Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 661–664, January 1989
Medical Sciences

Tissue-specific splicing mutation in acute intermittent porphyria

(porphobilinogen deaminase/tissue-specific expression)

B. GRANDCHAMP*, C. PICAT*, V. MIGNOTTE†, J. H. P. WILSON‡, K. TE VELDE§, L. SANDKUYL‡, P. H. ROMÉO†, M. GOOSSENS†, AND Y. NORDMANN¶

*Laboratoire de Génétique Moléculaire, Faculté de Médecine X. Bichat, 75018 Paris, France; †Institut National de la Santé et de la Recherche Médicale, Unité 91, Hopital Henri Mondor, 94010 Créteil, France; ‡Department of Internal Medicine, Erasmus University, Rotterdam, The Netherlands; §Department of Internal Medicine, Saint Geertruiden Hospital, Deventer, The Netherlands; and ¶Laboratoire de Biochimie, Hopital Louis Mourier, 92701 Colombes, France

Communicated by Rudi Schmid, September 19, 1988 (received for review May 18, 1988)

ABSTRACT **An inherited deficiency of porphobilinogen deaminase [porphobilinogen ammonia-lyase (polymerizing), EC 4.3.1.8] in humans is responsible for the autosomal dominant disease acute intermittent porphyria. Different classes of mutations have been described at the protein level suggesting that this is a heterogeneous disease. It was previously demonstrated that porphobilinogen deaminase is encoded by two distinct mRNA species expressed in a tissue-specific manner. Analysis of the genomic sequences indicated that these two mRNAs are transcribed from two promoters and only differ in their first exon. The first mutation identified in the human porphobilinogen deaminase gene is a single-base substitution (G → A) in the canonical 5′ splice donor site of intron 1. This mutation leads to a particular subtype of acute intermittent porphyria characterized by the restriction of the enzymatic defect to nonerythropoietic tissues. Hybridization analysis using oligonucleotide probes after *in vitro* amplification of genomic DNA offers another possibility of detecting asymptomatic carriers of the mutation in affected families.**

Acute intermittent porphyria (AIP) is a metabolic disorder characterized by attacks of neurological dysfunction with abdominal pain, hypertension, tachycardia, and peripheral neuropathy. Most attacks are precipitated by drugs, alcohol, caloric deprivation, infections, or endocrine factors (1). AIP results from a partial deficiency of the third enzyme of heme biosynthesis, porphobilinogen deaminase [PBGD; porphobilinogen ammonia-lyase (polymerizing), EC 4.3.1.8] that is inherited as an autosomal dominant trait (1). Early detection of gene carriers is important in the prevention of attacks, as they can be advised to avoid precipitating factors. Since asymptomatic carriers do not consistently excrete abnormal amounts of porphyrins and the porphyrin precursors, 5-aminolevulinic acid and porphobilinogen, the best method for detecting carriers in most AIP families is the determination of erythrocyte PBGD activity (1). There are, however, limitations to this approach as a screening method. Erythrocyte PBGD levels are affected by erythrocyte age and the presence of other diseases (2), and there is some overlap between values for normal individuals and AIP patients (1). In addition, some families have been described with clinical and biochemical criteria indicating AIP, but without the PBGD deficiency in the erythrocytes of the patients (3–5); in fact the distribution of PBGD activity in these AIP patients and their relatives is identical to that in healthy controls (3, 5), in contrast to most AIP families in which erythrocyte PBGD levels show a biphasic distribution. These features point to the existence of a subset of AIP families in whom the mutation is not expressed in erythrocytes.

We demonstrated (6) that two isoforms of PBGD, one found in nonerythroid cells and the other found only in erythroid cells, are translated from two mRNAs that differ solely in their 5′ termini. These mRNAs are produced through alternative splicing of primary transcripts arising from two promoters separated by 3 kilobases (kb) of DNA (7).

These peculiar features of PBGD gene expression suggested that the absence of the enzymatic defect in erythrocytes from patients with the subtype of AIP mentioned above might result from a mutation either in the nonerythroid sequences of the gene or in a distinct gene, the product of which would be important in the transregulation of PBGD expression in nonerythroid cells.

We have investigated a large kindred with this subtype of AIP by using restriction fragment length polymorphisms (RFLPs) of the PBGD gene (8, 9) to demonstrate the linkage between the porphyria and the PBGD locus in this family. Then by cloning and sequencing the nonerythroid part of the mutated PBGD gene from an affected individual, we demonstrated a point mutation within the donor splice junction of the first intron of the gene: a G → A transition at the first position of the intron. This observation documents the molecular abnormality responsible for the tissue-restricted defect of a ubiquitously expressed gene and provides another diagnostic tool for detecting asymptomatic carriers of this mutation.

