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Utilization of a two-standard system in real-time PCR for quantification of gene expression in the brain

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Abstract

In this study, we applied for real-time PCR the two-standard system that we had worked out previously for PCR with gel-detection of products. Genomic DNA of a known concentration was used as external standard and mRNA of the DNA-dependent RNA-polymerase II was used as internal standard. It was shown that PCR with gel-detection of products and real-time PCR provide similar results and demonstrate almost identical accuracy and repeatability when the two-standard system is used. With the help of the both methods and using the two-standard system we have confirmed the link between the genetically determined freezing reaction in mice and reduced 5-HT_{1A} receptor mRNA level in the midbrain. We have also found that the genetically determined freezing reaction in mice is not connected with changes in Tph2 gene expression.

Keywords: 5-HT_{1A} receptor; Tph2; mRNA level; RT-PCR; Real-time RT-PCR; Catalepsy

1. Introduction

At present, the most sensitive method for gene expression studies is RT-PCR (Jordan et al., 2007; Zamorano et al., 1996). During reverse transcription cDNA is synthesized on RNA template and the number of cDNA copies is evaluated with the help of PCR. One of the multiple varieties of this method that has been widely used recently is real-time PCR which allows detecting the accumulation of PCR-products immediately in the reaction process without using the additional electrophoresis stage. The quantity of amplicon is proportionate to the initial amount of template, which makes it possible to determine DNA quantitatively. One of the most commonly used real-time PCR variants consists in using an intercalating dye (for instance, SYBR Green I) that joins the newly synthesized amplicon, which leads to a multifold fluorescence growth that can be detected (Zannoni et al., 2007). A grave shortcoming of this method is the dye non-specificity. Since the intercalation occurs in any DNA-duplexes including, for instance, primer–dimers, this leads to an increase in fluorescence level and, consequently, to a greater measurement error (Ball et al., 2003). Furthermore, using an intercalating dye limits the application of both internal and external standards. For example, genomic DNA cannot be used as external standard because the dye non-specificity leads to an enormous background. As regards internal standard, intercalating dye does not allow to use competitor. Moreover, minor primers non-specificity leads to a substantial background growth and, hence, increases the error.

Another variety of real-time PCR is the amplification with fluorescently labeled hybridization probe (Abe et al., 1999). This is an oligonucleotide complementary to an area of the target PCR-product that is about 30 monomers long and contains a fluorophore on one end and a fluorescence quencher—on another. Taq-polymerase splits the probe during elongation due to its $5' \rightarrow 3'$ exonuclease activity. The increase in distance between the fluorophore and the quencher results in fluorescence growth that can be detected (Bustin, 2000). This method allows estimating the accumulation of products after each reaction step.

A system of internal or external standards is usually used for standardization. Listed below are the most commonly applied external standards: (1) artificially cloned fragment of the target gene DNA, containing annealing sites for the primers used for evaluation of the target gene expression (Lole and Arankalle, 2006); (2) genomic DNA (Cuscó et al., 2002; Kulikov et al.,

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2005); (3) amplicon (Zhao et al., 2006). Genomic DNA is the most universal since it represents all the genes of an organism (Cuscó et al., 2002; Kulikov et al., 2005). Furthermore, calibration curves that are obtained using genomic DNA are far more stable than those obtained using amplicons, which allows comparing results of different experiments. One of the shortcomings of genomic DNA as a standard is that it cannot be applied when using intron-spanning primers which anneal to two gene exons divided by an intron (Zamorano et al., 1996). In this connection, RNA samples should be checked for the absence of genomic DNA contamination (Naumenko and Kulikov, 2006).

Using an external standard that is completely identical in sequence to the target fragment supposes no difference in amplification efficacy of samples and standard and allows a more accurate determination of mRNA level. However, using an external standard does not enable one to control RNA extraction and the reverse transcription. The most effective solution for this problem is applying an endogenous internal standard. This is most often mRNA of a housekeeping gene that is expressed in a majority of tissues at almost equal levels (Cuscó et al., 2002; Yang et al., 2005). Only using such a standard enables controlling both RNA extraction and the reverse transcription reaction, which allows, along with using an external standard, as we have showed previously, a reliable and accurate quantitative determination of the target mRNA level (Kulikov and Naumenko, 2007; Naumenko and Kulikov, 2006).

