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## *FORMATION OF A DNA-SOLUBLE RNA HYBRID AND ITS RELATION TO THE ORIGIN, EVOLUTION, AND DEGENERACY OF SOLUBLE RNA*

BY HOWARD M. GOODMAN AND ALEXANDER RICH

DEPARTMENT OF BIOLOGY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY

*Communicated by Paul Doty, September 25, 1962*

It has been known for a long time that transfer or soluble RNA (sRNA*) molecules play a central role in the organization of amino acids into polypeptide chains during protein synthesis. Individual sRNA molecules combine with a particular amino acid to produce a complex which is active on the ribosomal particle. Recent experiments[1] make it likely that a sequence of nucleotides in sRNA carry the specificity for determining the position of the amino acid in the polypeptide chain. However, as yet little is known regarding the origin of sRNA. These molecules could arise from DNA in a manner similar to the production of messenger RNA. On the other hand, it has been demonstrated that the sRNA molecule is largely folded back upon itself with a regular system of hydrogen bonding,[2] and this has

given rise to the suggestion that the RNA may act as a template for manufacturing itself.[2, 3] These alternative possibilities have prompted us to carry out a series of experiments in which we look for the presence of a complementary sequence of bases in the DNA molecule by the formation of specific hybrids involving sRNA. This was stimulated by the work of Hall and Spiegelman,[4] who demonstrated that specific hybrids can be formed between T2 DNA and the RNA which is synthesized in *E. coli* during the T2 viral infection. In these experiments, the hybrids were formed by a heating and slow cooling process such as was first used by Doty, Marmur, and co-workers[5a] in their experiments on DNA. If hybrids are formed, the material can then be isolated through the use of density gradient centrifugation.

An important property of a DNA-RNA hybrid is the resistance which the RNA shows to ribonuclease digestion. This was first demonstrated by Schildkraut *et al.*[5b] using synthetic polynucleotides and has been employed by Yankofsky and Spiegelman[6] in their recent work on hybrid formation between ribosomal RNA and DNA. This property can be used to differentiate true hybrid formation involving systematic hydrogen bonding in a helical array from false hybrid formation such as may arise from "tangling" or other nonspecific interactions. In the work reported here, we have demonstrated the formation of *E. coli* sRNA-DNA complexes and, by saturating all the sRNA sites on the DNA, we have been able to obtain an estimate of their number. In addition, we have carried out a series of cross hybridizations between *E. coli* sRNA and a variety of bacterial DNAs. This is a technique for learning something about the persistence of the sRNA nucleotide sequence in other bacterial species which may be close or distant relatives of *E. coli*. Thus, the results tell us something about the evolutionary history of the sRNA molecules.

*Methods and Materials.—Preparation of DNA and $P^{32}$ sRNA:* An overnight culture of *E. coli* strain B was grown in H medium.[7] A portion of the culture was added to sterile H medium containing varying amounts of carrier-free $NaHP^{32}O_4$ (0.05 mc/ml, 0.5 mc/ml, 0.25 mc/ml in separate experiments) to bring the bacterial titer to 3–5 $\times$ $10^7$/ml. The flask was incubated at 37°C in a rotary shaker bath. When a bacterial density of 5 $\times$ $10^8$/ml was reached, the culture was flooded with unlabeled inorganic phosphate and the cells were allowed to grow for an additional 2–3 generations. After the cells were harvested and washed, sRNA was isolated by a phenol extraction of the whole cells.[8] The phenol-extracted sRNA was then carried through a further purification procedure using a methylated albumin column as described by Sueoka and Cheng.[9] Three different preparations were made in this way which had specific activities at the time of isolation of 1.4 $\times$ $10^5$, 1.2 $\times$ $10^6$, and 1.1 $\times$ $10^6$ cpm per $\gamma$ of sRNA respectively.

The unlabeled bacterial DNAs used in this investigation were prepared by the method of Marmur.[10] Calf thymus and salmon sperm DNA were obtained from Sigma Chemical Company and California Corporation for Biochemical Research, respectively. The DNAs from the bacteriophages were prepared by phenol extraction.[11] Prior to annealing, the DNA was denatured by heating at 95–98°C for 15 min in 0.015 *M* NaCl, 0.0015 *M* sodium citrate, pH 7.4, and then quickly chilled in an ice bath. Denaturation was followed by measuring the change in optical density at 260 m$\mu$.

