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Research paper

Characterization of specific cDNA background synthesis introduced by reverse transcription in RT-PCR assays

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ABSTRACT

To block expression of NMDA receptor NR1 subunit, we injected into rat hippocampus a Herpes Simplex Virus type 1 derived vector bearing a sequence for NR1 antisense. RT-PCR assays with RNA from hippocampus of animals injected either with NR1 antisense vector, control vector or vehicle, showed an amplification signal compatible with NR1 antisense which could be attributed either to an endogenous NR1 antisense or to an artifact. RT-PCR was performed either with different primers or without primers in the RT, using RNA from different tissues. RNAse protection assay was carried out to characterize the amplified signal nature. Our results show that the template for the unexpected amplified fragment was NR1 mRNA currently expressed in nervous tissue. We considered this basal amplification of a mRNA in a RT-PCR assay as "background amplification". After background subtraction, a significant signal only remained when samples from NR1 antisense vector injected animals were used, demonstrating that this was the only source for NR1 antisense. Background amplification at RT in the absence of primers, can constitute a troubling factor in quantitative nucleic acid determination, leading to major interference, particularly when both sense and antisense sequences are present in the sample. Since RT introduced a significant background signal for every gene analyzed, we propose that RT must be always performed also without primers. Then, this signal should be identified, quantified and subtracted from the specific reaction amplification signal.

1. Introduction

Reverse transcription polymerase chain reaction (RT-PCR) is the most common method for characterizing or confirming gene expression and comparing mRNA levels in different samples [1,2]. Furthermore, real-time RT-PCR is a sensitive and accurate technique for mRNA quantification [2].

To estimate the expression of a gene, its transcript must be first copied to cDNA by reverse transcription. Primers used for cDNA synthesis in reverse transcription (RT) assays are either oligo (dT), *random* oligonucleotides or sequence specific oligonucleotides. These are not natural primers for reverse transcriptases. Retroviruses and retrotransposons utilize specific cellular tRNAs as primers for reverse transcription of their own initiator RNA, resulting in a double stranded cDNA [3–5]. On the other hand, endogenous small RNAs could also be effective as primers for cDNA synthesis [6]. Frech and Peterhans (1994) reported that this cDNA could lead to a background signal in a subsequent PCR assay. To reduce this "background signal", cellular RNA was immobilized on membranes. However, further addition of either specific oligonucleotides or total RNA from different origins still served as primers for cDNA synthesis, resulting in an increase of PCR signal [7]. It had also been hypothesized that purification of mRNA could prevent background priming and, therefore, that background signal would be eliminated. However, mRNA preparations obtained using different purification methods could still be reverse transcribed, even in the absence of any added primer [8].

The occurrence of background amplification in the absence of primers at the RT reaction can constitute a significant troubling factor in quantitative nucleic acid determination. Moreover, it could lead to major interference when intending to detect a specific RNA

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present in both sense and antisense sequences in the same sample. This is relevant, for instance, in the field of virology, where viral RNA can be present as the positive and negative strand [9,10], or even in any sample where sense/antisense pairs of sequences are expressed [11–13].

In a previous study, we have blocked the expression of NR1 subunit of NMDA receptor using herpes simplex virus type 1 (HSV-1)-derived amplicon vectors bearing a sequence coding for a NR1 antisense. Then we intended to show NR1 antisense expression from the vector injected into rat hippocampus, where the NR1 is currently expressed [14]. However, using total hippocampal RNA, a RT-PCR product compatible with a NR1 antisense was obtained even in control animals. In the case of non-infected tissue, this unexpected fragment could be attributed either to an endogenous NR1 antisense RNA or to an artifact. In order to distinguish between these two possibilities, we carried out diverse RT-PCR assays, either with different primers or without primers in the RT assay. We also performed RNAse protection assay (RPA) in an attempt to characterize the nature of the unexpected amplification signal.

2. Material and methods

2.1. HSV-1-derived amplicon vectors

Amplicon vector stocks were prepared as already described by Zaupa et al. (2003), with minor modifications. Briefly, Vero 7b cells (which are modified Vero cells transcomplementing HSV-1 *ICP4* and *ICP27* genes) were maintained in culture in Dulbecco's minimum essential medium (DMEM) (Invitrogen, Paisley, UK) supplemented with inactivated Fetal Bovine Serum 10% (FBS) (Invitrogen), Penicillin (100 U/ml) and Streptomycin (100 μg/ml) (Invitrogen). Cell lines were transfected with the amplicon plasmid using LipofectAMINE Plus Reagent (Invitrogen), according to manufacturer's instructions. One day later, transfected cells were infected with helper HSV-1 LaLΔJ at a multiplicity of infection of 0.3 plaque forming units/cell. When cytopathic effect was maximum, cells were collected by centrifugation and disrupted by three cycles of freezing and thawing to release vectors particles. Media containing amplicon vectors were then centrifuged to pellet cell debris. Helper virus particles in the supernatant were titrated by plaque assay on Vero 7b cells [15]. To titrate vector particles, Gli36 cells [16] were infected with serial dilutions of the corresponding vector stock. After 24 h of incubation, cells expressing fluorescent EGFP were scored directly under a fluorescence microscope [17].

