Transcription of Bacteriophage T4 Deoxyribonucleic Acid *in vitro*

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1. RNA was synthesized *in vitro* from a template of bacteriophage T4 DNA, in the presence of Mn^{2+} . A comparison was made of the RNA synthesized by purified RNA polymerase from two sources, *Micrococcus lysodeikticus* and *Escherichia coli*; these are referred to as *Micrococcus* cRNA and *E. coli* cRNA respectively (where cRNA indicates RNA synthesized *in vitro* by using purified RNA polymerase and a DNA primer). 2. Both types of RNA were self-complementary as judged by resistance to digestion with ribonuclease after self-annealing, *Micrococcus* cRNA being more self-complementary (40%) than was *E. coli* cRNA (30%). The cRNA was found to be much less self-complementary if Mg^{2+} was present during RNA synthesis instead of Mn^{2+} . 3. *Micrococcus* cRNA hybridized with a larger part of bacteriophage T4 DNA than did *E. coli* cRNA. The *E. coli* cRNA competed with only part (70%) of the *Micrococcus* cRNA in hybridization-competition experiments. It is concluded that more sequences of bacteriophage T4 DNA are transcribed by *Micrococcus* polymerase than by *E. coli* polymerase. 4. The RNA sequences synthesized by *Micrococcus* RNA polymerase but not by *E. coli* RNA polymerase are shown by hybridization competition to compete with specifically late bacteriophage T4 messenger RNA sequences. The relevance of this finding to the control of transcription is discussed. 5. In an Appendix, new methods are described for the analysis of hybridization-saturation and -competition experiments. Particular attention is paid to the effects produced if different RNA sequences are present at different relative concentrations. 6. By using cRNA isolated from an enzymically synthesized DNA-RNA hybrid, it is estimated that, of the DNA that is complementary to cRNA, only about half can become hybridized with cRNA under the experimental conditions used.

The enzyme RNA polymerase catalyses the synthesis *in vitro* of RNA in the presence of a DNA primer and the four ribonucleoside triphosphates (Chamberlin & Berg, 1962; Nakamoto, Fox & Weiss, 1964). Homology between the DNA primer and the cRNA* product has been demonstrated by base-composition (Chamberlin & Berg, 1962) and nearest-neighbour analysis (Weiss & Nakamoto, 1961; Furth, Hurwitz & Goldman, 1961) and by DNA-RNA hybridization (Geiduschek, Nakamoto & Weiss, 1961).

The RNA polymerase purified from *Micrococcus lysodeikticus* catalyses the synthesis of a more self-complementary RNA product than does RNA polymerase purified from *Escherichia coli* (Colvill, Kanner, Tocchini-Valentini, Sarnat & Geiduschek,

1965). This suggests that the two enzymes have different sequence specificities for transcription. We have investigated this possibility further by directly comparing the RNA synthesized from the same template by the two types of RNA polymerase. Our results show that *Micrococcus* RNA polymerase transcribes more sequences of bacteriophage T4 DNA than does *E. coli* RNA polymerase.

We chose bacteriophage T4 DNA as the template because it has been shown that the transcription of bacteriophage T2 or T4 DNA by purified *E. coli* RNA polymerase *in vitro* is restricted to a particular group of sequences, the 'early' sequences (Khesin, Gorlenko, Shemyakin, Bass & Prozorov, 1963; Geiduschek, Snyder, Colvill & Sarnat, 1966). The sequences are also transcribed *in vivo* throughout the period of virulent infection of *E. coli* B by the bacteriophage. A second group of sequences, the 'late' sequences, are transcribed *in vivo* only during the later part of the infective period (Hall, Nygaard

*Abbreviations: cRNA, RNA synthesized *in vitro* by using purified RNA polymerase and a DNA primer; mRNA, messenger RNA; SSC, 0.15M-NaCl-15mM-sodium citrate, pH 7.5.

& Green, 1964; Bolle, Epstein, Salser & Geiduschek, 1968), and are not transcribed *in vitro* by *E. coli* RNA polymerase. We have used a DNA-RNA hybridization-competiton method to determine whether late sequences of bacteriophage T4 DNA are transcribed *in vitro* by *Micrococcus* RNA polymerase. Our results show that some late sequences are transcribed.

In this paper, several estimates are made of the homology between DNA and RNA species or between two samples of RNA. These were made by using the hybridization technique described by Gillespie & Spiegelman (1965), together with new methods of analysing the results, which are fully described in an Appendix to this paper.

