Genetic complementation in heterokaryons of human fibroblasts defective in cobalamin metabolism*

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ABSTRACT Inherited methylmalonic aciduria due to deficiency of methylnalonyl-CoA mutase (methylmalonyl-CoA CoA-carboxylase; EC 5.4.99.2) activity results from at least three classes of biochemically distinct defects affecting cobalamin (Cbl: vitamin B₁₂) metabolism (cbl A, cbl B, and cbl C mutants) and a fourth class producing a defective mutant apo-enzymone. We have obtained genetic evidence in support of this biochemical heterogeneity, using heterokaryons prepared by Sendai-virus-mediated cell fusion. Nine fibroblast lines from patients with defective Cbl metabolism (4 cbl A, 3 cbl B, and 2 cbl C), two from patients with defective mutase apo-enzyme, and two from controls were fused in pairwise combinations and tested for functional mutant holoenzyme using a radioautographic procedure which detects [¹⁴C]propionate incorporation into trichloroacetic-acid-precipitable material in fibroblast monolayers in situ. Each of the mutants incorporates negligible radioactivity compared to control cells. Activity is also negligible when different mutants are mixed without virus or when homokaryons are produced by self-fusion. Heterokaryons produced by fusing members of each of the four mutant classes with representatives of any other class recover the ability to incorporate [¹⁴C]propionate to levels comparable to those of control cells. However, heterokaryons produced between members of the same class fail to complement in all cases. We conclude that the mutants with defective Cbl metabolism (cbl A, cbl B, cbl C) comprise three complementation groups, that a fourth group corresponds to mutase apo-enzyme deficiency, and that all four classes of mutations are recessively inherited.

The utilization of cobalamin (Cbl: vitamin B₁₂) as a cofactor in enzyme-mediated reactions requires its conversion to two coenzyme forms. Each Cbl coenzyme participates in a single reaction in man (Fig. 1): 5′-deoxyadenosylcobalamin (AdoCbl) combines with L-methylmalonyl-CoA mutase (methylmalonyl-CoA CoA-carboxylase; EC 5.4.99.2) to catalyze the conversion of L-methylmalonyl-CoA to succinyl-CoA; and methylcobalamin (MeCbl) is required for 5-methyltetrahydrofolate-homocysteine methyltransferase (5-methyltetrahydropteroyl-L-tri-L-glutamate-1-homocysteine-S-methyltransferase; EC 2.1.1.13) activity which catalyzes the methylation of homocysteine to methionine and the formation of tetrahydrofolate from 5-methyltetrahydrofolate.

Individuals with inherited abnormalities of Cbl coenzyme synthesis have metabolic blocks at one or both of these reactions as a result of impaired holoenzyme formation. Two distinct clinical phenotypes have been reported. Rosenberg et al. proposed that an inherited defect of AdoCbl synthesis causes vitamin-B₁₂-responsive methylmalonic aciduria in patients lacking methylmalonyl-CoA homomutase activity despite a normal mutase apo-enzyme (1). Mudd and his associates suggested that patients with homocystinuria and methylmalonic acidemia due to combined deficiency of methyltransferase and mutase activities had impaired synthesis of both MeCbl and AdoCbl (2, 3).

Recent investigations of Cbl metabolism by Mahoney et al. in cultured fibroblasts from several patients with methylmalonic aciduria extend these previous observations (4, 5). Intact fibroblasts in monolayer were examined for the ability to synthesize AdoCbl and MeCbl from hydroxy-[⁵⁷C]cobalamin (OH-Cbl) added to the growth medium. In addition, conversion of OH-Cbl was studied in cell-free fibroblast extracts. These studies revealed three distinct classes of mutants, each with different defects in Cbl metabolism. Two classes, designated cbl A and cbl B mutants, failed to synthesize AdoCbl in intact fibroblasts but had normal synthesis of MeCbl. They could be distinguished from one another by examining crude cell extracts: cbl A mutants had normal synthesis of AdoCbl; cbl B mutants were defective. Both classes lacked methylmalonyl-CoA mutase activity in intact cells because of deficient holoenzyme formation. The third mutant class, designated cbl C, produced neither AdoCbl nor MeCbl in intact cells but had normal AdoCbl synthesis in crude extracts. Such mutants lacked both mutase and methyltransferase activities.

