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*RESTRICTION OF IN VIVO GENETIC TRANSCRIPTION TO ONE OF THE COMPLEMENTARY STRANDS OF DNA**

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Several recent investigations[1–3] demonstrate that when double-stranded DNA is employed as a template *in vitro*, the DNA-dependent RNA polymerase mediates the synthesis of RNA copies complementary to each of the two strands. The primary concern of the present paper is whether or not this situation obtains in the intact cell.

Existent evidence suggests that *in vivo* transcription may not involve both strands. Thus, the base composition of unfractionated T2 complementary RNA[4, 5] and the material collected by hybridization to DNA on columns[6, 7] all have shown a persistent and significant inequality of guanine to cytosine. This discrepancy would be explained if only one strand is transcribed and if it has a bias toward a low C:G ratio. Furthermore, it has been shown that sequences complementary to both ribosomal[8–10] and soluble[11, 12] RNA exist in homologous DNA, implying that DNA generates these molecular species. Nevertheless, G does not equal C nor does A equal U in ribosomal RNA, suggesting[8] that transcription in the corresponding region of the genome does not involve both strands. A similar statement can be made for the sRNA cistrons. The ability of 5-fluorouracil to restore[28] certain mutants of bacteriophage T4 also leads to the conclusion that only one strand of the r II region yields RNA messages.

None of these findings are decisive and they clearly do not define with certainty the mechanism which reads the entire genome. The following three possibilities can be entertained for genetic transcription: (1) all of both strands are transcribed into complementary RNA; (2) both strands are employed but never in the same regions; (3) only one of the two strands serves as a template for genetic messages. We are ignoring the possibility of regions which are not transcribed at all in either

strand. This would introduce three more possibilities, but they are not directly relevant to the central issue here nor to the experiments to be described.

Examination of the possibilities mentioned suggests immediately that the hybridization test of Hall and Spiegelman[13] can be employed to provide data useful for deciding which of the three mechanisms operates in the cell. A successful resolution requires a source of radioactively labeled homologous complementary RNA and the availability of at least one of the two strands of the relevant DNA in pure form.

To provide the experimental requisites, the authors turned to the DNA virus, ϕX174, which has been shown by Sinsheimer[14] to contain only one of the two complementary strands. Nature has thus provided one of the necessary experimental components in an easily accessible form. However, this useful feature can be fruitfully exploited only if the complementary strand can also be obtained. It has been found[15] that immediately after infection the complement to the injected strand is synthesized, and a duplex results which has been called the "replicating form" (RF). As a preliminary to the experiments to be described here, it was therefore necessary to devise a procedure for the isolation in pure state of the RF-DNA from the infected cells. This was accomplished[3] by repeated chromatography on columns of methylated albumin.

The availability of both RF-DNA and the single strand from the mature virus particle made possible appropriate hybridizations with the RNA message fraction of ϕX174. The hybridization tests and the base composition of the RNA hybridized revealed the presence in infected cells of RNA complementary to only one of the two strands in the RF-DNA. The data are consistent with the conclusion that one of the two possible complements is either the principal or sole source of translatable genetic information.

Materials and Methods.—Strains: The DNA virus ϕX174 and its host *E. coli*, strain *C*, were kindly provided by Dr. I. Tessman.

Media and buffers: Modified Penassay medium (MPM) was used as described by Nomura *et al.*:[5] SCXD medium is composed of Tris-HCl buffer 0.05 *M* pH 7.3; 0.5% glycerol; 10^{-3} *M* phosphate; 10^{-3} *M* $MgSO_4$; 0.5% casein hydrolysate and 1 μg/ml of $FeCl_3$. Adsorption medium (AD) is Tris-HCl buffer 0.05 *M* pH 7.3; 10^{-3} *M* phosphate; 10^{-3} *M* $MgSO_4$; 0.5% casein hydrolysate; 1 μg/ml of $FeCl_3$; 10^{-3} *M* $CaCl_2$; 10^{-2} *M* NaCl; 10^{-2} *M* KCl. 3×D medium is that described by Fraser and Jerrel;[16] SSC[17] is 0.15 *M* NaCl; 0.015 *M* Na citrate; TM is 3×10^{-2} *M* Tris; 5×10^{-3} *M* Mg^{++}; pH 7.4; TMS is 3×10^{-2} *M* Tris; 5×10^{-3} *M* Mg^{++}; 0.01 *M* NaCl; HMP is 0.01 *M* PO_4; pH 7.0.

