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Ornithine Transcarbamylase Deficiency: a Case with a Truncated Enzyme Precursor and a Case with Undetectable mRNA Activity

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The cell-free translation of ornithine transcarbamylase (OTC) mRNA from the livers of two heterozygous patients (from different families) with OTC deficiency was performed. The enzyme activities and the immunoreactive proteins in both patients were about 5% of those in controls. Immunoblotting assay of liver extracts from both patients showed decreased amounts of the OTC protein. The mRNA from the liver of patient 1 directed the synthesis of a very small amount of OTC precursor of normal subunit size (40 000 Da), whereas that from patient 2 directed the synthesis of small amounts of two distinct *in vitro* products; one was 40 000 Da and the other was about 30 000 Da. The *in vitro* product of normal precursor synthesized with mRNA from patient 2 was converted to mature-sized OTC by isolated rat liver mitochondria, whereas the smaller product was degraded during the incubation with the mitochondria. These results indicate that in both patients the translatable level of mRNA for active OTC from liver cells was much lower than that in the controls. The results also suggest that in patient 2, the smaller product presumably derived from an abnormal gene could not be transferred to the mitochondria.

Deficiency of ornithine transcarbamylase (OTC, ornithine carbamoyltransferase, EC 2.1.3.3) which is a urea cycle enzyme located in the mitochondrial matrix (Mizutani, 1968), is one of the most frequent causes of inherited ammonia intoxication; evidence indicates that the enzyme is X-linked (Short *et al.*, 1973; Ricciuti *et al.*, 1976). OTC deficiency is a disease with X-linked dominant inheritance, although different kinds of mutation in humans have been reported (Levin *et al.*, 1969; Matsuda *et al.*, 1971; Cathelineau *et al.*, 1972; Heiden *et al.*, 1977; Mori *et al.*, 1980; Yudkoff *et al.*, 1980; Yokoi *et al.*, 1981; Briand *et al.*, 1982). Most male patients therefore present severe symptoms and females show a wide variation of symptoms.

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Human OTC, a 110 kDa protein, is a trimer of identical 36 500 Da subunits (Pierson *et al.*, 1977). OTC is encoded by a nuclear gene and is synthesized on membrane-free polysomes in the form of a larger precursor with an amino-terminal extension of 3400–4000 Da; it is then proteolytically processed to the mature form of the enzyme in association with the transport into the mitochondria (Kraus *et al.*, 1981; Mori *et al.*, 1982).

We present here results on the cell-free translation of OTC mRNA from the liver of two heterozygous patients with OTC deficiency. We also describe the processing of the mutant enzyme precursor by isolated rat liver mitochondria.

MATERIALS AND METHODS

Materials

The liver samples from two female patients who suffered from hyperammonaemia due to an OTC deficiency were studied. The detailed clinical course of the condition of patient 1, who died when she was 10 years old, was reported previously (Kodama *et al.*, 1983). Patient 2, who was from a different family from patient 1, died at 5 years of age. The livers were obtained a few hours after their deaths and were kept at −80°C until use. The OTC activity (at several pH values) and the amount of cross-reactive material in the livers of both patients were about 5% of those in the control livers. Details of the enzymatic characteristics of OTC in these patients were given in a previous report (Kodama *et al.*, 1984). Control liver samples were obtained from infants who died from congenital heart disease.

Preparation of antibody

Bovine liver OTC was purified to homogeneity according to the method of Marshall and Cohen (1972). Antibovine OTC was raised in rabbits by injecting 0.5 mg of the purified enzyme mixed with Freund's complete adjuvant subcutaneously twice biweekly and then 3 times weekly. The antibody was purified by 3 successive ammonium sulphate precipitations at 40% saturation at 4°C.

Antirat catalase IgG was kindly provided by Dr T. Hashimoto (Shinshu University, Japan).

