Hybrid isolation by recovery of RNA-DNA hybrids from agar
using S₁ nuclease

J. Norman Hansen, Barbara H. Pfeiffer and Christopher J. Hough

Biochemistry Division, Department of Chemistry, University of Maryland,
College Park, Maryland 20742, USA

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ABSTRACT

A method for recovering RNA-DNA hybrids from agar employing a single strand specific nuclease is described. The procedure is suitable for large scale isolations, and immobilization of the DNA in agar prior to hybridization allows a high yield of hybrid without interference by DNA reannealing.

INTRODUCTION

Nucleases are useful for chromosome fractionation. For example, the single strand specific nuclease S₁ has recently been employed for isolation of repetitive DNA from mammals¹ as well as nuclease resistant regions of single stranded DNA². Mung bean nuclease has been similarly employed³. The neurospora enzyme has been used to isolate DNA corresponding to transfer⁴ and ribosomal⁵ RNA species. We have isolated pure ribosomal hybrids (unpublished results) using the nuclease described by Piñon⁶. Hybridization of DNA with RNA followed by treatment with nucleases which specifically degrade single stranded DNA while leaving hybrids intact is an important method of chromosome fractionation. A limitation to the effective exploitation of this technique is the DNA reannealing which occurs during the liquid hybridization step, resulting in contamination of the RNA-DNA hybrid with reannealed DNA. Reannealing interference in liquid can be reduced by some combination of lowering the DNA concentration, raising the RNA concentration, and shortening the hybridization time. Unfortunately, these reduce the potential yield of hybrid from a given amount of RNA, making recovery of hybrids corresponding to relatively rare message species difficult. Maximum yield of hybrids for a limited quantity of messenger RNA requires high DNA concentrations and long hybridization times⁷. Although DNA immobilized on nitrocellulose filter discs is routinely used for quantitative work, we have

encountered difficulty in recovering hybrids from filters. We therefore attempted to recover hybrids from agar in which DNA had been immobilized prior to hybridization. This paper describes a procedure which employs commercially available S_1 nuclease to liberate ribosomal RNA-DNA hybrids from agar. Subsequent ribonuclease treatment and MAK column purification yields a product with the properties of pure hybrid. The procedure was conducted on a relatively large scale, and further scaling up would be simple.

MATERIALS AND METHODS

S_1 nuclease from *Aspergillus oryzae* was obtained from Miles Laboratories, and proteinase K from EM Laboratories, Elmsford, New York. Methylated albumin, bovine pancreatic, and T_1 ribonucleases were obtained from Calbiochem, and deoxyribonuclease I from Worthington Biochemical Corp. Carrier free ^{32}P as orthophosphate was obtained from New England Nuclear, and tritiated thymidine was from Merck. *Bacillus cereus* T spores were a gift from H. O. Halvorson.

Labeled and unlabeled high molecular weight DNA was isolated from stationary phase *Bacillus cereus* T cells as previously described⁶. The cells were lysed in the presence of proteinase K, and the DNA was purified by ribonuclease treatment and phenol extraction. Ribosomal RNA containing ^{32}P (^{32}P rRNA) was isolated as previously described⁶ from cells grown in a low phosphate medium containing ^{32}P as orthophosphate. The RNA had a specific activity of 3×10^6 cpm/ μ g at the time of isolation. DNA agar was prepared as previously described⁸ using the method of Bendich and Bolton⁹. Trapping efficiency of the DNA was about 80%. The DNA agar used in these experiments contained about 25 μ g of DNA per g of agar. For hybridization, portions of the agar were weighed out and washed with 2 x SSC (SSC contains 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0). An equal volume of ^{32}P rRNA in 2 x SSC was added and the suspension was mixed thoroughly. Hybridizations were carried out for 16 hr at 60°C, and then washed several times with 2 x SSC to remove excess RNA.

