

*SEQUENCE COMPLEMENTARITY OF T2-DNA AND T2-SPECIFIC
RNA**

BY BENJAMIN D. HALL AND S. SPIEGELMAN

DEPARTMENTS OF CHEMISTRY AND MICROBIOLOGY, UNIVERSITY OF ILLINOIS

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Investigations of the functional interrelations among DNA, RNA, and protein are most conveniently performed under conditions which limit the synthesis of each macromolecular class to a few chemical species. A situation of this type obtains in *E. coli* cells infected with bacteriophage T2. Volkin and Astrachan¹ examined the nature of the RNA synthesized in the T2-coli complex by means of P³²-labeling. Estimation of the relative P³² content of the 2',3'-nucleotides isolated from an alkaline hydrolysate led Volkin and Astrachan to deduce that the RNA formed in the infected cell possessed an apparent base ratio analogous to that of T2-DNA. Subsequently, Volkin² obtained data suggesting that the synthesis of a specific RNA is a prerequisite for the intracellular production of bacteriophage.

Nomura, Hall, and Spiegelman³ confirmed the observations on the apparent base ratios. In addition, they offered independent evidence for the existence of a "T2-Specific RNA" by demonstrating that RNA molecules synthesized after infection differed from the bulk of the *E. coli* RNA in electrophoretic mobility and average sedimentation coefficient. Because the procedures employed (zone electrophoresis and sedimentation) led to a selective separation of T2-specific RNA from the normal RNA of *E. coli*, they open up possibilities of further experiments relevant to an understanding of the nature of T2-RNA.

The fact that "T2-RNA" possesses a base ratio analogous to that of T2-DNA is of interest because it suggests that the similarity may go further and extend to a detailed correspondence of base sequence. The central issue of the significance and meaning of "T2-RNA" is whether or not this is in fact the case. A direct attack on this problem by complete sequence determination is technically not feasible at the moment. However, some recent findings of Marmur, Doty, *et al.*^{4,5} suggest the possibility for an illuminating experiment. These authors demonstrated the specific reformation of active double-stranded DNA when heat-denatured DNA is subjected to a slow-cooling process. Such reconstitution of the double-stranded structure occurs only between DNA strands which originate from the same or closely related organisms. Presumably, the specificity requirement for a successful union of two strands reflects the need for a perfect, or near-perfect, complementarity of their nucleotide sequences. We have here then a possible method for detecting complementary nucleotide sequences. The formation of a double-stranded hybrid during a slow cooling of a mixture of two types of polynucleotide strands can be accepted as evidence for complementarity of the input strands.

We have used this procedure to examine for complementarity of sequence between "T2-RNA" and T2-DNA. Purified T2-RNA was used in order to provide an optimal opportunity for the T2-RNA to combine with its DNA complement, unhindered by non-specific interactions involving irrelevant RNA. Since the hybrid would have a lower density than uncombined RNA, a separation of the two might be attainable by equilibrium centrifugation in CsCl gradients.⁶ To insure a sensitive and unambiguous detection of the hybrid, should it occur, double labeling was used. The T2-RNA was marked with P³² and the T2-DNA with H³. Two isotopes emitting β -particles differing in their energies are conveniently assayed in each other's presence in a scintillation spectrometer.⁷ This device, coupled with the use of the swinging-bucket rotor for the equilibrium centrifugation, permits the actual isolation of the pertinent fractions along with a ready and certain identification of any hybrids formed.

The primary purpose of the present paper is to present the results of such experiments. The data obtained demonstrate that specific complexes are indeed formed between "T2-RNA" and its homologous DNA. Their occurrence offers strong presumptive evidence for a detailed complementarity of the nucleotide sequences in these two macromolecules.

