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Analysis of the transcriptional levels of guinea pig sines and lines

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Abstract

Short and long interspersed DNA elements (SINEs and LINEs, respectively) can “jump” in germ-line cells generating new copies in other chromosomal regions. There is a sharp contrast in the number of SINEs within various rodent genomes. The purpose of this investigation is to assess the relative paucity of SINEs in the guinea pig genome in relation to other rodents. RNA was isolated from brain and kidney tissues of guinea pigs, and from the brain tissue of mice, in order to develop a method to assess the transcriptional regulation of SINEs and the loci that serve as source genes. This involved the use of rapid amplification of cDNA ends (RACE) and C-RACE. The latter technique involved C-tailing the RNA prior to RT-PCR. It was verified that RT-PCR products were amplified. In the future, assaying in gonadal tissues will be contrasted to copy number estimates of SINEs within the genomes of guinea pigs, mice, hamsters, and rabbits.

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ANALYSIS OF THE TRANSCRIPTIONAL LEVELS OF GUINEA PIG SINEs AND LINEs

By

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Senior Honors Thesis

ABSTRACT

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Short and long interspersed DNA elements (SINEs and LINEs, respectively) can "jump" in germ-line cells generating new copies in other chromosomal regions. There is a sharp contrast in the number of SINEs within various rodent genomes. The purpose of this investigation is to assess the relative paucity of SINEs in the guinea pig genome in relation to other rodents. RNA was isolated from brain and kidney tissues of guinea pigs, and from the brain tissue of mice, in order to develop a method to assess the transcriptional regulation of SINEs and the loci that serve as source genes. This involved the use of rapid amplification of cDNA ends (RACE) and C-RACE. The latter technique involved C-tailing the RNA prior to RT-PCR. It was verified that RT-PCR products were amplified. In the future, assaying in gonadal tissues will be contrasted to copy number estimates of SINEs within the genomes of guinea pigs, mice, hamsters, and rabbits.

INTRODUCTION

Short and long interspersed DNA elements (SINEs and LINEs, respectively) are dispersed throughout rodent genomes (Kim et al. 1994; Kass et al. 1996; Dewannieux and Heidmann 2005). These sequences belong to a family of transposable elements referred to as retroposons. Retroposons make up the bulk of transposable elements in mammalian genomes (Deininger et al. 2003). This type of transposable element has the ability to move throughout the genome through the process of retroposition. During the process of retroposition, a DNA sequence is first transcribed into an RNA intermediate. This RNA functions as the template for synthesis of the complementary DNA (cDNA) using reverse transcriptase. The DNA copy is then integrated into another site in the genome, generating flanking direct repeats of host DNA (Kass et al. 1997; Deininger et al. 2003), and therefore increasing the number of elements within the genome.

There are several features that are characteristic of the majority of SINEs: an internal A box RNA polymerase III promoter sequence located downstream of the transcription start site, an internal B box RNA polymerase III promoter sequence located approximately 50 bp downstream of the A box, an adenine-rich 3' region (often termed an A-tail), and flanking direct repeats (Kass et al. 1996; Roy et al. 2000; Odom and Robichaux 2004).

SINEs are categorized as non-autonomous transposons because they do not encode protein and rely on the replication machinery of autonomous transposons for the process of retroposition (Kramerov and Vassetzky 2005). An ongoing hypothesis suggests that the retroposition of the non-coding SINEs is dependent on protein-encoding LINEs for their propagation (Deininger et al. 2003; Odom and Robichaux 2004; Rinehart

et al. 2005). It is also believed that the vast majority of SINEs are incapable of actively undergoing retroposition and that SINE amplification is instead controlled by a limited number of active elements that are termed master or source genes (Kim et al. 1995; Shen et al. 1997; Roy et al. 2000; Odom and Robichaux 2004).

