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Biochemistry

Mutation eliminating mitochondrial leader sequence of methylmalonyl-CoA mutase causes *mut*° methylmalonic acidemia

(molecular cloning/enzyme precursors/signal peptides/inborn error of metabolism/amino acid sequence)

FRED D. LEDLEY*†‡§, RUUD JANSEN*, SANG-UK NHAM*, WAYNE A. FENTON¶, AND LEON E. ROSENBERG¶

*Howard Hughes Medical Institute, and Departments of †Cell Biology and §Pediatrics, Baylor College of Medicine, Houston, TX 77030; and ¶Department of Human Genetics, Yale University School of Medicine, New Haven, CT 06510

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ABSTRACT **Methylmalonyl-CoA mutase (EC 5.4.99.2) is a mitochondrial matrix enzyme whose activity is deficient in the inherited disorder methylmalonic acidemia. Previous studies on primary fibroblast cell lines from patients with methylmalonic acidemia have delineated a variety of biochemical phenotypes underlying this disorder. One cell line with primary mutase apoenzyme deficiency exhibited a particularly unusual phenotype; it expressed an abnormally small and unstable immunoreactive protein, which was not imported by mitochondria. We now report cloning and sequencing of the cDNA encoding this mutant protein. The mutation is a single base change, a cytosine → thymine transition, which introduces an amber termination codon at position 17 within the mitochondrial leader sequence. The immunoreactive protein produced by these cells reflects translation from AUG codons downstream from this termination codon and, hence, lacks a mitochondrial leader peptide. This mutation represents a complex prototype for a class of mutations in which absence of the mitochondrial targeting sequence leads to absence of a functioning gene product.**

Methylmalonyl-CoA mutase [MCM; (*R*)-2-methyl-3-oxopropanoyl-CoA CoA-carbonyl mutase, EC 5.4.99.2] is a mitochondrial matrix enzyme encoded by a nuclear gene (1–3). It is translated on cytoplasmic ribosomes as a 742-amino acid (82,145 Da) precursor protein comprising a 32-amino acid mitochondrial leader sequence and a 710-amino acid (78,351 Da) mature apoenzyme (4). The leader sequence directs the precursor to the mitochondria where it is recognized, translocated across both mitochondrial membranes, and cleaved by a specific matrix endoprotease to form the mature subunit (3). The subunits then assemble into a homodimer and bind the adenosylcobalamin cofactor.

Inherited deficiency of MCM causes methylmalonic acidemia (MMA), an inborn error of organic acid metabolism in which failure of methylmalonyl-CoA degradation leads to widespread aberrations in organic acid, amino acid, and carbohydrate metabolism (5). MMA can result from two general classes of genetic defects: those termed *cbl*, which involve genes required for provision of the adenosylcobalamin cofactor (McKusick nos. 251100 and 151110) (5, 6), and those termed *mut*, which involve the gene encoding the MCM apoenzyme (McKusick no. 251000) (5, 6). *mut* MMA is further divided into phenotypic subgroups based on the presence (*mut*$^-$) or complete absence (*mut*°) of detectable enzyme activity in primary fibroblasts (5, 7). The *mut*° phenotype itself is pleomorphic, with some cells containing significant amounts of cross-reactive antigenic material (CRM), some expressing severely decreased CRM, some expressing unstable proteins, which can only be detected by pulse–chase or *in vitro* translation methods (8, 9), and some expressing no detectable CRM. More recent studies using the cloned MCM cDNA have shown that some *mut*° cells have extremely low levels of MCM mRNA, whereas other *mut*° and all *mut*$^-$ cells have mRNA that is grossly normal in quantity and size (10, 11).

One particularly interesting *mut*° cell line (FB552) has been described in which the mutant MCM gene expresses a protein that is smaller than the normal precursor and is highly unstable. This abnormal protein is not imported or cleaved by mitochondria (9). This cell line exhibits no detectable enzymatic activity and no stable CRM in culture (8, 9), despite having grossly normal hybridizing MCM mRNA (11). These data suggest that the mutant gene product is deficient in the mitochondrial targeting signal (9). We now report cloning and sequencing of the MCM cDNA from the FB552 cell line and identification of the mutation that gives rise to this phenotype.

