A single RNA species injected in *Xenopus* oocyte directs the synthesis of active tyrosine hydroxylase

Philippe Horellou, Bernard Guibert°, Vincent Leviel° and Jacques Mallet+

Laboratoire de Neurobiologie Cellulaire et Moléculaire and °*Laboratoire de Physiologie Nerveuse, Centre National de la Recherche Scientifique, F-91190 Gif-sur-Yvette, France*

Received 4 July 1986

Tyrosine hydroxylase, the rate limiting enzyme in the biosynthesis of catecholamine, is a tetramer composed of four subunits of the same molecular mass. A full length cDNA clone encoding tyrosine hydroxylase has been inserted into the SP6 expression system. Translation of the corresponding RNA in *Xenopus* oocyte results in enzymatic activity, demonstrating that a single gene contains all the necessary genetic information to code for a functional enzyme. The potential of this system in the analysis of posttranslational tyrosine hydroxylase modifications is discussed.

Tyrosine hydroxylase (Xenopus laevis) *SP6 polymerase* *Translation*

1. INTRODUCTION

Tyrosine hydroxylase (EC 1.14.16.2, tyrosine 3-monooxygenase, TH) catalyses the rate limiting step in the biosynthesis of catecholamines and plays a key role in the physiology of catecholaminergic neurones [1]. This enzyme is available in relatively high amounts from the rat pheochromocytoma PC 12 cell line, facilitating biochemical studies. TH purified from this source has an apparent molecular mass of 220 kDa on sucrose density gradients and migrates as a single band of 62 kDa when subjected to SDS-PAGE suggesting that it is a tetramer composed of four subunits of the same molecular mass [2]. We have recently isolated a full length DNA complementary to a mRNA coding for the rat TH antigen [3] and could deduce the amino acid sequence [4]. We report here that translation of the corresponding RNA in *Xenopus* oocyte yields active TH establishing that the cDNA clone previously described contains the necessary genetic information to direct the synthesis of a functional form of this enzyme. This study was performed by exploiting the SP6 system which allows the synthesis of large amounts of a single RNA species [5]. The potential of this SP6 system in the analysis of posttranslational TH modifications is discussed.

2. MATERIALS AND METHODS

2.1. *Enzymatic reaction conditions and transformations*

DNA was manipulated following standard procedures [6]. DNA fragments were purified on DEAE-cellulose paper [7]. Inhibition of blunt end ligation was performed by using ATP at concentration of 5 mM [8]. Bacterial transformations in both *E. coli* JM-101 using pEMBL 9+ [9] and DH-1 using pSP6 [5] were performed as described [10].

2.2. *In vitro transcription*

pSP6-TH was linearized with *Hin*dIII and trans-

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

cripts of TH RNA were synthesized with SP6 polymerase as described [5]. Capping was achieved using 7meGpppG, in an equimolar concentration to GTP, in the transcription assay [11]. Northern blot analysis of RNA was performed as described [12].

2.3. *In vitro translation studies*

Approx. 0.5 μg RNA was incubated with 50 μCi [^{35}S]methionine, in a final volume of 25 μl rabbit reticulocyte lysate as described [13]. After a 60 min incubation at 30°C, the radioactivity incorporated into synthesized proteins was estimated by liquid scintillation counting after precipitation with trichloroacetic acid. In addition, 10 μl lysate were separated by SDS-PAGE [14] and analysed by autoradiography using either Kodak X-omat AR5 or Fuji Rx X-ray film. Autoradiography intensities were quantified with a densitometric scanner.

2.4. *Injection of oocytes with RNA and immunoprecipitation studies*

Mature oocytes from *Xenopus laevis* were microinjected with 50 nl water containing 25 ng of either capped or uncapped TH RNA, or with 50 ng PC 12 mRNA. Control oocytes were injected with an equal volume of water. Injected oocytes were incubated individually for two days at 19°C in 200 μl modified Barth saline medium (MBSM) [15] before being pooled in groups according to the original injection and assayed for enzyme activity (section 2.5). Protein labeling was achieved by incubating injected oocytes for 24 h in 200 μl MBSM followed by a further 24 h incubation in 30 μl MBSM containing 25 μCi [^{35}S]methionine. Immunoprecipitation was performed according to Dobberstein et al. [16], starting with 20 oocytes lyzed by sonication in 200 μl Tris buffered saline.

