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# Determination of the protooncogene ets-2 gene transcript in human brain at the atto-gram-level by the use of competitive RT/PCR

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**Summary.** Protooncogenes (PO) play a crucial role for brain biology and pathology. Only the concerted action of protooncogenes enables normal brain development.

The reliable and sensitive quantification of brain PO is still holding centre stage in neurobiological research. The aim of our study was therefore the determination of PO in minute amounts of brain areas. For this purpose we decided to apply the most sensitive detection principle of competitive reverse transcriptase polymerase chain reaction using capillary electrophoresis and laser-induced fluorescence detection.

We selected the PO ets-2 for our studies as this transcription factor was shown to be involved in neurodegenerative disease. As little as 10 ng of total RNA each were extracted from 5 different regions of human postmortem brain and used in the assay system. Our results revealed that the ets-2 gene transcript was detectable at the atto-gram level in the brain (54.5 ± 17.7 ag/10 ng RNA in the occipital lobe, 34.2 ± 7.5 in temporal lobe, 40.2 ± 15.6 in the frontal lobe, 31.4 ± 15.7 in the cerebellum, and undetectably low in the parietal lobe).

This is the first report at this sensitivity level providing neurobiology with a powerful analytical tool.

**Keywords:** Amino acids – RT/PCR – Protooncogene – ets-2

## Introduction

The protooncogene ets-family is thought to play crucial roles in the transcriptional regulation of many genes. It is defined by a highly conserved DNA-binding domain which recognizes a nucleotide consensus sequence of GGAA and the DNA-binding motif has been found in the regulatory control regions of numerous genes (Bhat et al., 1987). Only ets-2, however, is ubiquitously expressed and serves important functions in several tissues during critical

stages of organ development, including the brain. Functionally, ets-2 is activating transcription through interaction with the transcription factors AP-1 and PEA 3, an interaction with c-Fos and c-Jun, required for efficient activation of transcription from different promotors by many individual PO, serum growth factors and the tumour promoter TPA (Wasylyk et al., 1990).

Differential expression of the ets-2 revealed by in situ hybridization technique during murine embryogenesis has been reported in 1994: ets-2 expression was not found in meningeal and capillary structures of the CNS. However, a moderate level of ets-2 was found in the medulla oblongata and throughout the entire length of the developing spinal cord from day 13–17. ets-2 expression within the brain is not significant until after birth. In the postnatal period, as brain structures continue to develop, ets-2 expression increases during the first three weeks of life in multiple structures. High levels of expression are detected in the dentate gyrus, the CA1, CA2 and CA3 areas of the hippocampus as well as in the granule layer of the cerebellum, while a weak to moderate expression are present in cerebral cortex, caudate putamen, septal nucleus, anterior and inferior calyculus. ets-2 expression in these multiple regions of the brain was maintained throughout life (Maroulakou et al., 1994).

In humans no systematic studies in the brain were reported although ets-2, which is encoded on chromosome 21, was proposed to be overexpressed in patients with Down syndrome (trisomy 21) and animal models of Down syndrome (Hernandez et al., 1996). The key role of ets-2 in the interaction between PO, growth factors with the biological meaning of growth, proliferation and differentiation made us create a highly sensitive assay system for the ets-2 transcript in brain.

## Materials and methods

### *Reagents*

RNAzol B from Molecular Research Center, Inc. was used for isolation of tRNA pSP64 Poly(A) Vector from Promega, TA Cloning Kit from Invitrogen BV, restriction enzymes and Rapid DNA Ligation Kit were purchased from Boehringer Mannheim GmbH. Oligo(dt) Cellulose Columns, DNA and RNA size standard were purchased from Gibco BRL and AmpliTaq DNA Polymerase, RNase Inhibitor, Oligo d(T)16, dNTPs, MuLV Reverse Transcriptase were purchased from Perkin-Elmer. PCR was performed on a Perkin-Elmer GeneAmp PCR System 9600. ABI-310 Genetic Analyzer with 47 and 61 cm (50 $\mu$m) capillaries. Performance Optimized Polymer 4 and GENESCAN-500 TAMRA as an internal lane standard were used.

### *Patients*

Postmortem brain samples (n = 8; 5 males, 3 females) from individuals with no history of neurological or psychiatric illness or brain (histo-)pathology were obtained from the MRC's London Brain Bank for Neurodegenerative Diseases, Dpt Neuropathology, Institute of Psychiatry. The mean age of individuals was 52 ± 11 (SD) years, the mean postmortem interval 37 hrs ± 12 hrs.