A 1/2 X methylated albumin kieselguhr (MAK) column with a bed volume of 15 ml was prepared by the procedure of Hayashi,

*et al.*¹⁰. Samples were applied to the column at concentrations below 50 µg/ml of nucleic acid in 0.1 M NaCl buffered with 0.05 M sodium phosphate at pH 6.5. After washing off unbound material with the same buffer, bound nucleic acid species were eluted with buffer containing 1 M NaCl.

A stock solution of S₁ nuclease was prepared by dissolving 10⁶ units of S₁ in 1 ml of a buffer similar to that of Vogt¹¹, which contained 0.03 M sodium acetate, pH 4.6, 0.3 M NaCl, 0.001 M ZnSO₄, and 5% glycerol (S buffer). One ml of glycerol was added, and the solution was stored at -20°C. Incubations were carried out in S buffer at 35°C for one hour unless indicated otherwise. Ribonuclease (RNAase) treatments were carried out at room temperature in 2 x SSC, using 50 µg of pancreatic and 50 units of T₁ RNAases per ml unless indicated otherwise. Deoxyribonuclease I (DNAase I) treatment was carried out at 35°C in 0.01 M magnesium acetate, pH 5 using 10 units/ml of DNAase I.

CsCl and sucrose density centrifugations were carried out as previously described⁸.

Radioactivity measurements were carried out in a Packard Tri-Carb scintillation spectrometer using a toluene based scintillation fluid containing 4 g of 2,5-diphenyloxazole (PPO) and 50 mg of p-Bis[2-(5-phenyloxazolyl)]-benzene (POPOP) per liter of toluene. Samples were counted after drying aliquots onto Whatman 3MM filter squares, or after collecting trichloroacetic acid (TCA) precipitates on nitrocellulose filters and drying. Radioactivity in agar samples was measured by adding an equal volume of water and heating in a boiling water bath to melt the agar which was then spotted onto filter squares, dried, and counted.

RESULTS

Formation and recovery of hybrids. A 7.5 g portion of DNA agar was hybridized with ³²P rRNA at a final concentration of 1 µg/ml. After washing several times with S buffer, 15 ml of a solution containing 1,000 units/ml of S₁ nuclease in S buffer was added and incubated at 35°C for an hour. After chilling in ice, the suspension was transferred to a 250 ml round bottom flask, and 75 ml of the MAK column buffer containing 5 mM ethylenediamine-tetraacetate (EDTA), pH 6.5, was added. The suspension was shaken

vigorously for 24 hr at 4°C. The supernatant was removed and applied to the MAK column. After loading, the column was washed with 30 ml of buffer to remove unbound material. Over 90% of the total radioactivity applied eluted in the wash, indicating that considerable degradation of unhybridized RNA had occurred. This was expected, since S_1 is active toward both RNA and DNA². The column was eluted with buffer containing 1 M NaCl and the radioactive peak was pooled. Ribonuclease was added and the mixture was incubated for an hour at 35°C, and diluted with 60 ml of MAK buffer. The sample was reapplied to the MAK column and about 65% of the counts eluted in the wash. The rest was subsequently eluted with 1 M NaCl. As discussed below, the material eluted by high salt has the properties of pure RNA-DNA hybrid.

Saturation curve. Hybridizations of DNA agar with various concentrations of ³²P rRNA were carried out in small tubes containing 0.1 g of agar. The hybridized agar was treated with RNAase and washed thoroughly. Blank agar samples retained less than 5% as many counts as did the DNA agar samples. The saturation curve obtained is shown in Figure 1. For the rest of the hybridization experiments, a ³²P rRNA concentration of 1 µg/ml was employed.

Activity of S_1 nuclease. Each sample contained 0.86 µg of tritiated DNA. Single stranded DNA was obtained by boiling native DNA for 5 min in S buffer and chilling in ice water. Incubations were carried out at 35°C in 0.5 ml volumes using various concentrations of enzyme. The reaction was stopped after 1 hr by addition of 100 µg of calf thymus DNA followed by 5 ml of cold 5% TCA. The TCA precipitates were collected by filtration through nitrocellulose filters, followed by washing with cold 95% ethanol. The filters were counted in the scintillation counter. The results are shown in Figure 2. From these results it can be concluded that this preparation of S_1 nuclease has approximately a 2,000-fold preference for single stranded DNA as compared to double stranded DNA.