2.2. Total RNA extraction

Tissue rat samples from cerebellum, cortex, hippocampus, spleen, heart, liver and kidney were collected and homogenized in Trizol Reagent (Gibco-BRL, Rockville, Md, USA) in a glass-teflon potter by 10 strokes, waiting 1 min and giving another 10 strokes. RNA isolation and purification was carried out following manufacturer protocols. Finally, RNA pellet was resuspended in DEPC (diethylpirocarbonate, Sigma Saint Louis, MO, USA) 0.2% RNAse free water. Total RNA samples were treated with DNAse I (Ambion Europe Ltd., Switzerland) to eliminate any genomic or vector DNA contamination. DNAse was inactivated by adding the inactivation mix (Ambion).

When indicated, RNA was extracted from a hippocampus fraction circumscribed to the injection area to avoid dilution of transgene expression.

2.3. DNA isolation

Rat hippocampus were homogenized in 10 mM Tris–HCl (pH 8), 0.1 M EDTA, 0.5% SDS, 50 μg/ml proteinase K (Roche Diagnostics GmbH, Mannheim, Germany); then, the homogenate was incubated 4 hours at 55 °C. DNA was isolated by extraction with phenol:chloroform:isoamyl alcohol (25:24:1 v/v). The resultant aqueous phase was extracted with chloroform; then, it was precipitated by centrifugation with four volumes of absolute ethanol and NaCl (final concentration 0.25 M). The pellet containing the DNA was washed with ethanol 70% and resuspended in water.

2.4. RNAse protection assay (RPA)

For NR1 riboprobes construction, pN60 plasmid was used as template [18]. pN60 contains the sequence of the NR1 subunit NMDA receptor. Sense and antisense riboprobes were generated by *in vitro* transcription of the NheI (New England Biolabs (NEB), Ipswich, MA, USA) and BssHII (NEB) linearized pN60 respectively, in the presence of 60μCi of [α-P^{32}]UTP (3000 Ci/mmol) (Amersham, Les Ulis, France). For RPA assay, 15μg of total RNA from cerebral cortex or hippocampus were co-precipitated with 10^5 cpm of sense or antisense riboprobe, resuspended in hybridization buffer (40 mM PIPES pH 6.7, 400 mM NaCl, 1 mM EDTA, 80%v/v deionized formamide) (all reactives from Sigma) and denatured at 90 °C. Annealing was carried out at 45 °C overnight. Then, RNaseA (MBI Fermentas, Germany) was added and digestion was performed at 37 °C. Reaction was stopped by addition of SDS (Sigma) and proteinase K (Roche) to a final concentration of 0.6% (w/v) and 0.1 mg/ml, respectively. Samples were then extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with absolute ethanol using yeast tRNA (10 mg/ml) as carrier. Denatured samples were analyzed by sequencing gels (6% polyacrylamide (Sigma), 42% urea (Sigma)).

2.5. Reverse transcription (RT)

1μg of total RNA was used as template in RT reaction, which was carried out in parallel with both RevertAidTM H(-) M-MuLV Reverse Transcriptase (MBI Fermentas), and Omniscript RT Kit (Qiagen Hilden, Germany), following instructions provided by the manufacturers. Briefly, total RNA was mixed with 10 μM primer (oligodT, random hexanucleotides or specific primers), incubated 5 min at 70 °C, and kept on ice for 2 min to allow hybridization. Then, RT reaction Mix (buffer 5X, dNTPs mix 10 mM each one, RNase inhibitor RNaseOUT (40 U/μl) (Invitrogen)) and Reverse Transcriptase were added following manufacturer instructions. After 60 min incubation at 42 °C (M-MuLV) or 40 °C (Omniscript) the RT enzyme was heat inactivated. In each case the total reaction volume was 30 μl.

2.6. Standard polymerase chain reaction (PCR) amplification

PCR was carried out with 1 μl of RT product, 1μM forward primer and reverse primer and Eppendorf® MasterMix (2.5×) (Eppendorf AG, Hamburg, Germany). In all cases, total reaction volume was 25 μl. Two PCR protocols were used: either (1) 2 min at 95 °C, followed by 30 s at 95 °C for denaturation, 1 min at 60 °C for hybridization and 1 min at 72 °C for extension (35 cycles) or (2) 15 s at 95 °C for denaturation, 15 s at 60 °C for hybridization, 30 s at 72 °C for extension (35 cycles). In all cases, 5 μl of PCR products were analyzed in 2% agarose gels containing ethydium bromide (both from Sigma).

2.7. Real time PCR amplification

Real time PCR was carried out using 1 μl of RT product, 1μM forward primer and reverse primer and Quanti Tect™SYBR® Green

PCR Master Mix (Qiagen). In all cases, total reaction volume was 20 μl. Real time PCR assays were carried out with a LightCycler system (Roche Molecular Systems, California, USA). Reactions were incubated 15 min at 95 °C for the DNA polymerase HotStarTaq activation. PCR cycle protocol was: 15 s at 95 °C for denaturation, 15 s at 60 °C for hybridization, 30 s at 72 °C for extension (45 cycles), followed by a progressive temperature increase of 0.1 °C/s, from 68 °C to 95 °C, finishing with 30 s at 40 °C for cooling. Specificity of amplification to each primer pairs was verified for the presence of one peak in the melting curve.

2.8. Primers

Primer sequences used were obtained from the literature or by Primer3 software [19] and are summarized in Table 1 for NR1 gene and in Table 2 for the other genes concerning the present work. Table 1 contains sequence, position and fragment size of primers used in the analysis of NR1 mRNA and genomic DNA. Table 2 contains primer sequences for the other genes analyzed by RT-PCR.

