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IN VITRO TRANSLATION OF mRNA FROM RAT PHEOCHROMOCYTOMA TUMORS, CHARACTERIZATION OF TYROSINE HYDROXYLASE

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SUMMARY: Studies are presented which demonstrate that rat pheochromocytoma tumors are a convenient material for the preparation of tyrosine hydroxylase mRNA. Total pheochromocytoma poly(A)$^+$mRNA has been extracted from tumors, then translated in a reticulocyte lysate cell-free system. Neo-synthesized tyrosine hydroxylase has been identified by direct immunoprecipitation followed by sodium dodecyl sulfate acrylamide gel electrophoresis. The proportion of this specific mRNA has been calculated; it represents 0.15 per cent of the total poly(A)$^+$mRNA. The molecular weight of the in vitro synthesized tyrosine hydroxylase is 62,000.

Tyrosine hydroxylase, the first enzyme of the metabolic pathway giving rise to noradrenaline from tyrosine (1), is a very important marker of differentiation for catecholaminergic neurons both in vivo and in vitro. It has been suggested, for some time, that the precursors of neuronal cells were not able to synthesize the specific proteins of mature neurons; it was therefore proposed that the arrest of cellular division was the signal for the expression of adrenergic phenotypes (2, 3). Recently, however, other authors have succeeded in characterizing the presence of catecholamine storage vesicles at early steps of embryonic development in cells which are still dividing (4, 5) and Rothman et al. (6) have described cells replicating their DNA and containing both TH and DβH.

Although TH has already been obtained in a highly purified state, little study has thus far been devoted to control mechanisms operating during its bio-

ABBREVIATIONS: TH, tyrosine hydroxylase, tyrosine 3 monooxygenase, EC (1.14.16.2); DβH, dopamine β hydroxylase; SDS, sodium dodecyl sulfate; βSH, β mercaptoethanol; IgG, immunoglobulin; NGF, nerve growth factor; poly(A)$^+$RNA, poly adenylate containing RNA.

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synthesis in terminally differentiated neural cells maintained in tissue culture. Rat pheochromocytoma, which are tumors of the adrenal medulla glands, are particularly rich in TH (7). The enzyme found in these tumors is a 220,000 molecular weight protein, which behaves as a 62,000 molecular weight polypeptide in a sodium dodecyl sulfate acrylamide gel electrophoresis (8).

In the present work, we describe an attempt to characterize the level of TH messenger RNA activity in a rat pheochromocytoma tumor system, based upon the capacity of its extracted total poly(A) containing mRNA to stimulate TH synthesis in a reticulocyte cell-free translation system. Characterization of the in vitro synthesized product was achieved by immunoprecipitation using an anti-TH specific rabbit antiserum. Calculation shows that TH mRNA amounts to about 0.15 per cent of the total poly(A)$^+$ messenger. This result represents a first step towards a further purification of TH mRNA by the genetic engineering techniques.

MATERIALS AND METHODS

Transplanted animals were rats of the New England Deaconess Hospital strain (Boston, Mass., USA). The original tumor (7) was a gift of Dr. Thoenen (Munich). It was transplanted subcutaneously every three weeks. Three week old tumors (total weight 45 g) were collected surgically, cleaned of conjunctive tissue, rinsed in a cold sterile isotonic solution and homogenized during 1 min at maximum speed with a Braun mixer in 400 ml of a buffer containing 0.2 M TRIS-HCl buffer, pH 8.8, 0.25 mM EDTA, 0.5 M LiCl, 1 per cent SDS and 100 ml of buffer saturated phenol. Then 200 ml of chloroform - isoamyl alcohol (96/4, v/v) were added and homogenization continued during 5 min at low speed. The purification procedure of Lomedico and Sanders (9) was then followed. Total RNA was kept under 66 per cent ethanol at minus 20°C. To discard contaminating DNA and tRNA, RNA was precipitated in 2 M LiCl. Poly(A) containing mRNA was further isolated by two successive chromatographies on oligo(dT)cellulose columns (10).

A Micrococcus nuclease-treated reticulocyte lysate was obtained according to (11, 12). Translation was carried out in the presence of optimal concentrations of spermidine (0.7 mM), Mg^{++} (4 mM) and K^+ (112 mM). Synthesis was for 1 hour at 30°C in a 100 µl assay containing 2.5 µg of pheochromocytoma poly(A)$^+$RNA in the presence of 5 µCi of ^{35}S-methionine (1170 Ci / mmole) (Amersham).

