Bovine Serum Albumin Reverses Inhibition of RT-PCR by Melanin

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Melanin contained in pigment cells in a variety of tissues co-purifies with nucleic acids in standard RNA (or DNA) extraction procedures. The presence of melanin in RNA (or DNA) templates can inhibit reverse transcription polymerase chain reactions (RT-PCR) and/or PCR (5). In the past, to circumvent this problem, melanin has been separated from DNA by either column-chromatography (5) or acid-precipitation (3). In this study, a facile approach is described to overcome melanin inhibition of RT-PCR. Specifically, the addition of >15 μg of bovine serum albumin (BSA) per 25 μL RT-PCR mixture is shown to reverse melanin inhibition (Figures 2, A and B). BSA has been used previously to prevent inhibition of PCR amplifications by hemin, iron chloride, tannic acids, fulvic acids, extracts from feces, freshwater or marine water (4).

In this study, we describe the dose-response effect of melanin on RT-PCR inhibition and the effect of BSA in overcoming melanin inhibition. The addition of 2 μg of synthetic melanin (Catalog No. M-8631; Sigma Chemical, St. Louis, MO, USA) to a 25-μL RT-PCR can completely inhibit RT-PCR of aldolase mRNA (Figure 1A). A similar degree of inhibition was observed when melanin-containing RNA preparations were prepared from either normal melanocyte cell strains (Figure 2A) or melanoma cell lines (data not shown). While RT-PCR of 0.5 μg total RNA will normally yield a product after 16 cycles of PCR following RT (2), when 2 μg of melanin were present, no RT-PCR product was obtained after 45 cycles of PCR. The addition of >15 μg BSA to the reaction mixture alleviated melanin inhibition and allowed detection of a PCR product by PCR cycle 16. The addition of 20–23 μg BSA per assay appeared to be optimum (Figure 1B) in overcoming melanin inhibition. Fatty acid-free BSA (Catalog No. A-7511; Sigma Chemical), >97% pure alcohol-precipitated BSA (Catalog No. A-4378; Sigma Chemical) and Fraction V 98%–99%-pure BSA (Catalog No. A-7906; Sigma Chemical) were found to be equally effective in reversing melanin-mediated RT-PCR inhibition (data not shown).

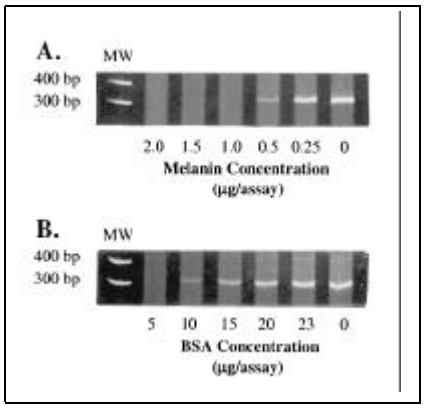

Figure 1. BSA reverses synthetic melanin inhibition of RT-PCR. (A) Melanin inhibition of RT-PCR. Aldolase mRNA levels were determined by RT-PCR as previously described (1,2). Each RT-PCR consisted of the same RT-PCR reagents, including 0.5 μg total RNA derived from the melanin-free A375 cell line. Other than the addition of the indicated amounts of melanin, each sample was identical to all other samples. PCR products were obtained from 25-μL RT-PCR assays containing various amounts of melanin after 16 PCR cycles. (B) BSA reversal of the inhibitory effect of 2 μg melanin per assay. With the exception of the positive control in lane 0 (rightmost), which contained no melanin or BSA, each 25-μL reaction consisted of the same RT-PCR reagents, including 0.5 μg total RNA (A375) and 2 μg melanin per assay. The only RT-PCR variable was the amount of BSA used. Increasing concentrations of BSA reversed the inhibitory effect of 2 μg melanin per assay. Lane MW shows the molecular weight DNA ladder.

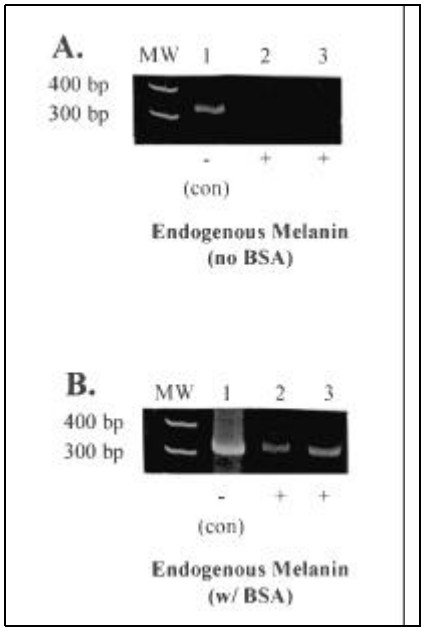

Figure 2. BSA reverses endogenous melanin inhibition of RT-PCR. (A) Endogenous melanin inhibition of RT-PCR. Aldolase mRNA levels were determined by RT-PCR as previously described (1). Each RT-PCR consisted of the same RT-PCR reagents, including 0.5 μg total RNA derived from the melanin-free A375 cell line (control, lane 1) and normal melanocyte cell strains (lanes 2 and 3). Other than the presence (+) or absence (-) of endogenous melanin of the RNA, each sample was identical. PCR products were obtained from 25-μL RT-PCR assays after one cycle of RT and 16 PCR cycles. (B) BSA reversal of endogenous melanin RT-PCR inhibition. Each RT-PCR consisted of the same RNA samples and conditions described in Panel A with the exception that 23 μg of BSA were added to each reaction at the RT step. Lane MW shows the molecular weight DNA ladder.

