

ing order. The ordinates show the mean suppression ratio during a given tone. This index compares the number of responses (tone R's) made in the 40-second tone period to the number of responses (pre-tone R's) made in the 40-second period prior to presentation of the tone.

Numerically, the relationship is expressed as a ratio:

(pre-tone R's — tone R's) / (pre-tone R's)

As can be seen in Fig. 1, during the earlier tests, generalization of suppression was broad at first, but as testing proceeded the gradients narrowed. In general, the interruption produced very little effect on the course of the gradients. For bird No. 21 the 1000 cy/sec tone exhibited no loss whatsoever, and the decrements for the other tones are no larger than would be expected had testing proceeded without interruption. The results of bird No. 9 are similar except that for this bird the tones on the wings of the gradient had ceased to cause suppression prior to the interruption.

An unexpected feature of the results is the finding that during the terminal stages of testing a disproportionately large number of negative ratios was obtained for tones on the wings of the gradient. For example, during the final eight sessions for bird No. 9 and during the final four sessions for bird No. 21 the several stimuli on the wings of the gradient yielded more than twice as many negative ratios as positive ratios. A *t*-test on the difference between the number of responses in the tone periods and in the pre-tone periods for these stimuli yielded  $t = 2.156$  with 23 degrees of freedom for bird No. 21 and  $t = 2.785$  with 47 degrees of freedom for bird No. 9. Since values of  $t$  as large as this would occur with  $p < .05$  on the basis of random sampling, we concluded that the observed tendency for the ratios to dip below zero represents a reliable effect. Apparently a stage was reached during the final phases of the extinction procedures in which many of the tones tended to facilitate pecking. Unfortunately we do not yet understand the mechanisms which underlie this curious phenomenon.

The main features of these results, however, are consistent with results of several other studies which show that conditioned responses are retained over long periods (5). In this respect, our results provide a dramatic illustration of

the proposition that the mere passage of time plays little, if any, role in the elimination of the negative effects of aversive training (6).

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### Homology Test between the Nucleic Acid of an RNA Virus and the DNA in the Host Cell

**Abstract.** Deoxyribonucleic acid in the host cell does not contain a sequence complementary to the nucleic acid of an RNA virus. Specific formation of hybrid between deoxyribonucleic acid and ribonucleic acid was used as a detecting device. The test was internally controlled and sensitive enough to reveal complementary stretches in the DNA corresponding to 10 percent of the viral ribonucleic acid. The implications of the results for replication and transcription of RNA are discussed.

In the last several years there has been an accumulation of data consistent with the concept that polyribonucleotides are the informed intermediaries between DNA-genomes and the cellular entities which carry out their instructions (1, 2). However, the problems posed by the existence of viruses which use RNA for the transmission and storage of genetic information remain for detailed resolution.

A definitive decision on the existence or nonexistence of homology between viral RNA and the DNA from its host would aid in delineating the mechanisms of replication of RNA and

transcription from an RNA genome.

For technical convenience the system chosen was *Escherichia coli* (Hfr strains, K-16 and K-10) and an RNA bacteriophage related to the one (f2) discovered by Loeb and Zinder (3). The virus used, MSφ2, (4) had biological, chemical, and physical properties similar to those reported for f2 (5).

A decision on whether or not there is homology between two nucleic acid molecules rests essentially on a comparison of base sequences. In practice what can be readily detected is sequence complementarity (6, 7). Experimentally, then, the question we seek to answer may be posed as follows: Does the DNA found in the host cell contain, either before or after infection, a sequence complementary to the nucleic acid of an RNA virus?

One answer to questions of complementarity is provided by specific hybrid formation. A sensitive test for hybridization of RNA and DNA was developed by Hall and Spiegelman (8) to demonstrate that the RNA synthesized in *E. coli* after infection by the bacteriophage T2 is complementary to the DNA of the virus rather than to that of the host. Equilibrium density centrifugation in swinging bucket rotors combined with isotopic labeling was used to identify the hybrid structures. The same procedures were successfully used to exhibit complementary RNA synthesized in cells during "step-down transitions" (9) or short pulses (2), as well as RNA formed in vitro by the DNA-dependent RNA polymerase (10).