Immunodetection of OTC-related protein after electroblotting

Liver was homogenized with 4 volumes of 1% Triton X-100 and centrifuged to remove insoluble materials. The liver extracts were mixed in 10 volumes of a solution containing 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol and 62.5 mmol l^{-1} Tris HCl, pH 6.8, and were heated for 3 min in boiling water. They were then subjected to polyacrylamide gel electrophoresis as described by Laemmli (1970). After electrophoresis, the proteins were electrophoretically transferred from gel to a nitrocellulose membrane according to the method of Towbin and colleagues (1979). OTC-related protein was located on the membrane with 4-chloro-1-naphthol and peroxidase after treatment with antibovine rabbit OTC IgG, using a BIO-RAD Immuno Blot Assay Kit.

Cell-free protein synthesis and immunoprecipitation

Total RNA was extracted from patients' and control liver by the guanidine-HCl procedure (Mori *et al.*, 1979). Total mRNA (0.3 mg ml^{-1}) was translated in a nuclease-treated rabbit reticulocyte system in the presence of [^{35}S]methionine (about 40 μCi) in a total volume of 50 μl as described previously (Mori *et al.*, 1979). The *in vitro* products were immunoprecipitated using antibovine OTC immunoglobulin and *Staphylococcus aureus* cells. The immunoprecipitates were subjected to SDS-10% polyacrylamide gel electrophoresis and then visualized by fluorography (Mori *et al.*, 1981).

The effect of RNA concentration on total protein synthesis was examined with [^{14}C]leucine (0.4 μCi in 25 μl).

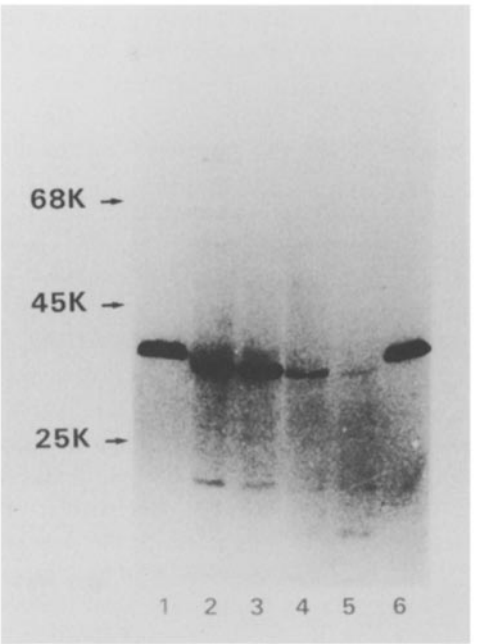

Figure 1 Immunodetection of OTC-related protein after electroblotting. After SDS-10% polyacrylamide gel electrophoresis of the liver extracts, the proteins were transferred to a nitrocellulose membrane. OTC-related protein was located on the membrane using anti-bovine OTC IgG (0.25 mg ml^{-1}) and BIO-RAD Immuno Blot Assay Kit. Lanes 1 and 6, purified bovine OTC (50 ng); lanes 2 and 3, 100 μg protein of control liver extracts; lane 4, 100 μg protein of liver extract from patient 1; lane 5, 100 μg protein of liver extract from patient 2. Marker proteins were bovine serum albumin (68 k), ovalbumin (45 k) and chymotrypsinogen (25 k).

Processing of OTC precursor by isolated mitochondria

Cell-free synthesis of OTC precursor was performed as described above. The translated mixture (50 μl) was incubated at 25°C for 60 min with freshly isolated rat

liver mitochondria (50 μg protein, 2 μl) as described previously (Morita *et al.*, 1982). The whole mixture was subjected to immunoprecipitation and the immunoprecipitates were analysed by SDS-gel electrophoresis and fluorography.

RESULTS

Immunodetection of OTC-related protein after electroblotting

We examined the subunit molecular weight of OTC-related proteins of both patients by immunobinding. The liver extracts of controls and patients each gave a band of 36 500 Da (Figure 1). However, the density of both patients' bands was much less than that of the controls. This result was compatible with the fact that the cross-reactive material of both patients' livers was significantly decreased (Kodama *et al.*, 1984). These polypeptides moved slightly faster than those of the bovine OTC. We have repeatedly observed that human OTC migrates slightly faster than bovine OTC in SDS gel electrophoresis (unpublished results).

