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A GENERAL METHOD FOR THE ISOLATION OF RNA COMPLEMENTARY TO DNA

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During the past few years evidence has accumulated that a class of RNA molecules which resemble DNA in base composition is synthesized in living cells. It has been proposed that this RNA is an intermediary in the transfer of genetic information from DNA to protein.[1] It is therefore of importance to have available a general method for the purification of such RNA molecules in order to study their chemical character and information content. Hall and Spiegelman[2] showed that T2-specific RNA could be hybridized with heat-denatured T2 DNA and the hybrid isolated by cesium chloride density gradient centrifugation. With this method Hayashi and Spiegelman[3] were able to demonstrate small amounts of DNA-like RNA in nongrowing bacteria. Base analyses of rapidly labeled RNA strongly suggest the existence of complementary RNA in several species of growing bacteria[4, 5] but attempts to purify such molecules have been only partially successful.[5]

Bautz and Hall[6] have made a notable advance toward purifying RNA molecules with a base sequence complementary to DNA. They used phospho-cellulose ace-

tate to immobilize denatured T4 phage DNA. The mechanism proposed for this attachment involved covalent bond formation between the glucosylic hydroxyls of the DNA and phosphate groups on the cellulose. The reaction therefore appeared to be limited to glucosylated DNA known to occur only in some bacteriophages. Incubation of RNA solutions with this preparation immobilized some RNA by specific hybridization. Noncomplementary RNA could then be washed away and complementary RNA reclaimed after decomposition of the hydrogen bonds between RNA and DNA.

Investigation of this reaction has led to a method for immobilizing any high molecular weight DNA by physical entrapment in cellulose acetate gels, or more readily, in agar gels. These preparations of immobilized DNA can form hydrogen bonds with complementary molecules. This paper describes the preparation of DNA-cellulose acetate and DNA-agar gels and their application to the isolation of complementary RNA.

Methods.—(a) *Preparation of DNA-cellulose acetate:* DNA was dialyzed successively against water, formamide, and anhydrous pyridine.[7] 2 gm cellulose acetate (Fisher Scientific Co., cellulose diacetate, "acetone soluble") were dissolved in 10 ml boiling pyridine and precipitated by the dropwise addition of 15 ml water. The precipitate was gathered on a glass rod and dissolved in 15 ml boiling pyridine containing 10 mg DNA. 7.5 gm dry cellulose powder were then added to soak up the liquid, and the crumbly mixture allowed to cool. One molar NaCl solution was poured onto the mass and gelation allowed to occur. When the gel was hardened it was ground in a mortar and washed with 2 × SSC (SSC = 0.15 *M* sodium chloride, 0.015 *M* sodium citrate) at 60°C. Trapping of DNA was greater than 70 per cent.

(b) *Preparation of DNA-agar:* 300 mg of agar (Difco Special Agar-Noble) were dissolved in 5 ml of water at 100°C. 5 mg DNA in 5 ml 0.01 × SSC were heated to 100°C for 5 min and poured into the agar. The hot solutions were thoroughly mixed and poured into an empty 250 ml beaker in an ice water bath. When the gel had hardened it was forced through a 35 mesh screen. The gel particles were washed with 2 × SSC at 60°C. Trapping of DNA was essentially quantitative.

(c) *DNA:* Bacterial DNA was prepared by the method of Marmur[8] and bacteriophage DNA according to Grossman *et al.*[9] Thymus DNA was obtained from Worthington.

(d) *Preparation of phage specific RNA:* Strain BB of *E. coli* growing in a tris/glucose medium[10] at 37°C was infected with T2 phage at a multiplicity of 10. Phage RNA was labeled through the addition of P^{32} (20 μc/ml) 5 min after infection. The cells were chilled 5 min later by pouring onto crushed frozen medium. The cells were washed, frozen, and lysed with lysozyme.[2] DNAase (20 μg/ml) was added for 2 min, followed by 1 per cent sodium dodecyl sulfate. RNA was extracted with phenol and purified by means of a Na^+ Dowex-50 Sephadex G25 column.[6]

(e) *Preparation of bacterial RNA:* RNA either pulse labeled with P^{32}, or randomly labeled with P^{32} and pulse labeled with C^{14}-uracil, was prepared from growing *Proteus vulgaris* as previously described.[5, 11] It was purified as described above for phage specific RNA.

