A GENETIC ASSAY FOR mRNA'S OF PHAGE T4*

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Abstract.—A method has been developed by which many gene-specific mRNA's in T4-infected cells can be quantitatively assayed. The method involves separation of complementary strands of phage T4 DNA, hybridization of the strands with RNA, digestion of nonhybridized regions of DNA with an endonuclease specific for single-stranded DNA, and assay of protected genetic markers by transformation. It has been shown that the gene *r*IIB is transcribed early from the light strand and that the gene 21 is transcribed late from the heavy strand.

Techniques of RNA-DNA hybridization and hybridization-competition have been very useful in studying genetic transcription.[1-4] In a few special cases, studies on transcription of specific genetic regions have been made possible by the availability of specific DNA segments from specialized transducing phages,[5-8] chromosomal segments partially purified by physical methods,[9] and specific deletion mutants.[10] We now report a method by which the amount of mRNA for almost any genetically specified region of phage T4 can be assayed. Our scheme (Fig. 1) is outlined below.

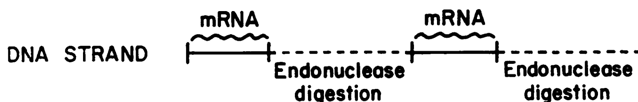


Fig. 1.

The complementary strands of the mature T4 phage chromosome are separated[11] and purified. RNA extracted from T4-infected cells is then hybridized separately with an excess of each of the strands, and the hybrids are digested with an endonuclease specific for single-stranded DNA.[12] The hybridized DNA is protected from degradation by its complementary RNA and is subsequently denatured and used as donor DNA in a genetic transformation system.[13] The frequency of transformation for a given marker should then be proportional to the amount of mRNA transcribed from that region. It should thus be possible to assay, from a single hybridization mixture, any mRNA complementary to a gene for which a suitable marker is available. We have investigated two genetic markers and found that each is protected from endonuclease digestion after hybridization with only one of the two DNA strands. The protection is proportional to the amount of RNA used, and the kinetics of production of each of these messengers is described.

Materials and Methods.—Bacterial and phage strains: *E. coli* B was used for preparing RNA. *E. coli* BB was used for making phage stocks. *A. aerogenes* (ATCC 8724) was used as host for the transformation assays. DNA strands were prepared from phage T4D$ac_{41}r^+$ (wild type). The following *r*II mutants (obtained from A. Doermann) were used as markers for transformation: T4Dr_{73}, T4Dr_{77}, T4Dr_{61}. The following *r*II extended deletion mutants (obtained from S. Champe) were used to make deletion RNA described below: T4Br_{638}, T4Br_{H88}, T4Br_{J3}.

A double mutant carrying the r_{73} marker (in the *r*IIB gene) and a temperature-sensitive mutation (*ts*N8, obtained from R. Edgar) in gene 21[14] was prepared in this laboratory by H. Zweerink.

Preparation and purification of complementary strands of T4 phage DNA: A modification of the method of Guha and Szybalski[11] was used. To 1.5 ml of ^{32}P-labeled phage (5×10^{12}/ml) 0.1 ml of 0.2 *M* disodium EDTA was added, followed by 0.5 ml of 0.2 *N* NaOH. After being mixed by gentle rotation, and allowed to stand at room temperature for 2 to 3 min, the mixture was diluted fivefold with cold 10^{-3} *M* disodium EDTA and dialyzed in the cold against 100 vol of 10^{-4} *M* Tris, 10^{-4} *M* disodium EDTA, 10^{-3} *M* NaCl, four times for 90-min intervals. After dialysis the mixture was added to a suspension of CsCl and poly U (approximately 4–5 times the weight of DNA) in about 10 ml of 0.01 *M* disodium EDTA. The density of this solution was adjusted to 1.72–1.73 by adding 0.01 *M* disodium EDTA. The strands were separated by sedimentation to equilibrium at 30,000 rpm in the Spinco type-50 rotor for 65 to 70 hr at 4°. Fractions of 0.2 ml were then collected from the bottom of the centrifuge tubes, and an aliquot of each fraction was assayed for radioactivity. Fractions containing the "heavy" (H) and "light" (L) DNA strands were separately pooled, dialyzed in the cold against 0.01 *M* Tris pH 7.0, 0.01 *M* NaCl, to remove CsCl, then against 0.1 *N* NaOH at room temperature for 15 to 18 hr, to degrade poly U, and finally against 0.01 *M* Tris pH 7.0, 0.01 *M* NaCl in the cold. The H and L strands were further purified by self-annealing in 0.3 *M* NaCl, 0.01 *M* Tris pH 7.0, at 66° for 6 to 7 hr. After dialysis against 0.22 *M* sodium phosphate buffer pH 7.0, one-fifth vol of a suspension of hydroxyapatite (Biogel HTp-Bio·Rad Labs, 1 gm/ml in 0.22 *M* sodium phosphate buffer pH 7.0) was added to bind the native but not denatured DNA,[15] and the mixture was shaken gently at room temperature for about 5 min. The hydroxyapatite was removed by centrifugation and the process repeated once more. The final supernatants were dialyzed against 0.01 *M* Tris pH 7.0, 0.01 *M* NaCl. After self-annealing and two hydroxyapatite treatments, each strand banded as a symmetrical peak in a CsCl gradient at a density characteristic of denatured T4 DNA.

