bodies by the lymphatic route is more speculative, but conventional therapy suggests several possibilities. Irradiation of regional nodes may improve the prognosis for patients with tumors spread predominantly through the lymphatics, for example in selected cases of Hodgkin's disease, non-Hodgkin's lymphoma, and seminomatous testicular carcinoma (*12*). Lymphatic flow might be distorted or blocked by large lymph node masses, but for occult metastases of such tumors, adjuvant therapy with antibodies delivered through the lymphatics could prove effective.

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12. See, for example, V. T. De Vita, Jr., S. Hellman, S. A. Rosenberg, *Cancer: Principles and Practice of Oncology* (Lippincott, Philadelphia, 1982).
13. We thank M. J. Talley and C. Botkin for valuable technical assistance; R. Blasberg, E. Owens, and M. Juhler for help with the autoradiography; K. A. Foon and M. I. Bernhard for advice and assistance with the L10 tumor and D3 antibodies; and G. Morstyn and E. Glatstein for critical reading of the manuscript.
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Biogenesis of Ornithine Transcarbamylase in *spf*^{ash} Mutant Mice: Two Cytoplasmic Precursors, One Mitochondrial Enzyme

Abstract. *Extracts of liver from hemizygous affected mice with the X-linked* spf^{ash} *mutation have 5 to 10 percent of normal ornithine transcarbamylase (OTC) activity, yet the homogeneous enzyme isolated from these extracts is identical to that in controls. The OTC messenger RNA from mutant livers programs the synthesis of two distinct OTC precursor polypeptides—one normal in size, the other distinctly elongated. Both precursors are imported and proteolytically processed by mitochondria, but only the normal one is assembled into active trimer. This novel phenotype may result from a mutation in the structural gene for OTC leading, primarily, to aberrant splicing of OTC messenger RNA and, secondarily, to formation of a structurally altered precursor whose posttranslational pathway is ultimately futile because its mature mitochondrial form is not capable of assembly and functional expression.*

Ornithine transcarbamylase (OTC; ornithine carbamoyltransferase; E.C. 2.1.3.3), a homotrimeric, mitochondrial matrix enzyme of the urea cycle in mammals, is of biological interest for several reasons. (i) Its structural locus is on the X chromosome and undergoes random inactivation in female cells (*1*); (ii) its subunit, of molecular size ~36 kD, is synthesized on free cytoplasmic polyribosomes as a larger precursor (~40 kD), designated pOTC (*2*), bearing an NH_2-terminal extension of amino acid residues (*3*), which is cleaved proteolytically concomitant with its posttranslational energy-dependent import by mitochondria (*4–6*); (iii) it is expressed almost exclusively in hepatocytes (*1*); and (iv) its inherited deficiency in man often produces lethal ammonia intoxication in affected males (*1*).

Our understanding of the nature and significance of mutations at the human OTC locus has been enhanced by the recent description of two X-linked, allelic OTC mutants in mice (*7*, *8*). One, designated *spf* (sparse fur) is almost certainly a point mutation, which affects the active site of OTC. When assayed in vitro, hepatic OTC activity in *spf* mice is reduced to ~20 percent of control at physiologic *p*H (*7*, *9*, *10*), affinity for

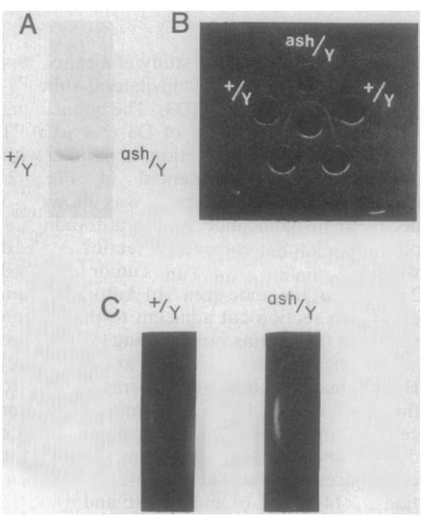

Fig. 1. Characteristics of homogeneous OTC from livers of +/Y and *spf*^{ash}/Y mice. (A) SDS-PAGE; enzymes (6 μg) were applied to SDS-polyacrylamide (10 percent) gel slabs after denaturation with 2-mercaptoethanol and SDS; the gel was stained with Coomassie blue. (B) Ouchterlony double diffusion in agar; 6 μl of rabbit antiserum to rat OTC was placed in the center well, and 1- to 2-μg of respective OTC's was added to the outer wells. (C) Immunoelectrophoresis; 4 μg of enzyme from +/Y or *spf*^{ash}/Y liver was placed in the wells; after electrophoresis, 200 μl of rabbit antiserum to rat OTC was placed in the channels and developed overnight.

