RNA Isolation from Cartilage Using Density Gradient Centrifugation in Cesium Trifluoroacetate: An RNA Preparation Technique Effective in the Presence of High Proteoglycan Content

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An efficient method for the isolation of RNA from cartilage is described. The difficulties in obtaining RNA from cartilage, a tissue of low cell density and high proteoglycan content, were overcome by making several modifications to the guanidine thiocyanate/cesium chloride method of RNA extraction. Cartilage tissue is frozen, crushed, and homogenized in a 4 M guanidine thiocyanate lysis buffer. The RNA is then pelleted by ultracentrifugation through a cesium trifluoroacetate density gradient. The use of cesium trifluoroacetate, rather than cesium chloride, for density gradient centrifugation improves both the yield and purity of total RNA isolated from cartilage. The ultracentrifugation has been adapted to the Beckman TL100 tabletop centrifuge and is complete in 3 h. This fast, simple method produces high quality RNA, suitable for use in RNase protection assays, polymerase chain reaction analysis, and Northern analysis. This purification procedure may be applicable to other sources, from which RNA isolation is complicated by the presence of abundant cell wall or matrix components.

MATERIALS AND METHODS

Materials

Guanidine thiocyanate was obtained from Boehringer-Mannheim. Cesium trifluoroacetate (CsTFA) was purchased from Pharmacia, as a solution at a density of 2 g/ml. Polysanomer centrifuge tubes (11 x 34 mm) were obtained from Beckman. Polysciences was the supplier of 1,9-dimethylmethylene blue.

Abbreviations used: CsTFA, cesium trifluoroacetate; DEPC, diethyl pyrocarbonate; Mops, 3-(N-Morpholino)propanesulfonic acid; SDS, sodium dodecyl sulfate; SSC, standard sodium citrate; ELISA, enzyme-linked immunosorbent assay; TFA, trifluoroacetate; PCR, polymerase chain reaction; SSPE, standard sodium phosphate.
Preparation of Cartilage Tissue

Articular cartilage was isolated from third trimester bovine fetuses, which had been obtained from a local slaughterhouse and immediately chilled on ice. Fetal hooves were kept chilled as cartilage was cut from the articular surface of the metacarpal phalangeal joint. The cartilage was weighed, frozen in liquid nitrogen, and stored at -70°C.

RNA Isolation

Frozen cartilage samples, each approximately 1 g, were crushed using a specialized mortar and pestle device (5). The device, consisting of a steel cylinder in a steel tube, was prechilled by placing on dry ice. The frozen sample was placed within and pulverized.

The frozen, powdered cartilage was homogenized in 4 ml of lysis buffer consisting of 4 M guanidine thiocyanate, 0.1 M Tris-HCl (pH 7.5), and 1% 2-mercaptoethanol (3). Tissue was homogenized using a Virtis ultramicrofine generator, at high speed for 1 to 2 min. Following homogenization, sodium lauryl sarcosine was added to 0.5% and the sample centrifuged for 15 min at 5000 rpm (3000g) in a Sorvall SS34 rotor, in order to remove insoluble material.

The supernatant was retained and layered over a 1.0 ml cushion of 1.60 g/ml CsTFA, 1 mM EDTA (pH 8.0) in Beckman polyclarimeter centrifuge tubes (11 × 34 mm). Samples were centrifuged 3 h at 55,000 rpm (201,247g), 18°C, in the Beckman TL100 tabletop ultracentrifuge using the TLS55 rotor (6). The capacity of each centrifuge run is limited to four tubes, or 4 ml of homogenate, and is ideal for small samples. This method, however, can be adapted for larger samples using conventional ultracentrifuges, though run lengths will increase. Conditions for conventional ultracentrifuges are supplied by Pharmacia, with the CsTFA solution. The temperature during ultracentrifugation was kept above 15°C to avoid any possible crystallization of the CsTFA solution.

