Ectopic Correction of Ornithine Transcarbamylase Deficiency in Sparse Fur Mice*

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Stephen N. Jones§§, Markus Grompe‡, M. Idrees Munir®, Gabor Veres‡‡, William J. Craigen‡, and C. Thomas Caskey§§

From the 1Institute for Molecular Genetics and 4Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas 77030 and the 2Hungarian Academy of Science, Biological Research Center, Institute of Genetics, P. O. Box 521, 6701 Szeged, Hungary

The sparse fur (spf) mutant mouse is a model for human X-linked ornithine transcarbamylase (OTC) deficiency. Human OTC cDNA placed under transcriptional control of the mouse OTC promoter was microinjected into fertilized oocytes of spf mice. Two founder lines of transgenic mice were phenotypically and biochemically corrected for OTC deficiency by the expression of the human gene at high levels in the small intestine with little or no expression occurring in the liver. The tissue pattern of expression of transgenic mice bearing the chloramphenicol acetyltransferase gene placed under the control of the mouse OTC promoter parallels these results. These experiments demonstrate that human OTC cDNA is selectively expressed in small bowel by a truncated OTC promoter, and such ectopic expression corrects the spf phenotypic and metabolic features of this inborn error. These data suggest that somatic gene therapy of OTC deficiency can be achieved by intestine-targeted gene transfer.

Ornithine transcarbamylase (OTC)1 (carbamoyl-phosphate: l-ornithine carbamoyltransferase, EC 2.1.3.3) catalyzes the condensation of ornithine and carbamyl phosphate to form citrulline (1). OTC is expressed primarily in liver, where it participates in the urea cycle and to a lesser extent in the mucosal layer of the small bowel where it has been proposed to aid in arginine economy (2). The enzyme assumes a homotrimeric structure in the mitochondrial matrix to form the active enzyme (3-5). The gene encoding OTC is located on the X chromosome in both man and mouse and is subject to random X inactivation (6). Some heterozygous females suffer episodic hyperammonemia, mental retardation, and possibly death in hyperammonemic coma (7). Treatment for OTC deficiency includes dietary restriction of protein intake coupled with the administration of citrulline and sodium benzoate, sodium phenylacetate, or sodium phenylbutyrate to provide alternate pathways for nitrogen excretion. Despite therapy, minor illnesses associated with protein catabolism can still lead to life-threatening hyperammonemnic episodes for these patients (8). For these reasons we seek to develop gene transfer methods for treating OTC deficiency and have elected to use the mouse mutant spf toward this goal.

The spf mouse OTC mutation results in a marked decrease in enzyme activity at physiologic pH, altered substrate affinity, and a change in pH optima from pH 7.7 to pH 9.5 (9). We have previously identified the mutation responsible for spf and found it to be a single base pair substitution which replaces a histidine residue with an asparagine residue at amino acid 117 (10). The spf mutation induces elevated blood ammonia levels, orotic aciduria, retarded growth, and an abnormal, sparse fur phenotype. A second OTC mutant mouse, sparse fur-abnormal skin and hair (spf-ash), is also available for study (11) but was not used in this investigation.

The spf mouse offers an attractive opportunity to develop and test gene therapy strategies since the genetic basis of the defect is known and the biochemistry of the system is well defined. As a first step toward somatic gene therapy, human OTC coding sequences were introduced into the germ line of fertilized spf oocytes via microinjection to determine if gene transfer would succeed in correcting the inborn error in the resulting transgenic mice. We have used in our studies the human OTC cDNA, placed under transcriptional control of 750 bp of mouse OTC 5'-flanking sequences. This promoter fragment has been shown to induce expression of a reporter gene in hepatoma cell lines and contains several sequence motifs which bind to proteins extracted from murine hepatocytes (12, 13). We report the establishment of two founder lines of transgenic spf mice which are corrected for OTC deficiency and describe the pattern of tissue-specific expression induced by the OTC promoter fragment in these mice. In addition, a 4-kbp fragment of the mouse OTC promoter region coupled to the chloramphenicol acetyltransferase (CAT) reporter gene was microinjected into non-mutant mouse oocytes to create two founder lines of transgenic mice. The tissue-specific pattern of bacterial CAT gene expression and human OTC cDNA expression in the transgenic mice are similar.