*Annealing of sRNA to DNA and density gradient centrifugation:* A variety of annealing experiments was carried out to determine optimal conditions. The following standard procedure was then adopted: 1 ml of 0.25 *M* NaCl, 0.015 *M* sodium citrate, pH 7.4, contained 45 $\gamma$ of heat-denatured DNA and varying amounts of $P^{32}$-labeled sRNA. This solution was sealed in a glass ampule, quickly heated to 70°C, and then maintained at this temperature for 2 hr, after which it was slowly cooled over a 15-hr period to room temperature. By this means, the solution was kept at a temperature over 50°C for approximately 8–10 hr. This solution was layered below 3 ml of cesium chloride solution of density 1.72 in a 5-ml Lusterloid centrifuge tube and then spun in a

Model L ultracentrifuge (35,000 rpm, 65 hr, 25°C). Using this layering procedure, the lighter DNA and attached sRNA rises in the tube as the gradient is established, leaving the residual sRNA at the bottom. At the termination of the centrifugation, the bottom of the tube was pierced and drops were collected to divide the entire tube into approximately 35 fractions. Each fraction was diluted to 1.1 ml with distilled water and its optical density read at 260 mμ. The fraction was then split and part saved for ribonuclease digestion. To the remaining portion of the fraction 0.1 mg of carrier bovine serum albumin was added, and the nucleic acid plus carrier was precipitated with TCA (final concentration 5%). The precipitate was collected on Millipore filters, (0.45 μ pore size), air-dried, and counted in a Nuclear-Chicago low-background counter.

*Ribonuclease digestion:* A standard procedure was adopted for ribonuclease digestion. The solution contained 0.18 *M* CsCl, $5 \times 10^{-3}$ *M* $MgCl_2$, 0.01 *M* Tris buffer, at pH 7.4, and 3.5 γ/ml of ribonuclease (crystalline pancreatic ribonuclease obtained from Worthington Biochemical Corporation). The incubations were carried out for 1 hr at 37°C. In the kinetic hydrolysis experiments, aliquots were removed from the reaction mixture at various time periods after the addition of ribonuclease and the digestion stopped by precipitation with TCA. The samples were then plated and counted as previously described.

*Base ratio analysis:* The $P^{32}$-labeled sRNA plus nonradioactive carrier sRNA prepared by the phenol method were hydrolyzed in 0.2 *M* KOH at 80° C for 45 min. The cooled hydrolysate was spotted on carboxymethylcellulose paper (Whatman CM50), and the nucleotides were eluted with distilled water. After suitable concentration, the hydrolysate was applied onto Whatman 3 MM filter paper and electrophoresis was carried out in 0.05 *M* citrate buffer, pH 3.4. The nucleotide spots were identified by their mobility, and the base ratios were determined by counting $P^{32}$ on the paper. Pseudouridylic acid, a minor component of sRNA, was isolated on a Dowex-1 formate column as described by Cohn.[12]

*Results.*—It has been shown that DNA hybrids can be formed both by messenger RNA and ribosomal RNA. Accordingly, great care was taken in the isolation of sRNA to be certain that it was pure. The first precaution was the use of a "cold chase" of inorganic phosphate to allow the rapidly metabolized messenger RNA to lose its $P^{32}$ label. In addition, two quite different isolation procedures were carried out sequentially. The sRNA base ratios are listed in Table 1, and it can be seen that they differ substantially from either ribosomal or messenger RNA. The possibility of trace $P^{32}$ DNA contamination was eliminated because the sRNA preparations were completely digested by ribonuclease and, in addition, no $P^{32}$ material banded in the DNA region when the sRNA was centrifuged by itself. $P^{32}$-labeled pseudouridylic acid isolated from the sRNA digest was identified by its characteristic absorption spectrum at pH 12.[12] This trace component has not been isolated from either ribosomal or messenger RNA and is therefore characteristic of sRNA alone. The labeled sRNA was centrifuged in a sucrose gradient, and the $P^{32}$ counts migrated as a single peak with a sedimentation constant of 4 svedbergs. Thus, the preparations have properties characteristic of *E. coli* sRNA.