There are three major SINE families specific to the rodent genome: ID, B1, and B2 elements (Kim et al. 1994; Kass et al. 1996; Kass et al. 1997; Deininger et al. 2003; Dewannieux and Heidmann 2005). B2 and ID elements are ancestrally derived from tRNA genes while B1 is ancestrally derived from the 7SL RNA gene (Deininger et al. 2003; Dewannieux and Heidmann 2005). The copy number of ID elements within different rodent species such as rat, mouse, hamster, and guinea pig varies by several orders of magnitude (Shen et al. 1997). It has been estimated that there are 150,000 copies of ID elements in the rat, 4,000 in the mouse, 1,500 in the gerbil, 1,000 in the hamster, and 200 copies in the guinea pig genome (Kass et al. 1996). The BC1 RNA gene is the presumptive master gene for ID amplification based on the observation that ID elements in various rodent species demonstrated sequence changes that coevolved with those at the BC1 RNA locus (Kim et al. 1994; Shen et al. 1997). The copy number of B2 elements within various rodent species also differed by several orders of magnitude: 80,000 copies in the rat, mouse, gerbil, and hamster, and less than 100 copies in the guinea pig genome (Kass et al. 1997). Similar variation in copy number was observed for B1 elements within different rodent species: 150,000 copies in rat and mouse, 200,000 in hamster, and 50,000 in guinea pig (Vassetzky et al. 2003).

There is clearly a paucity of SINEs in the guinea pig genome in relation to other rodents. The low copy number of rodent SINEs in the guinea pig genome may be due to

lack of RNA expression in the germ-line. SINEs must mobilize in germ cells in order to be passed on to future generations (Deininger et al. 2003). The expression of SINEs varies among tissues (DeChiara and Brosius 1987; Shen et al. 1997). Previous studies have indicated that B1 elements are transcribed in a variety of mouse cell types (Maraia 1991). Increased levels of B2 transcripts are observed in the testicles (Ryskov et al. 1985) and in early mouse embryos (Vasseur et al. 1985; Bachvarova 1988). ID transcripts have been detected in the brain and testes (Kim et al. 1995; Shen et al. 1997). It has been determined that ID transcripts are expressed in the brain via BC1 RNA transcripts which are highly abundant in neural tissue (DeChiara and Brosius 1987; Martignetti and Brosius 1993; Kim et al. 1994; Kim et al. 1995).

In this study, RNA was isolated from guinea pig kidney and brain tissue and mouse brain tissue to incorporate the C-RACE and RACE techniques for the purpose of determining transcriptional regulation. These tissues were readily available and the mouse brain had been shown to express high levels of a SINE master gene. Therefore, RNA isolated from these tissues will provide the basis for the development of an assay to determine the genes involved in SINE expression, as well as transcriptional levels. This will serve as a tool to assess transcriptional regulation as the key mechanism to explain varied copy numbers of SINEs among rodent genomes.

MATERIALS AND METHODS

RNA isolation

Total RNA was isolated from guinea pig brain and kidney tissue, and mouse brain tissue (Rockland, Inc.) using the SV Total RNA Isolation System (Promega). A 30 mg sample of each tissue was cut with a sterile razor blade, frozen in liquid nitrogen, and

ground in a mortar and pestle under liquid nitrogen. The tissue was transferred to a microcentrifuge tube containing 175 µL of SV RNA Lysis Buffer. 350 µL of SV Dilution Buffer was then added, mixed by inverting 4 times, and placed in a heating block at 70°C for 3 minutes. The sample was centrifuged for 10 minutes at 14,000 x g. The lysate solution was transferred to a new microcentrifuge tube, 200 µL of 95% ethanol were added, and the mixture was transferred to a spin column assembly, and centrifuged at 14,000 x g for one minute. The liquid was discarded from the collection tube, 600 µL of SV RNA Wash Solution was added to the column, centrifuged at 14,000 x g for one minute, and the collection tube was emptied. A DNase incubation mixture was prepared per sample by combining 40 µL Yellow Core Buffer (Promega), 5 µL 0.09M $MnCl_2$ and 5 µL of DNase I enzyme. The DNase incubation mixture was added directly to the membrane of the spin basket. The mixture was incubated for 15 minutes at 25°C, 200 µL of SV DNase Stop Solution was added to the spin basket, and centrifuged at 14,000 x g for one minute. Next, 600 µL of SV RNA Wash Solution was added and centrifuged at 14,000 x g for one minute. The collection tube was emptied, 250 µL of SV RNA Wash Solution was added, and centrifuged at 14,000 x g for two minutes. The spin basket was transferred from the collection tube to an elution tube, 100 µL of Nuclease-Free Water was added to the membrane and centrifuged at 14,000 x g for one minute. The spin basket was discarded and the purified RNA was stored at -70°C.

The RNA was quantitated using UV absorbance of 260 nm with a Beckman Coulter DU 530 Spectrophotometer.

