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An intron-based real-time PCR method for measuring vasopressin gene transcription

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Abstract

The hypothalamus contains distinct neuronal populations that express distinguishing neuropeptides. The supraoptic nucleus contains magnocellular neurons that predominantly express either vasopressin or oxytocin. Transcriptional activators of vasopressin and other neuropeptides have been the subject of much research. Here we present a method of measuring neuropeptide transcription by tailoring one-step quantitative real-time PCR (qRT-PCR) for the analysis of processed and pre-mRNA (heteronuclear RNA). Using moderate and strong hyperosmotic stimuli to induce transcription, we report an increase in vasopressin transcription (pre-mRNA) of 141% and 406% over control levels in response to a 2% injection of 900 mOsm saline or a 1% body weight i.p. injection of 2M NaCl, respectively. These results agree with a host of studies employing the more laborintensive method of in situ hybridization histochemistry by which investigators also measured intron-containing heteronuclear RNAs. Furthermore, these results confirm that qRT-PCR with intron-specific primers can be used to rapidly analyze transcription, and suggest an important further benefit of a real-time PCR analysis, such as the ability of measuring transcription of multiple neuropeptides along with other genes from a single sample.

Keywords

Vasopressin; Supraoptic Nucleus; quantitative PCR; Hyperosmotic stimuli; heteronuclear RNA

1. Introduction

The magnocellular neuroendocrine system has two principal neuronal phenotypes categorized by which neuropeptide is chiefly expressed, oxytocin (OT) or vasopressin (VP). Systemic and intranuclear release of these peptide hormones in response to specific physiological stimuli is well-documented and continues to shed valuable light on mechanisms of neurophysiology, transmitter release, osmosensation, and excitation-transcription coupling. Of the two cell types, VP neurons continue to be particularly well-studied, due in part to the relative ease by which these cells are activated both in vivo and in vitro. Increases in plasma osmolality or decreases in arterial pressure are effective stimuli to electrophysiologically excite VP neurons, and cause systemic VP release, and induce VP gene transcription (Burbach et al., 2001;Hayashi et al., 2006;Khanna et al., 1994;Kondo et al., 2004;Mohr et al., 1995;Shoji et al., 1993;Treschan and Peters, 2006;Wakerley et al., 1978;Zingg et al., 1986).

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Unlike classical neurotransmitters, neuropeptide protein precursors are synthesized de novo, directly from translation of mRNA and post-translational processing, and these are dependent on the transcriptional activity of the specific gene. The rat VP gene is composed of both non-coding (intronic) as well as coding (exonic) regions. Transcription of both introns and exons results in the formation of a pre-mRNA transcript containing both intronic and exonic sequences. Excising of the introns occurs either co-transcriptionally or within minutes of the completion of transcription, and the final mRNA product is then transported into the cytoplasm and serves as a template for translation (Clement et al., 1999;Wetterberg et al., 2001). Whereas levels of pre-mRNA are mainly determined by transcriptional rates, levels of mRNA are regulated by both transcription and degradation processes.

The process of transcription is known to be regulated by numerous signals, second messengers, and transcription factors. It is also now becoming clear that processes of RNA degradation involving microRNA and exosomes are also regulated (Houseley et al., 2006;Sontheimer and Carthew, 2005), further supporting the notion that analyses of nascent intron-containing transcripts in the nucleus focus more-specifically on transcription and are less affected by mRNA degradation processes.

Investigating the transcription of VP using intron-specific probes was first done by use of in situ hybridization histochemistry (ISHH) (Herman et al., 1991). Since that initial study, several laboratories including our own, have used this technique to examine molecular mechanisms of VP transcription. In this paper, we use PCR primers directed against both intronic and processed exonic regions of the newly-transcribed gene in order to measure and quantify changes in intron-containing pre-mRNA in response to hyperosmotic stimuli. Using quantitative reverse-transcription PCR (qRT-PCR), we confirm a graded increase in VP gene transcription in response to mild or strong hyperosmotic stimuli. This method, applied to the investigation of acute transcriptional events may benefit others in this and related fields interested in rapid assays for gene transcription.