Synthesis of OTC precursors with mRNAs from the patients and controls

Total RNAs were prepared from the livers of normal controls and the two patients with an A 260/A 280 ratio between 1.8–1.9. Total protein synthesis increased with increasing concentration of total hepatic RNA up to 0.4 mg ml^{-1} similarly with all RNA preparations from controls and patients (Figure 2). Figure 3(a) shows the pattern of newly synthesized total proteins analysed by SDS-polyacrylamide gel electrophoresis and fluorography. The polypeptides synthesized with hepatic RNA from both patients and controls migrated as numerous bands with molecular mass up to 100 000 Da. Catalase synthesized by cell-free translation with RNA from the patients' livers was compared with that from control livers. Catalase synthesized *in vitro* was isolated by immunoprecipitation with anticatalase and *Staphylococcus aureus* cells and was analysed by SDS-gel electrophoresis (Figure 3(b)). The amounts of catalase synthesized from the RNA of the patients were comparable to the amounts from the controls. These results as well as those in Figure 2 show that the hepatic RNA preparations from the patients and the controls were not much degraded, and were nearly equally active in protein synthesis.

Figure 4 shows the OTC-related protein synthesized *in vitro* with hepatic RNA of controls and patients. A major polypeptide band of about 40 000 Da was detected with the control hepatic RNA (Figure 4, lanes 2 and 5). This polypeptide is an OTC-related protein and apparently the OTC precursor, because this polypeptide band was not seen when the bovine OTC antibody was replaced by control IgG in the immunoprecipitation, or when an excess amount of purified bovine OTC was included during the immunoprecipitation (Figure 5, lanes 2 and 3). In the case of RNA extracted from patient 1, a very faint band corresponding to the normal OTC precursor was detected, although the band is not evident in Figure 4 (lane 3). In the case of RNA extracted from patient 2, on the other hand, two faint polypeptide bands were detected corresponding to molecular masses of 40 000 Da and 30 000 Da (Figure 4, lane 4). The band of normal precursor size was very weak while the

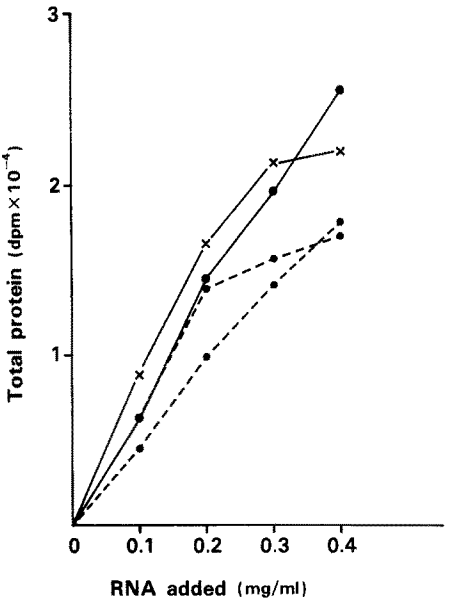

Figure 2 Cell-free translation of total RNAs from the livers of controls and patients with OTC deficiency. Various concentrations of RNA from the livers of controls (•- - -•), patient 1 (•——•) and patient 2 (×——×) were translated in a reticulocyte lysate system (25μl) containing [^{14}C]leucine (0.2μCi). 10μl aliquots of the reaction mixture were counted for acid-precipitable radioactivity.

smaller one was more evident. Neither smaller polypeptide nor the normal precursor size were seen when the bovine OTC antibody was replaced by control IgG at immunoprecipitation, or when an excess amount of purified bovine OTC was included during the immunoprecipitation (Figure 5, lanes 5 and 6). It is concluded, therefore, that both polypeptides in the RNA from patient 2 are OTC-related proteins; the larger one is the normal OTC precursor and the smaller one is presumably a truncated OTC precursor.

Processing of OTC precursor from patient 2 by isolated mitochondria

In the immunobinding assay of OTC-related protein of patient 2, no polypeptide band corresponding to the *in vitro* product of molecular mass 30 000 Da was detected (Figure 1, lane 5). We then examined the transport and processing by mitochondria of the putative truncated OTC precursor of molecular mass 30 000 Da in patient 2. We examined here only the processing, because the uptake and processing of the OTC precursor by the mitochondria are coupled (Morita *et al.*, 1982; Saheki *et al.*, 1984).