(f) *Hybridization of RNA with immobilized DNA:* Approximately 50 μg of RNA in 1 ml or less of 2 × SSC were incubated at 60°C with DNA-cellulose acetate or DNA-agar containing 0.5 mg DNA, either in a thick slurry in a screw top vial, or in a chromatograph tube heated by circulating water. The preparations heated in vials were then transferred to chromatograph tubes for subsequent operations. The column was washed with six to ten 5-ml aliquots of 2 × SSC and the aliquots separately collected. Recovery of the remainder of the labeled RNA was effected by washing with six to ten 5-ml aliquots of 0.01 × SSC which were also individually collected. The flow rate was approximately 1–2 ml per minute. Carrier RNA was added to the fractions and the total RNA precipitated with 5 per cent trichloroacetic acid. The precipitates were collected on membrane filters and assayed for radioactivity.[11]

(g) *Base compositions* were determined as described by Midgley.[10]

Results.—*Hybridization with DNA-cellulose acetate:* Phage T2 DNA in cellulose acetate (0.5 mg DNA in 1 gm cellulose) was incubated overnight at 60°C with 50

μg of the RNA labeled with P^{32} after phage infection. The preparation was then washed and the hybrid decomposed as described above. It was found that about 10 per cent of the phage specific RNA had been hybridized. When this RNA preparation was incubated with *P. vulgaris* —DNA in cellulose acetate or with a cellulose acetate preparation lacking DNA, 1–3 per cent of the phage specific RNA was eluted at the low salt concentration. When 20 μg of *P. vulgaris* P^{32} pulse labeled RNA were incubated with a homologous DNA-cellulose preparation, 17 per cent of the P^{32} label was affixed and this could be removed by lowering the salt concentration. The incubation of *P. vulgaris* RNA with T2-DNA, or blank, —cellulose acetate fixed a smaller fraction of the P^{32}. When 200 μg *P. vulgaris* RNA randomly labeled with P^{32} and labeled for 2 minutes with H^3-uracil were incubated with the homologous DNA preparation, about 20 per cent of the H^3, and 2 per cent of the P^{32} were fixed. Thus, this procedure yielded some purification of the pulse labeled RNA, and the hybridization process appeared to be specific for both T2-specific and *P. vulgaris* RNA. However, the yields of the apparent hybrids were disappointingly low, and the hybridized material was accompanied by a relatively high background of nonspecifically adsorbed nucleic acid. About 2 μg of RNA were adsorbed per gram of cellulose even when DNA was not present. The low yields of hybrid might well have been a result of trapping DNA so tightly that it was unavailable for hybridization. In support of this notion DNAase treatment of the DNA-cellulose acetate removed only about 10 per cent of the DNA in one-half hour at 20°C and only about 50 per cent overnight. In contrast DNAase treatment for one-half hour at 20°C removed over 80 per cent of the DNA in DNA-agar prepara-

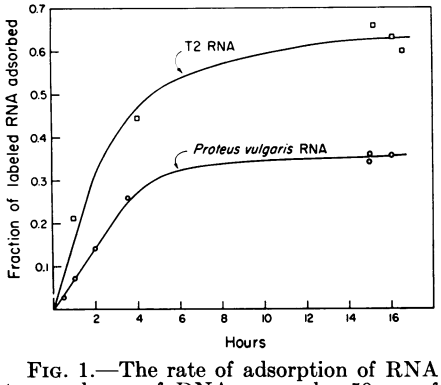

FIG. 1.—The rate of adsorption of RNA to a column of DNA-agar gel. 50 μg of P^{32} labeled RNA prepared from T2-infected *E. coli* or RNA of *P. vulgaris* labeled with P^{32} for one min were incubated in 2 × SSC for various times at 60°C in a 2 cm column of agar containing 0.5 mg of the corresponding DNA. At times indicated the unadsorbed RNA was removed by washing with six 5-ml fractions of 2 × SSC. The adsorbed RNA was recovered by elution with six 5-ml portions of 0.01 × SSC and the fraction of the labeled RNA adsorbed was recorded.

