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Step-wise Isolation of RNA and DNA from Mycobacteria[1]

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Advances in recombinant deoxyribonucleic acid (DNA) technology have stimulated interest in genetic studies of mycobacteria. Such studies demand the availability of high molecular weight DNA suitable for restriction by endonucleases and the isolation of ribonucleic acid (RNA) from which suitable probes may be prepared. The isolation of poly(A)+-RNA, if it is present in mycobacteria, would offer a facile route for gene cloning. Methods leading to the isolation of DNA and RNA of suitable quality from minimal quantities of cells would be particularly advantageous if they were generally applicable to mycobacteria which could not be cultivated *in vitro* or which are difficult to grow.

The strategy used in the present study was first to lyse cells in concentrated guanidinium salts (chloride or isothiocyanate) in order to inactivate the nucleases (both RNases and DNases) and to dissociate nucleoproteins ([6, 8]). Poly(A)+-RNA, non-polyadenylated RNA (mainly ribosomal RNA, rRNA) and DNA were then isolated in successive steps from the same cell lysate. This procedure was applied to two mycobacterial species, namely, *Mycobacterium phlei* and *M. smegmatis*. The same procedure was also applied to *Bacillus subtilis* and to BHK (baby hamster kidney) cells in order to provide a form of reference for the isolation of poly(A)+-RNA from mycobacteria.

MATERIALS AND METHODS

Growth of the organisms

M. smegmatis (NCTC 10265) and *M. phlei* (NCTC 8151) were grown in nutrient broth (Difco) containing 0.05% Tween 80. Actively growing cells (late log phase) were collected by centrifugation in a refrigerated centrifuge at 17,000 × *g* × 10 min. *B. subtilis* (strain BR 151) was grown in Nutrient Broth, and cells were harvested as described above. The mycobacteria and *B. subtilis* cells were labeled with [2,8 ^{3}H]-adenosine (100 μCi). The radiotracer was added to the medium 10 min before harvesting the *B. subtilis* ([11, 22]) and 24 hr before harvesting the mycobacteria. BHK cells were grown in tissue culture (Dulbecco's H21 medium), and labeled by the addition of [^{32}P]-PO_4 (1 mCi) to the medium and incubation for a further 7 hr.

Isolation of nucleic acids

Step 1—Cell lysis. The bacterial cells were suspended in (20 ml/g mycobacteria) lysis buffer consisting of 6 M guanidine hydrochloride (Sigma), 15 mM EDTA (BDH), and 1 mM β-mercaptoethanol (BDH) cooled to −15°C. Detergent, e.g., sarcosyl (0.5% w/v final concentration) or Tween 80 (0.1% v/v final concentration), was added to obtain the good suspension of mycobacteria required for effective lysis. The cells were passed once through a French Pressure Cell (American Instrument Co.) previously cooled to −15°C at 850 kg/cm^2. The capacity of the cell was approximately 0.75 ml (minimum) to approximately 3 ml (maximum). The lysate was centrifuged at 17,000 × *g* × 10 min at 0–4°C, and the supernatants were collected for Step 2. The BHK cells were lysed by the addition of lysis buffer cooled to −15°C to the monolayer of cells previously washed with Dulbecco's PBS medium. The radioactivity of the supernatants was measured in a Beckman counter (Model 7500).

Step 2—Purification of polyadenylated RNA. The debris-free lysates prepared in Step 1 were kept at 0°C in plastic (Falcon) tubes and poly(U)-Sepharose 4B ([15]) was added ([8]). The mixture was gently agitated overnight at 4°C (Stuart Tube Rotator TR-2). The poly(U)-Sepharose 4B was recovered by centrifugation at 3000 rpm for 10 min (1 MSE Centaur 2 centrifuge). Su-

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pernatants were saved for Step 3 and the poly(U)-Sepharose 4B was poured into a column (Amicon Wright), washed extensively (3 × 10 ml) with lysis buffer, and then with high salt buffer (3 × 5 ml of 0.5 M NaCl, 10 mM EDTA pH 7.5, 50% [v/v] formamide, and 50 mM Tris pH 7.5). Poly(A)+-RNA was recovered by washing the column with eluting buffer (90% v/v formamide, 1 mM EDTA, and 15 mM Tris HCl pH 7.5). The radioactivity of samples of the eluate was counted in a Beckman counter (Model 7500). It should be noted here that it is advisable to use plastic ware in handling poly(U)-Sepharose 4B because the gel sticks to glass. Silicone treatment of the glass reduces but does not eliminate the problem.