When the OTC precursor synthesized with RNA from control liver was incubated with the mitochondria isolated from rat liver, the OTC precursor was converted to a polypeptide which comigrated with the subunit of human mature OTC (Figure 6, lane 3). A similar result was obtained from the OTC precursor of molecular

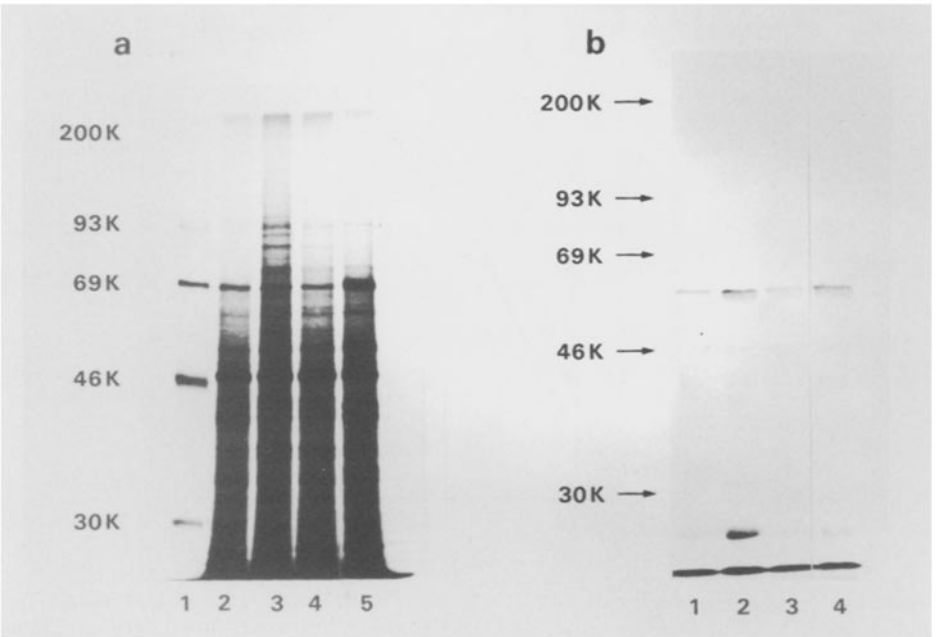

Figure 3 (a) Fluorogram of SDS-10% polyacrylamide gel electrophoresis of total proteins synthesized *in vitro* with total hepatic RNA of controls and patients. Cell-free translation was performed with 0.3 mg ml^{-1} of total hepatic RNA from controls (lanes 2 and 5), patient 1 (lane 3) and patient 2 (lane 4). Lane 1, ^{14}C-labelled marker proteins.

(b) Catalase synthesized *in vitro* with total hepatic RNA of controls and patients. Catalase was isolated by immunoprecipitation with antirat catalase IgG (100 μg) and fixed *S. aureus* cells (100 μl of 10% suspension) and SDS-10% polyacrylamide gel electrophoresis and detected by fluorography. Lanes 1 and 4, controls; lane 2, patient 1; lane 3, patient 2.

mass 40 000 Da synthesized with hepatic RNA from patient 2, although the band of mature-sized OTC was very faint and cannot be seen in Figure 6; apparently normal processing was seen when the film was exposed for a longer period (data not shown). The band of the putative truncated precursor from patient 2 became more faint with the processing, but the processed product was not found (Figure 6, lane 6).

DISCUSSION

Briand and colleagues (1982) reported that at least 5 different groups could be distinguished from the results of immunochemical examination of male patients with OTC deficiency: group 1, no residual activity and no detectable cross-reactive material; group 2, no residual activity but the normal amount of OTC protein in the liver; group 3, decrease in OTC protein with paralleled reduction of catalytic activity; group 4, a similar decrease of maximal enzyme activity and immunoprecipitable material as compared to controls, but shift of pH optimum far to the alkaline