2. Materials and methods

2.1 Physiological stimuli

All procedures were in accordance with NIH guidelines for animal use. Male rats (Sprague-Dawley, 110-180 g) were injected with either phosphate buffered saline (290 or 900 mOsm phosphate-buffered saline, pH 7.4, i.p. 2% body wt) or 2 M NaCl (1% body wt). A separate, untreated control group was also included. Following 30 minutes post-injection, a time point at which saline-induced changes in VP transcription can be measured (Herman et al., 1991), the rats were anesthetized with isoflurane and their brains were quickly removed. Coronal hypothalamic slices (thickness = 500-600 μm) containing the supraoptic nuclei (SONs) were collected using a vibratome (Hatton et al., 1980). The SONs were immediately dissected out of the slices and placed in lysis/RNA stabilization buffer. Care was taken to prevent contamination of the sample by the suprachiasmatic or paraventricular nuclei, two neighboring nuclei known to express the assayed transcript. Purified SON RNA was isolated using an Absolutely RNA kit (Stratagene, La Jolla CA), quantified in triplicate on a spectrophotometer (NanoDrop, Wilmington DE) and stored at -80 C until further use. All RNA used had a 260/280 nm ratio between 1.90 and 2.12, representing little if any contamination by proteins or organics.

2.2 Primers and quantitative RT-PCR procedure

To assay for transcription, two sets of primers were designed. One primer pair was used to amplify transcripts of VP RNA in which intron-1 had been removed (mRNA) while another set of primers was used to amplify intron 1-containing VP transcripts (pre-mRNA). Specific primers (Figure 1, Table 1) were designed with the help of genefisher software (Giegerich et

al., 1996).These primer sets were used in separate reactions at concentrations of 0.6 μM, and were included in a one-step quantitative RT-PCR (qRT-PCR) reaction (Qiagen, Valencia CA) along with 10 ng of the collected SON-enriched total RNA template. Procedures for qRT-PCR were done according to the manufacturer's protocol. The annealing temperatures were 60 °C and 66 °C for the exonic and intronic qRT-PCR reactions, respectively. Cycle threshold (Ct) values were measured and calculated by the real-time PCR apparatus (Smartcycler, Cepheid, Sunnyvale CA). Cycle threshold values indicate a PCR cycle number at which the measured fluorescence of the indicator dye (SYBR green), corresponding to the quantity of amplicon products, is increasing in a linear fashion above background. All qRT-PCR reactions were run in triplicate and an average Ct value was calculated for each PCR condition. An additional reaction using the pre-mRNA primers was done in the absence of reverse-transcriptases (-RT) to control for DNA contamination in the RNA template. Only templates giving Ct differences ≥ 9 for the +RT vs. -RT experiments, representing little if any DNA contamination, were included in the analysis.

2.3 Standard Curves

Standard curves were generated using two different templates, both in vitro transcribed RNA and double-stranded DNA. To generate the appropriate RNA, a PCR product amplicon (Table 1, Figure 1) was inserted into a plasmid vector containing a 5′ T7 polymerase binding sequence (TOPO-TA, Invitrogen, Carlsbad CA). The plasmid vector was then cloned and the inserted PCR product's sequence was verified. PCR amplification of the T7 binding site and insert was conducted using an annealing temperature of 50 °C. The insert + T7 binding sequence was PCR amplified. The PCR products were 130 and 220 bp for the exonic and intronic sequences respectively. These products were visually confirmed and purified on an ethidium bromide-stained 2.5% agarose gel and subsequently used for the *in vitro* generation of standard curve template RNA (MEGAshorscript T7, Ambion, Austin TX). The *in vitro*-transcribed RNA was purified from the DNA template (MEGAclear, Ambion) and subjected to electrophoresis on a denaturing 15% acrylamide gel to confirm the presence of the predicted transcript. The final RNA concentrations were then measured so that serial dilutions could be made and used as templates for generation of the RNA standard curves.