Preparation of RNA from phage-infected E. coli: *E. coli* B was grown to $OD_{550} = 0.6$ at 30° in the M9S medium of Bolle *et al.*[2] Then L-tryptophan (to give 50 μg/ml) and 2–3 min later, phage particles (to give $5–10 \times 10^9$/ml) were added. At the appropriate time the culture was chilled on an equal volume of a frozen slurry of 0.02 *M* Tris pH 7.0, 0.01 *M* $MgCl_2$, 0.02 *M* sodium azide, centrifuged at 10,000 rpm for 5 min in the cold, and resuspended in 1/30–1/60 the volume of 0.01 *M* acetate buffer, pH 5.2. To lyse the cells, SDS and EDTA were added to a final concentration of 2% and 0.01 *M*, respectively, and the mixture incubated at 37° for a few minutes until the solution cleared. The RNA was extracted four times with water-saturated phenol at 55° and the aqueous layer dialyzed at 4° against more than 500 vol of 0.01 *M* Tris pH 7.0, 0.01 *M* NaCl, 0.002 *M* $MgCl_2$. The dialysate was treated with RNase-free DNase (Worthington Biochemicals Corporation, 15 μg/ml) at 37° for 30 min, extracted once with phenol, and dialyzed against 0.01 *M* Tris pH 7.0, 0.01 *M* NaCl. The deletion-specific RNA's were made by the same procedure using appropriate deletion mutants for infection. In some preparations the RNA was labeled from 15 to 19 min after infection with ^{3}H-uridine.

RNA-DNA hybridization: Hybridization conditions were as described for self-annealing of DNA strands, in a final volume of 1 to 2 ml. To determine the amount of RNA hybridized, aliquots were diluted to 1 ml with 2 × SSC (0.3 *M* NaCl–0.03 *M* sodium citrate), treated with pancreatic RNase(25 μg/ml, 37° for 30 to 60 min), slowly filtered through nitrocellulose filters (Bact-T-Flex, type B-6, Schleicher and Schuell Co.), and

washed extensively with 2 × SSC; the filters were then assayed for radioactivity of the RNA.

Digestion with shark liver endonuclease: This enzyme, which is specific for single-stranded DNA, was prepared from shark liver acetone powder by a modification of the procedure of Ashe *et al.*[12] We are indebted to L. Corman for the acetone powder and to D. M. Trilling for the preparation of the enzyme. The ratio of endonucleolytic activity on denatured/native T4 DNA was 308/1. The reaction mixture (0.3 ml) contained the following: Tris buffer pH 8.0, 0.006 *M;* $MgCl_2$, 0.002 *M;* NaCl, 0.12 *M;* RNA-DNA hybrid containing about 1 μg DNA, and excess (13 units) enzyme. After incubation at 37° for 40 min, the tubes were chilled on ice, diluted with cold 0.01 *M* phosphate buffer pH 7.0 to 1 μg DNA/ml, and held at 100° for 7 min to inactivate the enzyme and denature the hybrid.

Transformation assay: The transforming activity of the DNA fragments was assayed using *Aerobacter aerogenes* spheroplasts as host.[16] Briefly, the assay consists of infecting spheroplasts with genetically marked urea-treated phage in the presence of wild-type donor DNA and scoring for transformed, wild-type phage particles among the progeny. Transformation frequency is transformed phage/total progeny. Specific transformation frequency is transformation frequency per microgram of original donor DNA.

Results and Discussion.—Table 1 shows the transforming activity of the two strands for markers in two genes. It can be seen that each of the two strands of T4 DNA can transform both r_{73} (*r*IIB) and *ts*N8 (gene 21) markers. Thus, specific protection of either of these markers can be determined on either strand. We do not know if the small variation of specific transformation frequencies in Table 1 is significant.