TABLE 1

BASE RATIOS OF sRNA

| | Zubay[18] | Preiss *et al.*[19] | Dunn, Smith, and Spahr[20] | This work | Ribosomal[20] RNA | DNA[20] (or messenger RNA) |
|---|---|---|---|---|---|---|
| A | 19.6 | 18.4 | 20.3 | 19.8 | 25.2 | 24.5 |
| C | 28.1 | 28.4 | 28.9 | 27.4 | 21.6 | 25.5 |
| G | 31.0 | 31.0 | 32.1 | 33.0 | 31.5 | 25.5 |
| U*(T) | 21.4 | 20.7 | 18.7 | 19.8 | 21.7 | 24.5 |
| Purine/pyrimidine | 1.00 | 0.99 | 1.12 | 1.12 | 1.30 | 1.0 |
| $\frac{A + U^*(T)}{G + C}$ | 0.69 | 0.66 | 0.64 | 0.66 | 0.88 | 0.96 |

U* includes pseudouridylic acid.

Figure 1*a* shows the results of annealing *E. coli* DNA with *E. coli* sRNA. It can be seen that almost half of the sRNA radioactivity is found associated with the DNA band. After ribonuclease digestion for 1 hr at 37°C, the sRNA at the bottom of the tube has been digested completely; however, less than 25 per cent of the sRNA in the band has been digested. In Figure 1*b* approximately ten times the amount of sRNA was annealed with the same amount of DNA as shown in Figure 1*a*. Under these circumstances a smaller proportion of the total radioactivity is found in the DNA band, and a larger proportion is digested off by ribonuclease. This suggests that the sRNA molecules may be competing for a limited number of annealing sites and consequently an increase in the number of sRNA molecules results in a larger number of imperfect annealings. Using a smaller amount of $P^{32}$ sRNA than is illustrated in Figure 1*a*, 85 per cent of the total counts are found in the hybrid band. This clearly shows that it is not a small contaminant which is binding to the DNA but the bulk of the preparation. Furthermore, base ratios were de-

