Investigative Ophthalmology & Visual Science

Articles

Gene Transfer and Expression of Human Ornithine Aminotransferase
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A generalized deficiency in the mitochondrial enzyme, ornithine aminotransferase (OAT), is present in the hereditary blinding disease, gyrate atrophy (GA). Because the OAT gene is a multi-gene family, and the native OAT enzyme is an oligomer, it would be important to identify the gene locus actually responsible for the OAT activity and affected in GA. A mammalian expression clone containing a previously characterized human OAT cDNA (pHOAT), corresponding to the OAT gene on chromosome 10, was prepared (pcDHOAT), transfected, and tested for expression in NIH3T3 cells and OAT(−) Chinese hamster ovary (CHO) cells. Incorporation of pcDHOAT and synthesis of human OAT mRNAs and active enzyme were demonstrated in both cell types, confirming the completeness of the coding sequence of pHOAT. The results indicated that the chromosome 10 gene corresponding to the cDNA is the functional gene fully capable of expressing the active oligomeric enzyme by itself and is, therefore, consistent with being the site of the molecular defect in GA.

Gyrate atrophy (GA) is an autosomal recessive degenerative disease of the retina and choroid of the eye that leads to blindness.1 An inborn deficiency of a mitochondrial matrix enzyme, ornithine aminotransferase (OAT; EC 2.6.1.13) and hyperornithinemia are present in patients with GA.2-4 Structural and expression defects of the OAT gene have been demonstrated in GA cases recently, confirming the importance of OAT in this disease.5-7

The native OAT is an oligomeric enzyme consisting of four to six identical subunits in the rat8,9 and most likely in the human.10 A cDNA for the human OAT mRNA has been cloned and characterized,11-13 and it has been used as a probe to demonstrate the presence of an OAT gene family and to map the OAT gene sequences to chromosomes 10 and X.14,15 A functional OAT gene had been shown to be present on human chromosome 10,16 and the OAT cDNA was recently shown to correspond to this gene.13 In considering the molecular basis of OAT defect in GA, it would be important to know which and how many of the genes in the OAT gene family are responsible for the OAT enzyme activity. We set out to determine if the OAT cDNA (pHOAT) and its gene on chromosome 10 contain all of the genetic information necessary for the expression of OAT enzyme activity by subcloning pHOAT into a mammalian expression vector (pcD), and transfecting and expressing it in mouse fibroblasts (NIH3T3) and OAT(−) Chinese hamster ovary cells (CHO),17 which have negligible OAT activity. Several transfectants of NIH3T3 were isolated that show stable integration of pHOAT sequences, and expression of human OAT mRNA, OAT enzyme activity and protein clearly distinguishable from those of the host. Furthermore, OAT(−) CHO cells containing the human OAT expression clones express the human OAT mRNA and active OAT enzyme protein.

材料和方法

细胞、酶和化学物质

NIH3T3 (慷慨的赠予H. Okayama) 和 OAT(−) CHO (American Type Culture Collection, Rockville, MD) 细胞用于转染。NIH3T3 细胞在 Dulbecco's Modified Eagle Medium 和 CHO 细胞在 F-12 营养基中培养，含有 10% 胎牛血清和

Materials and Methods

Cells, Enzymes and Chemicals

NIH3T3 (generous gift of H. Okayama) and OAT(−) CHO (American Type Culture Collection, Rockville, MD) cells were used for the transfection. NIH3T3 cells were grown in Dulbecco’s Modified Eagle Medium and CHO cells were grown in F-12 nutrient mixture containing 10% fetal calf serum and...
antibiotics (Gibco Laboratories, Grand Island, NY). Restriction enzymes were from Bethesda Research Laboratories (Gaithersburg, MD). DNA polymerase I and proteinase K were from Boehringer Mannheim (Indianapolis, IN). T4 DNA ligase was from New England Biolabs (Beverly, MA). 32P-labeled deoxythymidine 5'-triphosphate and 125I-labeled anti-rabbit immunoglobulin antibody were from Amersham (Arlington Heights, IL). Ornithine hydrochloride, α-ketoglutarate, pyridoxal phosphate and o-aminobenzaldehyde were from Sigma (St. Louis, MO). All chemicals were of reagent grade quality.