TABLE 1. *Transforming activity of the complementary strands of T4 DNA for two markers.*

Donor DNA	Specific Transformation Frequency × 10^6 for: r_{73}(*r*IIB)	*ts*N8 (gene 21)
Heavy strand	69	55
Light strand	50	35

Transformation was carried out at 28° using spheroplasts of *A. aerogenes* as host.[16] The recipient, urea-treated phage was a double mutant, T4Dr_{73}, *ts*N8. Total progeny phage were scored by plating on *E. coli* B at 28°, r^+ transformants were scored on *E. coli* K (λ) at 28°, and ts^+ transformants were scored on *E. coli* B at 42°. Specific transformation frequency is transformation frequency/μg DNA. Donor DNA was used at 0.03, 0.05, and 0.10 μg/ml. A background value for no donor DNA added was subtracted from each transformation frequency before the specific transformation frequency was calculated. This value was $<10^{-8}$ for r_{73} and $<5 \times 10^{-7}$ for *ts*N8. Specific transformation frequency was calculated for each DNA concentration and the mean value given in the table.

TABLE 2. *Selective destruction of transforming activity for two markers on different strands.*

Donor DNA	Specific Transformation Frequency × 10^6 for: r_{73} (*r*IIB)	*ts*N8 (gene 21)
H strand, undigested	160	88
H-strand hybrid, digested	2	60
L-strand hybrid, digested	43	1
H strand sham-annealed, digested	2	5
L strand sham-annealed, digested	9	6
H strand, digested	3	2
L strand, digested	1	1

12 μg of H strand and 10 μg of L strand were separately hybridized with 20-fold excess of RNA extracted at 19 min after infection (at 30°) and digested with endonuclease as described under *Methods*. After denaturation aliquots of 0.05, 0.10, and 0.20 μg were tested as donor DNA. Transformation was carried out as described under Table 1. Sham-annealed samples were treated in the same way as hybridized samples except that the RNA was added after annealing and before digestion.

Table 2 shows the effect of endonuclease digestion on the transforming activity of hybrids prepared from H and L strands. When the strands are treated directly with endonuclease, both markers on both strands are destroyed. However, when the strands are first hybridized with RNA from phage-infected cells and then digested with endonuclease, a selective destruction of transforming activity is observed. The transforming activity of the H-strand hybrid is greatly reduced for the r_{73} marker while that for the *ts*N8 marker is essentially unchanged. On the other hand the r_{73} marker is protected on the L-strand hybrid but the *ts*N8 is inactivated. This is consistent with the notion that each gene is transcribed from only one of the two complementary DNA strands. The sham-annealed controls demonstrate that the presence of mRNA *per se* cannot specifically protect markers from degradation by the endonuclease and that hybridization with the appropriate mRNA is required.

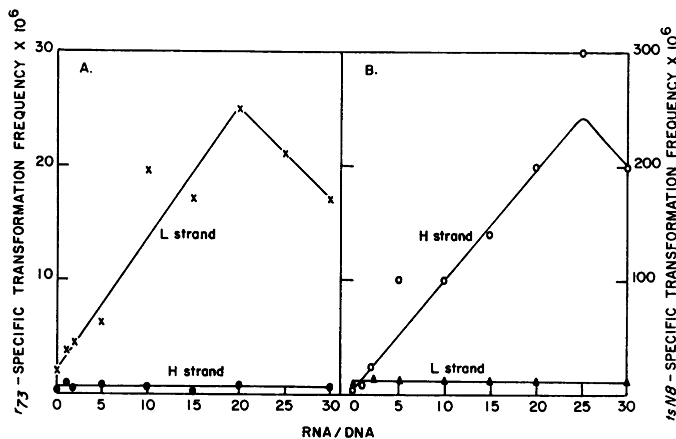


FIG. 2.—The relation between specific transformation frequency and RNA concentration. 5 μg each of heavy and light strands were hybridized with varying amounts of RNA extracted 19 min after infection at 30° to give the indicated RNA/DNA ratios. They were then digested with endonuclease as described under *Methods*. Specific transformation frequencies were determined as outlined in Table 1.

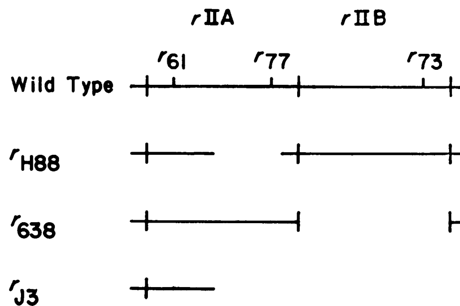
In order to utilize the protection of specific genetic markers by their respective mRNA's as a quantitative assay for the mRNA's, we studied the relation between RNA concentration and gene protection. We hybridized a fixed amount of H or L strand with varying quantities of RNA extracted late after infection. Figure 2 shows that the degree of protection of r_{73} on L strands and *ts*N8 on H strands is proportional to the ratio of RNA/DNA. The reciprocal experiments show that there is little or no protection of r_{73} on H strands or *ts*N8 on L strands for all the RNA/DNA ratios tested. We do not understand the decrease of specific transformation frequencies at high RNA/DNA ratios.

