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Genetics

Abnormal ornithine carbamoyltransferase in mice having the sparse-fur mutation

(X-chromosomal mutation/mouse genetics/human hereditary disease/urea cycle defect)

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ABSTRACT **Mice with the X-chromosomal sparse-fur (*spf*) mutation frequently have urinary bladder stones composed mostly of orotic acid, which was identified by the following criteria: ultraviolet and infrared absorption spectra, chromatographic behavior, melting point, and reactivity in a specific color test. This clue led to the discovery that *spf*-bearing mice have an abnormal form of liver ornithine carbamoyltransferase (carbamoylphosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3). Normal ornithine carbamoyltransferase has maximum activity at pH 7.6–8.0 and 80% of maximum activity at pH 10.0. The enzyme from *spf* males has 22% of normal specific activity at pH 7.6 but has almost twice the normal value at pH 10.0. The activities of normal and mutant enzymes as functions of the concentration of L-ornithine are also distinctly different. The apparent net deficiency of ornithine carbamoyltransferase activity in sparse-fur males is about 90%, which may account for the accumulation of orotic acid and other pathological traits of sparse-fur mice. No dissociation of the sparse-fur and abnormal ornithine carbamoyltransferase phenotypes has been observed, and it is likely that the *spf* locus determines the structure of liver ornithine carbamoyltransferase. Mixtures of normal and abnormal activities are found in the livers of heterozygous females. The proportion of abnormal enzyme has a large variance, indicating that the gene is subject to single-active-X control, but an explicit demonstration of single-allele-expression in individual cells has not been made. Since mice having the *spf* mutation on certain genetic backgrounds have greatly reduced fitness, sparse-fur mice may provide information about alleviating the consequences of ornithine carbamoyltransferase deficiency in humans. Heterozygous female mice should be useful in developing reliable methods for identifying heterozygous human females and in determining if spontaneous and induced hepatomas in mice are monoclonal or multiclonal.**

In 1970 (1) one of us proposed a procedure for creating strains of mice having X-chromosomal mutations corresponding to those causing the Lesch–nyhan syndrome (2) in humans. The proposal was outlined in a lecture at the Oak Ridge National Laboratory and it was pointed out that deposits of uric acid in various parts of the body, including the urinary bladder, were frequent consequences of the hypoxanthine phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) deficiency (EC numbers in this text are from ref. 3) in untreated patients with the Lesch–Nyhan syndrome. L.B.R. had discovered that male mice having the X-chromosomal sparse-fur (*spf*) mutation (4 and references cited therein) frequently (in 30 out of 43 males dissected) had bladder stones, which she had saved. Some stones were analyzed in Madison with results that both chagrined and pleased us: the bladder stones consisted primarily of orotic acid, a normal intermediate in the biosynthesis of pyrimidine nucleotides. The most familiar form of orotic aciduria in humans results from autosomal, recessive mutant alleles that cause associated deficiencies of orotate phosphoribosyltransferase (orotidine-5′-phosphate: pyrophosphate phosphoribosyltransferase, EC 2.4.2.10) and orotidine-5′-phosphate decarboxylase (orotidine-5′-phosphate carboxy-lyase, EC 4.1.1.23) activities (5). Therefore, the X-chromosomal location of the mutation causing accumulation of orotic acid in *spf*/Y mice puzzled us until we realized that orotic aciduria (6; references cited on p. 359 of ref. 7) is almost always among the consequences of X-chromosomal mutations that cause deficiencies of ornithine carbamoyltransferase (carbamoylphosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3.) activity in humans (8, 9). This transferase occurs most abundantly in liver mitochondria (10). It seems likely that in deficient patients the carbamoyl phosphate that ordinarily would be consumed in synthesizing citrulline leaks from the mitochondria and permits hypernormal function of aspartic carbamoyltransferase (carbamoylphosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2), the first and rate-limiting enzyme in the pathway of pyrimidine nucleotide biosynthesis. The clue of orotic aciduria in *spf*/Y mice led to the evidence, described below, that mice with the sparse-fur phenotype have an abnormal form of ornithine carbamoyltransferase, which functions poorly under normal physiological conditions. Since no dissociation of the two traits has been observed, it is likely that the abnormalities comprising the sparse-fur phenotype result from a partial deficiency of OCT activity.

Abbreviation: *spf*, X-chromosomal sparse-fur mutation.