MATERIALS AND METHODS

Materials. Special materials were obtained as follows: L-tryptophan, 'ribonuclease-free' deoxyribonuclease and three-times-crystallized ribonuclease from Sigma (London) Ltd., London S.W.6; unlabelled ribonucleoside triphosphates from Pabst Laboratories, Milwaukee, Wis., U.S.A.; [^{14}C]ATP and [^{32}P]P_i from The Radiochemical Centre, Amersham, Bucks.; Sephadex SE-50 from Pharmacia (G.B.) Ltd., London W.1; spray-dried cells of *Micrococcus lysodeikticus* from Cambrian Chemicals Ltd., Croydon; Sartorius nitrocellulose membrane filters (MF-50, weight constant, 3 cm. diam.) from V. A. Howe Ltd., London W.11; dithiothreitol from Calbiochem Ltd., London W.1. Frozen cells of *Escherichia coli*, strain M.R.E. 600, were very kindly provided by the Medical Research Council Microbial Products Division, Microbiological Research Establishment, Porton, Wilts.

Messenger RNA. *E. coli* B was grown overnight in a static culture of glycerol-casamino acids (casein hydrolysate) medium (3 × D medium; Frasset & Jerrel, 1953) and inoculated into a 400 ml. culture of the same medium to give an initial E_{650} value of 0.1. The culture was aerated at 30° until the E_{650} value reached 0.6. The cells were collected by centrifugation and resuspended in 10 ml. of minimal medium containing MgCl_2 (0.1%) and L-tryptophan (20 $\mu\text{g./ml.}$) but no carbon source. Purified bacteriophage T4 was added at a multiplicity of 5 and after 2 min. the suspension was reinoculated into 400 ml. of 3 × D medium. Under these conditions bacteriophage DNA synthesis began at about 10 min. Unlabelled mRNA was extracted after 4 min. or 20 min. mRNA was labelled by adding 100 μC of [^{14}C]uracil from 0 to 4 or 16 to 20 min. after reinoculation. Growth was stopped by pouring the culture on to frozen 10 mM- MgCl_2 -10 mM-tris-HCl buffer, pH 7.5 at 0°, and the cells were centrifuged and taken up in 5 ml. of 10 mM-sodium azide-5 mM-EDTA-0.1 M-NaCl-20 mM-sodium acetate buffer, pH 5. At this stage the cells could be frozen if necessary. To fresh or thawed cells 0.2 vol. of 10% sodium dodecyl sulphate was added. After 2 min. at 45° lysis was complete. An equal volume of water-saturated phenol (50°) was added and the mixture was shaken for 2 min. at 50° and 10 min. at room temperature and centrifuged, and the aqueous phase was recovered. An equal volume of ethanol was added and the DNA was spooled out and dissolved in 2 ml. of 5 mM- MgCl_2 -50 mM-tris-HCl buffer, pH 7.5. A second equal

volume of ethanol was then added and after 15 min. at 0° the precipitated RNA was collected by centrifugation and dissolved in 4 ml. of 5 mM- MgCl_2 -50 mM-tris-HCl buffer, pH 7.5. The 'DNA' and 'RNA' solutions were treated with 0.025 vol. of deoxyribonuclease ('ribonuclease-free') (2 mg./ml.) for 15 min. at 37°, then pooled and extracted twice with water-saturated phenol at room temperature. The final aqueous phase was extracted three times with redistilled ether, treated with N_2 to remove residual ether, adjusted to 0.3 M-NaCl-10 mM-sodium acetate buffer, pH 5, and passed through a 2.5 cm. × 40 cm. column of Sephadex SE-50 equilibrated and developed with the same buffer. The column separates the RNA from the DNA oligonucleotides, which are predominantly ethanol-precipitable. Appropriate effluent fractions were combined and neutralized with 3 M-tris base.

DNA. Bacteriophage T4 was grown on *E. coli* B in complete medium, and purified by differential centrifugation. ^{32}P -Labelled bacteriophage T4 was grown on *E. coli* B in low-phosphate medium (Mandell & Hershey, 1960) with 10–20 μC of [^{32}P]P_i/ml. added, and purified by differential centrifugation and banding in CsCl. Bacteriophage preparations were dialysed against 0.1 M-NaCl-10 mM-tris-HCl buffer, pH 7.5 at 0°, and extracted three times with water-saturated phenol to isolate DNA. After treatment with ether and nitrogen, the DNA was dialysed against 10 mM-NaCl-10 mM-tris-HCl buffer, pH 7.5.