Although the described system of standards can be used for investigation of any gene, its applying is particularly relevant when studying genes without introns. The latter include the genes encoding 5-HT_{1A} serotonin receptor, α2, β1 and β2 adrenergic receptors which play an important role in brain signal transduction and, therefore, are of a great interest to many neuroscientists. 5-HT_{1A} receptors mediate serotonin (5-HT) action and play the key role in the autoregulation of 5-HT neurons activity and neuromediator secretion (Barnes and Sharp, 1999). Serotonin is involved in regulation of various physiological functions and types of normal and pathological behavior: sleep, thermoregulation, central regulation of endocrine glands secretion, stress response, sexual and aggressive behavior, appetite and thirst (Jacobs and Fornal, 1995; Lucki, 1998; Olivier, 2005; Popova et al., 1978; Saudou and Hen, 1994). The recently discovered tryptophan hydroxylase 2 (Tph2) gene encodes the main enzyme of brain 5-HT synthesis and, hence, is likely to be implicated in the regulation of all the behavioral forms and mental processes controlled by this mediator (Walther et al., 2003).

The aim of the present study is applying the two-standard system that we have developed previously to real-time PCR in order to determine 5-HT_{1A} and Tph2 genes expression in mouse brain. To compare this method with the previously developed technique for quantitative determination of gene expression by means of gel-detection of PCR-products (Naumenko and Kulikov, 2006), we used both for studying 5-HT_{1A} receptor and Tph2 genes expression in the brain of mice. As a model for our investigation we have chosen the mice with genetically determined freezing reaction or catalepsy (CBA strain) and the non-cataleptic mice (AKR strain). Previously we have found a significant difference in 5-HT_{1A} receptor mRNA level between these two strains (Naumenko et al., 2006), but there was no data on Tph2 gene expression in brain of mice with genetically determined freezing reaction.

2. Materials and methods

2.1. Animals

Experiments were carried out on adult (aged 10–12 weeks) males of AKR/J and CBA/LacJ strains, 10 animals in each group. CBA strain is characterized by a high rate of cataleptic mice. About 54% of CBA mice demonstrate this kind of defensive behavior in response to a series of nap pinches, while there are no cataleptics among AKR mice (Kondaurova et al., 2006).

Animals were decapitated and the midbrain was taken. The samples were frozen in liquid nitrogen and kept at −65 °C until the RNA extraction. All experimental procedures were made in compliance with the Guidelines for Ethical Conduct in the Care and Use of Animals (developed by Committee on Animal Research and Ethics, 1991; http://www.apa.org/science/anguide.html).

2.2. Reagents

Taq-polymerase (30 U/μl) and M-MLV reverse transcriptase (750 U/μl) (Biosan, Novosibirsk, Russia). RNase-free DNase (1 U/μl) (Promega, USA). Set of dNTPs (100 mM each) and PCR buffer (60 mM Tris–HCl, 25 mM KCl, 10 mM 2-mercaptoethanol, 0.1% Triton X-100), $MgCl_2$ (SibEnzyme, Novosibirsk, Russia). Tris, guanidine isothiocyanate, SDS, EDTA, KCl, NaCl, $MgCl_2$, $MnCl_2$, DTT and Triton X-100 (Sigma, USA). Phenol, chloroform, isopropanol and ethanol (Reakhim, Moscow, Russia).

All the primers and hybridization probes containing fluorescein (FAM) at the 5′-end and fluorescence quencher (BHQ1) at the 3′-end used in the present work were synthesized on the synthesizer ASM-800 (Biosset, Novosibirsk) following a standard procedure. Nucleotide sequences and annealing temperatures for all the oligonucleotides used in PCR with gel-detection of products and real-time PCR are presented in Tables 1 and 2, respectively. The primers and hybridization probes for 5-HT_{1A} receptor, Tph2 and DNA-dependent RNA-polymerase II (rPol II) were designed on the basis of published sequences (Charest et al., 1993; Corden et al., 1985; Walther et al., 2003, respectively) using the EMBL Nucleotide database and GeneBank.

The primers for rPol II and Tph2 were designed in such a way that the corresponding fragments of genomic DNA were not divided by an intron.

In the presented technique, two standards, an internal and an external, were used. As internal standard we used rPol II mRNA, which allowed us to control RNA extraction, reverse transcription and served as the basis to calculate the receptor and Tph2 mRNA levels.