**EXPERIMENTAL PROCEDURES**

Transgene Constructions and Microinjections—Human OTC cDNA sequence bearing terminal EcoRI linkers was cloned into the EcoRI site of pUC8 to create pHOTC (14). An EcoRI-BamHI frag-

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§ To whom correspondence should be addressed.

1 The abbreviations used are: OTC, ornithine transcarbamylase; CAT, chloramphenicol acetyltransferase; bp, base pair(s); kbp, kilobase pair(s); SDS, sodium dodecyl sulfate; MOPS, 4-morpholinoresponsepropanesulfonic acid.
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Tissues were harvested from transgenic and non-transgenic mice following procedures described by Quereshi et al. (24). The tissues were polytron homogenized on ice in 0.25 M Tris-HCl, pH 7.7, and subjected to three rapid freeze-thaw cycles. Cell debris was removed via centrifugation at 19,000 rpm for 15 min at 4°C in an Eppendorf microcentrifuge. Supernatant was recovered and assayed for protein concentration (25). OTC activity in the tissue extracts was measured using a biochemical assay based upon the conversion of [14C] ornithine to [14C] citrulline. The reaction mix contained 250 mM Tris-HCl, pH 7.7, 0.15 μCi of [14C] ornithine (54.0 mCi/mmol), 2.5 mM uridine, 2.5 mM carbamyl phosphate, and 100–200 μg of protein extract in a final volume of 100 μl. The samples were incubated at 37°C for 30–60 min, and the reaction halted by the addition of 10 μl of 10% TCA. The reaction mixture was spotted onto a silica gel plate, and thin layer chromatography was performed using a chloroform, methanol, 17% ammonia solvent (40:40:20). Spots revealed on the plastic-backed thin layer chromatography plates by autoradiography were cut out and radioactivity determined by scintillation spectrophotometry. The reaction kinetics for this assay were examined and found to be linear over the range of substrate concentrations and time used in the experiment.

Orotic Acid Determination—Mouse urine was collected twice daily for 4 days starting on day 10 after birth from three phenotypically corrected 5F2 transgenic pups and from three phenotypically corrected 4F2 transgenic pups by placing the mice on Parafilm and gently stroking the abdomenal region. Urine was collected in a similar fashion from 6 C57/B6/C5H wild-type and 8 spf pups of the same age and receiving the same diet. Urinary orotic acid levels were measured in duplicate for each sample according to Stajner et al. (26). Creatinine levels were determined by the Jaffe method (27).

Assay of CAT Activity—Protein extracts were prepared from various tissues as described above. CAT assays were performed as described by Gorman et al. (16) with the following modifications. The extracts were heated at 60°C for 5 min, rapidly cooled, and the protein concentration determined (25). The reaction mix contained 250 mM Tris-HCl, pH 7.7, 0.2 μCi of [14C] chloramphenicol (60.0 μCi/mmol), 2 mM acetyl-CoA, and 5 μg of protein extract in a final volume of 100 μl. The mixture was incubated at 37°C for 30 min, and the reaction terminated by extracting with 1 ml of toluene. The ethyl acetate was evaporated, the pellets redissolved in 10 μl of ethyl acetate, and the reaction products analyzed by silica gel thin layer chromatography. Following autoradiography, the reaction products were quantitated by scintillography of the silica gel sections. The reaction kinetics for this assay are linear up to 50% conversion of substrate to products.

