Differential Diagnosis of *mut* and *cbl* Methylmalonic Aciduria by DNA-mediated Gene Transfer in Primary Fibroblasts

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Abstract

Methylmalonic aciduria can be caused by mutations in the gene encoding the methylmalonyl coenzyme A mutase apoenzyme (*mut*) or genes required for the provision of cofactor B_{12} (*cbl*). The *mut* and *cbl* forms are classically differentiated by somatic cell complementation. We describe a novel method for differential diagnosis of *mut* and *cbl* methylmalonic aciduria using DNA-mediated gene transfer of a methylmalonyl CoA mutase cDNA clone. Gene transfer of a functional methylmalonyl CoA mutase cDNA clone into *mut* fibroblasts reconstitutes holoenzyme activity measured by metabolism of [^{14}C]-propionate in culture. Identical gene transfers into *cbl* fibroblasts have no effect. This method is used for the differential diagnosis of *mut* and *cbl* genotypes in cells from patients with a clinical diagnosis of methylmalonic aciduria and is shown to be a facile, sensitive, and specific method for genetic diagnosis. This work establishes the principle of using DNA-mediated gene transfer to identify the genotype of diseases which can result from mutations at several different genetic loci. This type of differential genotypic diagnosis will be particularly important for establishing the applicability of somatic gene therapy in individual patients. (*J. Clin. Invest.* 1991. 87:915–918.) Key words: genetic complementation • gene expression • inborn errors of metabolism • organic acids • genotype

Introduction

Methylmalonic aciduria (MMA)[1] can result from mutations in the gene encoding the methylmalonyl coenzyme A mutase (MCM) apoenzyme (1) (*mut* MMA; McKusick No. 251000) or genes encoding proteins required for provision of the essential adenosylcobalamin cofactor (cofactor B_{12}) (*cbl* MMA; McKusick Nos. 251100, 251110). Differential diagnosis of *mut* and *cbl* forms of MMA is clinically important because *mut* MMA is not responsive to pharmacological administration of vitamin B_{12} and is generally associated with a worse prognosis than *cbl* MMA (2).

The *mut* and *cbl* forms of MMA are classically distinguished by somatic cell complementation in which cells with unknown defects are fused with prototypical cells whose genetic defects are known (3, 4). If the unknown cell has the same defect as the prototypical cell, then MCM holoenzyme activity measured by [^{14}C]-propionate metabolism in cultured cells remains deficient. If the unknown cell has a defect involving a different genetic locus, then fusion of the two cells reconstitutes the MCM holoenzyme and [^{14}C]-propionate metabolism. This differential diagnosis requires a battery of prototypical cell lines and is performed in few laboratories.

We have reported cloning of human and mouse MCM cDNAs (5–7) and identification of mutations associated with *mut* MMA (8–10). These studies suggest that there are many different mutations underlying *mut* MMA, and that allele-specific probes are unlikely to identify a sufficient fraction of the mutations at the MUT locus to provide a useful diagnostic discriminant between *mut* and *cbl* MMA (11).

Other studies have demonstrated that DNA-mediated gene transfer of the MCM cDNA into *mut* fibroblasts reconstitutes MCM holoenzyme activity and [^{14}C]-propionate metabolism to normal levels (7, Jansen and Ledley, unpublished data). These results confirmed that the genetic defect in *mut* MMA involves the gene encoding the MCM apoenzyme and suggested a novel method for differential diagnosis of *mut* and *cbl* MMA. We postulated that while gene transfer of the MCM cDNA into *mut* cells restores the deficient apoenzyme, gene transfer into *cbl* cells, which contain ample amounts of the apoenzyme but no cofactor, should not reconstitute holoenzyme activity or [^{14}C]-propionate metabolism. We now demonstrate that the ability of the MCM cDNA to complement [^{14}C]-propionate metabolism correlates with classical somatic cell and biochemical methods for differential diagnosis of *mut* and *cbl* MMA. This work introduces the principle of using DNA-mediated gene transfer for genotypic diagnosis of genetic diseases.

Methods

Primary fibroblasts from patients with a clinical diagnosis of MMA were obtained from the NIGMS mutant cell repository or investigators noted in the Acknowledgements. Somatic cell complementation data was ascertained from clinical records of diagnostic studies performed in the laboratories of Dr. Leon Rosenberg (Yale University, New Haven, CT) or Dr. David Rosenblatt (McGill University, Montreal, Quebec).