1. *Preparation and denaturation of DNA:* Tritiated phage were prepared by the addition of H³-thymidine to a T2-infected culture of *E. coli* B. The cells were treated with 5-fluorouracil deoxyriboside (0.5 μ g/ml) prior to infection. The phage were purified by treatment of the lysate with DNAase and RNAase followed by three cycles of high- and low-speed centrifugation. DNA was extracted from the purified phage by treatment with sodium dodecyl sulfate followed by chloroform-iso-amyl alcohol deproteinization and ethanol precipitation of the DNA.⁸ This preparation will be designated by H³-DNA(T2). DNA from other sources was similarly purified.

Tritiated *E. coli* DNA was prepared from cells of a thymineless mutant (15T⁻) grown in a synthetic medium supplemented with tritiated thymidine. This preparation will be designated by H³-DNA (*E. coli*).

DNA used for complex formation with RNA was first denatured by heating for 15 minutes at 95°C in 0.15 M NaCl + 0.01 M sodium citrate (pH 7.8), after which the tube containing the DNA was quickly placed in an ice bath. In all cases, the denaturation was carried out at a DNA concentration of 130 μ g/cc.

2. *Preparation of T2-specific RNA labeled with P³²:* P³²-labeled ribosome RNA was obtained from *E. Coli* B grown in synthetic medium, infected with T2 at a multiplicity of 3.8, and labeled with 10 millicuries of P³² between three and eight minutes after infection. The infection and radioisotope incorporation were done at 37°C in medium C (Roberts *et al.*)⁹ modified to include 5 gm NaCl, 0.37 gm KCl, and 1 gm casamino acids per liter. The phosphate concentration was lowered to 10⁻³ M and 0.1 M tris (hydroxymethyl) aminomethane (tris), pH 7.3, was used for buffering. The number of infective centers and uninfected survivors (2.5%) agreed with the multiplicity of infection. The procedures used for stopping incorporation, washing and disrupting cells, and preparing ribosomal RNA were those described previously (Nomura, Hall, and Spiegelman³).

3. *Purification of T2-specific RNA:* Enrichment of the ribosomal RNA preparation in its content of T2-specific RNA (as judged by an eightfold increase in specific activity of P³²) was obtained by zone centrifugation through a sucrose gradient. One ml of a 1.5% sucrose solution (w/w) + one ml of P³² ribosome RNA solution (1 mg/ml, 106,000 cpm/ml) were layered, with an inverted gradient of RNA on 20 ml of a 2 to 15 per cent sucrose gradient. All solutions were 0.05 M in KCl and 10⁻² M in tris buffer at a pH of 7.3. Following centrifugation for eight hours at 25,000 rpm in the SW-25 rotor of the Spinco preparative ultracentrifuge, the contents of the tube were removed by dripping through a hole punctured in the bottom of the tube. Fractions of 1.2 ml were collected by drop counting. The ultraviolet absorption at 260 m μ and P³² content of the fractions are shown in Figure 1. The two fractions at the peak of P³² activity (corresponding

to 18 and 19.8 ml) were used for hybrid formation. These will be referred to in the text as P³²-RNA(T2). In the experiments described below, the two fractions exhibited identical properties.

4. *Slow cooling of DNA and RNA*: Slow cooling of RNA and DNA was done in solutions 0.03 M in sodium citrate and 0.3 M in NaCl at a pH of 7.8. An insulated water bath having a capacity of 40 liters was used to provide slow cooling as follows:

Time (hr.)	Temperature (°C)
0	65
3.5	52
7.5	44
13.5	36
24.5	28.5
30.5	26

When the bath temperature reached 26°, the tubes containing RNA and DNA were removed and brought to a volume of 5.1 cc and a density of 1.74 gm/cc by addition of suitable amounts of water and saturated CsCl solution. Twenty-five µg of unlabeled, undenatured DNA were added to the solution as a reference density marker.

