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Validation of two reference genes for mRNA level studies of murine disease models in neurobiology

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Abstract

Reverse transcription of extracted cellular RNA combined with real-time PCR is now an established method for sensitive detection and quantification of specific mRNA level changes in experimental models of neurological diseases. To neutralize the impact of experimental error and make quantification more precise, normalization of test gene data using data from a constantly expressed gene, a reference gene that is tested along with the test gene, is required. There is no single gene constantly expressed under all experimental conditions. For a given set of conditions or a given disease model, identification of an unaffected reference gene is necessary. In this report, we present our findings from evaluation and validation of the genes encoding hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1) and glyceraldehyde phosphate dehydrogenase (GAPDH) as individual reference genes in mRNA level studies involving four murine neurological disease models. We find both genes are suitable as a reference gene with these four models, provided quantification of subtle changes are avoided. We furthermore demonstrate that above a certain threshold of test mRNA level changes and given high quality RNA processing, normalization to total RNA alone provides for equally reliable quantitative mRNA level results.

Keywords: Reference gene; Gene expression; Real-time PCR; PCR efficiency; Permanent middle cerebral artery occlusion; Perforant path lesion

1. Introduction

Reverse transcription (RT) of messenger RNA (mRNA) into complementary DNA (cDNA) followed by real-time PCR (rt-PCR) enables quantification of mRNA levels. For precise mRNA (transcript) level testing by RT rt-PCR, concomitant testing of a gene characterized by a constant mRNA level is generally considered necessary (Bond et al., 2002; Bustin, 2002; Dheda et al., 2004, 2005; Huggett et al., 2005). This is to enable normalization of experimental data to neutralize the impact of experimental error. Among the factors that may compromise the reliability of experimental data obtained in RT rt-PCR are degradation of the sample RNA, contamination of RNA by coextracted and coquantified genomic DNA (when optical density at 260 nm is used to quantify nucleic acid concentration of each extract), or the presence in the extracted RNA of reverse transcriptase inhibitors. Most often such genes are referred to as reference genes. It has been assumed that expression of so-called housekeeping or maintenance genes of the cell was unaffected by whatever insult, lesion or conditions the cell culture or experimental animal was subjected to. Housekeeping genes were generally considered suitable for application as reference genes. However, for many of the housekeeping genes and in various experimental settings, the assumption of unchanged expression has been shown not to hold true (Harrison et al., 2000; Schmittgen and Zakrajsek, 2000; Hamalainen et al., 2001; Bond et al., 2002; Bustin, 2002; Feroze-Merzoug et al., 2002; Tricarico et al., 2002; Dheda et al., 2004; Gutala and Reddy, 2004; Kobayashi et al., 2004; Trivedi and Arasu, 2005). Therefore, identification and proper validation of one or more suitable reference gene(s) is necessary prior to undertaking studies of mRNA levels by RT rt-PCR in any new combination of organism or cell culture and lesion or experimental treatment. An increasing fraction of reported rt-PCR based mRNA level studies encompass proper validation of the applied reference gene, but studies continue to be published without any validation of the reference gene applied (Lambertsen et al., 2004; Molteni et al., 2004).

There probably is no reference gene universally useful for mRNA level testing, not even when considering only murine

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neurobiological studies. The various reports indicate that which reference genes are suitable for a given study depends on what insult, lesion or experimental conditions is applied to the cell culture or experimental animal. Therefore, in each case, careful evaluation and validation is required (Schmittgen and Zakrajsek, 2000; Bond et al., 2002; Feroze-Merzoug et al., 2002; Vandesompele et al., 2002; Brunner et al., 2004; Kobayashi et al., 2004; Dheda et al., 2005; Trivedi and Arasu, 2005; de Kok et al., 2005). Examples of housekeeping genes applied as reference gene in neurobiological studies are glyceraldehyde phosphate dehydrogenase (GAPDH), hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1), cyclophilin, and β-actin (Harrison et al., 2000; Bond et al., 2002; Lambertsen et al., 2005). Furthermore, 18S ribosomal RNA has been applied as endogenous reference after 18S rRNA levels were found unchanged over the range of experimental conditions (Schmittgen and Zakrajsek, 2000; Burbach et al., 2003, 2004).

This report presents the details and findings from our validation of the genes encoding GAPDH and HPRT1 as individual reference genes in four murine models of CNS disease. The four models were: permanent middle cerebral artery occlusion (pMCAO) and perforant path (PP) axonal lesion for studies of brain inflammation and cytokine mRNA levels in stroke and anterograde axonal (Wallerian) degeneration, respectively, and lysolecithin injection into corpus callosum and experimental autoimmune encephalomyelitis (EAE) for demyelinating and inflammatory pathology. We demonstrate both genes are suitable as reference gene. Additionally, based on a comparative study of rt-PCR test data, we discuss the consensus that reference gene based normalization of test gene data always provides for more reliable quantitative mRNA level results.