MATERIALS AND METHODS

Porphyrin and Porphyrin Precursor Analysis. Erythrocyte PBGD activity was measured by the fluorometric assay of Strand *et al.* (10). Urinary 5-aminolevulinic acid and porphobilinogen were quantitated according to Mauzerall and Granick (11) and urinary and fecal porphyrins were measured either by solvent extraction techniques or by HPLC (5).

DNA Hybridization Analysis. DNA was prepared from peripheral blood nucleated cells (12) and digested to completion with the restriction endonucleases *Msp* I and *Pst* I (Boehringer Mannheim). Restriction fragments were separated on an agarose gel by electrophoresis and blotted to Zeta-Probe membranes (Bio-Rad) in alkaline conditions. A 0.9-kb *Pst* I fragment containing the fragment of the PBGD gene specific for nonerythroid cells and a 0.9-kb *Eco*RI–*Bam*HI fragment containing the erythroid promoter of the PBGD gene were used as hybridization probes to detect the *Msp* I and *Pst* I polymorphisms, respectively (Fig. 1). Labeling was performed by the random-priming method (13) (specific activity, $>10^9$ cpm/μg). Filters were hybridized and washed in a high-stringency buffer and then autoradiographed with intensifying screens for 48 hr.

Statistical Analysis. Linkage analysis was carried out with the aid of the MLINK computer program.

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: AIP, acute intermittent porphyria; PBGD, porphobilinogen deaminase; RFLP, restriction fragment length polymorphism.

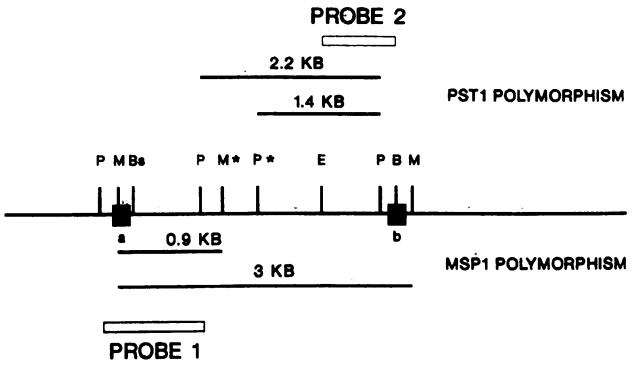

FIG. 1. Partial restriction pattern of the PBGD gene. P, *Pst* I; M, *Msp* I; E, *Eco*RI; B, *Bam*HI; Bs, *Bst*E2. Only those restriction sites are indicated that are relevant to the present study. The asterisks indicate the polymorphic sites. The *Msp* I RFLP was detected by hybridization with a 0.9-kb *Pst* I fragment (probe 1) and the *Pst* I RFLP was detected using a 0.9-kb *Eco*RI–*Bam*HI fragment as a probe (probe 2). The portion of the PBGD gene cloned from DNA of the patient is a 10-kb *Bam*HI fragment, the 3′ end of which is the *Bam*HI site represented on the figure. The *Pst I–Bst*E2 fragment of the mutated gene that contains exon a was sequenced. Exon a is the non-erythropoietic-specific exon of the gene; exon b is the erythropoietic-specific exon (7). Only the first two exons of the gene are represented by solid boxes.

Cloning the Mutant Gene. DNA prepared from a patient heterozygous for the *Msp* I and *Pst* I polymorphisms was digested to completion with *Bam*HI and then the restriction fragments were size-fractionated on a 0.8% agarose gel. DNA fragments in the range of 10 kb were recovered from the gel by electroelution and ligated to EMBL3 arms (14). Forty thousand plaques were screened by hybridization with a radiolabeled probe derived from a 0.9-kb *Pst* I restriction fragment containing the first exon (nonerythroid exon) of the PBGD gene (7). DNA from hybridizing clones was prepared and the haplotype restriction cleavage pattern was determined. Two clones with haplotype A (see *Results*) were further studied. A *Pst* 1–*Bst*E2 fragment was subcloned into M13mp18 and M13mp19 (15). The chain-terminator DNA sequence procedure (16) was then performed.

