is distinct rigid body movement with respect to the B pentamer. Residues A:1–222 (that is, the A1 fragment plus the long N-terminal helix of A2) move by a 5° rotation, with residues 216–222 functioning as a hinge (Fig. 3*a*). This type of rotation is also found in other crystal forms of LT without bound sugars (our unpublished results), but the remarkable flexibility may be important for translocation of LT and cholera toxin across the membrane.

One unexpected result was the well-defined density for five C-terminal residues of the A2 fragment (comprising residues A:196–240), which were poorly defined in the native map at 2.3-Å resolution[25]. In the LT-lactose complex, residues A2:232–235 adopt a helical conformation (Fig. 3*b*). Hence the unusual A2 fragment consists of (1) a long N-terminal helix, interacting with the A1 enzyme; and (2) an extended chain, comprising residues 227–231, protruding through the central pore of the B pentamer along the 5-fold axis; and (3) a one-and-a-half turn C-terminal helix (Fig. 3*c*). Temperature factors are relatively high for residues 232–235 (on average 45 Å^2 versus an average of 27 Å^2 for the entire A2, and 18 Å^2 for the B pentamer) and hence this helix may be poised for conformational change. The C-terminal helix is also found in a higher-resolution native LT structure based on 1.95 Å data (T.K.S. *et al.*, manuscript in preparation) and therefore seems to be an intrinsic feature of LT.

The orientation of the bound galactose (Fig. 3*a*) is such that the C1 linker atom is in the middle of the 'convoluted' surface of the B pentamer, about 8 Å horizontally away from the side of the pentamer and at least 25 Å vertically away from the opposite 'flat' or 'A-binding' surface. This distance is too far for the remaining sugar residues of the G_{M1} oligosaccharide to span because they extend 25 Å at most from the hydrophobic part of the membrane[26]. Binding with the A subunit facing the membrane would not only force the A subunit into the membrane but also insert the hydrophilic B pentamer into the hydrophobic part of the membrane by at least 8 Å, in contradiction to biochemical data[10,11,18]. As there is little or no evidence for any large conformational change in the B pentamer upon G_{M1} binding[9,14,20], the initial binding of LT or cholera toxin to G_{M1}-containing membranes most probably occurs with the A subunit pointing away from the membrane, when all five ganglioside binding sites are occupied. This mode of binding has already been proposed[5,10] but others have come to different conclusions[11,12,18]. It should be noted that here the C terminus of A2 interacts with the membrane (Fig. 3), in good agreement with photoaffinity labelling studies[10]. The C-terminal sequence of A2 (ArgAspGluLeu) closely resembles the LysAspGluLeu sequence in cholera toxin; this KDEL sequence may act as a retention signal in the endoplasmic reticulum membrane[27] and could be important for the interaction of both toxins with the cell membrane.

Finally, it has been pointed out to us that the topology of monomeric *Staphylococcus aureus* nuclease[28] is essentially the same as that of the B subunit of LT, although the relative orientation of the secondary-structure elements is different (A. Muzzin, personal communication). But the position of the galactose-binding site described here corresponds roughly with the active site of the nuclease, which may be of interest from the point of view of evolution in that LT, cholera toxin and the nuclease are all secreted by bacterial pathogens. □

Received 9 September; accepted 30 October 1991.