2.9. Animals

Adult male Wistar rats (2–3 months old; 200–250 g body weight) from our own animal house were used. All the procedures involving animals were carried out in accordance to the guidelines of the USA National Institutes of Health Guide for the Care and Use of Laboratory Animals (1996). Protocols were approved by the Animal Care and Use Committees from the University of Buenos Aires, Argentina (2004).

3. Results

3.1. Detection of NR1 antisense RNA

With the aim to find out if exogenous NR1 antisense RNA was expressed from an amplicon vector, three groups of rats were injected into the hippocampus with vectors expressing EGFP mRNA and one of the following sequences: 1) NR1 mRNA (NR1 vector, S), 2) NR1 antisense (NR1 antisense vector, AS) or 3) LacZ mRNA (Z) [20]. There was a fourth group which consisted in vehicle-injected rats (Vh). RT-PCR assays were carried out using total hippocampal RNA samples from these rats. Reverse transcription reaction was performed using a forward (5′) specific primer for NR1 sequence. In the subsequent PCR the cDNA was amplified by using specific primers for NR1. In all four samples, coming from animals either injected or not with NR1 antisense vector, we detected a band that could be interpreted as coming from an NR1 antisense (Fig. 1a), suggesting that this amplification could be originated from an endogenously expressed RNA molecule. Since the bands were rather similar in every case (Fig. 1a), RNA was then extracted exclusively from the hippocampus fraction circumscribed to the injection area. The corresponding RT-PCR assay showed that the expression of the putative NR1 antisense was higher in samples coming from NR1 antisense vector injected animals, although a band appeared in all cases (Fig. 1b). To further investigate the nature of this product, which appeared even when the transgene was not injected, we decided to perform an RT-PCR assay using RNA extracted from different tissues. Total RNAs from liver, spleen, heart, kidney, cerebral cortex and hippocampus from naive non-injected rats were used (Fig. 1c). A band similar to that reported above, matching with NR1 antisense, was exclusively present in samples coming from nervous tissue, i.e. cerebral cortex and hippocampus, where NR1 mRNA is endogenously and conspicuously expressed. This result raised the possibility that the amplified fragment could be originated from the NR1 mRNA. However, at this point we were not able to discard that the amplified fragment could have been originated from another transcript coming from a hypothetic genomic antisense sequence. Therefore, to elucidate if the amplified fragment was originated from an endogenous antisense or from a mRNA, three different approaches were followed: sequencing of PCR products, RT-PCR assay under different conditions and RNAse protection assay (RPA).

The first step was to sequence the products of PCR after RT from total hippocampal RNA samples performed either with reverse (3′) or forward (5′) primers. The corresponding sequences from both reactions resulted identical (data not shown). Furthermore, products sequences were identical to the NR1 cDNA sequence deposited at the Genbank (Acc. Number: NM_017010.1). This result confirmed that the amplified fragment was clearly related to the NR1 sequence and that the reverse transcription event appeared to be the same, disregarding the primer used.

As a second step to further clarify the origin of the amplified fragment, RT reaction were carried out with total RNA extracted from hippocampus of *naive* rats without primers added, followed by PCR assay using three different pairs of primers that covered almost the full length of NR1 mRNA. These primers corresponded to exon sequences P1, P2 and P3, as shown in Fig. 2a. In each one of the three reactions, a unique fragment was obtained having the expected molecular weight as for NR1 mRNA being the putative source spliced-out (Fig. 2b). The results were rather similar when different reverse transcriptases were used (see Materials and Methods). Furthermore, the ratio between products obtained with or without primers addition to the RT were similar at different temperatures (from 45.5° to 53.2°) and then, turning down at the highest temperature assayed (56.5°) (data not shown).

A feasible speculation was that any other molecule that could have served as the template, including a putative antisense RNA, would have produced larger fragments, due to the absence of splicing. Hence, these results provide evidence against the possibility that the unexpected amplified fragment could be originated from an endogenous NR1 antisense molecule and strongly suggest that it would be originated from the NR1 mRNA. However, the possibility for the existence of an antisense sequence equivalent to NR1 cDNA in the genome still remained. Thus, the following step consisted in performing PCR assays using genomic DNA from rat hippocampus as template, with different pairs of primers (Fig. 2a) that anneal in different *NR1* exons (Fig. 2c). If such an endogenous sequence for NR1 antisense exists, PCR assays with same primers pair would give a product of same size in spite of using either genomic DNA or cDNA. On the other hand, if the template for the amplified fragment of unknown origin was just the NR1 mRNA, PCR products obtained using genomic DNA and each pair of primers, would be different from the products obtained with same primers

Table 1
Sequence, position and fragment size of primers used in the analysis of NR1 mRNA and genomic DNA. (NR1: Rattus norvegicus glutamate receptor, ionotropic, *N*-methyl D-aspartate 1 (Grin1), mRNA. NM_017010).

Gene Target	Primer name	Primer sequence	Exon	Fragment size (bp)
NR1	P1	5′-aacctgcagaaccgcaag-3′	9	cDNA:334 gDNA: 4766
		5′-gcttgatgagcaggtctatgc-3′	12	
	P2	5′-tttttcctgctccttcgcc-3′	2	cDNA: 448 gDNA: 5318
		5′-gttccagttgtagactcgcatc-3′	4	
	P3	5′-caacagcaacaaaaaggagtgg-3′	13	cDNA: 558 gDNA:929
		5′-tctgaggggtttctgagcctg-3′	16/17	
	P4	5′-cagtgctgctatggcttctg-3′	12	cDNA: 150 gDNA:395
		5′-catcattccgttccactcct-3′	13	
	P5	5′-gacatgggttcggtatcagg-3′	19	cDNA: 151 gDNA:234
		5′-gtgtcgcttgtaggcgatct-3′	20	

Table 2
Primer sequences for the other genes analyzed by RT-PCR.

