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Genetics

Interallelic complementation in an inborn error of metabolism: Genetic heterogeneity in argininosuccinate lyase deficiency

(complementation map/urea cycle disorder/clinical heterogeneity)

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ABSTRACT **We used complementation analysis as a probe for the detection of genetic heterogeneity within a single locus affected in a human disease, argininosuccinate lyase (L-argininosuccinate arginine-lyase, EC 4.3.2.1) deficiency. Fibroblasts cultured from 28 unrelated patients were fused in all possible pairwise combinations, and the argininosuccinate lyase activity in heterokaryons was assayed by measuring the incorporation of ^{14}C from L-[*ureido*-^{14}C]citrulline into acid-precipitable material. Partial complementation was observed in fusions involving 20 of the 28 strains, with the lyase activity increasing from 2- to 10-fold. Thirteen of the mutants were identified by the complementation analysis as being phenotypically unique. Of the 20 complementing strains, 3 were remarkable because they participated in all but 2 of the 32 positive complementation tests; 2 others constituted a unique subgroup that produced the highest increases in argininosuccinate lyase activity of all fusions. The 8 strains that did not complement any others consisted of two types: 3 mutants with the highest residual argininosuccinate lyase activity of all strains and 5 mutants with low residual activity. All of the mutants mapped to a single major complementation group. The data could be summarized as a circular complementation map with an attached linear tail, the mutants being distributed among 12 subgroups in a complex pattern. We conclude that all of these mutants are affected at a single locus, that extensive genetic heterogeneity is present in the mutant population, and that the affected locus in argininosuccinate lyase deficiency is likely to be the structural gene coding for that enzyme.**

Argininosuccinate lyase (AS lyase; L-argininosuccinate arginine-lyase, EC 4.3.2.1) deficiency is a relatively common, autosomal recessive disorder of the urea cycle with significant unexplained clinical heterogeneity (1). To determine whether genetic heterogeneity was present and could be related to the clinical heterogeneity of this disorder, we performed complementation analysis with cells cultured from 28 patients. Complementation analysis has been used in human genetics primarily to establish the number of different loci responsible for an abnormal enzymatic (2–4) or clinical (5) phenotype—i.e., by the detection of nonallelic complementation. In contrast, there are few examples of interallelic complementation in human genetic disease (6, 7). However, AS lyase is a homotetramer (8, 9), and, in microorganisms, interallelic complementation has been found to be almost universal at loci coding for homomultimeric proteins (10). In such cases, complementation analysis has been a powerful approach to the study of genetic heterogeneity within a locus (10). Thus, it was likely that interallelic rather than nonallelic complementation would be observed in AS lyase deficiency. Such an observation would be of interest because interallelic complementation can be related to the subunit interaction of homomultimeric proteins (10, 11), allowing one to examine a functional phenotypic property of the mutant enzyme not assessed by any other technique presently in use. In this paper we demonstrate that AS lyase deficiency results from a large number of diverse mutations in a single gene. Our data also suggest that some of the clinical heterogeneity of this disease may derive from variation in the residual AS lyase activity in different patients.

MATERIALS AND METHODS

Fusion of Fibroblasts. Skin fibroblast strains were derived from 28 unrelated AS lyase-deficient patients. Each strain was cultured in α-minimal essential medium (α-ME medium) (12) and was free of mycoplasma as determined by direct culture and by a uridine phosphorylase assay. All three clinical phenotypes (1) were represented (9 neonatal onset, 2 subacute onset, and 17 late-onset). For complementation testing, 3×10^5 cells of each strain (or 6×10^5 cells for self-fusions) were plated in 35-mm culture dishes and fused 24 hr later by applying 1 ml of 40% polyethylene glycol 1000 for 60 sec, after which the glycol was rapidly diluted with serum-free α-ME medium. Multinucleate cells contained up to $\approx$12 nuclei, and $\approx$50% of the nuclei appeared to be in multinucleate cells (13). After fusion, the cells were incubated for 72 hr prior to the assay of AS lyase activity.