As external standard, we used genomic DNA extracted from hepatocyte nuclei of a C57BL/6 mouse (Moisan et al., 1996). Concentration of the obtained DNA was assessed by its optical density measured at 260 nm. The extracted DNA

Table 1
Sequences and annealing temperatures of primers used for PCR with gel-detection of products

Gene	Sequence	Annealing temperature (°C)	PCR-product size (bp)
5-HT$_{1A}$	F 5′-gactgccaccctctgccctatatc-3′; R 5′-tcagcaaggcaaacaattccag-3′	62	200
Tph2	F 5′-cattcctcgcacaattccagtcg-3′; R 5′-agtctacatccatcccaactgctg-3′	61	239
RNA-polymerase II	F 5′-gttgtcgggcagcagaatgtag-3′; R 5′-tcaatgagaccttctcgtcctcc-3′	63	188

Table 2
Sequences and annealing temperatures of primers and hybridization probes used for real-time PCR

Gene	Sequence	Annealing temperature (°C)	PCR-product size (bp)
5-HT$_{1A}$	F 5′-gccctatatctggaactttgagg-3′; R 5′-ggagaccagaatcagcagtgta-3′; FAM-5′-ctgctctgtctctccctccctccttc-3′-BHQ1	61	112
Tph2	F 5′-cgatctggcttcacagtgagac-3′; R 5′-tgggtgcagtggaatactctgtag-3′; FAM-5′-acctgagcccaagagacttcctggc-3′-BHQ1	59	92
RNA-polymerase II	F 5′-cctaacctatccattgaccaagtg-3′; R 5′-aagggtgtgacaatctctgc-3′; FAM-5′-tgccccgctccattgctgcca-3′-BHQ1	59	80

was stored at −20 °C. The size of mouse genome is known to be 2.493×10^9 bp (Mouse Genome Informatics, http://www.informatics.jax.org), which corresponds to a mass of 5 pg. In the present work, we used standard solutions containing from 2.5 to 60 ng/μl of genomic DNA (from 500 to 12000 copies/μl, respectively).

2.3. Total RNA

Total RNA was obtained using extraction with phenol, guanidine isothiocyanate and chloroform and then treated with RNase-free DNase. Total RNA was checked for the absence of genomic DNA admixture by means of amplification with beta-actin primers (Naumenko and Kulikov, 2006).

2.4. Reverse transcription

A 8 μl aliquot of total RNA (1 μg) was blended with 180 ng of a random hexanucleotide mixture, 2.25 μl of sterile KCl (1 M) and sterile water to a final volume of 16 μl, denaturated at 94 °C for 5 min and allowed to anneal at 41 °C for 15 min. After that 15 μl of mixture containing reverse transcriptase M-MLV (200 U), Tris–HCl (pH 8.3, 0.225 μmol), dNTPs (0.015 μmol), DTT (0.225 μmol) and $MnCl_2$ (0.03 μmol) were added. The obtained solution (with a final volume of 31 μl) was incubated at 41 °C for 1 h. The synthesized cDNA was stored at −20 °C.

2.5. PCR with gel-detection of products

A 1 μl aliquot of cDNA was mixed with 2 μl of PCR buffer, 0.3 μl of $MgCl_2$ (0.1 M), 1 μl of dNTPs (4 mM), 2.5 μl of mixture of forward and reverse primers for rPol II, Tph2 or 5-HT$_{1A}$ (2 μM of each; Table 1), 1 U of Taq polymerase and sterile water to a final volume of 20 μl. PCR was carried out according to the following protocol: 3 min at 94 °C, 1 cycle; 10 s at 94 °C, 30 s at 63 °C (rPol II), 61 °C (Tph2) or 62 °C (5-HT$_{1A}$), 15 s at 72 °C, 25 (rPol II), 26 (Tph2) or 27 (5-HT$_{1A}$) cycles; 2 min at 72 °C, 1 cycle. At the same time a series of mouse genomic DNA dilutions used as an external standard was amplified in separate tubes. Concentrations of 20, 30 and 50 ng/μl (4000, 6000 and 10,000 copies/μl, respectively) were used to evaluate the number of rPol II cDNA copies, concentrations of 10, 20, 30 and 40 ng/μl (2000, 4000, 6000 and 8000 copies/μl, respectively) were used for Tph2 and concentrations of 2.5, 5, 10 and 20 ng/μl (500, 1000, 2000 and 4000 copies/μl, respectively) were used for 5-HT$_{1A}$ receptor. Control of reagents was carried out under the same conditions but with template omitted. PCR was carried out in duplicate for the samples and in triplicate for the standards.