MATERIALS AND METHODS

Cloning of MCM cDNA by the Polymerase Chain Reaction (PCR) and Determination of the Nucleic Acid Sequence. MCM cDNA was cloned by reverse transcription and PCR amplification from total RNA by using the methods illustrated in Fig. 1*A* (12). Briefly, RNA was prepared by the hot phenol method (13), and first strand cDNA was prepared by priming reverse transcription with an oligonucleotide complementary to sequences in the 3′ untranslated region of MCM (14). Two overlapping portions of the cDNA were amplified by using oligonucleotides corresponding to the MCM cDNA sequence, subcloned directionally in pGEM7zf(+), and sequenced by dideoxy sequencing by using oligonucleotides complementary to vector or cDNA sequences as illustrated in Fig. 1*B*. PCR mistakes and heterozygosity were evaluated by sequencing single isolates of cloned material and pools containing 15–20 independent isolates. Recombinant DNA procedures were carried out by using standard methods (14). Oligonucleotide sequences will be given elsewhere.

***In Vitro* Transcription and Translation of MCM cDNA.** The full-length normal and mutant cDNA sequences were reconstructed by three-part ligation of the 5′ *Cla* I–*Acc* I and 3′ *Acc* I–*Mlu* I fragments into a *Cla* I–*Mlu* I-digested pGEM7zf vector. Subclones of the full-length MCM cDNA containing 5′ terminal exonuclease III deletions have been described (4) (see Fig. 3*A*). These include hMCM26 (bases 315–2770), hMCM1.1 (bases 424–2770), hMCM5.1 (bases 721–2770), and hMCM8.3 (bases 928–2770). Synthetic RNA was produced by transcription from the T7 promoter by T7 RNA polymerase (Promega) and was translated into protein by using reticulocyte lysate (Amersham). Incorporation of [^{35}S]methi-

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Abbreviations: MCM, methylmalonyl-CoA mutase; MMA, methylmalonic acidemia; PCR, polymerase chain reaction; CRM, cross-reactive antigenic material.

‡To whom reprint requests should be addressed.

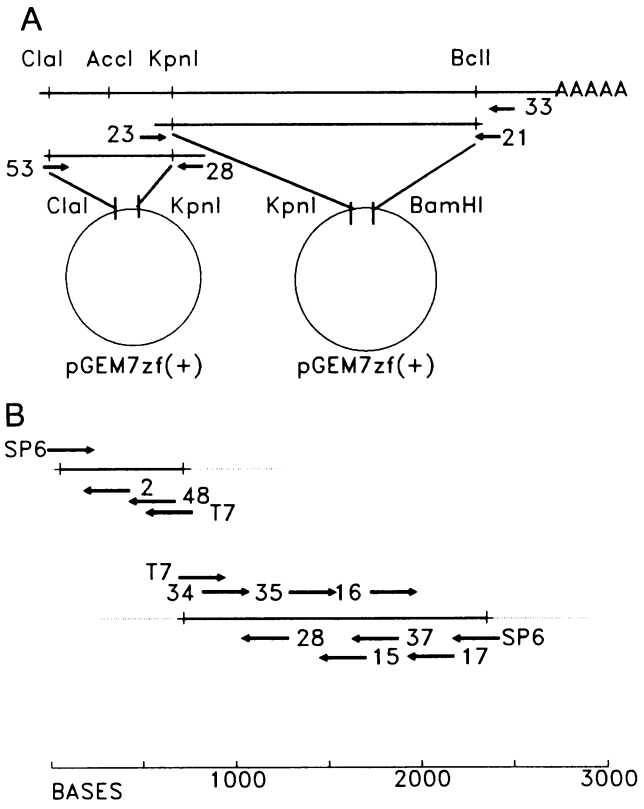

FIG. 1. Strategy for cloning and sequencing cDNA from FB552. (*A*) Schematic showing oligonucleotides used for reverse transcription and PCR amplification of FB552 cDNA. The restriction sites used for directional cloning of PCR products are shown. (*B*) Strategy for sequencing FB552 cDNA showing the positions of oligonucleotide primers internal to the cDNA sequence and the direction of sequencing. The full-length cDNA is described in ref. 4. The sequences of the numbered oligonucleotides will be given elsewhere.

onine (Amersham) into protein was assayed on SDS/7–15% polyacrylamide gradient gels.