Radioactive labeling: Pulse and uniform labeling carried out as described previously.[18] P^{32} from the Oak Ridge National Laboratory was hydrolyzed to remove inorganic pyrophosphate prior to use. H^3-uridine (500 μc/112 μg), H^3-thymidine (2 μc/μg), and C^{14}-uridine (5.7 μc/mg) were obtained from New England Nuclear Corp.

RNA and DNA purification: RNA was purified by the procedure of Hayashi and Spiegelman.[18] Bacterial DNA was prepared by Marmur's[17] method with the modification that, subsequent to treatment with RNAase, aqueous phenol was used to remove protein. DNA from virus particles was isolated according to Grossman *et al.*[19] The purification of ϕX174 replicating-form DNA was carried out as detailed by Hayashi, Hayashi, and Spiegelman.[3]

Preparation of methylated-albumin-Kieselguhr (MAK) columns: The protocol of Mandell and Hershey[20] was followed with one modification in the preparation of the methylated albumin. The mixture of albumin, methanol, and HCl was incubated at 37°C for 5 days. In our hands this resulted in preparations which yielded consistently superior separation of the DNA factors.

Conditions of infection: Log phase cells *E. coli* (strain C) were harvested at a density of 8–10 × 10^8/ml, washed with adsorption medium (AD) and resuspended in the same medium at 8–9 × 10^9/ml. Virus was added at a multiplicity of infection (m.o.i.) of about 20. The mixture was kept at 20°C for 30 min and then diluted 10 times with prewarmed SCXD medium. This is designated as 0-time of infection. Under these conditions, uninfected cells amounted to less than 0.5%. All experiments were carried out at 30°C.

Unlabeled and labeled virus: In general, the method of Hall *et al.*[21] was followed. On completion of the lytic cycle, DNAase (5 μg/ml) was added and allowed to incubate. Following this, $CHCl_3$ was added and cell debris centrifuged off. The supernatant was made 2.5 *M* with respect to $(NH_4)_2SO_4$, the precipitate collected, resuspended, and dialyzed against 0.01 *M* NH_4Cl. It was then loaded on a DEAE column which was washed with 0.01 *M* ammonium acetate, and the virus was then eluted with 0.1 *M* ammonium acetate. Virus labeled with P^{32} was purified in the same way. Growth and infection were carried out in SCXD medium to which 10 μc/ml of P^{32} was added per ml one generation prior to introduction of virus at an m.o.i. of 0.1. Incubation was continued to lysis. Labeling of viral DNA with H^3 was effected similarly with H^3-thymidine at 5 μc/ml.

Infectious DNA: Infectivity of DNA was measured in protoplasts of *E. coli* C by the procedure of Guthrie and Sinsheimer.[22]

Assay of radioactivity: Preparations of samples on millipore membranes and double channel counting in a Packard scintillation spectrometer was carried out as described previously.[13]

RNA base composition analysis: Bulk *E. coli* RNA was added as carrier to the P^{32}-labeled RNA being analyzed, and the mixture hydrolyzed with alkali (0.3 *M* NaOH) at 37°C for 15 hr. Chromatographic analysis of the resulting 2′–3′ nucleotides was performed using a Dowex-1-formate column.[8]

Results.—ϕX174 message production in the infected complex: Investigation[23] of the ϕX174 *E. coli* C complex quickly revealed that the ϕX174 was incapable of shutting off macromolecular synthesis specific to the host cell. Thus, it was found that the RNA synthesized after infection was indistinguishable in over-all base composition from that observed in uninfected cells. Further, induced enzyme formation could be instituted for considerable periods subsequent to infection. We were faced therefore with the problem of detecting message production from ϕX174 in the midst of the genetic messages generated by the host genome. This is, however, readily achieved by use of the two label and co-chromatographic procedure introduced and developed by Kano-Sueoka and Spiegelman.[24] The principle underlying the device can be simply stated. Consider two labeled RNA preparations, one identified by H^3 and the other by C.[14] If a mixture is loaded on a column, the elution profiles of the H^3 and C^{14} label should be identical if the two preparations are the same, and should differ if one contains some component absent from the other.