1. Eidels, L., Proia, R. L. & Hart, D. A. *Microbiol. Rev.* **47,** 596–620 (1983).
2. van Heyningen, S. in *Current Topics in Membranes and Transport* Vol. 18, 445–471 (Academic, New York, 1983).
3. Moss, J. & Vaughan, M. *Adv. Enzym.* **61,** 303–379 (1988).
4. Montesano, R., Roth, J., Robert, A. & Orci, L. *Nature* **296,** 651–653 (1982).
5. Janicot, M., Fouque, F. & Desbuquois, B. *J. biol. Chem.* **266,** 12858–12865 (1991).
6. Finkelstein, R. A. in *Immunochemical and Molecular Genetic Analysis of Bacterial Pathogens* (eds Owen, P. & Foster, T. J.) 85–102 (Elsevier, New York, 1988).
7. Sattler, J., Schwarzmann, G., Staerk, J., Ziegler, W. & Wiegandt, H. *Hoppe Seyler's Z. physiol. Chem.* **358,** 159–163 (1977).
8. Clements, J. D. & Finkelstein, R. A. *Infect. Immunity* **24,** 760–769 (1979).
9. De Wolf, M. J. S., Fridkin, M. & Kohn, L. D. *J. biol. Chem.* **256,** 5489–5496 (1981).
10. Tomasi, M. & Montecucco, C. *J. biol. Chem.* **256,** 11177–11181 (1981).
11. Wisnieski, B. J. & Bramhall, J. S. *Nature* **289,** 319–321 (1981).
12. Dwyer, J. D. & Bloomfield, V. A. *Biochemistry* **21,** 3227–3231 (1982).
13. Holmgren, J., Fredman, P., Lindblad, M., Svennerholm, A.-M. & Svennerholm, L. *Infect. Immunity* **38,** 424–433 (1982).
14. Ludwig, D. S., Holmes, R. K. & Schoolnik, G. K. *J. biol. Chem.* **260,** 12528–12534 (1985).
15. Tsuji, T., Honda, T., Miwatani, T., Wakabayashi, S. & Matsubara, H. *J. biol. Chem.* **260,** 8552–8558 (1985).
16. Griffiths, S. L., Finkelstein, R. A. & Critchley, D. R. *Biochem. J.* **238,** 313–322 (1986).
17. Fukuta, S., Magnani, J. L., Twiddy, E. M., Holmes, R. K. & Ginsburg, V. *Infect. Immunity* **56,** 1748–1753 (1988).
18. Ribi, H. A., Ludwig, D. S., Mercer, K. L., Schoolnik, G. K. & Kornberg, R. D. *Science* **239,** 1272–1276 (1988).
19. Goins, B. & Freire, E. *Biochemistry* **27,** 2046–2052 (1988).
20. Surewicz, W. K., Leddy, J. J. & Mantsch, H. H. *Biochemistry* **29,** 8106–8111 (1990).
21. Svennerholm, A.-M., Holmgren, J., Sack, D. A. & Bardhan, P. K. *Lancet* **i,** 305–307 (1982).
22. de Aizpurua, H. J. & Russell-Jones, G. J. *J. exp. Med.* **167,** 440–451 (1988).
23. Vyas, N. K., Vyas, M. N. & Quiocho, F. A. *J. biol. Chem.* **266,** 5226–5237 (1991).
24. Rutenber, E. & Robertus, J. D. *Proteins* **10,** 260–269 (1991).
25. Sixma, T. K. *et al. Nature* **351,** 371–377 (1991).
26. McDaniel, R. V. & McIntosh, T. J. *Biophys. J.* **49,** 94–96 (1986).
27. Pelham, H. R. B. *EMBO J.* **8,** 3171–3176 (1989).
28. Arnone, A. *et al. J. biol. Chem.* **246,** 2302–2316 (1971).
29. Read, R. J. *Acta crystallogr.* **A42,** 140–149 (1986).
30. Leong, J., Vinal, A. C. & Dallas, W. S. *Infect. Immunity* **48,** 73–77 (1985).
31. Pronk, S. E. *et al. J. biol. Chem.* **260,** 13580–13584 (1985).
32. Tronrud, D. E., Ten Eyck, L. F. & Matthews, B. W. *Acta crystallogr.* **A43,** 489–501 (1987).
33. Brünger, A. T., Kuriyan, J. & Karplus, M. *Science* **235,** 458–460 (1987).
34. Lesk, A. M. & Hardman, K. D. *Meth. Enzym.* **115,** 381–390 (1985).
35. Priestle, J. P. *J. appl. Crystallogr.* **21,** 572–576 (1988).
36. Sanchez, J., Hirst, T. R. & Uhlin, B. E. *Gene* **64,** 265–275 (1988).

