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Age-related changes in gene expression in the rat brain revealed by differential display

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Abstract. We have used the polymerase chain reaction (PCR)-based technique of differential display to analyse changes in gene expression during ageing of the rat brain. In this approach we have compared three young adult (6 months) with three old adult (20 months) animals. RNA preparations from the homogenised brains were subjected to reverse transcriptase (RT)-PCR using 36 different combinations of primer pairs. Any PCR product which was consistently found to be more prominent in the three young brains compared to the three old brains, and vice versa, was scored as potentially representing a gene which was differentially expressed during the ageing of this tissue. Out of a possible 2000+ PCR products we identified 44 that might represent genes that exhibit differential expression during ageing of the rat brain. An initial screen of these fragments, by Southern-blotting the PCR products and hybridising them with cDNA probes derived from either young or old brain RNA preparations, indicated that 40% of them represented genes that were differentially expressed. This approach is likely to prove invaluable for identifying cohorts of genes that show differential expression during the ageing process.
Key words. Ageing; rat; brain; gene expression; differential display.

The ageing process is observed in most multicellular organisms and yet very little is known of the underlying molecular mechanisms that regulate it. It has proven extremely difficult to devise experimental strategies to manipulate the rate of ageing in homeothermic animals and to establish model systems in which to investigate ageing. Those limited number of strategies that have been successful have always incorporated some form of restricted feeding [1, 2]. These have suggested that similar molecular mechanisms may underlie the ageing process in the majority of animals if its rate can be manipulated by a simple dietary strategy. The rapid development of diverse molecular genetic methodologies during the past decade means that we now have at our disposal a powerful set of tools with which to dissect the ageing process at the molecular level.

There seems little doubt that in most mammalian tissues there is a general decrease in total RNA synthesis with age [3], primarily due to a decrease in mRNA [4]. If synthesis of mRNA decreases as a function of age, but the total amount of mRNA remains the same, as most studies have suggested, then it appears that it is mRNA turnover that decreases with age [5]. While tissue content of mRNA shows little or no change with age, expression of different mRNA species could be differentially affected by the ageing process. A number of studies have attempted to address this issue, but the data are still very fragmented. For example, age-related changes in the mRNA species encoding for specific hepatic proteins in male Fischer 344 rats have been studied using an in vitro rabbit reticulocyte lysate translation system. Age-dependent changes were identified for three mRNA species (senescence marker proteins 1, 2, 3), senescence marker protein 3 being identified as α-globulin [6]. Also, examination of the mRNA levels and methylation patterns of the liver-specific tyrosine aminotransferase (TAT) gene in inbred female rats at 6, 24 and 36 months revealed a 65% decrease in the steady state transcript level of TAT in the liver of old rats as compared to 6-month-old animals [7].

The effects of ageing on the expression of several enzymes that are involved in free radical detoxification (superoxide dismutase, catalase and glutathione peroxidase) have been studied in liver tissue from male Fischer 344 rats. Both the enzymatic activity and mRNA levels of Cu-Zn superoxide dismutase and catalase decreased with age in rat liver [8]. Age-related and diet-induced mRNA changes in tissue levels and transcription rates for these enzymes correlated with changes in enzyme activity levels. An extension of this study to measure the effect of age on the expression of these antioxidant enzymes in brain, heart, intestinal mucosa and kidney of male Fischer 344 rats confirmed the

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observations for hepatic tissue [9]. Studies with female C3B10RF1 mice, a long-lived hybrid strain, revealed an age-associated decrease in mRNA levels in the liver for Mn-superoxide dismutase cytochrome P1 and P3-450 but not for mRNA for Cu-Zn superoxide dismutase, glutathione peroxidase and epoxide hydrolase. Other genes that have been reported to show age-related changes in expression include the insulin receptor and the transcription factor jun [10] and heat-shock proteins [11].

While the above reports have studied the effect of ageing on the expression of specific genes and the activity levels of the translation products of individual mRNA species, we have attempted to identify systematically widespread changes in gene expression during ageing. To do this we have adopted the PCR-based technique of differential display [12] and applied it to the study of age-related changes in gene expression in the rat brain.

Materials and methods

Adult 6- and 20-month-old male CFY strain rats were obtained from a closed colony of rats for which the life-time survival characteristics have been documented for a period of 20 years. Animals were maintained at 21 ± 1 °C, 50–70% relative humidity, 12 h light/dark cycle, and fed ad libitum a standard rat maintenance diet. The animals were sacrificed, the brains removed, immediately frozen in liquid nitrogen and stored at −70 °C until required. RNA was prepared from the frozen tissue by grinding to a fine powder under liquid nitrogen. The dry frozen powder was then added to RNAzol (Biogenesis) and further purification carried out according to the manufacturer's instructions.