Step 3—Purification of ribosomal RNA. Ethanol (0.5–0.75 vol) was added to supernatants recovered after poly(U)-Sepharose 4B treatment in Step 2 ([6, 7]). The ethanol was added dropwise with stirring. The mixture was kept at −20°C overnight when 0.5 vols of ethanol were added, or for 2–4 hr when 0.75 vols of ethanol were used. RNA was sedimented by centrifugation at 4000 × *g* × 10 min. The supernatants were saved for DNA purification in Step 4. The sediment was dissolved in lysis buffer and again precipated with 0.75 vols of ethanol at −15°C for 2 hr. The sediment was finally dissolved in TE buffer (10 mM Tris, 2 mM EDTA, pH 7.5) by briefly warming (e.g., to 37°C) and stored at −20°C.

Step 4—Purification of DNA. Equal volumes of chloroform–isoamyl alcohol (24:1) were added to supernatants recovered after Step 3, and the phases were separated by centrifugation ([17]). The procedure was repeated until a clear interphase was obtained. To the aqueous phase, 5 M NaCl was added to a final concentration of 0.3 M. DNA was precipitated with 0.75 vol of isopropanol ([21]) and was separated by centrifugation at 4000 × *g* × 10 min. The pellet was washed with 70% ethanol and dried. DNA was dissolved in TE buffer and stored at −20°C.

Spectrophotometry

Spectrophotometric measurements for DNA and RNA were carried out at 280 nm, 260 nm, and 230 nm in a double-beam UV spectrophotometer (Varian model 2200).

THE TABLE. *Measurements of nucleic acids isolated from* M. smegmatis *and* M. phlei.

		M. smegmatis	*M. phlei*
DNA	OD[a] ratio 260:280 nm	1.95	1.98
	OD ratio 260:230 nm	2.24	2.18
	Yield/g of wet weight of cells	1.1 mg	1.6 mg
Non-polyadenylated RNA	OD ratio 260:280 nm	2.15	2.24
	OD ratio 260:230 nm	2.35	2.30
	Yield/g of wet weight of cells	3.2 mg	6.0 mg

[a] OD = optical density.

The concentrations of DNA and RNA were calculated from the absorbance at 260 nm, and the levels of purification were estimated by their relative absorbance ratios.

Restriction of DNA and transfer to nitrocellulose membranes

Purified DNA was restricted with enzyme Bam HI and electrophoresed on 1% agarose gels by the method described by Maniatis, *et al.* ([16]). DNA isolated by the procedure of Imaeda, *et al.* which we used previously for restriction analysis ([7]) was used for comparison. The transfer to nitrocellulose filters was done by the method of Southern ([23]).

Labeling of RNA

RNA purified in Step 3 was radiolabeled with ^{125}I by the methods earlier described ([5, 9]). The radiolabeled RNA samples were counted in an Autogamma counter (Packard Autogamma Scintillation Spectrometer). The specific activity was 2–3 × 10^7 cpm/μg of RNA. This was used as a probe.

Hybridization

Prehybridization and then hybridization of radioactively labeled RNA to restricted DNA was done in a solution consisting of 5 × SSC (1 × SSC = 0.15 M NaCl plus 0.015 M sodium citrate). The nitrocellulose filters were soaked in 2 × SSC (+ 0.1% w/v sodium dodecyl sulfate) and incubated in

the above solution at 42°C for 2–4 hr. Ten μg/ml of transfer RNA (tRNA) was included to reduce nonspecific binding of ^{125}I-labeled RNA to the nitrocellulose filter. Then the ^{125}I-labeled RNA was added (0.5×10^5 cpm/ml), and incubation was carried out for 16 hr at 42°C. The filters were then washed with 4 × SSC (+ 0.1% w/v SDS) followed by 0.1 SSC (+ 0.1% SDS) for 60 min at 50°C. The filters were air dried, and autoradiographs were set up and developed by the method described earlier ([9]).