DNA Amplification and Hybridization with Oligonucleotides. DNA amplifications were carried out according to Saiki *et al.* (17) in the presence of *Taq* polymerase (Cambio). The two primers used were both 20-mer oligonucleotides, I (GTCTGGTAACGGCAATGCGG) and II (ATCGCTGCACGGCTCGTCCAG). They were designed to anneal to target sequences, 88 bases apart in genomic DNA, allowing the amplification of a segment containing the site of the mutation. The samples were denatured at 91°C for 1 min, cooled to 50°C over 1 min, and then heated to 65°C for 1 min. The reaction was performed 30 times.

For hybridization analysis, 10 μl of amplified sample was processed as described (18). Zeta-Probe nylon filters were prehybridized in 5× SSEP/5× Denhardt's solution/0.5% $NaDodSO_4$ for 1 hr at 55°C and then hybridized at the same temperature for 2–4 hr with radiolabeled oligonucleotide at 10^6 cmp/ml. (1× SSEP = 0.15 M NaCl/0.01 M NaH_2PO_4/0.001 M EDTA, ph 7.4; 1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin.) The probe was labeled at the 5′ end with [γ^{32}-P]ATP to a specific activity of 10^9 cpm/μg (18). Blots were washed at room temperature in 2× SSEP for 30 min and then in 5× SSEP/0.1% $NaDodSO_4$ at 68°C for 4 min and autoradiographed at −70°C with one Dupont Cronex Lightning Plus intensifying screen.

RESULTS

A large Dutch kindred with AIP and normal erythrocyte PBGD levels was studied (5). Genealogical studies of eight index patients with AIP in four families in the northern

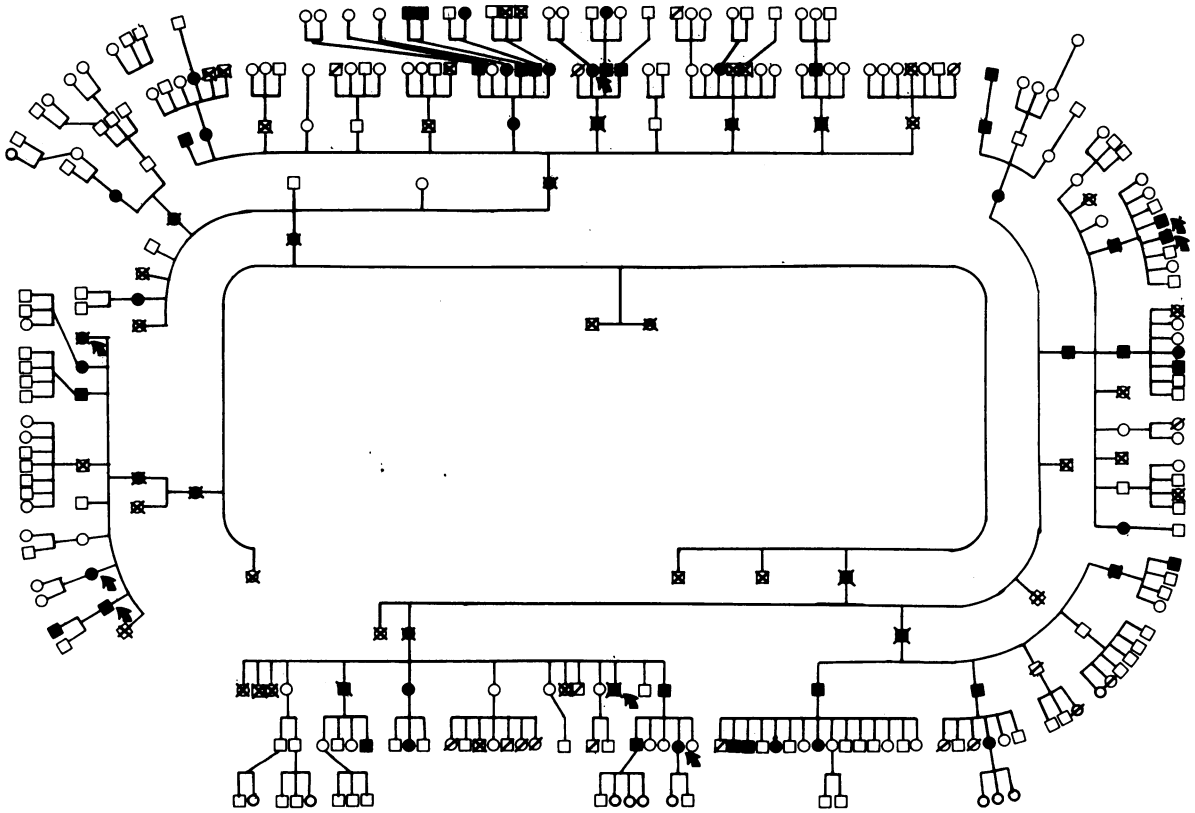

FIG. 2. Family tree of the Dutch family with AIP and normal erythrocyte PBGD activities. /, Not examined; \, dead. Solid symbols denote carriers of the AIP gene (individuals with biological abnormalities and obligate carriers). The arrows indicate AIP patients.