An expression vector containing the murine MCM cDNA (pCMV-MMCM) has been described (7). This vector, in the plasmid pNAssCMV (12), uses the cytomegalovirus immediate early promotor, SV40 late viral protein splice donor and acceptor signals, and SV40 polyadenylation sequences. DNA was introduced into primary fibroblasts by electroporation (13) using a Gene Pulser (Bio-Rad Laboratories, Inc., Richmond, CA), after a 5-min preincubation of DNA and cells. The range of conditions used included 0.3–0.6 ml of cells (density, 3×10^6/ml), 10–20 μg of DNA, and voltage pulses from 240 to 300 V (capacitance, 960 μF). MCM activity was assayed 2 d after electroporation, during the transient phase of gene expression.

This work was presented in part as an abstract to the Society for Pediatric Research, May 1990.

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Received for publication 11 September 1990.

1. *Abbreviations used in this paper:* MCM, methylmalonyl coenzyme A mutase; MMA, methylmalonic aciduria.

J. Clin. Invest.

0021-9738/91/03/0915/04 $2.00
Volume 87, March 1991, 915–918

Propionate metabolism was measured by incorporation of [^{14}C]-propionate into TCA-precipitable material (14), using the coincident incorporation of [^{3}H]-leucine as a control for cell number and rate of constitutive protein synthesis (8). Results are expressed as nanomoles propionate/micromoles leucine incorporated and are calculated as the mean and standard deviation of triplicate samples. Control experiments were performed with vectors containing the MCM cDNA in an antisense orientation or vector alone (pNAssCMV). Electroporation efficiency was monitored in control experiments using the *Escherichia coli* β-galactosidase gene (12). A discriminant of propionate stimulation is calculated as: $(S - AS)/AS$, where S = propionate incorporation after electroporation of pCMV-MMCM in the sense orientation, and AS = propionate incorporation after transformation under identical conditions with the control, antisense vector. Differences between the discriminants of *mut*°, *mut*$^-$, and *cbl* cells were analyzed using the Wilcoxon's two-sample rank test (15). Apoenzyme was measured in crude extracts in vitro as described (8, 16).

Results

The pCMV-MMCM expression vector was introduced into 20 fibroblast lines from patients with a clinical diagnosis of MMA which were also characterized by somatic cell complementation or in vitro apoenzyme assay (Table I). Propionate incorporation was increased in all *mut* cell lines with a discriminant value > 1. Propionate incorporation was not increased in *cbl*A, *cbl*B, *cbl*C, or *cbl*D cell lines or normal fibroblasts (Table I). There was no difference in the efficiency of electroporation into *cbl, mut,* and normal cells, and no difference in [^{3}H]-leucine incorporation which serves as an internal control for cell number and metabolic activity (data not shown).

The absence of increased [^{14}C]-propionate incorporation in *cbl* cells after electroporation was presumed to reflect lack of holoenzyme in the absence of the adenosylcobalamin cofactor. To confirm that the recombinant apoenzyme was expressed in *cbl* and normal cells, in vitro apoenzyme assays were performed on crude extracts (Fig. 1). Assayable MCM apoenzyme was increased in all cell types after gene transfer of the pCMV-MCM clone, indicating that the recombinant apoenzyme was expressed and potentially active even in *cbl* and normal cells.

To assess the reliability and reproducibility of DNA-mediated complementation, repetitive experiments were performed over a period of 6 mo using cell lines at different plating density and passage number, different DNA preparations, and minor variations in electroporation conditions (Fig. 2). Electroporation into *mut* cells was associated with a discriminant > 1 in 69 of 70 experiments (99% sensitivity). Electroporation into *cbl* or normal cells was associated with a discriminant < 1 in 76 of 78 trials (97% specificity). The difference between *mut* and *cbl* or normal cells is statistically significant (Z = 10.43 SD; $P < 10^{-5}$). A statistically significant difference was also observed in the degree of stimulation in *mut*$^-$ cells, which express low levels of kinetically abnormal enzyme, compared with *mut*° cells, which exhibit no constitutive enzyme activity (Z = 6.4 SD; $P < 10^{-5}$). Both *mut*$^-$ and *mut*° cells were statistically different from *cbl* cells (*mut*$^-$ vs. *cbl:* Z = 8.03, $P < 10^{-5}$; and *mut*° vs. *cbl:* Z = 8.79, $P < 10^{-5}$).

Discussion

Biochemical and somatic cell genetic studies over the past two decades predicted that *mut* forms of MMA resulted from mutations in the gene encoding the MCM apoenzyme (1). Gene transfer studies confirm this prediction by demonstrating that introduction of a functional MCM gene into *mut* cells by DNA-mediated gene transfer restores MCM enzyme activity measured either by [^{14}C]-propionate incorporation or in vitro assay of the MCM apoenzyme. In contrast, no increase in propionate incorporation was observed in cells exhibiting various *cbl* defects or normal fibroblasts. We used the differential response of *mut* and *cbl* cells to diagnose *mut* MMA in cells from patients with the clinical phenotype MMA and a discriminant value > 1. DNA-mediated gene transfer proved to be a specific and facile method for differential diagnosis of *mut* and *cbl* cell lines.

