Melanoma Research 2002, **12**, pp. 449–452

A versatile method for the removal of melanin from ribonucleic acids in melanocytic cells

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Melanin pigments often co-purify during preparation of nucleic acids from cells or tissues of melanocytic origin. Contaminating melanin can severely impede subsequent analyses of RNA. We attempted to eliminate melanin in RNA preparations using selected gel matrices. We show here that co-purified melanin pigments can be largely eliminated from RNA samples after passing through polyacrylamide-based beads (Bio-Gel P-60). After isolation from the pigment-containing cells or tissues, RNA was subsequently processed through batch or column purification under acidic pH conditions. The resulting RNA was devoid of contaminating melanin pigments and amenable to molecular reactions such as polymerase chain reaction and cDNA synthesis by reverse transcriptase. Although the process results in some loss of input RNA, this purification procedure is simple, robust and can easily be adopted in any laboratory for the molecular analysis of RNA that requires removal of melanin contamination. © 2002 Lippincott Williams & Wilkins

Keywords: Bio-Gel P-60, melanin, melanoma, removal, RNA

Introduction

Melanins are the natural biological pigments synthesized by melanocytes and actively transferred to neighbouring keratinocytes through special organelles named melanosomes. They are highly heterogeneous polymers of monomeric units of dihydroxyphenylalanine (dopa) and/or cysteinyldopa, and have molecular masses of 20–200 kDa. The oligomeric melanins can be eumelanin (dark brown to black), phaeomelanin (reddish brown to yellow), or a varying degree of mixture of both.[1]

Melanins are unstable in the presence of acid, alkali and oxygen, and moderately soluble in acidic and alkaline solutions. Hence during nucleic acid extractions there is a tendency for melanins to co-precipitate along with these macromolecules. In addition, melanin polymers contain a large number of free carboxylic acid residues, which are responsible for their strong anionic character and probably responsible for binding to charged molecules.[2,3] The presence of melanins in RNA can lead to problems with its subsequent use in cDNA synthesis and polymerase chain reactions. This has been attributed in part to the ability of melanins to bind to the thermostable DNA polymerases[4] and reverse transcriptases[5] usually used in these reactions. In addition, contaminating melanins contribute to inaccurate and misleading quantification of RNA using spectrophotometry and an abnormal migration rate during Northern analysis.

Over the years there have been a number of attempts to eliminate or circumvent the effects of melanins in these reactions. These include use of columns, multiple precipitation or the addition of an excess of bovine serum albumin (BSA) for competitive binding.[6–8] During the course of our studies on melanocytic cells, we encountered similar problems and attempted to overcome them using various physical and chemical means. In one set of experiments, we employed matrices used in column chromatography and found that Bio-Gel P-60 was superior in eliminating the melanin contamination from RNA. Since then, several laboratories have validated this and we now present an optimized method for routine use by the melanoma research community.

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Materials and methods

Isolation of RNA

Total RNA was extracted either from melanocytes, pigmented melanomas or tumour tissues derived from melanoma patients using TRI Reagent (Molecular Research Center Inc., Cincinnati, Ohio, USA). The cell pellets from the pigmented melanoma cell line WM75[9] were directly homogenized in TRI Reagent, whereas the tumour tissues were first cleared of debris, frozen in liquid nitrogen, ground to a powder and then subjected to TRI Reagent treatment according to the manufacturer's protocol. The tumour tissues were derived from vertical growth phase (VGP) and metastatic melanoma lesions. The precipitated RNAs were usually black to brown in colour.

Removal of melanin pigments

All precautions were taken that are routinely employed for the handling of RNA. Solutions were autoclaved after diethyl pyrocarbonate (DEPC) treatment. A 50% (w/v) mixture of Bio-Gel P-60 (Bio-Rad Laboratories, Hercules, California, USA) (exclusion limit 60,000 Da, wet mesh size 100–200 μM) in sterile 10 mM sodium acetate (pH 4.2) was prepared in DEPC-treated water. A prior repeated washing of Bio-Gel P-60 in DEPC-treated water is highly recommended. The protocol described is suitable for small-scale batch purification; however, it can be adapted for appropriate microfuge sterile filters or minicolumns. First, 300 μl of 50% Bio-Gel P-60 were placed in a micro-centrifuge tube, either in a 0.5 ml polymerase chain reaction (PCR) tube with a tiny hole in the bottom placed inside a 1.5 ml micro-centrifuge tube, or, for a batch protocol, directly into a 1.5 ml micro-centrifuge tube. The tubes were centrifuged at 1000 r.p.m. for 1 min. The supernatant was discarded and this step was repeated again. Total RNA extracted from 5×10^6 to 1×10^7 cells or equivalent tissues of melanocytic origin in 10 mM sodium acetate (pH 4.2) in a volume of 50 μl was placed on top of the Bio-Gel P-60 and allowed to enter the beads. The tubes were incubated in ice for 10 min, centrifuged at 1000 r.p.m. for 1 min, and the supernatant collected. The beads were mixed with an additional 100 μl of 10 mM sodium acetate (pH 4.2), incubated in ice for 10 min, and supernatant was collected by centrifugation as above and pooled. The melanin pigments attached to the Bio-Gel P-60 beads were clearly visible when compared with the supernatant. The supernatant can be subjected to Bio-Gel P-60 for further purification or concentrated by precipitation for further use.