The double-stranded DNA template standard curve was generated using the amplicons produced by the qRT-PCR process using the same primers shown in Figure 1 (also see Table 1). The PCR products were run on a 2% agarose gel and the ethidium bromide-labeled bands were purified to be used as template. Molecular weights for single-stranded RNA and double-stranded DNA templates were calculated online at: http://www.basic.northwestern.edu/biotools/oligocalc.html

2.4 Data Analysis

Cycle threshold values (each an average of triplicates) were pooled according to the experimental condition. An average ± S.E.M.was then calculated for each condition, and ultimately changes in gene expression were determined using a mathematical model for relative quantification (Pfaffl, 2001). Significance was tested using the unpaired Student's *t*-test and statistical significance was set at $p<0.05$.

3. Results

3.1 Verification of qRT-PCR products and PCR efficiency

The qRT-PCR reactions for the VP mRNA and pre-mRNA resulted in the generation of PCR products of predicted length. Figure 2 illustrates this point after electrophoresis on an ethidium bromide stained 2% agarose gel, where triplicates of single bands representing the 77 bp mRNA product and 171 bp pre-mRNA PCR product can be seen. As expected, little or no pre-mRNA

product can be detected in the absence of reverse transcription, indicating little if any contamination from genomic DNA. Automated DNA sequencing of the products shown in Fig 2 confirmed that only the intended DNA products were generated by the RT-PCR reaction.

Serial dilutions of in vitro transcribed RNA and purified PCR product were used to calculate RT-PCR and PCR efficiencies (E) respectively, according to the equation $E = 10^{-1/slope}$ (Rasmussen, 2001). Figures 3A & 4A show the raw data generated from the in vitro transcribed exonic (predicted exonic RNA product MW=36880.5 Da) and intronic RNA (predicted intronic RNA product MW=84508.8 Da) respectively. A logarithmic graph using this data was used to determine E values (Figs 3B and 4B). RT-PCR efficiencies were calculated to be 2.0 and 1.9, using the exonic and intronic RNAs respectively. Due to the, albeit small, differences in efficiencies, it was important to use a model for measuring relative changes in RNA amount that incorporates both RT-PCR efficiency values: $\text{Ratio} = (E_{target})^{\Delta Ct\ target}/(E_{reference})^{\Delta Ct\ reference}$ (Pfaffl, 2001). Thus, the equation for transcriptional change for these particular primer pairs is: $\text{Ratio} = (1.9)^{\Delta Ct\ intron}/(2.0)^{\Delta Ct\ exon}$.

The data and standard curve plots using PCR-generated amplicons as a template (MWs 69342.3 and 159119.8 Da for the double-stranded exonic and intronic amplicons respectively) are shown in figures 3 and 4, panels C and D. The PCR efficiencies were both calculated to be 2.0. Together these data indicate that both the reverse transcription step and PCR step are comparatively efficient over the range of templates used, and the slight difference seen with the intronic probe is likely to be due to the reverse transcription reaction.

3.2 Vasopressin transcription in Rat SONs induced by 2M NaCl injection

The total RNA from rats (150-180g) injected with 2M NaCl was isolated from SONs dissected 30 minutes post injection as described above. Average Ct values using the pre-mRNA probes were 27.04 ± 0.16 for the injected animals and 29.23 ± 0.12 for the non-injected animals (n=6, $p<0.01$). These same samples with the mRNA probes generated average Ct values of 18.28 ± 0.08 and 18.29 ± 0.14 for the injected and non-injected animals respectively (n=6, n.s.).The difference between Ct values (Ct_{intron} - Ct_{exon}) for the non-injected group was 10.94, representing a basal ratio of 439 mRNA (intron-1 removed) transcripts for every one intron-1 containing pre-mRNA transcript ($E_{intron}^{29.23}/E_{exon}^{18.29} = 1.9^{29.23}/2.0^{18.29}$). With the 2M NaCl stimulus, the ratio becomes 108 mRNA transcripts for every one intron-1 containing pre-mRNA transcript. These differences (Fig 5A,B) correspond to an increase in VP transcription of 406% in the injected animals over their control counterparts (Fig 5C).

