent dissociation constant K_d) of binding for FOdT15 to HL60 cells is ~22 nM (pH 7.0). The internalization of PO oligos by HL60 cells depends on the product of oligo concentration and time; thus, internalization in HL60 cells seems to depend primarily on fluid-phase pinocytosis. (Pinocytosis is the process by which cells constitutively engulf water and dissolved solute from the bulk phase.) This must be true when the external oligo concentration exceeds the K_m or apparent K_d (*15, 18, 23*) of oligo binding to the cell surface–binding protein. In those cases in which antisense effects are observed (oligo concentrations are routinely 1 to 50 μM), it invariably does.

Similarly, in the hepatoma lines Hep G2 and Hep 2215, PS oligos are internalized by the processes of adsorptive endocytosis and fluid-phase pinocytosis (*24*). The process of internalization is slowed by metabolic inhibitors, including deoxyglucose and cytochalasin (*15, 17*), and is temperature-dependent (*24*). In HL60 cells, inhibitors of PKC activity, including the isoquinoline sulfonamide H7, PKC pseudosubstrate polypeptide, staursporine, and tamoxifen, all drastically decrease oligo internalization. Furthermore, charged oligos, and particularly PS oligos, are potent, direct, non–sequence-specific inhibitors of PKC β1 activity. For HL60 cells, charged oligos, by virtue of their nonspecific interactions with cellular proteins, tend to inhibit their own internalization. These data, however, cannot be generalized. Several groups (*25–28*) have suggested that, in a variety of different cell lines including HeLa cells (*25, 27*), H9 cells (*27*), Hep G2 and Hep 2215 cells (*24*), K562 and U937 cells (*27*), and mitogen-stimulated peripheral T and B cells (*28*), internalization of both PO and PS oligos proceeds efficiently and rapidly. In some instances, the oligos appear to eventually localize in the nucleus or mitochondria or both (*27*).

The oligos must be retained by the target cell. Oligos undergo exocytosis (*15*) from H9 cells (*25*), HeLa cells (*29*), and HL60 cells (*30*). In HL60 cells, the rate of exocytosis of internally ^{32}P-labeled FOdT15 best fits a three-compartment model, where $C_T = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$. [$C_T$ is the internalized fluoroscence at any time (t).] The initial, or α, phase is rapid ($t_{1/2}$ = 10 min), and the products of exocytosis contain full-length oligos. The slower, β phase ($t_{1/2}$ = 30 min) may correspond to exocytosis of oligo truncation fragments. In HL60 cells the α and β phases account for >90% of labeled exocytosis. High molecular weight material can also be detected in the β exocytosis phase. Oligo chain extension has been seen in oligos extracted from the kidneys and intestines of mice after administration of PS oligos (*2*). The origins of this process and the effects of this process on antisense inhibition are unknown. In Hep 2215 cells, the process of oligo exocytosis is temperature-dependent, with the $t_{1/2}$ of the initial phase equal to 10 min. No chain extension was observed up to 24 hours (*31*). These data suggest that the metabolism of PS oligos may vary significantly in different tissues. The question of oligo exocytosis from the target cell must also be considered in the context of the rate of hybridization of the oligo to its target mRNA. If these rates become comparable, which they may under certain circumstances, then the ability to

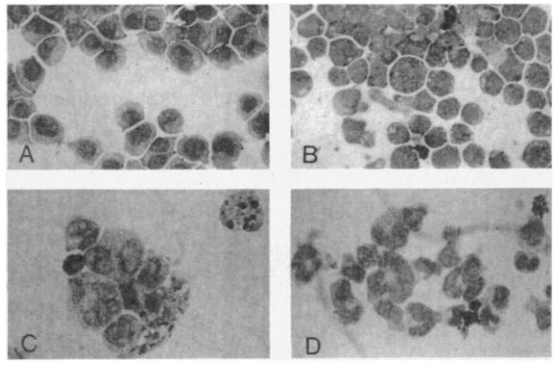

Fig. 2. Effects of an 18-mer phosphodiester antisense oligo targeted to the c-*fes* mRNA in HL60 cells. (**A**) Untreated, proliferating HL60 cells. (**B**) HL60 cells after treatment for 5 days with 10 μM antisense oligo. We could no longer detect c-*fes* mRNA by reverse transcriptase–polymerase chain reaction. (**C**) The cells described in (B) were treated with the differentiating agents retinoic acid and dimethyl sulfoxide for 36 hours in the presence of the c-fes antisense oligo. Cells show kypnosis and karyorrhexis, and significant cell death occurs. (**D**) HL60 cells in (B) after 5 days of treatment with retinoic acid and dimethyl sulfoxide in the presence of the c-fes sense oligo. The expected granulocytic differentiation occurred. [Reprinted with permission from (*118*), *Annals of the New York Academy of Sciences*]

produce the desired antisense effect may be compromised.

After internalization, by pinocytosis or adsorptive endocytosis or both, it is reasonable to expect that oligos will reside in vesicular structures. Punctate structures within cells may be observed by fluorescence microscopy (*32*) and by confocal microscopy, although assignation of subcellular localization by these methods may be complicated by technical difficulties. There is, however, little evidence that fluorescent oligos enter acidic compartments (late endosomes and lysosomes), although the fluorescein moiety may alter the subcellular distribution of the oligo. Treatment of HL60 cells that had been loaded with FOdT15 with monensin, which breaks down the pH gradient between the intracellular compartments and the external medium, did not increase pH-quenched fluorescein fluorescence. Furthermore, attempts to detect oligos in subcellular vesicular structures (such as endosomes and lysosomes) by differential centrifugation have not substantiated the belief that PS oligos are primarily localized in endosomes (*33*). Although it seems that it must be true that oligos can exit vesicular structures to interact with their intracellular or intranuclear targets, the mechanism for this process is unknown.