2.5. *Tyrosine hydroxylase activity*

TH activity was assayed by measuring the amount of tyrosine converted to DOPA as estimated by high-performance liquid chromatography (HPLC) using electrochemical detection (ECD) as modified from the technique of Buda et al. [17]. Typically, 20 oocytes were sonicated in 100 μl of ice-cold 5 mM potassium phosphate (pH 6.0) buffer containing 0.2% Triton X-100 before centrifugation, 10 000 $\times g$, 15 min at 4°C. Then 50 μl of the resulting supernatant was incubated for 15 min at 37°C with 50 μl assay cocktail to give a final reaction medium containing 100 mM potassium phosphate (pH 5.5), 0.5 mM NADPH, 1 mM 6-MPH$_4$, 2600 units catalase, 1 mM $FeSO_4$ and 40 μM L-tyrosine.

3. RESULTS

3.1. *Insertion of the tyrosine hydroxylase cDNA under the control of the SP6 promoter*

The full length TH cDNA was isolated from a pBR322 library in which the cDNA is inserted at the *Pst*I site by poly(dG)-poly(dC) tailing. The complete nucleotide sequence revealed that it contains one internal *Pst*I site [4]. The TH cDNA was first subcloned after partial digestion in the polylinkers of the pEMBL 9+ plasmid to facilitate its manipulation. Two clones pEM-TH 1 and pEM-TH 2 were isolated corresponding to the two possible orientations of the TH cDNA.

A series of manipulations were then performed to remove the poly(G) tail at the 5′-end of the TH cDNA by exploiting the unique *Alu*I restriction site contained within the 11 non-coding bp at the 5′-end. Since other *Alu*I restriction sites are present in the TH cDNA, the strategy presented in fig.1 was adopted. The pEM-TH 2 plasmid was first cleaved with *Apa*I and *Hin*dIII to generate a 1607 bp fragment that contains the entire coding sequence for TH. This moiety was then cleaved with *Hin*fI yielding a 5′- and a 3′-fragment containing 127 and 1480 bp, respectively. The single *Alu*I site present in the small fragment could then be used to remove the poly(G) tail. The resulting fragments and the 1480 bp moiety were then ligated together with the plasmid pEM-TH 1 previously cleaved with *Apa*I and *Hin*dII, to yield the recombinant plasmid pEM-TH 19. The correct construction of pEM-TH 19 was confirmed by restriction mapping with the enzymes *Pst*I, *Bam*HI, *Apa*I, *Eco*RI and *Hin*dIII.

The *Bam*HI, *Hin*dIII insert of pEM-TH 19 which contains both the coding and the entire 3′-non-coding regions of TH mRNA was then cloned into the corresponding site of the SP6 vector. Screening of the recombinant pSP6 clones was facilitated both by cleaving pEM-TH 19 with *Pvu*I, which inactivate the β-lactamase gene, and by further cleaving pSP6 with *Hin*dII in order to hamper the recloning of the linker moiety as ligation was carried out at 5 mM ATP. The restriction

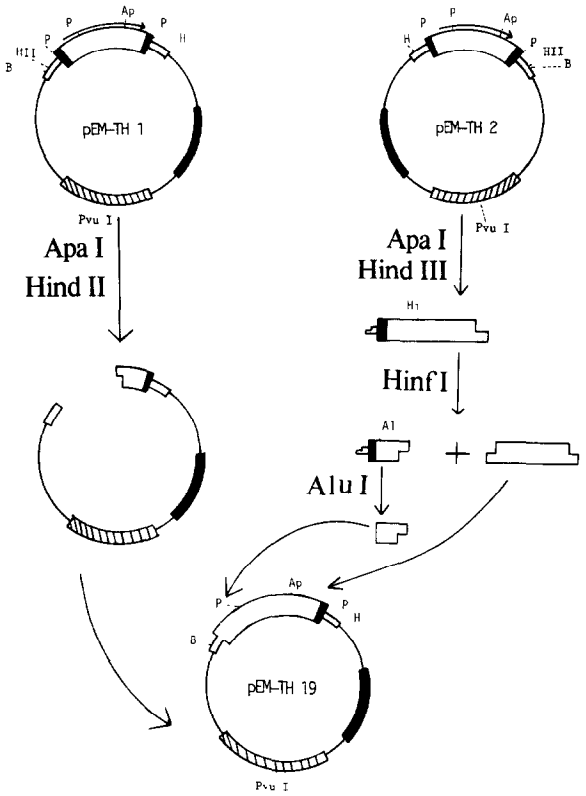

Fig.1. Subcloning the TH coding region in the pEMBL 9+ plasmid. Circles represent plasmids. Shaded boxes represent the intragenic region of phage F1 and hatched boxes represent the β-lactamase gene. Small open boxes represent the coding sequence for α-peptide of β-galactosidase containing a multiple cloning sites polylinker and large open boxes represent TH cDNA while the surrounding large shaded boxes correspond to poly(dG-dC) tails. Arrows indicate the relative orientation of TH cDNA. Symbols for restriction enzymes are as follows: P, *Pst*I; B, *Bam*HI; H, *Hin*dIII; HII, *Hin*dII; AI, *Alu*I; Ap, *Apa*I; Hi, *Hin*fI.

fragments of pEM-TH 19 and pSP6 were ligated and the correct pSP6 recombinant selected on the basis of its resistance to ampicillin and its restriction map.