3.3 Vasopressin transcription in isoosmotic 290 mOsm vs. hyperosmotic 900 mOsm-injected rat SONs

Since the qRT-PCR assay was able to detect the strong osmotic stimulus of a 2M NaCl injection, we then sought to determine if the assay was sensitive enough to detect changes in response to a more modest stimulus in a separate group of rats (110-130g). For this purpose we administered intraperitoneal injections of 900 mOsm (stimulus) or 290 mOsm (control) saline. Differences induced from these stimuli are detectible using ISHH (Arima et al., 1999), and by using qRT-PCR we also were able to detect these differences in transcriptional events. The average Ct values using the mRNA probes were 17.51 ± 0.41 and 17.25 ± 0.35 (n=8, n.s.) for the 290 (isoosmotic) and 900 mOsm (hyperosmotic) injected animals, respectively. The Ct values generated using the pre-mRNA probes were significantly different under the two conditions, 25.84 ± 0.16 for the 290 mOsm-injected and 25.03 ± 0.14 for the 900 mOsmo-injected (n=8 $p<0.02$). The relative increase of pre-mRNA in the 900 mOsmo-injected animals was calculated to be 141% above the 290 mOsmo-injected controls using the equation, Percent of Control = $[(1.9)^{Average\ \Delta Ct\ intron}/(2.0)^{Average\ \Delta Ct\ exon}] \times 100\%$ (Fig 5C).

4. Discussion

The magnocellular hypothalamo-neurohypophyseal system releases peptide hormones OT or VP in response to various physiological stressors. With respect to VP, its systemic release from the posterior pituitary has long been known to be induced by osmotic and volumetric challenges (Bisset and Chowdrey, 1988;Verney, 1947), and governed by magnocellular neuronal firing activity (Poulain and Wakerley, 1982). Studies using ISHH using intronic probes have shown that transcription of the VP gene also occurs in magnocellular neuroendocrine cells under these conditions (Arima et al., 1999;Herman et al., 1991;Kakiya et al., 2000;Kawasaki et al., 2005;Onaka et al., 2003;Yue et al., 2006).

Other conditions can also affect VP transcription. Noxious stimuli for example, or intracerebroventricular injection of certain neuromodulators can influence transcription (Kawasaki et al., 2006;Kurose et al., 2001;Nomura et al., 1999;Onaka et al., 2003), and the specific mechanism(s) by which either physiological stimuli or exogenous substances result in magnocellular VP transcription remains unknown. With regards to the stimulus used in the current study, it has been shown by several independent laboratories that transcription of VP increases in response to a hyperosmotic stimulus (see Supp Table 1). Those earlier results validate the method presented here for assaying gene transcription, and suggest an important further benefit of a real-time PCR analysis, such as the ability of measuring transcription of multiple neuropeptides along with other genes from a single sample. Along these lines, the gene structure of several neuropeptides lends itself to transcriptional analysis similar to that done here (Supp Table 2).

In summary, we present a new method for measuring gene transcription of the hypothalamic neuropeptide VP. One-step qRT-PCR using intron- and exon-specific primer pairs was used to study changes in VP transcription induced by hypertonic stimuli. We report that the osmotic stimuli increased VP transcription in a similar fashion to that previously reported using ISHH with intronic probes. This use of qRT-PCR for studying the relationship between processed and unprocessed RNAs will be usefully applied in investigations addressing dynamic changes in transcription.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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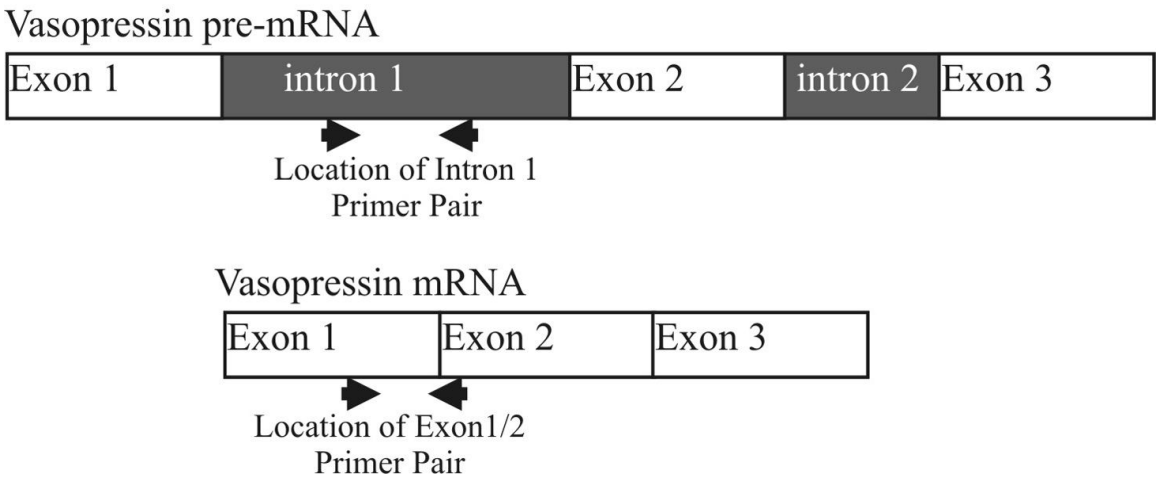