Netherlands had revealed a common ancestral pair in the early 19th century (Fig. 2). The diagnosis of AIP in the index patients was based on a history of an acute attack compatible with porphyria and associated with raised urinary 5-aminolevulinic acid and porphobilinogen levels, and the finding of raised urinary 5-aminolevulinic acid and porphobilinogen levels at follow-up examination. Variegate porphyria and coproporphyria were excluded by the absence of photosensitivity and by normal fecal porphyrin patterns in any of the family members studied.

We tested the linkage between the AIP phenotype and the PBGD locus in this family, using RFLPs of the human PBGD gene (8, 9). We first mapped the previously reported *Pst* I polymorphism (9) to the first intron of the PBGD gene by restriction analysis of genomic DNA from normal individuals, in close proximity to the *Msp* I polymorphism (8) (Fig. 1). Haplotype analysis in normal families demonstrated a strong linkage disequilibrium between the two polymorphic markers. From the 37 haplotypes determined, 17 were positive and negative for the *Msp* I and *Pst* I sites, respectively (haplotype A) and 20 were negative and positive for *Msp* I and *Pst* I sites, respectively (haplotype B). In all instances the different haplotypes segregated in a Mendelian fashion. From haplotype determination in 68 individuals from the AIP family under investigation, we calculated a Lod score 3.2 for linkage between the AIP phenotype and haplotype A (Fig. 3). This finding strongly suggested that the mutation responsible for AIP in this family lies within the PBGD gene and is likely to involve the sequences of the gene specific for nonerythroid cells if one considers the normal activity of PBGD in erythrocytes of these patients.

We next constructed a partial genomic library from the DNA of a patient heterozygous for the restriction haplotypes A and B. From this library we isolated PBGD clones and selected those carrying haplotype A. Then we sequenced parts of the gene specifically involved in its expression in nonerythroid cells (i.e., the nonerythroid promoter, the first exon, and the 5′ portion of the first intron). A G → A transition was observed at the first position of the intron modifying the normal consensus sequence of splicing from CGGTGAGT (7) to CGATGAGT. This base change was confirmed by direct study of genomic DNA from the patient. An 88-base-pair DNA fragment was amplified *in vitro* using the polymerase chain reaction and hybridized to radiolabeled oligonucleotides (17). One of the oligonucleotides matched the normal sequence and the other matched the mutated sequence (Fig. 4*A*). As expected, the amplified fragment from the patient hybridized to both probes in agreement with the