RESULTS

Creation of Transgenic spf Mice—Female mice homozygous for spf were mated with hemizygous spf males. 120 single cell embryos from each were injected with 500 copies of the 750-OTCSV construct. 60 embryos were transferred into 4 host mice (ICR strain). This experiment yielded 21 pups, 19 of which survived the newborn period. Offspring were maintained on normal diet and provided water supplemented with 7 mM sodium benzoate and 4 mM citrulline to ensure survival of any partially corrected or noncorrected mice. Two pups exhibited a striking phenotypic conversion to wild type like fur and body weight by day 9 (Fig. 1). Southern analysis of genomic DNA isolated from tail cuts revealed that the two mice with the phenotypic conversion bore the 750-OTCSV transgene. No other mice were found to have integrated the 750 OTCSV construct. 60 embryos were transferred into 4 host mice (ICR strain). This experiment yielded 21 pups, 19 of which survived the newborn period. Offspring were maintained on normal diet and provided water supplemented with 7 mM sodium benzoate and 4 mM citrulline to ensure survival of any partially corrected or noncorrected mice. Two pups exhibited a striking phenotypic conversion to wild type like fur and body weight by day 9 (Fig. 1). Southern analysis of genomic DNA isolated from tail cuts revealed that the two mice with the phenotypic conversion bore the 750-OTCSV transgene. No other mice were found to have integrated the 750 OTCSV construct into their genome. Allele-specific oligonucleotide hybridization experiments confirmed that the phenotypically converted mice possessed the spf allele (data not shown).

The two transgenic founder mice, F05 and F04, were mated with homozygous spf mice to establish F1 generations of each line. Southern analysis of genomic DNA from F05 probed with a fragment of the mouse OTC promoter present in the 750-OTCSV transgene determined that the transgene was inserted at a single site into the genome in a classical multi-copy, head to tail fashion. An example F1 offspring of F05 which inherited this insertion is presented in Fig. 2. Southern
analysis of genomic DNA from F04 and subsequent F1 mice revealed three sites of transgene insertions into the genome of founder mouse F04. Insertion a is composed of a low copy number, head to tail arrangement of the transgene. Insertion b contains to a high copy number, head to tail transgene concatenator with several copies of a head to head transgene arrangement included within this site. An example offspring bearing this event (F14-9) is shown in Fig. 2. Insertion c is a single copy integration of the transgene. F14-14 contains this insertion as well as insertion a described above.

Southern analysis performed on the offspring of F05 suggests that the 750-OTCSV transgene was inherited in a Mendelian fashion in the OTC(5) line and is required for phenotypic conversion (Fig. 3). Analysis of F1 progeny of F04 revealed that the three insertion elements were passed to the OTC(4) offspring in an unlinked manner. Phenotypic conversion from spf to wild type was observed only for those F04 progeny possessing the high copy number transgene insertion b suggesting that it is this insertion event which is responsible for correcting the inborn error in these mice.

Tissue-specific Expression of the Transgene—Total RNA was isolated from nine different tissues harvested from F1 progeny of F05 and F04. The results of Northern analysis of steady-state levels of RNA from a variety of tissues employing DNA sequences of the SV40 3′-coding portion of the transgene as a probe is presented in Fig. 4. The findings indicate that F15-9 and F14-9 (insertion b) expressed the 750-OTCSV transgene at high levels in the small intestine and at very low levels in the liver. Both F1 mice exhibited phenotypic conversion to wildtype. Analysis of RNA extracted from F14-14 which possessed both the a and c transgene insertions and which retained the spf phenotype failed to detect expression of the transgene in any tissue. Similar results were obtained for F14-10 which was negative for transgene insertion. The detected message migrated slightly in front of the 18 S ribosomal band in the 1% agarose-formaldehyde gel, consistent with the expected size (1900 bp) of a transcript produced from the 750-OTCSV transgene. Ethidium bromide staining of the gel prior to transfer of the RNA revealed intact RNA of equal amounts in all tissues (data not shown).

