ELSEVIER

Journal of Neuroscience Methods 123 (2003) 31–45

JOURNAL OF NEUROSCIENCE METHODS

www.elsevier.com/locate/jneumeth

Optimizing primer–probe design for fluorescent PCR

Dmitri Proudnikov *, Vadim Yuferov, Yan Zhou, K.Steven LaForge, Ann Ho, Mary Jeanne Kreek

Laboratory of the Biology of Addictive Diseases, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA

Received 30 July 2002; received in revised form 21 October 2002; accepted 25 October 2002

Abstract

TaqMan®, a variation of fluorescent PCR, is a powerful tool for gene expression and polymorphism studies. Here we describe the design and evaluation of 27 new TaqMan® primer-probe sets for rat genes that play a key role in neural signaling. These newly designed and synthesized probes were tested and then used for quantification of RNA isolated from rat brain. The usual length of common TaqMan® probes is 25 bases or less. In these studies we constructed probes with lengths of 25–39 bases to span exon–exon junctions of nucleic acids to avoid the influence of DNA contamination upon the RNA quantification. The specific sequences at these positions required probes of these lengths to optimize hybridization. We found that the relocation of the quencher from the traditional 3′ position to an internal one increases the sensitivity of probe up to 30 fold. Substitution of 6-carboxyfluorescein with Alexa Fluor® 488 as fluorophore and TAMRA with non-fluorescent quencher dabcyl was also investigated. We also describe the evaluation of part of a newly designed set of 27 TaqMan® primer-probes for the measurement of differences in gene expression levels in samples from the caudate putamen region of rat brain after 'binge' paradigm cocaine administration. Cocaine-induced alterations in expression of *c-fos* and preprodynorphin mRNAs measured by TaqMan® were confirmed by ribonuclease protection assay.

Keywords: TaqMan®; Fluorescent PCR; Energy transfer; 4-(4′-dimethylaminophenylazo)-benzoic acid; *N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine; 6-carboxyfluorescein; Gene expression; Cocaine; Rat; Brain

1. Introduction

The last several years have seen the publication of reports describing the use of fluorescent polymerase chain reaction (PCR) for different applications. High accuracy of measurement (Bustin, 2000), comparable with established methods like the ribonuclease (RNase) protection assay, in combination with extremely high sensitivity (Macdonald et al., 2001) were the main factors that promoted the technique for such sensitive applications as gene expression analysis (Medhurst et al., 2000), identification of microorganisms (Vet et al., 1999; Espy et al., 2000), polymorphism studies (Tyagi et al., 2000; Myakishev et al., 2001), and detection of a variety of genetic diseases (e.g. Parks et al., 2001).

In order to improve performance of this technique and its variations, several approaches in the experimental design have been suggested by different laboratories. These approaches include the use of fluorescent dyes other than 6-carboxyfluorescein (FAM), for example, BODIPY® dyes (Metzker and Gibbs, 1999) as fluorophores, and non-fluorescent quenchers (Tyagi and Kramer, 1996) for the improvement of physicochemical properties of fluorophore-quencher pairs, introduction of intercalating agents (Kutyavin et al., 2000) and modified bases (Meyer et al., 2000) for the reduction of probe lengths, the combination of PCR primer and fluorescent probe in the same molecule (Whitcombe et al., 1999), and the use of a double donor or a double

* Corresponding author. Tel.: +1-212-327-8234; fax: +1-212-327-8574.

E-mail address: proudnd@mail.rockefeller.edu (D. Proudnikov).

0165-0270/03/$ - see front matter
PII: S0165-0270(02)00325-4

acceptor to enhance the energy transfer effect (Okamura et al., 2000). Each of these approaches was generally developed as an independent method. Thus, non-fluorescent quenchers were used in the molecular beacon technique that utilizes PCR in the presence of specially designed fluorescent probes with hairpin formations (Tyagi and Kramer, 1996).

The use of BODIPY® instead of more common rhodamine dyes in fluorescent PCR has also been reported (Metzker and Gibbs, 1999). Being less sensitive to the influence of solvent or pH on their fluorescence, and more photostable, dyes of the BODIPY® group, along with more recently invented dyes of the Alexa Fluor® group, were reported to have improved spectral characteristics compared with conventional derivatives of fluorescein and rhodamine (Panchuk-Voloshina et al., 1999).

The influence of the distance between the fluorophore and quencher on fluorescent PCR assay performance is related to the fluorescent resonance energy transfer (FRET) effect (Foster, 1948)–the basis for all existing variations of real time optical PCR detection systems. FRET has been used for decades for measurement of the distance between biomolecules or specific parts of the same biomolecule (Stuhmeier et al., 2000; Hillisch et al., 2001). This technique has been effectively applied to the range of 20–100 Å (Lilley and Wilson, 2000) when the energy transfer effect is strongly dependent upon the proximity of fluorophores. The FRET effect disappears at a distance greater than 100 Å (for the fluorescein-tetramethylrhodamine pair).

TaqMan® is a variation of real time fluorescent PCR which utilizes the 5′–3′ exonuclease activity of Taq polymerase for enzymatic digestion of the fluorescent oligonucleotide probe during PCR amplification. Although the influence of the fluorophore-quencher distance upon the FRET effect has been known for years, optimization of fluorophore–quencher placement in TaqMan® probes has generally not been a part of design consideration. Thus, the construction of the fluorescent probes described in published TaqMan® reports traditionally has the fluorophore and the quencher at 5′ and 3′ positions, respectively, which generally is not optimal for probes longer than 20 bases.

In these studies we investigated the influence of the fluorophore–quencher distance on the performance of fluorescent probes in the TaqMan® assay. The substitutions of Alexa Fluor® 488 and the non-fluorescent quencher 4-(4′-dimethylaminophenylazo)-benzoic acid (dabcyl) for the more commonly used FAM and *N,N,N′,N′*-tetramethyl-6-carboxyrhodamine (TAMRA) were tested as well.

Based on the results of these studies, we designed TaqMan® probes for quantification of mRNA levels of 27 genes of interest to this laboratory (see Table 1). Then probes for opioid receptors (δ, κ, μ), peptides (preprodynorphin, preproenkephalin), dopamine D_2 receptor, and *c-fos* from this set were tested in an animal experiment to measure alterations in mRNA levels in the caudate putamen region of rat brain following 1 and 3 days of 'binge' paradigm cocaine administration. These genes have been shown to play key roles in the responses to cocaine treatment (e.g. Steiner and Gerfen, 1993; Spangler et al., 1996; Yuferov et al., 1999, 2001). Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA and 18S ribosomal RNA were used as 'housekeeping' RNAs for normalization of the results of measurements.

2. Methods

All oligonucleotides were custom synthesized by GeneLink (Hawthorne, NY). Unlabeled oligonucleotides used as PCR primers were made in 50 nmol scale synthesis; fluorescently labeled probes were made in 200 nmol scale synthesis followed by purification on polyacrylamide gel by the manufacturer. At our request, oligonucleotides were modified at 5′ with either 6-FAM-phosphoramidite, or 5′-amino modifier C6 followed by post-synthesis conjugation with Alexa Fluor® 488 carboxylic acid succinimidyl ester (Molecular Probes, Eugene, OR); 3′ modifications were introduced using 3′-TAMRA CPG, or 3′-dabcyl CPG 1000, or 3′-Phosphate CPG; internal TAMRA or dabcyl were introduced using TAMRA-dT or dabcyl-dT phosphoramidites. All modified phosphoramidites were from Glen Research (Sterling, VA).