PCR-products of cDNA, standards and reagent control were separated by electrophoresis in 2% agarose gel, stained with etidium bromide and photographed with a digital camera. Fluorescence intensity of the PCR-product bands was measured with Scion Image software (Scion Corporation, www.scioncorp.com). PCR-products of cDNA were calibrated against the corresponding standard curves, which allowed to determine the number of 5-HT$_{1A}$, Tph2 and rPol II cDNA copies in 1 μl of total cDNA. 5-HT$_{1A}$ and Tph2 genes expression was presented as number of the receptor cDNA copies with respect to 100 cDNA copies of rPol II.

2.6. Real-time PCR

A 1 μl aliquot of cDNA was mixed with 2.5 μl of PCR buffer, 2.5 μl of $MgCl_2$ (0.05 M), 2.5 μl of dNTPs (2 mM), 2.5 μl of forward and reverse primers for rPol II, Tph2 or 5-HT$_{1A}$ (4 μM of each; Table 2), 0.8 μl of the corresponding hybridization probe (5 μM, Table 2), 1 U of Taq polymerase and sterile water to a final volume of 25 μl. PCR was carried out on amplificator iCycler iQ (Bio-Rad, USA) according to the following protocol: 3 min at 94 °C, 1 cycle; 10 s at 94 °C, 30 s at 59 °C (rPol II, Tph2) or 61 °C (5-HT$_{1A}$), 15 s at 72 °C, 40 cycles. Fluorescence level was detected at the annealing stage of each cycle. A series of genomic DNA dilutions with concentrations of 20, 40 and 60 ng/μl (4000, 8000 and 12,000 copies/μl, respectively) for rPol II, concentrations of 10, 20, 40 and 60 ng/μl (2000, 4000, 8000 and 12,000 copies/μl, respectively) for Tph2 and

concentrations of 10, 20, 30 and 50 ng/μl (2000, 4000, 6000 and 10,000 copies/μl, respectively) for 5-HT_{1A} receptor were amplified in separate tubes and used as external exogenous standard for calibration curve building. PCR was carried out in triplicate both for the samples and standards. Control of reagents was performed under the same conditions but the template was omitted.

Calibration curve in the coordinates of Ct (threshold cycle value)–log *P* (decimal logarithm of the DNA standard quantity) was plotted automatically by the Bio-Rad iCycler software.

2.7. Statistical analysis

The results were presented as $m \pm$ S.E.M. and compared using the one-way ANOVA.