Demonstration of Mutation in Genomic DNA by PCR. Genomic sequences spanning the putative mutation site in exon II and a previously described *Hin*dIII polymorphism in exon III were amplified by PCR using oligonucleotides complementary to exon and intron sequences (S.-U.N., R.J., and F.D.L., unpublished data) (see Fig. 4*A*). The presence of the mutation was confirmed by direct sequencing of PCR products using methods described (16, 17). The *Hin*dIII polymorphism was identified by digestion of PCR products, separation of fragments on a 15% acrylamide gel, and staining with ethidium bromide (1 μg/ml) (S.-U.N., R.J., and F.D.L., unpublished data). In the direct analysis of PCR products by sequencing or restriction digestion, PCR mistakes represent minor species, which are not detectable.

RESULTS

Sequence of the FB552 cDNA. Several differences from the published reference MCM sequence (hMCM7.1) (4) were observed in the cDNA cloned from FB552. The mutation identified in the FB552 cDNA is a transition of $C^{128} \rightarrow T^{128}$, which changes the CAG codon encoding Gln-17 to a TAG (amber) termination codon. There was no evidence of the C^{128} base in the pooled sample, indicating that all of the mRNA contained the T^{128} variant (Fig. 2). We also observed a previously described *Hin*dIII polymorphism at base 712. Again, there was no evidence for heterogeneity in the mRNA; all of the cDNAs exhibited the *Hin*dIII(−) sequence.

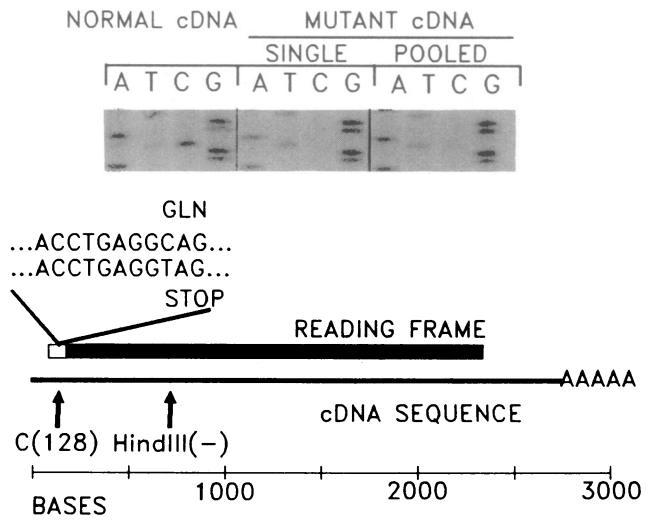

FIG. 2. Mutation in FB552 cDNA. The sequencing gel shows the sequence of a normal cDNA clone, an isolated single mutant cDNA clone, and a pool of 15 mutant cDNA clones. The $C^{128} \rightarrow T^{128}$ transition is apparent when comparing the normal and isolated clone sequences. The pooled sequence shows only T^{128}, indicating that all of the mRNA contains this mutation. This mutation introduces an amber termination codon at position 17 within the mitochondrial leader sequence. The position of the mitochondrial leader sequence (open box), mature apoenzyme sequence (solid box), and cDNA sequence are shown schematically along with the position of the mutation and *Hin*dIII polymorphic site.

***In Vitro* Transcription and Translation of cDNA Bearing the FB552 Mutation.** The 5′ segment of the FB552 cDNA, comprising bases 52–375, was recombined with a normal 3′ cDNA segment in the vector pGEM7zf(+). RNA was transcribed from this clone, from a control plasmid containing the normal 52–2774 sequence, and from a series of clones containing 5′ terminal deletions (Fig. 3*A*). These clones were selected such that the first AUG of each clone represents a different internal AUG (methionine) codon and potential initiation site (Fig. 3*A*).