The investigations just cited tested heterogeneous populations of RNA which represent between 10 and 100 percent of the available sequences in DNA. Consequently, the detection of hybrids did not strain the available sensitivity. However, the numerical situation inherent in the present problem is far less favorable. It demands finding one particular sequence in the DNA equivalent in length to the viral RNA. Since the molecular weight of the viral RNA is about  $8 \times 10^5$ , one must detect complexes including approximately 0.005 percent of the total length of *E. coli* DNA. However, a similar numerical difficulty was overcome by Yankofsky and Spiegelman (11, 12) when they established the existence of sequences in DNA complementary to sequences in homologous ribosomal RNA. These experiments

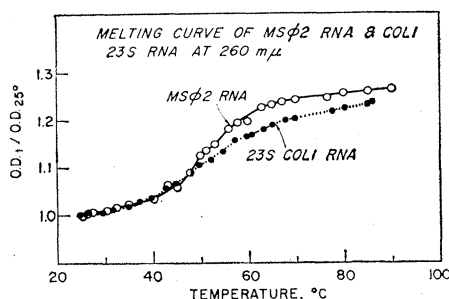


Fig. 1. Hyperchromic response of ribosomal and viral RNA to heat. Solutions containing nucleic acid ( $20 \mu\text{g/ml}$ ) in  $0.01M$  phosphate at  $pH$  6.8 were used. These were contained in closed quartz cuvettes held in a block wired to permit controlled electrical heating and monitored by a reference cell containing a thermistor. The block fits into the holder of the Opticon spectrophotometer which was used for these measurements. The ratios of the optical density (O.D.) at  $260 m\mu$  at the ambient temperatures ( $O.D.^\dagger$ ) to that at  $25^\circ\text{C}$  are plotted.

were designed so that hybrids including between 0.001 and 0.01 percent of the total DNA were readily detected. The necessary sensitivity was achieved by labeling the RNA to the required specific activity. Confusion with "noise" either in the form of mechanical trapping or chance coincidence in complementarity over short regions was avoided by use of the resistance of true hybrids to degradation with ribonuclease. Non-specific complexes are completely sensitive.

Our experience with the ribosomal complexes of RNA and DNA encouraged us to undertake the present study with viral RNA. It seemed highly probable that a definitive answer to homology, whether positive or negative, was experimentally attainable.

A further element of assurance was introduced into these experiments as an internal control. Ribosomal-23S and viral RNA are comparable in molecular weights ( $1.0 \times 10^6$  vs.  $0.8 \times 10^6$ ) and base composition (53 percent vs. 52 percent guanine-cytosine). These resemblances are reflected in their hyperchromic shift due to heat, as shown in Fig. 1. Since the responses of these two molecules to thermal disruption of their secondary structures are very much alike, their behavior during the annealing process should also be similar. Consequently, in addition to serving as a test for the sensitivity of hybrid detection, ribosomal RNA can also be used to monitor the adequacy of the hybridization test conditions. Thus, under conditions which yield

hybrids with ribosomal RNA, the absence of such complexes with viral RNA can be accepted with comparative confidence as evidence that there is no homology between viral RNA and cellular DNA.