To detect message specific to the genome of ϕX174, pairs of infected and noninfected cultures were pulse-labeled with H^3-uridine in one case and C^{14}-uridine in the other. The RNA was purified from each, and a mixture loaded on an MAK column. Figure 1 shows a series of the profiles obtained when such comparisons were carried out during different periods of the infection. There is virtually no discrepancy between the profiles of H^3 and C^{14} in Figure 1*A* suggesting that within 6.5 min little or no ϕX174 specific message has been produced in the infected cells. However, examination of the profiles obtained for the 35–36.5 min interval (Fig. 1*B*) reveals discordancies unique to the infected complex. Finally (Fig. 1*C*), the discrepancies between the H^3 and C^{14} become even more pronounced when the examination is extended to a 50–51.5 period. The greatest discordancies occur in the region to the right of the optical density peak corresponding to the 23S ribosomal RNA. That these discrepancies are indeed referable to ϕX174 specific messages

was tested by hybridizations along the column using RF-DNA. A pulse similar to that carried out in Figure 1*C* and the pooled material of the regions indicated in Figure 2 were hybridized to RF-DNA. The complexed RNA is indicated by the bar histogram. It is clear that there is a peak of RNA hybridizable to RF-DNA in the region corresponding to 4.

From these and similar experiments, one is led to conclude that prior to five min virtually all of the RNA messages found in the infected complex originate from the host genome. Viral specific messages accumulate later in the infection.

Hybridization with RF-DNA and with single-stranded mature DNA: Because of the small size of ϕX-DNA, some of the procedures which have been useful in the past for detecting and accumulating RNA-DNA hybrids proved inconvenient. Cesium chloride density centrifugations in swinging bucket rotors[13] yielded rather broad bands, particularly with heat-denatured material. Further, neither the DNA-agar columns of Bolton and McCarthy[7] nor the millipore filter technique of Nygaard and Hall[25] were found to be adequate for trapping DNA of this size. Consequently, a new procedure was devised[27] which stemmed from the observation[3] that single-stranded DNA of ϕX174 is very well separated from its double-stranded counterpart on MAK columns. RNA hybridized to DNA chromatograms in about the same position as the denatured DNA to which it is complexed.

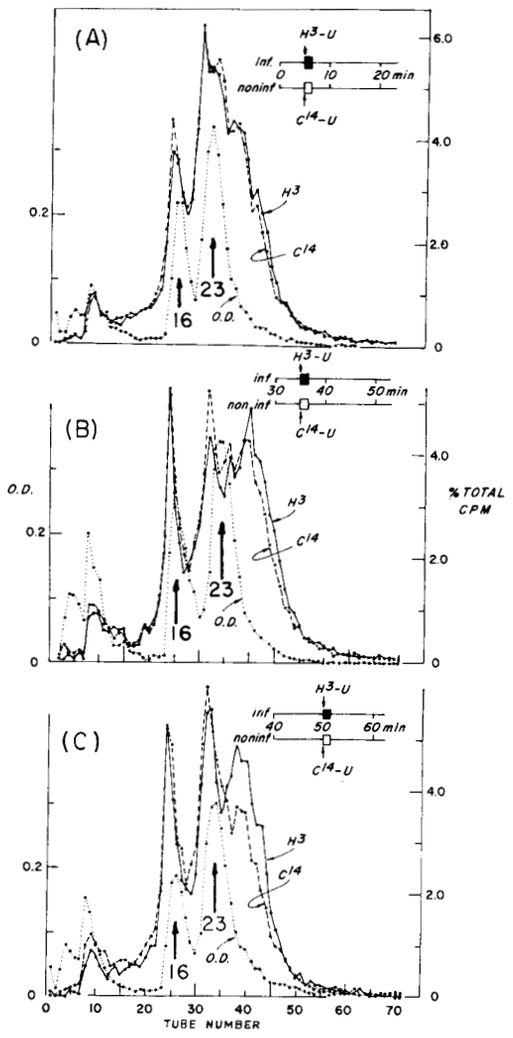

FIG. 1.—Detection of ϕX174 specific RNA by two-label co-chromatography on MAK column. The protocol and timing of each experiment is diagrammed. Time is measured from 0 min (see test). A noninfected culture of *E. coli* was pulse-labeled with C^{14}-uridine for 90 sec. Infected cells were labeled with H^3-uridine at the same time and for the same period. On termination of incorporation, total RNA from each culture was isolated and purified separately. A mixture of the two samples was chromatographed. The O.D. profile identifies pre-existent cellular stable components (16S and 23S indicated by arrows).