The suitability of this method for routine diagnostic applications is dependent upon its reproducibility, sensitivity, and

Table I. Differential Diagnosis of mut and cbl MMA by Somatic Cell Complementation, In Vitro Apoenzyme Assay, and DNA-mediated Gene Transfer

	Conventional diagnosis		DNA-mediated complementation[§]			
Cell	Somatic*	Enzyme[‡]	*S*	*AS*	Discriminant	Diagnosis[‖]
BEC		37.3	11.9	13.3	<0	*cbl*
BUS		39.1	3.0	3.0	<0	*cbl*
CD81	*cbl*B	39.7	4.6	5.1	<0	*cbl*
DS79	*mut*	5.9	40.9	17.2	1.37	*mut*
ESC		18.4	74.4	76.0	<0	Normal
ESP		36.7	31.9	32.8	<0	*cbl*
GM1673	*mut*	0.1	33.5	6.0	4.60	*mut*
GM1674	*cbl*A	38.3	61.9	61.3	0.01	*cbl*
GM212	*cbl*A	ND	35.2	28.5	0.23	*cbl*
GM2452	*cbl*D	43.2	36.5	28.9	0.26	*cbl*
GM2453	*cbl*C	ND	53.4	62.0	<0	*cbl*
GM306	*cbl*A	ND	19.3	22.8	<0	*cbl*
GM50	*mut*	1.0	47.0	10.3	3.55	*mut*
GM876	*cbl*B	ND	13.3	13.6	<0	*cbl*
HUL	*mut*	0.0	43.3	16.7	1.58	*mut*
PIN		2.8	60.1	20.9	1.87	*mut*
REG	*mut*	<0	22.4	6.8	2.27	*mut*
SMI		40.0	3.7	4.3	<0	*cbl*
SO82	*mut*	1.3	50.0	17.5	1.85	*mut*
ZUN		3.8	35.9	8.5	3.20	*mut*

* Complementation type determined by classical somatic cell genetic methods. Data reported in diagnostic studies performed by Dr. David Rosenblatt (McGill University) or Dr. Leon Rosenberg (Yale University).

‡ Apoenzyme activity determined in vitro. Units: nanomoles succinate formed per milligram per hour. The in vitro assay is performed in the presence of excess adenosylcoblamin cofactor and distinguishes *mut* MMA (apoenzyme activity < 10) from *cbl* MMA (normal apoenzyme activity). ND, not done.

§ DNA-mediated complementation was determined by electroporation of the pCMV-MMCM, in a sense orientation (*S*) and antisense control clone (*AS*) into fibroblasts and assay of [^{14}C]-propionate incorporation. Data from representative experiments are shown. Values reflect the mean of at least three independent determinations of [^{14}C]-propionate incorporation with standard deviations of < 5%. The discriminant is calculated as: $(S - AS)/AS$.

‖ The diagnosis of *mut* MMA was made in cell lines exhibiting a discriminant value > 1 and was uniformly concordant with the diagnosis by conventional somatic cell complementation or enzyme assays.

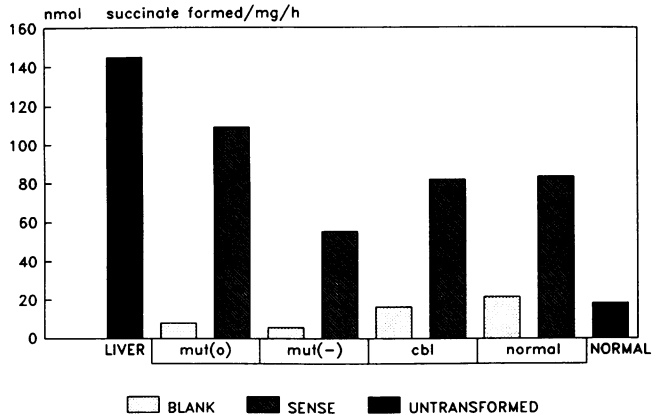

Figure 1. Constitution of MCM apoenzyme activity in *mut, cbl,* and normal fibroblasts by DNA-mediated gene transfer. MCM apoenzyme activity was measured in vitro in extracts of *mut°*, *mut⁻*, and *cbl* cells transformed with pMCV-MMCM or the control vector. Apoenzyme activity was increased in each cell type by the sense construct, indicating that the pCMV-MMCM vector was capable of directing production of the apoenzyme. Human liver and normal fibroblasts were assayed as positive controls.

specificity. We addressed these issues by performing repetitive assays in cells representing each *cbl* genotype, various phenotypic forms of *mut* MMA, and minor modifications of electroporation conditions, DNA quality, cell density, and passage number. We observed no reproducible stimulation of propionate uptake in any *cbl* cell line, indicating that the assay is specific. We observed no failures of complementation in *mut* cells when the gene transfer was technically successful (e.g., performed in conjunction with a positive control), suggesting the assay is sensitive.