To obtain TH as a reference standard, the enzyme was purified by a new rapid procedure, including ammonium sulfate

precipitation (13), hydrophobic chromatography (14) and affinity chromatography on agarose substituted heparin column (15). (The complete purification procedure will be published in detail elsewhere). Antiserum was raised in New Zealand rabbits by injection of 400 µg of pure TH in the presence of complete Freund adjuvant, followed 1 month later by a booster of the same amount of antigen. After 3 similar boosters, at 1 month intervals, the rabbit serum contained enough anti-TH immunoglobulin to react with TH. The equivalence point was determined using both the lot of TH activity of a TH solution after immunoprecipitation, and measuring the protein content of the immunoprecipitate. 25 µl of the crude antiserum were needed to immunoprecipitate quantitatively 1 µg of pure TH. After *in vitro* synthesis, the reticulocyte lysate was centrifugated 1 hour at 150,000 *g* at 4°C. The supernatant (105 µl) was collected, supplemented with 2 µg of pure TH, 1 µg of Soybean Trypsin Inhibitor, PMSF was added to a concentration of 10^{-4} M and NaCl to 0.15 M. 60 µl of freshly centrifugated antiserum were then added. The mixture was incubated for 1 hour at 37°C then overnight at 4°C. The pellet was recovered by centrifugation for 5 min at 5000 *g*, washed on a sucrose cushion as recommended by Palmiter *et al.* (15). The washed immunoprecipitate, first dissolved in 15 µl of 0.1 M NaOH, was neutralized by HCl, denatured at 90°C for 2 min in the presence of 2 per cent SDS plus 1 per cent βSH, was electrophoresed in a 15 - 0.1 per cent acrylamide - bis acrylamide (w/v) gel in the presence of 0.1 % SDS (16). After electrophoresis, the gel was stained, dried, autoradiographied, cut in 1.2 mM slices. Slices were then hydrolysed with 0.2 ml of H_2O_2 (120 vol) at 50°C during 24 h and radioactivity was measured by counting in Beckman HP scintillation liquid.

RESULTS

After SDS acrylamide gel electrophoresis, purified rat TH migrates as a major band with a molecular weight of 62,000; it is accompanied by a faint band with a 60,000 protein component (Fig. 1, lane A). This component is always present in preparations but its percentage varies among preparations. Preliminary date indicate (data not shown) that it is a partially proteolysed form of TH.

The specificity of the antiserum, prepared from immunized rabbits, has been determined. The double diffusion assay technique of Ouchterlony (17) only displays one precipitation line, at different antiserum dilutions, when purified TH is placed in the center well. The same picture was obtained when pheochromocytoma 100,000 *g* supernatant was used as a source of antigen (Fig.2). No precipitation band could be observed when serum from non-immunized rabbits was used. The direct

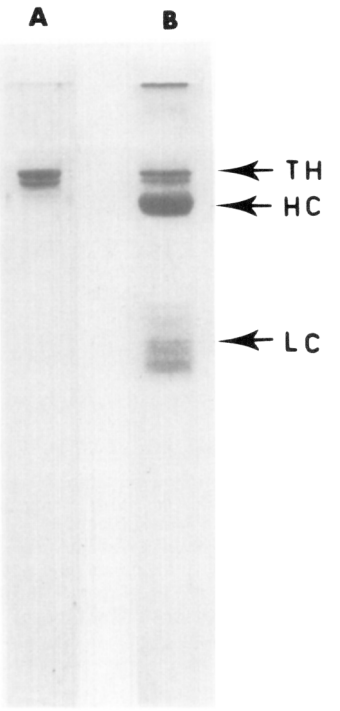

Figure 1

SDS gel electrophoregram of tyrosine hydroxylase.

Lane A : 2 µg of pure TH.
Lane B : 1 µg of pure TH immunoprecipitated by 30 µl of anti TH serum. The arrows indicate TH, HC (heavy chains), LC (light chains) of the immunoglobulin.

immunoprecipitation technique, performed with the pure TH, or with pheochromocytoma 100,000 g supernatant, as already mentioned in the section Materials and Methods, gave a pellet of TH-anti-TH-IgG complex. This pellet, analysed by electrophoresis on a SDS acrylamide gel (Fig. 1, lane B) shows only the two bands of the antigen plus the IgG heavy and light chains.