Modification of a PO oligo by poly-L-lysine (*34*), transferrin-polylysine (*35*), or a cholesteryl (chol)-moiety at the 5′ terminus (*36*) can increase its net intracellular internalization or retention (*37*) or both. The poly-L-lysine modification may mask the negative charge of the oligo, preventing the oligo from interacting with nonspecific cellular binding proteins, and it may help to destabilize endosomal membranes and thus permit transit of oligo into the cytoplasm. On the other hand, 5′-chol oligos can bind to low density lipoprotein (LDL) (*38*, *39*) and may be internalized, at least partially, by means of the LDL receptor (*39*). Intracellular internalization by adsorptive endocytosis may also be increased by a 5′-hydrophobic modification. This may lead to improved antisense efficacy: the 5′-chol–modified (as opposed to unmodified) PO oligos are effective antisense inhibitors of the expression of the MCF-7 murine endogenous retrovirus envelope protein in murine spleen cells (*39*). These oligos are also more potent non–sequence-specific inhibitors (see below) of HIV-1 reverse transcriptase (RT) (*40*) and HIV-1–induced syncytium formation than are the unmodified PS oligos. However, the chol modification tends to increase cellular cytotoxicity, perhaps by promoting extracellular calcium influx (*41*). Nevertheless, the augmentation in antisense potency makes these modified oligos attractive reagents for further development.

The oligos must be able to interact with their cellular targets. Intracellular targets for oligos (such as mRNA, pre-mRNA, and genomic DNA) are invariably protein bound, and many sites are probably not accessible for Watson-Crick base pairing. The location of the best targets for the antisense mRNA approach has been discussed (*42*). One approach, used to inhibit globin translation in the reticulocyte lysate system, has been to target the 5′ cap or initiation codon (AUG) regions. However, in HL60 cells the 5′ cap region of the c-*myc* mRNA is a significantly better target than the AUG region and about as useful as the first splice junction site (*43*). Daaka and Wickstrom (*44*) targeted a Ha-ras mRNA hairpin loop in the 5′ cap region and compared it to other targeted regions, including a region upstream of the initiation codon region. It was predicted that this latter region was a large bulge and that weak base pairing would occur with the 5′ cap region. The most useful target, as judged by the ability of the antisense oligo to decrease p21 Ha-ras protein production, was the 5′ cap.

Other studies have targeted 10-mer oligoribonucleotides to a 47-mer hairpin transcript corresponding to residues 18 through 64 of mutant Ha-ras mRNA. For targets at the 5′ terminus of the loop, values of the association constant (K_a) approximate those of the binding of 10-mer oligoribonucleotide to single-stranded complementary 10-mer targets. However, for 3′ loop and stem targets, the value of K_a may diminish by more than five orders of magnitude (*45*). This decrease is apparently caused by a decrease in the second-order association rate. Similar observations about hybridization rate have been made by Rittner *et al.* (*46*). For a series of oligoribonucleotides targeted to an RNA transcript that contained stretches of HIV-1 sequences, they demonstrated that the rate of hybridization could vary by more than two orders of magnitude. Furthermore, these rates changed dramatically as a function of oligoribonucleotide length. The hybridization rate also directly correlated with the ability

Table 1. Sequence-specific inhibition of mammalian mRNA translation by phosphodiester oligodeoxynucleotides. Ref., reference number.

Gene product	Ref.	Gene product	Ref.	Gene product	Ref.
c-myb	(*102*)	IL-1	(*102*)	hnrarα*	(*135*)
c-fes	(*119*)	IL-1 receptor	(*102*)	β-glucuronidase	(*136*)
c-fms	(*120*)	IL-2	(*102*)	PCNA†	(*102*)
c-fos	(*121*)	IL-2 receptor	(*102*)	Perforin	(*102*)
c-abl	(*102*)	IL-4	(*102*)	Tau	(*137*)
c-myc	(*102*)	IL-6	(*102*)	CFTR‡	(*138*)
c-kit	(*102*)	bFGF	(*102*)	Acetylcholine receptor subunits	(*139*)
c-mos	(*102*)	EGF	(*128*)	Phospholipase A2 activating protein	(*140*)
bcr-abl	(*102*)	TGFβ3	(*129*)	α tubulin	(*141*)
bcl-2	(*102*)	p120	(*102*)	Nonmuscle MLCK§	(*142*)
N-myc	(*102*)	cdc2	(*130*)	Troponin C	(*143*)
N-ras	(*122*)	Prothymosin α	(*102*)	Casein kinase IIβ	(*144*)
Ha-ras	(*123*)	PKC α and β	(*102*)	MZF-1‖	(*145*)
B-myb	(*124*)	lck	(*102*)	IGF-1	(*102*)
Retinoblastoma-1	(*102*)	mdr1	(*131*)	IGF-1 receptor	(*146*)
p53	(*102*)	Growth hormone	(*132*)	EGR-1¶	(*147*)
CSF-1	(*125*)	G proteins	(*102*)	MHC-1#	(*148*)
GM-CSF	(*102*)	cAMP-dependent protein kinase A, type IIβ	(*102*)	Angiontensinogen	(*149*)
G-CSF	(*102*)	Myogenin	(*133*)	LH receptor**	(*150*)
Myeloblastin	(*126*)	Cellular retinol-binding protein I	(*134*)	TNF-α	(*151*)
T cell receptor α and β	(*102*)			egr-1	(*102*)
Erythropoeitin	(*127*)				

*Human nuclear retinoic acid receptor α. †Proliferating cell nuclear antigen. ‡Cystic fibrosis transmembrane conductance regulator. §Myosin light chain kinase. ‖Myeloid zinc finger 1. ¶Early growth response gene 1. #Mixed histocompatibility complex 1. **Leutinizing hormone.

of the oligoribonucleotide to inhibit HIV-1 replication in vivo (46).