2.1. TAQMAN primer-probe design

Primers (Table 1) were designed using PRIMER EXPRESS® software (Applied Biosystems, Foster City, CA) to meet TaqMan® requirements. The lengths of amplicons were 90–250 bases. In order to avoid the possible influence of traces of DNA in RNA samples on the measurements, most probes were selected from the exon–exon junction regions of the mRNAs studied. In cases when the exon–intron gene structure for rat genes has not yet been reported, we assumed that it would be similar to that in the corresponding human genes, however, the existence of these junctions in the rat genes was not experimentally verified. If neither rat nor human exon/intron structure was known, this strategy could not be used. In general, fluorescent probes were designed so that the probe spans the exon–exon junction with the location of the junction in the middle of the probe. The second main limitation was the melting temperature of the probe that was chosen to be around 70 °C so that probes were effectively hybridized with target DNA. The third restriction is the presence of T base in the 9–13 position from 5′ end

Table 1
The list of designed TaqMan® primer–probe sets

Gene name	Gene bank accession number	Probe sequence (5′ → 3′)
μ opioid receptor [b,f]	L13069	FAM-TGGCAGTCTT(TAMRA)CATTTTGGTGTATCTTACAATCACAT-Ph (Probe) CAGCCCTTCCATGGTCACAG (Forward primer) TACTGGTCGCTAAGGCGTCTG (Reverse primer)
δ opioid receptor [b,e,f]	U00475	FAM-TTCAGCTTAGT(TAMRA)GTACCGGACGATTCCAAACAT-Ph (Probe) TGCTGGGCAACGTGCTC (Forward primer) GAAGGGCAGTGTGCTGGTG (Reverse primer)
κ 1 opioid receptor [b,f]	NM_017167	FAM-ATGTTTGTCAT(TAMRA)CATCCGATACACAAAGATGAAGACC-Ph (Probe) GGTGGGCTTAGTGGGCAAT (Forward primer) GACAGCACTCTGGAAGGGCA (Reverse primer)
Preproopiomelanocortin [a]	K01878; J00759	FAM-AAAGCAACCT(TAMRA)GCTGGCTTGCATCCG-Ph (Probe) CAGTGCCAGGACCTCACCA (Forward primer) GGGATTTTCAGTCAAGGGCTG (Reverse primer)
Preproenkephalin [a,f]	K02805; K02806	FAM-TCAACTTCCT(TAMRA)GGCATGCACACTCGA-Ph (Probe) AGTGCAGGCAGACTGCAGC (Forward primer) AGATCCTTGCAGGTCTCCCA (Reverse primer)
Preprodynorphin [c,f]	M32783; M32784	FAM-CCTCGGAGGAGTGGGAGACATGCC-TAMRA (Probe) TTCCCTGTGTGCAGTGAGGA (Forward primer) CAGAGGCAGTCAGGGTGAGAA (Reverse primer)
Orphanin FQ receptor [a]	AF216218	FAM-CAAGTGGAAGAT(TAMRA)GAAGAGATCGAGTGCCTG-Ph (Probe) GTTCGGACATCCAGCAAAGC (Forward primer) GAAGATGCAGATGGCGAATACA (Reverse primer)
Prepronociceptin [b]	NM_013007	FAM-CTTCAACCT(TAMRA)GAAGCTGTGCATCCTCCA-Ph (Probe) GTTCAGCAGCTGTCCCGAG (Forward primer) AAAGAGTCCAGAGAGGGCGG (Reverse primer)
Dopamine transporter [b]	NM_012694	FAM-TCCTGTCCT(TAMRA)GAAAGGTGTGGGCTTCACT-Ph (Probe) GCTGCTGGTGTCTGGAAGATC (Forward primer) GCCGTCGCTAGAGTTGCTG (Reverse primer)
Dopamine receptor D_1 [d]	M35077	FAM-TTTGGCCTTT(TAMRA)GGGTCCCTTTTGTAACATCT-Ph (Probe) CTGTCCTGGTCATGCCCTG (Forward primer) TGGACGCCGTAGAGCACAT (Reverse primer)
Dopamine receptor D_2 [a,f]	NM_012547	FAM-CCTGAAGACACCACT(TAMRA)CAAGGGCAACTGTA-Ph (Probe) CCAAGCGCAGCAGTCGAG (Forward primer) ATTCTCCGCCTGTTCACTGG (Reverse primer)
Dopamine receptor D_3 [a]	M69189; M69190; M69191	FAM-TGGTGTACTT(TAMRA)GGAGGTGACAGGTGGAGTCT-Ph (Probe) CCTGCAGACCACCACCAACT (Forward primer) TCATGACATCCAGGGTGACAA (Reverse primer)
Tyrosine hydroxylase [b]	L22651	FAM-CTGTCACGT(TAMRA)CCCCAAGGTTCATCGGA-Ph (Probe) CAGAGCAGGATGCCAAGCA (Forward primer) TTTACAGCCCGAGACAAGGAG (Reverse primer)
Serotonin transporter [d]	Y11024	FAM-CATCATAGCCT(TAMRA)GGGCGCTCTACTACCTCAT-Ph (Probe) AAAGGCATTGGTTACGCCAT (Forward primer) AGGAGTTCGTGCAGCTGGTC (Reverse primer)
Tryptophan hydroxylase [b]	X53501	FAM-ATGCCCTTGCT(TAMRA)AGAGTCAGCAGGTGGC-Ph (Probe) GGCATGACCTCGATGTCGT (Forward primer) CAAGAACTGGCCCTGGCTC (Reverse primer)
Norepinephrine transporter [b]	AB021971	FAM-TGAAGACAT(TAMRA)CGGGAAAGGTTGTCTGGATC-Ph (Probe) TGCCTGATGGTCGTTATCGTT (Forward primer) CAATGTGCAGGTAGGCGTTG (Reverse primer)
N-methyl-D-aspartate receptor type 1 (NMDAR1) [a]	L08228	FAM-CCATGTTCACT(TAMRA)CAGAGTATCCACCTGAGTTTCC-Ph (Probe) CAGTTGCAGAGGCGGTGAA (Forward primer) AGTAGGGCGGCACCGTG (Reverse primer)
Glutamate receptor GluR1 [b]	X17184	FAM-CCAACAATAT(TAMRA)CCAGATAGGGGGGTTATTTCCAAA-Ph (Probe) CTTTGCCTTTTTCTGCACCG (Forward primer) GCCGCATGTTCCTGTGATT (Reverse primer)
Glutamate receptor GluR2 [c]	AF164344	FAM-CACTTCGGAGT(TAMRA)TCAGACTGACACCCCATATC-Ph (Probe) CAGTGCATTTCGGGTAGGGA (Forward primer) GGGAGCAGAAAGCATTGGTG (Reverse primer)

Table 1 (*Continued*)

Gene name	Gene bank accession number	Probe sequence (5′→3′)
Substance P receptor [a]	M64234; M64235	FAM-ATCATCATTTT(TAMRA)GACCACCTTGCGTTTGG-Ph (Probe) GGCCAGTGAGATCCCCG (Forward primer) GGGTTGATGTAGGGCAGGAG (Reverse primer)
Preprotachykinin/Substance P [a]	M34159; M34160	FAM-TGACCAAAT(TAMRA)CAAGGAGGCAATGCCG-Ph (Probe) AGGAAATCGGTGCCAACG (Forward primer) CTGAAGAAGATGCACAAAGGGC (Reverse primer)
Corticotropin releasing factor [c,e]	M54987	FAM-TAATCTCCAT(TAMRA)CAGTTTCCTGTTGCTGTGAGCTT-Ph (Probe) CGCCCATCTCTCTGGATCTC (Forward primer) CCAAGCGCAACATTTCATTTC (Reverse primer)
Corticotropin releasing factor receptor type 1 [b,e]	NM_030999	FAM-TAGTGTACTT(TAMRA)TGCTCTTCTTCTCTTCGTTGAGAATCTCC-Ph (Probe) GGCAGCCCGTGTGAATTATT (Forward primer) ACCAGGGAGATGCAGTGACC (Reverse primer)
Glucocorticoid receptor [b]	Y12264	FAM-TTTTCTAAT(TAMRA)GGGTACTCAAGCCCTGGAATGAGA-Ph (Probe) GGACAGCCTGACTTCCTTGG (Forward primer) ACGAGCTGGATGGAGGAGAG (Reverse primer)
Mineralocorticoid receptor [b]	NM_013131	FAM-CCAAGGTACT(TAMRA)TCCAGGATTTAAAAACTTGCCTCTC-Ph (Probe) CGAAACAGATGATCCAGGTCG (Forward primer) CATCCCCTGGCACAGTTCATA (Reverse primer)
Ribosomal RNA 18S [c,f]	V01270	FAM-TGCCGACGGGCGCTGACC-TAMRA (Probe) CTTTGGTCGCTCGCTCCTC (Forward primer) CTGACCGGGTTGGTTTTGAT (Reverse primer)
Immediate early gene *c-fos* [b,f]	X06769	FAM-CTCCCCTGT(TAMRA)CAACACACAGGACTTTTGC-Ph (Probe) GCGGACTACGAGGCGTCAT (Forward primer) GGAGGAGACCAGAGTGGGC (Reverse primer)

Abbreviations: Ph, phosphate.
[a] Probe spans known exon–exon junction of gene.
[b] Probe spans assumed exon–exon junction of gene.
[c] Probe designed for gene with known exon–exon junction but directed at one exon only.
[d] Probe designed for intronless gene.
[e] Antisense probe.
[f] Primer–probe sets used in cocaine experiment.

to attach TAMRA quencher. We found that the lengths of fluorescent probes that met all three criteria are up to 39 bases. Primer sets were tested in reverse transcription PCR (RT-PCR) using in vitro synthesized RNA sense transcripts and total RNA from the caudate putamen, anterior pituitary or hypothalamus of drug-naïve rats as templates. RT-PCR was performed using TaqMan® EZ RT-PCR kit (Applied Biosystems) and the following temperature program: 50 °C for 2 min, 60 °C for 30 min, 95 °C for 5 min and then 40 cycles, 94 °C for 20 s, 62 °C for 1 min.