ornithine is decreased (*7*, *9*), the *p*H optimum is shifted from *p*H 7.7 to *p*H 9.5 (*7*, *9*), and the amount of immunologically, cross-reacting material (CRM) is increased (*11*). In the other mutant, designated *spf*^{ush} (sparse fur–abnormal skin and hair), both hepatic OTC activity and CRM are decreased to 5 to 10 percent of control (*9*, *10*), and affinity for ornithine and for carbamyl phosphate are normal (*9*).

We now present evidence that hepatic OTC from *spf*^{ush} hemizygotes is identical to that in controls. Moreover, we show that this novel mutation results in the formation of a reduced amount of translatable OTC messenger RNA (mRNA) that codes for the synthesis of two distinct pOTC species. Finally, we demonstrate that both pOTC's are taken up and processed by mitochondria but only the wild-type product is assembled to active trimer.

Having established a mouse colony from breeding pairs (*spf*^{ush}/+ by +/Y) (*12*), we confirmed that nine phenotypically affected males (*spf*^{ush}/Y) had hepatic OTC activities (*13*), only 7.8 percent of that in 15 controls (mean ± 1 S.D. (standard deviation) = 5.7 ± 1.6 U/mg in *spf*^{ush}/Y; 73.2 ± 8.5 U/mg in +/Y littermates]. Ten heterozygous females had hepatic OTC activities averaging 55.7 percent that of controls (the mean ± 1 S.D. was 40.7 ± 22.1 U/mg). The livers of five affected males had a correspondingly reduced amount of CRM (the mean ± 1 S.D. was 5.8 ± 0.8 percent of control) when assessed by radial immunodiffusion in agar (*14*). Next, we compared a number of characteristics of OTC purified to homogeneity by affinity chromatography from livers of +/Y and *spf*^{ush}/Y mice (*15*). The enzymes were uniformly identical with regard to native molecular size (108 kD); subunit molecular size (36 kD) (Fig. 1A); final specific activity (227 to 235 U/mg); *p*I (at *p*H 7.55); *p*H optimum (7.8 to 8.0); and K_ms for ornithine (1.0 to 1.3 m*M*) and for carbamyl phosphate (1.2 to 1.3 m*M*). Furthermore, the purified OTC's gave a reaction of identity when studied by Ouchterlony double diffusion in agar (Fig. 1B), and were indistinguishable by immunoelectrophoresis (Fig. 1C) (*16*).

These findings indicate that the livers of *spf*^{ush}/Y mice contain a much reduced amount of normal OTC enzyme, clearly distinguishing this mutation from its *spf* allele, and likening the *spf*^{ush} phenotype to that observed in the human thalassemias (*17*). Accordingly, we examined OTC biogenesis. We prepared polysomal RNA's from livers of +/Y, *spf*/Y, and *spf*^{ush}/Y mice, translated them in vitro in a rabbit reticulocyte lysate system containing [^{35}S]methionine, immunoprecipitated the translation products with antiserum to OTC, and identified the labeled products by fluorography after sodium dodecyl sulfate–10 percent polyacrylamide gel electrophoresis (SDS-PAGE) (*5*) (Fig. 2). Whereas *spf*/Y RNA (lane 2) programmed the synthesis of a single pOTC indistinguishable in size from that observed with +/Y RNA (lane 1), two prominent differences were noted with RNA from *spf*^{ush}/Y mice. First, the *spf*^{ush} RNA directed the formation of two distinct pOTC's (lanes 4 and 6), one identical in size to that seen with control RNA (lanes 3 and 5), the other at least 1 kD larger (compare lanes 5 and 6). Estimation of the relative amounts of these pOTC's by densitometry indicated a nearly constant ratio between the elongated and the normal one of 2:3. Second, when estimated by densitometry, RNA from *spf*^{ush}/Y mice programmed the synthesis of only 15 to 20 percent as much total pOTC as observed with an equivalent amount of +/Y RNA (compare lanes 3 and 4) (*18*). Each of these differences was observed with numerous RNA preparations from different animals (six +/Y livers; nine *spf*^{ush}/Y livers). Immunoprecipitations with control rabbit serum or in the presence of an excess of unlabeled OTC confirmed that both pOTC species programmed by *spf*^{ush}/Y RNA were OTC-specific. Because varying the duration of translation did not change the quantitative relationship between the two pOTC's, it is clear that the larger pOTC is not a precursor of the smaller one. Moreover, both precursors were observed when intact liver slices from *spf*^{ush}/Y mice were labeled with [^{35}S]methionine for 30 minutes prior to immunoprecipitation and SDS-PAGE (Fig. 2, lane 8), in contrast to the single pOTC noted in control slices (lane 7).