Gene Target	Primer sequence	Fragment size at cDNA
NR2A: Rattus norvegicus glutamate receptor, ionotropic, N-methyl D-aspartate 2A (Grin2a), mRNA. NM_012573	5′-tggctgccttcatgatcca-3′ 5′-tgcagcgcaattccatagc-3′	352 pb
NR2B: Rattus norvegicus glutamate receptor, ionotropic, N-methyl D-aspartate 2B (Grin2b), mRNA. NM_012574	5′-ggatctaccagtctaacatg-3′ 5′-gatagttagtgatcccactg-3′	563 pb
β-Actin: Rattus norvegicus actin, beta (Actb), mRNA. NM_031144	5′-tgcctgacggccaggtcatc-3′ 5′-cggatgtcaacgtcacactt-3′	145 pb
Bcl2: Rattus norvegicus B-cell CLL/lymphoma 2 (Bcl2), nuclear gene encoding mitochondrial protein, mRNA. NM_016993	5′-tgtggggagcgtcaacagg-3′ 5′-catgctggggccatatagtt-3′	145 pb
Bax: Rattus norvegicus Bcl2-associated X protein (Bax), mRNA. NM_017059	5′-gaggactccagccacaaaga-3′ 5′-cgagctgatcagaaccatca-3′	154 pb
EGFP: Cloning vector pEGFP-N1, enhanced green fluorescent protein (egfp). CVU55762 (U55762). Used as non related primer.	5′-cgaccactaccagcagaaca-3′	–

but using the cDNA. As it could be seen in Fig. 2c, PCR from genomic DNA led to different size products than the expected using cDNA. Actually, when we used the primers to perform those PCR from DNA extracted from rat hippocampus, the amplified fragment was larger than that obtained from the cDNA, since it contains the corresponding introns of *NR1* (Fig. 2c). Altogether, these results tend to exclude the possibility that the unexpected amplified fragment (Fig. 1) could be originated from an endogenous NR1 antisense molecule and strongly support the idea that the observed fragment was originated from NR1 mRNA.

Finally, to formally discard the existence of an endogenous antisense RNA, RPA was carried out. In this assay the RNA sample was hybridized with a specific riboprobe and then incubated with RNAse-A. RPA was performed using total RNA samples from hippocampus or cerebral cortex of naive rats (Fig. 3). Two riboprobes were used: one hybridizes with the NR1 mRNA (Rv) and the other, with the NR1 antisense transcript (Fw). RNAse protection was obtained only with the Rv probe, while there was no protection when samples were incubated with the Fw probe, specific for the antisense transcript, for both hippocampus and cerebral cortex. Hence, this confirmed our previous results and excluded the possibility of existence of an endogenous NR1 antisense transcript.

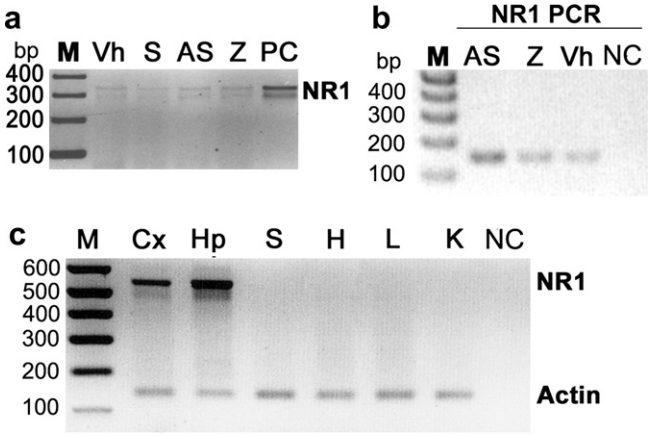

Fig. 1. RT-PCR for NR1 antisense. a. RT-PCR performed with total RNA from rat hippocampus using P1 as primers. The RT reaction was conducted with the sense primer (P1) for *NR1* gene, in order to evaluate the antisense expression. Rats were previously injected with amplicon vectors bearing one of the following sequences: NR1 sense (S), NR1 antisense (AS) and lacZ (Z). A fourth group was injected only with vehicle (Vh) to be used as negative control. As positive control (PC), total RNA extracted from Gli36 cells transfected with the antisense NR1 transcript, were included. M: 100 bp molecular marker. b. RT-PCR performed with total RNA extracted from rat hippocampus vector injection area. The RT was conducted with a sense primer for *NR1* (P4) in order to evaluate the antisense expression. In all cases, rats were previously injected either with vehicle (Vh) or amplicon vectors bearing NR1 antisense (AS) or lacZ (Z) sequences. A negative control for PCR, using water instead the sample, was included (NC). M: 100 bp molecular marker. c. To clarify the expression pattern of a possible endogenous NR1 antisense, we carried out the assay using as samples total RNA extracted from different tissues: cerebral cortex (Cx), hippocampus (Hp), spleen (S), heart (H), liver (L) and kidney (K). After PCR using P3 as primers for NR1, a band was obtained only with samples where NR1 mRNA is conspicuously expressed: cerebral cortex (Cx) and hippocampus (Hp), but not with samples coming from the other tissues. Actin 3′ primer was also added to the RT reaction to be used as an internal control. M: 100 bp molecular marker.