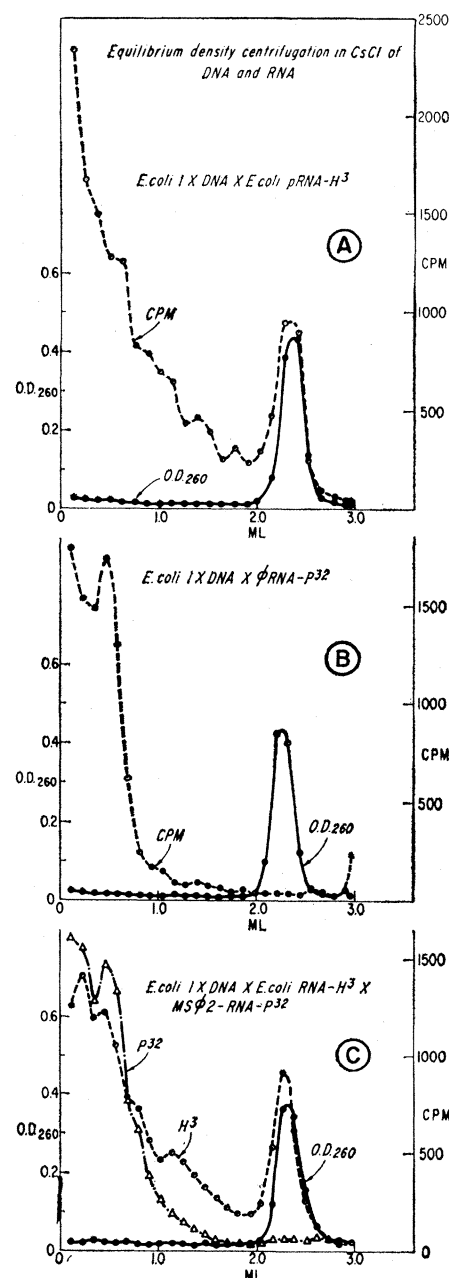
Isolated and purified DNA (13), purified  $H^3$ -23S RNA (40,000 count/min per  $\mu\text{g}$ ) (11), heat denatured DNA (11), and purified  $P^{32}$ -phage RNA (40,000 to 360,000 count/min per  $\mu\text{g}$ ) (14) were prepared. The relative specific activities of the ribosomal and viral RNA molecules were deliberately adjusted in some of the experiments to make the detection of hybrids with viral RNA 10 times more sensitive. The usefulness of two different labels will become apparent. The procedures of Hall and Spiegelman (8) for formation of hybrids, and their separation in CsCl gradients, were followed. Pertinent experimental details are found in the legends.

Results representative of a number of experiments are illustrated in Fig. 2. The top figure (2A) shows the usual hybrid structure which results when ribosomal RNA is incubated with homologous single stranded DNA. Figure 2B describes the completely negative outcome observed when viral RNA

Fig. 2 (right). Equilibrium density gradient centrifugation of incubated mixtures of RNA and DNA. In all cases the peak in O.D. identifies the position of DNA in the density gradient which decreases from left to right. In addition  $50 \mu\text{g}$  of heat denatured DNA of *E. coli*, the annealing mixtures contained the following: (A)  $2 \mu\text{g}$  of tritium- $(H^3)$  labeled ribosomal RNA (40,000 count/min per  $\mu\text{g}$ ); (B)  $2 \mu\text{g}$  of  $P^{32}$ -labeled viral RNA (85,000 count/min per  $\mu\text{g}$ ); (C) a mixture of A and B. The incubation solution was  $0.3M$  in NaCl, and  $0.05M$  in phosphate at  $pH$  6 to 8; the total volume was  $0.5 \text{ ml}$ . The mixtures were slow-cooled from  $55^\circ\text{C}$  to  $30^\circ\text{C}$  over a period of approximately 17 hours. The density was then adjusted to  $1.720$  with CsCl and the final volume was adjusted to  $3.0 \text{ ml}$ . The resulting samples were centrifuged for 72 hours at  $33,000 \text{ rev/min}$  at  $25^\circ\text{C}$  in the SW39 rotor of the Spinco model L ultracentrifuge. At the end, fractions of  $0.12 \text{ ml}$  were collected from the bottom of the tubes, diluted, and analyzed for optical density at  $260 m\mu$  and for radioactivity. Radioactivity of the acid insoluble fraction was assayed on aliquots, washed and dried on Millipore membranes, and counted in the Packard liquid scintillation spectrometer which measures simultaneously  $H^3$  and  $P^{32}$ . Optical Density at  $260 m\mu$  was measured on the whole sample; the radioactivity was assayed on a fraction. The activity given must be multiplied by 5.6 to convert to the activity of the total sample.

is substituted for ribosomal RNA in the reaction mixture. Since the two RNA molecules were labeled with different isotopes, they could be included in the same reaction mixture; and the extent of hybridization of the DNA with each RNA determined in the presence of the other. Figure 2C describes the results of such an experiment. Here one sees that the  $H^3$ -ribosomal RNA formed a hybrid structure and the  $P^{32}$ -labeled viral RNA did not.