RT-PCR was done in 10 μl volumes in 384-well plates using GeneAmp® PCR system 9700 and the Dual 384-Well Sample Block Module (Applied Biosystems). When a PCR cycling was complete, 4 μl of a solution of bromophenol blue in 50% glycerol containing 2 × TAE and 2 μl of aqueous PicoGreen® dye (Molecular Probes) (20 μl of the DMSO based stock solution was diluted with 1 ml of water) were added to each well and mixed. Six μl of the resulting mix from each well were loaded on a 2% agarose gel. Low DNA Mass™ Ladder (Gibco BRL, Gaithersburg, MD) was loaded on the gel as size control. The gel was run for 1 h (10 mA/cm) and examined under UV illumination using the EAGLE EYE® Still Video System and software (Stratagene, La Jolla, CA).

Primers that produced high levels of amplicon DNA following PCR were chosen for use in optical PCR. For such primer sets fluorescent probes labeled with 6-FAM or Alexa Fluor® 488 on 5′ position and TAMRA or dabcyl on 3′ or internal position were custom synthesized by GeneLink and tested in optical RT-PCR using the ABI Prism® 7900 Sequence Detection System (Applied Biosystems). Amplification was performed in 384-well clear optical reaction plates (Applied Biosystems) in 10 or 5 μl volumes using TaqMan® EZ RT-PCR kit and in vitro synthesized RNA sense transcripts or total RNA from the caudate putamen, anterior pituitary, or hypothalamus of drug naïve rats as the templates. Cycling was performed according to the program described above. Before loading to a 384-well plate, all solutions were degassed under low pressure (15 mm Hg) on ice for 5 min in order to prevent the formation of air bubbles during PCR. We found that

this step considerably improves reproducibility of measurements.

Fluorescent probes with internal quencher (TAMRA or dabcyl) were synthesized with 3′-phosphate in order to eliminate their extension during PCR amplification. The sequence for the GAPDH mRNA primer-probe set was taken from previously published reports (Medhurst et al., 2000). Probes for preprodynorphin and 18S mRNAs were designed with 3′-TAMRA as quencher.

Probes that showed low sensitivity were redesigned by using a different location on RNA sequence. Elements of predicted secondary structure, including formation of hairpins and dimers, of the 5′-end of such probes with low sensitivity were analyzed. No strong correlation between the performance of the probe in RT-PCR and the presence of such predicted structures was found.

2.2. Animal treatment and RNA preparation

The study protocol involving animals was approved by The Rockefeller University Institutional Animal Care and Use Committee.

For the design of primer-probe sets, RNA extracts from the caudate putamen, anterior pituitary or hypothalamus of six drug-naïve male Fischer rats (60 days old; Charles River, NY) were used. For studies of cocaine-induced alterations of mRNA levels in rat brain, four groups (28 animals each) of individually caged male Fischer rats were injected with 'binge' pattern saline or cocaine for one day (15 mg/kg per i.p. injection with three daily injections 1 h apart, beginning 30 min after lights turn on), or 'binge' saline or cocaine for 3 days as previously described (Spangler et al., 1996). Animals were sacrificed after the last injection in stress minimized conditions and brain regions promptly dissected.

Total RNA from caudate putamen was isolated by the phenol-acid method (Chomczynski and Sacchi, 1987) and pooled in order to exclude the influence of individual biological variations among rats upon the measurement of gene expression. Before use in fluorescent PCR, RNA samples were treated with DNase to reduce the possible effects of trace DNA on the TaqMan® measurements. Each measurement was performed in up to 24 replicates in either a 5 or 10 μl volume.

RNA sense transcripts (as standards) for most genes to be tested were prepared with an in vitro SP6 transcription kit (Ambion, Austin, TX) using cDNAs subcloned in pSP64/65 vector (Promega, Madison, WI) as the template. The sense RNA transcripts for preprodynorphin and GAPDH were made using in vitro T3 transcription kit (Ambion, Austin, TX) and PCR cDNAs prepared with T3 promoter-extended primer, as template. Using total RNA from caudate putamen from rat brain as the template, PCR amplification of cDNAs was performed employing TaqMan® EZ RT-PCR kit (Applied Biosystems) and the following primers: 5′-GTCCTTGTGTTCCCTGTGTG (preprodynorphin forward, GenBank accession #M32783), 5′-CATAGCGTTTCTGGTTGTCC (preprodynorphin reverse, GenBank accession #M32784), 5′-GCTGAGTATGTCGTGGAGTC (GAPDH forward, GenBank accession #X02231) and 5′-TGGGAGTTGCTGTTGAAGTC (GAPDH reverse). Forward primers were extended with the T3 promoter at the 5′ end.

2.3. Generation of standard curves

Calibration curves for fluorescence intensity plotted against number of PCR cycles were constructed using sense RNA transcripts as standards. In order to estimate the dynamic range of the method, 200, 60, 20, 6, 2, 0.6, and 0.2 fg of RNA transcripts or 150, 50, 15, 5, 1.5, 0.5, and 0.15 ng of total RNA per 10 μl of reaction mixture were used. The concentrations of total RNA as well as RNA transcripts found optimal in these preliminary studies were further used for gene expression analysis. Thus, 20 ng of total RNA from caudate putamen per 10 μl reaction volume was used for quantification of μ, κ, δ opioid receptors and *c-fos* mRNAs, 4 ng of total RNA was used for quantification of preprodynorphin and dopamine D_2 receptor mRNAs; 1 and 0.3 ng of total RNA were used for quantification of GAPDH and preproenkephalin mRNAs, respectively.

The calibration curves for 18S mRNA were plotted using 15, 5 and 1.5 ng from total RNA brain extracts from drug naïve rats per 10 μl of reaction volume. The values of signals obtained from 5 ng of total RNA from the caudate putamen of rats treated with cocaine or saline in 'binge' paradigm for 1 or 3 days were compared with calibration curves and were expressed in μg per μl of original mRNA extract.

Results of selected TaqMan® measurements were confirmed by RNase protection assay performed according to our previously published protocol (Spangler et al., 1996; Yuferov et al., 2001).

3. Results

3.1. TaqMan® primer-probe design for test of modification efficiency

In this study we tested the efficiency of the following probe designs: (1) probes with FAM and TAMRA as fluorophore and quencher on 5′ and 3′ positions, respectively; (2) probes with FAM and TAMRA as fluorophore and quencher on 5′ and internal positions, respectively; (3) probes with Alexa Fluor® 488 and TAMRA as fluorophore and quencher on 5′ and internal positions, respectively; (4) probes with Alexa

Table 2
Influence of different modifications for different probes upon the performance in TAQMAN

Probe name	Probe sequence (5′→3′)	Length of the probe	Fluorophore–quencher distance	$E = (I_{final} - I_{init})/I_{init}$	Coefficient of improvement
D_2-FT-Ter	FAM-CCTGAAGACACCACTCAAGGGCAACTGTA-TAMRA[b]	29	29	0.188	1
D_2-FT-Int	FAM-CCTGAAGACACCACT(TAMRA)CAAGGGCAACTGTA-Ph[b]	29	15	0.525	2.79
D_2-AT-Int	AL-CCTGAAGACACCACT(TAMRA)CAAGGGCAACTGTA-Ph[b]	29	15	0.584	3.11
D_2-AD-Int[a]	AL-ACAGT(DAB)GCCCTTGAGTGGTGTCTTCAGG-Ph[b]	29	5	0.106	0.56
D_2-FD-Ter	FAM-CCTGAAGACACCACTCAAGGGCAACTGTA-DAB[b]	29	29	0.302	1.61
MOR-FT-Ter	FAM-TGGCAGTCTTCATTTTGGTGTATCTTACAATCACAT-TAMRA[c]	36	36	0.319	1
MOR-FT-Int	FAM-TGGCAGTCTT(TAMRA)CATTTTGGTGTATCTTACAATCACAT-Ph[c]	36	10	0.880	2.76
MOR-AT-Int	AL-TGGCAGTCTT(TAMRA)CATTTTGGTGTATCTTACAATCACAT-Ph[c]	36	10	0.750	2.35
MOR-AD-Int	AL-TGGCAGT(DAB)CTTCATTTTGGTGTATCTTACAATCACAT-Ph[c]	36	7	0.112	0.13
MOR-FD-Ter	FAM-TGGCAGTCTTCATTTTGGTGTATCTTACAATCACAT-DAB[c]	36	36	0.509	1.60
ENK-FT-Ter	FAM-TCAACTTCCTGGCATGCACACTCGA-TAMRA[d]	25	25	0.264	1
ENK-FT-Int	FAM-TCAACTTCCT(TAMRA)GGCATGCACACTCGA-Ph[d]	25	10	0.896	3.40
ENK-AT-Int	AL-TCAACTTCCT(TAMRA)GGCATGCACACTCGA-Ph[d]	25	10	0.714	2.70
ENK-AD-Int	AL-TCAACTT(DAB)CCTGGCATGCACACTCGA-Ph[d]	25	7	0.079	0.30