We then studied the specificity of gene protection by hybridizing the L strand of r^+ DNA with different *r*II deletion RNA's (see *Methods*). Table 3 shows that

the capacity of a particular RNA to protect a specific marker depends on the non-deleted region. The r_{61} locus is included in all the mutants tested. The RNA of all these mutants when hybridized with wild-type DNA protected the r_{61} marker. The r_{77} locus is included only in the wild-type and r_{638} mutant. RNA from these phages, but not from r_{H88} or r_{J3}, protected the r_{77} marker. Failure of the r_{638} RNA to protect the r_{77} locus completely may be due to the proximity of the r_{77} locus to the left end of the r_{638} deletion. The r_{73} locus is included in the wild-type and r_{H88} mutant. Only RNA from these phages protects the r_{73} marker. It is still not clear why the markers not hybridized by deletion RNA's are not inactivated as much as sham-annealed strands. It is possible that nonspecific hybridization may protect these loci to a small extent. However, from the results in Table 3, it is evident that this method is specific enough to detect even fragments of mRNA.

TABLE 3. *Protection of different rII markers by deletion-specific RNA's.*

Hybrid preparation	Relative Transformation Frequency r_{61}	r_{77}	r_{73}
Wild-type hybrid	1.0	1.0	1.0
r_{H88} hybrid	0.7	0.2	1.0
r_{638} hybrid	1.1	0.5	0.1
r_{J3} hybrid	0.8	0.2*	0.2†
Sham-annealed wild L strand	<0.1	<0.01	<0.02



5 μg of L strand were hybridized separately with the wild-type RNA and the different deletion-specific RNA's as described under *Methods*. A genetic map of the point and deletion mutants[17] is given below the table. All RNA's were extracted 10 min after infection at 30°. After endonuclease digestion each preparation was tested at three DNA concentrations for transforming activity for the three rII markers. The ratio transformation frequency of deletion hybrid/transformation frequency of wild-type hybrid was calculated for each DNA concentration. The mean of the three values is given in the table.

* Mean of two values.

† Mean of five values.

We then applied this assay to the kinetics of appearance of rIIB mRNA and gene 21 mRNA. This was accomplished by hybridizing RNA extracted at different times after infection with both L and H strands at an RNA/DNA ratio of 15. After digestion and denaturation the specific transformation frequencies for r_{73} and *ts*N8 markers, corresponding to rIIB and gene 21, respectively, were determined as described earlier. Figure 3 shows that the amount of rIIB message starts to rise soon after infection. This finding is in agreement with the data of Kasai and Bautz.[10] The amount of gene 21 message, on the other hand, does not rise appreciably until after ten minutes. Thus, rII is an early gene (i.e., one

which is transcribed soon after infection), and gene 21 is a late gene (i.e., one which is transcribed after the onset of phage DNA replication).

The decrease in the *r*IIB specific transformation frequency after ten minutes suggests, among other possibilities, a net decrease in *r*IIB message, since the total RNA of the cells is almost constant during the course of infection.[18] It may, however, also reflect a decrease in the average size of *r*IIB mRNA fragments. Short mRNA fragments, after hybridization and digestion, would result in short DNA fragments, which may have less than the maximum transformation efficiency.[19] We are now investigating this point.

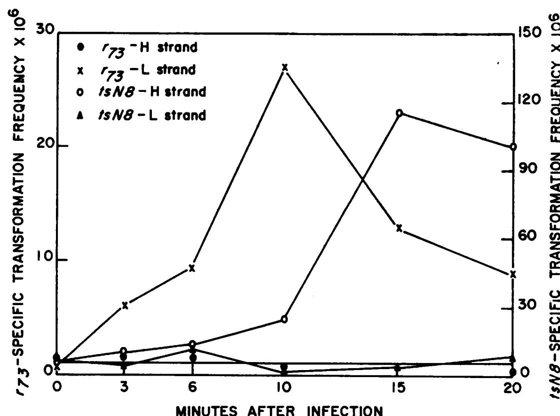


FIG. 3.—Kinetics of production of *r*IIB and gene 21 mRNA. 5 μg of heavy and light strands were hybridized separately with 15-fold excess of RNA extracted at various times after infection at 30°. Each hybrid preparation was digested and tested for transforming activity for r_{73} and *ts*N8 markers at two different DNA concentrations and the mean specific transformation frequency plotted.

We have demonstrated the usefulness of this method in determining, for one early and one late gene, the transcribing DNA strand, the time of initial mRNA production, and the variation of intracellular concentration with time. We expect this method to be applicable to determination of size of individual mRNA species, produced *in vivo* and *in vitro* and in gene purification. It may also be applied to determination of absolute rates of synthesis and degradation of individual mRNA species, and thus in elucidation of control mechanisms of protein synthesis.

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