MATERIALS AND METHODS

Genetic Background. The *spf* mutation arose spontaneously (nonirradiated X chromosome) in the progeny of an irradiated male at Oak Ridge (4) and has been maintained there on various genetic backgrounds that allow different frequencies of survival of *spf*/Y males. Almost all mice used in the present experiment were derived from the Oak Ridge SPFCP strain, which is maintained by alternate matings of *spf*/+ ♀ × +/Y ♂ (22A stock) and +/+ ♀ (22A stock) × *spf*/Y ♂. At Madison, the viability of this stock was found to be poorer and the gene was consequently transferred to a C57BL6J background (see *Results*).

Mouse Diet. The high mortality rate among *spf*/Y mice prompted us to try rearing them on a low-protein diet to reduce possible hyperammonemia. We used Teklad low-protein diet, which contains 3.2% casein as supplied (Teklad Test Diets, Madison, Wisc.). Each 300 g of the diet was supplemented with 17.2 g of casein, to give a final protein concentration of 9%, and with 3.0 g of L-arginine, 1.8 g of L-asparagine, and 0.9 g of L-methionine (11). The dry diet was mixed with 300 ml of 1.5% agarose, which had been dissolved in boiling water and cooled to 45–50° before mixing. The diet was refrigerated and replaced daily in the mouse cages because it dried rapidly. Pairs consisting of an *spf*/Y male and a littermate female were transferred to the artificial diet at the age of 20–21 days. Sparse-fur males began to survive weaning after adoption of

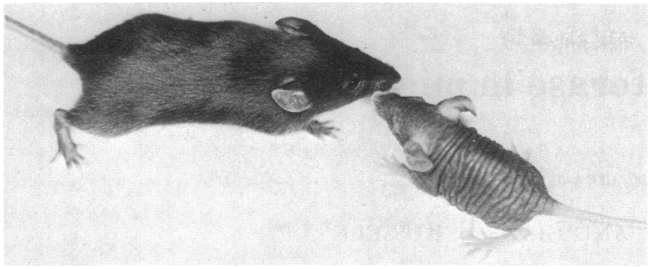

FIG. 1. A sparse-fur male and normal littermate at age 23 days.

the artificial diet but this may be coincidental and requires investigation.

Liver Extracts. Livers were removed from mice after light ether treatment followed by cervical dislocation and were then blotted dry and placed on ice. The livers were minced finely in a solution of 0.1% cetyltrimethylammonium bromide in 0.9% NaCl (2.0 ml of solution per 0.1 g of tissue). The cells were then disrupted with 12 strokes of a Potter-Elvehjem homogenizer and the extracts were spun at 16,000 rpm in a Sorval SS-34 rotor for 20 min. The pellet was discarded.

Ornithine Carbamoyltransferase Assays. We adapted procedures described in refs. 12–14 to our needs. Reaction mixtures (510 μl) contained 0.05 M maleate (pH 6.0–7.6) or 0.2 M triethanolamine (pH 8.0–10.0), 0.01 M L-ornithine, 10 μl of liver extract diluted 1:10 (*spf*$^+$) or 1:5 (*spf*) in water, and 0.01 M carbamoyl phosphate, which was freshly dissolved and added last. Reactions were stopped after 10 min at 37° by the addition of 1.0 ml of 5.0% trichloroacetic acid. A 0.5 ml aliquot was added to 0.5 ml of the following solution: H_2SO_4 (3 volumes), H_3PO_4 (3 volumes), water (4 volumes), and 35% (wt/wt) ferric ammonium sulfate (1 volumes). We then added 50 μl of a solution composed of equal volumes of 3% diacetyl monoxime and 8% antipyrine. The mixtures were mixed thoroughly and placed in a boiling-water bath for 20 min prior to determination of their absorbance at 465 nm. Enzyme-free blanks were processed through the entire procedure.

Paper Chromatography. Solutions (1 mg/ml) of hypoxanthine, xanthine, uric acid, orotic acid, and bladder stones from *spf*/Y mice were prepared in 0.1 M KOH. Samples containing 20 μg in 20 μl were applied to Whatman no. 1 paper and chromatographed (ascending) in isopropanol (65 volumes): water (18.4 volumes): HCl (16.6 volumes) for 22 hr (15) or in methanol (400 volumes): 90% formic acid (75 volumes): water (25 volumes) for 2.5 hr (16).