ACKNOWLEDGEMENTS. We thank B. Witholt and J. Kingma for purified LT, and C. Verlinde, A. Murzin and T. Hirst for discussion. This project has been sponsored by the Netherlands Foundation for Chemical Research (S.O.N.) and the Netherlands Organisation for Scientific Research (N.W.O.)

Selection of single-stranded DNA molecules that bind and inhibit human thrombin

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APTAMERS[1] are double-stranded DNA or single-stranded RNA molecules that bind specific molecular targets. Large randomly generated populations can be enriched in aptamers by *in vitro* selection and polymerase chain reaction[1–11]. But so far single-stranded DNA has not been investigated for aptamer properties, nor has a target protein been considered that does not interact physiologically with nucleic acid. Here we describe the isolation of single-stranded DNA aptamers to the protease thrombin of the blood coagulation cascade and report binding affinities in the range 25–200 nM. Sequence data from 32 thrombin aptamers, selected from a pool of DNA containing 60 nucleotides of random sequence, displayed a highly conserved 14–17-base region. Several of these aptamers at nanomolar concentrations inhibited thrombin-catalysed fibrin-clot formation *in vitro* using either purified fibrinogen or human plasma.

We synthesized a pool of ~10^{13} 96-mer oligodeoxyribonucleotides that share 18-nucleotide binding sites for polymerase chain reaction (PCR) primers at their 5′ and 3′ termini and also contain 60-nucleotide randomly generated sequences. Using a 5′-biotinylated primer for one strand, this pool was amplified and radiolabelled by PCR. The nonbiotinylated strand was isolated from its complementary strand after application to an avidin–agarose column and base denaturation[12]. Single-stranded DNA was then applied to a concanavalin A (con A)–agarose column to remove DNA with affinity for con A, and the eluent applied to human thrombin immobilized on con A. After washing unbound DNA from the column, thrombin–DNA complexes were eluted with a con A ligand, α-methylmannoside. Fractions containing thrombin were identified using a chromogenic substrate assay and DNA from those fractions was quantitated before PCR

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TABLE 1 Sequence specificity of thrombin inhibition

Sample	Sequence	Concentration	Clotting time (s)	
			Purified fibrinogen	Human plasma
No DNA	—	—	25 ± 1	25 ± 1
Clone 29	96-mer	100 nM	76 ± 3	50 ± 2
Unselected DNA	96-mer	100 nM	26 ± 1	26 ± 1
Consensus 15-mer	GGTTGGTGTGGTTGG	100 nM	169 ± 8	43 ± 2
Scrambled 15-mer	GGTGGTGGTTGTGGT	100 nM	26 ± 1	26 ± 1
Consensus 6-mer	GGTTGG	20 μM	42 ± 2	40 ± 2
Scrambled 6-mer	TGGGGT	20 μM	26 ± 1	26 ± 1

DNA was incubated for 1 min at 37 °C in either selection buffer (0.2ml; Fig. 1 legend) containing human fibrinogen (Sigma) or fresh human plasma. Thrombin (0.1 ml in selection buffer pre-equilibrated to 37 °C) was added to give a final concentration of 13 nM thrombin and the indicated concentrations of DNA; the final concentration of purified fibrinogen was 2 mg ml^{-1}. Clotting times were measured using an automated fibrometer. Human blood was obtained by venipuncture, anticoagulated with the addition of 0.1 volumes 3.8% sodium citrate and fractionated by centrifugation (at 2,000*g* for 5 min). Plasma was decanted and stored (for less than one day) at 4 °C. Clotting times are the average of three experiments.

amplification and repeated selection. The scheme for the selection and amplification cycle is shown in Fig. 1.