Several other mRNA regions have been useful as targets. The splice donor-acceptor site has also been the target for oligos complementary to the basic fibroblast growth factor pre-mRNA, and activity was observed in both melanoma and glial cell lines (47, 48). Useful targets in the mRNA polyadenylation signal region of HIV-1 include the internal *tat* splice acceptor site and the splice acceptor site upstream of the *env* initiator. Interestingly, the 3′ untranslated region of the mRNA of the intercellular adhesion molecule ICAM-1, which is predicted to exist in a stable stem loop conformation, was a far better target than the initiation codon region (49). This is precisely the opposite of what occurs in the reticulocyte lysate system (globin mRNA), in which only the 5′ and not the 3′ regions are useful targets. These data exemplify the intersystem variability in oligo technology and the need to avoid overgeneralization.

Reasonable questions have been raised about the specificity of oligos that are putatively sequence-specific. Woolf *et al.* (50) injected partially-matched oligos into *Xenopus* oocytes and noted partial destruction of the fibronectin mRNA target. This phenomenon could occur with as few as 10 complementary bases, implying, on a statistical basis, that many other mRNAs in addition to fibronectin could also be destroyed. It has been proposed that longer oligo sequences, instead of increasing specificity, may actually increase non–sequence-specific mRNA cleavage because of the length-dependent increase in potential hybridization sites. These data may help to explain why so-called "control" oligos (such as oligos with the same base composition but a scrambled sequence) frequently produce biological effects that are indistinguishable from the antisense oligo. The data also predict that the more control oligos that are used in an experiment, the greater the likelihood that such biological effects will occur.

The oligos should not interact in a non–sequence-specific manner with other macromolecules. Although this statement is self-evident, the fact that charged oligos are polyanions is frequently overlooked. Naturally occurring sulfated polyanions, such as the glycosaminoglycans heparan, dermatan, and chondroitin sulfates, play several vital physiologic roles. For example, these polyanions can bind to and sequester tumor (heparin-binding) growth factors. Glycosaminoglycans are also potent anti-angiogenesis agents. As discussed below, several proteins bind to both charged oligos and sulfated polyanions; these include CD4, HIV-1 RT, gp120, and PKC β1. The binding constants of PO oligos to these proteins are usually, although not always, significantly lower than those of the natural ligands. (MP oligos do not appear to exhibit non–sequence-specific binding to protein.) However, alteration of the polyanionic backbone of a charged oligo can alter the binding constants to protein, as occurs on PS substitution, and produce non–sequence-specific effects. But not all non–sequence-specific protein binding is deleterious; for example, the non–sequence-specific binding of PS oligos to albumin (51) may contribute to their relatively slow rate of total body clearance in experimental animals (see below). PS oligos of specific sequence may also have non–sequence-specific effects. This may occur, for example, when four contiguous guanine residues are present (52), although the mechanism of this is unknown. Oligo palindromes of six or more bases can induce interferon production (53), which may have unpredictable effects on measured experimental end points.

PS Oligos: Developing Charged, Therapeutic Oligos

As mentioned above, both the MP and PS oligos are either nearing or in clinical trials in humans. [For further information about MP oligos, see (54).] MP oligos are known to act in a sequence-specific manner against the herpes virus and to sequence-specifically suppress translation of a mutated Ha-ras allele in ras transformed NIH 3T3 fibroblasts without affecting the wild-type allele (55). They may also diminish c-*myc* translation in an animal model (56). This work with MP oligos may serve as a paradigm for the development of uncharged antisense agents, of which the polyamide DNA class may be representative (57). However, because of the amount of valuable information and the progress that has been made in advancing these compounds as therapeutic agents, the remainder of this article focuses on one class of antisense compounds, PS oligos. The development of these compounds may serve as the prototype for the development of charged oligos in general. Experimental work performed with PS oligos highlights many of the difficulties that must be overcome if antisense oligos are to become widely used therapeutics.

The substitution at phosphorus by sulfur for one of the nonbridging oxygen atoms produces a compound that retains its net charge and aqueous solubility. The substitution also, however, introduces chirality at the phosphorus atom, as each PS linkage can occur as either Rp or Sp diastereomers. Eckstein and co-workers (58) recognized that hydrolytic enzymes would accept only the Sp or Rp forms of PS mononucleotides. For example, snake venom phosphodiesterase will accept only the Rp configuration (59), and only the Sp configuration is accepted by S1 and P1 nucleases (60).

In 1984 PS oligos, in which each phosphorus was bound to a sulfur atom, were chemically synthesized by Stec *et al.* (61). Sulfurization of the P(III) intermediate in the growing oligo is now conveniently achieved with either tetraethylthiuram disulfide (62) or 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (63). Another automated synthesis that involves the hydrogen phosphonate method has been extensively used (64), and methods to radiolabel the oligo with ^{35}S (65) are available. However, because the automated synthesis of PS oligos is not stereospecific, 2^{n-1} diastereomers are likely to be produced in each nucleotide oligomer synthesis. This lack of stereoregularity is, for steric reasons, partially responsible for the lower melting temperature (T_m) of the PS DNA-RNA duplex relative to the PO DNA-RNA duplex (66–68). However, the ultimate biological significance of chirality at the phosphorus atom is not well understood. The recent chemical synthesis and separation of PS oligos of reasonable diastereomeric purity by Stec *et al.* (69) is an encouraging indication that this question may soon be addressed.