Abbreviations: AL, Alexa Fluor® 488; Ph, phosphate; DAB, dabcyl; E, efficiency of probe in fluorescent RT-PCR; I_{init}, fluorescence intensity of PCR solution at 530 nm before performance of amplification; I_{final}, fluorescence intensity of PCR mixture solution at 530 nm after performance of amplification. Coefficient of improvement was found as ratio between value E of probe of interest and value E of probe with 3′ TAMRA. TAMRA or dabcyl were attached to the probes either at 3′ through phosphodiether bond or at internal position through amino-modifier C6 dT.

[a] Antisense probe.

[b] Probes were designed for dopamine D_2 mRNA.

[c] Probes were designed for μ opioid receptor mRNA.

[d] Probes were designed for preproenkephalin mRNA.

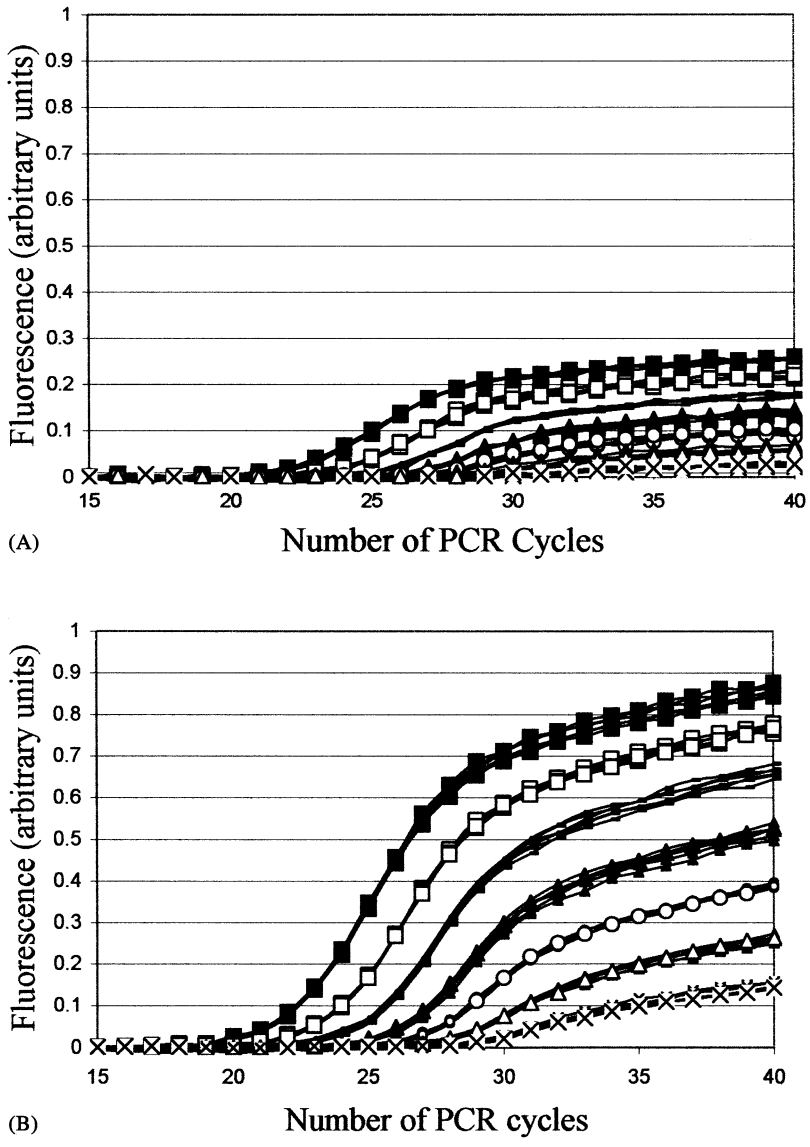

Fig. 1. Amplification plot for probe with (A) FAM and TAMRA at 5′ and 3′ positions of fluorescently labeled oligonucleotide, respectively (MOR-FT-Ter, for sequence see Table 2), and probe with (B) FAM and TAMRA at 5′ and internal positions of fluorescently labeled oligonucleotide, respectively (MOR-FT-Int). Different amounts of RNA transcript were used for amplification curve generation: 200 (■), 60 (□), 20 (▲), 6 (▲), 2 (○), 0.6 (⌂), and 0.2 (×) fg per 10 μl of reaction volume.

Fluor® 488 and dabcyl as fluorophore and quencher on 5′ and internal positions, respectively; (5) probes with FAM and dabcyl as fluorophore and quencher on 5′ and 3′ positions, respectively (see Table 2 for probe design).

To test efficiency of these modifications, mRNAs of dopamine D_2 receptor, μ opioid receptor and preproenkephalin were chosen. Fluorescent probes were selected to have an exon–exon junction in the middle and a predicted melting temperature around 69–71 °C as defined by the PRIMER EXPRESS® software (Applied Biosystems). The exon 1–exon 2 junction for both μ-opioid receptor and dopamine D_2 receptor mRNAs, and the exon 2–exon 3 junction for preproenkephalin mRNA were chosen (see Tables 1 and 2).

3.2. Introduction of TAMRA as quencher into an internal position

A comparison of amplification curves for probes containing quencher on the terminal or an internal position for μ opioid receptor mRNA is shown in Fig. 1 A, B. To quantify the efficiency of each modification within a group of probes for a particular mRNA, we selected a threshold level of 0.1 (arbitrary linear units) of fluorescence increment and plotted the number of PCR cycles at which fluorescence reaches this threshold level ('threshold cycle') as the dependence on concentration of mRNA transcript (Fig. 2A, B). The dependence of threshold cycle on mRNA concentration was found to

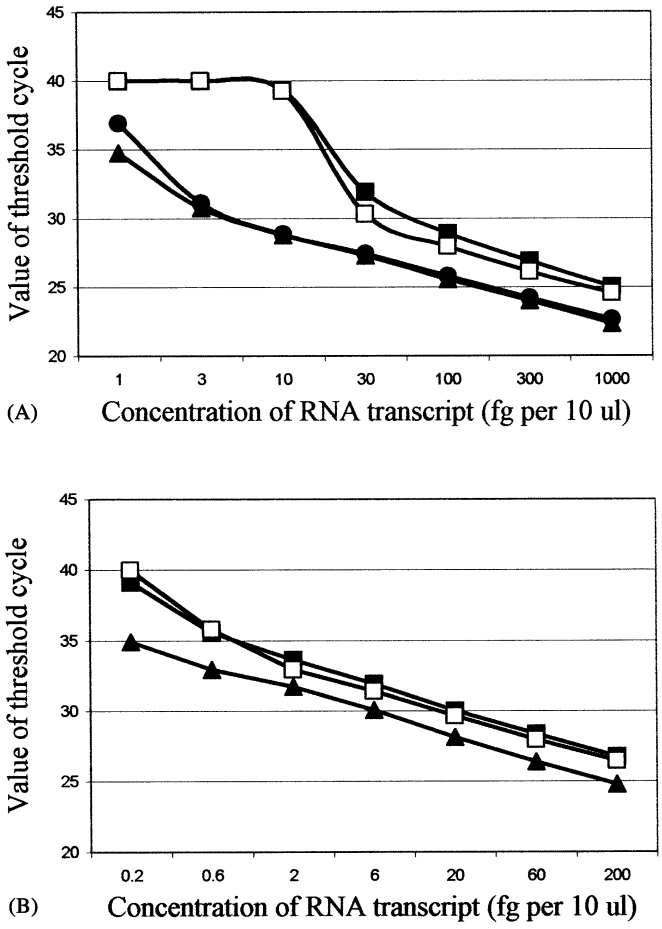

Fig. 2. Improvement of sensitivity and reduction of threshold cycle of TaqMan® probes by relocation of the fluorophore to an internal position. Sensitivity of detection for a specific probe is defined as concentration of specific mRNA template at which the linear dependence of threshold cycle on mRNA concentration breaks down. A. μ opioid receptor probes (36 bases long): FAM at 5′ and TAMRA at 3′ position (■), MOR-FT-Ter, for sequence see Table 2; FAM at 5′ and TAMRA at internal position (▲), MOR-FT-Int; Alexa-488 at 5′ and TAMRA at internal position (□), MOR-AT-Int; FAM at 5′ and Dabcyl at 3′ position (●), MOR-FD-Ter. B. Preproenkephalin probes (25 bases long): FAM at 5′ and TAMRA at 3′ position (■), ENK-FT-Ter; FAM at 5′ and TAMRA at internal position (▲), ENK-FT-Int; Alexa-488 at 5′ and TAMRA at internal position (□), ENK-AT-Int.