5. *Separation of RNA from DNA by density-gradient centrifugation*: The solutions of RNA and DNA containing CsCl were centrifuged at 33,000 rpm in the SW-39 rotor at a temperature of 25°C. At the end of each run, fractions corresponding to various density levels in the tube were obtained by piercing the bottom of the tube and collecting drops, 30 for each fraction. These were diluted to a volume of 1.2 cc for measurement of ultraviolet absorption and radioisotope concentration.

6. *Counting of H³-DNA and P³²-DNA*: To an aliquot from each swinging-bucket fraction 250 µg herring sperm DNA was added as carrier. The nucleic acid was then precipitated with trichloroacetic acid (final concentration 10%) in the cold, collected, and washed on a millipore filter (course, 50 mm dia.). The filter was air-dried for one hour and placed in a cylindrical glass vial filled with 15 ml redistilled toluene containing 1.5 mg of 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP) and 60 mg of 2,5-diphenyloxazole (PPO). P³² and H³ were counted in a Packard Tri-Carb liquid scintillation counter.

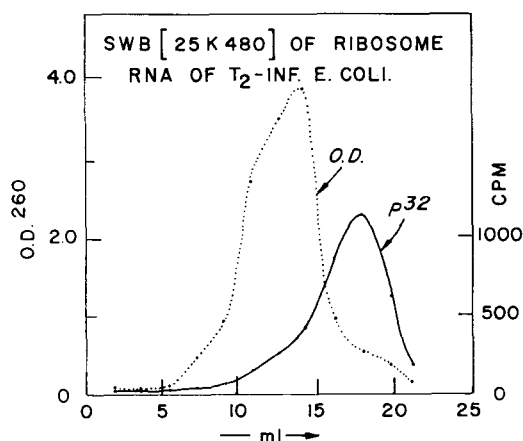


FIG. 1.—Separation of P³²-RNA(T2) from *E. Coli* RNA by sucrose-gradient sedimentation. One ml. of ribosome RNA solution containing 1 mg RNA and 106,000 cpm P³² + 1 ml 1.5% sucrose solution were layered on 20 ml of a 2–15% sucrose gradient. Centrifugation: 8 hours at 25,000 rpm. Cpm shown refer to 0.05 ml fractions of the swinging-bucket fractions.

Separation of T2-Specific RNA in a CsCl Gradient.—It was first necessary to establish the conditions required for an adequate separation of T2-specific RNA from T2-DNA. Whereas *E. coli* ribosome RNA formed a narrow band within two days, T-2 RNA, because of its smaller size, required five days to form a band near the bottom of the tube.

Figure 2 shows the result of a five-day run carried out under the conditions specified above. The mixture being separated consisted of 6.5 µg of heat-denatured H³-DNA(T2), 25 µg of unlabeled and undenatured T2-DNA, and 14 µg of the purified P³²-RNA(T2). Here, the three nucleic acids were not exposed to a slow-cooling operation but were mixed at 25°C, immediately put in the CsCl solution,

and centrifuged. It will be noted that there is no appreciable interaction between the RNA and DNA as evidenced by the absence of any appreciable overlapping of the P^{32} - and H^3 -containing regions. The small "tail" of P^{32} which extends to the top of the tube is presumably a consequence of the low molecular weight of the T2-specific RNA.

Hybrid Formation between Denatured T2-DNA and T2-Specific RNA.—The results described in Figure 2 show that CsCl density gradient centrifugation permits a clear separation of H^3 -DNA(T2) from P^{32} -RNA(T2) and provides, therefore, a test for interactions leading to the formation of RNA-DNA hybrids. Any distortion of the distribution of H^3 -DNA or P^{32} -RNA from that observed in Figure 2 which leads to regions of overlap between H^3 and P^{32} would be indicative of such interactions.

