Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 265–268, January 1990
Immunology

Type II hereditary angioneurotic edema that may result from a single nucleotide change in the codon for alanine-436 in the C1 inhibitor gene

(point mutation/serpin/dysfunctional protease inhibitors)

NANCY J. LEVY*, NARAYANASWAMY RAMESH*, MARCO CICARDI†, RICHARD A. HARRISON‡, AND ALVIN E. DAVIS III*§

*Department of Pediatrics, Harvard Medical School, and Divisions of Immunology and Nephrology, The Children's Hospital, Boston, MA 02115; †Cattedra di Clinica Medica Universita de Milano, Ospedale S. Paolo, Milan, Italy; and ‡Molecular Immunopathology Unit, Medical Research Council Centre, Cambridge, England

Communicated by Elkan Blout, August 11, 1989 (received for review May 26, 1989)

ABSTRACT **Identical single-base changes in the C1 inhibitor gene that may result in dysfunctional inhibitor proteins are described in two different families with type II hereditary angioneurotic edema. Initially, a restriction fragment length polymorphism was defined that resulted from loss of a *Pst* I site within exon VIII, which encodes the region containing the reactive center. Exon VIII from the normal and abnormal alleles was amplified by the polymerase chain reaction. Amplified DNA product was cloned into plasmid pUC18; clones representing normal and mutant alleles were distinguished by the presence and absence, respectively, of the *Pst* I restriction site. DNA sequence analysis revealed a G → A mutation in the codon for alanine-436, which would result in replacement with a threonine residue. This position is nine amino acid residues amino-terminal to the reactive-center arginylthreonine peptide bond. In contrast, previously defined mutations in type II hereditary angioneurotic edema result in replacement of the reactive-center arginine.**

C1 inhibitor is a serine protease inhibitor with activity against the C1r and C1s subcomponents of complement component C1, kallikrein, plasmin, and coagulation factors XI and XII (1–5). Genetic deficiency of C1 inhibitor results in hereditary angioneurotic edema (HANE) (6, 7). Since HANE occurs in individuals who are heterozygous for C1 inhibitor deficiency, it is inherited as an autosomal dominant trait. The disease is characterized by episodic localized swelling of the subcutaneous tissue or of the gastrointestinal or laryngeal mucosa. Two forms of the disease, types I and II, have been described. Type I HANE is characterized by low functional and low antigenic plasma levels of C1 inhibitor. In type II HANE, patients' plasma contains low levels of normal C1 inhibitor protein together with a dysfunctional mutant C1 inhibitor molecule (8, 9). Analysis of mutant proteins from type II HANE patients provided the first evidence for genetic heterogeneity of the disease: the proteins from different families differ in electrophoretic mobility, in size, and in function (8). More recently, restriction fragment length polymorphisms (RFLPs) of the C1 inhibitor gene (*C1NH*) have been observed in HANE (10, 11). In every kindred analyzed the polymorphism cosegregated with the disease. In type I HANE, the RFLPs thus far observed have resulted from partial deletions and/or duplications within the C1 inhibitor gene.

C1 inhibitor is a member of the serpin "superfamily" of serine protease inhibitors, which contains several plasma protease inhibitors including α_1-antitrypsin and antithrombin III (12). α_1-Antitrypsin is the only one for which the x-ray structure has been determined (13). Inactivation of proteases by serpins results from covalent binding to a site within the inhibitor that mimics the natural substrate of the protease. The substrate binding site of the protease recognizes a specific reactive-center amino acid (the P1 residue) and cleaves the peptide bond carboxyl-terminal to this residue. A major determinant of serpin specificity, therefore, is the P1 residue. As would be expected from the substrate specificity of its target enzymes, the P1 residue in C1 inhibitor is an arginine (residue 444). Mutations that replace this arginine with either histidine or cysteine result in functional impairment (14, 15).