The Indirect Quantitative Assay of AS Lyase. Intact cells were indirectly assayed for AS lyase activity by measuring the incorporation of ^{14}C from L-[*ureido*-^{14}C]citrulline into acid-precipitable material (14) and using ^3H incorporation from [4,5-^3H]leucine as a control. Cells were incubated for 22 hr at 37°C in 1 ml of arginine-free α-ME medium with 15% dialyzed fetal calf serum containing 0.04 mM citrulline and 0.11 μCi (1 Ci = 37 GBq) of L-[*ureido*-^{14}C]citrulline (specific activity, 51.7 mCi/mmol; New England Nuclear Canada). [^{14}C]Citrulline was purified prior to use by continuous TLC for 16 hr at 4°C in *t*-butanol/butanone/H_2O/NH_4OH, 4:3:2:1. Purification was necessary because up to 10% of the ^{14}C label was in $\approx$20 compounds other than citrulline, and the incorporation of ^{14}C in mutants was increased up to 50% when unpurified vs. purified [^{14}C]citrulline was used. After incubation, the cells were rinsed three times with phosphate-buffered saline (4°C) and then exposed to 5% CCl_3COOH (4°C) for 5 min. After rinsing off the CCl_3COOH, the cells were dissolved overnight in 0.2 M NaOH, and the ^3H and ^{14}C were assayed. Control strains generally incorporated 4000–7000 dpm of ^{14}C (vs. 280–2000 dpm for mutants) and 2800–4300 dpm of ^3H per 35-mm culture dish. All $^{14}C/^3H$ ratios were then multiplied by 10 to give a whole number.

The autoradiographic assay of AS lyase (15) was modified for complementation analysis (2). [^{14}C]Citrulline (10^6 dpm) and 4.0 mM citrulline (5 μl) were added to each 0.5 ml of arginine-free α-ME medium. AS lyase was assayed directly

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Abbreviations: AS lyase, argininosuccinate lyase; AS synthetase, argininosuccinate synthetase.

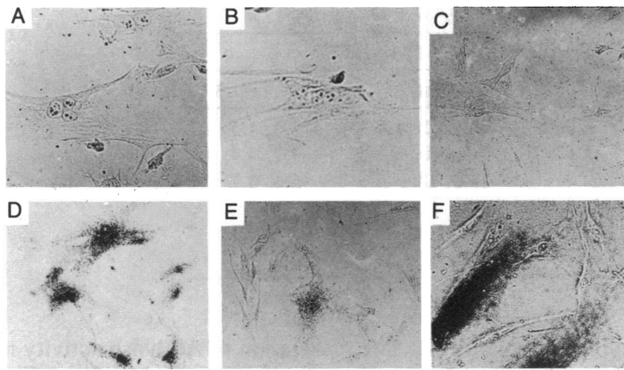

FIG. 1. Autoradiographic demonstration of AS lyase complementation in individual heterokaryons (×128). (*A*) Self-fusion of the AS synthetase-deficient strain used in the positive-control fusion. (*B* and *C*) Self-fusions of AS lyase-deficient strains 944 and 926, respectively. (*D*) Positive-control fusion of the AS synthetase-deficient strain fused with strain 926. (*E*) Test fusion of AS lyase-deficient strains 926 and 944. (*F*) Test fusion of AS lyase-deficient strains 926 and 1253.

by the method of O'Brien and Barr (8) except that the reaction was stopped by freezing, which minimized anhydride formation. Protein was measured by the method of Lowry *et al.* (16).

Statistical Analysis. All data of pairs of strains that were tentatively labeled as complementary after three consecutive complementation tests (see *Results*) were statistically evaluated to determine whether the increase in the $^{14}C/^{3}H \times 10$ ratio in the test fusions was significantly increased ($P < 0.001$) compared to the self-fusions of each cell strain. A 2-factor analysis of variance with unequal numbers of replications per cell was used to obtain an error-mean-square statistic using the Biomedical P Series software package (Department of Biomathematics, University of California at Los Angeles, University of California Press). The error-mean-square value was then used in a linear contrast analysis comparing the two self-fusion groups of each pair of strains to the test fusions of that pair.