It was reported previously that melanin inhibits PCR (5). We found that melanin slightly inhibits the RT step as well. This determination was made as follows: (*i*) two RT reactions were carried out: a control and an RT containing 2 μg of synthetic melanin, and (*ii*) PCR was carried out on 1/100 dilution of the RT-generated cDNAs. Note that 1/100 dilution was used as a means to eliminate the effects of melanin at the PCR step. A small reduction in PCR product was noted after 2 μg of synthetic melanin were added at the RT step. This amount of melanin present at the PCR step would have been completely inhibitory. Thus, melanin appears to have only a small effect on reverse transcription.

In summary, the simple addition of >15 μg BSA per 25 μL RT-PCR mixture effectively overcomes inhibition of RT-PCR by melanin. Addition of up to 23 μg BSA per RT-PCR appeared to have no deleterious effects on RT-PCR product yield. This modified procedure should allow more effective RT-PCR analysis in melanin-containing cells or tissues (e.g., skin, hair or eyes).

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Amplification of mRNAs from Single, Fixed, TUNEL-Positive Cells

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The ability to assess the expression of multiple genes in individual cells represents a powerful tool for studying the mRNA abundances within identifiable cell types. The amplified antisense RNA (aRNA) method (4) allows the simultaneous identification of relative messenger RNA (mRNA) levels for multiple genes within single cells. Recently, the aRNA procedure has been extended to characterize the expression of mRNA abundance for multiple genes within immunohistochemically labeled cells (3). This method allows additional phenotypic characterization of cells before single-cell amplification. Whereas this method is useful for identifying cells based on the expression of a particular protein, immunohistochemical detection is problematic for characterizing the genetic profile of degenerating or dying cells. Specifically, a frequent hallmark of damaged cells is disruption of protein turnover. Therefore, particular proteins' expression levels may be decreased or even absent in dying cells (7). The expression of immunohistochemical markers might reveal abnormal cell populations, whereas the expression of certain proteins in cells can be associated with cell death. However, without a specific marker of cell damage, positive identification of damaged/dying cells based on immunohistochemical criteria may be unreliable.

Here, we chose the terminal deoxynucleotidyl-transferase (TdT)-mediated biotinylated (dUTP) nick-end-labeling (TUNEL) technique (9) to identify dying cells in the developing brain. The TUNEL stain uses the enzyme TdT, which incorporates biotinylated nucleotides to the 3′ ends of fragmented DNA, and TUNEL has been used as a marker for dying cells. The TUNEL method is also useful for identifying cells that are undergoing programmed cell death (PCD), which occurs as a consequence of normal development (6). The purpose of this study was to adapt the single-cell aRNA amplification technique for use in TUNEL-positive cells from fixed tissue preparations.

Three Sprague Dawley rat pups at postnatal day 8 (P8) were used. This time point was chosen because PCD in the rat is maximal at P8 (6). Rats were anesthetized and decapitated. Brains were post-fixed in situ in 4% paraformaldehyde for 3–4 h before removal of the brain, which was then immersion-fixed for an additional 24 h. After fixation, brains were embedded in paraffin and cut into 6-μm sections. TUNEL was performed using previously described methods (9). Figure 1 presents an example of a TUNEL-positive cell selected for dissection and subsequent aRNA amplification. Apoptotic cells selected for analysis were detected based on the presence of at least two of the following characteristic features of apoptosis: cell shrinkage, membrane blebbing or intense nuclear and cytoplasmic staining. Briefly, coronal sections were adhered to poly-L-lysine-coated slides by brief heat treatment at 60°C for 15 min. After removal of paraffin and rehydration, the tissue was digested for 15 min with 20 μg/mL proteinase K (Sigma Chemical, St. Louis, MO, USA). The reaction was terminated with tap water, and the tissue was preincubated in Buffer A (25 mmol/L Tris-HCl, pH. 6.6, containing 200 mmol/L potassium cacodylate and 0.25 mg/mL bovine serum albumin) for at least 5 min. Sections were incubated at 37°C with labeling solution containing TdT (0.3 U/mL), biotinylated-16-dUTP (20 mmol/L; Boehringer Mannheim, Indianapolis, IN, USA) and 1.5 mmol/L cobalt chloride in Buffer A for 1 h in a humidified chamber. The reaction was terminated with 2× SSC (300 mM sodium chloride and 30 mM sodium citrate, pH 7.4). After vigorous washing with 0.1 M Tris, pH 7.4, the sections were blocked with 10% goat serum in 0.1 M Tris for 30 min. The labeled DNA was visualized by treating the tissue with a 1:40 dilution of streptavidin-conjugated alkaline phosphatase (BioGenex, San Ramon, CA, USA) and stained with Fast Red™ Tablets (Sigma Chemical). Sections were stored in diethyl pyrocarbonate (DEPC)-treated distilled (d) H_2O until further processing.

Following TUNEL, sections were placed in a humidified chamber and incubated in a mixture of 50% formamide, 5× SSC, DEPC-treated dH_2O and an oligo(dT) primer coupled to a

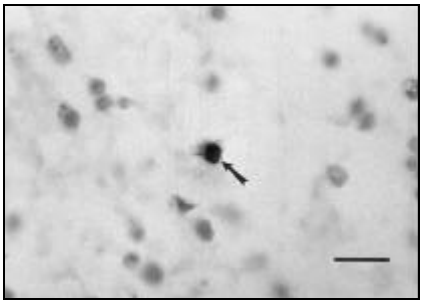

Figure 1. TUNEL-positive cell (arrow) from P8 rat cortex. (Bar = 20 μm) Note the presence of two apoptotic markers (cell shrinkage and intense nuclear and cytoplasmic staining).