In vitro (70) and in vivo (71) PS oligos are nuclease resistant and can be isolated virtually intact from some cell types after several hours of exposure. They can, however, be digested slowly by S1 and P1 nucleases (70) and probably by intracellular nucleases as well. This may lead to release of mononucleotide phosphorothioates which could then be reincorporated into and mutagenize cellular DNA. Chimeric oligos, which contain two or more PS moieties at the 3′ and 5′ terminals, also confer at least partial nuclease resistance (70) but may turn out not to retain all of the non–sequence-specific properties of all-PS oligos. Furthermore, PS oligos, at least when at a concentration lower than that of the complementary mRNA, will act as substrates for ribonuclease (RNase) H activity (72). This attribute may be extremely important, as it is believed that inhibition of protein translation, at least in cell-free systems and in *Xenopus* oocytes (73, 74), is highly dependent on RNase H cleavage of the DNA-mRNA duplex. (In contrast, MP oligos, which do not act as substrates for RNase H activity (75), remain active antisense compounds (55), perhaps by direct steric blockade of translation.)

The effects of PS oligos on human RNase H, however, are biphasic. In a non–sequence-specific and concentration-dependent manner, they competitively inhibit RNase H1 [inhibition constant (K_i) = 70 nM] and RNase H2 (K_i = 450 nM) with respect to poly(rA)·poly(dT) substrate

(*72*). This biphasic behavior implies that there may be only a very narrow concentration range in which PS oligos are maximally effective as antisense agents in human cells. Above this concentration range, the sequence-specific effects of the PS oligo may be severely vitiated by its non–sequence-specific inhibition of RNase H. Similar behavior has been observed with respect to PS oligo inhibition of rabbit globin mRNA translation in the cell-free reticulocyte lysate system (*76*): Sequence-specific inhibition of translation occurs at low concentration (<500 nM), and non–sequence-specific inhibition of translation at higher concentration.

PS Oligos as Antiviral Agents

Sequence-specific inhibition of HIV-1. In principle, antisense oligos should have a major therapeutic impact in the area of HIV-1 replication. These hopes have been heightened by the findings, as mentioned above, that PS oligos are nuclease-resistant, can hybridize with their mRNA targets at 37°C, can serve as substrates for RNase H activity (under some conditions), and appear to be adequately internalized in some cells of lymphoid origin (*25, 28*). In addition, an appropriate target for antisense therapeutics is available. In spite of the genetic variability of HIV-1, at least one region (*rev*) is highly conserved in the 16 isolates that have been evaluated [no more than single base variability (*77*)]. The protein product of the *rev* gene is critical for production of full-length viral transcripts (*78*), and the production of *rev* protein appears to be low.

Initially, Matsukura *et al.* (*79*) targeted 14-mer PS antisense oligos to the HIV-1 rev mRNA. Sense and random oligos were used as controls in a de novo infection assay in ATH8 cells. This T cell–derived line is sensitive to the cytopathic effects of HIV-1. If free virion is incubated with ATH8 cells, by 7 days almost all of the cells will die. All of the 14-mers were about equally effective at blocking the cytopathic effect of the virus. Indeed, in this system even homopolymers of cytidine and adenosine, such as $Sd(C)_{28}$ (3 μM), produced a cytoprotective effect. However, some sequence specificity with the 14-mers was also observed. A sequence identical to one of the antisense sequences was synthesized that contained two N^3-methyl thymidine residues. These residues disrupt duplex formation because of their inability to form Watson-Crick base pairs with their complements. These substituted oligos were ineffective at producing a cytoprotective effect, thus demonstrating the necessity for Watson-Crick base pairing and, hence, specificity.

These experiments also demonstrate the dual cytoprotective nature of PS oligos: Inhibition of viral replication may occur concurrently by both sequence-specific and nonspecific mechanisms. Agrawal *et al.* (*80, 81*), who used the Molt-3 and H9 cell lines which are tolerant to HIV-1 infection, showed that HIV-1–induced syncytia (giant cell) formation and p24 gag protein production were inhibited by 20-mer PS oligos targeted to the HIV-1 mRNA splice donor and splice acceptor sites. However, PS homopolymers [such as $Sd(C)_{20}$ and $Sd(T)_{20}$] were also effective inhibitors. It is probable that while some sequence-specific inhibitory effects were discernible, inhibition of viral protein production was predominately non–sequence-specific.

To conclusively demonstrate sequence specificity, Matsukura *et al.* treated the H9 cell line, which had been chronically infected by $HIV\text{-}1_{IIIB}$, with a 28-mer PS oligo complementary to the 5′ region of the *rev* gene (*82*). This compound, known as S-α*rev*, inhibited p24 gag protein production in a dose-dependent manner, with a 90% inhibitory concentration (IC_{90}) of 25 μM. There was no cellular cytotoxicity at this concentration. In addition to p24, synthesis of other viral proteins, including p33, p55, and gp120, was suppressed. The full-length, 9.2-kb mRNA was not detectable, but other viral mRNA species were observed. This pattern is identical to that found in mutants expressing low levels of *rev*. All control oligos (such as sense, random, N^3-methyl thymidine–containing, homopolymers, and PO) were ineffective. Also, S-α*rev* was effective against the RF strain of HIV-1 (*83*) but did not appear to be effective against some other viral strains or in other cell types, or both (*77*). In CEM cells chronically infected with $HIV\text{-}1_{IIIB}$, for example, the S-α*rev* 27-mer had substantial activity with respect to suppression of p24 gag protein production in culture supernatants; the activity was not significantly greater than that of a randomized 26-mer PS oligo (*84*). Sequence-specific inhibition of HIV-1 replication by PS oligos can be highly dependent on length. In infected Molt-3 cells, a 24-mer, unlike a 28-mer complementary to the HIV-1 gag mRNA, could not suppress viral reproduction in long-term culture (about 30 days as compared with 80 days) [Fig. 3 (*85*)].