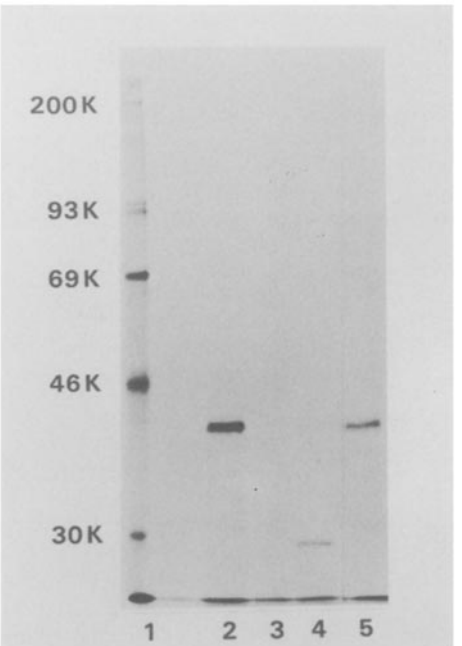

Figure 4 Total hepatic RNA (0.3 mg ml^{-1}) from controls (lanes 2 and 5), patient 1 (lane 3) and patient 2 (lane 4) was translated in a reticulocyte lysate cell-free system (50 μl) in the presence of [^{35}S]methionine. The cell-free products were isolated by immunoprecipitation with antibovine OTC IgG (150 μg) and fixed *S. aureus* cells (100 μl of 10% suspension) and SDS-10% polyacrylamide gel electrophoresis and visualized by fluorography. Lane 1, ^{14}C-labelled marker proteins.

side; and group 5, the OTC activity higher at pH 9.5 than the control values. Recently Saheki and colleagues (1984) have performed *in vitro* translation of hepatic RNA from an OTC-deficient patient with no detectable OTC protein, and found a normal level of OTC mRNA activity. Moreover, Briand and colleagues (1983) and Rosenberg and colleagues (1983) reported that in the spf-ash mouse with OTC mutation, the OTC mRNA from liver of hemizygous mice directs the synthesis of low amounts of two distinct OTC precursor polypeptides; one normal in size and the other distinctly elongated. These papers suggest that OTC deficiency is caused by various kinds of mutation.

Our patients were heterozygous for this X-linked dominant disorder because their mothers were detected as carriers by the examination of urinary orotate after protein loading (data not shown), and each of the patients had a brother who died from vomiting and coma during the neonatal period. Thus both patients were supposed to have normal and abnormal hepatocytes resulting from inactivation of either the abnormal or normal X chromosome, as shown for the female patients with OTC deficiency (Ricciuti *et al.*, 1976; Glasgow *et al.*, 1978). Residual enzyme activity and the amount of cross-reactive material in both patients' livers were about 5% of the respective values in control livers. The residual enzyme activity and

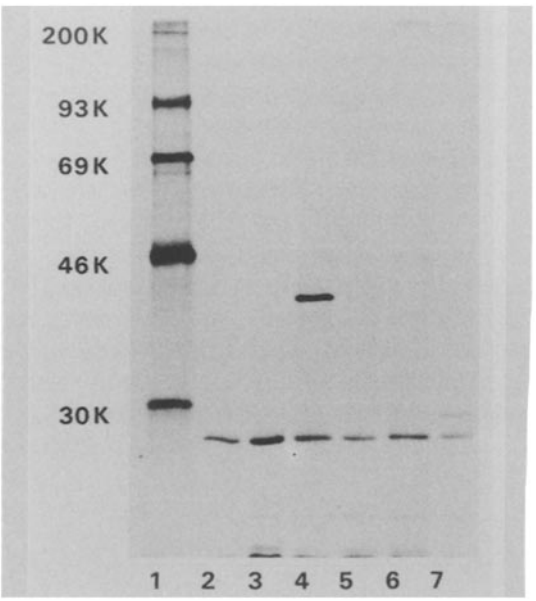

Figure 5 Identification of the *in vitro* products. Cell-free translation of total hepatic RNA from a control (lanes 2–4) and patient 2 (lanes 5–7) was performed as in Figure 4. Lanes 4 and 7, immunoprecipitation was performed with anti-OTC IgG (150 μg); lanes 2 and 5, immunoprecipitation was performed in the presence of 20 μg of purified bovine OTC; lanes 3 and 6, control rabbit IgG (150 μg) was used instead of anti-OTC for immunoprecipitation. The fastest moving polypeptide (molecular mass about 25 000) is due to non-specific absorption, because it appeared with control IgG and was not competing with bovine OTC for immunoprecipitation.