RNA synthesis *in vitro*. RNA polymerase was purified from *E. coli* by the method of Chamberlin & Berg (1962) and from *M. lysodeikticus* by the method of Nakamoto *et al.* (1964). The RNA synthesized *in vitro* is called simply *Micrococcus* cRNA or *E. coli* cRNA. Both enzymes were stored at –20° in 50% glycerol-50 mM-tris-HCl buffer, pH 7.5 at 0°, and 5 mM-dithiothreitol was added to the *E. coli* enzyme. RNA synthesis was carried out for 30–40 min. at 30° in 0.1 M-tris-HCl buffer, pH 7.5 at 30°, containing (final concentrations), 2.5 mM- MnCl_2 , 1.6 mM-spermidine, 0.8 mM-GTP, 0.8 mM-UTP, 0.8 mM-CTP and 0.4 mM-[^{14}C]-ATP (sp. radioactivity 1 c/mole). In some cases the MnCl_2 was omitted and 10 mM- MgCl_2 was added. The amounts of DNA and enzyme were adjusted to produce the synthesis of 1–1.5 times as much RNA product as DNA primer. After incubation, MgCl_2 (final concn. 5 mM) and 'ribonuclease-free' deoxyribonuclease (final concn. 40 $\mu\text{g./ml.}$), were added and digestion was carried out for 10 min. at 37°. Sodium dodecyl sulphate (final concn. 0.2%) and NaCl (final concn. 0.1 M) were added, and after 2 min. at 37° the solution was shaken for 10 min. at room temperature with an equal volume of water-saturated phenol and centrifuged. The aqueous phase was precipitated with 2 vol. of ethanol for 15 min., centrifuged and taken up in 0.15 vol. of 0.3 M-NaCl-10 mM-sodium acetate buffer, pH 5. This solution was passed through a 1 cm. × 15 cm. column of Sephadex SE-50, developed with the same buffer, to free the RNA of nucleotides and oligonucleotides. Appropriate fractions were pooled, neutralized with 3 M-tris base and stored at –20°.

Assay for self-complementarity. cRNA was heated at 90° for 10 min. to destroy secondary structure and quickly chilled. Uniform amounts of RNA (about 0.75 μg) in 2 × SSC were brought to different volumes with 2 × SSC and annealed for 16 hr. at 65°. All samples were then diluted to 3.5 ml. Six 0.5 ml. portions were removed from each sample. Three of these were treated for 15 min. at 37° with

ribonuclease (20 $\mu\text{g.}/\text{ml.}$) and then all six were precipitated by adding 0.05 ml. of carrier yeast RNA (500 $\mu\text{g.}/\text{ml.}$) and 0.1 ml. of 50% (w/v) trichloroacetic acid. After 20 min. at room temperature, acid-insoluble material was collected on Oxoid cellulose acetate filters, thoroughly washed with 5% (w/v) trichloroacetic acid, dried with ethanol and ether and assayed for radioactivity in a liquid-scintillation counter.

Hybridization. The hybridization technique of Gillespie & Spiegelman (1965) was followed closely. A 1.5 $\mu\text{g.}$ portion of ^{32}P -labelled bacteriophage T4 DNA, in 1 ml. of $6\times\text{SSC}$, was loaded on to each 3 cm.-diameter nitrocellulose membrane. The membranes were washed with $2\times\text{SSC}$ and dried as described by Gillespie & Spiegelman (1965). Hybridization was carried out at 65° in 20 ml. beakers with an inside diameter of 3 cm. Each membrane was annealed with 1 ml. of $2\times\text{SSC}$ containing ^{14}C -labelled bacteriophage T4-specific RNA and 100 $\mu\text{g.}$ of rat liver cytoplasmic RNA, added to decrease non-specific binding. To monitor non-specific binding, blank (but washed) membranes were annealed with RNA in the same way. The liquid in the beakers was covered with 1 ml. of paraffin wax (m.p. 50° , containing a little ceresin) to prevent evaporation. At the end of the specified hybridization time 20 ml. of $2\times\text{SSC}$ at 65° was added to each beaker and incubation at 65° was continued for 1 hr. longer. The beakers were chilled, and the membranes were removed and washed as described by Gillespie & Spiegelman (1965), including treatment with 1 ml. of ribonuclease in $2\times\text{SSC}$ (20 $\mu\text{g.}/\text{ml.}$) for 15 min. at 37° .

Calculation of results. We have used hybridization results to estimate two properties of various RNA preparations: (1) the complementarity, i.e. the proportion of the total DNA sequence represented by complementary RNA copies; (2) the proportion of hybridizing sequences competed against by a second RNA preparation. The methods are fully described in the Appendix (Bishop, Robertson, Burns & Melli, 1969).