pcDHOAT

Plasmids pL1 and pcDV1 were used to construct a recombinant pcDL, which can be used for the cloning and expression of cDNA in animal cells (Fig. 1).18 Procedure for the construction of pcDHOAT is shown in Figure I and described in the text. pcDHOAT1 is a recombinant shown in Figure 1 and pcDHOAT2 is a recombinant which has two OAT cDNAs in tandem (5'-3' 5'-3').

Transfection of NIH3T3 Cells

Transfection was performed according to the method of Wigler et al.19 Ten centimeter dishes were seeded with 5 x 10^5 cells 24 hr before transfection. Cells were either transfected with the neomycin resistance marker, pSV2neo,20 or cotransfected with pSV2neo plus pcDHOAT1 or pcDHOAT2. Mock transfections were also performed. Transfected cells were cultured in neomycin (G418) (Gibco Laboratories, 500 μg/ml) selection media 48 hr after DNA transfection, and drug-resistant colonies were isolated 14–21 days after DNA transfection.

Transfection of CHO Cells

Cells (5 x 10^7) were harvested with trypsin and resuspended in 5 ml media containing pcDHOAT1 (10 μg/ml). Electroporation was performed according to the method of Potter et al.21 using the Electroporation System (Bethesda Research Laboratory) at a setting of 200 V and 1980 μF. Cells were harvested for transient expression analysis 48 hr after DNA transfection.

Southern Blot Analysis

Ten micrograms each of genomic DNA isolated from the neomycin-resistant transfectants of NIH3T3 cells and 10 μg of nongenomic DNA isolated from the transfectants of CHO cells according to the method of Hirt22 were digested with EcoRI, subjected to electrophoresis on 0.8% agarose gel, transferred to nylon or nitrocellulose paper, hybridized with 32P-labeled human OAT cDNA, washed and autoradiographed as described before.23 Amount of pcDHOAT equivalent to one copy per genome was used for the calibration.

Northern Blot Analysis

Ten micrograms of total RNA, isolated from each of the NIH3T3 cell clones and transfectants of CHO cells by the guanidine thiocyanate method,24 were subjected to electrophoresis on 0.8% formaldehyde gel under denaturing condition,25 transferred onto nylon filter, hybridized with 32P-labeled human OAT cDNA or actin cDNA, washed and autoradiographed as described before.26 Hybridization with actin probe was performed to check the quality and quantity of RNA actually present on the blot for each lane.

Western Blot Analysis

Cells (6–8 x 10^6) were harvested, washed twice with phosphate-buffered saline and suspended in phosphate buffer (pH 8.0) at about 4 x 10^7/ml. Cells were disrupted by freezing and thawing three times. The cell lysate was centrifuged for 1 min at 14,000 rpm. Protein content was determined by the method of Lowry et al.27 with bovine serum albumin as standard. Thirty micrograms of the protein were subjected to electrophoresis on 10% SDS-polyacrylamide gel, transferred onto nitrocellulose filter, reacted with anti-human OAT rabbit antibody28 and with 125I-labeled anti-rabbit immunoglobulin donkey antibody and autoradiographed as described.29

OAT Assay

OAT activity was assayed using the cell lysates described in the method for the western blot, and under these conditions, the activity of OAT in the NIH3T3 cells and CHO cells was stable for at least 50 days. OAT activity was measured as described by Katunuma et al.20 A molar extinction coefficient of 2.7 x 10^3 was used to calculate the amount of pyrroline-5-carboxylate formed. Enzyme was prepared from each cell line at least twice and a total of ten assays was performed on each cell line.

