THE OCCURRENCE OF NATURAL DNA-RNA COMPLEXES IN E. COLI INFECTED WITH T2*

By S. Spiegelman, Benjamin D. Hall, and R. Storck†

DEPARTMENTS OF MICROBIOLOGY AND CHEMISTRY, UNIVERSITY OF ILLINOIS

In a previous publication, Hall and Spiegelman[1] showed that RNA-DNA complexes were formed when mixtures of single-stranded T2-DNA and purified[2] "T2-specific RNA" were subjected to slow cooling.[3, 4] It would appear from this that the Volkin and Astrachan[5] observation of a similarity in base composition between T2-RNA and DNA is a reflection of a more profound homology. The fact that hybrid formation was found to be unique to the homologous pair led to the conclusion that the nucleotide sequences of T2-RNA and DNA are complementary in the sense of the hydrogen bonding scheme proposed by Watson and Crick.[6]

An obvious implication of these findings is that the normal process of transferring information from DNA to the protein synthesizing machine involves a mechanism whereby single-stranded DNA serves as a template for the polymerization of a complementary ribopolynucleotide. If continued formation of complementary RNA is a necessary concomitant, it should be possible to find RNA-DNA hybrids in any cell actively engaged in protein synthesis.

The T2-*E. coli* complex was selected as the most suitable system in the initial search for native hybrid. This choice was dictated by the fact that RNA synthesis in a T2-infected cell is virtually confined to the class which is complementary to the viral DNA. The experimental devices employed were in essence similar to those used in the previous[1] investigation on artificially formed complexes. These involved the use of double labeling (H^3 and P^{32}) and equilibrium centrifugation in CsCl gradients[7] employing swinging bucket rotors. T2-DNA was labeled with P^{32} by growth of the virus in a medium containing this isotope. T2-specific RNA was marked with H^3 by introducing H^3-uridine in the early periods of infection. In this experimental setup, the presence of DNA-RNA hybrids would be signalled by the appearance of coincident peaks of the two isotopes. Further, these peaks should occupy a position in the CsCl gradient differing from those which characterize the densities of RNA and double-stranded DNA.

It is the primary purpose of the present paper to present the results of such experiments. The data demonstrate that native hybrids involving DNA and RNA strands do indeed exist.

Materials and Methods.—1. *Growth of cells and conditions of infection:* Cells of *E. coli* B were grown in a synthetic medium (medium C of Roberts *et al.*[8]). As a routine, cells were grown overnight in a glucose limiting medium. They were then harvested, washed, and resuspended in a fresh glucose medium at an O.D.⁵⁶⁰ of 0.270. Incubation was resumed and continued until logarithmic growth was established and an O.D.⁵⁶⁰ of between 0.285 and 0.300 attained. The cell suspensions were then centrifuged and concentrated tenfold in cold medium lacking glucose. A chilled concentrated bacteriophage suspension was then added in an amount to yield a multiplicity of between 10–30. The mixture was then held at 4°C for 30 minutes to allow for adsorption and then diluted tenfold into fresh glucose-synthetic medium prewarmed to 30°C. Aeration was immediately instituted and this moment was taken as the zero time of the experiment. In all experiments reported, cell survivors were less than 0.02%.

2. *H^3-uridine pulses of infected cells:* In the usual experiment, approximately 300 ml of in-

fected cells were pulsed at once. Tritiated uridine (1,600 μc–530 μc per μM) at final concentrations ranging from 0.3 μg–1.0 μg per ml was introduced at the appropriate time and the pulse continued for 3 minutes with vigorous aeration. At the end of this period, the entire suspension was poured on 120 grams of frozen Tris (0.01 M) + NaCl (0.005 M) to stop further incorporation. The cells were harvested by centrifugation, resuspended in 8 ml of the Tris-NaCl buffer, distributed in separate tubes, and frozen to await centrifugal analysis.