We have investigated the molecular genetic defect in two unrelated families with type II HANE. In a previous report (11), RFLPs had been detected by Southern blot analysis of the patients' DNA after digestion with *Pst* I and hybridization with a C1 inhibitor cDNA. Here, we report that the restriction site change is due to a point mutation, not in the reactive center, but within the DNA encoding the loop joining the reactive center to the remainder of the molecule. This mutation very likely is responsible for the dysfunctional C1 inhibitor in these two families.

METHODS

Patients. We studied two patients from different families with type II HANE (families A and C from ref. 11). In the previous report, family A was described as type I HANE. Initial measurements of C1 inhibitor antigen in several members of this family were below the normal range. Subsequent analysis of C1 inhibitor levels in members of this family has shown that they in fact have type II HANE. That is, they have normal levels of C1 inhibitor protein on most determinations as measured immunochemically, but this protein is dysfunctional. Preliminary data (M.C., unpublished data) suggest that the dysfunctional inhibitor in these two families is catabolized more rapidly than the normal inhibitor. All of the patients from both families had an abnormal 3.1-kilobase (kb) band when their DNA was digested with *Pst* I and probed with a 1227-base-pair (bp) cDNA probe that extended from nucleotide 554 to 1780 (numbered according to ref. 16) (11).

Preparation of Genomic DNA. Leukocytes were isolated from peripheral blood and high molecular weight genomic DNA was extracted as described (17). DNA samples were separated by electrophoresis in 0.8% agarose gels (Bethesda Research Laboratories) and blotted onto nitrocellulose (Millipore) after treatment according to Wahl *et al.* (18).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HANE, hereditary angioneurotic edema; RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction.

§To whom reprint requests should be addressed.

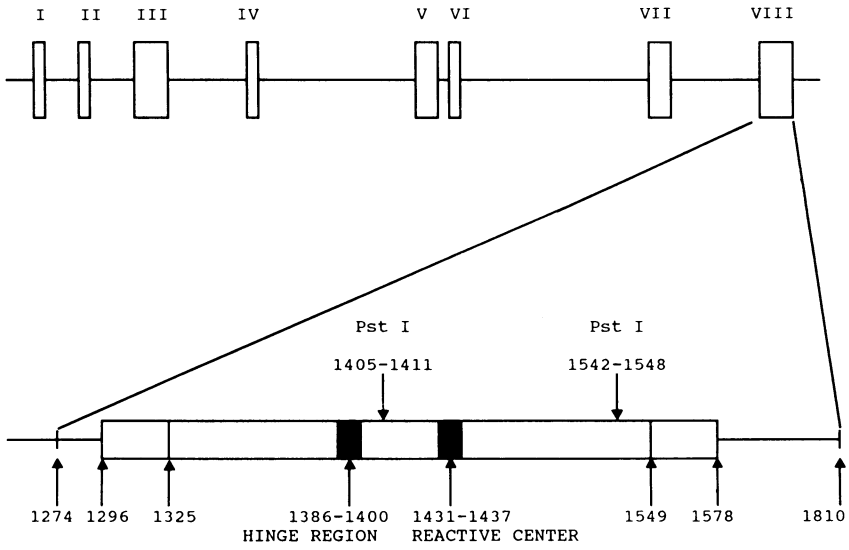

FIG. 1. PCR amplification of the region of the C1 inhibitor gene encoding the reactive center. (*Upper*) Intron–exon structure (23). Roman numerals indicate exon number. (*Lower*) Expanded representation of exon VIII. Numbers refer to cDNA nucleotide numbers from ref. 16. The two *Pst* I sites are indicated, as are the nucleotides encoding the hinge region and the reactive center. The sequences corresponding to the oligonucleotide primers are nucleotides 1296–1325 and nucleotides 1549–1578.