RESULTS

Autoradiographic Demonstration of Complementation in Individual Heterokaryons. Complementation between AS lyase-deficient strains was initially demonstrated with the autoradiographic assay. Six AS lyase-deficient strains were fused in all pairwise combinations ("test fusions") and also as "positive controls," with a strain deficient in argininosuccinate synthetase (AS synthetase; EC 6.3.4.5). Heterokaryons formed between AS lyase- and AS synthetase-deficient cells would be expected to convert citrulline to arginine at near-normal rates. Representative results are shown in Fig. 1. No silver grains were present in the self-fusions (Fig. 1, *A*–*C*), whereas every cell was labeled in the positive-control fusion (*D*), the larger multinucleate cells more so than mononucleate cells; the latter cells presumably become labeled by metabolic cooperation from complementing multinucleate cells. Test fusions in which positive complementation occurred (Fig. 1, *E* and *F*) show that the restoration of activity was considerably less than in the positive-control fusions—for example, the mononucleate cells were never labeled in the test fusions (*D* vs. *E* or *F*). Since the restoration of enzyme activity was only partial and all six strains mapped to one complementation group (data not shown), it seemed probable that the complementation was interallelic.

Complementation Analysis of 28 AS Lyase-Deficient Strains. To obtain an interallelic complementation map that was informative about this locus, we expanded our study to include 28 AS lyase-deficient strains. AS lyase was measured by the indirect quantitative assay. The AS lyase-deficiency of all strains was reflected in their reduced $^{14}C/^{3}H \times 10$ ratios, determined (in triplicate) in a single experiment to avoid interexperimental variation (see Fig. 3, right-hand column). Four control strains had a $^{14}C/^{3}H \times 10$ ratio of 201 ± 55.8 (mean ± SEM). The 9 neonatal strains had a $^{14}C/^{3}H \times 10$ ratio of 4.4 ± 2.0 (mean ± SD), a value significantly less than the late-onset patients (22.8 ± 39.8, $n = 17$). As suggest-

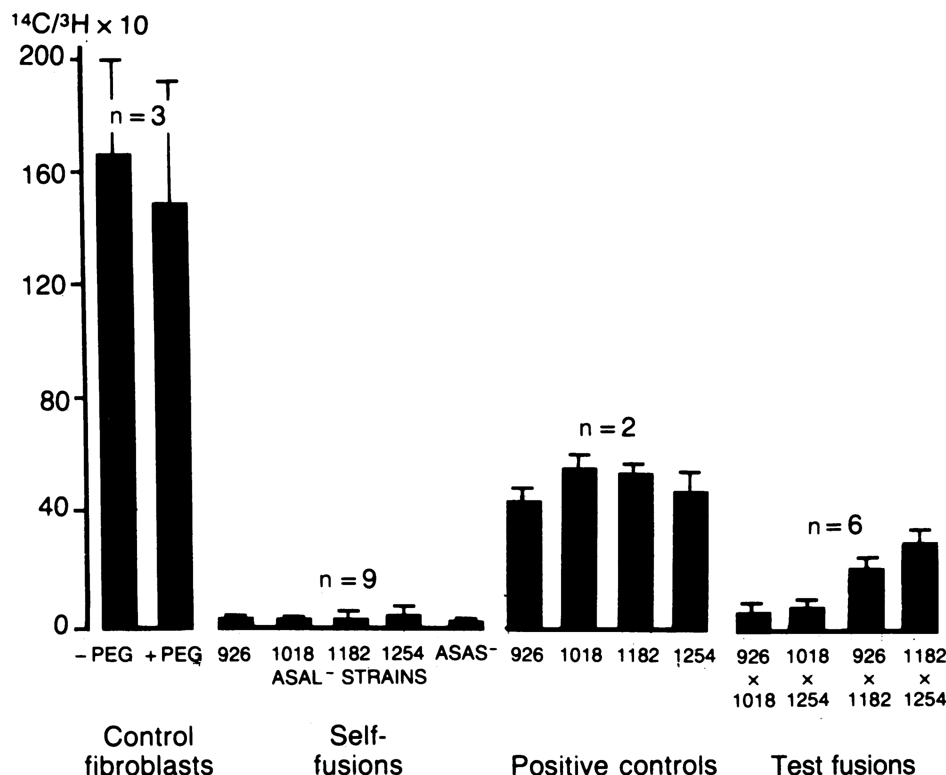

FIG. 2. Representative data from the quantitative *in situ* assay of three control strains and four representative mutants. ^{14}C and ^{3}H dpm from L-[*ureido*-^{14}C]citrulline and [4,5-^{3}H]leucine, respectively, were incorporated into 5% CCl_3COOH-precipitable material. *n* for the control strains is the number of strains studied in duplicate and for the mutants is the number of replicate dishes assayed. Data are expressed as the mean ± SD. The depicted test fusions (fusions of two AS lyase-deficient strains) are all positive for complementation. Positive controls are fusions of one AS lyase-deficient strain with an AS synthetase-deficient strain.