The outcome of chromatographic examinations of three different hybridizing experiments are shown in Figure 3. To identify the hybrid with certainty the DNA was labeled with P^{32} and the RNA with H^3. In all cases the H^3-RNA employed

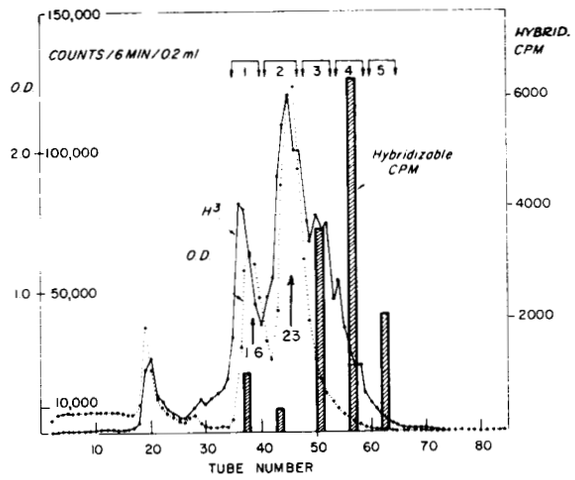

FIG. 2.—Column identification of ϕX174 specific RNA. ϕX174 infected *E. coli* culture was pulse-labeled with H^3-uridine for 90 sec, 50 min after infection. The total RNA was isolated and chromatographed. O.D. profile identifies pre-existing RNA. The pooled samples in the regions indicated in the figure were concentrated. The same number of count from each sample was hybridized with 20γ of RF-DNA which had been heat-denatured in 1/10 SSC at 97–98°C for 15 min. Hybridization was carried out in 2 × SSC at 42.5°C for 18 hr. The reaction mixture was chilled, and 30γ/ml of pancreatic RNAase, free of contaminating DNAase, was added. RNAase treatment was performed at 26°C for 30 min. The reaction mixture was then loaded on an MAK column as described previously. Counts in the hybrid region were summed up and are shown in the bar histograms.

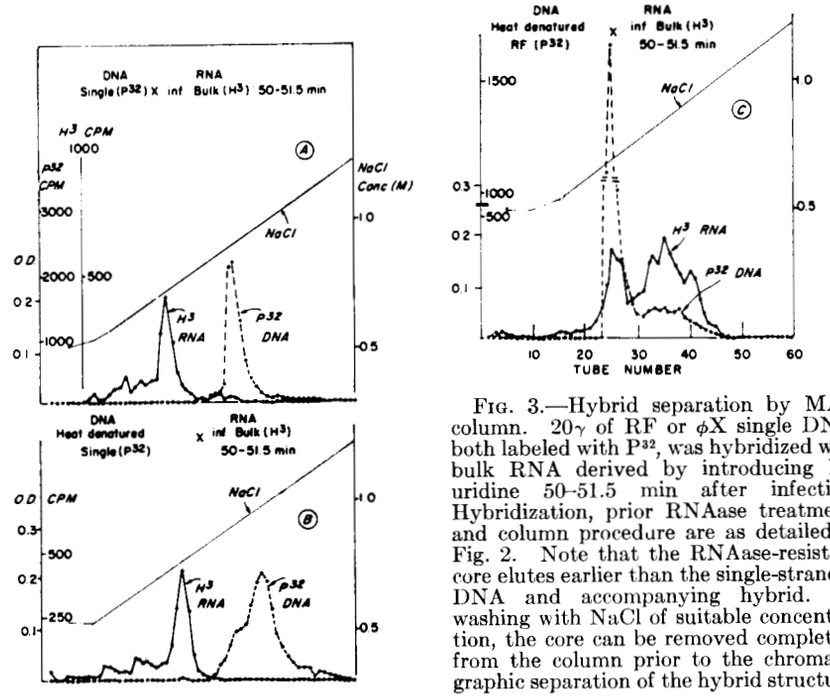

FIG. 3.—Hybrid separation by MAK column. 20γ of RF or ϕX single DNA, both labeled with P^{32}, was hybridized with bulk RNA derived by introducing H^3-uridine 50–51.5 min after infection. Hybridization, prior RNAase treatment, and column procedure are as detailed in Fig. 2. Note that the RNAase-resistant core elutes earlier than the single-stranded DNA and accompanying hybrid. By washing with NaCl of suitable concentration, the core can be removed completely from the column prior to the chromatographic separation of the hybrid structure.