Amplification of a DNA Segment by the Polymerase Chain Reaction (PCR). High molecular weight genomic DNA (10 μg) was sheared by repeated passage through a 26-gauge needle. The PCR primers were synthesized with an Applied Biosystems 380B DNA synthesizer. Both primers were 30 bases in length with *Bam*HI linkers at their 5′ ends. One oligonucleotide corresponded to nucleotides 1296–1325 in the C1 inhibitor cDNA, and the other was complementary to nucleotides 1549–1578. PCR amplification was performed using *Thermus aquaticus (Taq)* DNA polymerase according to the protocol described in the GeneAmp DNA amplification reagent kit (Perkin-Elmer/Cetus). The cycle of denaturation (94°C, 1 min), annealing (37°C, 2 min), and extension (72°C, 3 min) was repeated 25 times. Amplified DNA was visualized by ethidium bromide staining after electrophoresis in a composite gel of 3% NuSieve and 1% SeaKem agarose (FMC) (19–21).

DNA Sequencing. Amplified DNA was subcloned into plasmid pUC18 and DNA sequence analysis was carried out by a modification (DNA sequencing kit, Pharmacia) of the dideoxy chain-termination method of Sanger *et al.* (22).

RESULTS

PCR Amplification of the Abnormal DNA Segment. Previous investigation of these two families (A and C) with HANE revealed the presence of *Pst* I RFLPs that cosegregated with the disease (11). With the C1 inhibitor cDNA probe used in that study, *Pst* I digestion of normal DNA resulted in hybridized bands at 4.2, 2.9, 2.7, and <0.5 kb. Individuals with HANE in families A and C revealed a new band at 3.1 kb with a decrease in intensity of the 2.9-kb band. The mutation that resulted in the polymorphism was assigned to the 3′ end of the C1 inhibitor gene because only the polymorphic 3.1-kb band and the 2.9-kb band were detected with a probe for exon VII. This information, together with genomic sequencing data subsequently published by Carter *et al.* (23), shows that the 3′ end of the 2.9-kb *Pst* I fragment is within exon VIII at nucleotide 1409 (Fig. 1). There is a second *Pst* I site within exon VIII at nucleotide 1546. A likely explanation, therefore, for the increase in size of this fragment from 2.9 to 3.1 kb is a mutation resulting in loss of the *Pst* I restriction site at nucleotide 1409.

The DNA encoding the reactive center of C1 inhibitor is also located within exon VIII (23). A segment of exon VIII encompassing the two *Pst* I sites and the reactive center (nucleotides 1296–1578; Fig. 1) was amplified by PCR. Amplification of the patients' genomic DNA yielded a single DNA band at 282 bp, as expected (Fig. 1; Fig. 2, lane 1). *Pst* I digestion of the PCR-generated DNA yielded three visible fragments (Fig. 2, lane 2). Two of these fragments would be expected to have originated from the amplified normal allele: one extending from the 5′ end of the amplified DNA to the first *Pst* I site in exon VIII (112 bp) and one extending from the first *Pst* I site to the second *Pst* I site (137 bp) (Fig. 1). The 3′ DNA cleavage fragment could not be visualized easily due to its small size (32 bp). The PCR-generated segment from the putative mutant allelle yielded a fragment of 250 bp (Fig. 2, lane 2), slightly smaller than the full-length (282-bp) segment