Figure 1.
Diagram of vasopressin RNA species and PCR primer binding sites. Top, Pre-mRNA containing both introns and exons. Only the intronic primer pair can bind to this species. Bottom, Following the removal of both introns, the exonic regions are joined, thereby allowing the binding of the exonic primer pair.

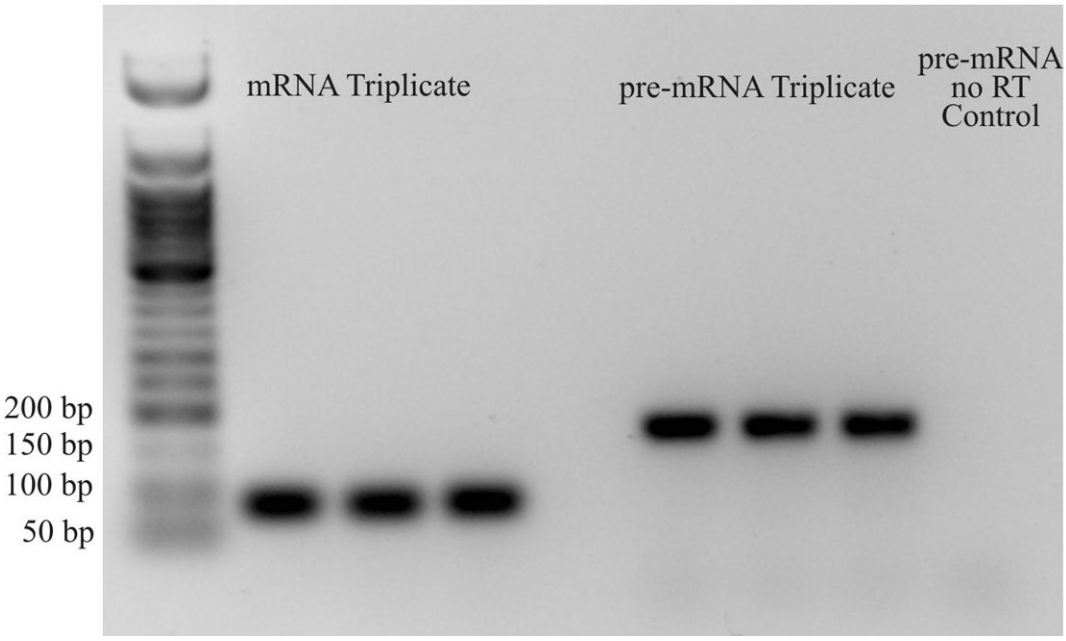

Figure 2.
Gel analysis of exonic and intronic PCR products generated from rat supraoptic nucleus. PCR triplicates were subjected to gel electrophoresis on a 2% agarose gel containing ethidium bromide. The products are the predicted sizes for the amplicons and are reproducible.