3. *Preparation of nucleic acid:* The following procedure was finally adopted for preparing material suitable for the detection of hybrid material in CsCl density gradients. A typical protocol is given. 2.0 ml of frozen cells (equivalent to 80 ml of the original pulsed suspension of infected cells) are thawed, 0.5 ml of 30% sodium dodecyl sulfate added, and the mixture shaken at room temperature for 10 minutes. 0.5 ml of NaClO$_4$ are then added and the shaking continued for 5 minutes. This is followed by the addition of 3 ml of chloroform and 0.3 ml of isoamyl alcohol. The mixture is then shaken for 15 minutes following which it is centrifuged in the Servall swinging bucket rotor (HS) for 20 minutes at 6,000. The top aqueous layer is removed and saved. To the residue are added 2.0 ml of Tris-saline and 0.5 ml of 5 M NaClO$_4$. The resulting mixture is then shaken for 10 minutes, centrifuged and the aqueous layer removed as previously described. The two aqueous layers are combined and again subjected to the deproteinization procedure by adding 6 ml of CHCl$_3$ and 1.2 ml of isoamyl alcohol. The mixture is then shaken, centrifuged again in the swinging bucket rotor (6K15′) and the aqueous layer removed. Two volumes of cold absolute EtOH are then added and the mixture held at 0°C for 10 minutes following which the nucleic acid precipitate is collected by centrifugation at 7,000 rpm for 10 minutes. The resuspended pellet is redissolved in Tris (0.001 M) and saline (0.005 M). After solution is complete, the buffer concentration is brought up to standard strength (0.15 M NaCl, 0.015 M Tris).

4. *Preparation of P³²-labeled T2:* Cells growing logarithmically in glucose-synthetic, low-phosphate medium (0.002 M PO$_4$ and 0.02 M Tris; pH 7.4) were infected with T2 (multiplicity of infection, 0.02) when an O.D⁶⁶⁰ of 0.150 was attained. Simultaneously, 10 mc of P³² were added per liter. The resulting suspension was incubated overnight with shaking at 37°C. After removal of cell debris from the lysate, the virus particles were purified by three cycles of low (3.5K30) and high (12K45) centrifugations. The preparation had 5 × 10⁻⁷ cpm/particle at the outset.

5. *Separation of nucleic acid components by density-gradient centrifugation:* The method of fractionation by CsCl density centrifugation is very similar to that described previously.[1] A layering technique was, however, introduced to cut the centrifugation time required to obtain adequate separation of free RNA from hybrid material. To 0.5 ml of the nucleic acid preparations described in paragraph 3 were added 0.8 gm of solid CsCl. The resulting solution was introduced as a bottom layer in a centrifuge tube containing a CsCl solution of density 1.770. This was accomplished by allowing the sample to run through a "dispo-pipette" to the bottom of the tube. Prior to its removal, the top of the pipette was plugged with a small stopper to eject the last bit of sample and prevent contamination of the upper portion of the tube when the pipette was withdrawn. The tubes were then placed in the SW-39 rotor of the Spinco model L and centrifuged at 33,000 rpm for 60 hours at 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, *ca.* 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-RNA and P³²-DNA:* Counting of the two isotopes was carried out as described previously[1] using trichloracetic acid washing on millipore membranes and the Packard Tri-Carb Scintillation Counter which permits counting of P³² and H³ in the same sample.

Results.—A variety of procedures was surveyed for obtaining material suitable for the reliable detection of DNA-RNA hybrids by CsCl density equilibrium centrifugations. These included methods (e.g. freezing and thawing in the presence of lysozyme[9, 10]) of cell rupture and fractionation which conserve the DNA and permit its isolation as a relatively intact fraction. However, none of these was reproducibly successful. Ultimately, attempts at preliminary fractionation of cell components were abandoned. Attention was focused on the use of detergents which lysed cells and simultaneously inactivated the enzyme activity of the lysate. It

was empirically established that removal of most of the protein was a necessary step prior to introducing the material into the CsCl. In the absence of this preliminary purification, much of the nucleic acid, including hybridized material, was trapped in the protein layer found floating at the top of the gradient.

The procedure described in *Methods* (section 3) was found to yield highly reproducible results. It consists essentially of the first few deproteinization stages employed for preparing[11] DNA from bacterial cells. Two modifications were made. None of the steps designed to remove RNA is included, and at the alcohol precipitation stage, centrifugation, rather than winding around a glass rod, is used to collect the nucleic acid. Such DNA preparations are, of course, heavily contaminated with RNA (50% and more) but served well the purposes of the present experiment.

The conditions used in the current investigation (synthetic medium, 30°C) were such as to yield a latent period of 35–40 minutes and an onset of DNA synthesis sometime after 15 minutes. It is known[12, 13] that some uridine ends up in the DNA once its formation begins in the T2-*coli* B complex. To avoid the complications which this would introduce in identifying radioactive peaks, experiments designed to detect hybrid formation were confined to the period (2–5 minutes) when no DNA synthesis can be detected.