Investigators have also evaluated PS oligos complementary to the TAR (transactivating responsive sequence) mRNA. The TAR mRNA forms a stem-loop structure that binds the tat protein, and tat binding is effectively inhibited by the PS oligos in vitro. In vivo, however ($HIV\text{-}1_{IIIB}$–infected CEM cells), random PS oligos were modestly effective, but inhibition could only be achieved by electroporation, calcium phosphate transfection, or lipofectin treatment (*86*). Lipofectin treatment alone, however, may have nonspecific effects on cell growth (*87*).

Non–sequence-specific HIV-1 inhibition. The anti-HIV properties of PS oligos are significantly influenced by non–sequence-specific effects, that is, the inhibitory effect is independent of the base sequence. Multiple, different mechanisms may be involved, including the following:

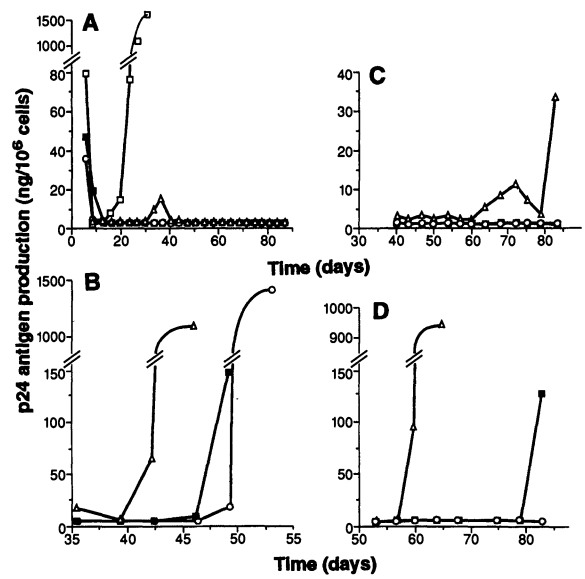

Fig. 3. The effects of PS oligos in long-term cultures of Molt-3 cells chronically infected with HIV-1. All oligos were 28-mers. The oligos rev1-28 (○) and rev2-28 (△) were targeted to the overlapping nucleotide sequences 5970 through 5997 and 5976 through 6003, respectively, in the HIV-1 genome, and the oligo gag-28 (■) was targeted to nucleotides 776 through 802. A random 28-mer (□) was synthesized by coupling a mixture of four bases at each position, giving a final product containing, theoretically, 4^{28} different sequences. (**A**) Inhibition of HIV-1 replication by oligo at a concentration of 1.0 μM, as measured by p24 antigen production. (**B**) Cells from (A) were split and maintained in the absence of antisense oligo, demonstrating the reversibility of the antisense inhibition. (**C**) Cells from (A) were treated with 0.1 μM antisense oligo after the 1.0 μM pretreatment dose. Suppression of HIV-1 replication was still present, but the rev2-28 (△) oligo was inactive after 60 days. (**D**) At day 53, cells that had been treated for 14 days with 0.1 μM antisense oligo were split and maintained with 0.01 μM oligo. The oligos rev1-28 and gag-28 suppressed HIV-1 replication for up to 79 days. [Reprinted from (*85*)]

1) HIV-1 RT may be inhibited by competition with template-primer binding [Fig. 4, (88)]. The value of K_i depends on the length of the PS oligo [2.8 nM for $Sd(C)_{28}$]. However, it is not known if inhibition of HIV-1 RT by PS oligos occurs in vivo. Human DNA polymerases (DNA pols) are also inhibited by $Sd(C)_{28}$ ($\gamma > \alpha > \beta$) (89, 90). The viral RT–associated RNase H activity was also sensitive to $Sd(C)_{28}$, but its associated DNA-dependent DNA polymerase activity was not (91).

2) The oligo may directly associate with cell surface CD4 and inhibit gp120 binding (20). Binding of monoclonal antibody to the CDR2-like loop of CD4 at or near the HIV-1 binding site is also inhibited by PS oligos [$Sd(C)_{28} > Sd(C)_{15} > Sd(C)_5$]. This property of PS oligos is shared with many other polyanions, including phosphoroselenoate oligos [$Sed(C)_{28}$, (92)], pentosan polysulfate (93), dextran sulfate (94), aurintricarboxylic acid (95), and organic dyes (96). Inhibition of the CD4-gp120 interaction prevents HIV-1–induced syncytia formation and is a cytoprotective process in the de novo infection assay. Inhibition depends on the PS oligo chain length.

3) The phosphorylating activity of PKC may be inhibited by PS oligos. It appears that PKC activity is required for virion internalization (97). In vitro, $Sd(C)_{28}$ inhibits PKC β1 activity competitively [K_i = 5.4 μM, (98)] with respect to substrate (octapeptide fragment of the epidermal growth factor receptor), but its inhibitory activity in vivo with respect to PKC is unknown.

4) The PS oligos may directly associate with the v3 loop of gp120. The competition constant K_c for $Sd(C)_{28}$ is 30 nM (45). Although the v3 loop does not appear to be involved in direct gp120-CD4 binding, it may play a critical role in HIV-1–induced syncytia formation and in the tropism of the virus for neural tissue (99).

Future directions in HIV therapeutics. There is evidence that some strains of HIV-1 may become resistant to the non–sequence-specific effects of PS oligos long before resistance to the sequence-specific anti-*rev* or anti-*gag* effects develop (85), if indeed such resistance ever does develop. Furthermore, it appears that the sequence-specific mode of inhibition is effective (at 2.5 μM) in viral isolates, that are both azidothymine (AZT) sensitive and resistant, and in macrophages as well as lymphocytes (100). These types of data are encouraging investigators to proceed with clinical therapeutic trials of the PS anti-*rev* or anti-*gag* compound in patients infected with HIV-1.