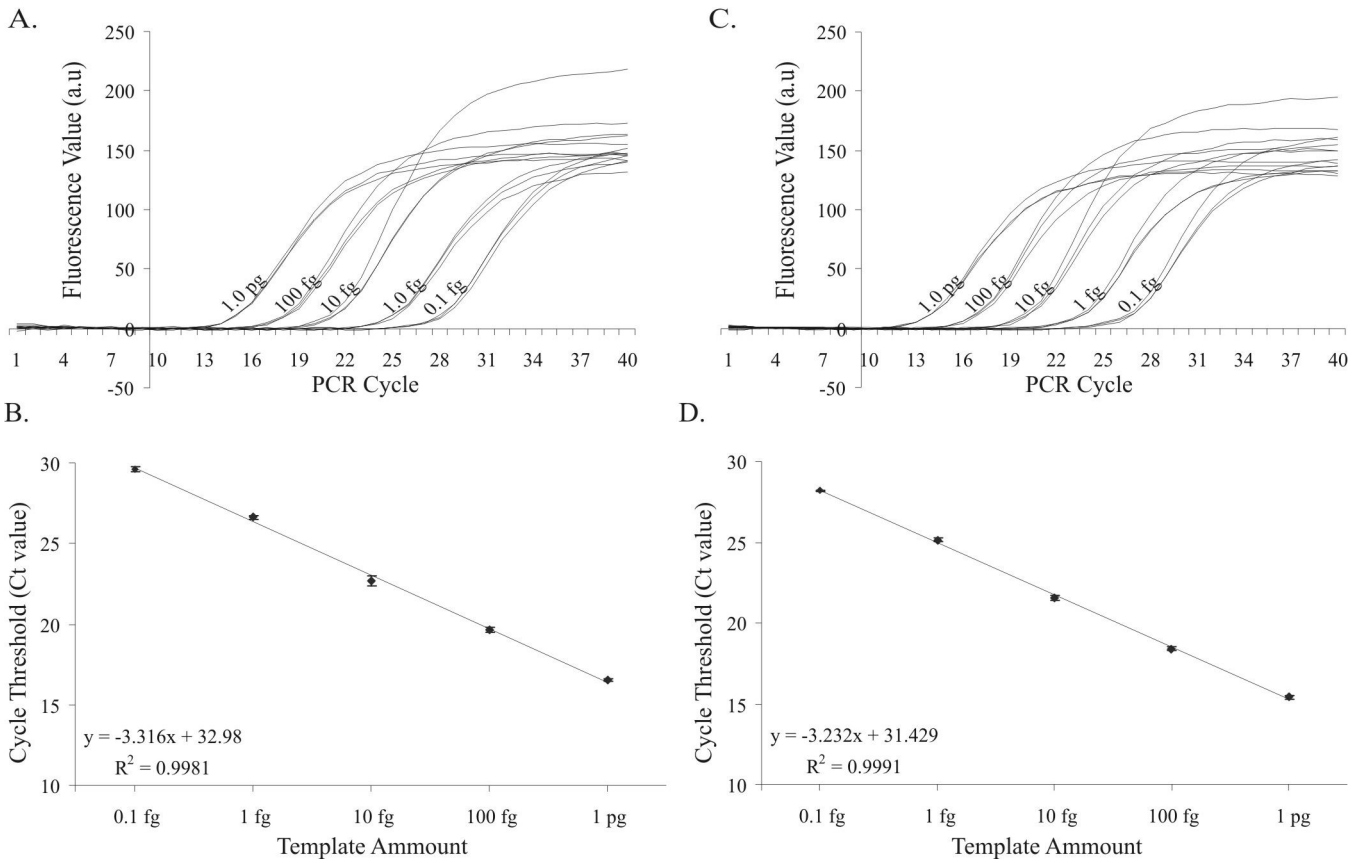

Figure 3.

RT-PCR and PCR-generated exonic probe standard curves. A, Known starting template amounts of in vitro-transcribed exonic RNA were subjected to quantitative RT-PCR. Samples were run in triplicate and an average Ct value was calculated for each starting template amount (shown in B). B, A standard curve by which one can calculate RT-PCR efficiency (E, see text) and absolute starting copy number. From this curve E equals 2.0. C, PCR-generated exonic amplicons were added as the starting double-stranded DNA template in various amounts. D, A standard curve on which is plotted the average Ct value from a set of triplicates and from which an efficiency of 2.0 was also calculated.