RNA tailing, cDNA synthesis, and amplification of B1 elements by RACE

A modified RACE technique referred to as C-RACE (C-tailed RNA rapid amplification of cDNA end) was used to isolate the 3' ends of the RNA transcripts (Kim et al. 1995). RNA was 3' tailed with CTP using RNA Poly(A)Polymerase (Ambion). The C-tailing reaction was carried out for 1 hour at 37°C in a 100µL reaction containing 1X Poly(A)Polymerase Buffer, 2.5 mM $MnCl_2$, and 1 mM CTP, and 0.04 u (units) Poly(A)Polymerase. cDNA was synthesized from C-tailed RNA by incubating at 70°C for 10 minutes. The reaction was carried out in a 20µL volume with 5 mM $MgCl_2$, Reverse Transcription 1X Buffer (Promega), 1 mM dNTP, Recombinant RNasin® Ribonuclease Inhibitor, AMV Reverse Transcriptase, and 0.025 µg C-RACE Adaptor primer (5'-GCCTTCGAATTCAGGTTGGGGGGGGGGG-3'). The reaction was incubated at 42°C for 15 minutes, 95°C for 5 minutes, and 4°C for 5 minutes. cDNA was also synthesized from total RNA using the above reaction conditions, substituting RACE primer (5'-GCCTTCGAATTCAGGTTTTTTTTTTTTT-3') for C-RACE Adaptor primer. The cDNA was amplified by the polymerase chain reaction (PCR) with 2 primers, B1F-AGE (5'-CCGACCGTAGCCGGGCATGGTGGCGCA-3') for the 5' end of B1 transcripts and C-RACE PCR (5'-GCCTTCGAATTCAGGTT-3') for the 3' end with the primer-adaptor. Ten microliters of cDNA was amplified in a 50 µL PCR reaction mixture containing 0.18 mM dNTP, 1.875 mM $MgCl_2$, Reverse Transcription 0.98X Buffer, 0.01µg C-RACE PCR primer, 1 mM B1F-AGE primer, and 5 u *Taq* polymerase (Promega). The reactions were carried out in an MJ Research thermocycler under the following conditions: 94°C 2 min.; 94°C 20 sec., 42°C 20 sec., 72°C 20 sec. for 32 cycles; 72°C 5 min.; and held at 4°C. The PCR reaction mixtures were separated by electrophoresis on a 2% agarose gel with 1XTAE buffer and 0.5µg ethidium bromide and visualized under UV light.

Subcloning and analysis of amplified DNA

For cloning, 2 µL of each PCR reaction mixture was ligated into the pGEM®-T Easy Vector (Promega) and transformed into competent *Escherichia coli* cells according to the manufacturer's protocol using heat shock. Four white colonies containing vectors carrying DNA inserts were chosen from each transformed bacterial culture using blue/white selection. DNA was isolated from the overnight cultures using the Wizard® *PLUS* Minipreps DNA Purification System (Promega). The colonies were analyzed for inserts by restriction digestion with 6 u *Eco*RI (Promega) using 7µL of DNA in a 20 µL reaction mixture. The reaction was carried out in an MJ Research thermocycler at 37°C for 16 hours and the DNA was analyzed by electrophoresis on a 2% agarose gel with 1XTAE buffer and 0.5µg ethidium bromide and visualized under UV light. The DNA samples were cleaned with Genetix *gen*CLEAN columns and the DNA sequences of two clones from guinea pig brain were determined with an Applied Biosynthesis ABI Prism 310 by the dideoxy method using a Big Dye Terminator V3.1 Kit.

RESULTS

RNA quantitation using UV absorbance

RNA was isolated from guinea pig brain and kidney tissue and mouse brain tissue to incorporate the RACE and C-RACE techniques for the purpose of analyzing B1 expression. Quantitation of RNA by UV spectrophotometry yielded a concentration of 0.36 µg RNA per µL for guinea pig brain. A concentration of 0.34 µg RNA per µL was obtained for guinea pig kidney and 0.06 µg RNA per µL for mouse brain. Therefore, a large amount of RNA was available for further analysis.