Herpes viruses. All known types of herpes viruses can induce a type-specific DNA pol that is critical for active viral DNA replication in virally infected cells. This DNA pol behaves differently from human DNA pols, and attempts to selectively inhibit the viral DNA pol are being made by many laboratories. In view of the observations that PS oligos are stable, can be internalized by at least some cell lines in tissue culture, and show potent activity against HIV-1 RT (which they competitively inhibit), PS oligos were also examined against several types of the herpes virus DNA polymerase. These included the herpes simplex virus (HSV) types 1, 2, and 6 and the Epstein-Barr virus (EBV) pols. All of these pols are more sensitive to the inhibitory effects of PS oligos than are the human DNA pols (89, 90). The degree of inhibitory activity of the PS oligo is dependent on the oligo chain length and the number of PS linkages within the oligo but is independent of sequence (91). For example, $Sd(C)_{28}$ was active against HSV1, HSV2, and EBV replication in cell culture, with a submicromolar median inhibitory dose (ID_{50}). However, different strains of HSV1 may have different sensitivities to the inhibitory effects of $Sd(C)_{28}$.

The mode of inhibition of viral replication is complex, and the mechanism of inhibition is not the same for HSV2 and EBV. In the case of HSV2, $Sd(C)_{28}$ may inhibit the interaction of the virus with its target cell, resulting in diminished viral internalization. This may result from the ability of $Sd(C)_{28}$ to bind directly to viral surface proteins, whose identity is unclear. At the same time, increased $Sd(C)_{28}$ internalization in infected versus uninfected cells has been observed. Once the virion-oligo complex is internalized, viral DNA synthesis may be blocked by inhibition of the viral DNA pol (31). In the case of EBV, however, there was no evidence of enhancement of $Sd(C)_{28}$ uptake in an EBV-producing cell line. Furthermore, $Sd(C)_{28}$ did not inhibit the uptake of virion in Raji cells.

Because $Sd(C)_{28}$ is active against the viral DNA pols while relatively sparing the mammalian enzymes, it may find therapeutic application. In vivo experiments with the HSV1 herpes keratitis rabbit model demonstrated that the compound was potent and selective, with no observed toxicity, when applied topically.

Influenza virus. Influenza RNA pol is critical for the replication of the influenza virus. PS oligos complementary to the polymerase PBI gene of either the influenza A/WSN/33 or the C/JJ/50 virus were evaluated for activity (101). Replication of both strains was inhibited in cell culture by 20-mer PS, but not PO, oligos, but the effect was non–sequence-specific for influenza A. In this case, the mechanism of inhibition is unclear, and it would be of interest to determine whether inhibition of the viral RNA pol by the PS oligo is involved. On the other hand, sequence-specificity was observed for inhibition of influenza C.

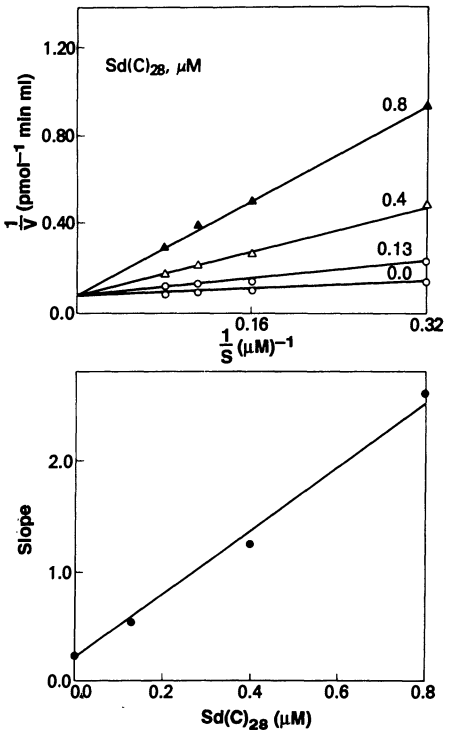

Fig. 4. A non–sequence-specific effect of a PS oligo; inhibition of the HIV-1 RT. DNA synthesis was measured with the template primer system r-A(810)·d(T)14. The dTTP concentration was 32 μM. The inhibitory PS oligo was $Sd(C)_{28}$. The top graph shows the Lineweaver-Burk plot of the inhibition kinetics at different concentrations of PS oligo. The bottom graph shows the replot of the slopes of the lines in the primary plot. The value of K_i is 2.8 nM. [Reprinted with permission from (88), © 1989 American Chemical Society]

Sequence-Specific Inhibition of Mammalian mRNA Translation by PS Oligos

Sequence-specific inhibition of mammalian mRNA translation in tissue culture by PS oligos has also been achieved, although not as frequently as for PO oligos. PS oligos have been successfully targeted to the mRNAs encoding bcl-2 (102); IL-1β (103); ICAM-1 (49); c-myc (104); c-myb (105); c-mos (102); nerve growth factor receptor (106); IL-1 receptor (102); Iγ2b (107); TGFβ1 and retinoblastoma-1 (Rb1) (108); cholinesterase-related cell division controller (CHED) (109); and DCC (110). Non–sequence-specific inhibition of transferrin receptor expression in HL60 cells has also been observed (111). The relative paucity of PS versus PO successes in tissue culture when targeted to mammalian mRNAs (as

opposed to viral mRNAs) is striking, particularly in light of the nuclease sensitivity of the PO oligo. This difference may be purely artifactual, because commercially available PS oligos tend to be more expensive than the PO congeners and thus available to fewer laboratories. However, it is also possible that the potential disadvantages of PS oligos, such as poorer hybridization with target mRNA and non–sequence-specific inhibition of RNase H and other cellular enzymes, can frequently overcome the single advantage of the PS oligo in tissue culture, its nuclease-resistant character. The use of chimeric oligos provides a basis for optimism that the useful properties of PS oligos can be retained without acquiring deleterious non–sequence-specific affects (see below).