In experiments which employ P^{32}-labeled T2-DNA and tritiated uridine as the RNA label, existence of hybrid would be detected as a tritium peak in the DNA region, somewhat heavier than the P^{32} peak corresponding to the T2-DNA. The P^{32}-peak should exhibit signs of bi-modality as evidence of a distribution of the T2-DNA between hybrid and unhybridized T2-DNA.

Several independent experiments were performed with essentially similar results. Figures 1 and 2 describe the results of two typical profiles obtained by subjecting nucleic acid preparations from different pulses to equilibrium gradient centrifugation. The general conditions of infection, pulsing, preparation of nucleic acid and separation in the CsCl gradients are all as described under *Methods*. Detailed differences are found in the legends. Three peaks are readily discernible. The lightest one corresponds to the input P^{32}-labeled DNA of T2. The optical density peak identifies the position of double-stranded *E. coli* DNA put in as a marker. The tritium peak locates the hybrid. It will be noted that in each instance one observes the existence of a shoulder on the heavy side of the P^{32} profile corresponding in position to the tritium peak region. The relative positions of the peaks and the bi-modality of the P^{32} distribution are all consistent with what would be predicted from the existence of a DNA-RNA hybrid.

If we assume that a small amount of T2-DNA can be synthesized even in this early period of infection, another interpretation of the optical density and radioactive profiles can be entertained. This would presume the appearance of new T2-DNA, all in the form of single-stranded material, along with the conversion of a portion of the input P^{32}-labeled DNA to single strands. To test for this possibility, the alkali lability of the H^3 and P^{32} in relevant fractions of a number of experiments was examined. The same results were obtained in all cases and are exemplified by the data summarized in Table 1. Here, the two fractions (7 and 26) indicated by arrows in Figure 2 were subjected to alkali digestion and the resultant effect on the acid precipitability of H^3 and P^{32} examined. Fraction 7 was included as a free RNA control. It is evident from the data in Table 1 that all of the H^3
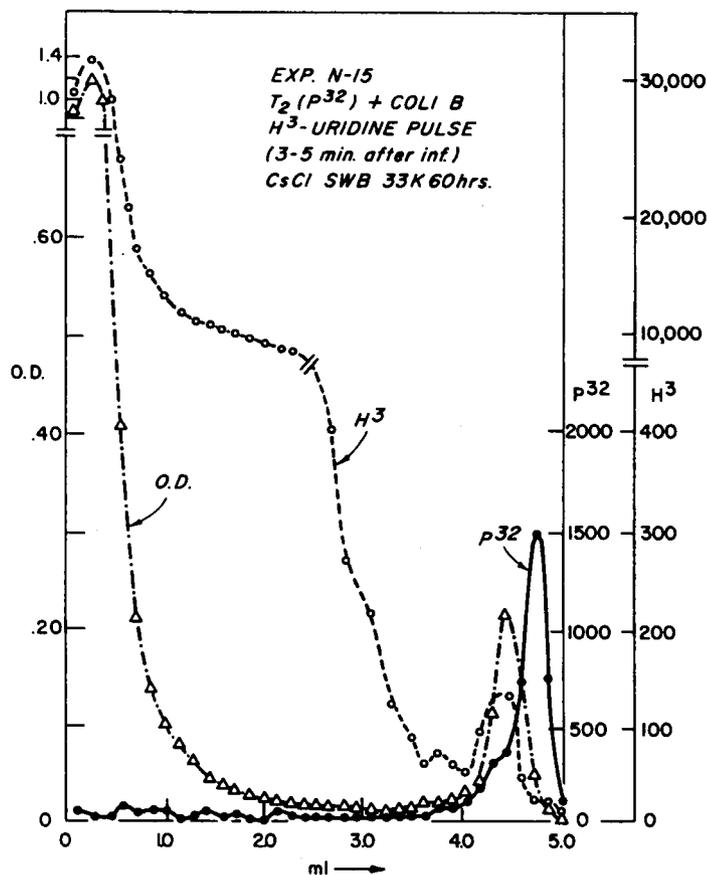
Fig. 1.—Adsorption of phage was carried out at a multiplicity of 45. P^{32}-labeled phage diluted 2-fold with unlabeled virus. Pulsing was done between 3–5 minutes after 0 time with H^3-uridine having a specific activity of 1,600 $\mu c/\mu M$ at a level of 0.3 $\mu g/ml$. All other details are as described under *Methods*. Note that the radioactivity scale is expanded in the hybrid region.