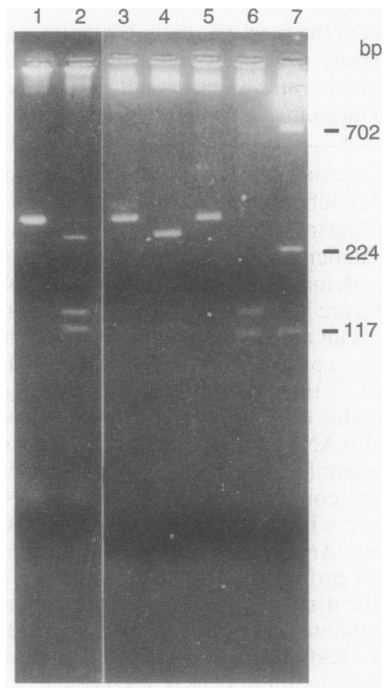

FIG. 2. Agarose gel electrophoresis of PCR-amplified DNA. Genomic DNA from one affected individual from each family was amplified by PCR, and the resulting DNA fragments were cloned into pUC18. Lane 1, amplified DNA from an individual from family C. Lane 2, DNA as in lane 1, but digested with *Pst* I. Lane 3, amplified DNA cloned into pUC18 from an individual from family A; the cloned DNA insert was excised with *Bam*HI. Lane 4, DNA from the same clone as in lane 3, digested with *Pst* I in addition to *Bam*HI. Lane 5, DNA from a different clone derived from the same amplified DNA preparation as shown in lane 3; the insert was excised with *Bam*HI. Lane 6, DNA from the same clone as shown in lane 5, digested with *Pst* I in addition to *Bam*HI. Lane 7, λ phage DNA digested with *Bst*EII.

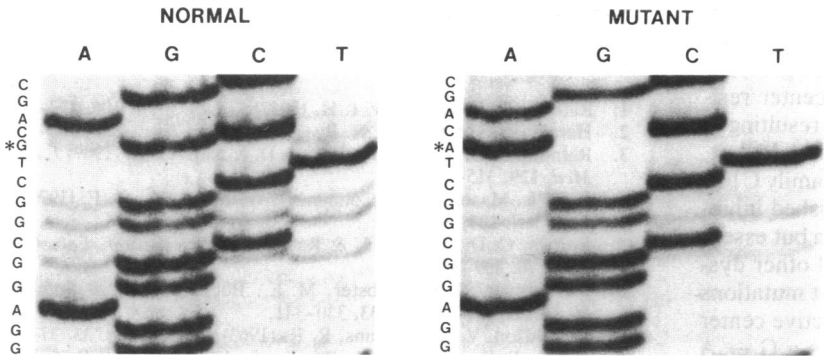

FIG. 3. DNA sequence of the PCR-amplified DNA derived from the normal and mutant alleles of an affected member of family A. The sequence of each is as indicated. The site of the mutation is marked with an asterisk.

(Fig. 2, lane 1) due to cleavage at nucleotide 1546 and loss of the 32-bp fragment of DNA.

The PCR-amplified DNA fragments were cloned into pUC18. Digestion of all clones with *Bam*HI yielded DNA inserts of the same size (Fig. 2, lanes 3 and 5). *Pst* I/*Bam*HI double digestion of some clones, putative normal clones, yielded two bands (110 and 140 bp; lane 6). Other clones yielded one band (250 bp) upon *Bam*HI/*Pst* I digestion (lane 4). These fragments from cloned DNA inserts were identical in size to those of the PCR-amplified DNA. Amplified DNA from the HANE patients from families A and C gave identical restriction patterns by these techniques.

Nucleotide Sequence Analysis of the PCR-Amplified DNA. DNA sequence analysis of clones from both families containing inserts with both *Pst* I sites intact revealed a sequence that was identical to the reported normal cDNA sequence between nucleotides 1296 and 1578. In those clones lacking one of the *Pst* I sites, there was a point mutation at nucleotide 1407 in which a guanine was replaced by an adenine (Figs. 3 and 4). Multiple independent clones from both families were sequenced and all revealed the same point mutation. This alters the sequence from a codon specifying an alanine to one encoding a threonine residue. The remainder of the DNA sequence was the same as the normal cDNA sequence over the equivalent nucleotides. As shown in Figs. 1 and 4, this mutation is located within the nucleotides encoding the loop that connects the reactive center with the remainder of the C1 inhibitor molecule.

DISCUSSION

Type II HANE is characterized by normal to elevated levels of C1 inhibitor protein in patient's plasma, as determined by immunochemical methods, together with reduced C1 inhibitor functional activity (8, 9). This results from the presence of a dysfunctional mutant protein in addition to diminished levels of normal protein. As with other plasma protease inhibitors, mutations resulting in amino acid substitution at the reactive center result in a dysfunctional C1 inhibitor molecule. Analysis of C1 inhibitor proteins from type II HANE patients has shown that approximately two-thirds have P1 mutations; all of these have resulted in substitution of either histidine or cysteine for arginine-444 (14, 15). The data described here clearly define, in two families, a point mutation that is near, but outside, the reactive center. The codon for the P1 arginine, in both families, is identical to the equivalent codon in the normal gene.

