Identification of an Active Site Alanine in Mevalonate Kinase through Characterization of a Novel Mutation in Mevalonate Kinase Deficiency

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Sequencing of polymerase chain reaction-amplified cDNAs from cultured cells of three patients with mevalonate kinase deficiency revealed a G → A transversion at nucleotide 1000 of the coding region, converting alanine to threonine at position 334 (A334T). To characterize this defect, we expressed wild-type and mutant cDNAs in Escherichia coli as the glutathione S-transferase fusion proteins, with purification by affinity chromatography. SDS-polyacrylamide gel electrophoresis analysis for wild-type and mutant fusion proteins indicated that the bacterial expression system for human MKase will provide a useful model system in which to analyze inherited mutations and identify the first active site residue in MKase associated with stabilization of mevalonate binding.

Inborn errors of human metabolism have served as useful sources of information, which have elucidated normal pathways of intermediary metabolism. Although the majority of these genetic diseases occur in catabolic pathways, several have been identified in well regulated anabolic pathways, such as the pathway of cholesterol and nonsterol isoprenoid biosynthesis. Recent work has identified three inherited defects within this pathway, Smith-Lemli-Opitz syndrome, desmosferolysis, and mevalonic aciduria due to mevalonate kinase (MKase)1 deficiency (1–3). Of these three defects, the former two occur beyond the branch point to isoprenoid biosynthesis at the level of farnesyl diphosphate and are therefore unique to sterol and bile acid biosynthesis. Conversely, MKase deficiency occurs just after the highly regulated step of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and, therefore, represents a defect potentially affecting both steroid and isoprenoid biosynthesis.

Mevalonate occupies a central role in intermediary metabolism and is an important precursor of membrane biogenesis, hormone production, protein glycosylation (dolichols), respiration (ubiquinone), and protein biosynthesis (isopenytenylated transfer RNAs) (4). As the precursor of farnesyl and geranylgeranyl diphosphates, mevalonate plays an important role in protein prenylation and intracellular signal transduction (5). MKase catalyzes the transfer of the ϕ-phosphorus of ATP to the 5-hydroxyl oxygen of mevalonate (6). The importance of maintaining mevalonate homeostasis is apparent in patients with MKase deficiency, who suffer an often fatal, multisystemic disease and excrete significant amounts of mevalonic acid in physiologic fluids (3). In human hepatocytes transfected with recombinant mevalonate kinase that has been induced to overexpress, cell cycle regulation is altered with concomitant increase in cellular transformation, presumably occurring through aberrant signal transduction (7). These data suggest a requirement for strict maintenance of mevalonate homeostasis.

Most evidence on cholesterol regulation has focused on the genes encoding HMG-CoA reductase and synthase, the low density lipoprotein receptor, and the genes encoding squalene and farnesyldiphosphate synthases (8–10). There has been little attention focused on the genes directly involved in early mevalonate disposition, even though these genes also appear to be regulated. The activity of MKase in animal liver fluctuates

* This work was supported by Grants 6-FY96-0309 and 6-FY97-0537 from the March of Dimes Birth Defects Foundation, Grant-in-aid 94010450 from the American Heart Association (to K. M. G.), and Grant Ho966/4-1 from the Deutsche Forschungsgemeinschaft (to G. F. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: MKase, mevalonate kinase; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; bp, base pair(s); PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; SLO, Smith-Lemli-Opitz.
in response to feeding with cholesterol and cholesterol deprivation (11). Evidence indicates that these fluctuations are paralleled by changes in MKase mRNA quantities, suggesting that regulation occurs at the transcriptional level (12). In addition, MKase may be regulated post-translationally through feedback inhibition by farnesyl diphosphate and potentially other non-sterol isoprenes (13). Despite these observations and a critical role in the control of mevalonate metabolism, little is known about the mechanisms through which MKase achieves catalysis or how regulation occurs at the gene and protein level.