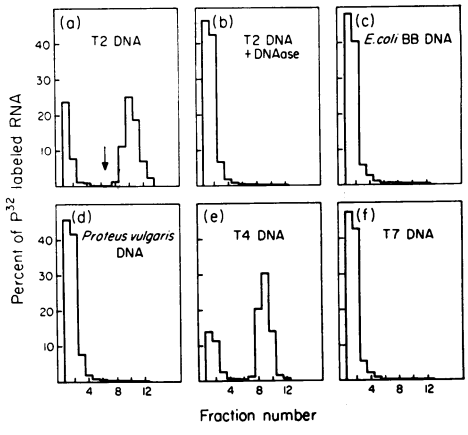

FIG. 2.—The specificity of the adsorption of T2-specific RNA to columns of DNA-agar gel. 50 μg of RNA prepared from T2 infected cells labeled after infection with P^{32} were incubated in 2 × SSC for 15 hr with 1 gm of agar gel containing 0.5 mg of DNA from various sources. Elution as in Fig. 1. Eluting solution changed to 0.01 × SSC at fraction 7. Fractions were assayed for the percentage of the P^{32} labeled RNA. (*a*) Agar containing T2 DNA. (*b*) T2 DNA trapped in agar and treated with DNAase at 20 μg/ml for 2 hr at 25°C. (*c*) *E. coli* BB DNA. (*d*) *P. vulgaris* DNA. (*e*) T4 DNA. (*f*) T7 DNA.

tions. Hence, the DNA in the agar appeared to be more readily available for contact with macromolecules.

Formation of RNA-DNA hybrids in agar: Figure 1 shows the time course of hybrid formation at 60°C in the homologous reactions: T2-specific RNA and T2 DNA-agar, and pulse labeled *P. vulgaris* RNA and *P. vulgaris* DNA-agar. In each case the reaction progresses smoothly and slowly as would be expected for a hybridization process. Under the conditions used about one-third of the *P. vulgaris* pulse labeled RNA and about two-thirds of the phage specific RNA can be hydridized. The latter result may be compared with that of Bautz and Hall[6] who found that 80 per cent of the T4-specific RNA could be hybridized with homologous DNA-cellulose. For convenience overnight incubations have been generally employed. The greater part of the reaction is, however, complete much earlier, especially if incubation is carried out in a column so that the concentration of reactants is as high as possible. Incubation as a thick paste in a vial produces rates of reaction lower by not more than a factor of two.

Specificity of hybrid formation: Figure 2 shows that the fixation of T2-specific RNA to DNA-agar depends upon the DNA. Thus *E. coli* BB DNA-agar (the host strain used for culture of T2 phage), *P. vulgaris* DNA-agar, and T7 phage DNA-agar do not allow fixation of the T2-specific RNA. No reaction occurs with preparations treated with DNAase. On the other hand, fixation is relatively efficient with the homologous T2 DNA preparation as well as with DNA-agar of the closely related bacteriophage, T4. A rerun of the adsorbed RNA from Figure 2(*a*) gave 70 per cent of the radioactivity in the back peak.

Figure 3 shows the specificity of reaction for the case of pulse labeled *P. vulgaris* RNA. This RNA hybridizes with the homologous DNA-agar but not with the unrelated DNA-agar of phage T2 which has the same base composition as *P. vulgaris* DNA. There is no reaction with the DNA-agar of *Pseudomonas aeruginosa* or calf thymus. Again a rerun of the back peak of Figure 3(*a*) allowed 70 per cent of the radioactive RNA to appear in the back peak.

Base composition of the adsorbed RNA: Analyses were made of the base compositions of the adsorbed and unadsorbed fractions of both the T2-specific RNA preparation and the one-minute P^{32} pulse labeled *P. vulgaris* RNA (Table 1).