Results

The pcD vector is a mammalian expression plasmid cloning vector consisting of the SV40 early region promoter, two introns that are normally spliced to form the 16S and 19S mRNAs, and the late region polyadenylation sequence of SV40, and pBR322 sequence containing the origin of replication and the ampicillin resistance gene.18 Construction of a cDNA
CONSTRUCTION OF pcDHOAT

Fig. 1. Construction of the expression clone pcDHOAT. pcDHOAT1 containing one copy of OAT cDNA and pcDHOAT2 containing two copies of OAT cDNA were constructed. See text for description.

in this vector, starting with the two precursor plasmids, pcDV1 and pL1, was previously described. For our purpose, the cloning strategy was slightly altered to allow for the insertion of EcoRI OAT cDNA insert from pHOAT directly into pcD. The appropriate HindIII-BamHI fragments from pcDV1 and pL1 were ligated to form pcDL1, which left intact one of the two spliceable introns (19S) in addition to the early region promoter and the late region polyadenylation sequence of SV40 (Fig. 1). The BamHI site was opened up, converted to an EcoRI site with adaptors, and used for the insertion of EcoRI OAT.
cDNA to form pcDHOAT. The insertion of the cDNA at this position maintains the proper physical relationship of the cDNA with respect to the promoter, spliced intron, and polyadenylation signal originally described as being important for the expression and proper processing of the transcript with pcD. Since NIH3T3 mouse fibroblasts, which we used to express the human OAT in, have endogenous OAT which may interfere with the examination of human OAT expression, a pcD clone containing two copies of the OAT cDNA (pcDHOAT2) was also constructed, which may express an oversized OAT mRNA easily distinguishable from the mouse OAT mRNA. A proper 5' to 3' orientation of the OAT cDNA in each of the pcDHOAT clones was checked by restriction digestion and gel analysis (data not shown).

NIH3T3 mouse fibroblasts (5 × 10⁵ per dish) were cotransfected with pcDHOAT1 or pcDHOAT2 and pSV2neo, a neomycin resistance gene marker, by the calcium phosphate method. Neomycin-resistant clones were isolated 14–21 days after the transfection. Six clones originally transfected with pcDHOAT1 and pSV2neo and six clones originally transfected with pcDHOAT2 and pSV2neo in addition to two clones transfected with pSV2neo alone were investigated.

The presence of the human OAT cDNA sequence in the genomes of the mouse cellular clones was confirmed by a Southern blot analysis. Hybridization of the human OAT probe to EcoRI-digested genomic DNA from the mouse clones demonstrated the presence of OAT sequences in a number of EcoRI fragments of different size in each of the clones (Fig. 2).
The only hybridizing sequences found in the original NIH3T3 cells and clones transfected with pSV2neo alone were the 17 Kbp, 15 Kbp, and 4.8 Kbp cross-hybridizing, mouse OAT genomic fragments except for the 5.6 Kbp fragment in control 2, which appears to be the linearized pSV2neo hybridizing to pBR322 contaminant of the OAT probe. The result indicated the integration of pcDHOAT sequence into multiple different sites in the mouse genome. All of the clones with the exceptions of 4, 7 and 13 also showed integration of intact copies of the human OAT cDNA, as represented by the 2.1 Kbp EcoRI fragment (Figs. 1, 2). Calibration of the hybridization intensities revealed integration of one (clone 6) to nine (clone 12) copies of the intact OAT sequence into the genomes of the mouse cellular clones. Clone 7, which was derived from clone 6, apparently had lost the one copy of pcDHOAT that it originally had, as shown by the loss of the 2.1 Kbp hybridizing fragment.