Amplification of B1 elements by C-RACE and RACE

A modified RACE technique was utilized to isolate the 3' ends of the RNA transcripts (Fig. 1). Analysis of the amplification of B1 elements from guinea pig brain tissue by C-RACE using agarose gel electrophoresis revealed a distinct 225 base pair fragment (lane #5, Fig. 2), and a distinct 300 bp fragment (lane #5, Fig. 2), as well as a less defined 100 bp fragment (lane #5, Fig. 2). Analysis of guinea pig kidney revealed a distinct 200 bp fragment (lane #6, Fig. 2) and a distinct 300 bp fragment (lane #6, Fig. 2) and a less defined 100 bp fragment (lane #6, Fig. 2). Analysis of mouse brain for amplification of B1 elements by C-RACE exhibited a bright, distinct band near 200 bp (lane #7, Fig. 2), a bright 225 bp fragment (lane #7, Fig. 2), a distinct 300 bp fragment (lane #7, Fig. 2), and a distinct 400 bp fragment (lane #7, Fig. 2). These ~200 bp fragments are in the expected range, as a B1 element is 140 bp and therefore, after C-tailing, we would expect a B1 element to be within the range of 150-250 bp.

Analysis of the amplification of B1 elements from guinea pig brain tissue by RACE using agarose gel electrophoresis exhibited a 225 bp fragment (lane #8, Fig. 2) and a 275 bp fragment (lane #8, Fig. 2). Analysis of guinea pig kidney tissue revealed a 75 bp fragment (lane #9, Fig. 2) and a 225 bp fragment (lane #9, Fig. 2). Analysis of mouse brain for amplification of B1 elements by RACE exhibited a bright, distinct 225 bp fragment (lane #10, Fig. 2) and a 400 bp fragment (lane #10, Fig. 2). These ~200 bp fragments are in the expected range for a B1 element.

Cloning B1 Transcripts

The RT-PCR products were directly cloned into the pGEM®-T Easy cloning vector for further characterization. 100 µL of transformed bacterial culture was plated for the cloned RT-PCR products from guinea pig kidney and brain, which yielded

approximately 100 colonies per plate. The first transformed bacterial culture for guinea pig brain RT-PCR products had a greater proportion of blue colonies, which do not carry any cloned DNA inserts, than the desired white colonies, which contain vectors carrying DNA inserts. The second bacterial transformation culture for guinea pig brain RT-PCR products had a larger proportion of blue colonies than white colonies concentrated along the outer edge of the plate and a few blue colonies in the center of the plate. The first transformed bacterial culture for guinea pig kidney RT-PCR products had a greater distribution of blue colonies compared to white colonies. The second bacterial transformation culture for guinea pig kidney RT-PCR products had a large number of blue colonies, particularly near the outer rim of the plate, and very few white colonies. The few white colonies obtained were further characterized by isolating the plasmid DNA.

Four white colonies containing vectors carrying DNA inserts were chosen from each transformed bacterial culture and grown overnight. Plasmids were isolated and digested with *Eco*RI. Analysis of the restriction-digested clones using 2% agarose gel electrophoresis (Fig. 3) revealed that two clones from the guinea pig brain RT-PCR products exhibited the expected insert sizes of 200 bp and 225 bp, allowing for the length of the A-tail. No inserts were observed for the clones from guinea pig kidney RT-PCR products. The two clones from the guinea pig brain RT-PCR products showing expected insert size were sequenced. The sequence for the first clone contained a large proportion of C's. The sequence for the second clone contained a large proportion of N's. These sequences were determined to be uninformative upon analysis of the electrophoretograms.

DISCUSSION

RNA was isolated from guinea pig brain and kidney tissue, as well as mouse brain tissue to incorporate the C-RACE and RACE techniques for the purpose of determining transcriptional regulation as a key mechanism to explain varied copy numbers of SINEs among rodent genomes. The length expected for B1 elements has been determined to be approximately 140 base pairs in length (Ryskov et al. 1985; Maraia 1991; Vassetzky et al. 2003). With an A-tail we would expect fragments to be within the range of 150-250 bp, which was observed in our RT-PCR analysis (Fig. 2). RNA isolated from mouse brain tissue served as the control because B1 elements are transcribed in a variety of mouse cell types (Maraia 1991).