Table 1. Complete data of the complementation analysis of strain 944 tested with strain 1254

	$^{14}C/^{3}H \times 10$ ratio*		
	Test 1	Test 2	Test 3
Self-fusion 944	2.8, 3.1, 3.1	4.2, 4.5, 4.6	1.5, 1.5, 1.7
Self-fusion 1254	4.6, 4.7, 4.7	7.5, 9.0, 9.2	3.7, 4.4, 4.6
Test-fusion 944–1254	16.9, 17.2	13.2, 18.1	6.6, 6.7
Ratio increase (test fusions/self-fusions)	4.5	2.4	2.3
Mean ratio increase ± SD		3.1 ± 1.2	

*Analysis of variance of tests 1–3 = $P < 0.001$.

ed by the large standard deviation, however, the late-onset patients were clearly of two types with respect to residual activity. One type, consisting of 13 of the 17 late-onset strains, had a residual AS lyase activity (3.9 ± 1.8, n = 13) indistinguishable from the neonatal patients. The second type, composed of 4 late-onset strains (928, 929, 945, and 1006), had $^{14}C/^{3}H \times 10$ ratios ranging from 31.3 ± 0.6 to 125 ± 22.5 (see Fig. 3, right-hand column)—up to 30-fold greater than the other 24 mutant strains we examined.

All possible pairwise fusions were made between the 28 AS lyase-deficient strains. Representative data are shown in Fig. 2. Polyethylene glycol 1000 had no significant effect on the $^{14}C/^{3}H$ ratio of control fibroblasts (Fig. 2) but was essential for complementation between AS lyase-deficient strains (data not shown). AS lyase-deficient cells generally had a $^{14}C/^{3}H$ ratio that was <3% of controls. In the positive-control fusions, the $^{14}C/^{3}H$ ratio increased to about one-third of the control value, an increase appropriate for intergenic complementation (2). The restoration of AS lyase activity in the positive test fusions was generally much less (e.g., Fig. 2, fusions 926–1018 and 1018–1254), although two exceptional test fusions with high $^{14}C/^{3}H$ ratios are shown in Fig. 2 (926–1182 and 1182–1254).

Criteria for Complementation. In each experiment complementation between a pair of strains was tentatively inferred if the $^{14}C/^{3}H$ ratio of all test fusions was greater than the $^{14}C/^{3}H$ ratio of any of the self-fusions. For example, strains 944 and 1254 were tentatively said to complement because the test-fusion $^{14}C/^{3}H \times 10$ ratios of 16.9 and 17.2 exceeded the highest self-fusion ratio of either strain (4.7 for strain 1254) (Table 1, test 1).

Two strains were said to complement definitively if the following three criteria were met. (*i*) By comparing the test fusion and self-fusion results as described above, three sequential positive tests were obtained. The results of all three tests between strains 944 and 1254 are shown in Table 1.