Only 0.01% of the input DNA eluted with thrombin during the first selection cycle; this percentage increased in subsequent rounds of selection to ~40% by selection cycle 5. DNA from selection cycle 5 was assayed for thrombin binding specificity on nitrocellulose filters. The results showed that a significant fraction (~30%) of aptamer DNA bound to thrombin but not to either ovalbumin or human fibrinogen (data not shown). Cycle-5 DNA was cloned in order to test the affinity of individual aptamers for thrombin, and radiolabelled single-stranded DNA from several clones was used to measure dissociation constants (K_d). Several clones gave $K_d \approx 200$ nM, whereas the original pool of DNA showed little affinity for thrombin (data not shown).

We determined the DNA sequence of the 60-nucleotide randomly generated region from 32 clones to examine both the heterogeneity of the selected population and to identify homologous sequences. Sequence analysis showed each of the 32 clones to be distinct, but there was a striking sequence conservation evident in every clone. The hexamer GGTTGG was found at a variable location within the 60-nucleotide randomized region in 31 out of 32 clones. The remaining clone contained the sequence TGTTGG, a match of five out of six bases. Additionally, in 28 of the 32 clones, a second hexamer GGNTGG was located 2–5 nucleotides 5′ or 3′ from the hexamer GGTTGG. The DNA sequence from the region of conservation from all 32 clones is presented in Fig. 2. Except for four clones that contain a close variation, the sequence $GGNTGGN_{2-5}GGNTGG$ is conserved. A compilation of the data relating base frequency to position is also shown in addition to the derived consensus sequence. DNA sequencing of clones (>20) from the unselected DNA population or from a population of aptamers selected for binding to a different target revealed no homology to the thrombin-selected aptamers (data not shown). These results indicate that this consensus sequence is responsible, either wholly or in part, for conferring thrombin affinity on the 96-mer oligodeoxyribonucleotide.

We next analysed whether the aptamer DNA could inhibit the thrombin-catalysed conversion of fibrinogen to fibrin using either purified human fibrinogen or adult human plasma. Using clone 29 DNA (Fig. 2) and purified fibrinogen, fibrin formation was detected at 76 seconds, compared with 26 s with unselected DNA or 25 s in the absence of DNA (Table 1). More impressively, with respect to oligonucleotide size and potency, fibrin formation using the 15-mer GGTTGGTGTGGTTGG (contained in clones 15 and 29) was detected at 169 s. The 6-mer GGTTGG also inhibited thrombin, although a higher concentration was required. No inhibition was seen with 15-mer or 6-mer scrambled sequence controls. The aptamers had anti-clotting activity in human plasma, although they were less potent (Table 1). This difference in potency may be attributable to nuclease

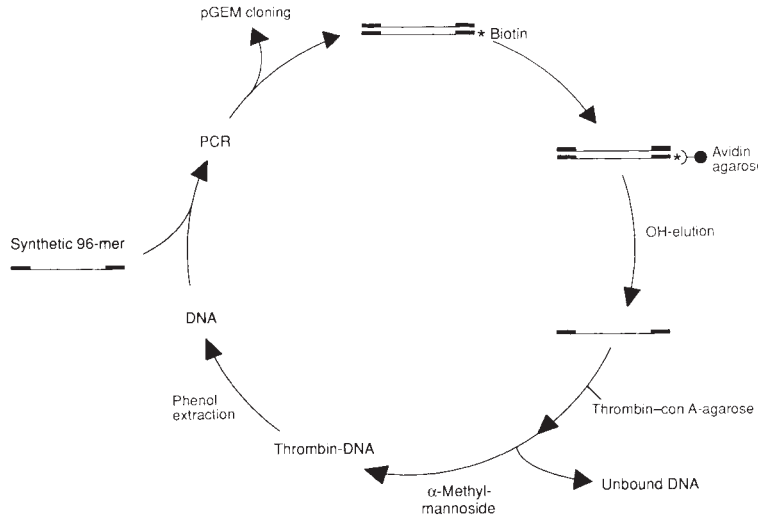

FIG. 1 Scheme for the selection of DNA aptamers to human thrombin.