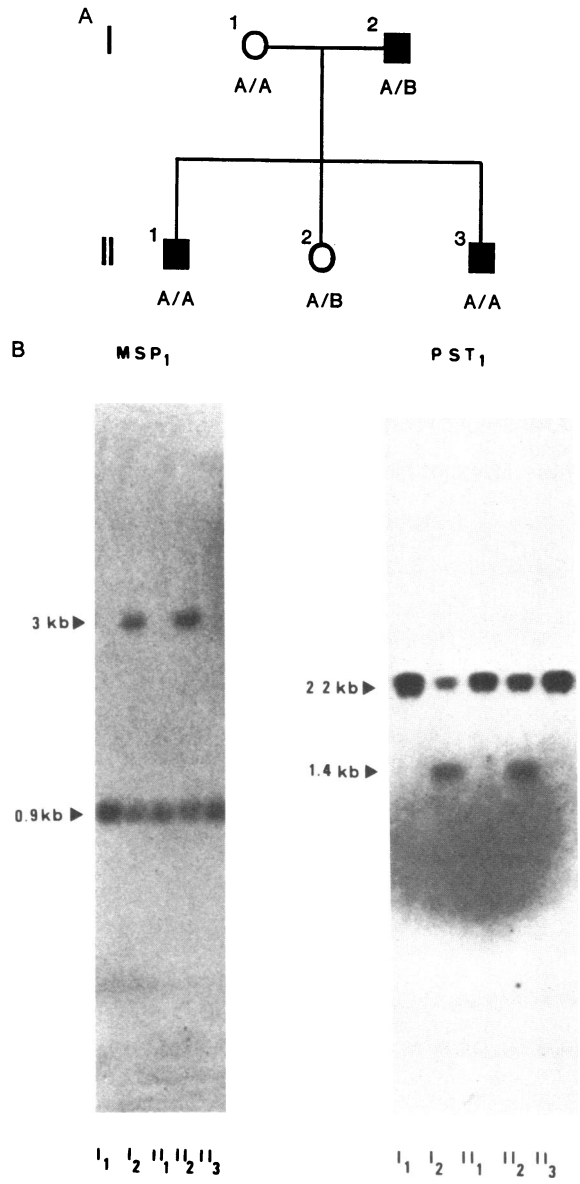

FIG. 3. (*A*) Section of the Dutch family illustrating the linkage between the AIP phenotype and haplotype A. Individuals were classified as carriers of the mutation if they (*i*) had a documented history of an acute attack, (*ii*) had raised 5-aminolevulinic acid and porphobilinogen levels in urine and raised porphobilinogen levels in plasma, or (*iii*) were an obligate carrier, as determined from the family tree. Only individuals related to the kindred by marriage were considered to be normal. All other family members were *a priori* considered as doubtful. Solid circles and squares represent carriers of the AIP trait, as defined above. (*B*) *Msp* I and *Pst* I restriction fragments of individuals from the pedigree are shown hybridized to probes 1 and 2, respectively (see Fig. 1). DNA (10 μg) was digested with the relevant restriction enzyme revealing the polymorphism for each PBGD probe. Lane labels are taken from family tree in *A*.

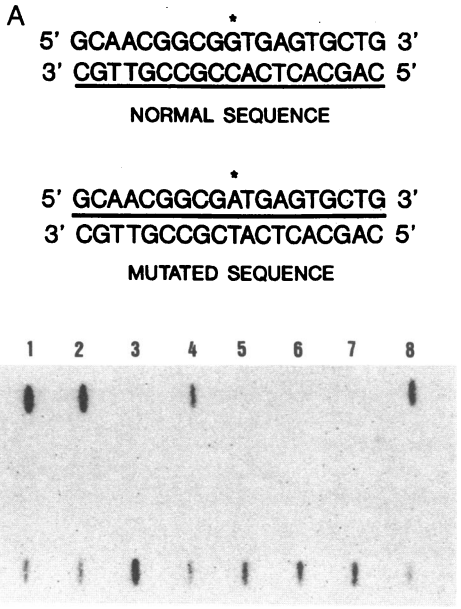

FIG. 4. (*A*) Probes used for hybridization of amplified genomic DNA. The sequences of the two oligonucleotides are underlined. The asterisks indicate the position of the base change. (*B*) Detection of carriers of the AIP mutation by hybridization with oligonucleotides of amplified sequences from family members. The amplified sequence from each individual was hybridized in duplicate with a probe corresponding to the normal sequence (N) and with a probe matching the mutated sequence (M). Individuals whose DNA is shown in lanes 1, 2, 4, and 8 were patients or obligate carriers; individuals whose DNA is shown in lanes 3, 5, 6, and 7 were unrelated controls.

fact that he is heterozygous for the mutation. All the other affected family members and obligate carriers that we tested also produced a similar pattern of hybridization. In contrast, amplified DNA from normal controls only hybridized with the oligonucleotide of normal sequence.