3.2. RT-PCR product from basal amplification

To further understand how the PCR fragment could be originated, and taking into account results reported above, total RNA samples from naive rat cortex were used to carry out RT reactions either with forward (5′), reverse (3′), or without primers (*wo*). Then, with the obtained cDNAs, PCR for NR1 was performed. A band of the expected size was observed when 3′, 5′, or no primers were

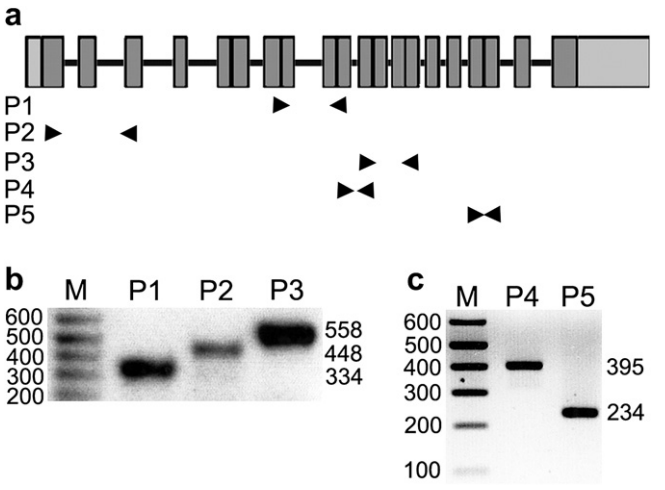

Fig. 2. RT-PCR for *NR1* with different pairs of primers and different templates. a. *NR1* subunit gene scheme. Primer pairs named P1, P2, P3, P4 and P5 were used in PCR assays when indicated. Arrowheads indicate primer annealing. b. RT-PCR for total hippocampal RNA extracted from naive rats. RT was performed without adding primers or oligodT. Then, RT products were amplified by PCR using primers P1, P2 and P3. In each one of the three reactions, a specific band of the expected molecular weight was obtained: 334 bp for P1, 448 bp for P2 and 558 bp for P3. M: 100 bp molecular marker. c. PCR performed with genomic DNA as template, using two different pair of primers. Lane 1 (using P4 primers): The presence of an intron in genomic DNA led to a 395 bp band (the band size of cDNA coming from the NR1 mRNA would be of 150 bp as it could be seen in Fig. 1b). Lane 2 (using P5 primers): The presence of an intron in genomic DNA led to a 234 bp band (83 bp longer than the corresponding cDNA coming from the NR1 mRNA, which would be a fragment of 151 bp as it could be seen in Fig. 5a).

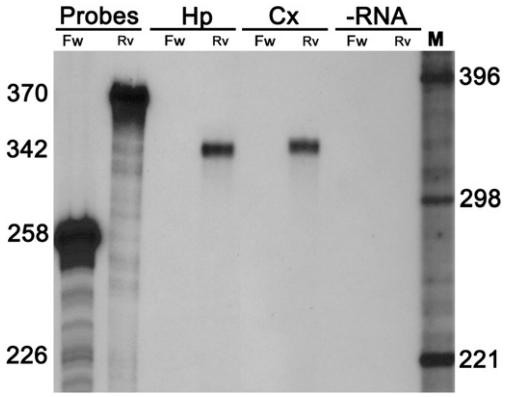

Fig. 3. RNase protection assay for NR1 and antiNR1. RPA was performed using total RNA from cerebral cortex (Cx) and hippocampus (Hp) of naive rats. Polyacrylamide gel revealed that probes run at its molecular size: 371b and 258b for reverse (Rv) and forward (Fw) probes respectively. RNA protection was positive only when Rv probe, which hybridizes with NR1 mRNA, was used (band of 342b in Hp and Cx). Since there was not protection with the Fw probe (there was no band at 226b), none NR1 antisense RNA was detected. As negative control, an assay without sample was included (-RNA). M: RNA marker.

added to the RT reactions (Fig. 4; *3′, 5′*and *wo* lanes). In contrast, there were no bands when the RT was carried out without the addition of reverse transcriptase to the sample, or when the PCR was performed with total RNA from cerebral cortex as sample (without performing RT reaction) (Fig. 4; *-RT, -S* and *NC* lanes). Hence, these results demonstrated that the RT reaction could take place even in the absence of added primers.

Because an active fraction of RT enzyme might be able to continue synthesizing cDNA when primers are added to the PCR assay, the enzyme was inactivated after reverse transcription, as described in Materials and Methods. In spite of RT inactivation, the band corresponding to that expected for NR1 was still obtained (data not shown). This result excluded the possibility that a remaining RT enzymatic activity could account for that amplified fragment.

Therefore, these results strongly suggest that the unexpected amplified fragment obtained by RT-PCR constitute a basal amplification or background signal, which can take place even in the absence of any primer.

3.3. Is background signal depending on the mRNA?

With the goal to find out if the background signal was restricted to our particular NR1 mRNA or if it could be a more general feature, a series of RT reactions for specific but different mRNAs were performed, either with specific 5′ or 3′ primer, *random* hexanucleotides or without adding any primer. Then, the cDNA was used as sample in an ordinary PCR assay. Transcripts analyzed