In Vivo Pharmacology of PS Oligos

When a 20-mer PS oligo was injected into the abdomens of mice, either intraperitoneally (IP) or intravenously (IV), ~30% of the dose was excreted in the urine within 24 hours (*2*). However, the PS oligo was significantly degraded (75%) when it was administered IP. The highest concentrations of oligo accumulated in the kidney and liver, with only very small amounts being found in the brain. Chain-extended oligos were also observed (*2*). When the PS 27-mer α*rev* oligo was given IV or IP to rats (*112*), the initial $t_{1/2\alpha}$ (transit out of the plasma) was 23 min, while the $t_{1/2\beta}$ of total body clearance was 33.9 hours. The long β half-life of elimination suggests that dosing could be infrequent and still maintain effective, therapeutic tissue concentrations. The plasma clearance was not affected by oligo length (21-, 27-, and 40-mer) or by base sequence. Toxicity included a two- to sixfold increase in lactate dehydrogenase, which normalized in 3 days. Smaller increases in serum hepatic transaminases were also seen. These data are consistent with the observation that PS oligos accumulate in the liver (40% of the administered dose within 12 hours) (*113*).

Evaluation of PS Oligos in Animal Models

The evaluation of PS oligos for their ability to inhibit translation of mammalian mRNAs is beginning in experimental animals. An antisense c-myb 18-mer locally delivered to a rat with an injured left common carotid artery suppressed c-*myb* mRNA concentrations 2 weeks after injury and blocked the accumulation of intimal smooth muscle cells (*105*). An identical oligo with a two-base mismatch was ineffective. Experiments of this type permit speculation that it may, in the future, be possible to elaborate this procedure into one that blocks the development of restenosis after coronary artery angioplasty. In another in vivo experiment (*114*), 24-mer PS oligos targeted to the human c-*myb* mRNA were infused, through a miniosmotic pump, into *scid* mice bearing the human K562 chronic myeloid leukemia cell line. Mean survival times of the mice treated with the antisense oligo were six- to eightfold longer than those of mice untreated or treated with the sense controls or treated with an oligo complementary to the c-*kit* proto-oncogene mRNA. Furthermore, significantly less tumor burden in the brain and ovary was observed histologically compared with the controls (Fig. 5). The potential use of antisense oligos in the treatment of solid murine tumors has recently been demonstrated. The HTLV-1–encoded *tax* gene, when expressed in transgenic mice, causes the development of fibrosarcomas that express NF-κB–inducible early genes. Kitajima *et al.* (*115*) developed a cell line from these tumors and transferred it into syngenic mice. Then, after injecting IP 3′-PS–modified PO chimeric oligos that were complementary to the initiation codon region of the NF-κB mRNA (p65), they observed complete tumor involution in 13 out of 13 antisense-treated mice. Untreated or sense-treated mice died by 12 weeks, whereas the treated animals had no recurrence for at least 5 months.

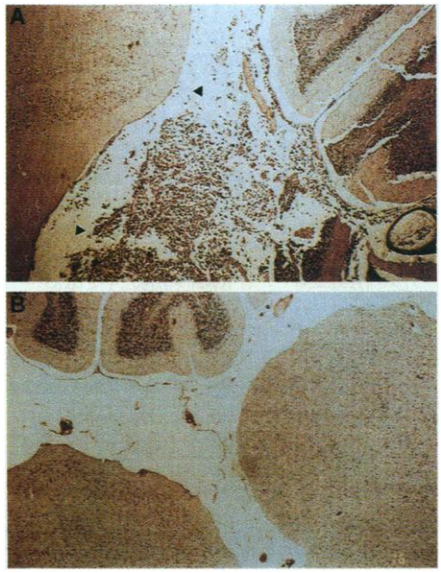

Fig. 5. In vivo efficacy of a PS oligo. Composite photograph (magnification, ×400) of a brain stained with hematoxylin and eosin. The brain was obtained from a leukemia-bearing *scid* mouse chimeric for the human K562 cell line that was treated with sense (**A**) or antisense (**B**) PS c-myb oligos. Extensive meningeal and subarachnoid infiltration of the meninges (arrows) is seen in (A) but not in (B). [Reprinted from (*114*)]

Conclusions

The example of PS oligos provides a paradigm for those factors that will drive the further development of antisense technology. Although touted as "informational drugs" (*116*), the implication that all of the "information" possessed by PS oligos resides solely in the base sequence is not correct. The dynamic interaction of sequence versus nonsequence specificity, although particularly pronounced for PS oligos, will probably occur to some extent with virtually any other newly synthesized class of charged oligo. If so, then the value of PS oligos as therapeutic agents will depend, as it does for all other drugs, on the therapeutic index in human beings. The data reported to date are too limited to assess this value. Some serious questions regarding the role of PS oligos in systemic therapy are emerging. These questions invariably are those of potency, targeting, cost, and reproducibility.

1) PS oligos are active, sequence-specific anti-HIV agents in cell culture. Can they penetrate different types of HIV-1–infected cells in concentrations high enough to exert their effects? Is viral strain specificity an important predictor of resistance to sequence-specific inhibition? Given the infidelity of the HIV-1 RT, the viral sequence may change with time. Fortunately, as described above, PS oligos have many potential anti-HIV mechanisms.

Furthermore, it seems clear that, if the viral-suppressive PS oligo is withdrawn from the chronically infected cell, viral replication recommences. Can PS oligos be administered (most likely this would have to be done IV) as chronic, suppressive agents in vivo without supervening toxicity? (One can speculate that PS oligos, like other polyanions such as suramin and heparin, may cause significant thrombocytopenia, especially in patients whose marrow has been compromised by disease infiltration or by prior therapy.) Can PS or other charged oligos be used for topical viral infections, perhaps after modification with a hydrophobic residue? Will these types of products be able to compete (medically and financially) with those already on the market?