In vitro translation of the normal sequence produces the expected precursor protein of ≈82 kDa as well as a series of smaller translation products (Fig. 3*B*, lane 3). The reconstructed mutant clone does not exhibit the 82-kDa peptide, as expected, although all of the smaller translation products are present (Fig. 3*B*, lane 2). The terminally deleted clones exhibit a sequential loss of the larger molecular mass translation products (Fig. 3*B*, lanes 5–8) corresponding to initiation at AUG codons within the cDNA, which can be individually identified by reference to the terminally deleted subclones (Fig. 3*A*). Specifically, clone hMCM26 lacks the first internal methionine codon at position 311 and does not make either the 82-kDa protein or the 70-kDa protein. Clone hMCM1.1 (Fig. 3*B*, lane 5) lacks the second internal methionine codon at position 371 and lacks the 63.9-kDa translation product. The dominant band of material translated from FB552 corresponds to the product of ≈45 kDa translated from clone hMCM8.3 (Fig. 3*B*, lane 7). It is not possible to correlate all of the bands observed by *in vitro* translation of the recombinant RNA with those observed from purified cellular mRNA, because plasmid and polylinker sequences at the 5′ end of the recombinant RNA transcript may affect translational efficiency (18). There are no other reading frames in the MCM cDNA that would encode proteins >10 kDa.

Identification of Mutation and Polymorphisms in FB552 Genomic DNA. To confirm the presence of the $C^{128} \rightarrow T^{128}$ mutation in the FB552 genome, DNA from the FB552 cell line was amplified by PCR and subjected to direct sequencing by

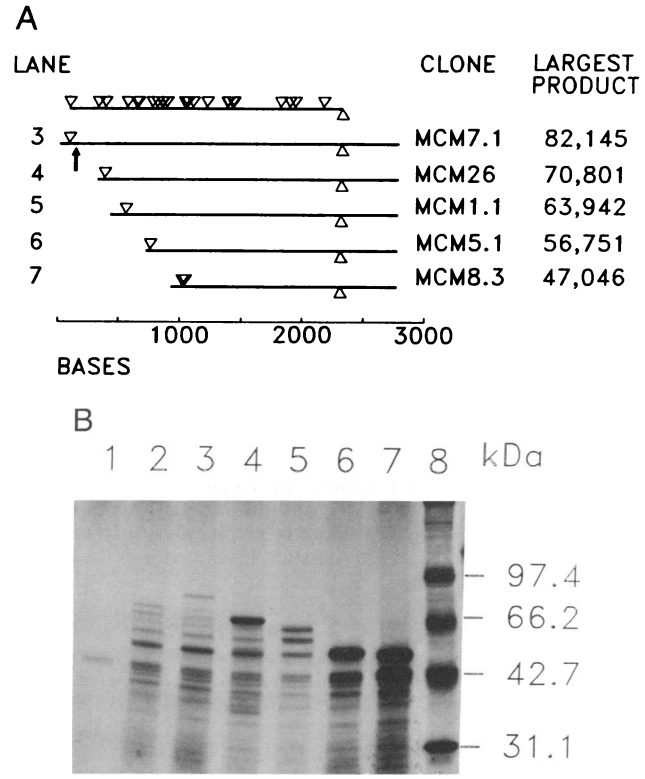

FIG. 3. *In vitro* transcription and translation of cDNA with the FB552 mutation and of controls. (*A*) Schematic of potential initiation sites within the long open reading frame of the MCM mRNA and the terminally deleted subclones used for *in vitro* transcription and translation. ▽, Position of "in-frame" methionine (AUG) codons; △, position of the normal termination codons; ↑, position of the amber mutation (position 128). No alternative reading frames that could code for translation products >10 kDa are present. (*B*) *In vitro* translation of clones containing normal MCM cDNA, of cDNA carrying the FB552 mutation, and of deletion subclones. Lane 1, no RNA; lane 2, translation of mRNA carrying FB552 mutation; lane 3, translation of normal MCM mRNA; lane 4, translation of mRNA from clone hMCM26; lane 5, translation of mRNA from clone hMCM1.1; lane 6, translation of mRNA from clone hMCM5.1; lane 7, translation of mRNA from clone hMCM8.3; lane 8, molecular size markers (sizes in kDa are indicated).