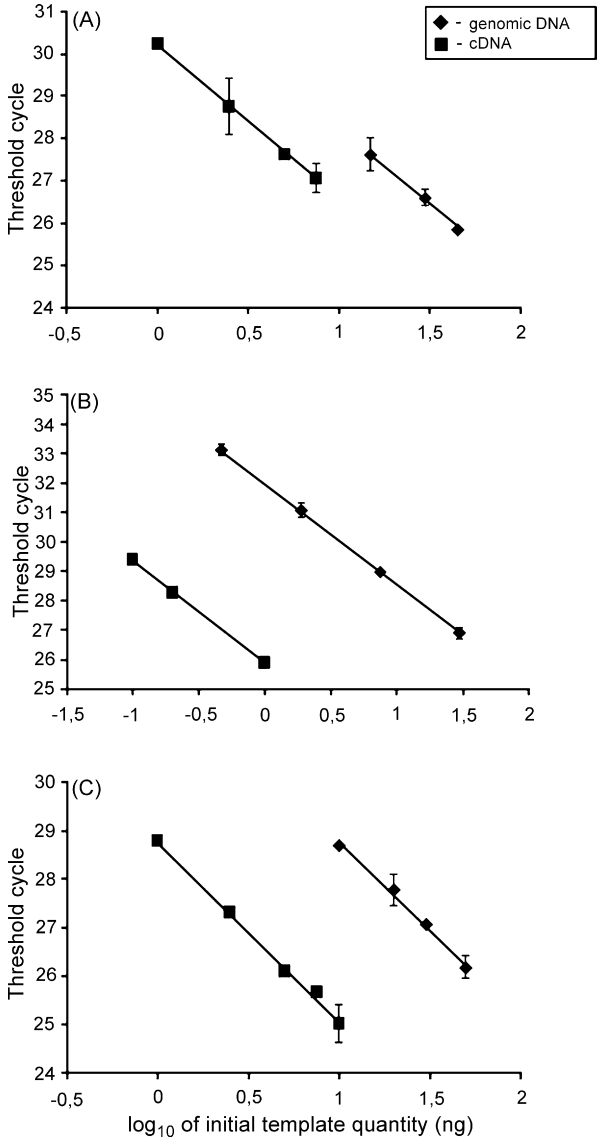

Fig. 1. Graphic chart of threshold cycle value as dependent on common logarithm of the initial template quantity of genomic DNA and cDNA for real-time PCR using 5-HT_{1A}, rPol II and Tph2 primer pairs (A, B and C correspondingly). The values are means of three replicates.

3. Results

In the present work, conditions were found for determination of 5-HT_{1A} receptor and Tph2 genes expression in mouse brain using real-time PCR. The two-standard system that we had developed earlier (Naumenko and Kulikov, 2006) was optimized for the application in real-time PCR. The primers used for real-time PCR differ from those developed for PCR with gel-detection of products (Tables 1 and 2) because a shorter amplicon was chosen for the real-time method.

It was shown that the threshold cycle depends on the common logarithm of initial template quantity in linear manner. The slope angles and, hence, the amplification coefficients were similar ($p > 0.05$) for genomic DNA or cDNA for all used primer pairs. The slope angles for cDNA and genomic DNA were 3.64 ± 0.115 and 3.92 ± 0.28 for 5-HT_{1A} primer pair (Fig. 1A); 3.51 ± 0.10 and 3.44 ± 0.07 for rPol II primer pair (Fig. 1B); 3.71 ± 0.16 and 3.67 ± 0.27 for Tph2 primer pair (Fig. 1C). The corresponding amplification coefficients for cDNA and genomic DNA were 1.88 and 1.80 for 5-HT_{1A} primer pair; 1.93 and 1.95 for rPol II primer pair; 1.86 and 1.88 for Tph2 primer pair.

Graphic charts of PCR-product fluorescence intensity as dependent on cycle number for PCR with gel-detection of prod-

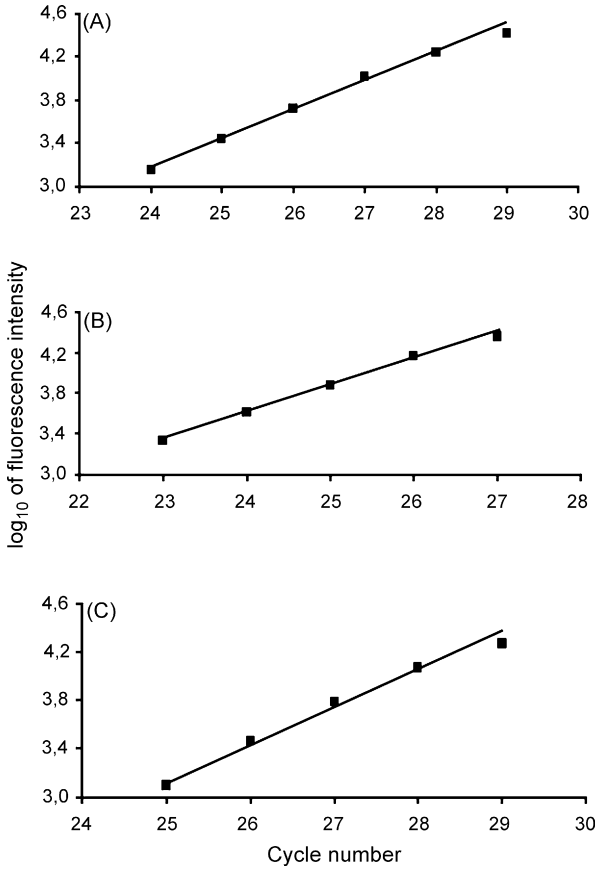

Fig. 2. Graphic chart of PCR-product fluorescence intensity as dependent on amplification cycle number for the PCR with gel-detection of products using 5-HT_{1A} receptor (A), rPol II (B) and Tph2 (C) primers. All curves were obtained for 1 μl of cDNA from midbrain of investigated mice. The values are means of two replicates.