Next, we used the cell-free system to study posttranslational uptake and processing of the pOTC's by intact hepatic mitochondria (*5*) (Fig. 3). The pOTC translated by RNA from *spf*/Y mice (lane 2) was processed by mitochondria identically to pOTC from +/Y littermates (lane 1). In each case, most pOTC was con-

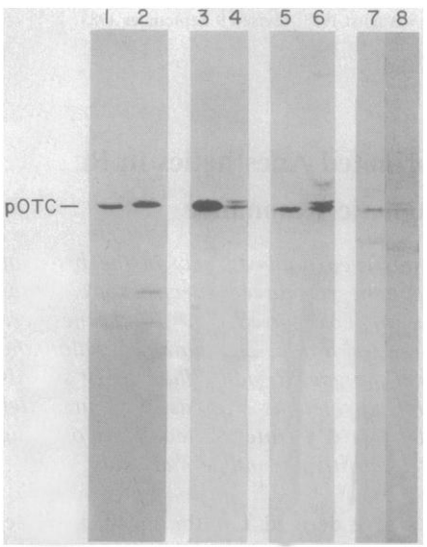

Fig. 2. Fluorograms of products of in vitro translated hepatic RNA's from +/Y, *spf*/Y, and *spf*^{ush}/Y mice. In lanes 1 to 6, cell-free protein synthesis with [^{35}S]methionine, immunoprecipitation, and SDS-PAGE were carried out as described (*5*). (Lane 1) +/Y littermate of *spf*/Y; (lane 2) *spf*/Y; (lanes 3 and 5) +/Y littermate of *spf*^{ush}/Y; (lanes 4 and 6) *spf*^{ush}/Y. In lanes 1 to 5, 7 μg of RNA was used to program the translation system; in lane 6, 42 μg of RNA was used. In lanes 7 and 8, intact liver slices were labeled with [^{35}S]methionine for 30 minutes, after which they were homogenized and centrifuged (17,000*g*; 5 minutes); the supernatants were then used for immunoprecipitation and SDS-PAGE; (lane 7), +/Y liver; (lane 8) *spf*^{ush}/Y liver. Duration of exposure was 18 hours for lanes 1 and 2; 120 hours for lanes 3 and 4; 13 hours for lanes 5 and 6; 30 days for lanes 7 and 8; pOTC, OTC precursor.

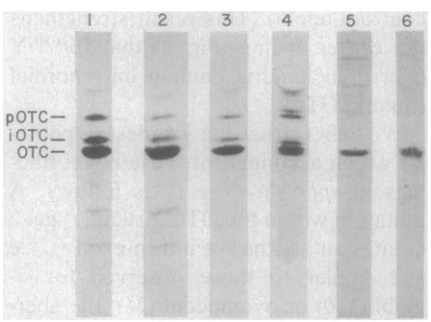

Fig. 3. Products formed after posttranslational processing of OTC precursors from control and mutant mice by intact mouse liver mitochondria. The conditions for cell-free protein synthesis, posttranslational incubation with mitochondria, immunoprecipitation, and SDS-PAGE used in lanes 1 to 4, have been described (*5*). (Lane 1) +/Y littermate of *spf*/Y; (lane 2) *spf*/Y; (lane 3) +/Y littermate of *spf*^{ush}/Y; (lane 4) *spf*^{ush}/Y. The volume of translation mixture used for processing was adjusted such that the number of disintegrations per minute in labeled pOTC was approximately equal in each pair of littermates. In lanes 5 and 6, products formed during posttranslational processing of control (lane 5) and *spf*^{ush}/Y (lane 6) pOTC's were treated with Triton X-100 (final concentration, 0.5 percent) at 4°C for 30 minutes, diluted to 10 ml with 10 m*M* Hepes buffer, *p*H 7.4, applied to an affinity column containing δ-*N*-(phosphonacetyl)-L-ornithine, and eluted with carbamyl phosphate (*15*). Samples were then concentrated, lyophilized, and analyzed by SDS-PAGE without immunoprecipitation. For +/Y control, 75 μg of RNA was used to program the translation mixture; for *spf*^{ush}/Y mutant, 230 μg of RNA was used. pOTC, OTC precursor; iOTC, intermediate-sized OTC species; OTC, mature OTC subunit.