In patients from the two families, an adenine replaces a guanine in the codon (GCA) specifying the third of four consecutive alanines, alanine-436, which is at the P9 position. The reactive-center mutations in C1 inhibitor occur within a CpG dinucleotide and result in alteration of the arginine codon from CGC to either TGC (cysteine) or CAC (histidine) (14, 15). This type of change accounts for 35% of coding-region single base-pair mutations causing human disease (24) and probably results from deamination of 5-methylcytosine to thymidine within the CpG dinucleotide in either the coding or the noncoding strand (25, 26). The biochemical mechanism producing the mutation described here has not been defined. Although an identical mutation, in terms of the nucleotide substitution and codon involved, has been described in antithrombin III (see below), these do not lie within a sequence with a known propensity toward point mutation (24).

Formal proof that this mutation alone is responsible for dysfunction will require functional analysis of expressed protein with this mutation induced in an otherwise normal C1 inhibitor cDNA. Since the complete mRNA sequence from these patients has not been determined, it is theoretically possible that some unidentified mutation is, in fact, responsible for dysfunction. Several lines of evidence suggest that the defined mutation results in abnormal function. First, the G → A mutation leads to loss of a *Pst* I restriction site. The consequent RFLP has never been observed in a normal individual, and it has been shown to cosegregate with HANE in both families (11). This linkage of the mutation with dysfunction strongly supports the above suggestion. Support is also provided by the fact that two unrelated families have the same mutation linked to the disease.

An additional argument that the observed point mutation may be responsible for dysfunction is that the mutation is within a highly conserved region near the reactive center. However, it is not immediately apparent why substitution of a threonine for an alanine at residue 436 should give rise to a dysfunctional protein. An alanine at the position equivalent to alanine-436 in C1 inhibitor is present in about two-thirds of

Normal C1 INH	Glu Thr Gly Val Glu Ala Ala Ala Ala Ser Ala Ile Ser Val Ala *Arg Thr
Nucleotide sequence	GAG ACT GGG GTG GAG GCG GCT GCA GCC TCC GCC ATC TCT GTG GCC CGC ACC
Mutant nucleotide sequence	- - - - - - - ACA - - - - - - - - - -
Inferred mutant sequence	Glu Thr Gly Val Glu Ala Ala Thr Ala Ser Ala Ile Ser Val Ala Arg Thr

FIG. 4. DNA and inferred amino acid sequences of the normal and mutant alleles through the hinge and reactive-center region. The P1 arginine (Arg-444) is indicated by the asterisk. INH, inhibitor.

the serpins, while other amino acids, including threonine (in heparin cofactor II; ref. 27), are present in others. Apart from the critical contribution of the P1 residue, target protease specificity must be influenced by non-reactive-center residues. These could differ with different proteases, resulting in altered inhibitory profiles for some mutant proteins. In fact, in a previous study, the C1 inhibitor protein from family C [C1 INH (Mo) in ref. 28], was shown to have diminished inhibitory capacity toward C1s, kallikrein, and plasmin but essentially normal activity against factor XII. Several other dysfunctional serpins have been described with point mutations resulting in amino acid substitutions near the reactive center (29–32). Antithrombin III-Hamilton (29) also has a G → A point mutation resulting in a threonine-for-alanine substitution, at the P12 position; this mutation results in a dysfunctional protein. In addition, an alanine insertion between the P9 and P10 residues of α_2-antiplasmin results in loss of antiplasmin activity (31). These observations suggest that functional inhibitors have precise residue requirements distinct from those involved in determination of target specificity, in the region amino-terminal to the P1 residue.