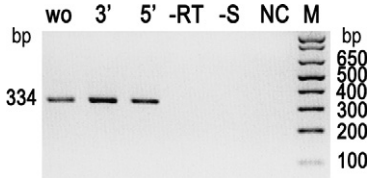

Fig. 4. PCR amplification for *NR1* after RT carried out either with forward (5′), reverse (3′) or without primers (wo). P1 primers for NR1 were used both in the RT and in the PCR assay. In all three lanes: 3′, 5′ and wo, appeared a band corresponding at PCR products of the expected molecular weight for P1 (334 pb). 5′: RT using the 5′ primer. 3′: RT using 3′ primer. wo: RT without primers added. Controls: -RT: RT-PCR without M-MuLV reverse transcriptase; -S: RT-PCR without sample; NC: PCR negative control. M: Molecular marker (1 kb plus, Invitrogen). In this case, RT-PCR assays were performed using total RNA from cerebral cortex of naive rats.

corresponded to three different gene categories: (1) typical neuronal genes such as NR1 (GenBank acc. Number: NM_017010), NR2A (GenBank acc. Number: NM_012573) and NR2B (GenBank acc. Number: NM_012574), (2) highly regulated genes such as Bcl-2 (GenBank acc. Number: NM_016993) and Bax (GenBank acc. Number: NM_017059), and (3) ubiquitous cellular transcripts like β-actin (GenBank acc. Number: NM_031144) (Fig. 5). For each of the selected messengers, the cDNAs obtained with 5′ or 3′ specific primer, random hexanucleotides primers (RP) or no primers (wo) were amplified by PCR. In all cases, a band corresponding to the expected molecular weight for the specific mRNA was obtained after amplification (Fig. 5a; 3′, 5′, *RP* and *wo* lanes). There were no amplification products after PCR when reverse transcriptase was not included in the RT reaction, neither when the sample was replaced by water (Fig. 5a; *-RT* and *-S* lines). Furthermore, other samples (i.e. RT reaction without primers followed by PCR for fibronectin or using samples from other tissues and cell types) gave similar results (data not shown). These results indicate that the RT-PCR background signal obtained without adding primers could be a highly prevalent phenomenon.

The cDNAs obtained by RT with or without addition of primers, using total RNA from rat cerebral cortex, were quantified by real time PCR. For this analysis, NR1, Bcl-2, Bax and β-actin mRNAs were selected (Fig. 5b). For the different messengers selected, real time amplification of RT using 3′ primer ranged between 3.5 and 30 fold the background signal. On the other hand, there were significant differences between the background signal and amplification of RT products using 5′ primer.

Moreover, RT reactions with total RNA from cortex of naive rat were carried out using EGFP primer as rat genome non-related primer. *NR1, Bcl2* and *Bax* gene were analyzed by RT performed with 3′ specific primer (3′), the non related primer (EGFP) or without primer (wo) addition. There were no significant differences when the EGFP primer was added to the RT reaction compared to RT without primers (Fig. 6).

Taken together, these results indicate that RT-PCR brings a background signal that is inherent to the cDNA synthesis process by the reverse transcriptase in the absence of any added primer, and that this could be a very common circumstance.

3.4. How to discriminate specific from background signal

To discriminate exogenous antisense expression *in vivo* from background signal, total RNA was extracted exclusively from rat hippocampus, though circumscribed to the injection area. These RNA coming from hippocampus either injected or not with NR1 antisense vector, were analyzed by RT-real time PCR. The RT was carried out using 5′ primer which hybridizes with the NR1 antisense transcript. Samples from animals injected with NR1 antisense vector (Fig. 7; *AS*) showed a 16 fold increase in PCR amplification compared with animals injected with vehicle or with control vectors bearing the LacZ gene (Fig. 7; *Vh* and *Z* respectively). After background subtraction, PCR product signal remained positive only in samples from rats injected with the NR1 antisense vector (Fig. 7, *inset*). This result showed that the presence of the NR1 antisense RNA was due to its exogenous expression and that there was no evidence for any endogenous NR1 antisense.

4. Discussion

This work came after the need to explain the results obtained while we were intending to demonstrate the expression of an exogenous antisense RNA into rat hippocampus. The corresponding transgene for the antisense against NR1 subunit of the NMDA receptor was cloned in an amplicon vector which was injected into