In this report we present genetic evidence supporting the observed biochemical heterogeneity of Cbl mutants. Such evidence is based on complementation tests in heterokaryons formed from mutant fibroblast strains. Our work was facilitated by the report of Hill and Goodman (6) that mutase activity can be monitored in fibroblast monolayers in situ using a radioautographic procedure which detects [¹⁴C]propionate incorporation into trichloroacetic-acid-precipitable material.

MATERIALS AND METHODS

Source and Growth of Cells. All strains are subcultures of skin fibroblasts maintained in Eagle's minimal essential medium (Gibco) supplemented with 1% nonessential amino acids (Gibco), 10% fetal calf serum (Flow Laboratories), and 100 μg/ml of kanamycin. Cells were grown at 37° in a 5% CO₂/95% air atmosphere, washed with Dulbecco's phosphate buffered saline without calcium or magnesium, and harvested with 0.25% trypsin (Gibco).
Table 1. Source and phenotype of fibroblast strains

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Patient's initials</th>
<th>Phenotype†</th>
<th>Refs. ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1f</td>
<td>R.P.</td>
<td>cbl A</td>
<td>(1, 4, 5)</td>
</tr>
<tr>
<td>214</td>
<td>K.B.</td>
<td>cbl A</td>
<td>(5, 15)</td>
</tr>
<tr>
<td>221</td>
<td>M.O.</td>
<td>cbl A</td>
<td>(5)</td>
</tr>
<tr>
<td>245</td>
<td>A.M.</td>
<td>cbl A</td>
<td>(5)</td>
</tr>
<tr>
<td>209</td>
<td>T.S.</td>
<td>cbl B</td>
<td>(5, 15)</td>
</tr>
<tr>
<td>215</td>
<td>M.R.</td>
<td>cbl B</td>
<td>(5)</td>
</tr>
<tr>
<td>224</td>
<td>K.B.</td>
<td>cbl B</td>
<td>(5)</td>
</tr>
<tr>
<td>78</td>
<td>E.M.</td>
<td>cbl C</td>
<td>(3, 4)</td>
</tr>
<tr>
<td>178</td>
<td>M.M.</td>
<td>cbl C</td>
<td>(24)</td>
</tr>
<tr>
<td>77</td>
<td>L.A.</td>
<td>Mutase apoenzyme defect</td>
<td>(7)</td>
</tr>
<tr>
<td>184</td>
<td>M.M.</td>
<td>Mutase apoenzyme defect</td>
<td>(7)</td>
</tr>
<tr>
<td>87</td>
<td>M.K.</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>237</td>
<td>D.N.</td>
<td>Normal</td>
<td></td>
</tr>
</tbody>
</table>

* Refers to number of times the cultures had been split 1:2 or 1:3 by the beginning of this study; cultures were split an additional three to five times by the end of the study.
† Includes previous publications dealing with the biochemical or clinical description of the individual cell strains in patients.
‡ Includes previous designations as described in the Introduction.

The individual fibroblast strains used in these experiments are shown in Table 1. Using the designations described in the introduction, 11 mutant strains were employed: 4 cbl A mutants; 3 cbl B mutants; 2 cbl C mutants; and 2 mutants with a primary defect of the mutase apoenzyme (7). Two normal strains were used as controls.

The strains were studied between the 5th and 29th passage in culture (Table 1). Such passages appear not to affect the ability of the cells to fuse or to demonstrate complementation. Prior to this study, three strains (209, 214, and 224) tested positive for mycoplasma by broth or agar assay (8). For this reason all strains were maintained in kanamycin. No subsequent evidence of mycoplasma was obtained by examination of cell appearance, growth rate, or microscopic assay.

Cell Fusion. Cells were fused according to the procedure of Klebe et al. (9). Freshly confluent cells were harvested, mixed 1:1 in sterile tubes, and seeded into 35 mm Falcon petri dishes, 4 × 10⁵ cells per dish. One or 2 days later, the cells were grown with washed medium and incubated for 30 min at 4° with 0.4 ml per dish of β-propiolactone-inactivated Sendai virus, 250 hemagglutinating units/ml (10). In early experiments 10⁶ cells were seeded into 25 cm² flasks (Falcon) and 1 ml of virus was used. Two milliliters of growth medium were added to each dish and incubation was continued for an additional 90 min at 37°. The cells were treated briefly with 0.25% trypsin, washed with medium, and incubated overnight in growth medium. On the following day the cells were tested for [14C]propionate incorporation as described below. In two representative fusions, the proportion of cells demonstrating multiple nuclei was 25% and 26%. Multinucleate cells were scored in the complementation tests without regard to the number of nuclei present.