The C-RACE and RACE techniques seem to be potentially useful to analyze expressed loci and levels of RNA within tissues. The C-RACE technique could prove to be particularly useful for isolating the 3' unique sequence of a B1 transcript, which can be utilized to isolate the genomic loci. For example, the analysis of the amplification of B1 elements from guinea pig brain and kidney tissue and mouse brain tissue by C-RACE and RACE using agarose gel electrophoresis exhibited differences in mouse and guinea pig expression of B1 elements. There are four distinct bands exhibited for the amplification of B1 elements by C-RACE in mouse brain, each band potentially corresponding to a distinct genetic locus. Conversely, there appears to be two distinct genetic loci for guinea pig. If B1 transcripts are derived from more loci in mice than in guinea pigs, it could explain the increased expression of B1 elements in mice compared to guinea pigs. Unfortunately, due to the inability to obtain useful sequence data, it has not been confirmed whether B1 elements were indeed amplified from guinea pig brain and kidney

tissue or mouse brain tissue by C-RACE and RACE. Possible reasons for the lack of quality sequence data could include low concentration of DNA, reduced purity of template DNA, or insufficient removal of excess dye terminators. In order to rectify these problems, cloning of the guinea pig brain and kidney RT-PCR products will be repeated and the mouse brain RT-PCR products will be cloned as well. Informative sequence data will allow for determination of expression of B1 elements.

It has been determined that the amplification of SINEs is dominated by a limited number of master genes (Kim et al. 1994; Kim et al. 1995; Kass et al. 1996). A previous study concluded that the rat ID family consists of at least four subfamilies. These ID subfamilies appear to be derived from at least two master genes. An increase in active master genes could allow for a change in expression pattern and could result in an increase of germ-line transcription and retroposition (Kim et al. 1994). The large increase in ID element copy number within the rat genome has been attributed to the multiple master genes of the four ID subfamilies (Kim et al. 1994). A previous analysis of B1 retroposons in the genus *Mus* determined that there were four B1 subfamilies which were concurrently active between 1 and 3 million years ago (Kass et al. 2000). Therefore, based on the findings from the previous study by Kass et. al. (2000), it is possible that the four distinct bands exhibited for the amplification of B1 elements by C-RACE in mouse brain, could potentially correspond to four source loci.

In the future, the newly cloned RT-PCR products for guinea pig brain and kidney, and mouse brain will be sequenced to verify expression of B1 elements, to assess whether various genetic loci are expressing B1, and to potentially identify master genes. Next,

RNA will be isolated from germ-line tissue (ovaries and testes) of guinea pigs, mice, hamsters, and rabbits. A northern blotting technique will be employed to characterize and quantify the transcriptional activity of B1. The amplification patterns of the B2 and ID transcripts will also be assessed by analyzing transcriptional activity in guinea pig germ-line cells in relation to other rodent germ-line cells. These data will be used to further study transcriptional regulation as a key mechanism to explain varied copy numbers of SINEs among rodent genomes.

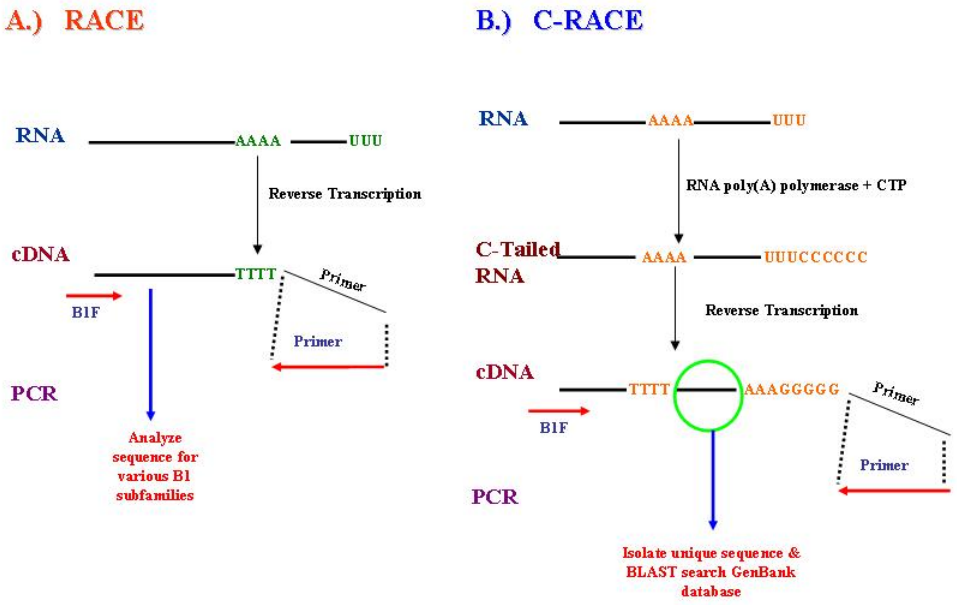

FIGURE 1: Schematic representation of RT-PCR Technique (A.) and C-RACE Technique (B.) For the C-RACE technique, isolated RNA was tailed with CTP using RNA poly(A)polymerase. The tailed RNA was subject to reverse transcription with an adaptor primer. The cDNA from both RT-PCR and C-RACE was amplified using an identical PCR protocol.