METHODS. 96-mer DNA was prepared by solid-phase phosphoramidite chemistry on a Biosearch 8600 synthesizer using an equimolar mixture of the four bases for the 60-nucleotide random portion of the sequence which is flanked by defined 18-nucleotide regions that allow for PCR priming by the following oligonucleotides: 5′-CGTACGGTCGACGCTAGC-3′ and 5′ biotin-GGATCCGAGCTCCACGTG-3′ (biotinylation reagent from NEN). The synthetic DNA was purified by PAGE and amplified 100-fold by large-scale PCR to generate a sequence library with a complexity $>10^{13}$ individual sequences[1]. DNA from this library was then amplified and radiolabelled by PCR under standard conditions in the presence of 60 μCi [α-^{32}P]dNTPs. The resulting biotinylated (asterisk) double-stranded DNA was applied to an avidin–agarose (Vector Labs) column (●) equilibrated with 0.1M Tris–HCl, pH 7.5, 0.1M NaCl. ssDNA was then eluted in 0.15N NaOH (ref. 12). The sample was neutralized with acetic acid, concentrated, and the DNA precipitated with ethanol. Following centrifugation, DNA was redissolved in selection buffer (20 mM Tris–acetate, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) and 100 pmol applied to 1 ml concanavalin A–agarose (Vector Labs) equilibrated in selection buffer. The flow-through was applied to 6 nmol of human thrombin (Sigma) immobolized on 1 ml concanavalin A–agarose equilibrated in selection buffer. After washing with several column volumes of selection buffer, thrombin–DNA complexes were eluted by the addition of 0.1M α-methylmannoside to the selection buffer wash. Fractions containing thrombin were identified using a chromogenic substrate assay (KabiVitrum), extracted with phenol, and the DNA precipitated by addition of 20 μg glycogen and three volumes of ethanol. DNA was pelleted by centrifugation, redissolved, and subjected to further cycles of amplification and selection. After the fifth selection cycle, DNA was amplified by PCR using the primers 5′-CGTACGGTCGACGCTAGC-3′ and 5′-TAATACGACTCACTATAGGGATCCGAGCTCCACGTG-3′, and subsequently cloned using the plasmid pGEM3Z (Promega). Single-stranded 96-mer DNA was then prepared from the clones using the primers 5′ biotin-GGATCCGAGCTCCACGTG-3′ and 5′-CTGCAGGTCGACGCTAGC-3′.