RESULTS

The results are presented in The Table and in Figures 1 and 2. The principal results are:

Although the incorporation of [2,8 ^{3}H]-adenosine into mycobacterial cells was low compared with incorporation into *B. subtilis,* significant radioactivity was eluted from poly(U)-Sepharose 4B in the same position as authentic poly(A)+-RNA from BHK cells and from *B. subtilis.*

The profiles obtained for the elution of poly(A)+-RNA from poly(U)-Sepharose 4B are compared in Figure 1. [2,8 ^{3}H]-Adenosine-labeled RNA from *M. phlei* and *M. smegmatis* was found to elute from the columns after treatment with approximately the same volume of eluting buffer as that needed to elute poly(A)+-RNA from BHK cells and from *B. subtilis.* The similarity in the elution profile for all four of the species studied indicates that poly(A)+-RNA was present in each of them.

The yield of poly(A)+-RNA from a 100 ml culture of exponentially growing *B. subtilis* was a total of 340 μg. Part of the poly(A)+-RNA fraction (230 μg, 5.1% of total RNA), the fraction with the shortest poly(A) tails, was eluted from poly(U)-Sepharose 4B with high salt buffer and the remaining 110 μg (2.4% of total RNA) was obtained by treatment with elution buffer. This yield of poly(A)+-RNA compares favorably with that obtained by other methods ([11]). The yield of poly(A)+-RNA isolated from *M. phlei* and *M. smegmatis* was approximately 0.8% of total RNA (measured by spectrophotometry) in each case. The incorporation of [2,8 ^{3}H]-adenosine into mycobacterial RNA was low (0.29×10^6 cpm for *M. phlei* and 0.15×10^6 for *M. smegmatis*) compared with the incorporation of the radiotracer in *B. subtilis* RNA (16×10^6 cpm).

Non-polyadenylated RNA (mainly rRNA) purified by the step-wise procedure was found to be comprised of two principal components on electrophoresis in formamide denaturing gels corresponding to 16S and 23S RNA. The preparation was readily iodinated yielding ^{125}I-labeled rRNA probes ($4–6 \times 10^6$ cpm/μg) suitable for hybridization studies (Fig. 2).

The purified DNA was found to have a sufficiently high proportion of DNA (40% or more) which entered a 1% agarose gel to about the same extent as phage-λ DNA. Treatment of the mycobacterial DNA preparations with restriction endonucleases led to the production of a range of products which could be resolved as clear bands on 1% agarose gels. After its restriction, DNA was transferred to nitrocellulose film and probed with ^{125}I-labeled rRNA. Distinct bands were detected by autoradiography corresponding to fragments of DNA containing sequences coding for rRNA. This demonstrates that the DNA isolated by the procedures described above is suitable for use in recombinant DNA procedures.

The yield of DNA was 1.6 mg DNA/g (wet weight) of *M. phlei* and 1.1 mg DNA/g (wet weight) of *M. smegmatis.* These values are comparable with the yields of DNA obtained from mycobacteria by Baess ([1]) (1 mg DNA/g cells [wet weight]), Clark-Curtiss, *et al.* ([4]) (1–2 mg DNA/1×10^{12} cells [roughly equivalent to 1 g, wet weight, of cells]), Mizuguchi and Tokunga ([18]) (1.34 mg DNA/g cells [wet weight]), Patel, *et al.* ([20]) (0.3–2.0 mg DNA/g cells [wet weight]), and Wayne and Gross ([26]) (0.16–2.1 mg DNA [average 0.67 mg DNA] /g cells [wet weight]).

The procedure described above was based on 50 mg cells (wet weight)/ml which is approximately 5×10^{10} cells/ml, assuming that 1 g wet weight is equivalent to approximately 1×10^{12} cells. The French Pressure Cell requires a minimum of approximately 0.75 ml of cell suspension so that a minimum of approximately 3.7×10^{10} cells can be treated exactly as described above, except that the amount of poly(U)-Sepharose 4B may be reduced tenfold.

The cell concentration of 50 mg cells (wet

weight)/ml is presented as a guide. A less-concentrated cell suspension may be used within the limits that rRNA can be precipitated from the lysate with ethanol and that DNA can be precipitated from the chloroform-treated lysate by isopropanol. Both rRNA and DNA were successfully isolated from cell suspensions (10 ml) of 1–5 mg cells (wet weight)/ml lysis buffer (Dr. I. Lamb, unpublished work).

DISCUSSION

The step-wise procedure described above provides a method for the isolation of poly(A)+-RNA, non-adenylated RNA, and high molecular weight DNA from the same mycobacterial lysate. This economy in the use of lysates could be of advantage in the study of mycobacteria such as *M. leprae,* which are available in very limited quantities.