RESULTS

Sparse-Fur Mice. All but two of the mutant mice we have studied are descendants of three SPFCP strain heterozygous females from Oak Ridge. No sparse-fur males produced by mating these females with 22A males survived weaning in Madison. Therefore, the heterozygous females were mated with C57BL males in an attempt to obtain viable mutants. This approach was partially successful and the mutant allele is now maintained in Madison on a mostly C57BL genetic background. Thus far 15 out of 75 *spf*/Y males with this still variable background have survived weaning but only one has produced progeny.

The most obvious superficial characteristics of young *spf*/Y mice we have studied are small size, absence or relative paucity of fur, and wrinkled skin (Fig. 1). These traits are clearly evident by 5–7 days, but we have observed great diversity in subsequent development. Depending on genetic background, *spf*/Y mice may be almost fully furred and of normal size at weaning age, or may remain dwarfed and relatively hairless for several weeks after weaning. At Oak Ridge, several *spf*/*spf* and *spf*/O females have been observed to have a phenotype similar to *spf*/Y males of the same background. At Madison we have so far produced only one female with the sparse-fur phenotype (presumed *spf*/*spf*). She died when 10 days old but proved to have only abnormal ornithine carbamoyltransferase (see below), maintaining the, so far, invariable association of the sparse-fur phenotype and abnormal ornithine carbamoyltransferase.

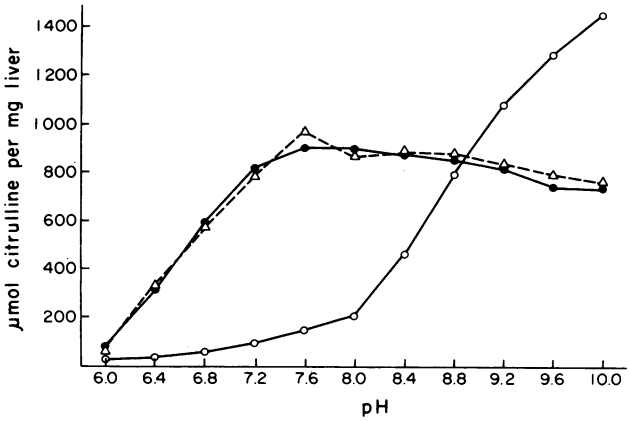

FIG. 2. The activity of liver ornithine carbamoyltransferase as a function of pH. Average of 20 normal males: ●—●; average of 10 normal females: Δ- - -Δ; average of 7 *spf*/Y males: ○—○. Buffers were: 0.05 M maleate for pH 6.0–7.6; 0.2 M triethanolamine for pH 8.0–10.0. Enzyme-free blanks were included over the entire range of pH.

Sparse-Fur Bladder Stones Consist Mostly of Orotic Acid. The bladder stones recovered from sparse-fur males at Oak Ridge were brownish-white and weighed 9–62 mg. Stones were powdered and recrystallized once from hot water. The UV absorption spectra in 0.01 M KOH and in 0.01 M HCl suggested that the main constituent of the bladder stones was neither uric acid nor xanthine but was probably orotic acid. The correctness of this hypothesis was supported by several additional kinds of evidence: (*i*) ***Melting points.*** Once-recrystallized orotic acid, which was white, became discolored at 331° and melted at 332.5°; once-recrystallized bladder stone material became discolored at 327° and melted at 331°. We regarded this as fairly good agreement since the recrystallized bladder stone material was still slightly colored. (*ii*) ***Chromatography.*** Bladder stone material chromatographed on Whatman no. 1 paper just as orotic acid did in isopropanol–HCl and in methanol–formic acid (***Materials and Methods***). (*iii*) ***Specific color reaction.*** Bladder stone material produced a colored product with the same absorption spectrum as that produced by orotic acid when the procedure of Rogers and Porter (17) was followed. Neither uric acid nor xanthine produced the colored product. (*iv*) ***Infrared absorption spectra*** were essentially similar for orotic acid and bladder stone material, both of which had been purified by chromatography with a Dowex-1 column (18).