Clone																		
1	g	G	G	t	T	G	G	-	-	g	t	c	G	G	t	T	G	G	t
2	g	G	G	a	T	G	G	-	-	t	t	t	G	G	t	T	G	G	g
3	a	G	G	t	T	G	G	-	-	-	g	a	G	G	g	T	G	G	g
4	t	G	G	t	T	G	G	-	-	c	g	a	G	G	a	T	G	G	a
5	a	G	G	t	T	G	G	-	g	t	a	g	t	G	t	T	G	G	t
6	a	G	G	t	T	G	G	-	-	g	c	t	G	G	t	T	G	G	g
7	g	G	G	t	T	G	G	-	-	-	g	a	G	G	t	T	G	G	a
8	t	G	G	t	T	G	G	-	-	g	t	c	G	G	t	T	G	G	g
9	g	G	G	a	T	G	G	-	-	t	g	t	G	G	t	T	G	G	c
10	t	G	G	t	T	G	G	-	-	c	a	g	G	G	a	T	G	G	g
11	t	G	G	a	T	G	G	-	-	t	g	a	G	G	t	T	G	G	a
12	g	G	G	g	T	G	G	-	-	t	t	a	G	G	t	T	G	G	t
13	a	G	G	g	T	G	G	-	-	t	t	a	G	G	t	T	G	G	t
14	c	G	G	t	T	G	G	-	g	t	t	g	G	G	a	T	G	G	a
15	c	G	G	t	T	G	G	-	-	t	g	t	G	G	t	T	G	G	t
16	a	G	G	t	T	G	G	-	-	t	g	t	G	G	g	T	G	G	g
17	c	G	G	g	T	G	G	-	-	a	t	a	G	G	t	T	G	G	a
18	g	G	t	g	T	G	G	t	a	g	t	t	t	G	t	T	G	G	g
19	t	G	G	t	T	G	G	t	t	a	c	t	G	G	t	T	G	G	g
20	g	G	G	t	T	G	G	-	-	t	c	t	G	G	g	T	G	G	a
21	t	G	G	t	T	G	G	-	-	g	t	t	G	G	g	T	G	G	a
22	t	G	G	t	T	G	G	-	-	c	c	a	G	G	t	T	G	G	a
23	c	t	a	g	c	G	G	-	c	a	g	t	G	G	t	T	G	G	g
24	t	G	G	g	T	G	G	-	-	g	g	a	G	G	t	T	G	G	t
25	a	G	G	t	T	G	G	-	-	t	t	t	G	G	g	T	G	G	t
26	a	G	G	t	T	G	G	-	t	t	a	g	G	G	t	T	G	G	t
27	g	G	G	a	T	G	c	-	-	g	g	t	G	G	t	T	G	G	g
28	t	G	G	t	T	G	G	-	t	t	a	t	G	G	t	T	G	G	t
29	a	G	G	t	T	G	G	-	-	t	g	t	G	G	t	T	G	G	c
30	a	G	G	t	T	G	G	-	-	t	g	t	G	G	g	T	G	G	g
31	t	G	G	t	T	G	G	-	-	-	g	a	G	G	t	T	G	G	t
32	g	G	G	t	T	G	G	t	g	g	g	t	G	G	a	T	G	G	t
G		31	30	6	0	32	31						30	32	6	0	32	32	
A		0	1	4	0	0	0						0	0	4	0	0	0	
T		1	1	22	31	0	0						2	0	22	32	0	0	
C		0	0	0	1	0	1						0	0	0	0	0	0	
Consensus sequence																			
		G	G	t	T	G	G			(N)3			G	G	t	T	G	G	

FIG. 2 Thrombin aptamer sequence homology. DNA from the fifth cycle of thrombin selection was amplified by PCR and cloned (see Fig. 1 legend). The sequences of the randomly generated 60-mer inserts were determined for 32 clones using dideoxynucleotide chain termination. A region of homology was identified shown in bold upper case. Below is a tabulation of the data and the deduced consensus sequence.

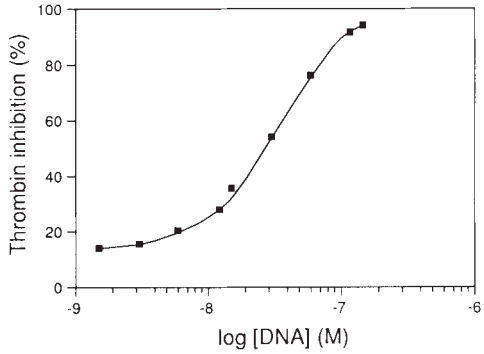

FIG. 3 Per cent thrombin inhibition versus DNA concentration.
METHODS. Selection buffer containing human fibrinogen (2mg ml^{-1} final) and varying concentrations of DNA (~1 nM–150 nM) was incubated for 1 min at 37 °C before adding thrombin as described in Table 1 legend. Clotting times were measured using an automated fibrometer. The extent of thrombin inhibition was then calculated using a thrombin standard curve generated by measuring clotting time versus thrombin concentration.

action or to binding of prothrombin or other plasma proteins present in much higher concentrations than thrombin. When the 6-mer was tested at much higher concentrations it was still as potent, perhaps because plasma nucleases and/or binding proteins were saturated. Thus, this protocol enabled us to isolate single-stranded DNA molecules that inhibit catalysis by thrombin. Moreover, this activity is retained by a short consensus sequence derived from the selected 96-mers.