In the current report, we describe the identification of a novel mutation in MKase deficiency, detected in three patients from two families. The nucleotide transversion results in substitution of threonine for invariant alanine at position 334 of the wild-type recombinant enzyme and a >30-fold increase in $V_{\text{max}}$ in comparison with the wild-type recombinant enzyme and a 1.4% of control $V_{\text{max}}$. Comparable results were obtained using extracts of cultured lymphoblasts, which were homozygous for this mutation. These data represent the first identification of a residue in MKase directly involved in stabilization of mevalonate binding at the active site.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotide primers, Moloney murine leukemia virus reverse transcriptase, and deoxyribonucleotides were obtained from Life Technologies, Inc. Restriction endonucleases, $T_4$ DNA ligase, RNase inhibitor, $Taq$ DNA polymerase, and Expand™ polymerase were obtained from Boehringer Mannheim. DNA purification kits were purchased from Qiagen. DNA was sequenced using $T_7$-Sequenase 7-deaza-dGTP sequencing and Thermosequenase kits. ECL Western blotting kits were obtained from Amersham Life Science, Inc. BCA protein quantitation kits were purchased from Bio-Rad. All other reagents were of the highest purity obtainable.

**Reverse Transcription-Polymerase Chain Reaction (PCR)**—Standard molecular biologic methods were used throughout. RNA was extracted from cultured lymphoblasts, and first strand cDNA was synthesized using a downstream gene-specific antisense primer (5'-GGGACTTCAGGATGTCGACACAGGTCAGGAGCTC-3'). Two sets of PCR were performed using gene-specific primers. One set of primers (sense, 5'-GGGAATTCAGGATGTCGACACAGGTCAGGAGCTC-3' antisense, 5'-CAGCTTGTGTTGAGTCCGGCG-3') was employed to amplify the entire 1010 bp of the cDNA. The second primer set (sense, 5'-GGATCCTCTCTTGAGTGCACGAGG-3' antisense, 5'-CTGGACACCTGAGCTTGAGGACCCGTCAGGG-3') amplified an 815-bp product containing the 3' end of the cDNA.

**PCR Amplification of the MKase cDNA for HhaI Digestion**—An additional round of PCR was performed to produce a 714-bp fragment for digestion with the restriction endonuclease HhaI. The primers used were sense, 5'-GTTGAGGTAGTCGCTGTAATCCCG-3' and antisense, 5'-GTTAAGCCACAGCAGGTGCACAGG-3'. This 714-bp product encompassed alanine 334 and contained only three of the six HhaI sites within MKase. As the A334T transversion deleted a HhaI site, digestion of this 714-bp amplicon provided an easily distinguishable pattern for the A334T mutation.

**Cloning and Sequencing of Wild-type and Patient cDNAs**—Amplified products were cloned (EcoRI/SacI and AccII/Xhol, respectively, for 5'- and 3'-portions of the cDNA) by substitution of PCR products directly into wild-type MKase cDNA using the pGEX 4T-3 vector (Pharmacia Biotech Inc.). Clones were sequenced using the dyeoxy chain termination method. Mutations were confirmed by sequencing multiple clones from several different reverse transcription-PCR reactions as well as direct sequencing of the PCR products.

**Protein Expression and Purification**—No additional cloning steps were required for bacterial expression analysis. Plasmids were expressed in E. coli DH5a, and the recombinant proteins were chromatographically purified as described (13).

**Lymphoblast Culture**—Human lymphoblasts, transformed with Epstein-Barr virus, were cultured in RPMI 1640 medium supplemented with 1-glutamine and 20% fetal bovine serum (14).

**HhaI Digestion**—The A to T transversion at nucleotide 1000 (A334T) resulted in the loss of a HhaI restriction site. Purified PCR products derived from patients and family members were digested with 20 units of HhaI at 37 °C for 2 h and analyzed on a Bio-Rad 3% NuSieve gel.

**Enzyme Assay, Kinetic Determinations, SDS-Polyacrylamide Gel Electrophoresis (PAGE), and Protein Alignment for Purified Recombinant Proteins**—For studies on lymphoblast extracts, MKase activity and kinetic constants were determined using a standard radiometric assay (14). Purified wild-type and mutant recombinant proteins were assayed spectrophotometrically using a standard assay and analyzed using 10% SDS-PAGE gels with Coomassie Blue staining (13). Protein alignments and Chou-Fasman secondary structure predictions were performed using the Hitachi MacDNAAsia version 3.6.