The critical step in the isolation of nucleic acids described above is the requirement for the cells to be lysed by chemical or mechanical methods in the presence of a 4 M solution of a guanidinium salt (e.g., 4 M guanidinium chloride at −15°C). Provided that this condition is met poly(A)+-mRNA, non-polyadenylated RNA (mainly rRNA), and DNA may be isolated from the lysate. Our step-wise procedure is based on simple manipulations. However, once lysis in guanidinium salts is achieved, RNA and DNA may be isolated by other methods, for example, by centrifuging through CsCl gradients ([10]).

A range of chemical and physical methods have been used to lyse mycobacteria. The chemical procedures ([3, 18, 26, 27]) are designed to weaken the cell wall, but they vary in their effectiveness when applied to a wide range of mycobacteria and the methods available are not suitable for nonculturable or difficult-to-grow mycobacteria. Several physical methods of cell rupture ([1, 2, 4, 13, 25, 30, 31]) have been tried and, of these, we chose to use the French Pressure Cell because it has been shown to break a wide range of mycobacteria, including *M. tuberculosis* ([1, 2]). Imaeda, *et al.* ([13]) isolated DNA from *M. leprae* and *M. lepraemurium* by lysis at 1062 kg/cm^2 pressure using the Sorvall cell fractionator which is based on the same principle as the French Pressure Cell.

Polyadenylated RNA has been shown to be present in a range of bacteria including *B. subtilis.* The poly(A)+-RNA fraction of *B. subtilis* is polyadenylated at or near the 3′ end, functions as messenger RNA

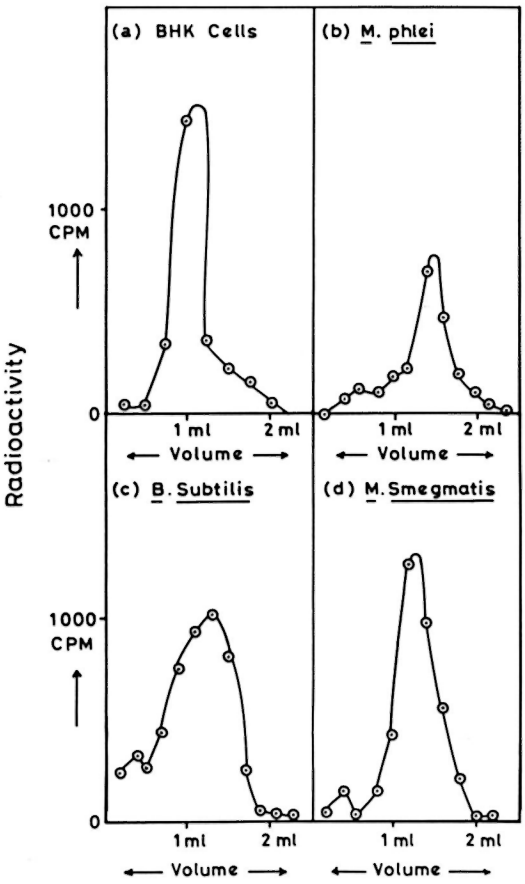

FIG. 1. Elution of poly(A)+-RNA from poly(U)-Sepharose 4B. Cells grown in the presence of a radioactive tracer were lysed at −15°C in the presence of lysis buffer. The French Pressure Cell was used to break the cells in (b)–(d). Poly(U)-Sepharose 4B (0.3–0.5 g) was added to the lysate, and poly(A)+-RNA was absorbed overnight. The gel was recovered and washed with lysis buffer to remove impurities including rRNA and with high salt buffer to remove poly(A)+-RNA with tracts of less than 20 adenine residues, then with eluting buffer to release poly(A)+-RNA with tracts of more than 20 adenine residues. Fractions of 200 μl were collected. Profiles of the radioactivity of a sample of each fraction against volume of eluting buffer are presented for (a) BHK cells labeled with ^{32}P Na phosphate, (b) *M. phlei,* (c) *B. subtilis,* and (d) *M. smegmatis.* In (b), (c), and (d) the label was [2,8 ^{3}H]-adenosine. Radioactivity measurements presented are for a sample (1 μl) of each fraction in (a), for the whole 200 μl fraction in (b) and (d), and for a sample (2 μl) of each fraction in (c).