was bulk RNA obtained by introducing H^3-uridine 50–51.5 min after infection. It is clear from Figures 3*A* and 3*B* that vegetative single-stranded DNA, whether heated or not, cannot hybridize significantly to any of the H^3-RNA included in the reaction mixture. However, when heat-denatured RF-DNA is employed, one observes an RNAase-resistant H^3-component in the region of the single-stranded DNA and containing P^{32}. The fact that the radioactivity is alkali labile

and yields the expected 2′–3′, ribotides proves that it is RNA. These results suggest that ϕX174-RNA message is incapable of complexing with the single-stranded DNA but can hybridize with the RF-DNA.

Table 1 summarizes a series of hybridization experiments carried out with labeled RNA derived from different periods of the infection. Comparatively little hybridization is observed between RF-DNA and RNA labeled between 5 and 6.5 min. However, at later periods of infection the amount of hybridization observed with the RF-DNA increases strikingly. In contrast, the single strand from the mature virus exhibits virtually no capacity to hybridize with RNA from any interval. The results in Table 1 agree with the profiles observed in Figure 1. Both indicate that ϕX174 message is to be found in considerable amounts only late in the infectious cycle.

TABLE 1

HYBRIDIZATIONS OF RNA WITH RF AND SINGLE-STRANDED DNA

RNA/DNA pulse-labeled between (min)	RF	Single-strand
5– 6.5	253	<60*
35–36.5	3,261	<160*
50–51.5	3,473	<160*

* These represent upper limits estimated from summation over all tubes in the hybrid regions of the MAK column (cf. Fig. 3). The actual number is undoubtedly much lower.

20γ of heat-denatured RF and 20γ of single-stranded DNA were hybridized with bulk RNA pulse-labeled with H^3-uridine in the intervals indicated in the first column. The conditions of hybridization, RNAase treatment, and subsequent isolation on an MAK column are as detailed in Fig. 2.

Base composition of RNA hybridizable to RF-DNA: The results described indicate that RNA messages produced in the *E. coli*-ϕX174 complex hybridize effectively only with RF-DNA and not with single-stranded mature DNA of ϕX174. This outcome offers strong support for the assertion that the component of the RF-DNA which serves as a template for message production corresponds to the complement of the DNA strand found in the vegetative particle. To complete the proof it is necessary to demonstrate that the base composition of the RNA hybridized to RF-DNA corresponds to that which this assertion predicts; the complexed RNA should be complementary to the complement of the mature vegetative strand. As a consequence, it should possess a base composition which mimics that of the vegetative DNA. The numerical situation is such as to make an experimental test of this prediction easily attainable.

The necessary P^{32}-labeled RNA was prepared by 3-min labeling of the injected complex at various times. As an added precaution a simple device, detailed in Table 2, was used to make a preparation which would provide an adequate sample of all ϕX174 messages. The labeled RNA thus obtained was hybridized to RF-DNA and the hybrid mixture subjected to RNAase. The product was then chromatographed on MAK columns as in Figure 3. The hybrid region was collected, hydrolyzed with alkali, and the base composition of the labeled nucleotides determined. The results are summarized in Table 2. The base composition of the RNA hybridized coming from the 50–51.5 min period is in good agreement with that obtained from the preparation expected to contain equivalent amounts of all messages synthesized during the infection. Comparison of the three DNA base compositions listed reveals that the hybridized RNA is similar to the vegetative single strand and

TABLE 2

BASE RATIO OF HYBRIDIZABLE RNA

	C	A	U(T)	G
RNA				
all stage-pulsed (0–54 min)	17.5	23.8	33.1	25.6
short pulse (50–53 min)	17.5	25.5	34.0	23.0
DNA				
ϕX174 single*	19	25	33	23
ϕX174 complementary†	23	33	25	19
ϕX174 RF‡	21	29	29	21

* Taken from Sinsheimer.[14]

† Assumed complementary to the original strand.

‡ Assumed double-stranded with single and complementary strands.