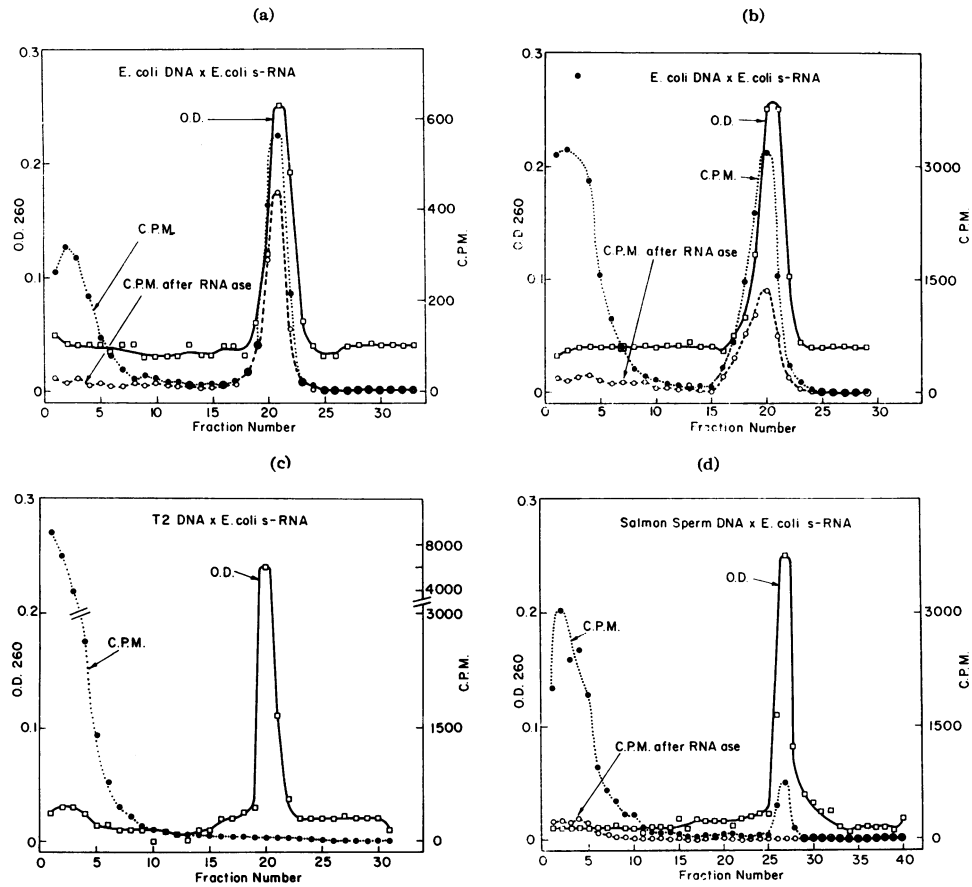


FIG. 1.— Cesium chloride density gradient centrifugations (35,000 rpm, 65 hr, 25°C) are shown for annealed *E. coli* sRNA and a variety of DNAs. Fractions were collected, diluted to 1.1 ml and the $O.D._{260}$ was read. The fractions were then split. One part was counted directly; the other part was treated with ribonuclease (3.5 $\gamma$/ml, 1 hr, 37°C) as described under *Methods*.
(*a*) 45 $\gamma$ *E. coli* B DNA + 0.011 $\gamma$ $P^{32}$ sRNA. (*b*) 45$\gamma$ *E. coli* B DNA + 0.13 $\gamma P^{32}$ sRNA. (*c*) 45 $\gamma$ T2 DNA + 0.13 $\gamma$ $P^{32}$ sRNA. (*d*) 45 $\gamma$ Salmon sperm DNA + 0.13$\gamma$ $P^{32}$ sRNA.

termined on the sRNA in the hybrid band, and these gave results similar to those shown in Table 1. It should be noticed in Figure 1*a* and *b* that the radioactivity peak is not found directly under the peak of optical density but is shifted toward higher density. Since the sRNA molecules are comparatively small, we can use shifts of this type to draw some conclusions concerning the grouping of sRNA annealing sites on the DNA. If they were randomly distributed, the optical density band would be symmetric with the count band. Because of the shift, we have evidence that the sRNA positions are located near each other. This subject will be explored more fully in another publication.

In experiments using materials of high specific activity, one has to be very careful about potential artifacts, among which may be included nonspecific trapping due to the presence of short sequences of nucleotides in the DNA which are the same as those found in sRNA. Negative controls are thus very important. In addition, it is of great importance to carry out the ribonuclease digestions, as has been emphasized by Yankofsky and Spiegelman[6] in their work on ribosomal RNA. An example of a negative result is shown in Figure 1*c*, in which the sRNA was heated and slowly cooled together with T2 viral DNA. It can be seen that none of the sRNA is banding with the DNA. Similar results were obtained with DNA from the bacteriophages T7 and $\phi$X 174. Another kind of negative result is indicated in Figure 1*d*, which shows an annealing experiment with salmon sperm DNA. A small peak of radioactivity is found in the DNA region, but it is digested away by the treatment with ribonuclease, thereby indicating that it was an artifact rather than a true complex. It should be noted that in this case the band of radioactivity is symmetric with the band of optical density which implies that the sRNA molecules may be randomly attached to the DNA. Similar negative results were obtained with calf thymus DNA and DNA obtained from the P22 virus. However, in the latter two cases the amount of radioactivity in the band before digestion was less than 20% of that observed with the salmon sperm DNA.

The kinetics of ribonuclease digestion are shown in Figure 2. It can be seen that the control sRNA, to which DNA had been added without annealing, is rapidly di-

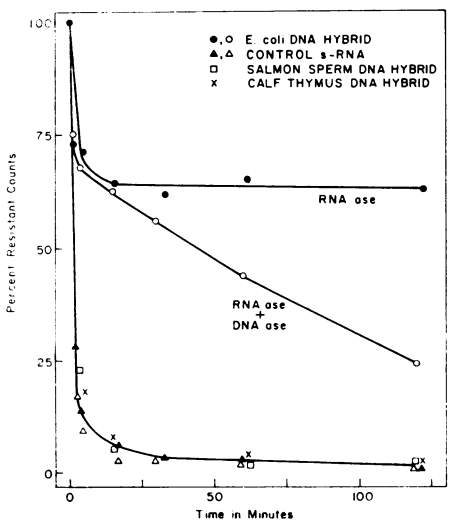


FIG. 2.—The time course of enzymatic digestion. All solutions contained 0.18 *M* CsCl, $5 \times 10^{-3}$ *M* $MgCl_2$, 0.01 *M* Tris buffer, at pH 7.4 and 3.5 $\gamma$/ml ribonuclease. Aliquots were collected and precipitated for counting at various time periods. Resistant counts are shown for the *E. coli* DNA + $P^{32}$ sRNA complex with ribonuclease alone (●) and with added DNAase, (○). Control $P^{32}$ sRNA with added (but not annealed) *E. coli* DNA is shown with ribonuclease alone (▲) and with added DNAase (△). Ribonuclease digestion of salmon sperm DNA hybrid (□) and calf thymus DNA hybrid (×) is also shown.