ucts were obtained in a preliminary experiment. It was shown that these curves are exponential in the range from 24 to 28 cycles for 5-HT_{1A} receptor (Fig. 2A), from 23 to 27 cycles for rPol II (Fig. 2B) and from 25 to 28 cycles for Tph2 (Fig. 2C). Basing upon the obtained charts we chose the optimal, in respect to signal intensity, cycles in the exponential area of the curves: 27 cycles for 5-HT_{1A} receptor, 25 cycles for rPol II and 26 for Tph2. Graphic charts of fluorescence intensity as dependent on the initial amount of genomic DNA obtained for these cycle numbers using PCR with gel-detection of products were linear within the range of 2.5–20 ng for 5-HT_{1A} receptor (Fig. 3A), 20–50 ng for rPol II (Fig. 3C) and 10–40 ng for Tph2 (Fig. 3E). As for the real-time PCR, the curves showing the dependence of threshold cycle on common logarithm of initial template quantity were linear within the range of 10–50 ng for 5-HT_{1A} receptor (Fig. 3B), 20–60 ng for rPol II (Fig. 3D), and 10–60 for Tph2 (Fig. 3F). The correlation coefficient (R^2) was not less than 0.995 in all the cases.

To compare the two methods, we used them to determine 5-HT_{1A} receptor and Tph2 genes expression in the midbrain of AKR and CBA mice. It was shown with the help of PCR with gel-detection of products that 5-HT_{1A} receptor gene expression in the midbrain of mice of the cataleptic strain CBA is substantially decreased as compared to the control AKR strain ($p < 0.05$). Thus 5-HT_{1A} receptor mRNA level in the midbrain of AKR mice amounted to 28.8 ± 2.23 copies per 100 copies of rPol II mRNA, whereas it was as low as 22.6 ± 1.86 copies per 100 copies of rPol II mRNA in the midbrain of CBA mice (Fig. 4A).

Similar results were obtained when using real-time PCR. 5-HT_{1A} receptor gene expression in the midbrain of CBA mice was substantially lower than that of the AKR strain ($p < 0.05$). 5-HT_{1A} receptor mRNA level amounted to 34.9 ± 2.13 and 26.9 ± 2.44 copies per 100 copies of rPol II in the midbrain of AKR and CBA mice, respectively (Fig. 4B).

We have not revealed any differences in Tph2 gene expression in the midbrain between mice of AKR and CBA strains using