The translation products of pheochromocytoma poly(A)$^+$ RNA synthesized in a reticulocyte lysate and then analysed by SDS gel electrophoresis exhibit the autoradiographic pattern shown on Fig. 3, lane A. The immunoprecipitate of 200 µl of these products in the presence of 2 µg of pure TH was also subjected to SDS-acrylamide gel electrophoresis. The autoradiogram (Fig. 3, lane B) indicates that

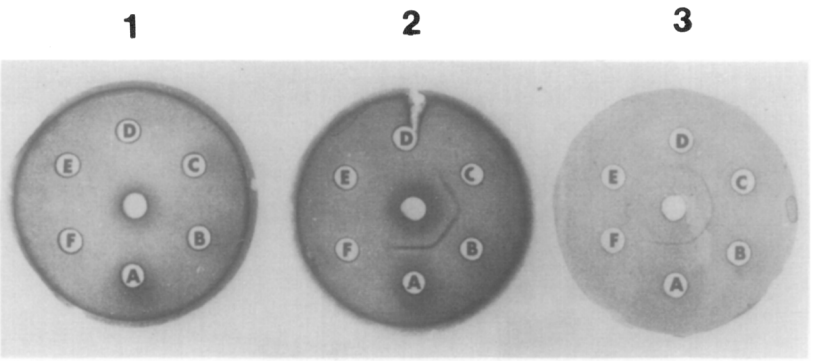

Figure 2

Ouchterlony, double diffusion pattern of TH with antiserum.

(1) The central well contained 15 µl of 100,000 g supernatant of pheochromocytoma containing 5 mg per ml of protein. Surrounding wells A, B, C, D, E, F contained respectively 15 µl of pure and diluted 2, 4, 8, 16, 32 times in isotonic buffer preimmune serum.
(2) Identical to (1) but with dilutions of the anti TH serum.
(3) The central well contained 15 µl of pure TH 100 µg/ml. The surrounding wells contained the same dilution of anti-serum as (2).

the major radioactive component comigrates with the cold carrier TH (molecular weight 62,000). The band corresponding to 60,000 component is exceedingly faint. A control experiment using non-specific antiserum or after formation of an heterologous complex between rat albumin and rabbit anti-rat albumin serum indicates only a trace of radioactivity at the level of the 62,000 component, due to non-specific adsorption on the carrier TH (data not shown).

In other experiments, after electrophoresis, the gels were cut into slices and the radioactivity was determined on each slice (Fig. 4). The ratio of radioactivity found in the 62,000 band of the immunoprecipitate compared with that of the total synthesized proteins as determined before immunoprecipitation was calculated to be approximately 0.15 per cent (Table I). This value is an approximate minimal estimate for the percentage of TH mRNA present in the tumors.

DISCUSSION

The preparation of pure rat pheochromocytoma TH and of its cognate antiserum has permitted us to quantitatively monitor the amount of TH synthesized in a

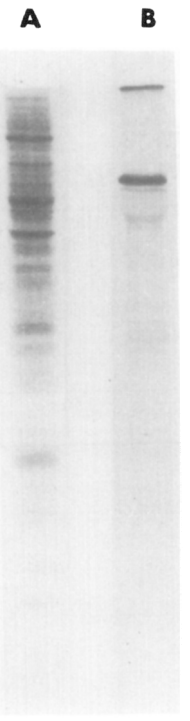

Figure 3

Autoradiography of reticulocyte lysate electrophoresis in a 15% acrylamide gel in the presence of SDS

A. Total protein pattern (5 µl).
B. Immunoprecipitate of in vitro synthesized TH. 100 µl of the total protein synthesis mix were immunoprecipitated in the presence of 2 µg of pure TH. 60 µl of the sample were loaded onto the gel. Autoradiography was for 48 h.

reticulocyte cell-free system by exogenous poly(A) containing pheochromocytoma mRNA. The use of a direct immunoprecipitation technique, applied to the supernatant fraction of the cell-free system, allows an unambiguous electrophoretic characterization of a material migrating as a single band, with the same mobility as that of the major TH subunit. This technique is sufficiently sensitive to permit detection of very small amounts of newly synthesized TH. The number of methionine residues in TH is unknown: the minimal supposition would be 1 residue per 62,000 molecular weight polypeptide. Thus, as little as 10^{-15} mole of TH could be detected. Assuming that the TH specific mRNA is translated with the