**Western Blotting of Lymphoblast Extracts**—Total protein was isolated from cultured lymphoblasts by sonication and subsequent centrifugation. Protein content of the cell extract was quantified using the BCA kit by Bio-Rad. Ninety micrograms of protein were loaded onto a 10% SDS-polyacrylamide gel. Following electrophoresis, the gel was blotted and probed according to the manufacturer's protocols for the ECL chemiluminescence-based Western blotting kit. The primary antibody was a polyclonal antipeptide MKase antibody produced in rabbit.

**RESULTS AND DISCUSSION**

**Expression, Purification, and Kinetic Characterization of Wild-type Human Mevalonate Kinase**—To undertake molecular genetic analysis in patients with MKase deficiency, it was necessary to obtain a rapid system for characterization of mutations in expressed recombinant proteins. The cDNA encoding rat MKase had been expressed in E. coli, and an expression plasmid was prepared for preparation of human MKase in COS-7 cells (15, 16). For rapid analysis of MKase cDNAs harboring mutations, however, we sought to express human MKase in bacteria and develop a rapid and simple purification system. For this, we engineered the expression plasmid of MKase fused to the pGEX vector, which produces the fusion protein of MKase attached to glutathione S-transferase. With this chimera, isolation of pure, recombinant MKase is achieved using one-step affinity chromatography on glutathione-Sephrose beads (Fig. 1). The estimated molecular mass of the MKase fusion protein was 74 kDa, with unfused glutathione S-transferase migrating at an estimated molecular mass of 32 kDa. These data yielded non-fusion protein molecular masses of 42 kDa for MKase, consistent with earlier estimations for rat and human (17).

**Kinetic analysis of wild-type recombinant human MKase is depicted in Table I.** When analyzed as the fusion protein or as the free enzyme following cleavage of the fusion protein with the site-specific protease thrombin, we found little difference for kinetic constants and enzyme-specific activities (13). However, we found the fusion protein to have a considerably longer shelf life, so we chose to work with this form of the protein. The mean specific activity for MKase was 13.6 ± 1.4 units/mg of protein.
Kinetic constants for wild-type and mutant mevalonate kinase fusion proteins purified from sonicates of E. coli and in crude extracts of cultured human lymphoblasts

| Mevalonate kinase protein | Source | \( V_{\text{max}} \) (\( \mu \text{mol/min/mg protein} \)) | \( K_m \) (\( \text{RS}-\text{mevalonate} \)) | \( K_m \) (\( \text{ATP} \)) |
|--------------------------|--------|-----------------|-----------------|-----------------|
| Wild type                | Recombinant | 13.6 ± 1.4 (n = 22) | 150 ± 23 (n = 4) | 440 ± 190 (n = 5) |
| A334T mutant             | Recombinant | 2.86 ± 0.02 (n = 6) | 4,623 ± 1,167 (n = 4) | 680 ± 226 (n = 3) |
| N301T mutant             | Recombinant | 0.19 ± 0.04 (n = 4) | 151 ± 16 (n = 4) | 293 ± 17 (n = 4) |
| Wild type                | Lymphoblast extract | 2.15 ± 0.35 (\( \times 10^{-3} \)) (n = 8) | 12,378 ± 1,793 (n = 5) | 161 ± 36 (n = 4) |
| A334T mutant             | Lymphoblast extract | 0.09 ± 0.001 (\( \times 10^{-3} \)) (n = 8) | 12,378 ± 1,793 (n = 5) | 161 ± 36 (n = 4) |

\( a \) n = number of determinations, with data presented as mean ± S.E.

\( b \) p < 0.0001.

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**FIG. 2.** 
HhaI digestion of cDNAs derived from cultured cells of three patients (siblings BK and PK and unrelated patient WS) and family members for the A334T allele. The size of the original PCR-derived amplicon was 714 bp as described under “Experimental Procedures.” Restriction digestion of this amplicon yields four products at 391, 170, 100, and 53 bp, corresponding to three HhaI sites. The A334T allele results in the loss of one HhaI site creating a new product at 270 bp with the loss of the 170- and 100-bp bands. The heterozygous pattern reveals the 270-bp band along with the wild-type pattern. Lane contents of the gel included: 1, DNA molecular weight standard; 2, wild-type cDNA; 3, father of WS; 4, mother of WS; 5, patient WS; 6, sibling of WS; 7, father of patients BK/PK; 8, patient BK; 9, patient PK; 10, DNA molecular weight standard. The digest pattern for the mother of patients BK/PK was identical to that of the father (data not shown). Clinical details on all three patients have been reported (3, 18).