TABLE 1

EFFECT OF ALKALI DIGESTION ON ACID PRECIPITABILITY

Fraction No.	Isotope	Acid Precipitable Counts/ml Control	After alkali treatment
26	H^3	230	5
	P^{32}	735	720
7	H^3	1950	10
	P^{32}	. . .	. . .

0.4 ml aliquots of Fractions 7 and 26, indicated by arrows in Fig. 2, were made 0.3 N with respect to NaOH and incubated for 24 hours at 30°C. Equivalent aliquots were held as controls under the same conditions. Following the incubation, the alkali was neutralized, carrier herring sperm DNA added, and the contents precipitated with TCA; washing and counting was then carried out as described under *Methods*.

and none of the P^{32} counts are alkali labile. These data appear to eliminate the possibility that the displaced tritium peak can be ascribed to newly synthesized single-stranded DNA. It is also evident that the displaced P^{32} cannot be ascribed to a conversion of the input viral DNA to an RNA polynucleotide. We may, there-
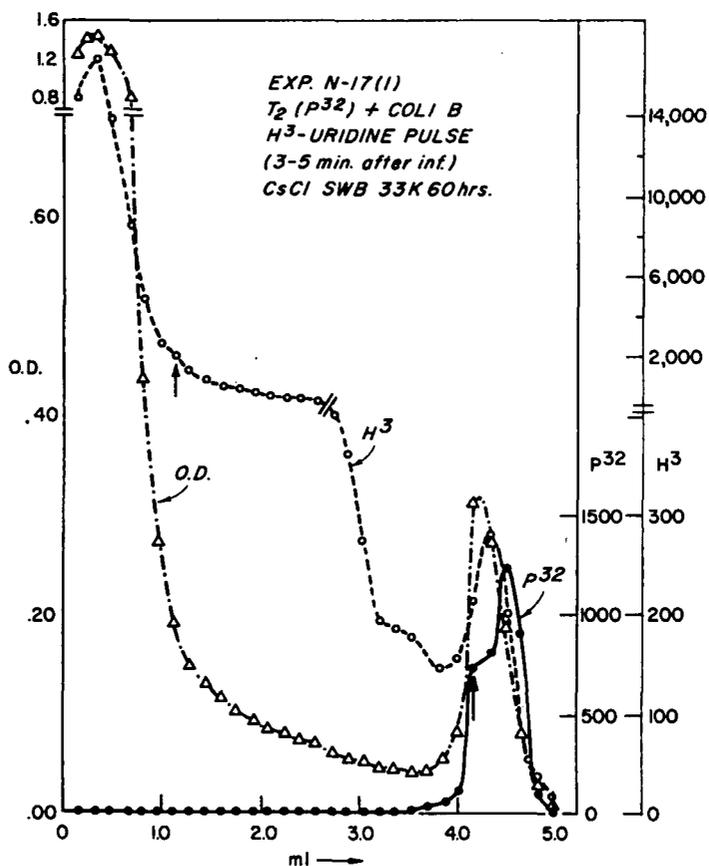
Fig. 2.—Adsorption of phage was carried out at a multiplicity of 15 of undiluted P^{32}- labeled virus. Pulsing was done between 3-5 minutes after 0 time with H^3-uridine having a specific activity of 530 $\mu c/\mu M$ at a level of 1 $\mu g/ml$. All other details are as described under *Methods*. Note that the radioactivity scale is expanded in the hybrid region.

fore, conclude that a hybrid region exists containing newly synthesized T2-specific RNA complexed with some of the input P^{32}-labeled DNA.