A northern blot analysis of RNAs from the cell clones was performed with the human OAT probe. A 2.1 Kbp OAT mRNA was demonstrated in the control human fibroblast and all of the mouse cell clones (Fig. 3A). The endogenous OAT mRNA in the mouse cells was detected by weak cross-hybridization with the human OAT probe. In addition to the endogenous mouse mRNA, stronger-hybridizing human OAT transcripts were present in the transfected cell clones 6, 12 and 15. Several human OAT transcripts of varying size were present in each of these clones, including OAT mRNAs approximately double the normal size (5 Kbp) corresponding to transcripts coming from the pcDHOAT2 in clones 12 and 15. While the human OAT transcripts were present in clone 6, they were completely absent in clone 7, which had lost the transfected pcDHOAT from its genome, clearly demonstrating the origin of these stronger-hybridizing transcripts to be the transfected human OAT sequence. A quantitative and qualitative determination of the RNA actually present on the northern blot by actin probing (Fig. 3B) confirmed the results stated above.

A western blot analysis of proteins from the cell clones was performed with anti-human OAT rabbit antibody. Mouse OAT protein of approximately 45 kD, cross-reactive with the anti-human OAT antibody, is present in all of the mouse cell clones; six representative clones along with controls are shown in Figure 4 (upper arrow). Although the size of the mouse OAT is very similar to the human OAT, there
is present, in addition to the endogenous mouse OAT, a band of immunoreactive OAT protein representing the human enzyme and migrating slightly faster than the mouse OAT in the human OAT mRNA-producing cell clones 6, 12 and 15 (Fig. 4, lower arrow). The extra human OAT band is not present in clone 7, which had lost the human OAT cDNA sequence.

OAT activity of the cell clones was assayed to determine if expression of the human OAT mRNA and protein in the mouse fibroblasts can result in active OAT enzyme. Significant increase in OAT activity (49-95%) was observed for the cell clones shown to contain human OAT transcripts and protein (clones 6, 12, 15) (Table 1). The source of the OAT activity increase in these cells was clearly demonstrated to be the transfected human OAT sequence, again by the obliteration of the activity increase (originally seen in clone 6) in clone 7, which had lost the human OAT sequence.

Finally, a transient expression experiment was also performed using pcDHOAT and OAT(−) CHO cells, which have negligible OAT enzymatic activity,17 to confirm the de novo and independent synthesis of human OAT from pcDHOAT in heterologous cells. After transfection an average of 25 copies of pcDHOAT were detected per cell with expression of high levels of the human OAT mRNA and active OAT enzyme protein confirmed by Southern, northern, and western analysis and OAT enzyme activity assay (data not shown).

Discussion

Complementary DNAs have been cloned into various mammalian expression vectors and used to express the corresponding gene products.1831 The pcD vector has been specifically used to express α-globin and hypoxanthine-guanine phosphoribosyltransferase in mammalian cells.1832 We used this expression vector to express human ornithine aminotransferase from an OAT cDNA that we had previously constructed and characterized.11 Our results indicate that we were successful in expressing the human OAT mRNA and protein with enzymatic activity in the NIH3T3 mouse fibroblasts and OAT(−) CHO cells, therefore demonstrating that our human OAT cDNA (pHOAT) contains all of the sequence information necessary for the expression of intact OAT gene product. The presence of endogenous OAT in the NIH3T3 cells did not interfere with the analysis of human OAT expression since: (1) the quantity and quality of human OAT mRNAs, especially the pcDHOAT2-coded mRNA of double the normal size, in the transfectants were easily distinguishable from the faintly hybridizing mouse OAT mRNA; (2) a clearly distinguishable human OAT protein was detected immunologically in cells producing the human OAT mRNA; and (3) the increase in OAT activity was observed only in cells containing the human OAT protein and was significant enough to be clearly attributable to the human OAT sequence. The fortuitous presence of cell clone 6 containing one copy of pcDHOAT and its derivative clone 7, which had lost this copy of pcDHOAT, offered a direct proof that the increase in OAT mRNA and enzyme activity and the extra band of OAT pro-