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**FIG. 3.** 
Activity of recombinant and rat enzymes. These values were compared with data presented as mean ± S.E.

- Alanine 334 is invariant in all species of MKase thus far analyzed.
- The A334T mutation occurred in the wild-type sequence, suggesting he was a compound heterozygote.
- For further analysis, we prepared cDNAs encoding wild-type cDNA; the unrelated patient, WS, demonstrated a composite of mutant and wild-type patterns (Fig. 2). In the family of patient WS, the father contributed the A334T allele, whereas the mother did not contribute this allele and carries an undefined mutation; the clinically unaffected female sibling of patient WS manifested only the wild-type HhaI digestion pattern, consistent with earlier enzyme results indicating that she was not a carrier of an MKase mutation (18). Both parents of patient PK and BK contributed the A334T (Fig. 2). These results verified the Mendelian inheritance of the A334T allele in an autosomal recessive fashion. PCR products derived from cDNAs from the patients and family members were directly sequenced to further confirm the results of restriction digestion. Restriction digestion verified that no other MKase-deficient patient carried the A334T mutation.

**Residual MKase Activity and Kinetic Characterization of the A334T Mutation**—Determination of MKase activity in recombinant enzymes harboring the A334T allele revealed a residual activity of 0.26 ± 0.02 unit/mg of protein (± S.E., n = 6), representing a decrease to 1.4% of control MKase activity (Table I). This lack of enzyme activity was not the result of inability to produce the fusion enzyme, as the amount of protein produced with the A334T allele was comparable with wild type when analyzed by SDS-PAGE (Fig. 1). As a control, we assessed the activity of the fusion protein harboring the first documented mutation in MKase, the N301T allele, which converts an invariant asparagine to threonine at amino acid 301 of the polypeptide sequence (16). The level of activity for this allele was comparable with that for the A334T allele at 0.19 ± 0.04 unit/mg of protein (± S.E., n = 4). These data and the inheritance pattern depicted in the family verified that the A334T allele contributed to MKase deficiency in the three patients under study.

We next characterized kinetic constants for the A334T allele. Whereas the \( K_m \) for substrate ATP was comparable with control for the mutant protein at 680 ± 226 \( \mu \text{M} \) (± S.E., n = 3), the \( K_m \) for substrate (RS)-mevalonate was >90-fold elevated in comparison with control at 4,623 ± 1,167 \( \mu \text{M} \) (± S.E., n = 4). Alanine 334 is invariant in all species of MKase thus far analyzed and lies in a glycine-rich region near the C terminus of protein (± S.E., n = 22, range 7.3–32.8 units/mg of protein).

These values are consistent with activities for the recombinant rat enzyme of 30.2 ± 5.7 and 32 units/mg of protein for MKase purified from rat liver (15, 16). Kinetic analysis of the recombinant human protein revealed \( K_m \) values for substrates (RS)-mevalonate and ATP of 150 ± 23 (n = 4) and 440 ± 190 \( \mu \text{M} \) (n = 5), respectively. For the recombinant and purified rat enzymes, the same values were 271–288 \( \mu \text{M} \) for (RS)-mevalonate and 1240–1750 \( \mu \text{M} \) for ATP. These values were comparable with those found in the current study using the human enzyme (Table I), indicating that the expressed human fusion protein was a useful system in which to model inherited mutations in MKase.