Following ultracentrifugation, the RNA was recovered by first aspirating the supernatant, leaving approximately 0.25 ml of the CsTFA cushion. The upper part of the tube was cut off with a centrifuge tube slicer (Beckman) to avoid contamination by any proteins adhering to the wall of the tube. The remainder of the CsTFA cushion was decanted and the RNA pellet at the bottom of the tube was dissolved in 100 to 200 µl of DEPC-treated water (7). The RNA sample was transferred to an Eppendorf tube and precipitated by the addition of ammonium acetate to 0.25 M and 2 vol of ethanol. Samples were chilled at -20°C for 15 min and the RNA pelleted by centrifuging at 10,000 rpm for 15 min in a microfuge at 4°C. Pellets were rinsed with 70% ethanol, dried, dissolved in DEPC-treated water, and stored at -70°C. RNA was quantified by absorbance at 260 nm.

Agarose Gel Electrophoresis and Northern Blot Analysis

RNA was electrophoresed on a 1.5% formaldehyde–agarose gel using a running buffer of 20 mM Mops, pH 7.0, 5 mM sodium acetate, 1 mM EDTA (8). The gel was stained with 5 µg/ml ethidium bromide and photographed under uv illumination. For Northern blot analysis, RNA was transferred to nitrocellulose, baked for 2 h at 80°C under vacuum, and the filters were prehybridized for 2 h at 42°C in 5 × SSPE buffer (9), 2 × Denhardt’s solution (9), 0.1% SDS, 5 µg/ml poly (A), 100 µg/ml yeast tRNA, 200 µg/ml sheared, denatured hammer sperm DNA, and 50% formamide. Filters were hybridized for 16 h at 42°C with 32P random-primed labeled probe at 106 cpm/ml. Probes included the clone for the human α1(I) procollagen gene, pCOL2A1 (10), the human γ actin cDNA clone pHF1 (11), the human biglycan cDNA clone P6 (12), and the human α1(I) procollagen gene, HF677 (13). Filters were washed at a final stringency of 0.1% SDS, 0.1 × SSC buffer (9), at 65°C for the collagen and biglycan hybridizations and at 45°C for the actin hybridization. Northern blots were exposed to Kodak XAR-2 film for 2 to 24 h at -70°C in the presence of an intensifying screen.

Proteoglycan Analysis

Proteoglycan content of the samples was determined using the dimethylmethylene blue assay (14), adapted to the ELISA format. A solution of the dye reagent was prepared as described by Farndale et al. (14). Aliquots of 25 µl of each sample were mixed with 250 µl of dye reagent and the absorbance at 595 nm determined. Shark chondroitin sulfate, obtained from Sigma, was used to generate a standard curve.

RESULTS AND DISCUSSION

We have developed an efficient method to isolate RNA from cartilage and from other tissues with a high proteoglycan content in general. The procedure is outlined in Fig. 1. Frozen articular cartilage samples were crushed in a chilled steel mortar and pestle and rapidly homogenized in guanidine thiocyanate lysis buffer, as described under Materials and Methods. This section of the procedure utilizes the standard protocol recommended by MacDonald et al. (3). Immediate, high speed homogenization is required to ensure a good yield of intact RNA.

Physical separation of the RNA from other macromolecular components of the homogenate was achieved by selective sedimentation through CsTFA. We have substituted CsTFA for the CsCl cushion recommended in the MacDonald method (3). The salting-in effect of the TFA anion enables the RNA pellet to dissolve easily. This eliminates the need for extensive vortexing and heating typically required to dissolve RNA pelleted
Crush in mortar + pestle
Homogenize in 4M guanidine thiocyanate, 100 mM Tris HCl pH 7.5, 1% 2-mercaptoethanol

**4M guanidine thiocyanate homogenate**

Add sarcosyl
Centrifuge 5,000 rpm, 15 min
Layer over CsTFA

**CsTFA gradient**

Centrifuge 3 hours, 55,000 rpm
Dissolve RNA pellet in water
Ethanol precipitate
Dissolve in DEPC water
Quantitate and store at -70 °C

**RNA**

FIG. 1. Protocol for isolation of RNA.

Through CsCl. As an additional modification, we utilized a Beckman TL100 tabletop ultracentrifuge for this step, enabling completion of centrifugation in 3 h. The method can be used with conventional ultracentrifuges, though run lengths will increase.