Properties of the hybrid. RNA-DNA hybrids were prepared using the preparative scale approach outlined above. CsCl gradients of the material obtained at two different stages of the preparation

FIGURE 1

Saturation curve of DNA in agar with ^{32}P rRNA. Measured as RNA counts bound to DNA.

FIGURE 2

Activity of S_1 nuclease measured by disappearance of TCA precipitable DNA counts. Native DNA (●). Denatured DNA (○).

are shown in Figure 3. The radioactivity profile after the first MAK column step shows a very broad peak at high density. The same material after RNAase treatment and rechromatography on the MAK column is also shown. The peak is smaller, much sharper, and bands with a single peak at a density of 1.78. Since the material from the first MAK column had been exposed only to S_1 nuclease, it is possible that RNA fragments remained which were retained by the column. After RNAase treatment, however, these were removed by the second MAK column. The density of 1.78 is that expected for pure rRNA-rDNA hybrid⁵.

A melting curve of the hybrid material after the second MAK column is shown in Figure 4. Aliquots of the hybrid were diluted to 1 ml of 2 x SSC. Samples were placed in a water bath at various temperatures for 15 min and chilled rapidly in ice water. The samples were treated with RNAase for an hour followed by addition of 100 μ g of carrier calf thymus DNA and 1 ml of cold 10% TCA. The precipitates were collected on nitrocellulose filters and counted. A very sharp melting curve with a T_m of about 78-80°C was obtained.

The hybrid was tested for sensitivity toward specific nucleases. Retreatment of the material from the second MAK column with RNAase rendered no more than about 1% of the 32 P counts TCA soluble. In contrast, treatment with DNAase I before the RNAase produced more than 99% TCA soluble counts. These results are expected for RNA which is bound solely as double strand hybrid. The effect of retreatment of the purified hybrid with S_1 nuclease was also determined. This was of significance, since data presented below indicates that DNA trapped in agar is not as susceptible to nuclease attack as it is in free solution. Although Figure 2 shows that single stranded DNA exposed to 1,000 units/ml of S_1 is completely degraded to TCA soluble fragments, the possibility remains that the hybrid from the agar may have single stranded DNA regions. If so, then retreatment of the hybrid in solution with S_1 should produce observable shifts in the buoyant density and the molecular weight. An aliquot of the hybrid from the second MAK column step was dialyzed against S buffer and incubated with 100 units/ml of S_1 nuclease for an hour at 35°C. According to Figure 2, this concentration of nuclease should remove all single stranded regions.

FIGURE 3

CsCl density gradient profiles of RNA-DNA hybrid measured as ^{32}P in RNA. Agar hybridization, after the first MAK column (○). Agar hybridization, after the second MAK column (●). Liquid hybridization (X).

FIGURE 4

Melting curve of purified RNA-DNA hybrid.

Figure 5 shows the radioactivity profiles from CsCl gradients of hybrid before and after retreatment with S_1 nuclease. Figure 6 shows the profiles obtained from sucrose gradients prepared as previously described⁸. They were centrifuged in a Beckman SW27.1 rotor at 25,000 rpm for 20 hr at 20°C. No significant differences can be discerned between the two in either the CsCl density profiles or in the sedimentation rates in sucrose gradients. It does not seem likely that any single stranded DNA regions remain in the material from the second MAK column, and it can be concluded that retreatment with S_1 is unnecessary.