Differential display was performed as described by Liang and Pardee [12]. Reverse transcription (RT) was carried out using 0.2 µg total RNA with one of the four anchored primers (T1–T4) described in the table. The reaction volume was 20 µl and Superscript reverse transcriptase (Gibco) was used, as it was found to give the most consistent data. Each RT reaction provided a template for 10 PCR reactions, which were performed with the primer used for the RT reaction and one of the random 10mer primers (table). The PCR reactions involved 40 cycles of 94 °C for 30 s, 40 °C for 2 min, 72 °C for 30 s and a final cycle at 72 °C for 5 min. In each gel a negative sample (no DNA) was also run. ^{35}S-labelled nucleotides were added to the PCR reactions to enable the products to be visualised after size fractionation on a sequencing gel.

After PCR amplification was completed, 10 µl of product per reaction was dried in a speedvac (Savant) and resuspended in 2–3 µl of loading buffer. The PCR products were size-fractionated on a standard 6% acrylamide sequencing gel by electrophoresis at 1800 v, 100 ma, 50 W for approximately 1.5 h. The gel was then dried and the products visualised by autoradiography. Bands of interest were cut from the gel by aligning the gel with the exposed autoradiographic film. These were then rehydrated in 100 µl of water, which was boiled to elute the DNA. The DNA was recovered by precipitation. This DNA was used as a template for a second round of PCR, using the same primer pair, to provide sufficient material for further analysis.

For each reamplified product, 5 µl of amplified DNA was analysed by Southern blotting. The filter was hybridised with a cDNA probe representing mRNA species expressed in the old adult rat brain. Hybridisation and washing conditions were as previously described [13]. After exposure to an autoradiographic film, the filter was stripped [13] and then reprobed with a cDNA probe representing mRNA species present in a young adult rat brain.

Table. Sequences of primers used in PCR amplification.

Primer	Sequence (5′-3′)
T1	A
	TTTTTTTTTTTTCA
	G
T2	A
	TTTTTTTTTTTTCG
	G
T3	A
	TTTTTTTTTTTCC
	G
T4	A
	TTTTTTTTTTTCT
	G
R1	GTTGCGATCC
R2	GACCGCTTGT
R3	AGCCAGCGAA
R4	AGGTGACCGT
R5	CTAGCAGTCG
R6	GGTACTCCAC
R7	GCAATCGATG
R8	CCGAAGGAAT
R10	CGTGGCAATA

Results

We have analysed gene expression in young adult (6 months) and old adult (20 months) rat brains. RNA preparations from each of the homogenised brains were subjected to RT-PCR using 36 different combinations of random primer pairs (table). It was calculated that this should allow for the identification of 15–20% of the

genes expressed in brain tissue [12]. The PCR products generated by the use of each primer pair were separated on acrylamide gels. In these experiments we compared three young adult with three old adult animals to avoid problems associated with the identification of false positives [12]. Therefore, any PCR product which was consistently found to be present in the three young brain preparations but not in the three old brain preparations, or vice versa, was scored as potentially representing a gene which was differentially expressed during the ageing of this tissue.

Examples of two of the differential display gels are shown in figure 1. In each case the PCR products derived from young brain RNA preparations are in lanes 1–3 and those from old brain RNA preparations in lanes 4–6. On average we could detect approximately 50 PCR products per lane. In the case of figure 1a, three bands are indicated as being more prominent in old brain than in young brain, whereas in figure 1b two bands are indicated which appeared to be more prominent in the young brains. We ran a total of 36 gels using different primer pair combinations and identified over 2000 PCR products. Out of these we identified 17 that appeared to represent genes that were upregulated in the young brain and 27 that appeared to represent genes that were upregulated in the old brain.

To further assess whether the PCR products genuinely represented genes which were differentially expressed during the ageing process, the products were cut from the gels, reamplified using the appropriate primer pair and Southern-blotted onto nylon filters. These were then probed with cDNA prepared from total RNA which had been derived from one of the young brains. The filters were then stripped and reprobed with cDNA prepared from total RNA which had been derived from one of the old brains. An example of this type of analysis is shown in figure 2. A negative control, which was a region of the gel that was clear of PCR products but was subjected to the reamplification procedure, was demonstrated to be negative with both probes (figure 2, lane 1). A PCR product which had been identified using young brain mRNA hybridised with the young brain probe but not the old brain cDNA probe (figure 2, lane 2). Conversely, two PCR products which had been identified using old brain mRNA hybridised only to the old brain cDNA probe (figure 2, lanes 3 and 4) the PCR product in lane 3 being the same as the smaller of the three products identified on figure 1a. A positive control, which was a PCR product that had been observed