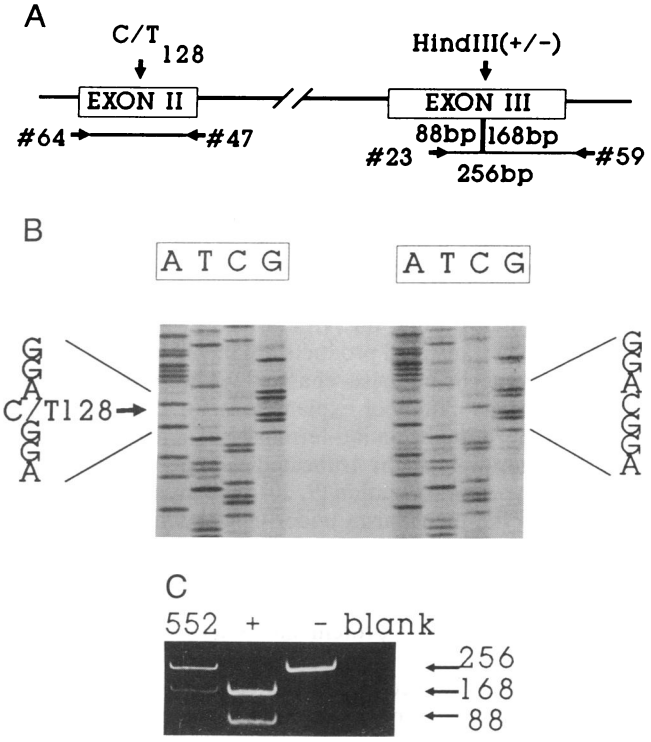

FIG. 4. Demonstration of heterozygosity in FB552 genomic DNA. (*A*) Scheme for amplification of exon II sequences containing the FB552 mutation and exon III sequences containing the *Hin*dIII polymorphism. The position of oligonucleotides and regions amplified by PCR are indicated. Oligonucleotide sequences are as follows: no. 23, CCGCTCGAGTTTCCATGACTATGA; no. 47, CATACTGGCGGATGGTCCAG; no. 59, AATTCCTACATTCAAGGAACTATAG; no. 64, TACTCTATGTTTCTTTTCTAGGTCAG. (*B*) Sequence analysis of exon II PCR products. Heterozygosity is apparent by the presence of both the cytosine and thymine residues at position 128 as indicated. In contrast, normal DNA shows only the cytosine at this position. (*C*) Ethidium bromide-stained PCR products from exon III showing heterozygosity for *Hin*dIII polymorphism. The *Hin*dIII(−) allele is identified in genomic DNA by the presence of a 256-bp PCR fragment ("−"). The *Hin*dIII(+) allele is identified by cleavage of the PCR products into 168- and 88-bp fragments ("+"). Heterozygosity is established in FB552 genomic DNA ("552") by the presence of the 256-, 168-, and 88-bp bands.

using the scheme illustrated in Fig. 4*A*. Direct sequencing revealed the presence of the $C^{128} \rightarrow T^{128}$ change within exon II but also uncovered heterozygosity at this position; the normal cytosine base was also detected (Fig. 4*B*). The *Hin*dIII polymorphic site within exon III was assayed after PCR amplification (Fig. 4*A*) and was similarly found to be heterozygous (Fig. 4*C*).