be linear for up to five orders of magnitude (data not shown). At concentrations of specific mRNA template of 0.01–20 fg per well, the linear dependence of threshold cycle on concentration breaks down, thereby defining the sensitivity of detection for the specific probe. This level was variable for different probes and presumably depends upon the secondary structure of the template as well as physicochemical properties of the specific fluorescent probe.

We found that for all tested probes the positioning of the quencher in the internal site in the probe reduces the value of threshold cycle by 2–3 for high concentrations of the template (200–20 fg per 10 μl) and by up to 10 for low concentrations of the template (2–0.6 fg per 10 μl). This modification considerably improves the accuracy of measurements at low template concentrations (Fig. 2A, B). The effect was strongest in the longest probe (μ-opioid receptor probe, 36 bases) where the fluorophore-quencher distance was reduced from 36 to ten bases. As a result, the sensitivity of the detection was improved up to 30-fold with the value of threshold cycle reduced by up to 12. Reduction of the number of bases between fluorophore and quencher from 25 to ten bases (pre-proenkephalin probe) or from 29 to 15 bases (dopamine D_2 receptor probe) also reduced the value of threshold cycle.

The efficiency E (Table 2) of each probe was also measured as the increment of the reporter fluorescence before and after PCR performance. This measurement was made with high concentrations of template (200 fg of RNA transcript per 10 μl reaction volume) in order to minimize errors in measurement and to increase the accuracy and precision of calculations. The results were expressed as a normalized ratio of increment of final to starting fluorescence: $E = (I_{final} - I_{init})/I_{init}$, where I_{init} and I_{final} are fluorescence of the PCR solution at 530 nm (fluorescein emission maximum in TaqMan® probes) before and after amplification, respectively. Coefficient of improvement was found as ratio between value E of probe of interest and value E of probe with 3′-TAMRA. In all tested primer–probe sets relocation of the quencher from the 3′ position to an internal one led to considerable improvement of probe efficiency E (Table 2). The value E increased by up to 3-fold in all three probe sets tested.

3.3. Data analysis and reproducibility of the results

The choice of the appropriate range of measurement for analytical studies is essential. The use of a large amount of sample may provide higher accuracy of measurements, although impurities in crude sample preparation may affect assay performance. In our measurements we used amounts of RNAs 100 times higher than the approximate sensitivity level of probe described above.

The choice of an appropriate number of replicates for TaqMan® studies is also a critical issue. Too few replicates will result in unacceptably high error of measurement, but excessive numbers of replicates reduce the total number of samples that can be analyzed simultaneously in the same plate and significantly increases the cost and complexity of an experiment. We found that the mean coefficient of variation for a number of replicates from seven to 24 are in the range 6.5–8% for 10 μl and 9–10% for 5 μl reaction volume (data not shown). For the further studies described

Table 3
Influence of cocaine administration upon gene expression in caudate putamen during 1 and 3 day treatment

Gene name (Lengths of RNA transcripts)	Treatment group (day)	TaqMan® measurements[a]			RNase protection assay measurements[a]		
		Saline treatment	Cocaine treatment	Fold change	Saline treatment	Cocaine treatment	Fold change
Preproenkephalin (971 b)	1	30.33±0.94	30.11±0.59	0.99	39.16±0.19	47.20±1.44	1.21*
Preproenkephalin (971 b)	3	39.61±1.20	32.87±1.17	0.83*	58.16±1.35	53.96±0.63	0.93*
Preprodynorphin (593 b)	1	3.268±0.086	3.671±0.135	1.12	5.28±0.05	6.54±0.13	1.24*
Preprodynorphin (593 b)	3	3.820±0.150	5.147±0.131	1.35*	6.98±0.05	7.72±0.16	1.11*
μ opioid receptor (1520 b)	1	0.156±0.003	0.142±0.003	0.91*	0.32±0.04	0.39±0.06	1.22
μ opioid receptor (1520 b)	3	0.159±0.008	0.147±0.003	0.92	0.48±0.03	0.42±0.03	0.86
κ opioid receptor (1734 b)	1	0.192±0.007	0.173±0.004	0.90*	N/A	N/A	
κ opioid receptor (1734 b)	3	0.217±0.011	0.265±0.005	1.22*	N/A	N/A	
δ opioid receptor (1342 b)	1	0.198±0.003	0.214±0.005	1.08*	N/A	N/A	
δ opioid receptor (1342 b)	3	0.259±0.007	0.276±0.008	1.06	N/A	N/A	
Dopamine D_2 receptor (970 b)	1	6.339±0.082	6.493±0.149	1.02	2.63±0.16	3.07±0.06	1.17
Dopamine D_2 receptor (970 b)	3	9.052±0.371	8.116±0.199	0.90	4.00±0.44	2.88±0.09	0.72
c-fos (1255 b)	1	0.110±0.003	0.593±0.025	5.38*	0.17±0.02	0.89±0.02	5.29*
c-fos (1255 b)	3	0.116±0.003	0.426±0.004	3.68*	0.19±0.02	0.68±0.02	3.50*
GAPDH (599 b)	1	42.97±1.03	46.15±1.88	1.07	N/A	N/A	
GAPDH (599 b)	3	46.89±2.24	53.04±2.49	1.13	N/A	N/A	
18S (μg/μl)	1	2.02	2		1.83	1.77	
18S (μg/μl)	3	2.29	2.24		1.76	1.83	

*Changes in expression are significant according to two-tail t-tests ($P < 0.05$), N/A- data not available.
[a] Data is expressed in atommoles of mRNA of interest per μg of total RNA±standard error of measurement except 18S RNA that was measured in μg per μl of stock solution.

below we used six replicates of 10 μl volume which had a mean coefficient of variation 6.6%.

Since the technique described here employs manual preparation and pipetting of the samples, errors in loading cannot be completely avoided, which raises the question of identification of outliers. We used the method of Dean and Dixon (1951), which is appropriate for small numbers of observations (from three up to 25). Tests for outliers were performed for all calibration curves as well as for the results of measurements and outliers were trimmed from data sets. The significance of differences of expression of RNA levels between treatment groups was evaluated by two-tail *t*-tests.

3.4. Measurement of cocaine-induced alterations in gene expression in the rat caudate putamen

TaqMan® measurements of mRNA levels were performed in six replicates of 10 μl reaction volume (Table 3). Significance in change of the gene expression level between treatment groups (1 day saline-1 day cocaine and 3 day saline-3 day cocaine) indicates technical variability of measurement but not biological variations since the samples from individual animals were pooled. We found with this technique that preprodynorphin as well as *c-fos* mRNAs are increased by cocaine administration both for 1 and 3 day administration, which is in agreement with previously published data (Hope et al., 1992; Steiner and Gerfen, 1993; Spangler et al., 1996; Yuferov et al., 2001).

The reproducibility of measurements between plates within the same experiment was examined for dopamine D_2, δ, κ and μ opioid receptors and preproenkephalin mRNAs. Reaction mixtures were loaded in three identical plates and run consecutively. The average coefficient of correlation between results of plate reading was found to be 0.992.

The level of gene expression in RNA samples measured by TaqMan® was also confirmed by commonly employed RNase protection assay using the same pooled extracts (Table 3).

4. Discussion

4.1. Influence of different modifications upon the fluorescent probe performance in TaqMan® experiments

4.1.1. Introduction of TAMRA as quencher to an internal position

The optimal distance between quencher and fluorophore in energy transfer primers has been reported to be six–14 bases (Ju et al., 1995, 1996). We chose a ten base long spacer in order to keep fluorophore and quencher at the shortest possible distance from one another, while not disturbing the process of digestion of the oligonucleotide between these two labels by polymerase.