In the case of the T2-specific RNA it is clear that essentially all of the labeled RNA has a composition close to that of the virus DNA. The composition of the unadsorbed material is not significantly different from that in the back peak. The disparity between the contents of cy-

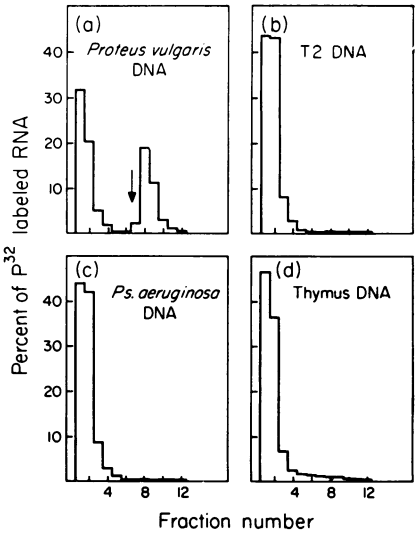

FIG. 3.—The specificity of the adsorption of RNA from *P. vulgaris* labeled with P^{32} for 1 min to columns of DNA-agar gel. Conditions as for Fig. 2. (*a*) Agar containing *P. vulgaris* DNA. (*b*) T2 DNA. (*c*) *Ps. aeruginosa* DNA. (*d*) Calf thymus DNA.

tidylic acid and guanylic acid has also been noted by other workers.[6, 12] Evidently the conditions of the incubation are not sufficient to hybridize more than about 70 per cent of the specific RNA.

The base composition of the RNA synthesized in the first 3 minutes after the addition of P^{32} to growing bacteria is intermediate between that of the bacterial DNA and ribosomal RNA.[4, 5] This could result from the entry of P^{32} into 2 different types of RNA molecule, one DNA-like (D-RNA) and the other ribosomal. Even with the shortest (10 seconds) exposure to P^{32} there seemed to be a partition of the label between 2 types of newly synthesized molecule.[5] Fractionation of the pulse labeled RNA of *P. vulgaris* on the *P. vulgaris* DNA-agar column demonstrates the validity of this interpretation. The labeled RNA is separated into 2 components of very different composition. The adsorbed material has a composition closely resembling that of the DNA and the unadsorbed RNA is very like ribosomal RNA. The rapidly labeled RNA would therefore appear to be only about 30 or 40 per cent D-RNA and the remainder precursor of ribosomal RNA.[13]

TABLE 1

BASE COMPOSITION OF P^{32} LABELED RNA SEPARATED BY DNA-AGAR

	Mole Fraction				
	C	A	G	U(T)	% GC
T2-specific RNA:					
Unadsorbed RNA 34%	18.4	28.6	24.1	28.9	43
Adsorbed RNA 66%	17.4	31.0	21.9	28.7	39
Bacteriophage DNA	18	32	18	32	36
Pulse labeled *P. vulgaris* RNA:					
Unadsorbed RNA 65%	22.5	23.3	32.1	22.1	55
Adsorbed RNA 35%	19.8	30.1	20.9	29.2	41
Purified 14*S* RNA fraction of *P. vulgaris* labeled for 5 min with P^{32} (ref. 5)	22.7	26.7	27.6	23.0	49
P. vulgaris ribosomal RNA[10]	21.7	26.2	31.4	20.7	53
P. vulgaris DNA	19	31	19	31	38

Kinetics of labeling of DNA-like RNA: The separation of pulse labeled bacterial RNA into 2 components and the consequent purification of the D-RNA makes it possible to study the rate of synthesis of the latter component. Previously all studies of the kinetics of labeling of the rapidly labeled 14*S* fraction have been concerned with a mixture of ribosomal RNA precursor (R-RNA) and D-RNA.[11, 14] As a result estimates of the quantity and half-life of the D-RNA molecules could be made only by correlation of the results of kinetic studies and the changes in base composition of the total RNA with time of labeling.[5] The present method permits the measurements to be made directly.