[14C]Propionate Incorporation. Mutase activity was monitored by the radioautographic procedure of Hill and Goodman (6) which detects [14C]propionate incorporation into trichloroacetic-acid-precipitable material by fibroblast monolayers in situ. The [14C]propionate is taken up, converted to its CoA derivative, and metabolized in two steps to L-methylmalonyl-CoA (Fig. 1). Mutants with defective AdoCbl synthesis or a primary defect of the mutase apoenzyme cannot metabolize [14C]propionate further, while normal cells incorporate the isotope into labeled macromolecules via succinyl-CoA and the Krebs cycle. [14C]Propionate incorporation was monitored in the unfused strains described in Table 1 and in the complementation tests. Four chambered slides (Lab-Tech Products) were seeded with 10⁵-10⁶ cells per chamber and incubated overnight in growth medium at 37°. The attached cells were washed with phosphate-buffered saline and incubated for 10 hr at 37° in Puck's saline F (0.5 ml per chamber; Gibco) containing 0.05 M glucose, 15% (v/v) fetal calf serum which had been dialyzed overnight against phosphate buffered saline, and 10⁻⁴ M sodium [1-14C]propionate, 10 Ci/mol (New England Nuclear). At the end of incubation, the cells were washed with 0.9% NaCl, fixed in 10% formalin in 0.9% NaCl for 10 min, washed four times in cold 5% trichloroacetic acid (1–2 min per wash) and washed in water for 30 min. The slides were then air dried and coated with NTB-2 Nuclear Track Emulsion (Kodak).

After 4–14 days exposure in the dark at 4° (depending on the thickness of the emulsion), the slides were developed and stained for 10 min with Giemsa (Fisher) diluted 1:10 with water. The silver grain patterns were examined by light microscopy, and the relative number of silver grains observed over the cells was taken as an index of mutase activity.

Protocol and Scoring of Results. The strategy of each experiment was to test each mutant strain in three kinds of fusions: self-fusion as a "negative control", fusion with a line with which it was expected to complement as a "positive control", and the test fusions. For the Cbl mutants, the positive control was usually a fusion with strain 184 which has a primary defect of the mutase apoenzyme (Table 1). These controls insured that each strain being tested was competent to complement in each experiment and provided upper and lower limits of expected silver grain patterns. Normal cells were also self-fused as controls in early experiments but such
RESULTS

Initially we examined [14C]propionate incorporation in 13 unfused fibroblast strains: 11 mutant and two control. Nine mutant strains have defective Cbl metabolism (4 cbl A, 3 cbl B, and 2 cbl C mutants) and two have a mutase apoenzyme defect (Table 1). As shown in Fig. 2a, cells of the control strain were completely covered by large numbers of silver grains which were so densely distributed as to obscure the cell architecture. In contrast, each of the mutants accumulated only a few grains. The strains with a mutase apoenzyme defect demonstrated almost complete absence of silver grains and consistently scored 0 (Fig. 2b). Most of the cbl mutants were scored 0 or 1+ while a few mutants, demonstrating a more ‘leaky’ phenotype, tended to be scored 2+ (Fig. 2c). Some variability was noted in the scoring. It was often difficult to distinguish between 0 and 1+; and cells scored against a high background produced by a thick coating of emulsion were most often read 1+ or 2+. Nevertheless, in no case did a mutant strain ever score higher than 2+, and mutant strains were easily distinguished from controls. These results confirm the findings of Hill and Goodman (6) in tests of similar mutant and control strains.

Individual strains were treated with inactivated Sendai virus to produce multinucleate homokaryons. Such ‘self-fusions’ always gave silver grain patterns similar to those of the unfused parent strain. The cells in Fig. 2d are from the self-fusion of a normal strain. As with the unfused cells in Fig. 2a, these multinucleate cells were completely covered with a dense blanket of silver grains. Most self-fusions of mutant strains gave scores of 0 or 1+, comparable to their unfused counterparts. Fig. 2e and f show the silver grain patterns of representative self-fusions of mutant strains. Nuclei are readily discernible in these photographs because there are very few silver grains over the cells. As with unfused cells, some self-fusions produced scores as high as 2+. Three mutants consistently giving 2+ scores were strains 221 and 245, both cbl A mutants, and strain 215 (Fig. 2f), a cbl B mutant. The leaky character of these mutants did not preclude their use in complementation tests, since normal cells were always far more densely covered with silver grains.