The simplest way to reduce the length between the 5′ fluorophore and the 3′ quencher is to introduce the quencher into an internal position of the nucleic acid chain during oligonucleotide synthesis, using deoxythymidine phosphoramidite modified with TAMRA through a polyether linker at 5 position in the pyrimidine moiety (Glen Research). This linker was specially designed for the introduction of bulk fluorophores and other modifiers into oligonucleotides with minimal disturbance of the double helix structure (Haralambidis et al., 1987). We avoided post synthesis introduction of TAMRA into aminopropyl modified purine bases of deoxyguanosine or deoxyadenosine (Petrie et al., 1991) since these approaches are more complicated technically and more expensive for high output synthesis.

Although some groups use fluorescent probes with the quencher in an internal position (Kalinina et al., 1997), the exact location of the quencher within the oligonucleotide has been reported to be insignificant for the probe fluorescent properties (Livak et al., 1996). However, since that experiment was performed using relatively short (24–28 bases long) oligonucleotides, this conclusion might not apply to all lengths of probes. Thus, in the design of 27 primer–probe sets for this study we found that lengths of fluorescent probes up to 39 bases were necessary (Table 1), which is considerably longer than the probe lengths in previously published data. In all our experiments, the relocation of TAMRA as quencher from 3′ to an internal position positively affected the efficiency of the probe in TaqMan® assays (Table 2).

No strong dependence of the value of efficiency *E* on the number of reduced bases between fluorophore and quencher was observed (see Table 2). Thus, the reduction of the fluorophore–quencher distance for the μ-opioid receptor probe from 36 to ten bases increases the *E* value 2.76-fold, while the reduction of the fluorophore–quencher distance for preproenkephalin probe from 25 to ten bases increases the *E* value 3.40-fold. However, improvement of the sensitivity level as determined by reduction of the threshold cycle value at a given RNA concentration for the μ-opioid receptor probe (see Fig. 2A; 30-fold at 36 bases long) was considerably greater than that of the preproenkephalin probe (see Fig. 2B; 3-fold at 25 bases long). Therefore, both sensitivity level and efficiency *E* are important in characterization of the influence of modification upon the performance of the probe in the fluorescent PCR and these values should be considered and analyzed simultaneously.

Apparently, along with the strong influence of the position of the quencher within the probe sequence, other factors may influence the performance of the probe in fluorescent PCR. One such factor recently

reported is quenching of the fluorophore (fluorescein or BODIPY® FL) by a neighboring guanine residue that is located in the fluorescent probe or in the complementary sequence of the amplicon (Crockett and Wittwer, 2001; Kurata et al., 2001). Since we were constrained to specific exon–exon boundary sequences, we could not completely avoid this potential problem. As a result, the probe for dopamine D_2 receptor that started with 5′-CC dimer (GG in the complementary amplicon) showed the least efficiency E among probes tested (Table 2). This phenomenon was observed within the group of oligonucleotides that contained quencher at 3′ position as well as within the group of oligonucleotides with an internal quencher.

Since the probe for dopamine D_2 receptor does not have a thymine base in the tenth or nearby position of sequence, we used a thymine in the 15th position for modification of the probe with an internal quencher. This modification resulted in decreasing the fluorophore–quencher distance from 29 to 15 bases, in turn increasing the efficiency E of probe 2.79-fold.

Introduction of a minor groove binder (MGB) into fluorescently-labeled oligonucleotide has been reported to stabilize the oligonucleotide–DNA duplex (Kutyavin et al., 2000). With the addition of this modification, the length of the probes can be decreased to as few as ten bases. Such probes are reported to have better mismatch discrimination properties and have been recommended for allele discrimination. The introduction of the MGB increases probe sensitivity by reducing fluorophore–quencher distance, similar to the introduction of quencher into an internal position of the probe as reported herein. Additionally, simultaneous reduction of the size of the oligonucleotide and incorporation of three bulk moieties (two fluorophores and MGB) could potentially raise sterical tensions that would disturb the formation of the duplex and decrease sensitivity of detection. Being commercially unavailable themselves, MGB phosphoramidites are accessible only in oligonucleotides custom-made by one vendor thus preventing the possibility of easy ‘in house’ synthesis of probes labeled with this type of compound. These considerations along with high cost of MGB conjugated probes led us to avoid the use of such probes in these experiments.

4.1.2. Substitution of FAM with Alexa Fluor® 488

Alexa Fluor® 488 fluorescent dye was developed to substitute for fluorescein and FAM. It has been demonstrated to be much more resistant to photobleaching, and its fluorescent properties are much less temperature-and pH-dependant than those of fluorescein (Panchuk-Voloshina et al., 1999). In our experiments the fluorescein moiety was substituted with Alexa Fluor® 488 in order to increase the stability of the probe and decrease the decomposition of the label due to exposure of the probe to light or heat.

Our studies showed, however, that this modification does not improve the fluorescent properties of the system both with the reference to the value E (Table 2) and to the value of threshold cycle (Fig. 2A, B). We conclude that the time of exposure of reaction mixture to the direct light during PCR cycling (600 exposures for 40 cycles, 10 ms each) is shorter than the half-time of FAM bleaching and that the difference in chemical structure makes energy transfer in the Alexa Fluor® 488–TAMRA pair less, not more, efficient than in the FAM-TAMRA pair.

4.1.3. Introduction of nonfluorescent quencher at 3′ or an internal position

Introduction of a fluorescent quencher into the oligonucleotide probe produces a broad peak of fluorescence in addition to the fluorescence of the fluorophore itself and, therefore, the changes in fluorescence of the fluorophore during PCR amplification may be partially masked, leading to a loss of sensitivity. The substitution of a non-fluorescent quencher for a fluorescent one eliminates this problem and, potentially, may improve the sensitivity.

In order to test this hypothesis, a set of oligonucleotide probes with the dabcyl moiety, a well-known quencher of fluorescein (Tyagi and Kramer, 1996; Tyagi et al., 1998), introduced into an internal (attached to a deoxythymidine residue) or 3′-terminal position was synthesized (Table 2) and used in fluorescent PCR. In contrast to the fluorescein–tetramethylrhodamine pair, there is not much data available regarding the properties of fluorescein–dabcyl or Alexa Fluor® 488–dabcyl pairs. In the most common technique that uses the dabcyl moiety as a quencher, molecular beacons, the quencher and fluorophore are close to one another being located at the terminal positions of the hairpin, so the optimal fluorophore–quencher distance is of no concern. Fluorescent probes with dabcyl were previously reported for use in the TaqMan® technique, but neither the properties of these probes nor their efficiency were compared directly with properties or efficiency of probes that use conventional fluorescent quenchers (Kreuzer et al., 2001).

In order to suppress fluorescence of the fluorophore completely, we reduced the distance between fluorophore and nonfluorescent quencher to seven bases for the μ opioid receptor and preproenkephalin probes. Since the sequence of the probe used for the dopamine D_2 receptor did not allow us to place the quencher on the seventh or any nearby position, we used a fluorescent probe with reverse sequence and dabcyl at the fifth position from the 5′ end.

Efficiency E (Table 2) and calibration curves of threshold cycle of probes containing dabcyl and probes

with TAMRA as a quencher were compared. As expected, according to the raw fluorescence spectrum data, the fluorescence of the fluorophore in the probe with internal dabcyl was completely suppressed by the presence of the neighboring quencher. However, no increase in fluorescence was observed during the performance of PCR.

This last phenomenon was observed in all tested probes and may not be explained by different rates of digestion of different nucleotides within the fluorescent probe by Taq polymerase. More likely it is caused by inhibition of polymerase activity by the dabcyl moiety located close to the 5′ position, which prevents fluorescently-labeled oligonucleotide from enzymatic digestion. The placement of dabcyl at the 3′ position increases the ability of the enzyme to digest the fluorescent probe. At the same time, this construction of the probe reduces the efficiency of quenching of fluorophore resulting in considerably higher fluorescence of the oligonucleotide probe, which lessens the advantage of using a nonfluorescent quencher. Thus, the sensitivity level of such probes in our studies is about the same as for probes with internal TAMRA as quencher (Fig. 2A), while the value of E is about half compared with probes with internal TAMRA (Table 2). The use of recently developed nonfluorescent nitro substituted cyanine dyes as fluorescence quenchers (Lee et al., 2000) may offer a promising approach.