(mRNA) in *in vitro* protein synthesizing systems, serves as a substrate for reverse transcriptase, and hybridizes with a high proportion of genomic DNA sequences ([11]). The mRNA fraction of *Escherichia coli* is also polyadenylated at the 3′ end, although no more than 3% of this fraction (0.007% of total RNA) has tails of 20 adenine residues or more ([19]).

The presence of poly(A)+-RNA in mycobacteria is indicated by the absorption of ^{3}H-adenosine labeled RNA to poly(U)-Sepharose 4B and its elution with 90% (v/v) formamide, 0.01 M Tris HCl pH 7.5, in a manner that is characteristic of authenticated poly(A)+-RNA from BHK cells and with poly(A)+-RNA from exponentially growing *B. subtilis*. Poly(A)+-RNA species (e.g., globin mRNA) isolated by this procedure have poly(A) tracts of 20–150 adenine residues ([14]), indicating that a minimum of 20 adenine residues are required for binding to immobilized poly(U) (see also [19]). We infer that tracts of at least 20 adenine residues are present in the mycobacterial RNA species retained on poly(U)-Sepharose 4B and eluted as described in Figure 1. It remains to be shown that the tracts of adenine residues present in mycobacterial poly(A)+-RNA are located at the 3′ end. *M. phlei* and *M. smegmatis* each has a level (approximately 0.8% of total RNA) of poly(A)+-RNA with tracts of at least 20 adenine residues that is lower than the level (2.4% of total RNA) found for *B. subtilis* (Fig. 1) but which is higher than the level (0.007% of total RNA) found in *E. coli* ([19]).

The rRNA preparation obtained by the step-wise method was found to be useful as ^{125}I-labeled radioactive probes as seen by hybridization to restriction DNA (Fig. 2). The iodination of RNA proceeds smoothly to high specific activities only when they are free of contaminants such as proteins, which themselves react with iodine and therefore with the reaction. The properties of rRNA isolated by this procedure compare favorably with those of rRNA isolated from other mycobacteria by other methods ([24, 28, 29]).

Previously, the use of the French Pressure Cell was thought to lead to the DNA being sheared to low molecular weight fragments ([1, 12]). The results presented above show that damage to DNA arising from the passage of cells through the French Pressure Cell is

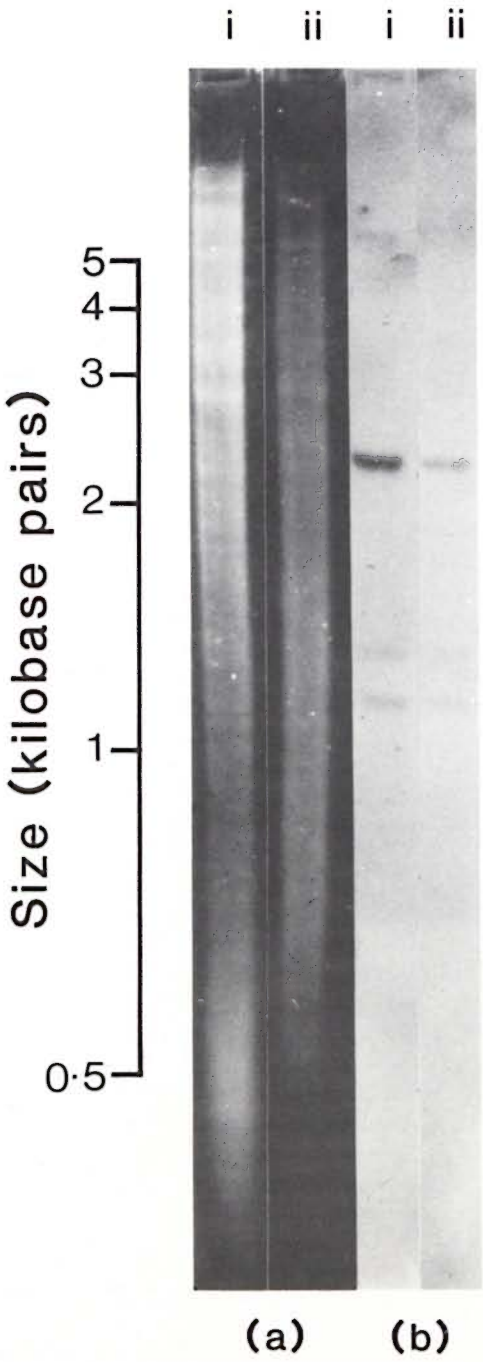

FIG. 2. Hybridization of *M. phlei* DNA restricted with Bam HI endonuclease, with ^{125}I-labeled *M. phlei* rRNA. DNA fragments were separated by electrophoresis through 1% w/v agarose gels, transferred to nitrocellulose filter, and hybridized with ^{125}I-labeled *M. phlei* RNA. (a) = ethidium-bromide-stained gel; (b) = autoradiograph; (i) = DNA isolated by phenol/chloroform treatment; (ii) = DNA isolated by the step-wise method.