cDNA synthesis

About 3–5 μg of total RNA was used for cDNA synthesis. All solutions used were RNase-free, having been previously treated with DEPC. The cDNA synthesis was performed as suggested by the manufacturer (Clontech Laboratories, Inc., Palo Alto, California, USA) for array analysis using gene specific primers. For oligo-deoxythymidine (dT)-directed cDNA synthesis, the reaction buffer contained 50 mM Tris-HCl, pH 8.2, 70 mM KCl and 5 mM $MgCl_2$, 1 mM each of the deoxyribonucleotide triphosphates (dNTPs), 5 mM dithiothreitol (DTT), 1 μg of oligo-(dT) and 50 U of Moloney murine leukaemia virus (MMLV) reverse transcriptase. The reverse transcription (RT) reaction was performed at 42°C, and was terminated after 45 min with 10 mM ethylene diamine tetra-acetic acid (EDTA) solution. Radioactively labelled dATP was included wherever necessary. The rate of incorporation was measured, compared with that of controls, and analysed by agarose gel electrophoresis for size distribution. For the RT-PCR reactions, first strand cDNA was synthesized as described above and PCR was performed as described by the manufacturer (Stratagene, Austin, Texas, USA) using specific primers for β-actin.

Northern analysis

Pigmented and non-pigmented RNA were separated on a formaldehyde–agarose gel, transferred to nylon membrane and hybridized to radioactively labelled probes as described elsewhere.[10] Radioactive labelling with [α-^{32}P]dATP (Amersham, Piscataway, New Jersey, USA) of microphthalmia-associated transcription factor (MITF) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes was performed using a random labelling kit from Roche Pharmaceuticals, Indianapolis, Indiana, USA.

Results and discussion

A strategy for the removal of melanin contamination in RNA preparations was devised. This was deemed necessary due to frequent failures of experiments involving cDNA synthesis, PCR, formaldehyde–agarose gel electrophoresis, and even simple spec-

trophotometric quantification when RNA preparations were contaminated with melanin. A search for a solid matrix led to the use of Bio-Gel P-60 as an ideal medium to remove contaminating melanins in RNA. Fig. 1 shows the total RNA isolated from the highly pigmented melanoma cell line WM75 before and after Bio-Gel P-60 purification. The melanin pigments retained by the Bio-Gel P-60 beads (tube B) and RNA eluted from the beads (tube A) are shown. There is a loss of approximately 25–30% of the RNA during the preparation, including the precipitation step.

The samples were then separated on a formaldehyde–agarose gel and hybridized to examine low abundance (MITF) and high abundance (GAPDH) messages from the RNA, with or without melanin contamination. Fig. 2a shows the separation of 18S and 28S rRNA on a formaldehyde–agarose gel after loading equal amounts. These samples were either before (–) or after (+) Bio-Gel P-60 treatment. Northern analysis of RNA hybridized to a MITF probe (low abundance message) and a GAPDH probe (high abundance message) are shown in Fig. 2b. The normalized autoradiographic scan of these two messages in RNA with or without melanin did not reveal significant differences.

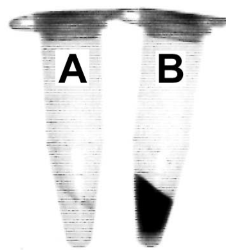

Figure 1. Removal of melanin pigments by Bio-Gel P-60. The tubes contain RNA after extraction with Bio-Gel P-60 (**A**) and the melanins retained by the Bio-Gel P-60 (**B**).