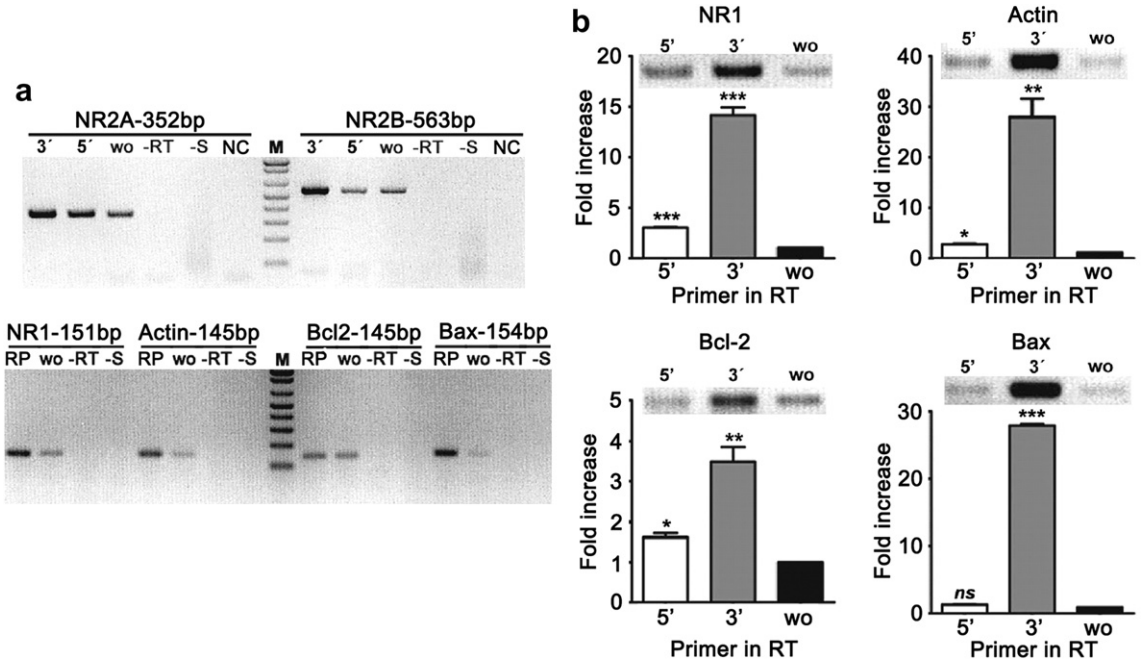

Fig. 5. RT-PCR background amplification for different genes. a. RT was performed with random primers (RP), 3′ or 5′ specific primers, or without (wo) primer addition. Total RNA from cerebral cortex of naïve rat was used to analyze background amplification of *NR2A*, *NR2B*, *NR1* (P5), *Actin*, *Bcl2*, and *Bax* mRNA. After RT, the obtained cDNA for each gene in every condition was used as sample in a standard PCR assay. In all cases, a specific band of the expected molecular weight for the gene analyzed was obtained. Controls: -RT: RT-PCR without adding M-MuLV reverse transcriptase; -S: RT-PCR without sample; NC: PCR negative control. M: Molecular marker (1 kb plus, Invitrogen). b. mRNA quantification by RT-real time PCR for *NR1* (using P5 primers), *Actin*, *Bcl-2* and *Bax*. Results are expressed as fold, arbitrarily considering RT-PCR without primers (wo) as 1. Gel bands of RT- real time PCR products for each mRNA analyzed are shown above the corresponding bar. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; ns: no significant differences (One Factor ANOVA. Post-test Dunnet).

the hippocampus. RT-PCR products using specific primers for NR1 displayed a band that would have come from an NR1 antisense RNA, not only in animals injected with the NR1 antisense vector but also, and most surprisingly, in control vehicle injected rats (Fig. 1a). Hence, that product could be interpreted either as being originated from an endogenous antisense molecule or as a contaminating background signal. Looking for the source of this RNA molecule, total RNA from different tissues of naive rat was analyzed by RT-PCR. A band corresponding to the putative antisense was only present in samples from tissues where the NR1 mRNA was endogenously expressed, leading to the idea that the NR1 mRNA could be responsible for the generation of that amplification signal. After RT performed with 5′ and 3′ specific primers for NR1, PCR amplification fragments exhibited sequences matching to NR1.

To clarify the origin of that putative antisense fragment, RT-PCR was performed using three different pairs of primers that covered almost the full length of NR1 mRNA, corresponding to different NR1 exon sequences; a fragment of the expected molecular weight for each NR1 mRNA portion was always obtained (Fig. 2b). This was the first evidence against the existence of an endogenous NR1 antisense molecule as the origin of the amplified fragment and strongly suggested that it was originated from the NR1 mRNA.

Then, taking into account that the possibility for the existence of an antisense sequence equivalent to NR1 cDNA in the genome still remained, two pairs of primers that anneal in different NR1 exons were used in PCR assays performed with genomic DNA as template. PCR from genomic DNA led to a product which molecular weight was that of NR1 mRNA fragment plus the intron included,

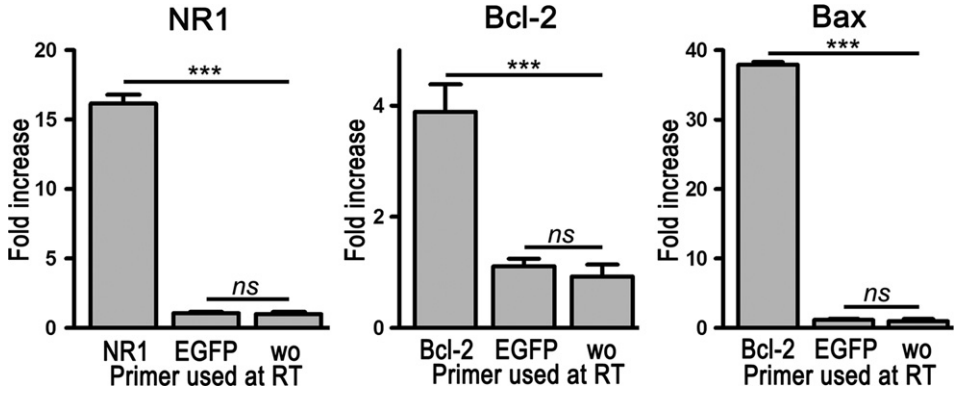

Fig. 6. Real time PCR using a rat genome-non related primer. RT-real time PCR for *NR1*, *Bcl-2* and *Bax* was performed using a specific 3′ primer or a non related primer (EGFP) in the RT. A third RT reaction without primers (wo) was included as control. As could be seen, there are not significant differences using EGFP primer or no primers at RT for each gene analyzed. *** $P < 0.001$; * $P < 0.05$; ns: no significant differences (One Factor ANOVA. Post-test Dunnet).