### *Tissue preparation and RNA extraction*

2–4 mg tissue from different brain regions including frontal lobe (F), temporal lobe (T) parietal lobe (P), occipital lobe (OC) and cerebellum (C) were immediately frozen in

liquid nitrogen and stored at −70°C. Total cellular RNAs were extracted from the samples by the RNAzol B reagent following the protocol established by Chomczynski and Sacchi (1987) using an acid Guanidinium Thiocyanate-Phenol-Chloroform extraction method and precipitation by isopropanol. Total RNA was rinsed twice with 75% ethanol and then treated with DNAse I (Gibco BRL) for 15 minutes at room temperature before reextraction using the same protocol. Pellet was air dried and resuspended in RNase free water. The amount of RNA recovered was quantified by UV absorbance spectrophotometry at 260/280 nm stored at −70°C for 6–8 weeks.

### *Synthesis of RNA internal standard*

We produced a synthetic RNA internal standard using a Competitor DNA-cloning strategy. The primer sequences were selected with the OLIGO Program from Rychlik and Rhoads (1989). Bluescript vector containing the Ets2-cDNA (wich was a gift from R. H. Scheuermann, Southwestern Medical Center, Dallas) was linearized by the restriction enzyme EcoR I.

In the first step two DNA fragments each with an overlapping end of 43 bp was amplified separately. Primer pair 1 with a 21 bp linker at the antisense primer (5′GCAGCGGCAGGATGAATGAT3′ and 5′**CTCTGTGCCAAAACCTAATGT**AGGAACGGACCTGAGGTGTGAA3′) amplified a 596 bp fragment and primer pair 2 with a 22 bp linker at the sense primer (5′**TTCACACCTCACCTCCGTTCCT**ACATTACCTTTTGGCACAGAG3′ and 5′GGCTTATTGAGGCAGAGAGAC3′) amplified a 420 bp fragment. Both amplified DNA fragments were extracted from 1% low melting agarose gel with the GELase Agarose Gel-Digesting Preparation Kit from Epicentre Technologies and quantificated by UV absorbance spectrophotometry.

Both DNA fragments were mixed in equal concentrations and used as templates in the PCR reaction to amplify one DNA fragment of 1,006 bp length. Therefore the sense primer from primer pair 1 linked with a Hind III restriction enzyme site and the antisense primer from primer pair 2 linked with a BamHI restriction enzyme site were combined as primer pair. PCR conditions were: 10× reaction buffer, 2 mM MgCl2, 150 mM each dNTP, 300 nM each primer and 1 U Taq Polymerase in a 50 ml reaction volume. The amplification procedure was performed in a Perkin Elmer thermocycler 9600 starting with a 3 min. denaturing step at 94°C, followed by a 35 cycle step with denaturing 30 sec. at 94°C, annealing 15 sec. at 60°C and elongation 30 sec. at 72°C and ending with a 7 min. elongation step at 72°C. The sequence of the 1,006 bp DNA fragment lacked a 18 bp fragment between position 856 and 874 to the original sequence of the cDNA of ets-2 which was confirmed by sequencing the synthetic DNA fragment with fluorescent labeled nucleotides using the ABI Prism 310 Genetic Analyzer from Perkin Elmer. The 1,006 bp DNA fragment was cut with the restriction enzymes HindIII and BamHI and purified with the PCR Purification Kit from Qiagen.

The DNA fragment was ligated into the pSP64 Poly(A) Vector from Promega between the Hind III and BamHI restriction enzyme sites.

### *In vitro RNA-synthesis*

After transforming E.coli cells, grown in culture medium and purification with the Qiagen Plasmid Extraction System (Qiagen, Inc) the pSP64 Poly(A) Vector containing our constructed insert, it was linearized with the restriction enzyme EcoRI. For in vitro RNA synthesis 3 $\mu$g linearized plasmid, 5× Gibco buffer (200 mM Tris-HCl, 30 mM MgCl2, 10 mM spermidine hydrochloride), 10 mM DTT, 20 $\mu$M each rNTP, 10 U human placental RNase Inhibitor, 35 U SP6 polymerase were incubated in a 25 $\mu$l volume at 40°C for 1 hour.

After treatment with RNase-free DNase I (Gibco BRL) for 15 min the in vitro synthesized RNA fragment was recovered by ethanol precipitation, resolved in DNase and RNase free water and an aliquot was visualized on a 1% agarose gel electrophoresis

by ethidiumbromide staining (fragment size was seen nearby the 500 bp ladder as single band).