OTC protein probably resulted from the normal liver cells in which the abnormal X chromosome was inactivated, because the enzymatic and immunochemical properties of the OTC in the patients are similar to those in the controls.

We observed that the RNA from the liver of patient 1 directed the synthesis of a very small amount of normal OTC precursor. It must be produced from the normal gene, though we could not quantitate the amount. These results suggested that the translatable level of mRNA for OTC in the abnormal liver cells of patient 1 was zero. The mutant OTC in patient 1 could be due to a mutation affecting either transcription of the gene, processing of the mRNA precursor, stability of mRNA or its translatability.

The OTC mRNA from the liver of patient 2 directed the synthesis of two distinct OTC precursor polypeptides; one was normal in size (subunit of molecular mass 40 000 Da) and the other was distinctly smaller (subunit of molecular mass about 30 000 Da) (Figure 4, lane 3). It is unlikely that the polypeptide of molecular mass 30 000 Da is a proteolytic fragment, because this polypeptide was also detected even when a mixture of protease inhibitors was added during the *in vitro* translation

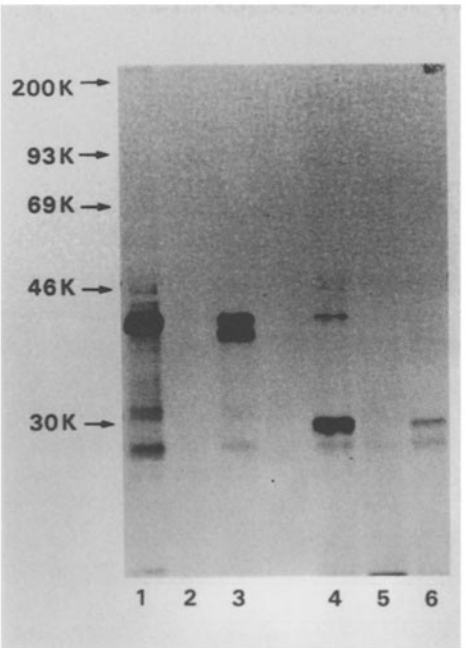

Figure 6 Processing of cell-free synthesized OTC precursors by isolated rat liver mitochondria. Total hepatic RNA from a control (lanes 1–3) and patient 2 (lanes 4–6) was translated in a reticulocyte lysate system (50 μl) in the presence of 25 and 50 μCi of [^{35}S]methionine respectively. The translated mixture was incubated at 25°C for 60 min with (lanes 2, 3, 5 and 6) or without (lanes 1 and 4) rat liver mitochondria (50 μg protein, 2 μl). The whole mixture was subjected to immunoprecipitation with anti-OTC and the immunoprecipitates were subjected to SDS-10% polyacrylamide gel electrophoresis and fluorography. In lanes 2 and 5, anti-OTC was replaced by control IgG. The fastest moving polypeptide (molecular mass about 25000) is due to non-specific absorption (see Figure 5).

and immunoprecipitation (data not shown). When the OTC precursors from patient 2 were incubated with isolated rat liver mitochondria, only the normal sized OTC precursor was converted to the mature subunit, whereas the putative truncated precursor was degraded during the incubation. It is very likely that the normal sized OTC precursor was derived from the normal gene and the truncated precursor was derived from the abnormal gene. The results of *in vitro* processing experiments suggested that the truncated OTC precursor could not be transferred to the mitochondria, or even if it was transferred to mitochondria, the transferred protein was rapidly degraded.

This is the first report of human OTC deficiency to show that translatable mRNA encoding the enzyme protein was markedly decreased (patient 1) and that a truncated enzyme precursor was detected by *in vitro* translation (patient 2). Complementary DNAs for human OTC (Horwich *et al.*, 1984) and rat OTC (Horwich *et al.*, 1983; Takiguchi *et al.*, 1984) have been isolated and the complete amino acid sequences of the OTC precursors have been determined (Horwich *et al.*, 1984;

Takiguchi *et al.*, 1984). Studies on OTC mRNAs and genes in the patients using cDNA probes are in progress in our laboratory.

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