Cells of *P. vulgaris* were grown in C medium containing P^{32} for 3 generations. The generation time was 72 minutes. C^{14}-uracil was then added and samples taken for the isolation of RNA after 1, 2, 3, 5, 7, and 10 minutes. 30 μg of each of the RNA samples were then incubated with agar gel containing 0.5 mg of *P. vulgaris* DNA for 15 hours at 60°C. The fractionation of the 1-minute sample is shown in Figure 4. It is evident that about one-third of the C^{14}-labeled RNA is in the hybrid peak and is accompanied by only a small fraction of the total RNA as represented by the P^{32} label. The C^{14} and P^{32} radioactivity in the front and back peaks were computed for each time point. After normalization to the same size sample of RNA by means of the total P^{32} eluted, the C^{14} radioactivity in the total

RNA and in the adsorbed peak were plotted (Fig. 5). A small correction was applied to the latter series of numbers to account for the small amount of ribosomal RNA appearing in the back peak. This may be due to specific hybridization between ribosomal RNA and DNA.[15, 16] The correction can be easily estimated from a knowledge of the total RNA adsorbed in the hybrid peak as measured by the P^{32} radioactivity.

The rate of entry of C^{14}-uracil into total RNA produces a straight line through the origin as in *E. coli*.[11] Apparently the initial rate of entry of label into the D-RNA accounts for about one-third of this flow. The entry of C^{14}-uracil into D-RNA soon levels off indicating an average lifetime of these molecules of about 2 minutes. Estimation of the quantity of D-RNA from these kinetics is made somewhat inaccurate by the large correction at late times, but the curve suggests an amount corresponding to the RNA synthesized during 1 minute; i.e., approximately 1 per cent of the total RNA content.

Discussion.—Once it is realized that single-stranded DNA can be immobilized by trapping in a gel, a large number of variants on the methods described above become available. The long length of the DNA molecules makes it possible to confine them in a three-dimensional structure even where the holes in the structure are relatively large. Of the two types of gels here employed it seems clear that the agar gels are the most permeable. This is certainly true with respect to availability to the action of DNAase. DNA trapped in cellulose acetate by the method outlined above showed disappointingly low efficiency although this is not true of the preparations used by Bautz and Hall.[6]

In addition there remains the possibility that the DNA in the preparation of Bautz and Hall is bound covalently to the cellulose as they indicated. In our experience, however, very similar quantities of DNA are bound to the column whether or not the conditions for phosphate ester formation are met. Thus, the yield is apparently unaffected by the presence of the condensing agent, dicyclohexylcarbodiimide, and certainly fixation is not limited to those bacte-

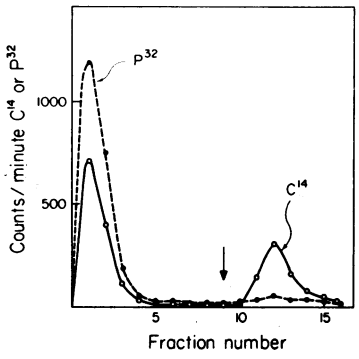

FIG. 4.—The adsorption of *P. vulgaris* RNA labeled for 3 generations with P^{32} and for 1 min with C^{14}-uracil to a column of DNA-agar gel. Incubation 15 hr at 60°C. Elution with eight 5-ml portions of 2 × SSC followed by eight 5-ml portions of 0.01 × SSC.

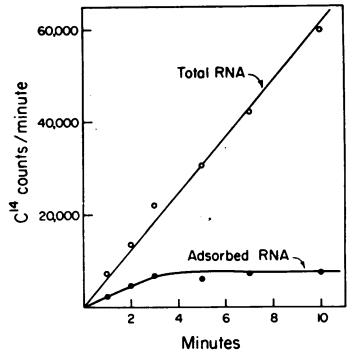

FIG. 5.—The rate of synthesis of RNA adsorbable by a DNA-agar gel column. 6 samples of RNA from *P. vulgaris* cells grown for 3 generations in P^{32} with a generation time of 72 min were taken after exposures of 1, 2, 3, 5, 7, and 10 min to C^{14}-uracil. Each was incubated with an agar gel containing *P. vulgaris* DNA 30 μg RNA/0.5 mg DNA (see Fig. 4). The C^{14} cpm in the total RNA and the adsorbable material was normalized for the amount of RNA employed by means of the P^{32} cpm and plotted against time. Data from Fig. 4 and five additional analyses.

riophage DNA's having glucosylated residues containing reactive hydroxyl groups.