Thereafter the synthetic RNA fragment was purified by oligo(dt) affinity chromatography using Oligo(dt) Cellulose Columns (Gibco BRL). The quantity and purity of the synthetic RNA was determined by OD 260/280 absorbance spectrophotometry. Reverse transcription PCR (RT-PCR) amplification from the synthetic RNA was confirmed with the original primer pair (sense primer from primer pair 1 and antisense primer from primer pair 2) with an RNAse-treated sample used as a negative control to rule out contamination of any DNA template.

Several synthetic RNA aliquots ranging from 12 pg/$\mu$l to 0.012 fg/$\mu$l were diluted in yeast tRNA (1 mg/ml) in sialinised tubes (Sialinising Solution from SERVA) to prevent absorption (adhesion) to plasticware. Several PCR reactions performed with yeast tRNA as template were negative and therefore interferences between the synthetic RNA and yeast tRNA during transcription and amplification could be excluded.

### *Competitive RT-PCR*

First-strand synthesis of cDNA was accomplished in sialinisated Eppendorf tubes by adding serial dilutions of the synthetic RNA (0.012 fg to 1.2 pg) to equal quantities of total RNA (10 ng) extracted from brain samples to a total volume of 20 $\mu$l of 4 mM MgCl2, PCR Buffer II from Perkin Elmer, 1 $\mu$M dGTP, 1 $\mu$M dATP, 1 $\mu$M dTTP, 1 $\mu$M dCTP, 1 U/$\mu$L RNase Inhibitor, 2.5 $\mu$M Oligo d(T) 16 primer and Moloney murine leukemia virus reverse transcriptase 2.5 U/$\mu$L (all components from Perkin-Elmer). The reaction was carried out at room temperature for 10 min. and then at 42°C for 15 min., followed by heat inactivation of the enzyme at 99°C for 5 minutes and rapidly cooled to 5°C for 5 min.

Nested PCR reactions was performed by adding 5 $\mu$l of cDNA to a total volume of 50 $\mu$l of 10×PCR Buffer II of Perkin-Elmer, 2 mM MgCl2, 150 $\mu$M each dNTP, 400 nM each primer (forward 5′TGGAGTGAGCAACAGGTATG3′ and reverse 5′GGCTTATTGAGGCAGAGAGAC3′) and 2 U Taq Polymerase. For amplification the temperature profile started with denaturing for 3 min. at 94°C, followed by 20 cycles with denaturing at 94°C for 30 sec., annealing at 60°C for 30 sec. and elongation at 72°C for 1 min. and stopped with elongation for 7 min. at 72°C.

The first step PCR-products were of a length of 665 bp for the ets2 cDNA and of 647 bp for the synthetic cDNA fragment. The second step was a nested-PCR with a FAM-labeled primer and the products were of a length of 284 bp for the ets2 cDNA and of 266 bp for the synthetic cDNA fragment. Second nested-PCR was performed by adding 2 $\mu$l of first nested-PCR product to a total volume of 50 $\mu$l of PCR buffer of Perkin Elmer, 2 mM MgCl2, 150 $\mu$M each dNTP, 400 nM each primer (forward FAM-labeled 5′CTGGAGCTGGCACCTGACTT3′ and reverse 5′GACTTGGGGAACATCTGAAACT3′) and 1 U/$\mu$L AmpliTaq DNA polymerase.

### *Quantitation of PCR products*

For quantitation 0.5 $\mu$l of each FAM-labeled nested-PCR product were mixed with 20 $\mu$l Formamide and 0.5 $\mu$l GENESCAN-500 TAMRA internal lane standard solution. GENESCAN-500 TAMRA provides 16 single-stranded fragments and is designed for sizing DNA fragments in the 35–500 bp range.

These fragments are uniformly spaced to provide extremely accurate base calling. After denaturation at 92°C for 5 min. samples were placed in a 48 sample-tray and automatically analyzed on the ABI PRISM 310 Genetic Analyzer. The results were analysed with the GeneScan Analysis 2.1 Software from Applied Biosystem.