The Ornithine Carbamoyltransferase Activity of Sparse-Fur Mice Varies Abnormally with Variation in pH and in the Concentration of Ornithine. The ornithine carbamoyltransferase activity of *spf*$^+$/Y males, *spf*$^+$/*spf*$^+$ females, and of *spf*$^+$/Y littermates of *spf*/Y males has maximum activity at pH 7.6–8.0 and has about 80% of maximum activity at pH 10.0. In contrast, the specific activity of the enzyme in sparse-fur male livers is only 22% of the normal value at pH 7.6–8.0 but is about twice the normal value at pH 10.0 (Fig. 2). The mutant activity does not increase further at pH 10.4–12.00 (not shown).

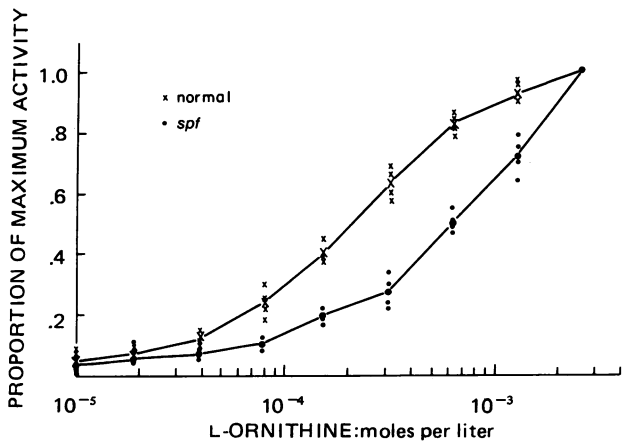

FIG. 3. The activity of liver ornithine carbamoyltransferase as a function of the concentration of L-ornithine in reaction mixtures containing 10^{-2} M carbamoyl phosphate at pH 8.4. Larger symbols represent the means.

Fig. 3 shows that the dependence of liver carbamoyltransferase activity on the concentration of L-ornithine is distinctly different in *spf*/Y and normal mice.

We did not detect significant differences between enzyme activities from *spf*/Y and *spf*$^+$/Y mice in preliminary studies of heat inactivation, inhibition by L-norleucine (14), and dependence on the concentration of carbamoyl phosphate. It is possible that a more sensitive procedure for determining activity at lower carbamoyl phosphate concentrations would reveal differences between normal and mutant enzymes that could contribute to the pathological consequences of having abnormal ornithine carbamoyltransferase. Even without this contribution, the summed reductions in activity resulting from abnormal pH and ornithine dependence cause a net deficiency of about 90% in *spf*/Y males at pH 7.4.

The Sparse-Fur and Abnormal Ornithine Carbamoyltransferase Phenotypes Have Been Inseparable, So Far, and May Be Different Expressions of the *spf* Allele on the X Chromosome. When the criterion of pH dependence was used, only normal enzyme was detected in nonsparse-fur males and females (two of each sex) of each of the following mouse strains obtained from R. Auerbach's colony: C3H, C57BL, BALB/c, CBA, AKR, TL, 129. This suggests that abnormal enzyme of the sort described herein is not a fairly common trait that has previously been overlooked and that can be expressed independently of the sparse-fur phenotype. So far, abnormal transferase has been observed only in descendants of the three *spf*/+ heterozygotes from Oak Ridge and in two *spf*/Y males provided by Dr. Douglas Grahn from the Argonne National Laboratory.

Nonsparse-fur females that were descended from the original *spf*/+ heterozygotes and that produced at least 10 sons, none of them with the sparse-fur phenotype, were judged to be *spf*$^+$/*spf*$^+$ homozygotes with high probability. Only normal ornithine carbamoyltransferase was observed in each of two such females and in all 10 nonsparse-fur daughters produced by one of them.

In contrast, each of the three *spf*/+ females from Oak Ridge and their female descendants that produced at least one sparse-fur male had a mixture of normal and abnormal ornithine carbamoyltransferase (Fig. 4). The unmistakable presence of abnormal enzyme was also detected in livers of four out of 10 daughters of a proven *spf*/+ heterozygote. Only abnormal activity was detected in the single phenotypically sparse-fur female produced at Madison by mating a *spf*/Y male and a *spf*/+ female.