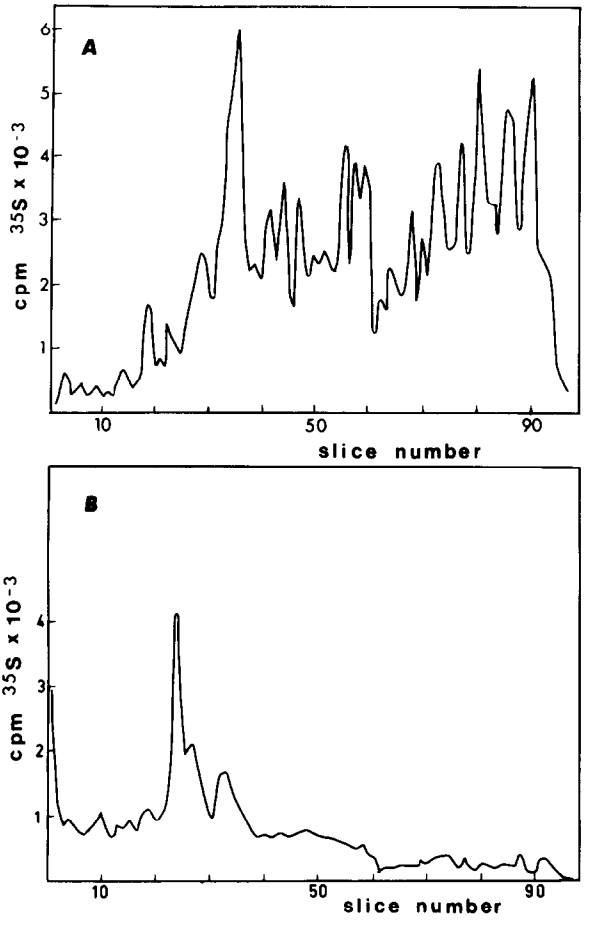

Figure 4

SDS gel electrophoresis of in vitro protein synthesis in the reticulocyte lysate system

The conditions are the same as described in Materials and Methods except the gel was made with 12% acrylamide. After coloration the gel was cut in slices and each slice counted.
A. 5 µl of the mixture before immunoprecipitation.
B. Immunoprecipitate of 60 µl of the 105 µl incubation mixture.

same efficiency as other pheochromocytoma species, its relative amount can be estimated to be of the order of 0.15%.

Interestingly, under our electrophoretic conditions (15% acrylamide, 0.1% bis acrylamide), the purified preparation of TH clearly migrated as a doublet, differing by no more than 12-13 amino acid residues, our technique being sufficiently precise to detect two proteins differing by 3 to 4 amino acid residues.

Table I

Immunoprecipitation of the cell-free products by TH antiserum

	cpm	%
Total protein synthesis	4.54×10^6	100
Total TH immunoprecipitated	6.861×10^3	0.15

Calculations were based on the data of Figure 4.
Background (650 cpm per slice) have been subtracted.

The complete superposition of the neosynthesized TH as revealed by autoradiography with the Coomassie blue coloured 62,000 molecular weight carrier TH would therefore lead us to think that under our in vitro translational conditions TH does not undergo processing of its polypeptide chain. Lack of post-translational modification under in vitro conditions is supported by competition experiments between in vitro synthesized TH and carrier TH (data not shown) showing that the affinity for antiserum of the neosynthesized TH is weaker suggesting that actually occurring TH is probably modified after its synthesis. A post-translational modification such as glycosylation or, more probably, phosphorylation since TH can be phosphorylated (18) seems likely.

The possibility of titrating TH specific mRNA will facilitate the further analysis of the control of TH gene expression in adrenergic clones of the nervous tissue. TH induction has been reported to accompany terminal differentiation in the rat embryo (19), in superior cervical ganglia (20), or in pheochromocytoma cells following stimulation by NGF or glucocorticoids (21,22).

Work is presently underway in our laboratory to further characterize the poly(A) containing TH messenger RNA fraction, and to obtain a cloned cDNA sequence.

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