In general, however, it appears that preparations of DNA agar have advantages over those in cellulose. The method of preparation has the great advantages of ease and rapidity and the material prepared has properties suitable for packing in a column and rapid equilibration with the ionic environment. The gel network is a very open structure since Steere and Ackers[17] have shown that even gels made from 4 per cent agar have holes large enough to accommodate southern bean mosaic virus (molecular weight, 6.6×10^6). There is, however, a failing of the agar materials which may be of consequence; the 3 per cent agar preparations are not resistant to temperatures above 70°C and in cases where higher temperatures are necessary for the formation or dissociation of hybrid molecules, other means of immobilizing DNA must be employed. Probably other gels such as polyacrylamides or related compounds will prove useful for this purpose.

The high specificity shown in the reaction between RNA and DNA immobilized in agar suggests that relatively long regions of complementary sequences are necessary for attachment under the conditions of these experiments. Thus the reaction does not occur between molecules of DNA and RNA derived from different sources, e.g., T2 and *P. vulgaris* nucleic acids, even though they have the same average base composition. Likewise, bacterial nucleic acid has no reaction with thymus DNA which is presumably highly diversified in base sequences. It is probable that the high temperature maintained throughout the separation precludes adventitious pairing of short regions to form spurious hybrids. It is not clear at the moment, however, how long the paired regions must be to survive incubation and washing at 60°C.

It is of considerable interest that there exists a high degree of cross reaction between the RNA of T2-infected cells and the DNA of T4 phage, while no reaction with phage T7 DNA is noted. Clearly, the method here employed appears to have potential for exploring quantitatively the genetic relatedness among species. In this connection we have preliminary results indicating the feasibility of such studies with several bacterial species.[16] Schildkraut *et al.*[18] have already demonstrated formation of interspecific hybrid DNA-DNA molecules by means of the cesium chloride method. The ease and economy of the present method may make possible a quantitative taxonomy based on homologous sequences of nucleotides in nucleic acids.

Application of the method to the rapidly labeled RNA of bacteria results in considerable clarification of some questions relating to messenger-RNA. The labeled RNA, which is all present in the 14*S* peak in extracts made after short exposures to P^{32}, can be cleanly separated into 2 types of molecule easily distinguishable by their base composition. The 14*S* RNA comprises 3 per cent of the total RNA.[11] This establishes that at least half of that RNA commonly referred to as "messenger" as judged by its sedimentation coefficient and its high rate of labeling[14] is actually ribosomal RNA presumably in some precursor stage before entering ribonucleoprotein particles.[11] Moreover, the fact that such a high proportion of the P^{32} enters ribosomal RNA in 1 minute shows that the rate of synthesis of the D-RNA fraction cannot be higher than that of the bulk RNA. Rather, it seems that approximately one-third of the total cellular synthesis of RNA is in the form of D-RNA. The final level attained by the C^{14}-uracil in the D-RNA peak

demonstrates that about 1 per cent of the cellular RNA of growing cells is in this form. This estimate depends upon quantitative removal of the D-RNA by the DNA agar column. The base analysis of the separated R-RNA and D-RNA (Table 1) shows that the removal was highly effective.

Measurement of the rate of labeling of the hybridizable RNA with C^{14}-uracil indicates an average lifetime of these molecules of about 2 minutes which is similar to that of the β-galactosidase forming unit.[19, 20] As pointed out in more detail elsewhere,[5] such a period of activity on the part of template or messenger-RNA molecules, which should form part, at least, of the D-RNA fraction, would allow the direction of the synthesis of perhaps 50 protein molecules. The participation of template RNA in protein synthesis would therefore appear to be catalytic rather than stoichiometric.

Summary.—Methods are described for the immobilization of single-stranded DNA by trapping in gels of cellulose acetate or agar. The immobilized DNA retains the ability to form specific hybrids with complementary RNA. This provides a general method for the isolation of RNA molecules having sequence homology with DNA molecules from any given source. The specificity and kinetics of the hybridization reaction are examined, and the method is illustrated by a study of the rate of synthesis of complementary RNA in *Proteus vulgaris*.

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