Table 1. Ornithine aminotransferase assay of cell lines

<table>
<thead>
<tr>
<th>Cell</th>
<th>Specific activity*</th>
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<tbody>
<tr>
<td>1</td>
<td>291 ± 15</td>
</tr>
<tr>
<td>2</td>
<td>315 ± 52</td>
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<tr>
<td>3</td>
<td>293 ± 11</td>
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<tr>
<td>4</td>
<td>288 ± 48</td>
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<tr>
<td>5</td>
<td>290 ± 18</td>
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<tr>
<td>6</td>
<td>386 ± 122</td>
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<tr>
<td>7</td>
<td>289 ± 19</td>
</tr>
<tr>
<td>8</td>
<td>267 ± 38</td>
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<tr>
<td>9</td>
<td>373 ± 26</td>
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<tr>
<td>10</td>
<td>295 ± 25</td>
</tr>
<tr>
<td>11</td>
<td>279 ± 42</td>
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<tr>
<td>12</td>
<td>546 ± 73</td>
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<tr>
<td>13</td>
<td>286 ± 11</td>
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<tr>
<td>14</td>
<td>233 ± 14</td>
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<tr>
<td>15</td>
<td>448 ± 44</td>
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</table>

* nmol of pyrroline-5-carboxylate formed/mg of protein/h ± SD as measured by the method of Katunuma et al30; protein concentration was assayed by the method of Lowry et al19; 1, NIH3T3; 2-15, the same as described for Figure 2.
tein seen in the transfected cell clones are due to expression of the integrated human OAT cDNA, since all of these, present in clone 6, were obliterated in clone 7. Ability to express the human OAT mRNA, protein and enzyme activity in OAT(−) CHO cells, which have negligible endogenous OAT activity, confirmed our conclusions derived from the NIH3T3 cells that there is a de novo and independent expression of human OAT from pCDHOAT. The results with the CHO cells indicate that one does not have to postulate the formation of a human–rodent OAT hetero-oligomer or activation of the endogenous rodent OAT gene to explain the appearance of OAT activity since these are very unlikely in the OAT(−) cells.

Since OAT is a mitochondrial matrix enzyme, the results also indicate that our human OAT cDNA contains all of the sequence information necessary to code for an OAT precursor molecule that can be transported to the mitochondrial matrix and processed correctly to form the active oligomeric enzyme. Only one type of OAT subunit has been described in the rat that forms the active homo-oligomeric enzyme, and our results also indicate that only one form of human OAT protein, expressed by pCDHOAT in the NIH3T3 and CHO transfectants, is involved in the formation of the active human OAT enzyme, which is presumably homo-oligomeric. Formation of an active hetero-oligomeric enzyme consisting of human and mouse OAT subunits cannot be ruled out in the NIH3T3 transfectants but it is not necessary to explain the increase in OAT activity, as mentioned above, and is unlikely since one would have to assume compatibility of the mouse and human subunits to combine and form an active oligomer and also an abundance of free, unassociated mouse subunits in the mitochondrial matrix. If significant amounts of mouse subunits were combining with the human subunits to form an active hetero-oligomer, the net amount of OAT activity in the NIH3T3 transfectants would probably be unchanged instead of the observed 95% increase.

The demonstration that the chromosome 10 OAT gene corresponding to pHOAT is indeed the functional OAT gene responsible for the expression of active OAT enzyme has significant implications for investigation of the molecular genetic basis of gyrate atrophy. The finding identifies the correct gene to focus on among the OAT gene family members in studying the gene defect of gyrate atrophy. This conclusion is borne out by the recent demonstration of a GA case with a partial heterozygous deletion and no mRNA of this functional OAT gene. The ability to express active OAT in mammalian cells using an expression clone of OAT cDNA also opens up the possibility of considering a replacement gene therapy for gyrate atrophy.

Key words: gyrate atrophy, ornithine aminotransferase, cDNA, gene transfer, expression

Acknowledgment

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References