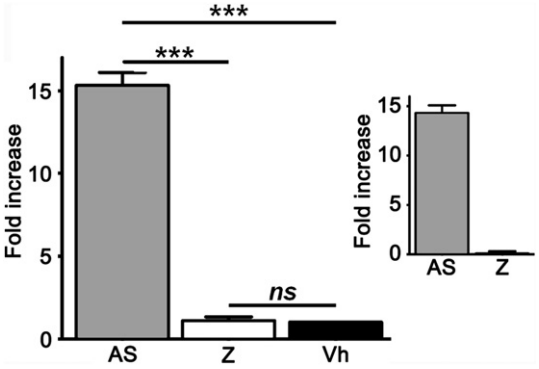

Fig. 7. NR1 antisense detection by real time PCR. RT-real time PCR were performed using P5 primers for NR1, from total RNA from rats injected into dorsal hippocampus with amplicon vectors bearing sequences either coding for NR1 antisense (AS) or control vector LacZ (Z). A third group, injected only with vehicle (Vh), was included. When RNA extracted from the injection area was used as sample, a 15 fold amplification was obtained in samples from animals injected with antisense vectors (AS), compared with control rats (*** $P < 0.001$). There were no significant differences between rats injected with control vectors and with vehicle (ns: no significant differences, One Factor ANOVA. Post-test Dunnet). Inset: Same analysis, but subtracting amplification level corresponding to vehicle injected animals from data of AS injected animals.

according to the primers used (Fig. 2c). That means that the sequence of origin for the amplified fragment was not present in the genome.

The idea of a putative endogenous antisense was fully discarded after RPA. Protection from RNAse activity was only obtained when the probe that hybridizes with the NR1 mRNA was used. Instead, no protection occurred when the specific riboprobe hybridizing with antisense transcript was used (Fig. 3).

Taken together, these results discarded the expression of an endogenous NR1 antisense RNA, and let to the conclusion that the unexpected fragment was due to the amplification of a background signal coming from the mRNA.

Since products of the expected size were obtained when either 3′, 5′ or no primer were added to RT reaction under different conditions (i.e., with two different RT, at several temperatures, with different RNA extraction procedures, after RT inactivation), it let us to conclude that cDNA synthesis can take place even without adding any primer to the RT reaction. That phenomenon should be considered as “background amplification” in the PCR assay.

Previous studies had already reported that some cDNAs could be synthesized without primer addition to the RT reaction [6,7], suggesting that the amplified fragments could constitute a basal amplification or background signal. Our results further confirm and extend these observations, and provide a plausible explanation for them. No matter which set of primers have been used or which RNA has been analyzed, a specific product always appears in the RT-PCR reaction; moreover, the sequence of these PCR product was identical to the cDNA for the gene analyzed (data not shown). For this reason, we can conclude that RT introduces some background amplification in RT-PCR assays that increases the expected signal. Taking into account that amplification without the addition of primers did not occur if the RNA came from tissues or cells where the mRNA of interest is not expressed, we propose that the corresponding mRNA is the source for that background amplification signal. A possible explanation is that the RNA could hybridize either with oligonucleotides present in the sample [6,7] or that mRNAs could self-hybridize [21] and hence, such association would work as primer for the RT enzyme. Other authors have suggested that background priming is the result of small RNAs or DNAs molecules present in the sample that are able to hybridize with the RNAs [6,7]. Our results are in line with this putative explanation. Furthermore, preliminary results showed that mRNA purification decreased the background signal, increasing from 3 to 9 fold the ratio between PCR products obtained after RT performed either with or without added primers (data not shown). In this work we also showed that the addition of forward primer, but not a non-related EFGP primer, could slightly increase background amplification signal introduced by the RT reaction, as could be seen in a real time PCR assay (see Figs. 5 and 6). Relationship between cDNA synthesis with and without added primers to RT is variable and seems to depend on each mRNA considered. We suggest that the variability of this relationship could be due to primer specificity or efficiency, and/or to hybridization capability and efficiency for every analyzed RNA.

Background signal becomes a very relevant issue, particularly when both sense and antisense RNA are present in the same sample or when looking for antisense regulatory molecules. In this case, each molecule would introduce a background signal which could interfere with the quantification of the other. Hence, it is necessary to determine and quantify the specific background signal for each strand and then, to subtract it from the total signal to obtain the genuine values of PCR amplification.

The RNA from hippocampus of animals injected either with NR1 antisense vector, control vectors or just vehicle showed a specific amplification signal by RT-PCR. This signal was slightly higher in NR1 antisense vector injected animals (Fig. 1b). Here, we were able to discriminate the expression of the exogenous antisense *in vivo* from the endogenous NR1 mRNA, thanks to the determination and quantification of that specific background signal by real-time RT-PCR (Fig. 7). After background signal subtraction there was a significant remaining signal only in samples from animals injected with the NR1 antisense vector, while there was no remaining signal in control samples. This indicates that the NR1 antisense RNA was only expressed from the amplicon vector bearing that sequence. The existence of an endogenous antisense molecule was definitively discarded by RPA assay. Taken together, results presented in this work strongly indicate that the template for the unexpected amplified fragment was the NR1 mRNA normally expressed in the nerve tissue.

5. Conclusions

As the RT reaction introduces a significant background signal in every RT-PCR assay, it would be necessary to determine the identity of the amplified signal; particularly when a couple of sense/antisense RNA is expressed in the same sample. Since background signal is introduced by the RT reaction, we suggest that another control should always be included: the background amplification signal control (BASC), for both standard and real time RT-PCR assays. To this end, RT reaction for this control has to be performed in the absence of any primer, and then, the product of this RT has to be used as sample in the subsequent PCR assay. Finally, the background amplification signal obtained in BASC after PCR should be identified, quantified and subtracted from the amplification signal corresponding to the specific PCR analyzed.

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