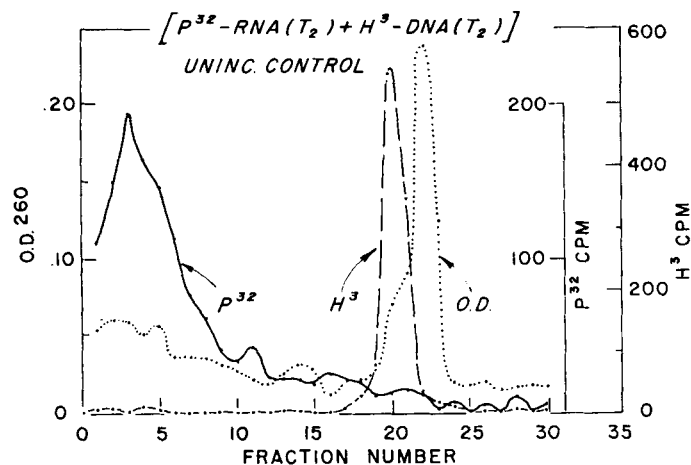


FIG. 2.—Separation of P^{32} -RNA(T2) from H^3 -DNA(T2) by CsCl-gradient centrifugation. A mixture of 6.5 μ g heat-denatured H^3 -DNA, 14 μ g P^{32} -RNA and 25 μ g undenatured, unlabeled T2 DNA was made at 25°C, immediately diluted with CsCl, and then centrifuged for five days at 33,000 rpm.

1. *The effect of temperature during slow cooling on hybrid formation:* The influence of the starting temperature of the slow-cooling process was examined in a number of runs. In all cases, the nucleic acid mixture incubated consisted of 6.5 μ g of heat-denatured H^3 -DNA(T2) and 14 μ g of P^{32} -RNA(T2). The rate and conditions of the cooling were as described earlier.

Three tubes containing this RNA-DNA mixture were placed in the slow-cooling bath at starting temperatures of 65°, 52°, and 40°C respectively. Slow cooling was followed by CsCl gradient centrifugation.

Figure 3 shows the optical density profiles and distributions of H^3 and P^{32} obtained at the three temperatures. Comparison of the profiles of H^3 and P^{32} with those of the control (Fig. 2) shows clearly that in all three cases, slow cooling of the DNA and RNA has produced a new peak of P^{32} approximately centered on the band of H^3 (denatured DNA). This new P^{32} -containing band must contain an RNA-DNA hybrid having approximately the same density as denatured T2-DNA. The amount of complex formed on cooling from the three temperatures was the