Identification and Inheritance of the A334T Mutation in Three Patients with MKase Deficiency—During our analysis of PCR-derived cDNAs from previously documented patients with MKase deficiency, we identified a G \( \rightarrow \) A transversion at nucleotide 1000 of the MKase coding region in cDNAs derived from three patients in two families. This allele results in conversion of alanine to threonine at amino acid 334 (A334T). A total of 24 cDNA clones encoding MKase, obtained using PCR amplification of RNA derived from cultured cells of these patients, was isolated and sequenced. All 16 clones from male and female siblings demonstrated the A334T transversion, suggesting their status as homozygotes. For the third unrelated patient, four of six clones revealed the A334T mutation with two clones showing wild-type sequence, suggesting he was a compound heterozygote. For further analysis, we prepared cDNAs encoding MKase from parents and siblings of all patients and screened for the A334T allele utilizing restriction digestion with HhaI endonuclease. Fig. 2 shows the results of these restriction digestions. Siblings BK and PK demonstrated only the mutant pattern following HhaI digestion, gaining a 270-bp band and lacking both the 170- and 100-bp bands. This corresponded to the loss of one HhaI site as a result of the A334T allele. The unrelated patient, WS, demonstrated a composite of mutant and wild-type patterns (Fig. 2). In the family of patient WS, the father contributed the A334T allele, whereas the mother did not contribute this allele and carries an undefined mutation; the clinically unaffected female sibling of patient WS manifested only the wild-type HhaI digestion pattern, consistent with earlier enzyme results indicating that she was not a carrier of an MKase mutation (18). Both parents of patients PK and BK contributed the A334T (Fig. 2). These results verified the Mendelian inheritance of the A334T allele in an autosomal recessive fashion. PCR products derived from cDNAs from the patients and family members were directly sequenced to further confirm the results of restriction digestion. Restriction
the enzyme. Alignment of MKase poly sequence from six species (Homo sapiens, Rattus norvegicus, Arabidopsis thaliana, Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Mycobacterium thermotuberculosis) reveals the following near consensus sequence of KLTGAGGGGC (single-letter amino acid code, alanine 334 underlined) spanning residues 330–339. Only in M. thermotuberculosis are there differences in amino acid sequence at positions 331 (leucine to isoleucine) and 339 (cysteine to serine). The remaining residues are invariant, including alanine 334.

**Kinetic Characterization of MKase in Extracts of Lymphoblasts Homozygous for the A334T Allele**—To verify results obtained with recombinant fusion proteins, we assessed the size, amount, and kinetic characteristics of MKase protein in extracts of lymphoblasts homozygous for the A334T allele (patient BK). Western blotting of wild-type and mutant lymphoblast extracts using a polyclonal antipeptide antibody raised in rabbit against the C-terminal region of the MKase polypeptide revealed a normal size and amount of MKase protein in mutant cells (Fig. 3). Kinetic characteristics of wild-type and mutant MKase in lymphoblast extracts are shown in Table I. As expected, the activity of MKase in crude lymphoblast extracts decreased to 0.4% of control. We observed an even larger decrease in (RS)-mevalonate Km in mutant lymphoblast extracts (12.37 ± μm as compared with 151 μm in control), representing a >80-fold increase in (RS)-mevalonate Km. As for recombinant proteins, there was no significant difference in Km for ATP between the wild-type and mutant lymphoblast cell lines. These data in lymphoblast extracts homologous for the A334T mutation corroborated our results obtained with purified wild-type and mutant recombinant fusion proteins.

Modeling of the A334T allele using the Chou-Fasman algorithm suggests that substitution of threonine for alanine at position 334 results in loss of predicted coiled probability in the secondary structure of MKase, with insertion of predicted β-sheet probability (Fig. 4). The region spanning the A334T allele is predicted to contain only turns and coils, consistent with the high glycine content of this region. These observations in conjunction with the decreased affinity for (RS)-mevalonate binding, which is observed when threonine replaces alanine, suggest a role for alanine 334 in the stabilization of mevalonate binding, placing alanine 334 at the MKase active site. One possibility is that alanine stabilizes mevalonate binding through hydrophobic interaction. This would be consistent with the difference in free energy change for transfer of alanine or threonine when each is moved from the interior of a lipid bilayer to water (alanine, 1.0 kJ mol–1; threonine, -0.75 kJ mol–1). Exact modeling of this mutation must await crystal structure analysis of MKase.