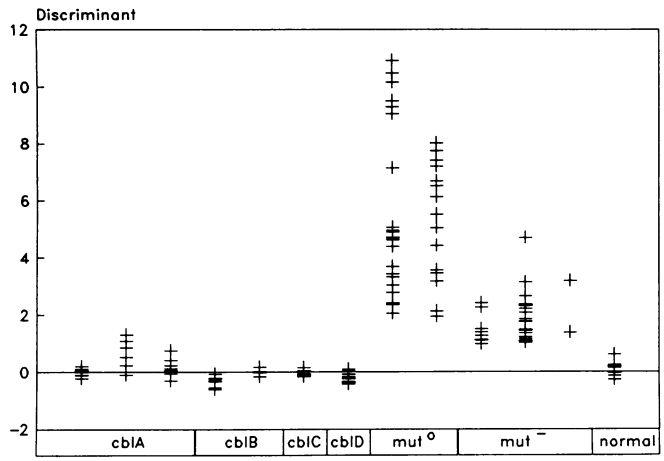

Figure 2. The reproducibility of DNA-mediated gene transfer in the differential diagnosis of *mut* and *cbl* MMA. DNA-mediated gene transfer was performed into *mut°*, *mut⁻*, *cbl,* and normal fibroblasts using the murine MCM expression vector pCMV-MMCM (*S*) or antisense (*AS*) control. The discriminant of propionate incorporation is calculated as $(S - AS)/AS$ described in the text. Each column represents a different cell line and each point represents an independent electroporation. Data is calculated as the mean of triplicate [^{14}C]-propionate uptake assays using the sense and antisense vectors. Experiments were performed over a period of 6 mo using minor modifications in cell density, passage number, and electroporation conditions described in the text.

The discriminant of propionate incorporation was quantitatively lower in *mut⁻* cells than in *mut°* cells. This may reflect decreased activity of the recombinant gene product when multimers are formed between this normal protein and the residual, mutant enzyme (17) present in *mut⁻* cells. Whereas such a suppression of recombinant enzyme activity could theoretically interfere with this diagnostic procedure, we did not observe any significant overlap in the discriminant values of *mut⁻* and *cbl* cells in the present work. Moreover, the autosomal recessive pattern of inheritance of MMA, and the qualitatively normal metabolism in MMA carriers, suggests that naturally occurring mutations are unable to significantly inhibit the activity of heterozygous normal alleles. Thus, we believe this assay will be a sensitive discriminant for *mut⁻* as well as *mut°* forms of MMA.

This work introduces the principle of using DNA-mediated gene transfer and complementation assays for genotypic diagnosis of pathological phenotypes involving multimeric proteins or multistep pathways. This assay can be performed using conventional tissue culture methods, commercial electroporation equipment, and clones which are generally available. While genotypic diagnosis is commonly construed as involving the identification of specific mutations with allele-specific probes, recent experience demonstrates that the enormous pleomorphism in most genetic diseases and the rate of new mutations may limit the sensitivity of this approach except in select populations. DNA-mediated gene transfer, in contrast, unequivocally identifies the mutant gene locus in a patient and does not depend on prior knowledge of specific mutations. As vectors for expression of other genes are developed, this method may be useful for predicting the prognosis and determining the optimal therapy for many genetic diseases.

This diagnostic approach will be particularly important when techniques for somatic gene therapy become clinically available (18, 19). Whereas in many instances the specific genotypic defect can be inferred with confidence from phenotypic (biochemical, enzymatic, or clinical) tests, DNA-mediated gene transfer represents a more specific discriminant of the genotype. Furthermore, differential diagnosis by DNA-mediated gene transfer would specifically confirm that the molecular defect in an individual patient can be reversed by gene transfer and would delineate rare or previously unknown variants involving other genetic loci which would require alternative approaches to somatic gene therapy.

Acknowledgments

The authors would like to thank Amira Elgawly for her technical assistance with this project and Tammy Reid for preparation of the manuscript. Cell lines used in this work were kindly provided by Drs. Arthur Beaudet, Stephen Cederbaum, Bruce Dowton, Stephen Goodman, Flemming Güttler, Harvey Levy, William Nyhan, David Rosenblatt, Joel Rutman, Vivian Shih, and David Valle.

Supported by National Institutes of Health grants HD-24186 (F. D. Ledley) and HD-24064 (MRRC, Dr. Edward McCabe). F. D. Ledley is also Assistant Investigator of the Howard Hughes Medical Institute.

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