While little is known directly about the tertiary structure of C1 inhibitor, its sequence homology with α_1-antitrypsin, together with a common reaction mechanism (33), susceptibility to proteolytic inactivation within the reactive-center loop (34), and differential stability of native and reactive-center-cleaved inhibitors to a variety of denaturants (35, 36), makes it likely that it has a similar folded structure. The x-ray structure of α_1-antitrypsin cleaved at the P1-P'1 reactive-center peptide bond shows that the P1 and P'1 residues are separated by about 65 Å, and the region amino-terminal to P1 is buried in the center of a six-stranded β-sheet (13). Presumably, in the active inhibitor these residues (P1–P17) are looped over the β-sheet to connect with the P'1 residue. While the hydrophobic nature of many of the residues within this putative solvent-exposed loop makes it an energetically unfavorable structure, the proteolytic sensitivity of residues P10 through P'2 indicates that these residues are at least more accessible in the native molecule than in the cleaved molecule (37).

The hydrophobic nature of the reactive-center loop and the enhanced stability of the cleaved inactive inhibitor to denaturants have led to the concept that the active protein is an intermediate in the folding pathway that is trapped in a "stressed" state (34–36). Stress is released by cleavage within the reactive-center loop, permitting attainment of the stable structure. It is probable that certain residues near the junction of the loop with the subsequent strand of the underlying β-sheet are critical to maintenance of a highly specific stressed conformation of the reactive center. The high degree of sequence conservation among the serpins through this region supports this hypothesis. Further, the data from the mutant proteins described here, together with the data from the other mutant serpins described above, suggest that maintenance of this conformation is mediated by specific interactions between these residues and residues in the underlying β-sheet. Any disruption of these interactions, as with replacement of alanine-436 with the bulkier, more polar threonine, may generate an unreactive inhibitor. In this regard, it will be of particular value if dysfunctional inhibitors are found with mutations affecting residues underlying the reactive-center loop.

We wish to thank Dr. Zuheir Audeh for his generous assistance and advice and Ms. Leslie Rhubin for excellent technical assistance. This work was supported by the U.S. Public Health Service Grant HD22082 and by the March of Dimes Birth Defect Foundation Grant 1-775. This work was done during the tenure of an Established Investigatorship of the American Heart Association (A.E.D.) and with funds contributed in part by the American Heart Association Massachusetts Affiliate, Inc.