2) PS oligos are active, sequence-specific inhibitors of translation in many mammalian tumor cell lines. However, cell death is rarely a consequence of monogenetic suppression, although exceptions [such as *bcl-2* (*102*)] are known. Can systemic therapy with antisense PS oligos be formulated to yield clinical responses, especially in the more common solid tumors (breast, lung, prostate, and colon) which tend to be genetically heterogeneous? Can antisense oligos be used to specifically sup-

press the production of an autoantibody, thus expanding their therapeutic potential to autoimmune diseases? Will antisense oligos be useful agents in combination with other drugs, and what would those other drugs be? How does the cellular pharmacology (that is, the rate and mechanisms of exocytosis and intracellular compartmentalization) of oligos affect their antisense efficacy? Can cells exhibit de novo resistance to oligos, as they do with many other cytotoxic agents, and can resistance be acquired?

It is not unreasonable to state that some therapeutic success with PS or other classes of oligos, perhaps in systemic antiviral therapeutics, may be in the offing. Other therapeutic antisense oligos that exert their effects after topical administration, or that have activity in the extracorporeal purging of bone marrow or in malignant disease found in closed cavities (such as the intrathecal space), will likely be in clinical trials soon. However, as with any other drug in the developmental process, the relevance of the preclinical models to the human condition is not clear. The test of the validity of the concept that antisense oligos can be therapeutic agents will come only from clinical therapeutic trials and from their full and open evaluation by the entire medical and scientific community.

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RESEARCH ARTICLE

Labyrinthine Pattern Formation in Magnetic Fluids

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A quasi two-dimensional drop of a magnetic fluid (ferrofluid) in a magnetic field is one example of the many systems, including amphiphilic monolayers, thin magnetic films, and type I superconductors, that form labyrinthine patterns. The formation of the ferrofluid labyrinth was examined both experimentally and theoretically. Labyrinth formation was found to be sensitively dependent on initial conditions, indicative of a space of configurations having a vast number of local energy minima. Certain geometric characteristics of the labyrinths suggest that these multiple minima have nearly equivalent energies. Kinetic effects on pattern selection were found in studies of fingering in the presence of time-dependent magnetic fields. The dynamics of this pattern formation was studied within a simple model that yields shape evolutions in qualitative agreement with experiment.

Several distinct physical systems form strikingly similar labyrinthine structures. These include thin magnetic films (*1*, *2*), amphiphilic "Langmuir" monolayers (*3*–*5*), and type I superconductors in magnetic fields (*6*). Similarities between the energetics of these systems suggest a common mechanism for pattern formation. In each case, the labyrinth is formed by the boundary between two thermodynamic phases (oppositely magnetized domains, expanded and condensed dipolar phases, or normal and superconducting regions), and has an associated surface tension favoring a minimum interface length. Each also has long-range dipolar interactions. These may be electrostatic, as in amphiphilic monolayers (the dipolar molecules of which are perpendicular to the air-water interface), or may be due to bulk magnetization (permanent or induced, as in superconductors). These interactions are repulsive, favoring an extended interface. From a dynamical point of view, the evolution of each of the patterns from some unstable initial state occurs in the presence of a global constraint such as prescribed magnetization, constant domain area, or fixed magnetic flux, respectively. In each case, the shape evolution is also dominated by dissipation.

It has been recognized for some time, both in the context of amphiphilic systems (*7*) and superconductors (*8*), that the competition between long-range forces and surface tension can result in a variety of regular patterns such as lamellar stripe domains and hexagonal arrays. The more widely encountered irregular, or disordered, patterns are poorly understood. In analyzing these shapes, a number of general questions naturally arise. (i) Is an observed time-independent shape a unique energetic ground state or does the energy functional contain multiple minima? (ii) If the latter, are the minima roughly equivalent in energy? (iii) Might kinetic considerations force a relaxing system into a metastable minimum instead of the true ground state? Such questions are of course not confined to these particular examples of pattern formation, but also arise in systems such as spin glasses (*9*) and protein folding (*10*).

Motivated by the above-mentioned similarities among labyrinthine pattern forming systems, we have investigated the fingering instabilities of macroscopic domains of magnetic fluids (also known as "ferrofluids"), which are colloidal suspensions of microscopic magnetic particles in a hydrocarbon medium (*11*). Ferrofluids are known to produce complex labyrinthine patterns when trapped between closely spaced glass plates (a "Hele-Shaw cell") and subjected to a magnetic field normal to the plates (*11*–*13*). Here, as in the systems described above, there is a competition between the ferrofluid-water surface tension and bulk induced magnetic dipole interactions. The motion satisfies a global constraint (fixed fluid volume) and is dominated by viscosity. The macroscopic nature of this system affords distinct experimental advantages, including ease of visualization and direct con-

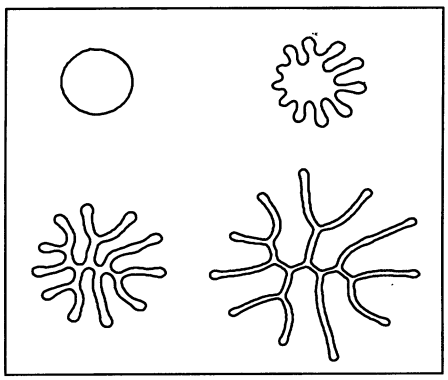

Fig. 1. Stages in the fingering instability of a magnetic fluid drop of initial diameter 2.1 cm, in a field of 87 gauss, as seen from above.

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