4.2. Measurement of gene expression level in RNA samples

For this study we chose the caudate putamen as a brain region in which dopamine levels are known to be elevated following 'binge' cocaine administration (e.g. Maisonneuve and Kreek, 1994). The list of genes to be tested included those of the opioid system (δ, κ, μ opioid receptors, preproenkephalin, and preprodynorphin), dopamine receptor D_2 and the immediate early gene *c-fos*.

Cocaine administration rapidly increases extracellular synaptic dopamine level by blocking monoamine reuptake by the dopamine transporter. Cocaine-mediated stimulation of dopamine receptors (D_1 and D_2 receptor types) leads to alterations in the activity or expression of diverse types of cellular proteins of specific neurons in specific brain regions, which are part of an increasingly well identified and expanding network of neurotransmitters and neuropeptides altered by stimulant exposure in an abuse pattern. Upregulation of immediate early genes (e.g. c-*fos, zif*/268) as well as preprodynorphin mRNAs in the rat brain following acute, repeated, and 'binge' pattern cocaine administration has been extensively studied as well (e.g. Hope et al., 1992; Steiner and Gerfen, 1993; Spangler et al., 1996, 1997). The induction of *c-fos* and other immediate-early genes is thought to reflect increased neuronal activity. Some, though not all, studies, reported that cocaine administration increases levels of preproenkephalin mRNA in striatal brain regions, including caudate putamen (e.g. Hurd and Herkenham, 1992; Steiner and Gerfen, 1993; Spangler et al., 1997). Previous reports demonstrated that chronic cocaine administration causes a significant increase in μ and κ opioid receptor density in specific brain regions including the cingulate cortex, nucleus accumbens, basolateral amygdala and caudate putamen (Unterwald et al., 1992, 1994; Yuferov et al., 1999).

The results of our studies are presented in Table 3. The level of expression of each gene was normalized on the amount of 18S RNA and expressed as attomoles per μg of total RNA. Since the measurements were performed using pooled samples, significance in gene expression change showed variability of the technique of measurement but not biological variability within the treatment groups. Changes in expression of almost all genes were found in the range $\pm 10\%$ with exception of *c-fos*, preprodynorphin (after both 1 and 3 day cocaine treatment), and κ opioid receptor (after 3 day cocaine treatment). Some of these measurements were confirmed by RNase protection assay (Table 3) using precisely the same pooled samples. We found that absolute values of preproenkephalin and *c-fos* mRNAs established by TaqMan® and RNase protection assay are close to one another. Twofold difference between RNase protection assay and TaqMan® measurements were found for μ opioid receptor, dopamine D_2 receptor and preprodynorphin mRNAs.

In comparing these findings, it should be noted that each of these techniques has its potential strengths and weaknesses. For example, the extent of degradation of either sense–strand transcript or mRNA in extracts from tissue might be expected to influence the two assays differently. In the traditional RNase protection assay, gel electrophoresis is used to separate protected duplex RNA–RNA hybrids (specific mRNA and labeled antisense probe) from unhybridized probe molecules that have been degraded by RNase. Autoradiography and film densitometry are then employed to quantify radioactivity in protected hybrids. In the modification of the RNase protection assay used in these studies, protected RNA–RNA hybrids are collected by acid precipitation followed by filtration through glass fiber filters and liquid scintillation counting of radioactivity on filters. This technique would be expected to collect and count duplex RNA fragments greater than approximately 25 nucleotides in length.

In an earlier study, we compared three methods of detection in RNase protection assays: (1) the filtration assay used in this study, or using gel electrophoresis followed by either (2) autoradiography and densitometry or (3) direct liquid scintillation counting of dried gels (Pham et al., 1998). We found no significant differences

in accuracy of measurements between the three methods (although those techniques that use gel electrophoresis are substantially more time-consuming, labor intensive, and expensive). We additionally found no significant difference in results if the single major band in the autoradiogram was measured compared with the entire lane of the gel (from directly above the position of the major protected species to a position at approximately 50 bases). This is analogous to the procedure of collection of all macromolecular-protected hybrids by acid precipitation and filtration. If sense strand transcript or mRNA in tissue extracts is partially degraded (at least, to a limited extent), we would expect minimal influence on the performance of the RNase protection assay. This is not the case for the TaqMan® assay, in which any single strand break in an unknown RNA sample within the 200–300 bases spanned by the primers would absolutely prevent amplification of that molecule by RT-PCR, and would lower the apparent amount of the specific mRNA measured by the assay. Conversely, if in the construction of calibration curve the sense strand standards were partially degraded, the assay would report values higher than actually present in the sample. Any determination of mRNA quantity based on RT-PCR is, therefore, more sensitive to the effects of partial degradation of RNA compared with the RNase protection assay.

Also, the presence of inhibitors of DNA polymerase in the analyzed sample (e.g. tRNA as RNA stabilizer) could potentially reduce activity of the enzyme and, therefore, reduce the value of RNA measured by TaqMan®.

These factors might explain observed differences in measurements obtained by RNase protection assay compared with TaqMan®. Nevertheless, in the analysis of induced alterations in gene expression, the question of absolute quantification of mRNA level might not be the most critical issue. Not estimation of absolute mRNA quantity, but determination of whether expression of gene of interest was changed under the influence of the factors investigated, becomes the question to study.

Thus, fold change in mRNA level numerically expressed in comparative units determined by TaqMan® assay was found to be close (less than 20% difference) to that of RNase protection assay for seven measurements out of ten, with the exception of preproenkephalin and μ opioid receptor mRNAs quantified for 1 day saline–cocaine treatment group, and preprodynorphin quantified for 3 day saline–cocaine treatment group.

One of the new alternative approaches for quantification of gene expression that has been developed over the last decade is microarray technology. The number of genes that may be simultaneously analyzed is much greater for microarrays than for real time PCR, but the sensitivity of the arrays is several 1000-fold lower than that of real time PCR. The average amount of total RNA extracted from nucleus accumbens of a single rat, for instance, is 5 μg, which is 4-fold lower than that required for the analysis on a single microarray. Our recent microarray studies (article under review) show that gene expression measurements on microarrays are more reliable if studies are done using replication of microarrays, which requires additional RNA and cannot be performed using tissue from brain regions of individual rats. This imposes certain restrictions on the use of the method since samples must be pooled and biological variations of individual animals cannot be measured. Even with pooled specimens and, thus, with more RNA, many genes are still below the detection limit of microarray analysis.

On the other hand, in our recent studies (article in preparation) we demonstrate the quantitative measurement of expression of as many as 25 genes in six replicates in nucleus accumbens using as little as 10% of total RNA extracted from individual rat brain regions using real time PCR. Therefore, less than 0.5% of total RNA from a single animal was used for measurement of expression of a single gene.

Another limitation of microarray studies is the dynamic range of measurement, which is two orders of magnitude for CCD camera and three orders of magnitude for photoelectronic multiplier-based detection systems. All signals higher or lower than this limit cannot be analyzed. Fluorescence PCR operates with the number of PCR cycles when fluorescence of the solution reaches a certain level, but it does not compare fluorescence of the solutions itself. Therefore, the dynamic range of real time PCR is not restricted by the properties of a detector and reaches up to seven to eight orders of magnitude.

5. Conclusions

We used the TaqMan® technique for the analysis of the influence of different probe designs upon their performance in real time PCR. In terms of sensitivity, the highest benefit from probe modification was obtained by placing the fluorescent quencher TAMRA in a central position of the oligonucleotide. A similar effect was obtained using probes with nonfluorescent quencher at the 3′ position. Other variations of fluorescent probe design led to lower levels of sensitivity of measurements. Based on the results of these studies, the design, synthesis and evaluation of 27 optimized primer–probe sets for genes of importance for neurobiological studies were performed. This set of probes is routinely used in our laboratory for quantification of mRNA level in samples of rat brain. In this report we demonstrated the use of seven probes of this set for measurement of alterations in mRNA levels following cocaine administration. The set of designed probes

might be also used for investigation of molecular mechanisms of neural pathways to control pain and behavioral studies, or for analysis of psychiatric disorders on a molecular level using animal models. Compared with RNase protection assay, real time optical PCR requires up to a 200-fold lower amount of starting RNA template and may be successfully applied for multigene expression analysis in small samples of tissue.

Acknowledgements

This work was supported by the National Institutes of Health-National Institute on Drug Abuse Research Scientific Award Grant K05-DA00049 and the National Institutes of Health-National Institute on Drug Abuse Research Center Grant P60-DA05130.

References

Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000;25:169–93.

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chlorophorm extraction. Anal Biochem 1987;162:156–9.

Crockett AO, Wittwer CT. Fluorescein-labeled oligonucleotides for real-time PCR: using the inherent quenching of deoxyguanosine nucleotides. Anal Biochem 2001;290:89–97.