1. Ratnoff, O. D. & Lepow, I. H. (1957) *J. Exp. Med.* **106,** 327–343.
2. Harpel, P. C. & Cooper, N. R. (1975) *J. Clin. Invest.* **55,** 593–604.
3. Ratnoff, O. D., Pensky, J., Ogston, D. & Naff, G. B. (1969) *J. Exp. Med.* **129,** 315–331.
4. Gigli, I., Mason, J. W., Colman, R. W. & Austen, K. F. (1970) *J. Immunol.* **104,** 574–581.
5. Forbes, C. D., Pensky, J. & Ratnoff, O. D. (1970) *J. Lab. Clin. Med.* **76,** 809–815.
6. Landerman, N. S., Webster, M. E., Becker, E. L. & Ratcliffe, H. E. (1962) *J. Allergy* **33,** 330–341.
7. Donaldson, V. H. & Evans, R. R. (1963) *Am. J. Med.* **35,** 37–44.
8. Rosen, F. S., Alper, C. A., Pensky, J., Klemperer, M. R. & Donaldson, V. H. (1971) *J. Clin. Invest.* **50,** 2143–2149.
9. Gadek, J. E., Hosea, S. W., Gelfand, J. A. & Frank, M. M. (1979) *J. Clin. Invest.* **64,** 280–286.
10. Stoppa-Lyonnet, D., Tosi, M., Laurent, J., Sobel, A., Lagrue, G. & Meo, T. (1987) *N. Engl. J. Med.* **317,** 1–6.
11. Cicardi, M., Igarashi, T., Kim, M. S., Frangi, D., Agostoni, A. & Davis, A. E., III (1987) *J. Clin. Invest.* **80,** 1640–1643.
12. Carrell, R. W. & Boswell, D. R. (1986) in *Proteinase Inhibitors*, eds. Barrett, A. & Salvesen, G. (Elsevier, Amsterdam), pp. 403–420.
13. Loebermann, H., Tokuoka, R., Diesenhofer, J. & Huber, R. (1984) *J. Mol. Biol.* **177,** 531–557.
14. Aulak, K. S., Pemberton, P. A., Rosen, F. S., Carrell, R. W., Lachmann, P. J. & Harrison, R. A. (1988) *Biochem. J.* **253,** 615–618.
15. Skriver, K., Radziejewska, E., Siebermann, J. A., Donaldson, V. H. & Bock, S. C. (1989) *J. Biol. Chem.* **264,** 3066–3071.
16. Bock, S. C., Skriver, K., Nielsen, E., Thogersen, M. C., Wiman, B., Donaldson, V. H., Eddy, R. L., Marrinan, J., Radziejewska, E., Huber, R., Shows, T. & Magnussen, S. (1986) *Biochemistry* **25,** 4292–4301.
17. Whitehead, A. S., Woods, D. E., Fleishnick, E., Chin, J. E., Yunis, E. J., Katz, A. J., Gerald, P. S., Alper, C. A. & Colten, H. R. (1984) *N. Engl. J. Med.* **310,** 88–91.
18. Wahl, G. M., Stern, M. & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3683–3687.
19. Mullis, K. B. & Faloona, F. A. (1987) *Methods Enzymol.* **155,** 335–350.
20. Saiki, R. K., Schart, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. & Arnheim, N. (1985) *Science* **230,** 1350–1354.
21. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239,** 487–491.
22. Sanger, F., Nicklen, S. & Coulson, R. A. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
23. Carter, P. E., Dunbar, B. & Fothergill, J. E. (1988) *Eur. J. Biochem.* **173,** 163–169.
24. Cooper, D. N. & Youssoufian, H. (1988) *Hum. Genet.* **78,** 151–155.
25. Coulondre, C., Miller, J. H., Farabaugh, P. J. & Gilbert, W. (1978) *Nature (London)* **274,** 775–780.
26. Wang, R. Y.-H., Kuo, K. C., Gehrke, C. W., Huang, L.-H. & Ehrich, M. (1982) *Biochim. Biophys. Acta* **697,** 371–377.
27. Ragg, H. (1986) *Nucleic Acids Res.* **14,** 1073–1088.
28. Donaldson, V. H., Harrison, R. A., Rosen, F. S., Bing, D. H., Kindness, G., Canar, J., Wagner, C. J. & Awad, S. (1985) *J. Clin. Invest.* **75,** 124–132.
29. Devraj-Kizuk, R., Chiu, D. H. K., Prochownik, E. V., Carter, C. J., Ofosu, F. A. & Blajchman, M. A. (1988) *Blood* **72,** 1518–1523.
30. Stephens, A. W., Thalley, B. S. & Hirs, C. H. W. (1987) *J. Biol. Chem.* **262,** 1044–1048.
31. Holmes, W. E., Lijnen, H. R., Nelles, L., Kluft, C., Nieuwenhuis, H. K., Rijken, D. C. & Collen, D. (1987) *Science* **238,** 209–211.
32. Bock, S. C., Marrinan, J. A. & Radziejewska, E. (1988) *Biochemistry* **27,** 6171–6178.
33. Davis, A. E. (1988) *Annu. Rev. Immunol.* **6,** 595–628.
34. Pemberton, P. A., Harrison, R. A., Lachmann, P. J. & Carrell, R. W. (1989) *Biochem. J.* **258,** 193–198.
35. Bruch, J., Weiss, V. & Engel, J. (1988) *J. Biol. Chem.* **263,** 16626–16630.
36. Carrell, R. W. & Owen, M. C. (1985) *Nature (London)* **317,** 730–732.
37. Carrell, R. W., Pemberton, P. A. & Boswell, D. R. (1987) *Cold Spring Harbor Symp. Quant. Biol.* **52,** 527–535.