The hybrid prepared using immobilized DNA was also compared with hybrid formed in free solution. DNA (25 μ g) was denatured in 2 ml of 2 x SSC by heating in a boiling water bath for 5 min and chilling in an ice bath. RNA labeled with 32 P was added to a final concentration of 1 μ g/ml and the mixture was hybridized at 60°C for 16 hr. After dialysis against S buffer, the hybrid was treated with 1,000 units/ml of S_1 nuclease for an hour at 35°C. The sample was then centrifuged in a CsCl gradient which was fractionated as above. The result is shown in Figure 3 along with purified hybrid obtained from DNA agar. All the RNA counts band at a density equal to that of reannealed DNA, showing that immobilization of the DNA is required to prevent renaturation interference, and that renaturation does not occur in the agar.

Molecular weight of hybrid. It has been estimated that the molecular weight of an intact ribosomal cistron of *Bacillus subtilis*¹² is approximately 1.65×10^6 , and the corresponding hybrid about 3.3×10^6 . Ribosomal hybrids have been isolated from *B. subtilis* with molecular weights estimated to be as high as 3.5×10^6 , although the DNA moiety⁵ of those hybrids did not exceed 8×10^5 . The sucrose gradient profiles shown in Figure 6 of hybrids recovered from agar after S_1 nuclease treatment indicate they are not intact. Using intact 16 S rRNA as a standard these hybrids have a sedimentation coefficient of about 7-8 S, from which a molecular weight of about $4-5 \times 10^5$ can be calculated¹³ assuming that double stranded hybrids have sedimentation properties comparable to native DNA. The validity of this estimate is supported by Figure 7 which shows the chromatographic behavior of the hybrid on A-5 agarose (Bio-Rad) and G-100 Sephadex (Pharmacia).

FIGURE 5

CsCl density gradient profiles of purified RNA-DNA hybrid before (○) and after (●) retreatment with S_1 nuclease.

FIGURE 6

Sucrose gradient profiles of purified RNA-DNA hybrid before (○) and after (●) retreatment with S_1 nuclease. Sedimentation is from right to left.

A-5 agarose has an exclusion limit of the order of 2×10^6 daltons for linear polymers¹⁴, while G-100 Sephadex has an exclusion limit¹⁴ of 10^5 . Both columns gave a single peak at fraction 11 when denatured DNA which had not been treated with nuclease was chromatographed. This was assumed to correspond to the excluded volume in both columns. When the hybrid was passed over the G-100 column in 2 x SSC, the counts emerged with a single peak at fraction 11, although some tailing was observed. For the A-5 column, only a small portion of the hybrid emerged in the excluded column, while most of the counts emerged in later fractions. The result from the G-100 column indicates the hybrid exceeds a molecular weight of 100,000, while the A-5 column indicates that the molecular weight is well below 2×10^6 . This evidence shows that the hybrid isolated by this procedure is degraded, although a small portion corresponding to the front peak of the A-5 column may be relatively intact. This is not surprising inasmuch as the hybrid was liberated from the agar using an S_1 concentration of 1,000 units/ml, and Figure 2 shows that this concentration approaches that which yields TCA soluble fragments from double stranded DNA. It is not unexpected that RNA-DNA hybrids would be attacked at this concentration of nuclease as well. Experiments employing various concentrations of S_1 to release hybrid from agar are presented below.

Kinetics of release of nucleic acid species from agar. DNA agar which had been hybridized with ^{32}P rRNA and washed with 2 x SSC was suspended in the MAK column buffer and shaken vigorously in the cold for 24 hr. The supernatant was treated with RNAase and precipitated with TCA. Only 6% as many counts were precipitable as compared to the final yield of hybrid recoverable from the complete MAK column procedure after treatment of the same amount of agar with S_1 nuclease. This shows that dissociation of hybrid from the agar is completely dependent upon prior nuclease treatment.