## Results

We found ets-2 concentrations in the atto-gram range expressed as atto-grams per 10 ng of total RNA. ets-2 concentrations were comparable in occipital

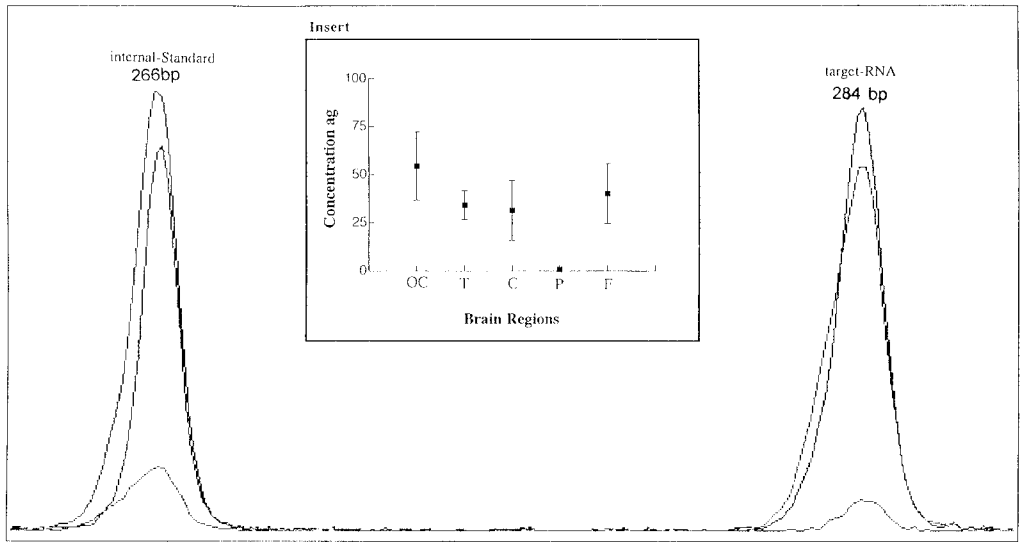


**Fig. 1.** The linearity of the competitive RT/PCR is shown: peaks on the left hand represent the peak height/area of the internal standard (exogenous template of 262 bp = target-RNA – 18 bp); peaks at the right hand represent peak height/area of the endogenous template = target-RNA of 280 bp. Linearity of the assay within the range of 1,2 fg–12 ag was documented

lobe (54.5 ± 17.7 SEM), temporal lobe (34.2 ± 7.5), frontal lobe (40.2 ± 15.6) and cerebellum (31.4 ± 15.7) but undetectably low in parietal lobe (insert, figure 1). There was no significant correlation between ets-2 levels with age ($r = 0.61$, $p = 0.1$) or postmortem interval ($r = 0.2$, $p = 0.5$). Only total RNA from brains with comparable beta-actin, used as a housekeeping gene and reflecting integrity of gene transcripts were used (Oyama et al., 1994). Methodologically, linearity of the assay is shown in Fig. 1, the coefficient of variation was 2.5%. Congruent results were obtained when either peak height or peak area were used for calculations.

## Discussion

Methodologically, our results show the determination of the ets-2 gene transcript in the atto-gram level using 10 ng of total RNA extracted from brain regions.

The method of RT/PCR using co-amplification of a standard-template originated with early work by Wang et al. (1989) and was subsequently further developed by a series of others (Personett et al., 1996). In this quantitation principle, a known amount of a "standard sequence", possessing primer annealing sites in common with the native sequence to be quantitated, is added as RNA and the target DNA (the "endogenous template") is co-amplified with a known amount of this "external DNA standard or exogenous template": Thus a competitive reaction is used for the determination of a gene

transcript. The rapid technical development of the capillary electrophoretic detection principle lead to already enormous sensitivity and specifity which was further perfected by the introduction of laser-induced fluorescence detection of PCR products on capillary electrophoresis (Vincent et al., 1996).

Investigating the technological and chemical basis for the higher sensitivity of our competitive RT/PCR-LIF principle (we used 10 ng of total RNA extracted from brain rather than others who used a minimum of 1 microgram RNA) was not the aim of this study but apart from column length, applied voltage, gel and camera type and dimensions of the instrument, the fluorophore used seems to be the major determining factor (Issaq et al., 1997).

FAM-(5-carboxyfluorescein) labeling in contrast to other fluorescence dyes is not intercalating with DNA but linked to the 5′ end of DNA and is therefore sequence – independent and stoechiometric as only one FAM molecule is attached to one DNA molecule. Biologically, the meaning of the undetectably low gene transcript level in the parietal lobe cannot be explained. Transcription activity in parietal lobe per se as evaluated by the determination of the housekeeping gene, beta-actin (data not shown), was comparable to the other brain regions studied. Comparable housekeeping gene transcription also rules out destruction of RNA in the parietal lobe.

Now, the sensitive tool and methodology is available, individual areas, however small they are, can be easily, rapidly and reliably studied to map the brain for PO in general and ets-2 in particular.

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