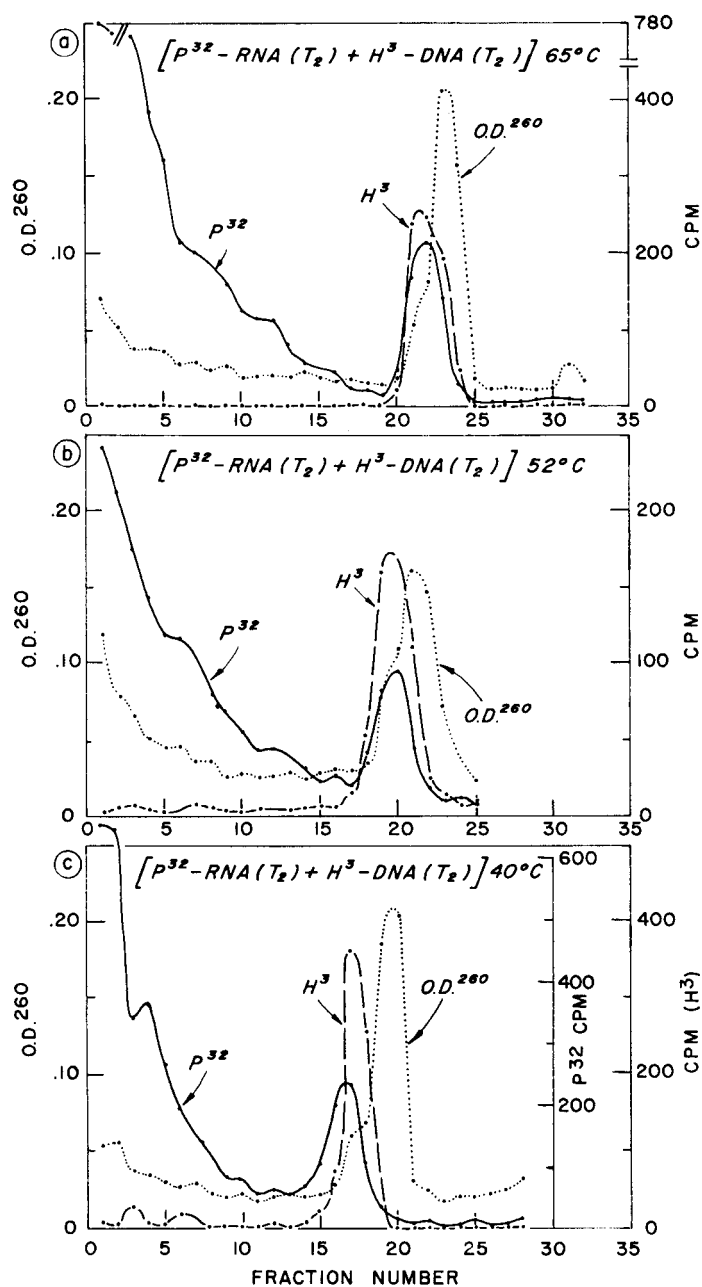


Fig. 3.—Formation of DNA-RNA hybrid at various temperatures. CsCl-gradient centrifugation analysis. P^{32} -RNA(T₂) (14 μ g) and H^3 -DNA(T₂) (6.5 μ g) were mixed in 0.6 ml 0.3 M NaCl and 0.03 M Na citrate, (pH 7.8); then the solution was immediately placed in the slow-cooling bath. Three identical solutions were made; (a) was placed in the bath at 65°, (b) at 52°, and (c) at 40°C. When the bath temperature reached 26°, CsCl and 25 μ g T₂ DNA were added to each solution; then they were centrifuged for five days at 33,000 rpm.

same within experimental error. The three differ slightly in the density of the complex relative to DNA, the complex formed at low temperature being apparently more dense. This may be explained by the occurrence of partial renaturation of the H^3 -DNA at the higher temperatures.

2. *Requirement for presence of single-stranded DNA during cooling:* In order to successfully complex with T2-RNA, the molecules of T2-DNA must be present in the single-stranded state. This was shown by an experiment in which a mixture of native H^3 -DNA(T2) (13 μg) and P^{32} -RNA(T2) (15 μg) was subjected to slow cooling, starting from 40°C . No evidence of hybrid formation is observed (Fig. 4). In a companion run (a repetition of the experiment of Fig. 3c) with denatured H^3 -DNA(T2), approximately 10 per cent of the P^{32} -RNA was included in the hybrid region.

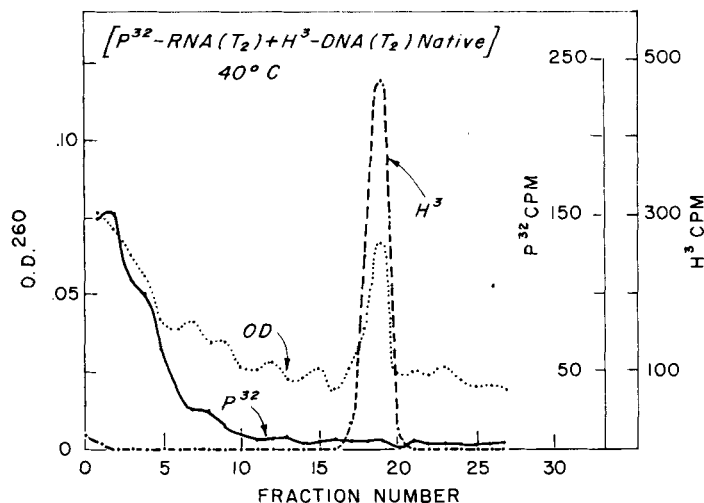


FIG. 4.—CsCl-gradient centrifugation of a slowly cooled mixture of native H^3 DNA (T2) with P^{32} -RNA(T2). 13 μg DNA and 15 μg RNA were mixed in 1.2 ml 0.3 M NaCl, 0.03 M Na citrate, slowly cooled from 40° to 26° , diluted with saturated CsCl solution, and centrifuged.