After ultracentrifugation, the CsTFA was decanted and the RNA pellet dissolved in DEPC-treated water. Samples were ethanol-precipitated and centrifuged, and the RNA was redissolved in DEPC-treated water. Yields and purity of RNA were determined by absorbance at 260 nm. The 260/280 ratio of RNA preparations was typically in the range 1.9 to 2.0.

We obtained the highest yield of RNA from cartilage using this method. In comparison, RNA isolated by other standard isolation techniques gave unsatisfactory results (Table 1). Isolation by the guanidine thiocyanate/CsCl method of MacDonald et al. (3) gave lower yields and the RNA pellet was difficult to dissolve. The method of Adams et al. (2), selective precipitation of RNA from guanidine hydrochloride, was tried but yielded no detectable RNA. This method is not generally recommended for tissues of low cell density and high extracellular matrix content, such as cartilage. The single step isolation procedure of Chomczynski and Sacchi (1) gave low yields and the RNA was highly contaminated with proteoglycans. When phenol:chloroform (1:1) extractions were performed to eliminate proteoglycans, there was a large precipitate at the interface. RNA was trapped in the interface, the yield was further reduced, and the proteoglycan contamination persisted even after phenol extraction. Similarly, precipitation of the RNA by high salt, as recommended in the modified Chomczynski and Sacchi method (15), also drastically reduced yield and still resulted in proteoglycan contamination.

In addition, we tested the method developed by Nemeth et al. (4) for RNA isolation from skeletal tissue. This method recommends omitting the reducing agent from the guanidine hydrochloride lysis buffer. The homogenate is then centrifuged through a CsCl density gradient. In our experiments, RNA obtained from articular cartilage using this method was often degraded. Repeated applications of the technique, using fresh reagents each time, yielded variable results.

Thus, the guanidine thiocyanate/CsTFA method gave the highest yield of intact RNA. However, some proteoglycan contamination of the RNA did persist after ultracentrifugation through CsTFA. As discussed above, we have observed proteoglycans copurifying with cartilage RNA through other standard RNA isolation methods, including the one step acid phenol method (1), as well as sedimentation through cesium salts.

In order to decrease proteoglycan contamination of the RNA, we increased the density of the CsTFA cushion above the 1.50 g/ml recommended by the supplier. This was done in order to determine conditions under which RNA would pellet while the majority of proteoglycans would remain suspended in the gradient. RNA sediments at a slightly higher density than proteoglycans in CsTFA, according to the observations of McBain and Mueller (16). In isolating proteoglycans from cultured colon cells, they observed the peak of proteoglycans to sediment at a density of 1.65 g/ml while RNA sediments at a density of 1.75–1.80 g/ml. Therefore, we used increasing densities, from the recommended 1.50 g/ml CsTFA up to 1.75 g/ml, to isolate RNA.

As the CsTFA density was raised, the purity of the RNA preparations increased (Fig. 2). Proteoglycan content of the RNA samples was determined using a dye binding assay specific for proteoglycans (14). The best yields of RNA, with the lowest proteoglycan contamination, were obtained with CsTFA densities in the range of 1.50 to 1.60 g/ml. As the CsTFA density was increased further, the yield of RNA decreased. At a density of 1.75 g/ml the RNA no longer pelleted. Therefore, a CsTFA density of 1.60 g/ml was routinely used to give the highest yields of RNA while enhancing the exclusion of proteoglycans.