Complementation tests were carried out with all 11 mutant strains fused in pairwise combinations. The results of these experiments are shown in Table 2. Heterokaryons produced by fusing members of each of the four mutant classes with mutants of any other class uniformly recovered the ability to incorporate [14C]propionate to 3+ or 4+ levels. For example, Fig. 3a shows the silver grain pattern of cells from

![Fig. 2. Silver grain patterns obtained after radioautography of fibroblasts incubated in the presence of [14C]propionate. The presence of a dense cover of silver grains over cells corresponds to functional methylmalonyl-CoA mutase. The cells in the left column are unfused fibroblasts. The cells in the right column are fibroblasts of the strain shown immediately to the left which have been treated with inactivated Sendai virus to produce multinucleate homokaryons. The fibroblast strains are 87, a normal strain, in frames a and d; 184, with a defect of the methylmalonyl-CoA mutase apoenzyme, in frames b and e; and 215, a cbl B mutant, in frames c and f. Magnification: ×180.

Table 2. Results of complementation tests in heterokaryons formed from pairs of mutant cell lines

<table>
<thead>
<tr>
<th>MUTANT CLASS</th>
<th>cbl A</th>
<th>cbl B</th>
<th>cbl C</th>
<th>mutase</th>
</tr>
</thead>
<tbody>
<tr>
<td>cbl A</td>
<td>1f</td>
<td>2f</td>
<td>3f</td>
<td>4f</td>
</tr>
<tr>
<td>cbl B</td>
<td>2f</td>
<td>4f</td>
<td>3f</td>
<td>1f</td>
</tr>
<tr>
<td>cbl C</td>
<td>3f</td>
<td>1f</td>
<td>4f</td>
<td>2f</td>
</tr>
<tr>
<td>mutase</td>
<td>4f</td>
<td>2f</td>
<td>1f</td>
<td>3f</td>
</tr>
</tbody>
</table>

The mutant cell lines (1f, 214, 221, etc.) are listed according to mutant class across the top and down the left ordinate. Each self-fusion and test fusion was scored from 0 (no grains) to 4+ (dense grain pattern obscuring cell outline) as described in the text. The intraclass fusion results are enclosed by the heavy black line, while the interclass fusions are reported in the shaded area. Blank spaces designate tests which were not carried out. Results noted with an asterisk denote fusions which were repeated on two or more occasions.
a fusion between a cbl A and a cbl B mutant. The cells are completely covered with silver grains and the test was scored 4+. Since self-fusions of these strains were scored 1+ or 2+ (Table 2), these results show correction of the mutant phenotype in the heterokaryons and provide evidence of complementation between the mutant genomes. Similar results were observed for fusions between a cbl B and a cbl C mutant (Fig. 3b) and a cbl A and cbl C mutant (Fig. 3c). Table 2 shows that all interclass fusions (shaded area), including fusions with mutase apoenzyme mutants, resulted in complementation as shown by the 3+ or 4+ scores recorded in every test.

No evidence of such increased $[^{14}C]$propionate incorporation was obtained by mixing different mutant strains in the absence of Sendai virus. Thus, cell to cell cross-feeding was not observed in mixtures between mutants from different cbl groups (cbl A and cbl B; cbl B and cbl C; cbl A and cbl C). This indicates that mutants and those with a mutase apoenzyme defect. The existence of cross-feeding cannot be excluded by these experiments, but seems most unlikely, since the number of cells used was purposefully small and since mixed cultures were maintained for only short intervals before testing.

Significantly, no complementation was observed when fusions were carried out between mutants of the same class. Fig. 3d shows the silver grain pattern for a fusion between two cbl A mutants, strains 1f × 221. These heterokaryons show some silver grains and were scored 2+. As noted in Table 2, the apparent self-fusions produced scores of 1+ for strain 1f and 2+ for strain 221. These results differ markedly from the scores produced by these same mutants in "positive control" fusions with strain 184. Table 2 shows that fusion between strains 1f and 184 was scored 3+ and that fusion between strains 221 and 184 was scored 4+. Since both strains (1f and 221) were competent to complement, the failure of the test fusion (1f × 221) to incorporate normal levels of $[^{14}C]$propionate demonstrates that these mutants do not complement in heterokaryons. Fig. 3e and f shows the results of complementation tests between two cbl B mutants and between two cbl C mutants, respectively. All possible intraclass complementation tests were performed and the results are shown in Table 2 (unshaded areas). In no instance was the score for the silver grain pattern produced between two mutants of the same class greater than that obtained for each mutant alone. Thus, the four cbl A mutants complement mutants of all other classes but not each other. The same was true for the cbl B and cbl C mutants, and the two mutants with a defective mutase apoenzyme.