3. *Stoichiometry of hybrid formation:* Assuming the specific activity of P^{32} to be equal in all RNA molecules, one can estimate the amount of RNA which formed hybrid. From the data of Figure 3, this would be 1.4 μg RNA. (This figure is a maximum value, for some of the RNA which failed to form hybrid may be pre-existing *E. coli* RNA and, therefore, devoid of P^{32} .) The amount of DNA in the hybrid cannot exceed 6.5 μg , the total amount of denatured T2-DNA present. Because the hybrid and denatured T2-DNA have the same density, no more precise estimate can be made. From these considerations, it appears probable that the ratio of DNA to RNA in the hybrid does not exceed 5. That the complex does in fact contain considerably more DNA than RNA is suggested by its density, which is very nearly that of T2-DNA. A further indication that the entire 6.5 μg of T2-DNA has participated in complex formation is the proportionality observed between the amount of hybrid formed and the amount of DNA present when the ratio of DNA to RNA is varied. Experiments completely comparable to those

described by Figure 3 were carried out with the same concentration of P^{32} -RNA(T2) but with $1/5$ the quantity of denatured T2-DNA. In these cases, the amount of P^{32} -RNA found associated with the denatured DNA band was approximately two per cent of the input RNA which compares with the average of ten per cent observed when five times as much T2-DNA is included in the cooling mixture.

4. *On the specificity of the interaction leading to hybrid formation:* Having established the existence of the phenomenon and the conditions required for its occurrence, it became of obvious interest to examine the specificity requirements of hybrid formation. This was tested by carrying out the cooling process with mixtures of P^{32} -RNA(T2) and denatured DNA from heterologous sources. These included DNA of *Ps. aeruginosa*, *E. coli*, and bacteriophage T5. The DNA of the *E. coli* was labeled with H^3 -thymidine whereas the others were unlabeled. The mixtures of P^{32} -RNA(T2) and denatured DNA preparations were subjected to a 52°C slow-cooling incubation under conditions identical to those described for the experiment of Figure 3b. Upon completion of the incubation, unlabeled native DNA was added to each tube as a density marker.

Figure 5 gives the optical density profiles and distributions of radioactivities. There is a suggestion of a very slight peak of P^{32} in the DNA region of the mixture containing *E. coli* DNA. It corresponds to $1/30$ the amount of hybrid produced with T2-DNA in a similar experiment. This may reflect the presence of the small amount of non-infected cells present during the P^{32} -labeling of the material from which the T2-RNA was obtained. However, it is too small to be considered seriously without further investigation. None of the other heterologous mixtures tested yielded detectable amounts of hybrid. It is of interest to note that although T5 has the same over-all base ratio as T2, no evidence of interaction between T2-RNA and T5-DNA was observed.

Interpretation of the Results.—The data presented here show that RNA molecules synthesized in bacteriophage-infected cells have the ability to form a well-defined complex with denatured DNA of the virus. That this interaction is unique to the homologous pair is shown by the virtual absence of such complexes when T2-specific RNA is slowly cooled with heterologous DNA. The fact that T2-RNA and DNA do satisfy the specificity requirement must reflect a correspondence in structure between the two. Structural specificity of this order in single polynucleotide strands can only reside in definite sequences of nucleotides. We conclude that the most likely interrelationship of the nucleotide sequences of T2-DNA and RNA is one which is complementary in terms of the scheme of hydrogen bonding proposed by Watson and Crick.¹⁰