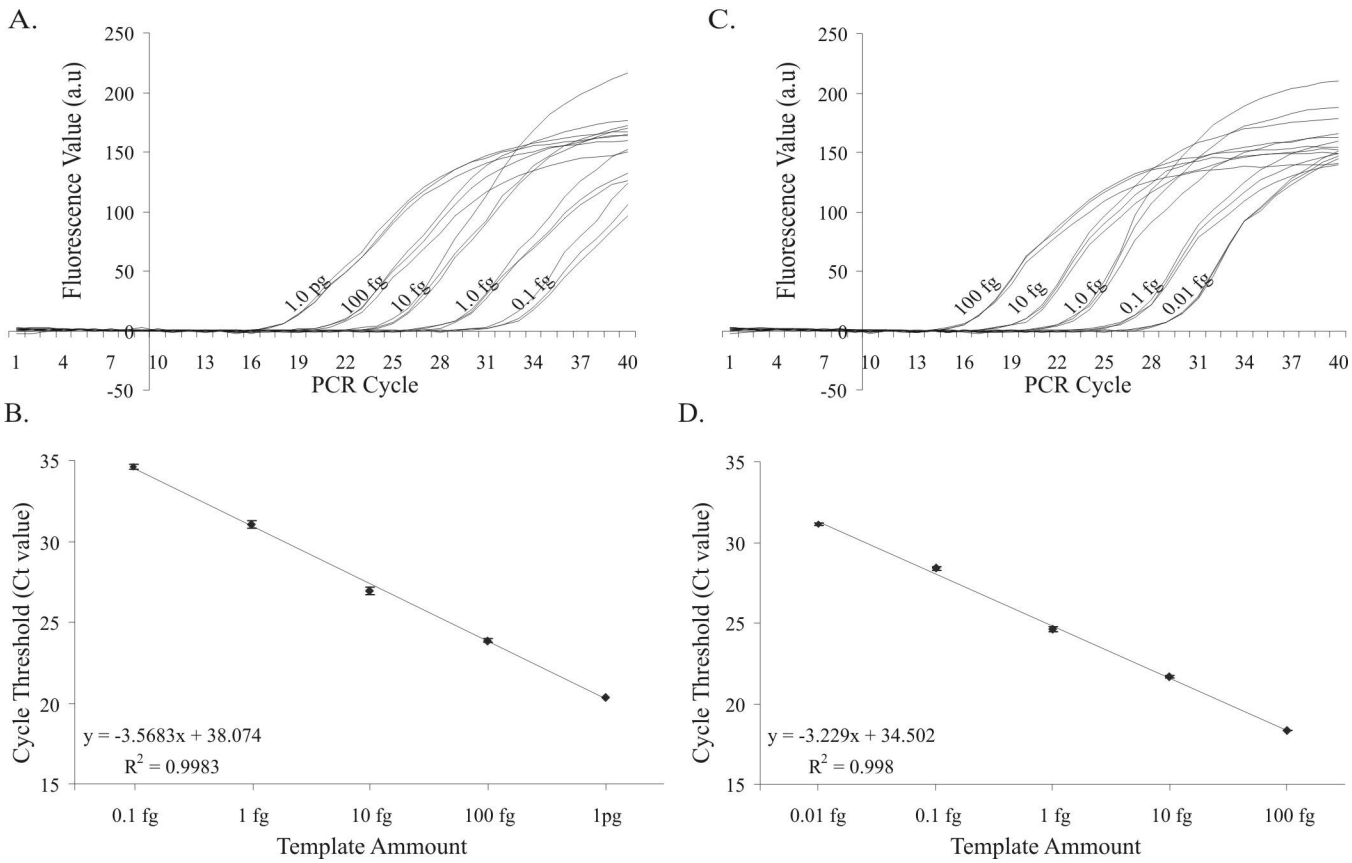

Figure 4.

RT-PCR and PCR generated intronic probe standard curves. A, Known starting template amounts of in vitro-transcribed intronic RNA were subjected to quantitative RT-PCR. Samples were run in triplicate and, as done previously, an average Ct value was calculated for each starting template amount. B, The resulting standard curve from the data shown in (A) by which one can calculate RT-PCR efficiency (2.0) and absolute starting copy number. C, PCR-generated intronic amplicons were added as the starting double-stranded DNA template in various amounts. D, A standard curve on which is plotted the average Ct value from a set of triplicates and from which an efficiency of 1.9 was calculated.