E. coli C in log phase was concentrated to 10^{10}/ml in AD. θX174 was added at m.o.i. $\sim$ 20. For the complete message sample, adsorption of the phage was performed at 20°C for 30 min; 1 ml of this complex was added every 3 min into prewarmed SCXD (150 ml) at 30°C under aeration. When the 18th complex was added (51 min after 0 time), 20 mc of P^{32} was pulsed for 3 min. The RNA was then isolated, purified, and designated as "all stage-pulsed" RNA. A short P^{32} pulse 50–53 min after infection was also performed and RNA was isolated and purified.

6 mg of each bulk RNA was hybridized with 70γ of heat-denatured RF; hybridization and RNAase treatment are as detailed in Fig. 2. The hybrid was isolated on a MAK column and the base composition determined as described in *Methods*.

is complementary to its complement. The prediction that the complement to the mature strand generates genetic message would appear to be confirmed.

Discussion.—It will be recalled that analysis in cesium chloride gradients[15] and on MAK[3] columns have shown that the injected strand of ϕX174 is incorporated into the RF duplex structure. The buoyant density and melting temperature[3] of RF-DNA both agree that it is a double-stranded molecule containing mature viral DNA as one component and its complement as the other. The fact that ϕX174 message hybridizes to the RF-DNA and not to the mature single-stranded DNA means that it is the complementary component of the RF-DNA which is involved in the hybrid complex. The base composition of the hybridized RNA adds further strong support to the validity of this conclusion. We obviously cannot categorically state that the other strand does not produce any messages. However, if it does, they represent less than 5 per cent of the population. There is also the unlikely possibility that both are transcribed and that one of the transcriptions is preferentially destroyed.

All of the results reported are consistent with the inference that one of the two strands of the DNA duplex is predominantly, or solely, used to generate genetic messages. This conclusion does not deny the possibility[27] that effective transcription of only one of the strands requires the presence of both strands.

It may perhaps be proposed that deductions derivable from the study of a single-stranded DNA virus may not be generally applicable to organisms which normally possess both complementary strands. However, it must be noted that message production does not begin in this system until the double-stranded structure is constituted. One is inclined therefore to believe that the situation being examined is not so abnormal as to be completely unique. Nevertheless, general acceptance obviously requires confirmation with other DNA systems.

The fact that only one strand is used possesses an interesting implication for the problem of genetic inversions. Sequence inversion in a DNA molecule requires in addition a 180° rotation of the inverted stretch in order to reconstitute the antiparallel 5′–3′-internucleotide linkage. This exchanges sequences between the

strands. Thus, inverted sequences will be lost to the transcription mechanism, so that inversion necessarily results in a deletion. We predict therefore that transcribable inversions resulting in nondeletion phenotypes will not be observed in organisms which contain a continuous DNA duplex structure as the sole component of their chromosomal apparatus. The corollary to this, for organisms which do exhibit nondeletion inversions, is obvious.

We should like to conclude by noting briefly the discovery in uninfected host cells of a strange genetic message of peculiar base composition, and complementary to a restricted section of ϕX174 DNA. This RNA disappeared within 5 min after infection of the host cell and thus did not complicate the experiments described here. The significance of this unusual finding is at present under investigation.

Summary.—The experiments described were designed to determine whether *in vivo* transcription of genetic information involves only one or both strands of the DNA-duplex.

The experiments used DNA from the single-stranded virus ϕX174 and its purified replicating form which contains the original strand and its complement. Appropriate hybridization tests with these two DNA preparations and the RNA message fraction were carried out. The results of the hybridization tests and of the base composition of the RNA complex revealed the presence of RNA complementary to only one of the two strands of the RF-duplex. The data are consistent with the conclusion that only one of the two complements in a DNA duplex is either the principal or sole source of translatable genetic information. Among other conclusions, these results imply that transcribable genetic inversions resulting in nondeletion phenotypes are not to be expected within a continuous DNA duplex structure.

Note added in proof: The desirability of extending the conclusions reported here to DNA which is normally double-stranded in the vegetative state has been met rather rapidly. C. Greenspan and J. Marmur (personal communication) have separated the two strands of the SP-8 virus of *B. megaterium* and have shown that only one of them hybridized extensively with homologous message. G. P. Tocchini-Valentini, M. Stodolsky, A. Aurisicchio, F. Graziosi, M. Sarnat, and E. P. Geiduschek (personal communication) have accomplished the same result with phage alpha of *B. subtilis* and have obtained similar results.

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