Extension to Other Systems.—The bulk of the RNA in *E. coli* corresponds to the 18S and 25S¹¹ components of the ribosomes. These are metabolically stable,¹² remain firmly attached to ribosome protein at 10^{-4} M Mg^{++} ,¹³ and have a base composition¹⁴ not related in any obvious way to the DNA of the cells. In addition to the lack of correspondence in base ratio, two other reasons can be advanced for doubting the suitability of the large ribosomal RNA molecules for directing the synthesis of proteins as specified by the genetic material. First, the experiments of Riley *et al.*¹⁵ suggest that the intermediary between the genome and the protein-synthesizing mechanism is metabolically unstable. Second, the formation of the larger RNA components is virtually absent in T2-infected cells (cf. Fig. 1) despite

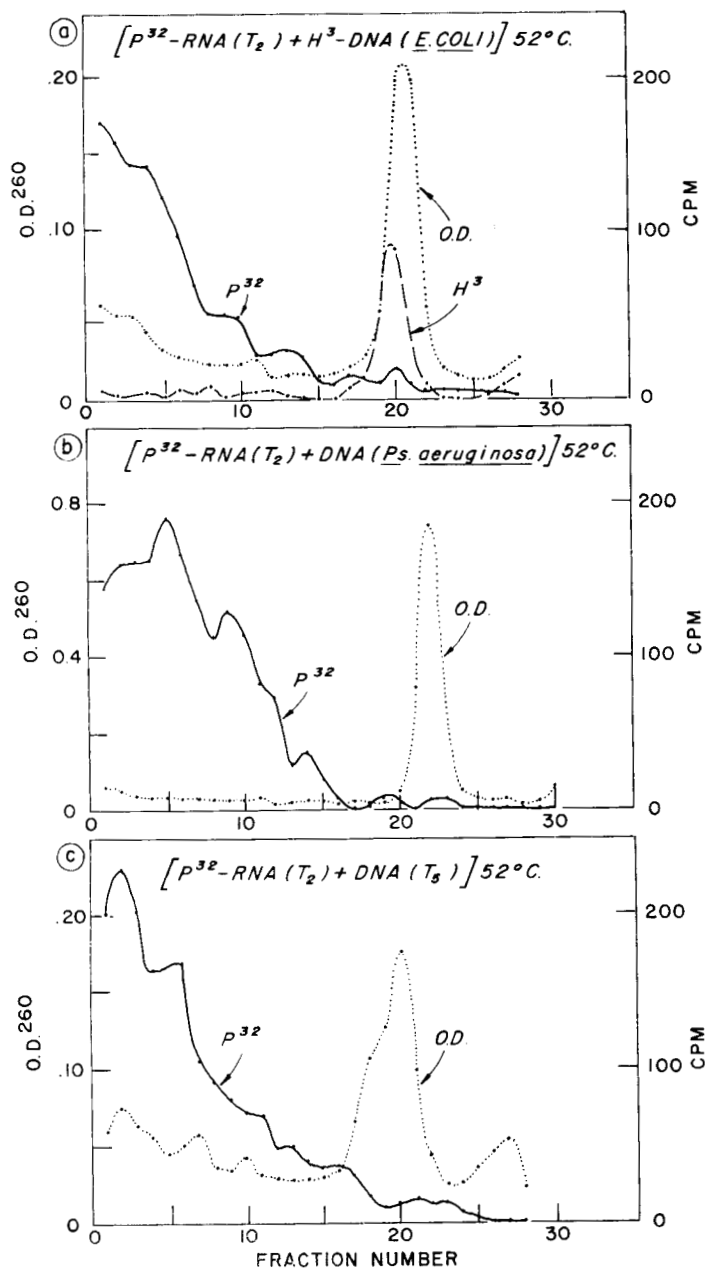


FIG. 5.—CsCl-gradient centrifugation of slowly cooled heterologous DNA-RNA mixtures. (a) P^{32} -RNA(T_2) + H^3 -DNA (*E. coli*), (b) P^{32} -RNA(T_2) + DNA (*Ps. aeruginosa*), (c) P^{32} -RNA(T_2) + DNA (T_5).