Similar experiments have been carried out under a variety of other conditions with identical results. In some experiments the specific activity of the viral RNA used was 360,000 count/min per microgram. Thus, if one sequence complementary to viral RNA



existed per genome, 1800 count/min would have been observed in the DNA density region of the gradient and none were found. Other variations included raising the initial temperature to 65°C in the annealing process, increasing viral RNA to amounts five times above saturation for ribosomal RNA, a preliminary heat denaturation of viral RNA, and the use of DNA preparations from a variety of strains of *E. coli*. Finally, DNA was isolated from host cells after infection at various intervals from 20 to 45 minutes. No hybrid formation was detected with any of these preparations. The absence of hybrid formation could not be ascribed to the fact that only one of the two possible complementary RNA strands was being tested. The DNA which was being challenged contained both strands and one of the two would have satisfied the condition of complementarity, if it existed.

It is evident that neither before nor after infection can one find sequences in the DNA which are complementary to the viral RNA. In evaluating these negative findings several features of these experiments must be recalled. First, the hybridization process was monitored internally. Second, the relative specific activities in counts per minute was such that the sensitivity per strand of detecting hybrids with viral RNA was ten times that possible with the ribosomal RNA. Finally, the procedure used was adequate to detect, with ease, complementary stretches in the DNA corresponding in length to 10 percent of the length of viral RNA.

These results have a number of interesting implications. They obviously lend no support to the rather attractive speculation that the RNA viruses might represent "escaped" genetic messages of the host. The data do suggest that RNA viruses do not employ the "DNA to RNA to protein" path of information transfer. This in turn implies that the viruses have evolved a mechanism of transcription and replication at the level of RNA. We would then predict the existence of an enzymatic mechanism involving an RNA-dependent RNA polymerase. It seems highly unlikely that an enzyme of this sort pre-exists in the cell. All recognized nucleic acid components, including "informational" RNA (9), ribosomal RNA (11, 12), and RNA which transfers amino acid (15) have been shown to be complementary to some sequences in homologous DNA. Further, actinomycin-D, which inhibits the DNA-dependent

RNA polymerase (16), prevents synthesis of RNA in both bacterial (17) and animal cells (18) but does not inhibit production of RNA virus (18). One must conclude, therefore, that the incoming viral RNA contains the structural program for this new polymerase. Since this enzyme must be synthesized before replication, the viral RNA must be conserved during its translation into protein (19). Results of experiments confirming some of these predictions are in preparation for publication.

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## Photographic Cytophotometry with a Dual Microscope

**Abstract.** A modified comparison microscope assembly was developed for quantitative cytochemical analysis of stained or naturally pigmented objects. Test and control objects were photographed simultaneously on the same frame of color film. Pigments in the film image were then extracted and analyzed in a recording spectrophotometer. The total procedure is an extension and simplification of existing methods.

Cellular or tissue inhomogeneity was first recognized by Caspersson as a major obstacle in microscopic photometry (1). In succeeding early work, the problem demanded tedious photographic densitometry, now replaced by two modern techniques involving use of photoelectric receivers. Complicated scanning-recording instrumentation permits area measurements and yields total extinctions (2). Less complicated apparatus is satisfactory for spot measurements (3) which meet certain inhomogeneity situations if the "two-wavelength method" is applied (4). As a simple solution to distributional error, Ornstein also suggested a novel photographic method in which absorption is measured in terms of reduced silver in a specially prepared positive image. It is the purpose of this report to describe an assembly which exploits the photographic method, uses color film, and greatly simplifies the technique.

There are two fundamental opera-

tions in the entire procedure (Fig. 1). The details are described in terms of the instrumental components as they now exist.

The light source is a Mikrark 100-watt zirconium arc (Sylvania C-100/DC) whose power supply is operated from a Sola constant-voltage transformer through a variable transformer that is usually set for 115 volts. Köhler illumination is strictly maintained and is attenuated only by neutral density filters, with or without a Corning blue filter (5800, melt 528).

The beam-splitter is a complex of one half-aluminized optical flat (Bausch and Lomb No. 45-10) and three first-surface mirrors. The mirrors are mounted on double ball-and-socket clamping joints.

The triocular microscopes (Bausch and Lomb Dynazoom) have apochromatic objectives, compensating eyepieces, achromatic centering condensers (1.40 numerical aperture), and first-surface mirrors. Visual and photographic coupling is effected by a Bausch and Lomb comparison eyepiece between the vertical microscope tubes. Specific design of the microscopes is not critical, although focusing by movement of the stage eliminates the eyepiece binding that occurs with movements of the body tube.

Photomicrographs were made on