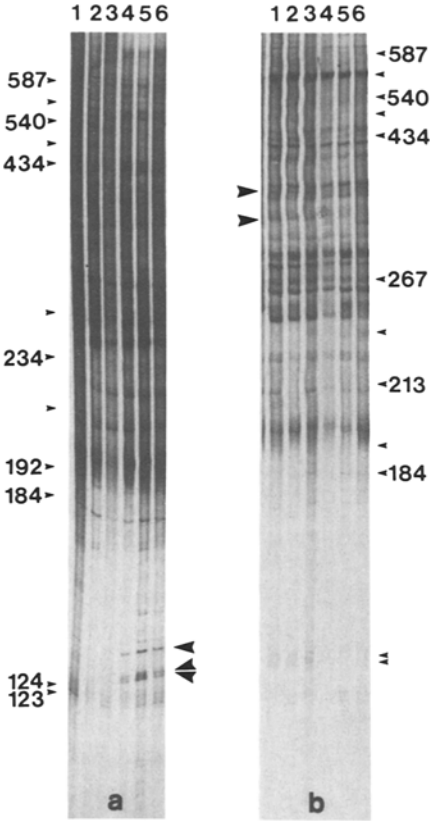

Figure 1. Differential display comparison of gene expression in young and old rat brains. The PCR products produced by the use of PCR primers T2/R8, are shown in (*a*) and those produced by the use of PCR primers T2/R7 are shown in (*b*). Lanes 1–3, young adult (6 month) brains; lanes 4–6, old adult (20 month) brains. The PCR products which are prominent primarily in either young or old brains are indicated by large arrowheads. Size markers (pBR322 Hae III digest fragments) are indicated at each side of the gels by the small arrowheads.

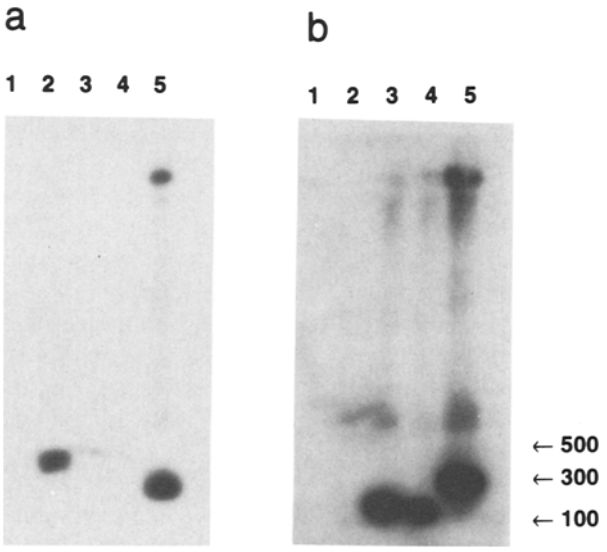

Figure 2. Southern blot analysis of PCR products isolated from differential display gels. After blotting PCR products onto a filter, it was probed first with a cDNA probe from young rat brain RNA (*a*), then stripped and reprobed with a cDNA probe from old rat brain cDNA (*b*). Lanes (1) negative control, (2) PCR product identified from young rat brain, (3) PCR product identified from old rat brain – see smallest arrowed band in figure 1a, (4) PCR product identified in old brain, (5) positive control. Molecular weight sizes indicated at right of gel.

using both young and old brain mRNA samples, hybridized with both cDNA probes (figure 2, lane 5). It should be noted that although figure 2a represents a five-day exposure, we carried out a further exposure of 4 weeks, but still failed to reveal signals in lanes 3 and 4. Therefore, the PCR products in these lanes appear to represent genes that are switched on during the ageing process, and do not simply change their level of expression.

It appeared that at least 14 of the 44 PCR products represented genes that showed differential expression during ageing of the brain. It is not certain, however, whether these represented 14 genes, as more than one PCR product might represent different regions of the same gene.

Discussion

Two previous studies have reported attempts to systematically identify widespread changes in gene expression with ageing. Differential screening of whole mouse cDNA libraries prepared from young (3 month) and old (27 month) C57BL/6J inbred mice allowed 5 clones to be isolated which showed an age-related pattern of expression. Four of these were identified by computerised matching sequence to be MUP2 (a major urinary protein), Q10 of the MHC locus, a cytoskeletal actin gene and creatine kinase. The fifth gene whose expression increased with age and was found to be most abundant in spleen has not been identified [14]. A similar approach was carried out by screening a cDNA library constructed from 24-month-old rat liver mRNA, with cDNA probes from young and old rat livers. Most of the clones were found to represent T-kininogen, whose expression increases two to four months before death and may be involved in the disruption of intracellular protein degradation [15]. We have now demonstrated that a third approach, using differential display, also offers the possibility of identifying genes which exhibit age-related changes in expression. Although more than one PCR product may be obtained from the same gene, this approach appears to be useful not only in identifying differences in the pattern of gene expression that occurs during ageing but also in providing the possibility of isolating and characterising these genes by cloning the relevant PCR products. The ability to retard the rate of ageing in rats by caloric or dietary restriction feeding regimes provides an additional approach by which to confirm that genes identified as exhibiting differential expression with age are indeed relevant to the ageing process. It would therefore be of interest to determine whether the differential gene expression identified in the present study was present in animals maintained on caloric restricted feeding regimes and whether the transition of differential gene expression occurred at a slower rate commensurate with the extended survival times of such animals.

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