**DISCUSSION**

Nine mutant fibroblast lines with defective Cbl metabolism and two with a mutase apoenzyme defect were grouped into four complementation groups by examining $[^{14}C]$propionate incorporation in Sendai-virus-mediated heterokaryons. These data confirm the separation of cbl A and cbl B mutants into distinct classes as demonstrated biochemically by Mahoney et al. (5). The four cbl A mutants, which have in common a block in AdoCbl synthesis in intact cells but normal activity of the final steps of AdoCbl synthesis in crude extracts, make up a single complementation group; and the three cbl B mutants, with deficient synthesis of AdoCbl in crude extracts as well as intact cells, comprise a second complementation group. The third complementation group includes the two cbl C mutants. They have a combined defect of AdoCbl and MeCbl synthesis. The two mutants with a de-

*Fig. 3.* Results of representative complementation tests. The cells in each frame were produced by fusion of different Cbl mutants in pairwise combination. The intensity of the silver grain pattern observed over multinucleate cells by radioautography following incubation with $[^{14}C]$propionate is taken as a measure of complementation (see text for details). The fibroblast strains used in each frame are as follows: 1f (cbl A)/215 (cbl B) in frame a; 215 (cbl B)/78 (cbl C) in frame b; 1f (cbl A)/178 (cbl C) in frame c; 1f (cbl A)/221 (cbl A) in frame d; 209 (cbl B)/215 (cbl B) in frame e; and 78 (cbl C)/178 (cbl C) in frame f. Magnification: ×180.
that these two classes reflect mutations at the same locus and that the complementation observed between them is intragenic. For instance, one step in AdoCbl synthesis could be catalyzed by an enzyme composed of two identical subunits and bound to the mitochondrial membrane. In intact cells or homokaryons, different mutations of this enzyme (i.e., cbl A or B) could produce an indistinguishably severe block in AdoCbl synthesis, whereas, when the mitochondrial membrane is altered or destroyed during the preparation of cell extracts, the subunits of the cbl A-type mutant enzyme could assume a configuration restoring near normal enzymatic activity. To complete this formulation, complementation in heterokaryons between cbl A and cbl B mutants could reflect formation of hybrid enzyme with activity far greater than that of either parent line. Clearly, an unambiguous distinction between intragenic and intergenic complementation of these mutants must await their biochemical characterization.

The occasional demonstration of affected sibs and the approximately equal numbers of affected males and females has suggested that each of the methylmalonic acidemias is inherited in Xcys0 as an autosomal recessive trait. This assumption has not yet been tested by statistical analyses in pedigrees or by heterozygote detection. The results of our complementation tests confirm that the three defects of Cbl metabolism and the defect of the mutase aepoenzyme are expressed as recessive mutations in heterokaryons, since each can be complemented by fusion with cells of a class other than their own. It is unlikely that the expression of a dominant allele could have been masked by an excess of normal alleles, because complementation was observed regularly in dikaryons as well as heterokaryons containing several nuclei.

Some patients with methylmalonic acidemia respond to administration of pharmacologic doses of vitamin B12 with decreased accumulation of methylmalonic acid and clinical improvement (7, 14). Such responsive patients appear to be restricted to those with defects of Cbl metabolism. Among the nine patients with Cbl defects whose fibroblast lines were examined in this study, seven have been tried on vitamin B12 therapy. Three of the cbl A and one of the cbl B patients are responsive to vitamin B12 (ref. 14, and unpublished observations) while one patient from each group (cbl A, K.B.; cbl B, T.S.) showed no improvement after short-term therapeutic trials (15). If these results are confirmed with long-term therapeutic trials, it would imply that mutations of the same Cbl gene can lead to different clinical expression of the defect. This would underscore still additional heterogeneity and preclude the use of complementation analysis to determine the likelihood that vitamin B12 therapy would be effective.

Beginning with the demonstration of complementation between mutations of independent loci on the X chromosome in human diploids (16), several metabolic defects have been successfully subjected to complementation tests. Genetic heterogeneity has been demonstrated in cells from patients with galactosemia (17), xeroderma pigmentosum (18-20), maple syrup urine disease (21), and hexosaminidase deficiency (22, 23). It is apparent that genetic complementation is proving to be an effective tool for the elucidation of the genetic basis of inherited metabolic disease.

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