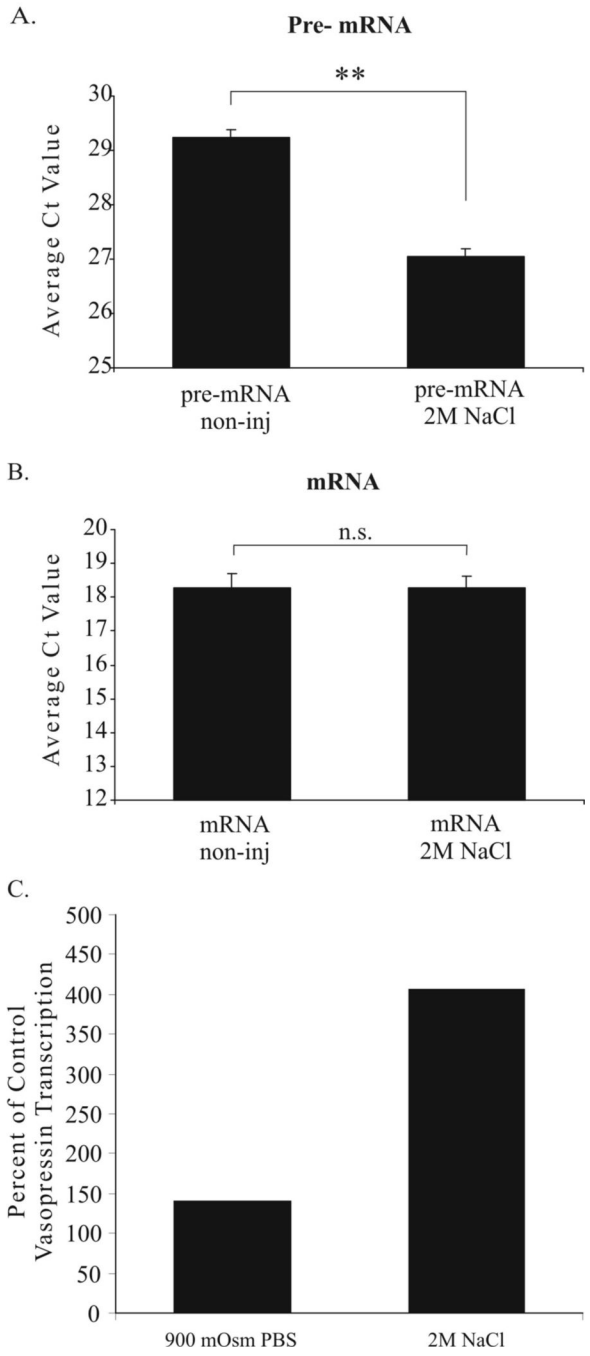

Figure 5.

Comparison of exonic and intronic Ct values in SONs from normosmotic and hyperosmotic animals. A, Average ± SEM Ct values for the intronic probe (pre-mRNA) were significantly different in SONs from normosmotic vs. the saline-injected animals, $p<0.01$, n=6. B, Exonic Ct values (mRNA) for both groups of animals were not significantly different. C, Comparison of two hypertonic stimulus intensities. The mild stimulus of an injection of 900 mOsm PBS solution increases VP transcription to 141% of control ($p<0.05$), and the stronger stimulus of a 2M NaCl injection increases VP transcription to over 406% of control values. The formula used for calculating transcription is described in the text.

Table 1

Real-time PCR primers used to assay vasopressin transcription.

Primers	Sequence	Product	T_A	T_M
VP Intron 2 forward VP Intron 2 reverse	5′-GCAGGGCTGAAGGGAATCCAGTCA 5′-TTAGGCTGATGGGTGCAAGACACA	171 bp	66 °C, 30 s	83.3 °C
VP Exon 1 forward VP Exon 1/2 bridge rev.	5′-TGCCTGCTACTTCCAGAACTGC 5′-AGGGGAGACACTGTCTCAGCTC	77 bp	60 °C, 30 s	81.2 °C
T7 forward	5′-TAATACGACTCACTATAGGG	220_i,130_e bp	50 °C, 30 s	-

T_A, annealing temperature; T_M, PCR product melting temperature; 220_i and 130_e products from separate plasmids using the T7 forward primer with either VP Intron 1 reverse or Exon 1/2 bridge reverse primers.