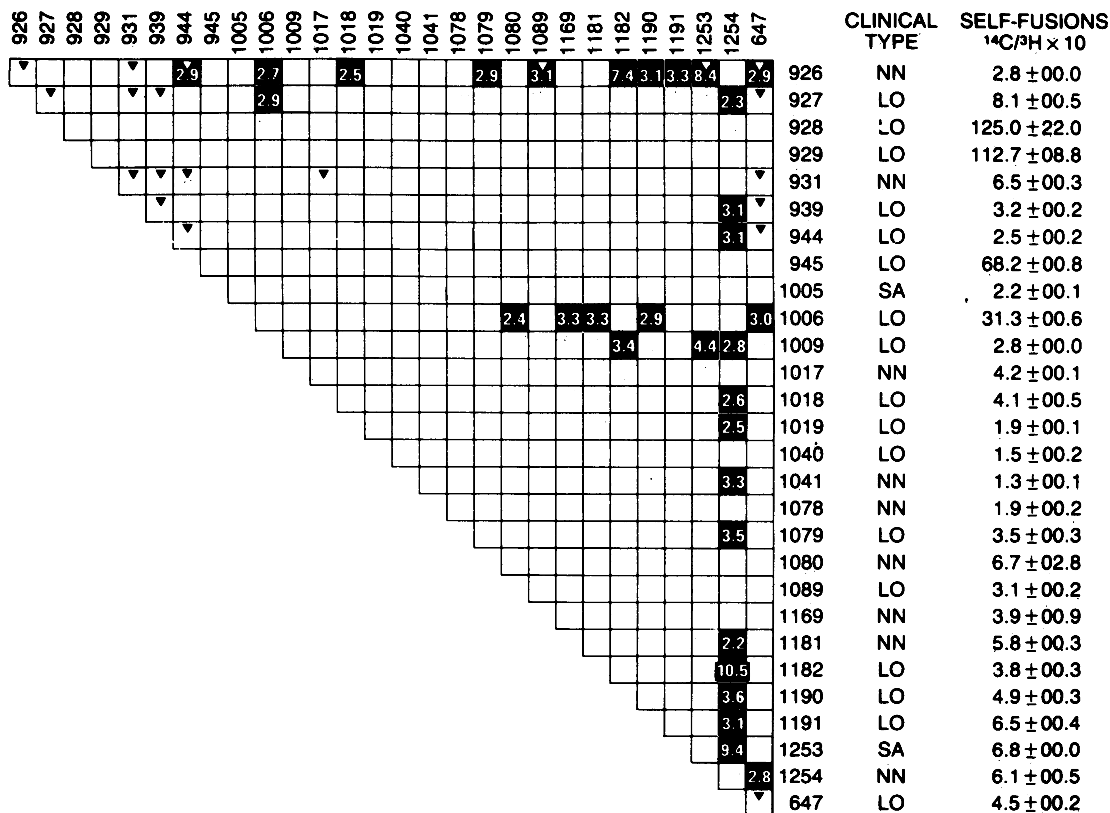

FIG. 3. A matrix summarizing the complementation data of 28 AS lyase-deficient strains. The numbers along the top and right side of the matrix designate the individual fibroblast strains. In the boxes, complementing pairs of strains are identified by a number that is the mean increase in AS lyase activity from all three tests of a pair of strains (e.g., 3.1 for strains 944 and 1254; see Table 1). Noncomplementing pairs of strains are indicated by the blank boxes. The triangles in some boxes designate pairs of strains for which the complementation status has been confirmed by the autoradiographic assay. The columns on the right side of the matrix list the clinical type of the patient from which each strain was derived (NN, neonatal; SA, subacute; LO, late-onset) and the $^{14}C/^{3}H \times 10$ ratio of each strain (self-fused) (data are the mean ± SD of triplicate dishes).

Strains that did not tentatively complement in the initial test fusion were permanently classified as noncomplementing. (*ii*) Statistical analysis of the data from the three tests (nine self-fusions and six test fusions, as in Table 1) showed the test fusions to differ significantly from the self-fusions ($P < 0.001$). (*iii*) Use of the data from all tests of a pair of strains showed that the increase in the $^{14}C/^{3}H \times 10$ ratio in the test fusions exceeded the ratio of the self-fusions by 2.2-fold or more. Thus, for strains 944 and 1254, the increase in the $^{14}C/^{3}H \times 10$ ratio ultimately used in the complementation matrix (Fig. 3) was 3.1, the mean of the increases (Table 1) obtained in the three tests of these two strains. The cutoff of 2.2-fold was chosen because it included all but three pairs of strains that met the first two criteria and because it provided an informative complementation map (10) with multiple overlapping segments (see Fig. 4).

Summary of the Complementation Analysis. The complementation data are summarized in a complementation matrix of all strains (Fig. 3). Of the total of 378 pairwise fusions, 32 showed complementation. The majority (20 of 28) of the strains complemented at least once but, more interestingly, 30 of 32 of the pairs of complementing mutants involved 1 of only 3 strains (926, 1006, and 1254) (Fig. 3). The increase in AS lyase-activity from complementation was generally 2- to 4-fold, except that strains 1182 and 1253, when fused with either 926 or 1254, increased the $^{14}C/^{3}H \times 10$ ratio 7- to 10-fold (Fig. 3).