DISCUSSION

The mutations that produce AIP have not been characterized at the DNA level. Four mutant classes were previously identified by immunological and enzymatic studies of PBGD in erythrocytes from AIP patients (4, 5). In the majority of affected families, immunodetectable protein was decreased proportionally to the enzymatic activity. Recent studies using PBGD probes detecting a *Msp* I RFLP suggested the molecular heterogeneity of the mutation in this group (8). Two groups of mutations have been described with different ratios between immunological and enzymatic activity (4, 5). More surprisingly, in a few families, the enzymatic deficiency of PBGD appeared to be restricted to nonerythroid cells (3–5). We studied a family with this form of porphyria by first addressing the question of the involvement of the PBGD gene in the pathology of the disease. Evidence for linkage between the phenotype and a RFLP haplotype at the PBGD locus prompted us to clone and sequence the mutant allele of a patient from this family. We demonstrated a point mutation in the canonical 5′ splice donor site of the first intron of the PBGD gene (G → A). Similar mutations have been shown (19, 20) to completely abolish normal splicing. Since the first intron interrupts the sequence coding for the nonerythroid isoform of PBGD (7), it is to be expected that an abnormal splicing leads to detrimental effects on that gene product. In contrast, in erythroid cells, transcription of the gene starts 2.8 kb downstream from the identified mutation and it is, therefore, logical that this mutation has no consequence for the expression of the PBGD gene in these cells, in keeping with the normal PBGD activity in erythrocytes from patients of this family. This documents an example of a mutation of a ubiquitously expressed gene that affects its function only in certain tissues in relationship with the mechanism of the differential transcription and splicing of this gene determined by tissue specificity. This finding has also practical implications: early detection of gene carriers is important in the prevention of attacks, as they can be advised to avoid precipitating factors. In most AIP families, the detection of asymptomatic carriers is achieved by the determination of erythrocyte PBGD (1). However, this diagnostic test cannot be utilized in families where the mutation does not affect the enzyme activity in erythrocytes. In some instances, linkage of the mutation to common RFLP detected with PBGD probes provides a means of identifying carriers of the AIP gene, but this approach is limited to informative pedigrees (8). In the family reported here, the identification of the mutation allows another approach: namely the direct detection of the mutation in family members using oligonucleotide-mutation-specific probes after *in vitro* amplification of the target DNA sequence by the polymerase chain reaction (17). Such probes can be used to determine whether the same mutation is present in other families with the subtype of AIP described above.

This work was supported by grants from Institut National de la Santé et de la Recherche Médicale (CRE 87.1006) and from Agricultural Research Council (6702).

1. Kappas, A., Sassa, S. & Anderson, K. E. (1983) in *The Metabolic Basis of Inherited Disease*, eds. Stanbury, J. B., Wyngaarden, J. B., Frederickson, D. S., Goldstein, J. L. & Brown, M. S. (McGraw-Hill, New York), pp. 1301–1384.
2. Anderson, K. E., Sassa, S., Peterson, C. M. & Kappas, A. (1977) *Am. J. Med.* **63**, 359–374.
3. Mustajoki, P. (1981) *Ann. Int. Med.* **95**, 162–166.
4. Desnick, R. J., Ostasiewicz, L. T., Tishler, P. A. & Mustajoki, P. (1985) *J. Clin. Invest.* **76**, 505–509.
5. Wilson, J. H. P., de Rooy, F. W. M. & te Velde, K. (1986) *Neth. J. Med.* **29**, 393–399.
6. Grandchamp, B., de Verneuil, H., Beaumont, C., Chretien, S., Walter, O. & Nordmann, Y. (1987) *Eur. J. Biochem.* **162**, 105–110.
7. Chretien, S., Dubart, A., Beaupain, D., Raich, N., Grandchamp, B., Rosa, J., Goossens, M. & Romeo, P. H. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6–10.
8. Llewellyn, D. H., Elder, G. H., Kalsheker, N. A., Grandchamp, B., Picat, C., Nordmann, Y., Romeo, P. H. & Goossens, M. (1987) *Lancet* **ii**, 706–708.
9. Jin-Sung, L., Anvret, M. (1987) *Nucleic Acids Res.* **15**, 6307.
10. Strand, L. J., Meyer, U. A., Fesher, B. F., Redeker, A. G. & Marver, H. S. (1972) *J. Clin. Invest.* **51**, 2530–2536.
11. Mauzerall, D. & Granick, S. (1956) *J. Biol. Chem.* **219**, 435–436.
12. de Martinville, B., Wyman, A. R., White, R. & Francke, U. (1982) *Am. J. Hum. Genet.* **34**, 216–226.
13. Feinberg, A. F. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13.
14. Maniatis, T. E., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
15. Messing, J. & Vieira, J. (1982) *Gene* **19**, 269–276.
16. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
17. Saiki, K. R., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239**, 487–491.
18. Kogan, S. C., Doherty, M. & Gitschier, J. (1987) *N. Engl. J. Med.* **317**, 985–990.
19. Treisman, R., Orkin, S. H. & Maniatis, R. T. (1983) *Nature (London)* **302**, 591–596.
20. Dilella, A. G., Marvit, J., Lidsky, A. S., Guttler, F. & Woo, S. L. C. (1986) *Nature (London)* **322**, 799–803.