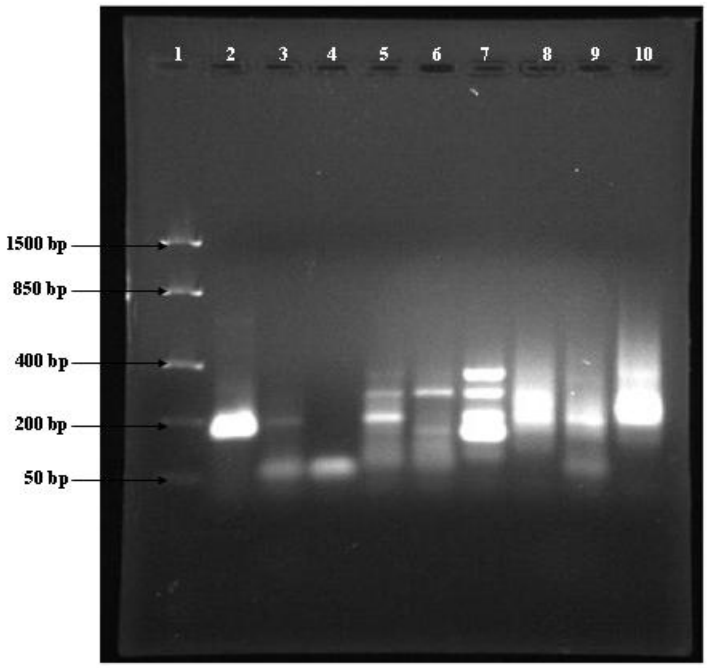

Figure 2. Analysis of RT-PCR products on a 2% agarose gel stained with ethidium bromide and visualized with UV light. Lane 1: Low Range Fast RulerTM(Promega). Lane 2: Kanamycin + Control RNA. Lane 3: 1:20 dilution of amplification of B1from guinea pig brain by C-RACE. Lane 4: 1:20 dilution of amplification of B1 from mouse brain by C-RACE. Lanes 5-7: amplification of B1 from guinea pig brain, kidney & mouse brain, respectively by C-RACE. Lanes 8-10 amplification of B1 from guinea pig brain, kidney, & mouse brain, respectively by RACE.

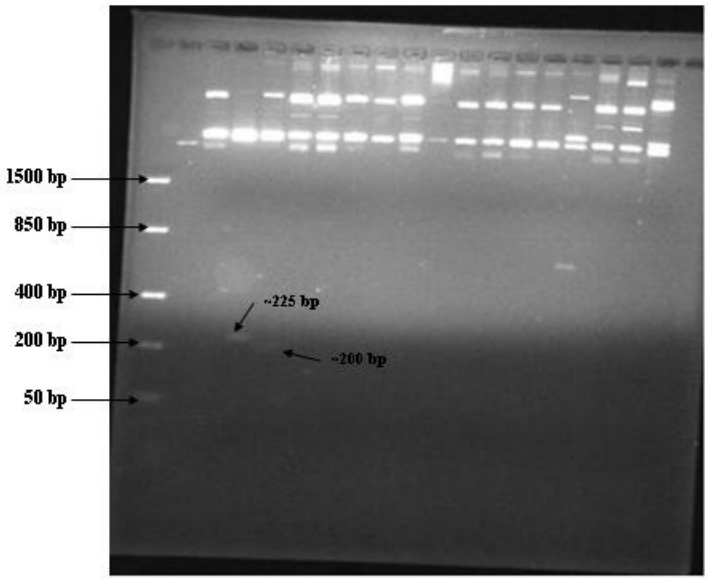

Figure 3. Analysis of clones by restriction digestion with *Eco*RI on a 2% agarose gel stained with ethidium bromide and visualized with UV light. The guinea pig brain RT-PCR products exhibited insert sizes of 200 bp and 225 bp as indicated by the arrows.

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