gested down to a very low level. The rate at which it is digested is indistinguishable from the rate at which the false positives are digested, i.e., the salmon sperm and calf thymus DNA-*E. coli* sRNA complexes. In the true hybrid (Fig. 2), about 30% of the radioactivity is digested away almost immediately and thereafter there is a plateau of radioactivity even after 2-hr incubation at 37°C. However, the addition of DNAase to the system yields a slow digestion of the sRNA, as the DNA is digested enzymatically and the sRNA is liberated. These results are quite similar to those obtained with true and false hybrids involving ribosomal RNA.[6]

*Saturation experiments:* The experiments described in Figure 1*a* and *b* showed that more sRNA could be annealed to the DNA if a larger amount was added to the annealing mixture. This immediately suggests the possibility of attempting to saturate the sRNA sites on the DNA by increasing the amount of sRNA in the annealing mixture. The results of experiments of this type are shown in Figure 3. A constant amount of DNA (45 $\gamma$) was annealed with varying amounts of sRNA over a 300-fold range in concentration. The dashed curve shows that the amount of attached sRNA in the hybrid band goes up steadily while the solid curve shows that the ribonuclease-resistant part saturates. A plateau appears as a mass ratio of sRNA to DNA of 0.025 per cent. Thus, only a very small portion of the DNA is able to accept an sRNA molecule in hybrid formation. Furthermore, these results show that the preparation does not contain ribosomal RNA, since DNA-ribosomal RNA hybrids contain six times more RNA.[6] If cold ribosomal RNA is added to the annealing mixture, it does not compete with the bonding of sRNA, thereby suggesting that the ribosomal RNA sites are different from the sRNA sites.

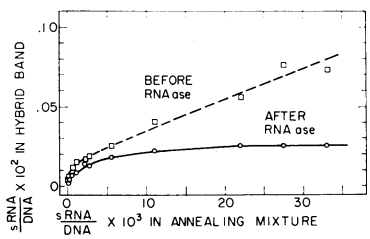


FIG. 3.—The amount of *E. coli* $P^{32}$ sRNA found in the *E. coli* DNA band is plotted as a function of increasing amounts of $P^{32}$ sRNA in the annealing mixture. All preparations were annealed with 45 $\gamma$ DNA and varying amounts of $P^{32}$ sRNA (0.005 to 1.50 $\gamma$ $P^{32}$ sRNA, specific activity = $5.6 \times 10^5$ cpm/$\gamma$). The $O.D._{260}$ and cpm before and after ribonuclease digestion were measured through the band region as described in *Methods*.

The genome in *E. coli* contains a DNA molecular weight equivalent of $4 \times 10^9$.[13] Knowing this, and using the molecular weight of *E. coli* sRNA (25,500), we may calculate from the plateau in Figure 3 that there are approximately 40 sRNA sites in the *E. coli* genome. If we assume that there is one site per sRNA molecule, this number provides a direct estimate of the degeneracy of the amino acid code.

*Inter-species hybrid formation:* Having shown that it is possible to form hybrids between sRNA and its homologous DNA, we thought it would be of interest to examine the formation of hybrids between *E. coli* sRNA and other bacterial DNAs both from closely related and more distantly related species. These DNA preparations were all annealed with the same amount of *E. coli* sRNA under identical conditions in order to compare the amount of ribonuclease-resistant material produced by these cross hybridizations. Figure 4 shows that more hybrid is formed when the sRNA is annealed with the DNA from a closer relative than from a more distant relative. These initial experiments prompted us to survey a series of bacterial DNAs to see whether any trends could be observed as a function of varying guanine-cytosine (G-C) content in the DNA or familial proximity.