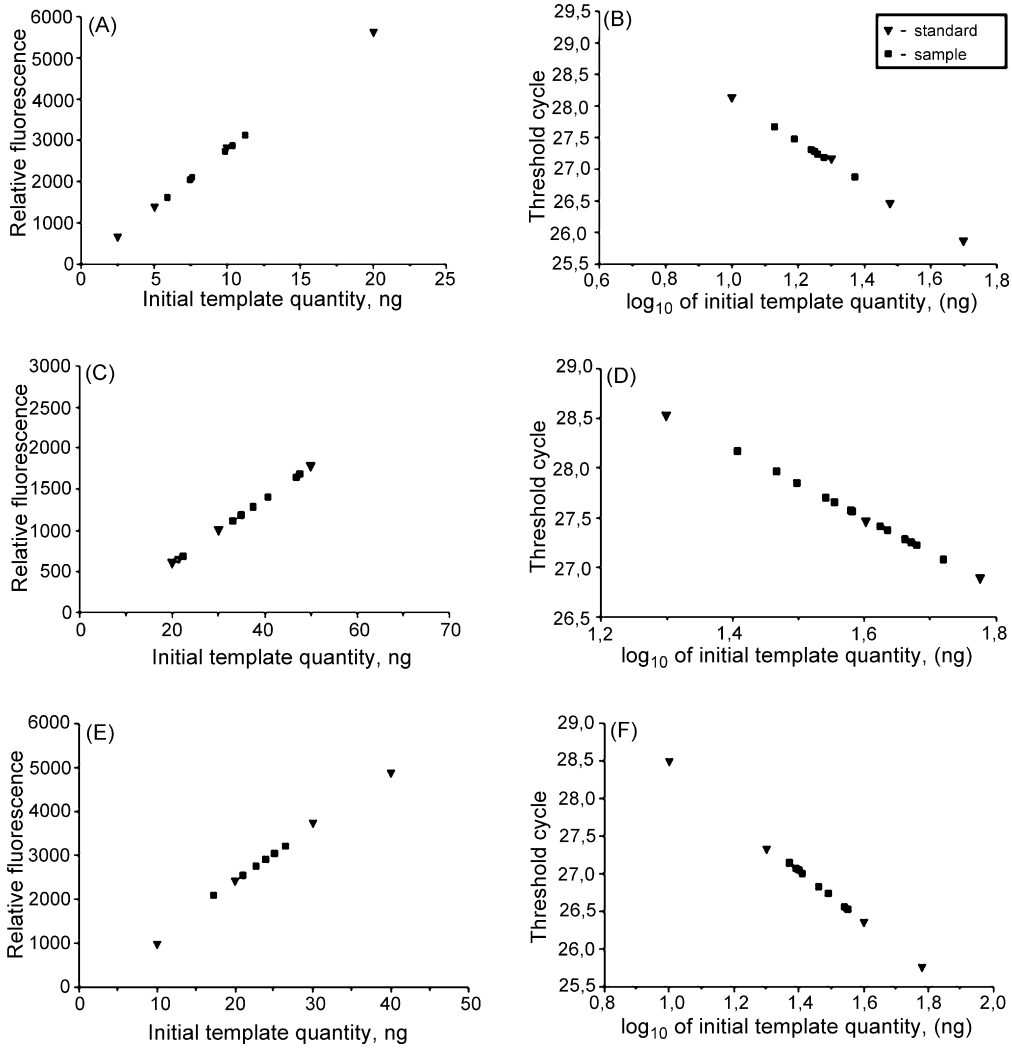

Fig. 3. The curves describing dependence of PCR-product fluorescence intensity on the initial template quantity. Variation of fluorescence intensity as a function of the initial genomic DNA quantity for PCR with gel-detection of products using 5-HT_{1A}, rPol II and Tph2 primer pairs (A, C and E correspondingly). Graphic chart of threshold cycle value as dependent on the common logarithm of genomic DNA quantity for real-time PCR using 5-HT_{1A}, rPol II and Tph2 primer pairs (B, D and F correspondingly). The values are means of three replicates.

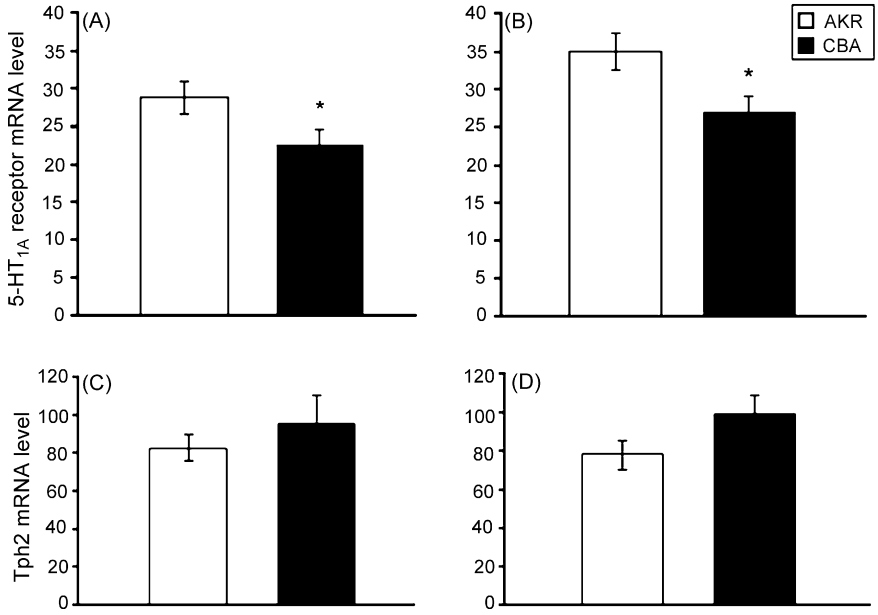

Fig. 4. Expression of 5-HT_{1A} receptor gene estimated using PCR with gel-detection of products (A) and real-time PCR (B). Expression of Tph2 gene estimated using PCR with gel-detection of products (C) and real-time PCR (D). The 5-HT_{1A} and Tph2 genes expression is presented as 5-HT_{1A} receptor or Tph2 cDNA copy number per 100 copies of rPol II cDNA. *$p < 0.05$ as compared to AKR.

both methods ($p > 0.05$). By means of PCR with gel-detection of products we have found that the Tph2 mRNA level in the midbrain of mice of AKR strain amounted to 82.11 ± 6.67 copies per 100 copies of rPol II mRNA, whereas in the midbrain of CBA mice it was 95.19 ± 14.87 copies per 100 copies of rPol II mRNA (Fig. 4C).