OTC Activity in Tissues of Corrected Mice—The level of OTC activity present in the liver and small bowel of OTC(5) mice was examined to determine if the large amount of transgene expression occurring in the small intestine of corrected spf mice correlates with an increase in OTC enzyme activity. Transgenic mouse 5F12 was mated with a homozygous spf female to establish F2 generation offspring for the F05 line of corrected mice. Liver and small bowel mucosal cells were harvested from three 14-day-old 5F2 corrected mice and from three non-transgenic spf and C57B6/C3H mice of similar age. The C57B6/C3H hybrid strain is the background strain of the spf mice and was used as wild-type controls in these experiments to avoid any strain-specific differences which may exist in levels of tissue-specific OTC activity.

Tissue extracts were prepared and assayed for OTC activity using a radiochemical assay based upon the conversion of [14C]ornithine to citrulline ("Experimental Procedures"). No significant difference in the level of hepatic OTC activity was found between transgenic spf mice and non-transgenic spf mice (Table I). Hepatic OTC activity in both sets of mice was reduced compared with wild-type levels, consistent with previous reports that spf mice possess approximately 20% of the level of wild-type hepatic OTC activity (9, 24). No significant difference in OTC activity was recorded for wild-type and spf intestinal mucosa. A 17-fold increase in intestinal OTC activity was measured in OTC(5)F2 mice relative to non-transgenic spf mice. Thus, the large amount of steady-state transgene message present in the small intestine of these animals corresponds with a large increase in intestinal OTC activity.

Orotic Acid Levels in Corrected spf Mice—A pronounced orotic aciduria is one of the hallmarks of OTC deficiency both in man and in the spf mouse. A decrease in OTC activity results in an accumulation of the substrate carbamyl phosphate. When the carbamyl phosphate present in the mitochondria reaches high levels the substrate exits the organelle and enters the pyrimidine biosynthesis pathway (28). This induces urinary orotic aciduria which in adult spf mice approaches levels 15-20 times greater than that of normal mice (29).

Measurements of the levels of orotic acid present in urine specimens of phenotypically corrected spf mice were performed in an effort to determine if the transgenic spf mice...
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were biochemically corrected for OTC deficiency. Transgenic spf males F15-2 and F14-12 were mated with homozygous spf male mice and homozygous spf female mice, respectively. Southern analysis of F05 progeny confirmed that inheritance of the transgene conferred phenotypic conversion. Only those offspring of F04 possessing transgene insertion b were found to be phenotypically corrected. Several litters of F2 mice from this line have been similarly examined and confirm this finding (data not shown).

Fig. 3. F1 generation established from transgenic mice. F05 and F04 were mated with hemizygous spf male mice and homozygous spf female mice, respectively. Both breeding pairs were placed on nonsupplemented water at the time of pairing since sodium benzoate would likely reduce the amount of excess nitrogen present in the bloodstream of the offspring thereby decreasing the level of carbamyl phosphate. Urine was collected twice daily for 4 days starting on day 10 after birth from three phenotypically corrected F2 transgenic pups and from three phenotypically corrected F2 transgenic pups. Urine was also collected in a similar fashion from C57B6/C3H wild-type and spf pups of the same age and diet. The results of this experiment reveal that the transgenic spf mice possess urinary orotic acid values similar to those of wild-type mice and are greatly reduced relative to the levels obtained with non-transgenic spf mice (Fig. 5), indicating that both lines of transgenic spf mice are metabolically corrected for OTC deficiency.