DISCUSSION

The phenotype of the FB552 cell line has been characterized in several previous publications. This cell line exhibits no detectable MCM enzymatic activity either by *in vitro* enzymatic assays or by propionate uptake in cultured cells and is hence designated *mut*° (8, 9, 11). Steady-state CRM was not detected in this cell line in an initial survey (8), although detailed analysis of CRM production by using pulse–chase methods revealed the presence of an abnormally sized and unstable translation product, which was neither translocated into mitochondria nor proteolytically processed, either in cells or *in vitro* (9). Subsequent studies demonstrated that the FB552 cell line contained a qualitatively normal MCM mRNA (11).

Cloning and sequencing cDNA from the FB552 cell line revealed a nonsense (amber) mutation at position 17, which terminated translation from the proper AUG in the midst of the leader sequence. The previously described immunoreactive proteins are apparently produced by translation from AUG codons internal to the normal reading frame 3′ of the mutation. These truncated peptides, lacking the mitochondrial leader sequence and a portion of the amino terminus of the mature apoenzyme, are not transported into the mitochondria and do not express MCM activity *in vivo*.

These studies describe the genotype of a complex mutation in a nuclear-encoded mitochondrial matrix protein. It has been suggested that this mutation represents a prototype for a class of mutations in which the absence of a mitochondrial targeting sequence leads to the absence of a functioning gene product (9). The failure of mitochondrial transport, however, is only one of several consequences of this mutation.

First, normal translation of the long open reading frame terminates after amino acid 17. This premature termination of translation is the ultimate cause of the deficiency of steady-state immunoreactive protein and assayable enzyme activity in this cell line. It is interesting that sensitive methods for assaying translation indicate that immunoreactive protein is produced in cultured cells and can be produced by *in vitro*

methods. When the FB552 cDNA is translated *in vitro*, the truncated proteins appear to be produced by coincident translation from internal AUG sequences rather than by reinitiation of translation following premature termination, because the smaller translation products are generated in roughly equivalent amounts from the normal and FB552 cell lines. In contrast, when terminally deleted subclones are used as controls, greater translational efficiency is seen from the internal initiation sites. These observations are consistent with previous data indicating that the translational efficiency of mRNA purified from the FB552 cell line is significantly lower than that of other mRNAs (9).

Second, the translated products are unstable and can only be detected in cells by pulse–chase or *in vitro* translation (9). There are many potential explanations for this instability, including the primary amino-terminal sequence (19) or tertiary configuration of the truncated peptide or a failure of proper compartmentalization (9, 20). The present work does not address the mechanism underlying this instability. The impaired translation of the long open reading frame distal to the amber mutation and the relative instability of this abnormal protein product constitute the proximal cause of the absence of steady-state protein and assayable enzyme activity.

Third, in the absence of a leader sequence, the truncated translation products are not transported into mitochondria. Even if these proteins retained determinants for necessary catalytic activity, it is doubtful that this gene product would be biologically active in the cytoplasmic space. The adenosylcobalamin cofactor, required for holoenzyme formation and activity, is synthesized within the mitochondria and is not transported into the cytoplasm. In addition, the methylmalonyl-CoA substrate is also synthesized within the mitochondria (5). In addition, protein factors responsible for proper assembly of mitochondrial proteins are not present in the cytosol (15).

Finally, although only one cDNA species was cloned from this cell line, analysis of genomic DNA indicates that this cell line is heterozygous for both the C^{128} mutation and a polymorphic *Hin*dIII site. It was previously suggested that the multiple bands of immunoreactive protein observed in the FB552 cell line represented translation products of mRNA species arising from heterozygous alleles. Analysis of polymorphisms within the MUT genomic locus indicates that this cell line is indeed a compound heterozygote. One allele contains the $C^{128} \rightarrow T^{128}$ mutation. The second allele, however, apparently does not express a clonable mRNA because pools of amplified cDNA contain only T^{128}/*Hin*dIII(−) transcripts. Thus, it is likely that the multiple translation products observed previously arose from coincident usage of alternative AUG codons within the mutant mRNA, as seen in the *in vitro* translation experiment, rather than from translation from heterologous alleles.