limited and that further degradation can occur by nuclease action. These DNases are inhibited by phenol ([13]) and also, as we have shown, by guanidinium salts. Provided the DNases are inhibited, the French Pressure Cell can be used to isolate DNA of a size sufficient to yield unique fragments on treatment with restriction endonucleases (Fig. 2).

It is concluded that this step-wise procedure can be a useful method for the isolation and purification of poly(A)+-RNA, non-polyadenylated RNA (mainly rRNA), and DNA from mycobacteria, especially the difficult-to-grow or noncultivable species.

SUMMARY

Mycobacterium phlei and *M. smegmatis* were lysed at −15°C in the presence of guanidinium salts and poly(A)+-RNA. Nonpolyadenylated RNA (mainly rRNA) and DNA were isolated in successive steps from the same lysate. DNA isolated by this procedure was sufficient to yield distinct bands on treatment with restriction endonucleases as shown by hybridization to ^{125}I-labeled rRNA. The successive isolation of poly(A)+-RNA, nonpolyadenylated RNA, and high molecular weight DNA from the same lysate by this procedure, which is being reported for the first time, provides a very economical approach for isolation and purification of nucleic acids from mycòbacteria and other organisms. This could be of special value for various genetic recombination studies, particularly in the case of mycobacteria which are available in very limited quantities, e.g., noncultivable or difficult-to-grow mycobacteria, especially *M. leprae.*

RESUMEN

Se prepararon lisados de *Mycobacterium phlei* y de *M. smegmatis* a −15°C y en presencia de sales de guanidina y de poli(A)+-RNA. A partir del mismo lisado y en etapas sucesivas se pudieron aislar RNA poliadenilado (principalmente rRNA) y DNA. El DNA aislado fue suficiente para producir varias bandas por tratamiento con enzimas de restricción como se demostró hibridazación con rRNA marcado con I^{125}. El exitoso aislamiento de poli(A)+-RNA, RNA no poliadenilado y DNA de alto peso molecular a partir del mismo lisado, usando el procedimiento descrito aquí por vez primera, constituye una manera muy económica de aislar y purificar los ácidos nucleicos de micobacterias y de otros microorganismos. Esto podría ser de un valor muy especial en estudios de recombinación genética con micobacterias que se obtienen en cantidades limitadas, por ejemplo, las micobacterias de difícil crecimien to o aquellas no cultivables como el *M. leprae.*

RÉSUMÉ

Mycobacterium phlei et *M. smegmatis* ont été lysés à une température de −15°C en présence de sels de guanidine et de poly(A)+-ARN. De l'ARN non-polyadénylé, principalement du rARN, de même que le l'ADN, ont été isolés, par étapes successives, à partir du même lysat. La quantité d'ADN isolé par ce procédé a été suffisante pour démontrer des bandes distinctes au traitement par les endonucléases de restriction, ainsi qu'on a pu le montrer par hybridation avec du rARN marqué à 125 I. C'est la première fois que l'on rapporte l'isolement successif par cette procédure, à partir du méme lysat, de poly(A)+-ARN, d'ARN non-polyadénylé, et d'ADN de poids moléculaire élevé. Cette méthode constitue une manière très peu coûteuse d'isoler et de purifier les acides nucléiques chez les mycobactéries et chez d'autres organismes. Elle pourrait être particulièrement précieuse dans des études de recombinaison génétique, surtout lorsque les mycobactéries ne sont disponibles qu'en très petites quantités, par exemple, lorsqu'il s'agit de mycobactéries difficiles à cultiver, ou même non cultivables tel que *M. leprae.*

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Note added in proof. Our colleague Dr. F. I. Lamb has used the procedures described above to isolate both ribosomal RNA and DNA from *M. leprae.* The ribosomal RNA preparation comprised two components, one corresponding to 16S and the other to 23S RNA when analyzed by electrophoresis under denaturing conditions. The DNA preparation was a suitable substrate for restriction endonucleases.

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