Tissue Pattern of CAT Expression in Transgenic Mice—To determine whether the tissue pattern of human OTC expression in the spf mice was due to positional effects of transgene integration into the host genome, we created non-mutant, transgenic mice which harbored the bacterial CAT reporter gene placed under transcriptional control of a mouse OTC promoter fragment containing 750 bp of 5'-flanking sequence plus an additional 3250 bp of upstream DNA sequence. Two founder lines of transgenic mice (CS/ICR strain) were generated. Southern blot analysis of genomic DNA isolated from the two founder mice (F04 and F06) revealed each founder to possess multiple copies of the 4KCAT transgene in a concatenated fashion at a single site of insertion within the recipient's genome. Transmission frequencies of the transloci to the respective offspring of these animals was consistent with a single integration site (data not shown). F1 generation offspring heterozygous for the transloci to the respective offspring of these animals was consistent with a single integration site (data not shown). F1 generation offspring heterozygous for the transloci to the respective offspring of these animals was consistent with a single integration site (data not shown). F1 generation offspring heterozygous for the transloci to the respective offspring of these animals was consistent with a single integration site (data not shown).
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FIG. 4. Northern RNA analysis of transgene expression in F1 mice. Five μg of total cellular RNA isolated from various tissues were electrophoresed through a 1% formaldehyde-MOPS agarose gel and transferred onto nylon membrane. A portion of the SV40 3'-transcribed region (BglI-BamHI fragment) used in construction of the transgene was radiolabeled with [32P]dCTP to yield a probe specific for the transgene message. The filter was autoradiographed for 5 days at -70 °C with an intensifying screen to permit visualization of the trace amounts of liver transgene expression in the corrected mice.

FIG. 5. Urinary orotic acid levels in transgenic and non-transgenic mice. Urine was collected from phenotypically corrected OTC(4)F2 and OTC(5)F2 pups and from wild-type (wt.) and spf pups of equal age. Solid and stippled bars represent the mean values of urinary orotate documented for wild-type mice and mice bearing the spf allele, respectively. Results are expressed as micromoles of orotic acid/milligram of creatinine with bars representing SD. Number of mice sampled (n) is shown at bottom. F2 offspring of both founder lines of corrected mice possess reduced levels of urinary orotate relative to the value recorded for non-transgenic spf mice.

TABLE I

| Protein extracts were prepared from liver and small bowel mucosal epithelium of three wild-type, sparse fur, and transgenic OTC(5)F2 mice, and assayed for OTC activity as described under “Experimental Procedures.” Mean values and SD are expressed as picomoles of radiolabeled citrulline formed/milligram of protein in 1 h. |
|---|---|---|
| Liver | Small bowel |
| Wild-type | 74,882 ± 4,860 | 76 ± 25 |
| Sparse fur | 11,886 ± 2,692 | 136 ± 101 |
| OTC(5)F2 | 9,296 ± 3,708 | 2,380 ± 1,505 |

of CAT assays performed on tissue extracts from F14-3 and F16-1 reveal that the two lines of mice express the reporter gene at high levels in the small bowel with modest or no transgene expression occurring in the liver (Fig. 6). Transgene expression was not detected in any other tissue examined. This pattern of expression mimics the pattern of OTC cDNA expression in the transgenic spf mice and suggests that positional effect of transgene insertion is not responsible for the tissue pattern of transgene expression observed in these mice.

DISCUSSION

We have shown that transfer and expression of DNA complimentary to the human OTC gene succeeds in correcting OTC deficiency in spf mice. Two founder lines of transgenic spf mice were established which were phenotypically corrected for spf. These founder mice and their respective progeny were also approximately one-third greater in body weight and were capable of breeding much sooner than non-transgenic littermates. Measurement of urinary orotic acid levels, a standard indicator for OTC deficiency, confirmed that the two lines of mice were biochemically corrected for OTC deficiency.