DNAs from five different bacterial families were used. The largest group is from the family Enterobacteriaceae to which *E. coli* belongs. In Figure 5, the results of the cross hybridization experiments are shown. The most striking feature of the figure is the fact that the results clearly fall into two different classes. Those points shown below the dashed line are all ribonuclease-resistant sRNA annealed to DNA from a different bacterial family than that of *E. coli* B. These are all at the level of 5 to 15 per cent of the amount of *E. coli* sRNA which is annealed to its homologous DNA. Furthermore, the figure shows that the G-C content is not an important distinguishing parameter. Thus, for example, *Brucella abortus* retains approximately 10 per cent of the radioactivity in its DNA band even though it has a G-C content which is reasonably close to that found in *E. coli* B. It is interesting that the three highest points in Figure 5 all belong to the same genus, *Escherichia*. Most of the Enterobacteriaceae fall in the region 50 to 70 per cent of *E. coli* B. *Proteus vulgaris* is known to be a quite distant relative of *E. coli* B and these results are consistent with the comparatively low level (30%) of ribonuclease-resistant hybrid which is formed when its DNA is annealed with *E. coli* sRNA.

*Discussion.*—DNA-RNA hybrids have now been demonstrated in several systems. The initial demonstrations by Rich[14] and by Schildkraut *et al.*[50] involved synthetic polynucleotides and led to later experiments by Spiegelman and his collaborators which demonstrated the formation of these hybrids with both T2-specific messenger RNA,[4] naturally occurring messenger RNA,[15] and ribosomal RNA.[6] The present experiments deal with a much smaller molecular species, soluble RNA. The conclusion from these experiments is that the DNA is also the primary site for the manufacture of sRNA. This does not, of course, rule out the possibility

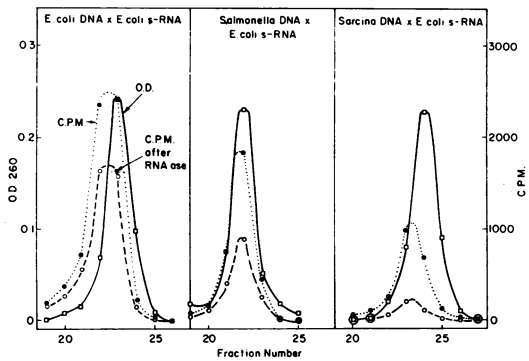


FIG. 4.—The results of cesium chloride density gradient centrifugations are shown for annealing experiments involving *E. coli B* $P^{32}$ sRNA (0.11 $\gamma$) and 45 $\gamma$ of DNA from (*a*) *E. coli B*. (*b*) *Salmonella typhimurium* and (*c*) *Sarcina lutea*. Only the fractions containing the DNA band region are shown. Solid curve is $O.D._{260}$; dotted curve is cpm before ribonuclease digestion; dashed curve is cpm after ribonuclease digestion under conditions described in *Methods*.

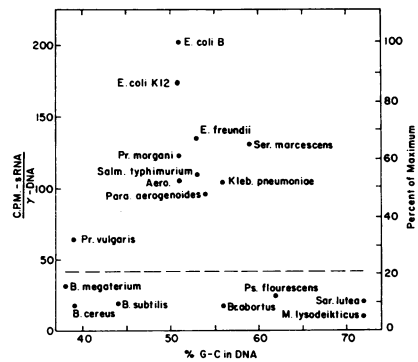


FIG. 5.—The amount of ribonuclease resistant $P^{32}$ sRNA per $\gamma$ of DNA in the band after annealing is shown for a variety of different bacterial DNAs. Each annealing mixture contained 45 $\gamma$ of the DNA named in the figure and 0.11 $\gamma$ of $P^{32}$ sRNA. The results are plotted against the guanine-cytosine content of the DNA in the annealing mixture.[21] The dashed line separates the family Enterobacteriaceae from the four other bacterial families: Bacillaceae, Brucellaceae, Pseudomonadaceae, and Micrococcaceae. Aero is "Aerobacter" (1041).

that sRNA molecules may also be produced by RNA-RNA replications but rather demonstrates that the sequences involved originate from the DNA.

The quantitative interpretation of these experiments rests heavily on the ability of ribonuclease to digest away the parts of the sRNA molecule which are not systematically hydrogen-bonded to DNA. This resistance was first demonstrated in synthetic polynucleotides and has now also been shown in double-stranded RNA-RNA helices which have been annealed together.[16] We would like to know the extent to which irregularities in the hydrogen bonding would make the RNA strand susceptible to enzymatic degradation. Perhaps some indication of this can be seen in the fact that sRNA itself is readily digested by ribonuclease even though it has considerable internal hydrogen bonding. This suggests that a high degree of regularity may be necessary to bring on this resistance to enzymatic digestion.