Dean RB, Dixon WJ. Simplified statistics for small numbers of observations. Anal Chem 1951;23:636–8.

Espy MJ, Teo R, Ross TK, Svien KA, Wold AD, Uhl JR, Smith TF. Diagnosis of Varicella-Zoster virus infections in the clinical laboratory by LightCycler PCR. J Clin Microbiol 2000;38:3187–9.

Foster T. Zwischenmolekulare Energiewanderung und Fluoreszenz. Annalen der Physik 1948;2:55–75.

Haralambidis J, Chai M, Tregear GW. Preparation of base-modified nucleosides suitable for non-radioactive label attachment and their incorporation into synthetic oligodeoxyribonucleotides. Nucleic Acids Res 1987;15:4657–76.

Hillisch A, Lorenz M, Diekmann S. Recent advances in FRET: distance determination in protein-DNA complexes. Curr Opin Struct Biol 2001;11:201–7.

Hope B, Kosofsky B, Hyman SE, Nestler EJ. Regulation of immediate early gene expression and AP-1 binding in the rat nucleus accumbens by chronic cocaine. Proc Natl Acad Sci USA 1992;89:5764–8.

Hurd YL, Herkenham M. Influence of a single injection of cocaine, amphetamine or GBR 12909 on mRNA expression of striatal neuropeptides. Mol Brain Res 1992;16:97–104.

Ju J, Ruan C, Fuller CW, Glazer AN, Mathies RA. Fluorescence energy transfer dye-labeled primers for DNA sequencing and analysis. Proc Natl Acad Sci USA 1995;92:4347–51.

Ju J, Glazer AN, Mathies RA. Cassette labeling for facile construction of energy transfer fluorescent primers. Nucleic Acids Res 1996;24:1144–8.

Kalinina O, Lebedeva I, Brown J, Silver J. Nanoliter scale PCR with TaqMan® detection. Nucleic Acids Res 1997;25:1999–2004.

Kreuzer KA, Bohn A, Lupberger J, Solassol J, Le Coutre P, Schmidt CA. Simultaneous absolute quantification of target and control templates by real-time fluorescence reverse transcription-PCR using 4-(4′-dimethylaminophenylazo)benzoic acid as a dark quencher dye. Clin Chem 2001;47:486–90.

Kurata S, Kanagawa T, Yamada K, Torimura M, Yokomaku T, Kamagata Y, Kurane R. Fluorescent quenching-based quantitative detection of specific DNA/RNA using a BODIPY®-FL-labeled probe or primer. Nucleic Acids Res 2001;29:e34.

Kutyavin IV, Afonina IA, Mills A, Gorn VV, Lukhtanov EA, Belousov ES, Singer MJ, Walburger DK, Lokhov SG, Gall AA, Dempcy R, Reed MW, Meyer RB, Hedgpeth J. 3′-Minor groove binder–DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res 2000;28:655–61.

Lee LG, Graham RJ, Mullah KB, Haxo FT. Nitro-substituted non-fluorescent asymmetric cyanine dye compounds, 2000; US patent 6,080,868.

Lilley DMJ, Wilson TJ. Fluorescence resonance energy transfer as a structural tool for nucleic acids. Curr Opin Chem Biol 2000;4:507–17.

Livak KJ, Flood SA, Marmaro J. Method for detecting nucleic acid amplification using self-quenching fluorescent probe, 1996; US patent 5,538,848.

Macdonald R, Bingham S, Bond BC, Parsons AA, Philpott KL. Determination of changes in mRNA expression in a rat model of neuropathic pain by TAQMAN quantitative RT-PCR. Mol Brain Res 2001;90:48–56.

Maisonneuve IM, Kreek MJ. Acute tolerance to the dopamine response induced by a binge pattern of cocaine administration in male rats: an in vivo microdialysis study. J Pharmacol Exp Ther 1994;268:916–21.

Medhurst AD, Harrison DC, Read SJ, Campbell CA, Robbins MJ, Pangalos MN. The use of TaqMan® RT-PCR assays for semi-quantitative analysis of gene expression in CNS tissues and disease models. J Neurosci Methods 2000;98:9–20.

Metzker ML, Gibbs RA. Substituted 4,4-difluoro-4-bora-3A,4A-diaza-s-indacene compounds for homogenous amplification/detection assays, 1999; US Patent 5,994,063.

Meyer JRB, Afonina IA, Kutyavin IV. Oligonucleotides containing pyrazolo[3,4-D]pyrimidines for hybridization and mismatch discrimination, 2000; US Patent 6,127,121.

Myakishev MV, Khripin Y, Hu S, Hamer DH. High-throughput SNP genotyping by allele-specific PCR with universal energy-transfer-labeled primers. Genome Res 2001;11:163–9.

Okamura Y, Kondo S, Sase I, Suga T, Mise K, Furusawa I, Kawakami SH, Watanabe Y. Double-labeled donor probe can enhance the signal of fluorescence resonance energy (FRET) in detection of nucleic acid hybridization. Nucleic Acids Res 2000;28:e107.

Panchuk-Voloshina N, Haugland RP, Bishop-Stewart J, Bhalgat MK, Millard PJ, Mao F, Leung W-Y, Haugland RP. Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. J Histochem Cytochem 1999;47:1179–88.

Parks SB, Popovich BW, Press RD. Real-time polymerase chain reaction with fluorescent hybridization probes for the detection of prevalent mutations causing common thrombophilic and iron overload phenotypes. Am J Clin Pathol 2001;115:439–47.

Petrie CR, Adams AD, Stamm M, Van Ness J, Watanabe SM, Meyer RB, Jr. A novel biotinylated adenylate analog derived from pyrazolo[3,4-d] pyrimidine for labeling DNA probes. Bioconjug Chem 1991;2:441–6.

Pham K, LaForge KS, Kreek MJ. Comparison of methods for quantitation of radioactivity in protected hybrids in RNAse protection assays. Biotechniques 1998;25:198–206.

Spangler R, Ho A, Zhou Y, Maggos CE, Yuferov V, Kreek MJ. Regulation of κ opioid receptor mRNA in the rat brain by 'binge' pattern cocaine administration and correlation with preprodynorphin mRNA. Mol Brain Res 1996;38:71–6.

Spangler R, Zhou Y, Maggos CE, Schlussman SD, Ho A, Kreek MJ. Prodynorphin, proenkephalin and κ opioid receptor mRNA responses to acute 'binge' cocaine. Mol Brain Res 1997;44:139–42.

Steiner H, Gerfen CR. Cocaine-induced c-fos messenger RNA is inversely related to dynorphin expression in striatum. J Neurosci 1993;13:5066–81.

Stuhmeier F, Hillisch A, Clegg RM, Diekman S. Fluorescent energy transfer analysis of DNA structures containing several bulges and their interaction with CAP. J Mol Biol 2000;302:1081–100.

Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol 1996;14:303–8.

Tyagi S, Bratu DP, Kramer FR. Multicolor molecular beacons for allele discrimination. Nat Biotechnol 1998;16:49–53.

Tyagi S, Marras SAE, Kramer FR. Wavelength-shifting molecular beacons. Nat Biotechnol 2000;18:1191–6.

Unterwald EM, Horne-King J, Kreek MJ. Chronic cocaine alters brain mu opioid receptors. Mol Brain Res 1992;584:314–8.

Unterwald EM, Rubenfeld JM, Kreek MJ. Repeated cocaine administration upregulates κ and mu, but not δ, opioid receptors. Neuroreport 1994;5:1613–6.

Vet JAM, Majithia AR, Marras SAE, Tyagi S, Dube S, Poiesz BJ, Kramer FR. Multiplex detection of four pathogenic retroviruses using molecular beacons. Proc Natl Acad Sci USA 1999;96:6394–9.

Whitcombe D, Theaker J, Guy SP, Brown T, Little S. Detection of PCR products using self-probing amplicons and fluorescence. Nat Biotechnol 1999;17:804–7.

Yuferov V, Zhou Y, Spangler R, Maggos CE, Ho A, Kreek MJ. Acute 'binge' cocaine increases mu-opioid receptor mRNA levels in areas of the rat mesolimbic mesocortical dopamine system. Mol Brain Res 1999;48:109–12.

Yuferov V, Zhou Y, LaForge KS, Spangler R, Ho A, Kreek MJ. Evaluation of guinea pig brain preprodynorphin mRNA expression and hypothalamic-pituitary-adrenal axis activity by 'binge' pattern cocaine administration. Mol Brain Res 2001;55:65–70.