The kinetics of release of nucleic acid species after treatment with different nucleases provides insight into the mechanism of DNA trapping and the accessibility of the DNA by RNA and nucleases. We have found that the kinetics of release of hybrid are dependent on whether S_1 treatment precedes or follows RNAase treatment. After an hour of incubation of hybrid agar with S_1 ,

FIGURE 7

Gel permeation chromatography of purified RNA-DNA hybrid. Bio-Rad A-5M agarose (O). Sephadex G-100 (●). Measured as ^{32}P counts in RNA.

no more than about 5% of the hybrid counts can be recovered from a 2 x SSC wash carried out immediately after the incubation. Extraction overnight by shaking with the EDTA buffer in the cold released about 60%. About half of the remainder could be recovered after an additional week of shaking. CsCl and sucrose gradient centrifugation revealed no differences between the material dissociating within 24 hr and the material dissociating during the subsequent week (data not shown), suggesting that the difference is not due to molecular weight. In contrast, treatment with RNAase prior to S_1 treatment gave a different result. Figure 8 shows the release of hybrid from agar which had been treated with RNAase prior to treatment with various concentrations of S_1 . In all cases, the counts represent hybrid released into the 2 x SSC wash immediately after incubation with S_1 . Also shown in Figure 8 are the kinetics of release of tritium counts from DNA agar containing DNA labeled by tritiated thymidine. It can be seen that DNA fragments are released with kinetics which are indistinguishable from release of hybrid. This shows that prior treatment with RNAase dramatically increases the ease with which hybrids are released from agar after S_1 treatment, and that this release is as rapid as for unhybridized DNA. One explanation for this observation is that RNAase treatment increases the number of sites which are susceptible to S_1 . Another possibility is that RNA "tails" which slow diffusion are removed by the RNAase, allowing more rapid elution of the hybrid from the agar. Figure 9 shows a time course of release of tritium from agar containing tritiated DNA in the presence of 1,000 units of S_1 nuclease. Total tritium counts in both the supernatant and the agar are shown. A time course experiment was also carried out in which TCA precipitable counts released into the supernatant were measured. The appearance of TCA precipitable counts followed the same kinetics as total counts when differences in counting efficiencies were taken into account. This is surprising when the results of Figure 2 are considered, since most of the counts should have been TCA soluble under these conditions. This suggests that the agar interferes with the action of the nuclease.

FIGURE 8

Effect of S₁ concentration on release of RNAase treated RNA-DNA hybrid from agar measured as ³²P RNA counts disappearing from agar (●) or appearing in the supernatant (○); and on the release of single stranded DNA from agar measured as disappearance of ³H counts from agar (▲). Value in parenthesis was obtained in the absence of nuclease.

FIGURE 9

Time course release of ³H DNA from agar measured as total DNA counts in the agar (●) or in the supernatant (○), and as TCA precipitable DNA counts appearing in the supernatant (▲).

DISCUSSION

Hybridization of RNA to DNA which is immobilized in agar followed by treatment with a single strand specific nuclease liberates RNA-DNA hybrids which can be purified on a MAK column. Using ribosomal RNA as a model system, a 60% yield of rRNA-rDNA hybrids was obtained. CsCl density gradient profiles showed that recovered hybrid was not contaminated by reannealed DNA; the hybrids giving a single peak at a density of 1.78. This density is that expected for pure hybrids⁵. A control experiment in which non-immobilized DNA was hybridized with RNA gave hybrid counts which banded only at a density of 1.7, which is the density of reannealed DNA. Immobilization in agar eliminated reannealing interference. Profiles obtained from gel filtration columns combined with sedimentation through sucrose gradients indicate that the purified hybrids have a molecular weight of the order of 4-5 hundred thousand, which is less than that expected for an intact ribosomal cistron^{5,12}.

The kinetics of release of unhybridized DNA from the agar in the presence of S₁ indicates that the immobilized DNA strands are attacked less actively than is the same DNA in solution. Whether this is due to inaccessibility of the DNA or to inhibition of activity by the agar is not clear.

The results presented in this paper show that this method is suitable for obtaining RNA-DNA hybrids in high purity and high yield. The procedure is readily scaled up, and hybridization conditions can be adjusted to achieve maximum yield of hybrid with limited amounts of RNA. Although intact cistrons cannot be obtained without further technical improvements, it should be possible with this method to isolate large amounts of any DNA regions for which complementary RNA species can be obtained.

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