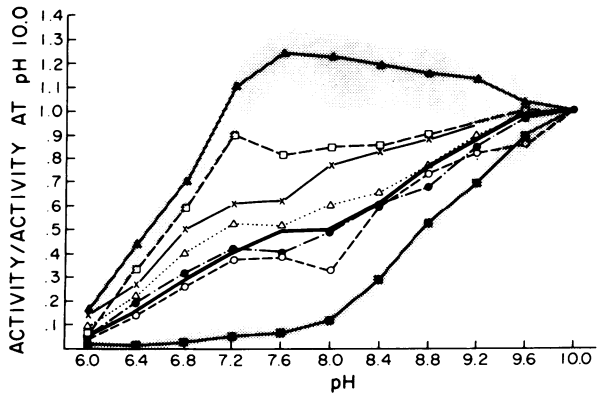

FIG. 4. The activity of liver ornithine carbamoyltransferase as a function of pH in *spf*/+ females. For each extract the activity at a given pH is expressed as a fraction of the activity of the same extract at pH 10.0. The average of 20 normal males and 10 normal (+/+) females: ▲—▲; the average of 7 *spf*/Y males: ■—■; 5 individual *spf*/+ heterozygous females: □- - -□, ×—×, △· · ·△, ●— · —●, ○- - -○. The curve for a sixth *spf*/+ female was determined after this figure was prepared and was not clearly different from ●— · —●. Shaded zones describe the ranges of variation. The bold continuous line without data points describes the behavior of a hypothetical mixture of equal amounts of normal and *spf* liver.

Mixtures of normal and abnormal enzyme were not detected in males. Each of 18 males born to 12 mothers and judged to have the sparse-fur phenotype had the same kind of abnormal enzyme. Abnormal carbamoyltransferase was not detected in 22 nonsparse-fur male littermates of sparse fur males.

These observations indicate that a locus determining the structure of ornithine carbamoyltransferase is located on the X chromosome in the mouse. If the locus is not *spf* itself, the results with male and female progeny produced so far by proven heterozygotes at the University of Wisconsin indicate a 99% probability that the locus is 11 map units or less distant from *spf*. However, the presence in both *spf*/Y males from the Argonne Laboratory of the same kind of abnormal enzyme as occurs in Madison *spf*/Y males makes it almost certain that the *spf* locus itself determines the structure of the abnormal enzyme. Although the *spf* stocks at Argonne and Oak Ridge are descended from the same original *spf* mutant, they have been maintained separately on different genetic backgrounds for many generations. Clinching evidence that the *spf* locus determines the structure of ornithine carbamoyltransferase would be provided by the demonstration of abnormal activity in *spf*^{ash} mice, which bear an independent mutation that is structurally and functionally allelic to the *spf* locus (19)‡.

Direct Evidence for Single-Allele-Expression of the Ornithine Carbamoyltransferase Locus in Heterozygous Cells Is Not Yet Available But the Relative Amounts of Normal and Abnormal Enzyme in Livers of Heterozygous Females Varies Greatly. Genes on the X chromosomes in somatic cells of female mammals exhibit single-allele-expression: the allele on the single active X (20) is expressed while alleles on the other, inactive, X are phenotypically repressed (21). We have attempted to determine if individual liver cells of heterozygous females are of two phenotypic classes, "normal" and "mutant",

‡ **Note Added in Proof.** We have found a second abnormal form of liver ornithine carbamoyltransferase in an *spf*^{ash}/Y mouse kindly provided by Dr. D. Doolittle of Purdue University. The specific activity is less than 5% of normal and is almost invariant in the pH range 6.0–10.0.

with regard to ornithine carbamoyltransferase activity. Our attempts to apply the Mizutani (22) procedure for the cytological demonstration of this activity to sections of liver prepared with the cryostat have been unsatisfactory: livers from mice that had only normal enzyme consistently yielded cryostat sections that contained groupings of negative as well as of positive cells. Therefore, the observation of groups of deficient cells in livers of heterozygous females would not be critical evidence for single-allele expression[§]. Some observations do suggest that the ornithine carbamoyltransferase locus is subject to single-active-X control. Fig. 4 displays activities as a function of pH in a special manner. For each liver extract, its activity at a given pH was plotted as a fraction of the activity of the same extract at pH 10 to display most clearly the activities of heterozygous females. The activities of males and females as a function of pH were apparently indistinguishable (Fig. 2). Therefore, the averaged values of the activity ratios for 20 normal males and 10 normal females are plotted in Fig. 4, and the range of variation of the activity ratios is displayed by a shaded zone. Similarly, the activity ratios for seven *spf*/Y males were averaged and plotted with their range of variation indicated by a shaded zone. The values for our sole *spf*/*spf* female were determined after preparation of Fig. 4, but fell within the range of variation for males.