Independent validation of the complementation analysis was obtained by comparing the complementation response of strain 1465, which was from an affected sibling of the patient from whom strain 944 was obtained, with the results that had been obtained with 944 itself. In two separate studies, complete concordance was observed between 944 and 1465 for the 10 test fusions we compared (8 negative and 2 positive tests with strains 926 and 1254) (data not shown).

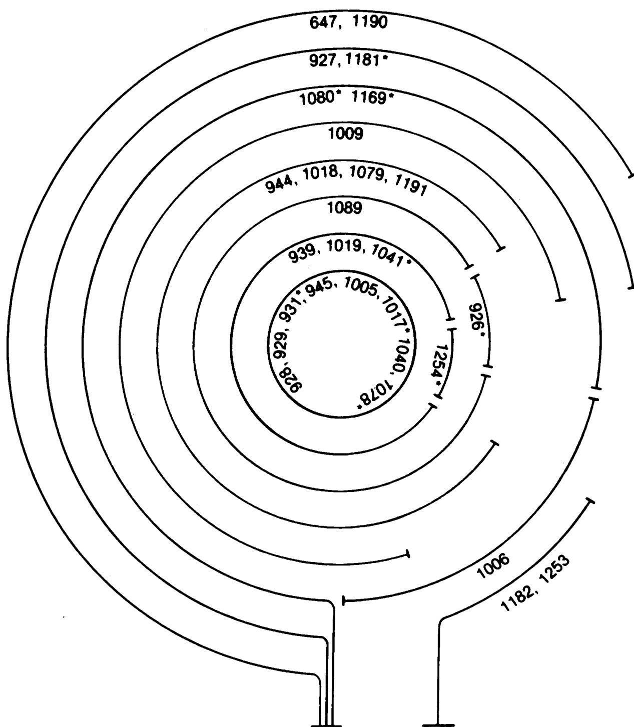

FIG. 4. The human AS lyase-deficiency complementation map for 28 mutants. The numbers identify individual strains. Mutants grouped together below a line (e.g., 647 and 1190) belong to the same subgroup. Neonatal group strains are indicated by an asterisk. All other strains are from late-onset patients except 1005 and 1253, which are from the subacute-onset group.

The AS Lyase-Deficiency Complementation Map. The most remarkable feature of the complementation analysis is that all 28 of the mutants mapped to one complementation group (Fig. 4). The circular form of the map, with the attached linear tail, is the simplest two-dimensional shape that can represent the data without showing some mutants as discontinuous lines. Each number in the map identifies a mutant strain. Mutants grouped together below a line (e.g., 647 and 1190) belong to the same subgroup. Thus, they do not complement each other but behave identically with respect to their ability to complement other specific mutants. Noncomplementing mutants have overlapping lines (e.g., 926 and 1254), while complementing mutants have nonoverlapping lines (e.g., 926 and 647). The linear arrangement of the mutants is determined by mutants with overlapping lines. The single complementation group is defined most explicitly by the eight strains (the innermost circle) that were incapable of complementation with any other strain. The remainder of the mutants are distributed amongst 11 subgroups in a complex pattern. One particular subgroup, that of strains 1182 and 1253, not only occupies a novel position on the map (Fig. 4) but is also noteworthy because these two strains, as noted above, produced the greatest increase in the $^{14}C/^{3}H \times 10$ ratio when fused with either 926 or 1254 (Fig. 3). No relationship was present between the complementation behavior of a strain and the clinical phenotype or the residual enzyme activity of that strain (Figs. 3 and 4).

DISCUSSION

We have shown that extensive genetic heterogeneity characterizes human AS lyase deficiency. This heterogeneity occurs entirely within a single gene because all 28 of the mutants we examined could be mapped to one complementation group. Because interallelic complementation in lower organisms has invariably been demonstrated with mutants affecting the structural locus of homopolymeric proteins (10) and because AS lyase is a homotetrapolymer (8, 9), our observations suggest that the structural locus for AS lyase is affected in this disease. Furthermore, using protein blotting, we have obtained direct evidence for variation in the amount and/or stability of the AS lyase monomer in all of these mutants (unpublished data). *Cis*-regulatory and coding-region mutants cannot be distinguished by complementation. Thus, while some mutants may be *cis*-regulatory, our data offer no support for separate regulatory gene mutations in this disease, as has been suggested by the finding of normal brain or kidney AS lyase activity in some patients with deficient hepatic AS lyase activity (17, 18). Interallelic complementation of AS lyase mutants has been observed in *Chlamydomonas reinhardi* (19) and in fibroblasts from four AS lyase-deficient patients (20).