Interpretation of the saturation data in Figure 3 depends upon the extent to which the enzymatic digestion is carried out to completion without denuding DNA stretches of sRNA which should be annealed there. It is not likely that this occurs, but we have no direct evidence on this point. Bearing in mind these assumptions, we can ask how much reliance may be placed on the calculation of 40 sRNA sites from the data in Figure 3. Although the molecular weight of sRNA is only slightly uncertain, the uncertainty in the total amount of DNA in the *E. coli* genome may be considerable. Thus, we cannot regard the figure of 40 sRNA sites as a very accurate determination. Nonetheless, this result suggests that there is considerable degeneracy in the amino acid code, a finding which is given indirect support from the genetic experiments of Crick *et al.*[17]

The cross species hybrid experiments suggest the existence of some common nucleotide sequences in *E. coli* B sRNA and a variety of closely related bacterial DNAs. Thus they give us some information about the evolutionary history of the sRNA molecules. Closer relatives are able to form a greater stretch of regular hydrogen bonding than are more distant neighbors. When experiments of this type are carried out on individually purified sRNA molecules, we will be able to trace the extent of the modifications which have occurred in the course of evolution.

*Summary.*—It is possible to form ribonuclease-resistant hybrid complexes between sRNA and DNA. This suggests the existence of a sequence of nucleotides in the DNA complementary to the sRNA. When this complex is formed with an excess of sRNA, the DNA in one *E. coli* genome is saturated with approximately 40 sRNA molecules. If there is one site per sRNA molecule, this suggests that there is considerable degeneracy in the amino acid code. Hybrids have been formed between *E. coli* sRNA and DNA from a variety of bacterial species. Closer relatives form larger amounts of ribonuclease-resistant hybrid than distant relatives.

*Note added in proof:* We have learned that specific *E. coli* DNA-sRNA hybrids have also been made by D. Giacomoni and S. Spiegelman. They observed a saturation plateau value similar to that shown above.

We wish to thank J. Marmur for his generous gift of several DNAs and R. L. Sinsheimer for his gift of $\phi$X 174 DNA. We acknowledge Melissa Quinn's capable technical assistance. This research was supported by grants from the U.S. Public Health Service and the National Science Foundation.

* The following abbreviations are used: DNA, deoxyribonucleic acid; sRNA, soluble ribonucleic acid; Tris buffer, tris (hydroxymethyl) aminomethane buffer; $O.D._{260}$, optical density at

260 mμ; cpm, counts per minute; DNAase, deoxyribonuclease; RNAase, ribonuclease; γ, microgram; mc, millicurie; TCA, trichloracetic acid.

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## *A GENETIC LOCUS IN E. COLI K12 THAT CONTROLS THE REACTIVATION OF UV-PHOTOPRODUCTS ASSOCIATED WITH THYMINE IN DNA*

BY P. HOWARD-FLANDERS, RICHARD P. BOYCE, EVA SIMSON, AND LEE THERIOT

DEPARTMENT OF RADIOLOGY, YALE UNIVERSITY SCHOOL OF MEDICINE

*Communicated by Alexander Hollaender, October 9, 1962*

A radiosensitive mutant *E. coli* $B_{s-1}$ was discovered by Hill.[1, 2] It exhibits the same plating efficiency as the parental strain *E. coli* B for normal T1 bacteriophage, but a greatly reduced efficiency with ultraviolet (UV) irradiated T1 phage.[3] The sensitive mutant apparently lacks some factor capable of reactivating certain photoproducts in the UV-irradiated DNA of T1 phage.

Sensitive mutants of *E. coli* K12 which show the same low efficiency for scoring UV-irradiated T1 phage were induced by nitrous acid treatment.[4] This paper reports experiments on genetic crosses between a sensitive $F^-$ strain and several resistant Hfr strains of K12. The inheritance of resistance to UV-irradiation among the progeny from the crosses was analyzed following methods similar to those previously reported,[5] and a locus on the male chromosome was identified that can transmit UV resistance to the zygote. This locus will be referred to as $UV^R$ or $UV^S$ ac-