The A334T mutation represents only the second mutation responsible for MKase deficiency and the first identification of an amino acid residue in MKase specifically associated with (RS)-mevalonate binding. Using site-directed mutagenesis, Miziorko and co-workers (15) identified lysine 13 as an active site residue in rat MKase. These investigators employed site-directed mutagenesis to convert lysine 13 to methionine (K13M). This mutation resulted in a significantly decreased binding affinity for ATP, most likely through interaction of the e-amino moiety of lysine with negative charges on the phosphate backbone of ATP. As noted by these investigators, MKase achieves catalysis most likely through an “in-line” transfer mechanism, which places mevalonate and ATP in close proximity at the active site. In the case of alanine 334, the pocket proposed by Chou-Fasman analysis would appear to specifically accommodate mevalonate at the active site of the enzyme, with little effect on the binding of ATP.

Alanine 334 lies in a region with proposed ATP binding motif homology, with a consens sequence GXGXXGXX15–21AXK, in which X represents any amino acid (16). Although the homology to this element is maintained in rat and human MKase, it is not maintained in other species of MKase (15). In addition, the location of alanine 334 in the MKase active site suggests that cysteine 339 is the likely candidate for the active site cysteine previously identified in MKase from hog liver (6). Cysteine 339 is almost invariant and is conserved in five of six species when amino acid sequences are aligned. In bacteria, cysteine 339 is a serine residue, although two additional cysteines are found within eight residues of serine 339.

**Pathophysiologic Mechanisms in MKase Deficiency**—In 16 patients thus far studied, we have observed considerable phenotypic heterogeneity in MKase deficiency (3). The disease has been fatal for six patients, whereas other patients have survived through childhood and even into adulthood. Despite this heterogeneity, MKase activity is uniformly absent in extracts of cultured cells derived from all patients. One mechanistic approach toward understanding this phenotypic variation is to define the underlying genetic alterations in the disease. In the current report, the three patients identified with the A334T allele represent the most mildly affected patients we have studied thus far. In cells of these three patients (and others), we previously documented a significantly up-regulated activity of HMG-CoA reductase (19). Up-regulated HMG-CoA reductase activity may reflect the loss of a downstream regulator not produced in patients with MKase deficiency.

Patients with defects beyond the branch point for isoprenoid biosynthesis, such as Smith-Lemli-Opitz (SLO) syndrome or desmosterolosis, do not have demonstrable mevalonate acid accumulation in physiologic fluids. This would imply that HMG-CoA reductase regulation is intact, perhaps down-regulated by
intermediates that accumulate in these diseases, such as 7-dehydrocholesterol or desmosterol. Whatever the cause for constantly up-regulated HMG-CoA reductase activity in patients with MKase deficiency, resulting mevalonic aciduria may represent a cellular response designed to partially compensate the defect. Such a response, in which intracellular pools of mevalonate are increased beyond physiologic levels, may have particular relevance for patients with a $K_a$ alteration, such as those described in the present report. Overaccumulation of intracellular mevalonate acid may enable increased turnover of mutant MKase in these patients, although it is still likely that shortages along the isoprene pathways may occur and contribute to pathology (20).

Important information regarding the regulation of cholesterol metabolism in humans may be gleaned by a comparison of the biochemical findings in MKase deficiency with those in another well studied disease of cholesterol biosynthesis, SLO syndrome. The latter is characterized by a defect in 7-dehydrocholesterol reductase, resulting in greatly lowered levels of 7- and 8-dehydrocholesterol. However, the combined levels of total serum and tissue cholesterol and elevated levels of 7- and 8-dehydrocholesterol, resulting in greatly lowered levels of cholesterol reductase, are generally found to be less than those described in the present report. Overaccumulation of intracellular mevalonate may enable increased turnover of mutant MKase in these patients, although it is still likely that shortages along the isoprene pathways may occur and contribute to pathology (20).

The levels of both nonsterols and sterols are depleted, and the dehydrocholesterol that accumulate can apparently serve as the sterol suppressor. Patients with MKase deficiency provide an interesting model system in which to study normal and altered regulation of cholesterol synthesis. Characterization of additional defects in patients may provide further insight into normal, and aberrant, pathway function and regulation.

Acknowledgments—We are indebted to Dr. Barbara Goebel-Schreiner for assistance with mutation analysis, Dr. G. P. A. Smit for contributing urine samples from patients with Smith-Lemli-Opitz syndrome, and the Bristol-Myers Squibb Company for contribution of the human mevalonate kinase cDNA.

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