We next examined the presence of the two messages in poly(A)$^+$ RNA extracted from melanoma tissue samples after depigmentation with Bio-Gel P-60. Both tissues were heavily pigmented as measured by Fontana staining. There was no significant loss of the two messages after removal of pigments (data not shown). We next performed RT-PCR for the housekeeping gene β-actin on total RNA from the cell line WM75 before and after Bio-Gel P-60 treatment (Fig. 2c). The positive control using primers for β-actin cDNA showed a strong band (lane 1) when compared with a negative control (lane 2). The sample contaminated with melanin (lane 3) showed inefficient amplification of the product compared with the sample devoid of melanin (lane 4).

From the poly(A)$^+$ RNA extracted from VGP and metastatic melanoma tissue samples after pigment removal with Bio-Gel P-60, ^{32}P-labelled cDNA was synthesized using reverse transcriptase with gene-specific primers (588 genes) according to the manufacturer's instructions (Clontech Laboratories, Inc.), and separated on a 4% polyacrylamide gel. The autoradiogram was scanned and the mean average length of cDNA synthesized was calculated as 1.1 kb from two different experiments (Fig. 3), indicating successful labelling of RNA for microarray analysis. We have also successfully performed oligo(dT)-mediated cDNA synthesis reactions and successfully used the products for Affymetrix (Santa Clara, California, USA) arrays (data not shown).

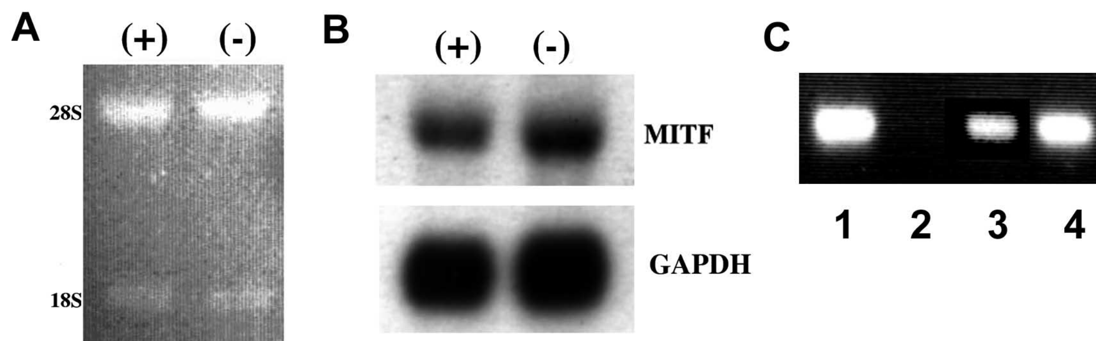

Figure 2. Properties of RNA before and after the removal of melanins. **A** Total RNA from the pigmented melanoma cell line WM75 was separated on a formaldehyde–agarose gel. The positions of 18S and 28S RNA after (+) and before (–) removal of melanins are shown. **B** Northern analysis of total RNA from the WM75 melanoma cell line after (+) and before (–) removal of melanins, using MITF and GAPDH cDNA for hybridization. **C** RT-PCR of WM75 RNA with β-actin primers. Lane 1, positive control; lane 2, negative control (no reverse transcriptase); lane 3, RNA extract prior to removal of melanins; lane 4, RNA extract after removal of melanins.

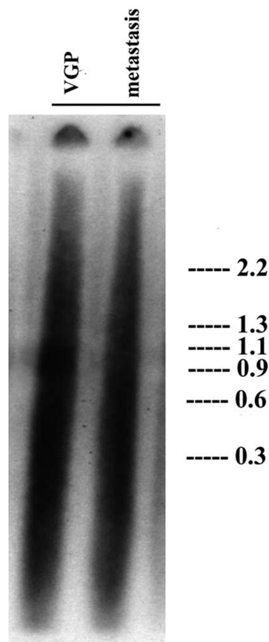

Figure 3. Separation of labelled RNA from VGP and metastatic melanoma tumour tissues after subjecting to Bio-Gel P-60 treatment to remove melanin pigments. The cDNAs were synthesized using primer sets for known genes (Clontech) in the presence of radioactive dATP, separated on agarose gel and autoradiographed. Measurements are in kb (kilobasepair).

In conclusion, a simple method is described here to eliminate contaminating melanins in RNA preparations from cells/tissues of melanocytic origin. The resulting RNA was found to be amenable to molecular manipulations.

Acknowledgements

K. Satyamoorthy is grateful for the start-up funding provided by MAHE, Manipal, India. This work was supported by grants CA-25874, CA-47159 and CA-80999 from the National Institutes of Health.

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(Received 22 November 2001; accepted 6 February 2002)