Expression of the urea cycle enzyme ornithine transcarbamylase has been shown to be restricted to the liver and, to a lesser extent, the mucosal layer of the small bowel (30, 31). Previous studies have reported extreme variations in the level of intestinal OTC activity in rodents, ranging from 0.02 to 10% of that of the liver (31-33), while the intestinal OTC mRNA concentration is approximately 50% of that of the liver (30). The reason for the low level of OTC activity present in the intestinal epithelium in light of the relatively large amount of steady-state OTC message present in this tissue is not clear. Hepatic OTC has been calculated to possess a half-life of 6-9 days (34). Since the epithelial mucosa in rodents has a life span of approximately 5 days, it has been suggested that OTC is synthesized and degraded at a much higher rate in this tissue than in liver and therefore requires a relatively higher level of message production to maintain even low levels of OTC enzyme activity (30).

The tissue pattern of transgene expression in the two lines of corrected spf mice reflects the endogenous tissue pattern of OTC gene expression. The tissue specificity of CAT gene expression in the non-mutant, transgenic animals also parallels the tissue restricted pattern of expression of the endogenous OTC gene, indicating that tissue specificity of OTC expression is conferred by the DNA sequences adjacent to the structural gene. The relative levels of transgene expression detected in the liver and intestine in both sets of transgenic
mice do not reflect the relative levels of OTC activity found in these tissues in wild-type mice, suggesting that additional DNA elements are required for the quantitatively correct expression of OTC in the liver. Alternatively, the large amount of CAT activity found in the intestinal mucosa of the 4KCAT transgenic mice may reflect dissimilarities between the rates of protein turnover for the bacterial CAT protein and murine OTC protein. However, the human OTC enzyme is very closely related to the mouse OTC enzyme in structure and function (34–37). Thus, it is unlikely that different kinetics of protein turnover are responsible for the elevated level of OTC activity found in the intestine of the transgenic spf mice.

Cavard and co-workers have performed microinjection experiments on a second mouse model of OTC deficiency, spf-ash, using an SV40 promoter-driven copy of the rat OTC cDNA and obtained one line of transgenic mice corrected for the spf-ash-induced defect (38). Northern blot analysis of various tissues from the corrected spf-ash mice indicated that the SV40 promoter induced OTC transgene expression only in the liver (50% of normal value), and liver OTC enzyme activity was reported to be 80% of the level of activity documented for control mice. The authors proposed that expression of the rat OTC cDNA in the liver of these mice was responsible for correcting the spf-ash OTC deficiency and suggested that regulatory sequences contained in the rat OTC cDNA acting jointly with the SV40 promoter resulted in proper tissue specificity of transgene expression.

The similarity in the tissue pattern of transgene expression in the 4KCAT and 750-OTCSV mice used in our study suggests that tissue specificity is controlled by the promoter region in these mice and is not a function of transgene integration site or of regulatory signals which may exist within the expressed sequences. The results of our study further suggest that expression of human cDNA in the small bowel of spf mice will correct the inborn error.

The role of OTC in the liver is to provide citrulline thereby cycling nitrogen into urea. In contrast to the liver, the intestinal epithelium does not possess a complete urea cycle. Citrulline is synthesized in the small bowel, via carbamyl phosphate synthetase and OTC, at a rate approximately one-twentieth of the hepatic rate and is considered to be the end product of ammonia metabolism in the small intestine, acting primarily as a nitrogen sink for glutamine, the major respiratory fuel of the bowel (39, 40). Citrulline synthesized in the small intestine is released into the circulation and taken up by peripheral tissues, predominately the kidneys, where it is converted into arginine (41). Our findings indicate that a large increase in intestinal mucosa OTC activity will facilitate conversion of enough glutamine to relieve the hyperammonemia associated with the inborn error in spf mice, thereby correcting the phenotype. In addition, increased conversion of ornithine to citrulline induced by the exogenous OTC utilizes the excess carbamyl phosphate present in the bowel thereby reducing urinary orotic acid levels.

The ectopic correction of the inborn error in transgenic spf mice suggests that somatic gene therapy of OTC deficiency may be achieved by intestine-targeted gene transfer. The ease with which the small bowel may be accessed and the susceptibility of this tissue to viral infection provokes further interest in the intestine as a target for somatic gene therapy of OTC deficiency.

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