Discussion.—The existence of native DNA-RNA hybrids in T2-infected *E. coli* is consistent with the assumption that DNA serves as a template for the synthesis of complementary informational RNA. This view would require the existence of a DNA-dependent enzymatic mechanism for synthesizing polyribonucleotide. Evidence suggesting the presence of such a pathway emerged from a study[14] of cell-free preparations derived from *E. coli* which possessed considerable capacity to synthesize polyribonucleotide. The observed synthetic activity exhibited a requirement for riboside tri-phosphates and Mn, and was sensitive to DNAase. More recently a number of laboratories[15-17, 20] have independently achieved considerable purification of an RNA polymerase capable of synthesizing polyribonucleotide from ribose triphosphate in the presence of DNA. Furthermore, the base ratio of the polyribonucleotide synthesized bears a striking homology to that of the DNA used as a primer.[18, 19] It would appear from the preliminary reports available that considerable information will soon be available on the enzymological

aspects of DNA-mediated RNA synthesis. It will be of obvious interest to see whether hybrid formation is the first step in this process.

The experiments described in the present paper were designed primarily to detect the presence of native RNA-DNA hybrids. The use of isotopic labeling combined with CsCl gradients in swinging buckets provides a sensitive method for the certain detection of hybrid even when it is present in minute quantities. However, these methods are too laborious to permit the rapid accumulation of the kind of detailed quantitative and kinetic information we would like to possess. Other more efficient and less time-consuming methods for both the detection and estimation of hybrid material will undoubtedly be developed as we learn more of its nature. It may perhaps be of value for further efforts if we summarize briefly here the experience we have thus far accumulated with the T2-*E. coli* complex. As determined by their relative position, the densities of the natural hybrids are very similar to those formed artificially by the slow cooling process. In seven experiments involving pulses between 3 and 5 minutes after infection, the proportion of the newly synthesized RNA found in the hybrid region ranged between 1 and 0.1 per cent. As measured by the amount of P^{32} shifted into the hybrid region, the proportion of input DNA hybridized varied from 15–30 per cent in all cases where approximations could be made.

Our experience with other stages of infection are not as extensive. However, it may be noted that hybrids involving the input DNA were observed in pulses covering both 9–12 minutes and 19–22 minutes after infection.

Summary.—Experiments are described exhibiting evidence for the existence of natural DNA-RNA hybrids in *E. coli* cells infected with T2. The use of double labeling (P^{32}-DNA and H^3-RNA) coupled with equilibrium centrifugation in CsCl gradients in swinging bucket rotors permitted the isolation and identification of the hybrid regions.

[1] Hall, B. D., and S. Spiegelman, these PROCEEDINGS, **47**, 137 (1961).

[2] Nomura, M., B. D. Hall, and S. Spiegelman, *J. Molec. Biol.*, **2**, 306 (1960).

[3] Marmur, J., and D. Lane, these PROCEEDINGS, **46**, 453 (1960).

[4] Doty, P., J. Marmur, J. Eigner, and C. Schildkraut, *op. cit.*, **46**, 461 (1960).

[5] Volkin, E., and L. Astrachan, *Virology*, **2**, 149 (1956).

[6] Watson, J. D., and F. H. C. Crick, *Nature*, **171**, 964 (1953).

[7] Meselson, M., F. Stahl, and J. Vinograd, these PROCEEDINGS, **43**, 581 (1957).

[8] Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten, *Studies of Biosynthesis in E. coli* (Washington: Carnegie Institution of Washington, 1957), p. 5.

[9] Kohn, A., *J. Bacteriol.*, **79**, 697 (1960).

[10] Hall, B. D., R. Storck, and S. Spiegelman, *J. Biophys.* (in press).

[11] Marmur, J., *J. Molec. Biol.* (in press).

[12] Cohen, S. S., H. D. Barner, and J. Lichtenstein, *J. Biol. Chem.* (in press).

[13] Ben-Porat, T., "RNA Metabolism in Phage Infected Protoplasts," Doctoral Thesis, University of Illinois (1959).

[14] Spiegelman, S., *Recent Progr. Microbiol.*, **7**, 82 (1959).

[15] Weiss, S. B., these PROCEEDINGS, **46**, 1020 (1960).

[16] Hurwitz, J., A. Bresler, and R. Dinger, *Biochem. Biophys. Research Commun.*, **3**, 15 (1960).

[17] Stevens, A., *op. cit.*, **3**, 92 (1960).

[18] Furth, J. J., J. Hurwitz, and M. Goldmann, *op. cit.*, **4**, 362 (1961).

[19] Weiss, S. B., and T. Nakamoto, these PROCEEDINGS, **47**, 694 (1961).

[20] Ochoa, S., D. P. Burma, H. Kröger, and J. D. Weill, *op. cit.*, **47**, 670 (1961).