Previous studies have shown that up to one-third of *mut*^{0} cell lines express a "low message" phenotype in which the levels of hybridizable mRNA are significantly lower than in normal cell lines (11). This observation is consistent with protein data, which predicted the presence of "null" alleles among the CRM^+ cell lines (9). It is likely that these low message alleles are common contributors to compound heterozygosity in both *mut*$^-$ and *mut*^{0} forms of MMA. The nature of this mutation (or mutations) in genomic DNA has yet to be determined.

This work represents an application of molecular cloning to the characterization of mutations in *mut* MMA. These studies confirm the predictions made by somatic cell complementation and analysis of the biochemical phenotype of MMA cells that the *mut* complementation group represents cells with allelic mutations within the MUT locus. A variety of mutations have now been described, which underlie *mut* MMA (R.J., S.-U.N., and F.D.L., unpublished results). There is no evidence from population genetic data or molecular cloning that MMA represents a discrete set of common mutations. Rather, this relatively rare disorder appears to result from a large variety of mutations as would be expected if a genetic equilibrium exists between new mutations and the generally lethal nature of MCM deficiency.

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1. Kolhouse, J. F., Utley, C. & Allen, R. H. (1980) *J. Biol. Chem.* **255,** 2708–2712.
2. Fenton, W. A., Hack, A. M., Willard, H. F., Gertler, A. & Rosenberg, L. E. (1982) *Arch. Biochem. Biophys.* **214,** 815–823.
3. Fenton, W. A., Hack, A. M., Helfgott, D. & Rosenberg, L. E. (1984) *J. Biol. Chem.* **259,** 1–21.
4. Jansen, R., Kalousek, F., Fenton, W. A., Rosenberg, L. E. & Ledley, F. D. (1989) *Genomics* **4,** 198–205.
5. Rosenberg, L. E. & Fenton, W. A. (1989) in *The Metabolic Basis of Inherited Disease*, eds. Scriver, C. R., Beaudet, A. L., Sly, W. S. & Valle, D. (McGraw-Hill, New York), 6th Ed., pp. 822–844.
6. McKusick, V. A. (1988) *Mendelian Inheritance in Man* (Johns Hopkins Univ. Press, Baltimore).
7. Willard, H. F. & Rosenberg, L. E. (1980) *J. Clin. Invest.* **5,** 90–98.
8. Kolhouse, J. F., Utley, C., Fenton, W. A. & Rosenberg, L. E. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7737–7741.
9. Fenton, W. A., Hack, A. M., Kraus, J. P. & Rosenberg, L. E. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1421–1424.
10. Ledley, F. D., Lumetta, M., Nguyen, P. N., Kolhouse, J. F. & Allen, R. A. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3518–3521.
11. Ledley, F. D., Crane, A. M. & Lumetta, M., *Am. J. Hum. Genet.*, in press.
12. Vrieling, H., Simons, J. W. I. M. & vanZeeland, A. A. (1988) *Mutat. Res.* **198,** 107–113.
13. Scherrer, K. (1969) in *Fundamental Techniques in Virology*, eds. Habel, K. & Salzman, N. P. (Academic, New York), pp. 413–432.
14. Berger, S. B. & Kimmel, A. R. (1987) *Guide to Molecular Cloning Techniques* (Academic, New York).
15. Cheng, M. Y., Hartl, F. U., Martin, J., Pollock, R. A., Kalousek, F., Neupert, W., Hallberg, E. M., Hallberg, R. L. & Horwich, A. L. (1989) *Nature (London)* **337,** 620–625.
16. Scarf, S. J., Horn, G. T. & Erlich, H. A. (1986) *Science* **233,** 1076–1078.
17. Gibbs, R. A., Nguyen, P. N., McBride, L. J., Koepf, S. M. & Caskey, C. T. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1919–1923.
18. van der Werf, S., Bradely, J., Wimer, E., Studier, F. W. & Dunn, J. J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2330–2334.
19. Bachmair, A. & Varshavsky, A. (1989) *Cell* **56,** 1019.
20. Horwich, A. L., Fenton, W. A., Figaira, F. A., Fox, J. E., Kolansky, D., Mellman, I. S. & Rosenberg, L. E. (1985) *J. Cell Biol.* **100,** 1515–1521.