2. Materials and methods

2.1. Surgical procedures

Mice were purchased from Bomholtgaard (Laven, DK) or bred in the Laboratory of Biomedicine, University of Southern Denmark. All animal protocols were approved by and complied with the ethical guidelines of the Danish Animal Health Care Committee.

2.1.1. Permanent middle cerebral artery occlusion (pMCAO)

Permanent occlusion of the distal part of the left middle cerebral artery (MCA) was performed on male SJL mice as described in detail in Lambertsen et al. (2004). Mice were anaesthetized by subcutaneous injection of 0.21 ml/10 g body weight of a 1:1:2 mixture of Hypnorm™ (fentanyl citrate 0.315 mg/ml and fluanisome 10 mg/ml, Janssen-CILAG, Birkerød, Denmark), Stesolid™ (5 mg/ml Diazepanum, Dumex, Copenhagen, Denmark) and distilled H_2O. Mice were placed on a 37.5 °C heating pad during surgery. The MCA was exposed and coagulated by bipolar electrocoagulation applying forceps coupled to an electrosurgical unit (ICC50 from ERBE, Germany). Mice were kept in a recovery room at 28 °C for 20 h before return to the animal facility. In sham operated mice the electrocoagulator was applied in the CNS parenchyma just next to the MCA. The incision was sutured. Post surgical pain treatment consisted of supplying the mouse s.c. with 0.15 ml Temgesic™ diluted 1:30 (stock: 0.3 mg Buprenorphinum, Reckitt & Colman, Hull, England) three times with an 8 h interval starting immediately after the surgery.

2.1.2. Perforant path axonal transection

Male C57BL/6 mice were subjected to perforant path axonal transection using the method described by Drøjdahl et al. (2004) with slight modifications. The procedure was performed using a closed wire-knife (David Kopf Instruments) and a Kopf stereotactic frame. The mouse was anasthetized by an intraperitoneal injection with a 1:1:1 mixture of xylazine (Rompun VET, Bayer), ketamine (Ketaminol VET, Intervet) and sterile water (SAD, Denmark), dosage was 0.06 ml/g mouse. The mouse was fixated in the stereotactic device with the nose-bar 3.0 mm below zero. A burr hole was made in the skull 0.4 mm behind lambda and 2.0 mm lateral to the midline. The wire-knife was inserted at an angle of 10° lateral and rotated 15° anteriorly. The knife was moved 3.6 mm ventrally from the dura. The knife was unfolded, retracted 3.3 mm for transection of the PP, refolded and withdrawn from the brain. Mice were supplied s.c. with Temgesic™ for postsurgical analgesia.

2.1.3. Experimental allergic encephalomyelitis

Experimental allergic encephalomyelitis was induced in female SJL mice according to the method described in Lambertsen et al. (2004). Subcutaneous injections were given 2 weeks apart with 400 μg MBP (Sigma–Aldrich) emulsified in Freunds complete adjuvant containing 50 μg *Mycobacterium tuberculosis* H37RA (ICN, Aurora, OH, USA) in combination with i.p. injections of 200 ng pertussis toxin (PTX: Sigma–Aldrich, P7208) day 0 and 2. Unimmunized mice only receiving PTX on day 0 and 2 served as controls. The mice were monitored daily and scored clinically as follows: 0, no symptoms; 1, flaccid tail; 2, hindlimb paresis; 3, hindlimb paralysis; 4, complete fore/hind limb paralysis; 5, moribund.

2.1.4. Induction of demyelination in corpus callosum by way of lysolecithin injection

Female C57BL/6 mice were anaesthetized as described for the pMCAO-operated mice using a 1:1:2 mixture of Hypnorm, stesolide and sterile isotonic 0.9% NaCl (0.1–0.2 ml per 10 g body weight). The mouse was positioned in the stereotaxic frame, with the nose-bar placed 3.0 mm below zero, and a burr hole was made 0.9 mm anterior to Bregma, and 1.5 mm lateral to the sagittal fissure. A 5 μl Hamilton syringe containing 0.75 μl lysolecithin (1%, w/v, L-lysolecithin (L5004, Sigma) diluted in 0.15 M phosphate buffered saline, pH 7.4) was inserted 1.3 mm profund to dura, and left for 1 min. The needle was then withdrawn 0.25 mm and 0.25 μl lysolecithin was injected every 2 min to reduce reflux up the needle track. Mice were supplied with Temgesic™ for postsurgical analgesia. Controls were injected with 0.75 μl saline only.