Using real-time PCR we have obtained similar results. Tph2 gene expression was 77.75 ± 7.63 for mice of AKR strain and 98.75 ± 9.48 for mice of CBA strain (Fig. 4D).

4. Discussion

In this paper, we demonstrate that the previously developed two-standard system (Naumenko and Kulikov, 2006) can be successfully applied in real-time PCR. As in the case of PCR with gel-detection of products, we used genomic DNA solution of a known concentration as an external standard in real-time PCR. Earlier we had practiced the normalization on fluorescence intensity of the standard of a particular concentration, but in the present work, we used a series of genomic DNA dilutions both for PCR with gel-detection of products and real-time PCR. This modification makes it possible to control the linearity of the dependence between amplification intensity and the initial template amount. Besides, it allows to determine the amount of cDNA copies in a sample more accurately, since a linear approximation of the calibration curve is used instead of a simple normalization.

Given the absence of differences in primary sequence and length between the PCR-products of genomic DNA and cDNA, we had supposed the amplification efficiency to be the same for both templates. The detection of fluorescence level after each cycle allowed us to verify this suggestion. Indeed, the amplification coefficients for cDNA and genomic DNA did not differ significantly for all primer pairs in the range of standard concentrations used in the present study.

Previously we had used mRNA of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous standard, but recent findings have shown that there are several pseudogenes in mouse genome, closely homologous to GAPDH (European Bioinformatics Institute Database; accession numbers: AC158345; AC163335; AL935328; AC156283). This might have led to an overestimation of the absolute (but not relative) results, so we chose another housekeeping gene to serve as internal standard—the gene encoding the largest subunit of the DNA-directed RNA polymerase II (European Bioinformatics Institute Database; accession number: M12130). This gene does not have close homologues in mouse genome. Furthermore, its expression has been shown to be relatively stable in different tissues (Radonic et al., 2004).

To compare the two methods we used them to determine 5-HT_{1A} receptor and Tph2 genes expression in the midbrain of mice belonging to the cataleptic CBA strain and non-cataleptic AKR strain. It was shown with the help of PCR with gel-detection of products that 5-HT_{1A} receptor gene expression is decreased by 22% in the midbrain of CBA mice as compared to the AKR strain. Similar results were obtained by means of real-time PCR. These data are in a good agreement with the previously obtained results (Naumenko et al., 2006) that have shown a 24% difference between these strains.

The 5-HT_{1A} and Tph2 genes expression levels assessed with the help of real-time PCR are, respectively, 20% and 5% higher than those measured using PCR with gel-detection of products. The significant difference in the 5-HT_{1A} gene expression values assessed by means of PCR with gel-detection of products and real-time PCR may be related to a higher sensitivity of real-time PCR for this particular primer–probe system or to diverse PCR

conditions as all the values obtained were reproduced with an error less than 10% when the experiment was repeated. These factors may be less crucial for Tph2 primer–probe system since the discrepancy between Tph2 mRNA levels assessed using both methods is as low as 5%. The difference in 5-HT_{1A} values between the present data and those obtained earlier (Naumenko et al., 2006) obviously results from the different endogenous standards used.

The obtained results on decreased 5-HT_{1A} receptor gene expression in the brain of cataleptic mice confirm the involvement of 5-HT_{1A} receptors in the regulation of catalepsy. This is in a good agreement with the published data on anti-cataleptic action of 5-HT_{1A} receptor selective agonists (Haleem et al., 2004) and on localization of the main QTL for catalepsy in the region of the 13 chromosome that includes 5-HT_{1A} receptor gene (Kulikov et al., 2003).

Important is the advantage of PCR with gel-detection of products that it does not require producing expensive hybridization probes and, consequently, studies using this method can be performed almost in any laboratory. As the present work has shown, PCR with gel-detection of products, when applying the two-standard system, does not yield in accuracy and reproducibility to one of the most up-to-date methods for quantifying gene expression—real-time PCR. Moreover, it was shown that the two-standard system that we had previously developed can be successfully applied in real-time PCR.

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