3.2. *Synthesis of RNA from pSP6-TH*

Transcription was carried out using pSP6-TH linearized with *Hin*dIII. Analysis of the resulting transcripts by Northern blot revealed a single RNA species which migrates as PC 12 TH mRNA. The first 37 nucleotides originate from the SP6 vector with the following corresponding to the TH cDNA, beginning 7 nucleotides away from the initiation AUG codon and extending to the poly(A) and poly(C) tails. About 10 μg RNA was obtained from 5 μg pSP6-TH plasmid. These RNA could be capped during transcription using ^{7}meGpppG as substrate, with an efficiency of $\simeq$50% and these conditions did not modify either the yield of RNA synthesis or the apparent length on Northern blot (not shown).

3.3. *Translation of RNA transcribed from pSP6-TH*

The translation of RNA derived from the pSP6-TH template was first examined using the rabbit reticulocyte lysate. Translation occurred with uncapped RNA but was increased 5-fold in the presence of capped RNA giving a 5% incorporation of [^{35}S]methionine into precipitable material. The translation products were analyzed by SDS-PAGE followed by autoradiography as described in section 2. Short time exposure of the autoradiograms revealed a single band corresponding to a 62 kDa

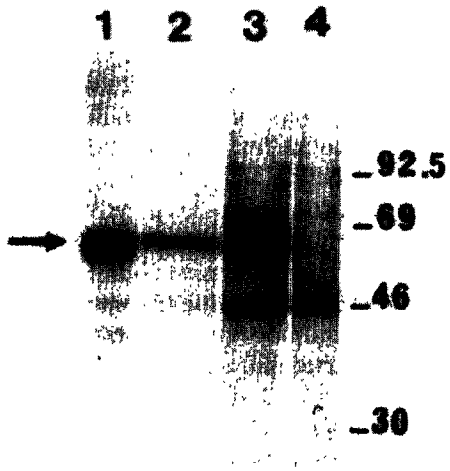

Fig.2. Detection of TH RNA translation products. Samples are all heated for 5 min at 100°C and subjected to SDS-PAGE (10% polyacrylamide). Lanes: 1, translation products from the reticulocytes lysate; 2 and 3, translation products from 4 and 16 oocytes injected with TH RNA, respectively, following immunoprecipitation; 4, immunoprecipitation of translation products from 16 control oocytes injected with water. Molecular mass markers are given in kDa. The arrow indicates the position of TH antigen (62 kDa).

Table 1

TH activity in injected oocytes

	TH enzymatic activity
Capped TH RNA	99.0 ± 1.5 (n = 5)
Uninjected	0.7 ± 0.1 (n = 5)
Uncapped TH RNA	0.8
PC 12 mRNA	0.7
Water	0.7

Enzyme assays were as described in section 2. The results are expressed in pmol DOPA formed/h for 20 oocytes. In the case of capped TH RNA injected and uninjected oocytes, values correspond to mean ± SE where $n = 5$. In the case of uncapped TH RNA, PC 12 mRNA and water injected oocytes, values were each from one pool of 20 oocytes

protein, resulting from the translation of both capped and uncapped RNA. However, the addition of capped RNA led to a five-fold increase in the intensity of the autoradiographic signal confirming the above results. This protein comigrates with native TH immunoprecipitated from PC 12 mRNA translation products (not shown). Longer exposure time of the autoradiograms revealed other bands of higher and smaller molecular mass (fig.2). However, only the major band could be identified when translation products were analyzed with a specific TH antibody. No enzymatic activity could be detected when the translation products were assayed for TH.

As shown on fig.2 a band equivalent to a 62 kDa protein was also detected after immunoprecipitation by TH antiserum of oocyte proteins following microinjection with capped TH RNA. However, in sharp contrast with the reticulocyte system significative TH enzymatic activity was assayable in the injected oocytes as shown in table 1. Control oocytes and oocytes injected with both mRNA from rat PC 12 cells or with uncapped RNA gave no detectable activity.