We next determined the extent of thrombin inhibition with varying DNA concentration and found that thrombin was inhibited by 50% at 25 nM (Fig. 3). This is not a true inhibition constant, but it demonstrates the potency of the thrombin aptamer. Also, the data indicate that stoichiometry could be 1 : 1 and that each bound thrombin is largely, if not completely, inhibited.

Our findings show that a population of $\sim 10^{13}$ molecules of 96-mer single-stranded DNA can be selected for aptamers that bind human thrombin, a protein with no known nucleic acid-binding function. As thrombin is a glycoprotein, we have been able to use lectin–agarose to immobilize it and α-methylmannoside to elute the thrombin–DNA complexes. Initially we bound DNA to thrombin that was covalently linked to agarose. Denaturing elution with EDTA gave single-stranded DNA with affinity for the matrix and hence only a modest enrichment of thrombin aptamers. These results suggest that the conditions under which aptamers are eluted from a covalently bound target are crucial to the successful isolation of high-affinity aptamers. One way to circumvent these problems may be to elute aptamer–target complexes from the matrix. Once isolated from matrix-associated oligonucleotides, the complexes can be fully denatured and the aptamers with the highest affinity recovered. In view of our success in isolating high-affinity aptamers to thrombin, we believe that lectin immobilization may provide an aptamer selection technique applicable to a large variety of glycoproteins.

The basic nature of thrombin may also have facilitated the isolation of aptamers; apart from the catalytic site, thrombin also contains an anion-binding exosite which binds fibrinogen[13–15], and a binding site for heparin, a polyanion. *In vitro* selection should allow the isolation of aptamers to most proteins, although the affinity may be highest for proteins rich in basic residues. The strong sequence dependence shown in Fig. 3 and in Table 1 suggests, however, that electrostatic interactions may be necessary but not sufficient for high-affinity thrombin binding.

It may be that the affinity of an aptamer for its ligand is comparable to that of an antibody for its antigen. The chemical nature, size and mode of isolation of aptamers may sometimes offer advantages over existing antibody technology. We are at present investigating the aptamer-binding site on thrombin and analysing the binding sequences of individual aptamers in an effort to understand the base relationships that mediate binding and inhibition, our long-term interest being to develop diagnostics and therapeutic agents. □

Received 28 August; accepted 15 November 1991.

1. Ellington, A. D. & Szostak, J. W. *Nature* **346,** 818–822 (1990).
2. Blackwell, T. K. & Weintraub, H. *Science* **250,** 1104–1110 (1990).
3. Blackwell, T. K. & Weintraub, H. *Science* **250,** 1149–1152 (1990).
4. Kinzler, K. W. & Vogelstein, B. *Nucleic Acids Res.* **17,** 3645–3653 (1989).
5. Thiesen, H. J. & Bach, C. *Nucleic Acids Res.* **18,** 3203–3208 (1990).
6. Kinzler, K. W. & Vogelstein, B. *Molec cell. Biol.* **10,** 634–642 (1990).
7. Chittenden, T., Livingston, D. M. & Kaelin, W. G. Jr *Cell* **65,** 1073–1082 (1991).
8. Joyce, G. F. *Gene* **82,** 83–87 (1989).
9. Robertson, D. L. & Joyce G. F. *Nature* **344,** 467–468 (1990).
10. Green, R., Ellington, A. D. & Szostak. J. W. *Nature* **347,** 467–468 (1990).
11. Tuerk, C. & Gold, L. *Science* **249,** 505–510 (1990).
12. Hultman, T., Stahl, S., Moks, T. & Uhlén, M. *Nucleosides and Nucleotides* **7,** 629–638 (1988).
13. Fenton, J. W. *Semin. Thromb. Hemostat.* **14,** 234–240 (1987).
14. Bode, W. *et al. EMBO J.* **8,** 3467–3475 (1989).
15. Rydel, T. J. *et al. Science* **249,** 277–280 (1990).

ACKNOWLEDGEMENTS. We thank H. Weintraub and D. Melton for review of the manuscript, V. Collins for administrative assistance, and all members of the Gilead team.