verted to a polypeptide identical in size to the mature subunit (denoted OTC); a small portion was found as an intermediate-sized species (iOTC) whose precise physiologic significance remains unclear (*3–6*). When pOTC's programmed by spf^{ash}/Y RNA were processed, a far more complex pattern was observed (lane 4). Six distinct bands were noted, two each corresponding to mutant and normal pOTC's, iOTC's, and OTC's. These results were obtained with mitochondria from either control or mutant mice. Both mature-sized OTC species were found in mitochondrial fractions prepared from [^{35}S]methionine-labeled spf^{ash}/Y liver slices (not shown).

Finally, we asked whether both mature-sized OTC species observed with spf^{ash}/Y RNA can be assembled to trimeric form. After cell-free translation and mitochondrial processing, soluble products were applied to and eluted from a ligand affinity column (Fig. 3, legend) which, we have shown recently, binds only trimeric OTC composed of mature subunits (*19*). Significantly, only a single species was isolated from the spf^{ash}/Y programmed mixture (Fig. 3, lane 6), identical in mobility to that noted for the control (lane 5). This result strengthens our earlier finding (Fig. 1) that spf^{ash}/Y liver mitochondria contain only normal trimeric OTC.

We believe that the simplest hypothesis which accounts for these novel findings in spf^{ash}/Y mice is as follows. A mutation within the OTC structural gene creates an alternative intron-exon splice site similar to those observed for β-globin (*20*) or ovomucoid (*21*); the aberrant nuclear processing of pre-mRNA resulting therefrom leads to the formation of two distinct and translatable mRNA's; each mRNA directs the synthesis of a specific pOTC—one normal in size, the other elongated; both pOTC's are imported and processed by mitochondria but only the wild-type subunit is assembled to active trimeric enzyme. We cannot, however, exclude the possibility that the processed, mutant subunit undergoes assembly to homotrimeric, catalytically inactive enzyme that does not bind to the affinity ligand. Because we have recently succeeded in isolating a rat OTC complementary DNA (*22*), it should soon be possible to explore the mutation directly by analyzing mRNA and genomic DNA from spf^{ash}/Y mice.

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18. We recognize that this result is an accurate quantitative reflection of translatable OTC mRNA only if the mutant and control RNA's and pOTC's are equally stable, and if the pOTC's have the same affinity for our antiserum to OTC.
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Noninvasive Observations of Fluorinated Anesthetics in Rabbit Brain by Fluorine-19 Nuclear Magnetic Resonance

Abstract. *Fluorinated anesthetics were observed noninvasively in the brain of intact rabbits with fluorine-19 nuclear magnetic resonance spectroscopy. High-resolution fluorine-19 spectra of halothane, methoxyflurane, and isoflurane were obtained with a surface coil centered over the calvarium. Elimination of halothane from the brain was also monitored by this technique. Residual fluorine-19 signals from halothane (or a metabolite) could be detected as long as 98 hours after termination of anesthesia. These observations demonstrate the feasibility of using this technique to study the fate of fluorinated anesthetics in live mammals.*

It is generally believed that anesthesia is produced when an appropriate partial pressure of a volatile anesthetic agent is reached in the brain (*1*). Since only indirect methods have been available to study this phenomenon, information on the uptake and distribution of these agents in the brain has been extremely limited. Morever, there is no information on the environments anesthetics occupy in the brain or on their residence times in this organ. Fluorinated hydrocarbons and ethers are among the most commonly used inhalation anesthetic agents. We used ^{19}F nuclear magnetic resonance (NMR) spectroscopy to observe fluorinated anesthetic agents in the brain of a live mammal and monitored its uptake and elimination. In this technique the ^{19}F signals derived from the anesthetic molecule itself are used to detect its presence and to assess the environment in which the molecule resides. The approach is noninvasive, since ^{19}F nuclei of the anesthetic molecule serve as the probe used to monitor the anesthetic-brain interaction.

We followed the incorporation of three different anesthetic agents into the rabbit brain (*2*): halothane (CF_3-CHBrCl), methoxyflurane ($CHCl_2$-CF_2-O-CH_3), and isoflurane (CF_3-CHCl-O-CF_2H). New Zealand White rabbits (3 to 4 kg) were subcutaneously injected with a combination of ketamine and xylazine, intubated (*3*), and positioned in the bore of the magnet. Each anesthetic agent was delivered with a "nonrebreathing" system