In each case, 14 μg P^{32} -RNA and 6.5 μg heat-denatured heterologous DNA were mixed in 0.6 ml 0.3 *M* NaCl, 0.03 *M* sodium citrate and slowly cooled from 52° to 26°C. Before CsCl-gradient centrifugation, 25 μg T_2 DNA was added to (a) and (c) and 25 μg *Pseudomonas* DNA to (b).

the fact that they are actively synthesizing a variety of new protein species.

It seems more likely that the RNA molecules directly concerned with specifying protein synthesis in normal cells would have a base ratio corresponding to DNA and would possess other properties analogous to those found for T2-specific RNA. Its principle characteristics may be summarized as follows:^{1, 3} (1) a weak linkage with the ribosome fractions since it can be broken by dialysis against $10^{-4} M$ Mg^{++} , (2) an active metabolic turnover, (3) an average sedimentation coefficient of about 8S, (4) a base composition which is closely analogous to its homologous DNA (considering thymidine equivalent to uridine and similarly for cytidine and hydroxymethyl cytidine), and (5) a sequence complementary to its homologous DNA.

The detection of the complementary RNA in T2-infected cells was greatly facilitated by the fact that the larger ribosomal components are not synthesized. Indeed, it would appear as if RNA synthesis in the T2-coli complex is largely confined to the class which is complementary to DNA. This advantage is not present in uninfected cells. Consequently the search for normal complementary RNA will be technically more difficult. That it is nevertheless feasible is suggested by the experiments of Yčas and Vincent¹⁶ with yeast. These authors used P^{32} in a manner comparable to the procedures of Volkin and Astrachan and, despite surprisingly long pulses, they were able to detect the formation of a fraction with a high metabolic turnover and possessing a base composition analogous to yeast DNA.

Ultimately, attempts at establishing the presence in normal cells of RNA complementary to the genetic material will require that it be separated from the other RNA components. If all complementary RNA molecules possess physical chemical characteristics analogous to those of T2-specific RNA, the same methods which effected a successful isolation in this case may well serve in others. Once isolated, sequential complementarity to relevant DNA can be examined by the methods described above.

Some Implications of Complementary RNA.—An increasing amount of attention is currently being focused on the possibility of forming hybrid helical complexes composed of DNA and RNA strands. Interest in this originates quite naturally from its obvious implication for translating the genetic information coded in DNA to a functional RNA complement. Previous experiments^{17, 18} had already demonstrated that paired helices were generated in mixtures of the synthetic polyribonucleotides of uridylylate and adenylylate. More recently,^{19, 20} this has been extended to combinations involving synthetic polydeoxyribonucleotides and polyribonucleotides. It is of some interest that the experiments reported in the present paper lend support to the concepts underlying such model experiments by exhibiting hybrid formation between natural polynucleotides which are complementary and biologically related.

The demonstration of sequence complementarity between homologous DNA and RNA is happily consistent with an attractively simple mechanism of informational RNA synthesis in which a single strand of DNA acts as a template for the polymerization of a complementary RNA strand.

Summary.—Experiments are described showing specific complex formation between single-stranded T2-DNA and the RNA synthesized subsequent to infection of *E. coli* with bacteriophage T2. No such hybrid formation is observed with

heterologous DNA even if it has the same over-all base composition as T2-DNA. It is concluded that T2-DNA and T2-specific RNA form hybrids because they possess complementary nucleotide sequences. The generality of the existence of complementary RNA and its possible role as a carrier of information from the genetic material to the site of protein synthesis is briefly discussed.

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