The criteria which we used to define complementation were both pragmatic and stringent but, as is always the case, arbitrary (10). Because fusions that were negative on the first analysis were not studied further, some false-negative complementation data may be a feature of the map. This is not of great significance, however, because it merely means that no assertion is made that two particular mutants are different and does not affirm that they are identical. The use of stringent criteria to identify complementing strains, in contrast, assures that the claim that these strains are genotypically different has been firmly substantiated.

Although it accurately reflects the AS lyase genotype of fibroblasts, the *in situ* ^{14}C-incorporation assay for AS lyase activity used in the complementation tests is indirect. Direct evidence for an increase in AS lyase activity in fused populations of complementing strains was obtained by assaying AS lyase in cell lysates. In two separate studies, the enzyme activity in the populations of fused 926–1253 increased signifi-

cantly over the mean activity of the self-fusions (unpublished observations), validating the use of the ^{14}C-incorporation assay in this work.

The tailed circular complementation map, which accommodates the AS lyase-mutants in continuous lines, is complex but not without precedent in interallelic complementation (21–23). Similar maps were obtained for the *ad-7* locus of *Saccharomyces cerevisiae* (21, 22), and even more complex figures have been required for other loci—for example, the two circles connected by a line for the *ad-6* locus of *Schizosaccharomyces pombe* (23). Whether the AS lyase-deficiency complementation map has any structural meaning relative to the enzyme cannot be determined without some knowledge of the three-dimensional structure of the mutant, complementing, and normal AS lyase proteins (10) or at least of the complementing fragments of the mutant polypeptides (11, 24).

Since the majority of the human AS lyase mutants we studied may be genetic compounds (25) (only strains 944, 1017, 1078, and 1254 are from known consanguineous matings), a complementation map of such mutants cannot be interpreted as simply as the maps of microorganisms, in which only two alleles are present in the cells tested for complementation (10). As many as four allelic polypeptides may be present in the heterokaryons formed between two fibroblasts, and the consequences of this diversity of genotypes are 3-fold. First, the frequency of complementation is likely to be greater, resulting in an increased number of segments in the map (i.e., a mutant with two different alleles is more likely to complement any other allele than is a mutant with only one type of allele). The complexity of some maps in microorganisms (10) indicates, however, that the complexity of the AS lyase-deficiency map does not necessarily derive from the compound nature of the human mutants. Second, a complementation map of nonconsanguineous human mutants is only a minimal representation of all the complementation that may be occurring. The map is the same whether the complementation occurs with only one or all four of the possible combinations of alleles in a heterokaryon of two genetic compounds. Third, in the absence of a preferential association of subunits, the restoration of enzyme activity resulting from complementation between any two specific alleles will be less in a tetraploid heterokaryon containing one or two other noncomplementing alleles than in a diploid cell with only those two alleles. In the tetraploid heterokaryon, the noncomplementing alleles will reduce the specific activity of the enzyme. Consequently, complementation may occur more frequently between genetic compounds, but the restoration of enzyme activity may be less than between cells truly homozygous at the locus of interest.

The identification of the subset of four late-onset strains with high residual enzyme activity suggests that, at least in some patients, the clinical heterogeneity in this disease may be attributed to variation in the amount of residual AS lyase activity in the liver. That these four patients are also amongst the most mildly affected supports this hypothesis.

The present significance of the map, apart from demonstrating genetic heterogeneity at a single locus, is that it summarizes the complementation data, giving significance to certain mutants that neither enzymatic studies nor clinical assessment would confer. On the basis of the complementation results, one may speculate about the nature of the mutations in certain strains. The three frequent complementers (926, 1006, and 1254), for example, are likely to have abnormalities that impair the active site of the protein while affecting less its capacity to aggregate or to correct other mutants conformationally (10, 26). Active-site mutants that remain highly competent in their interaction with other monomers have been reported, the glutamate dehydrogenase mutant *am*^{1} (26) being one example. Therefore, the strains shown to be unique by the complementation studies will merit special attention in molecular analysis of the AS lyase protein, mRNA, and gene.

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