Extracts from normal mice always had greater activity at pH 7.6–8.0 than at pH 10.0. In contrast, the activities of extracts from all five heterozygous females that we studied were higher at pH 10.0 than at pH 7.6–8.0, but the five curves differed significantly. There are two ways of interpreting the curves.

(*i*) Both ornithine carbamoyltransferase alleles are expressed in individual cells. The curves reflect the relative amounts of normal, mutant, and, possibly, mutant–normal hybrid enzymes [bovine ornithine carbamoyltransferase is a trimer (23)]. The variability in the curves for heterozygotes represents distinctly different degrees of expression of the normal and mutant alleles in cells of different livers. We can't interpret the curves in detail on this basis because we don't know the properties of the possible mutant–normal hybrid enzymes.

(*ii*) Only one ornithine carbamoyltransferase allele is expressed in individual cells. The curves reflect the relative numbers of cells expressing normal and abnormal enzymes. According to this interpretation, the curves depict a fairly large variance in these proportions, as has been observed in females heterozygous at other X-chromosomal loci that show single-allele-expression. The bold line without individual data points in Fig. 4 represents a hypothetical liver with equal numbers of cells expressing the normal and the mutant alleles. This standard indicates that one half or more of the liver cells in most *spf*/+ females produce normal ornithine carbamoyltransferase. The largest proportion of normal enzyme in an *spf*/+ liver depicted in Fig. 4 corresponds to about 78% of the cells producing normal carbamoyltransferase. The number of *spf*/+ females studied is small and females with larger proportions of abnormal enzyme than those shown may occur. Heterozygous livers with a great excess of phenotypically mutant cells could result in *spf*/+ females that have the *spf* phenotype. No such female has been definitely observed by us, but they have occasionally occurred at Oak Ridge and elsewhere. Some human females who are heterozygous for alleles causing ornithine carbamoyltransferase deficiency have pathological expressions of the enzymatic defect.

[§] **Note Added in Proof.** Ricciuti *et al.* (27) have detected two phenotypic classes of cells in the liver of a heterozygous human female by using the Mizutani procedure (22).

DISCUSSION

The retarded growth and reduced fitness of sparse-fur mice having a strain 22A background may be consequences of partial ornithine carbamoyltransferase deficiency. If so, the mice may provide a useful model for learning how to alleviate the serious, even lethal, effects of ornithine carbamoyltransferase deficiency in humans. For instance, blood ammonia concentrations, orotic acid concentrations, and growth rates as functions of diet and genetic background should now be studied.

The key to reducing the occurrence of new cases of this enzyme deficiency in humans is the identification of heterozygous females, a diagnosis that can then be followed by the avoidance of conception or by the early abortion of male conceptuses. Readily available *spf*/+ female mice may be valuable tools in the perfection of accurate, convenient methods for the detection of heterozygotes.

It was realized early that the single-active-X phenomenon provided an opportunity to determine if tumors were monoclonal in humans that were heterozygous for alleles of the *gpd* locus, since monoclonal tumors would express only one allelic form of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase, EC 1.1.1.49) or the other, whereas multiclonal tumors might express both forms of the enzyme (24). This approach has been fruitful (25, 26); its potential application to *spf*/+ mice is obvious, provided single-allele-expression of the locus for ornithine carbamoyltransferase can be demonstrated. It should then be possible to compare results obtained with spontaneous hepatomas, with tumors induced by different carcinogens, and with different genetic backgrounds.

The *spf* allele may be useful in studying several aspects of the regulation of ornithine carbamoyltransferase activity, including that imposed by the single-active-X control scheme. If the extrahepatic activities reported (10) are of the same sort as the activity present in liver, the same characteristic pH dependence we have demonstrated for liver extracts should be found in other tissues of sparse-fur mice. Our preliminary studies indicate this is true for ornithine carbamoyltransferase of the small intestine, but assay procedures more sensitive than ours are needed for studying other tissues. Finally, the ability to detect mixtures of normal and mutant enzymes should be useful in studying factors regulating the expression of the enzyme in hybridized somatic cells. For instance, it would be interesting to determine if factors that permit the expression of this enzyme in hepatocytes would result in the new expression of normal ornithine carbamoyltransferase in cell hybrids formed by fusing *spf*$^+$ fibroblasts and *spf* hepatocytes.

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