4. DISCUSSION

In this paper we have shown that the injection in *Xenopus* oocytes of a single RNA species yields TH activity. This result also gives further weight to the previous report that the native enzyme is formed of a homotetramer [2].

A cDNA clone encoding immunoreactive TH was subcloned in the SP6 vector system following removal of the poly(G) tail at the 5′-end since we have shown, in several cases (unpublished), that this stretch of nucleotides inhibits the progression of ribosomes. The RNA obtained by in vitro transcription contains coding sequences that are identical to native TH mRNA and differ only in the 5′-non-coding portion.

Translation of the corresponding capped mRNA in both the reticulocyte lysate and in the *Xenopus* oocyte leads to the synthesis of a protein whose size is identical to that of the native enzyme. In agreement with previous reports [11], the translation efficiency was very poor using uncapped RNA. Interestingly, TH enzymatic activity could only be detected after injection in the oocyte. It is possible that this simply reflects the better efficiency of the oocyte translational machinery as compared with that of the reticulocyte lysate. However, it is most probable that enzyme activity results from posttranslational modifications which are more likely to occur in the oocyte. More particularly the activity of this enzyme has been found to be modulated by a variety of kinases [18–20] including cAMP-dependent protein kinase, an active form of which is found with cAMP, in the oocyte.

Clearly the availability of a pure RNA species encoding TH sequences should greatly facilitate the characterization of the phosphorylation and other posttranslational events that initiate and modulate the activity of the TH enzyme.

ACKNOWLEDGEMENTS

We thank Danielle Grégoire and Gisèle Peudevin for excellent technical assistance; François Blanot, Nicole Faucon Biguet, Brigitte Grima, Annie Lamouroux and Georges Lutfalla for helpful discussions; Philippe Kahn for providing rabbit polyclonal TH antibody; Kathleen Metters, John Powell and Philippe Vernier for critical reading of the manuscript; Marceline Feigel for typing the manuscript. This work was supported by grants from the Centre National de la Recherche Scientifique, the Fondation pour la Recherche Médicale Française, the Association pour la Recherche sur le Cancer and Rhône-Poulenc Santé.

REFERENCES

[1] Nagatsu, T., Levitt, M. and Udenfriend, S. (1964) J. Biol. Chem. 239, 2910–2917.
[2] Markey, K.A., Kondo, S., Shenkman, L. and Goldstein, M. (1979) Mol. Pharmacol. 17, 79–85.
[3] Lamouroux, A., Faucon Biguet, N., Samolyk, D., Privat, A., Salomon, J.C., Pujol, J.F. and Mallet, J. (1982) Proc. Natl. Acad. Sci. USA 79, 3881–3885.
[4] Grima, B., Lamouroux, A., Blanot, F., Faucon Biguet, N. and Mallet, J. (1985) Proc. Natl. Acad. Sci. USA 82, 617–621.
[5] Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984) Nucleic Acids Res. 12, 7035–7056.
[6] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1983) in: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[7] Dretzen, G., Bellard, M., Sassone-Corsi, P. and Chambon, P. (1981) Anal. Biochem. 112, 295–298.
[8] Ferretti, L. and Sgaramella, V. (1981) Nucleic Acids Res. 9, 3695–3705.
[9] Dente, L., Cesareni, G. and Cortese, R. (1983) Nucleic Acids Res. 11, 1645–1655.
[10] Hanahan, D. (1983) J. Mol. Biol. 166, 557–580.
[11] Contreras, R., Cheroutre, H., Degrave, W. and Fiers, W. (1982) Nucleic Acids Res. 20, 6353–6362.
[12] Faucon Biguet, N., Buda, M., Lamouroux, A., Samolyk, D. and Mallet, J. (1986) EMBO J. 5, 287–291.
[13] Merrick, W.C. (1983) Methods Enzymol. 101, 606–615.
[14] Laemmli, U.K. (1970) Nature 227, 680–685.
[15] Barth, L.G. and Barth, L.S. (1959) J. Embryol. Exp. Morph. 7, 210–222.
[16] Dobberstein, B., Garoff, M., Warren, G. and Robinson, P.J. (1979) Cell 17, 759–769.
[17] Buda, M., Roussel, B., Renaud, B. and Pujol, J.F. (1975) Brain Res. 93, 564–569.
[18] Joh, T.H., Park, D.H. and Reis, D.J. (1978) Proc. Natl. Acad. Sci. USA 75, 4744–4748.
[19] Vulliet, P.R., Woodgett, J.R. and Cohen, P. (1984) J. Biol. Chem. 259, 13680–13683.
[20] El Mestikawy, S., Glowinski, J. and Hamon, M. (1983) Nature 302, 830–832.