Cartilage RNA isolated by this method was intact, with no evidence of degradation. Samples were electrophoresed on a formaldehyde–agarose gel and stained with ethidium bromide (Fig. 3). The intact ribosomal 28S and 18S RNA are clearly visible (Fig. 3, lane A). In addition, messenger RNA transcripts were intact, as demonstrated by Northern analysis (Fig. 4). Utilizing probes for actin and for the core protein of the small leucine-rich proteoglycan, biglycan, transcripts of expected sizes were detected as sharp bands (Fig. 4, lanes A, B). Cartilage-specific transcripts were also intact, as demonstrated by the hybridization of the α1(II) procollagen probe to mRNA in the expected size range (Fig. 4, lane C). An additional, higher molecular weight band was detected by the α1(II) procollagen probe and has been seen previously by other investigators (17). Bovine
TABLE 1
Comparison of RNA Isolation Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Typical yield of RNA/gram cartilage (µg)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Guanidine thiocyanate lysis, CsTFA density gradient</td>
<td>136</td>
<td>RNA easily dissolved</td>
</tr>
<tr>
<td>2. MacDonald method (3): guanidine thiocyanate lysis, CsCl density gradient</td>
<td>10</td>
<td>RNA difficult to dissolve</td>
</tr>
<tr>
<td>3. Adams method (2): guanidine HCl lysis, selective RNA precipitation</td>
<td>Not detectable</td>
<td>Poor recovery, RNA too dilute</td>
</tr>
<tr>
<td>4. Chomczynski method (1): guanidine thiocyanate lysis, acid phenol extraction</td>
<td>14</td>
<td>High proteoglycan content, pellet insoluble</td>
</tr>
<tr>
<td>5. Nemeth method (4): guanidine HCl lysis-reducing agent, CsCl density gradient</td>
<td>33</td>
<td>RNA quality inconsistent, often degraded</td>
</tr>
</tbody>
</table>

Note. Articular cartilage was used for all preparations. The protocol for method 1 is described under Materials and Methods. References for the remainder of the methods are indicated in the table. Values are averages of three experiments, with the exceptions of methods 3 and 4 which gave unsatisfactory results and were not repeated. RNA yields were determined by absorbance at 260 nm.

tendon fibroblast RNA was included in the collagen type II blot (Fig. 4, lane D) as a negative control for cross-hybridization to collagen type I transcripts. The integrity of the fibroblast RNA, which was prepared from cultured cells by the guanidine thiocyanate/CsTFA method, was demonstrated by hybridization to α1(I) procollagen transcripts (Fig. 4, lane E).

In using this method on cartilage, we have noted a requirement for the presence of strong denaturing agents to protect the RNA from degradation. We obtained variable results when using the RNA isolation method described by Nemeth (4), which recommends the elimination of reducing agent from lysis buffer in order to increase RNA yields from bone and cartilage. Repeated applications of this method, using a lysis buffer composed of guanidine hydrochloride and lacking 2-mercaptoethanol, resulted in RNA preparations that were degraded (Fig. 3, lane B). The extent of degradation appeared to depend on the source of cartilage, growth plate cartilage RNA being even more susceptible to degradation than RNA from articular surfaces (data not shown). To ensure the integrity of cartilage RNA preparations, we find a lysis buffer of guanidine thiocyanate to be preferable to guanidine hydrochloride and the presence of a reducing agent is required.

CONCLUSIONS

We have described a method for the isolation of RNA from cartilage, a tissue with high proteoglycan content. This procedure is rapid and requires minimal manipula-
FIG. 4. Northern analysis confirms the presence of intact mRNA transcripts in cartilage RNA isolated by guanidine thiocyanate/ CsTFA. A 1.5% formaldehyde-agarose gel was electrophoresed with 5 μg total RNA from bovine articlar cartilage (A, B, and C) and cultured bovine tendon fibroblasts (C and D). RNA was blotted to nitrocellulose and the filter cut into sections. Each lane was hybridized with the following radiolabeled probes: (A) γ actin cDNA, (B) biglycan cDNA, (C and D) probe for the α(II) procollagen gene, (E) the blot of lane D was washed and rehybridized with probe for the α(III) procollagen gene. The migration of the 28S and 18S ribosomal RNA is indicated by large arrows. The size of the mRNA transcripts are shown, in kb, to the right of each lane.

In comparison to other standard RNA isolation techniques, this method produces the highest yield and purity of total RNA isolated from cartilage. The RNA isolated by this technique is intact and has proven suitable for analysis of gene expression using the methods of Northern blotting, PCR analysis, and RNase protection. The capacity to obtain good total RNA yields from tissue with high proteoglycan content might make this method applicable to other sources of RNA, sources from which RNA extractions are typically difficult.

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REFERENCES