(1) A constant amount of DNA is annealed with different concentrations of RNA. The observed hybridization (r $\mu\text{g.}$ of RNA/ $\mu\text{g.}$ of DNA, or c c.p.m. of radioactive RNA/ $\mu\text{g.}$ of DNA) is related to the RNA concentration (h $\mu\text{g.}/\text{ml.}$) by the following linear equations (Bishop, 1969b; Bishop *et al.* 1969)

$$h/r = h/r_s + K$$

$$h/c = h/c_s + K^1$$

where K and K^1 are constants, and r_s ($\mu\text{g.}$ of RNA/ $\mu\text{g.}$ of DNA) or c_s (c.p.m. of radioactive RNA/ $\mu\text{g.}$ of DNA) is the hybridization extrapolated at infinite RNA concentration. We call r_s the saturation value. Our experiments [see the Appendix (Bishop *et al.* 1969)] suggest that the saturation value is approximately half the complementarity of cRNA.

(2) A constant amount of DNA is annealed with a constant amount of labelled RNA (h $\mu\text{g.}/\text{ml.}$) giving hybridization c_0 (c.p.m.) in the absence of unlabelled RNA. Different amounts of unlabelled RNA (g $\mu\text{g.}/\text{ml.}$) are also added. As the amount of unlabelled RNA is increased, the amount of RNA hybridized increases, but its specific radioactivity falls, producing a decreased hybridization, c c.p.m. This is expressed as the competition, C :

$$C = (c_0 - c)/c_0$$

The competition is related to the amount of unlabelled RNA through two important characteristics of the RNA

preparations, p_c and F . p_c is the proportion of c that would be competed against by an infinite concentration of the unlabelled RNA. F is the concentration factor. Only part of each RNA preparation may be participating in hybridization. F is related to the ratio (proportion of labelled RNA competed against)/(proportion of unlabelled RNA competing). C , p_c and F are related through the linear equation:

$$g/C = g/p_c + F(h + Kr_s)/p$$

Here we evaluate p_c from the slope of the plot of (g/C) against g . p_c is an estimate of p , which is defined as the hybridizing RNA sequences common to labelled and unlabelled preparations, expressed as a proportion of the hybridizing RNA sequences of the labelled RNA.

These estimates are subject to uncertainty because in some cases the different sequences of an RNA mixture will be present at different relative frequencies. The effects of RNA frequency distributions are discussed in the Appendix (Bishop *et al.* 1969).

RESULTS

Self-complementarity of cRNA. Self-complementarity was estimated by measuring the proportion of ribonuclease-resistant RNA after self-annealing of the cRNA (Robinson, Hsu, Fox & Weiss, 1964). A comparison of *Micrococcus* cRNA and *E. coli* cRNA is shown in Fig. 1. Both types of cRNA became ribonuclease-resistant upon self-annealing. The greater self-annealing of the *Micrococcus* cRNA suggests that it is rather more self-complementary than is *E. coli* cRNA. The self-complementarity of *Micrococcus* cRNA is in agreement with previous reports (Geiduschek, Moohr & Weiss, 1962; Colvill *et al.* 1965), although previously a higher degree of ribonuclease-resistance was observed.

Fig. 1. Self-complementarity of cRNA. $\circ$, *Micrococcus* cRNA; $\bullet$, *E. coli* cRNA. Controls, heated to destroy secondary structure and immediately treated with ribonuclease, showed 4.8% and 5.2% resistance respectively.

Table 1. Ribonuclease-resistance of annealed cRNA

The ribonuclease-resistance of a heated and quenched, but not annealed, sample was in every case within the range 2.5–6.5%.

Source of RNA polymerase	Condition of template	RNA synthesis		Assay for self-complementarity	
		Bivalent cation	Highest concn. of RNA ($\mu\text{g./ml.}$)	Highest observed ribonuclease-resistance (%)	
<i>E. coli</i>	Native	Mn ²⁺	12.6	29.0	
	Native	Mg ²⁺	10.0	4.8	
<i>Micrococcus</i>	Native	Mn ²⁺	15.3	39.2	
	Native	Mg ²⁺	19.3	18.0	
	Denatured	Mn ²⁺	13.2	40.9	

Earlier work on the transcription of T-even bacteriophage DNA by *E. coli* RNA polymerase has been interpreted as showing asymmetrical synthesis (Green, 1964; Luria, 1965; Colvill *et al.* 1965). However, Luria (1965) found 15% ribonuclease-resistance of the labelled cRNA after annealing a small amount with a large amount of unlabelled mRNA. This is consistent with up to 30% self-annealing. Green (1964) obtained a slightly higher degree of ribonuclease-resistance in a similar experiment, but found very little resistance after self-annealing of the cRNA. This may be explained if a very low cRNA concentration was used in the self-annealing experiment. In the work of Colvill *et al.* (1965) the cRNA clearly shows only about 2% ribonuclease-resistance after self-annealing. Thus the results of Luria (1965) and Green (1964) are largely consistent with our result, whereas those of Colvill *et al.* (1965) are not. Both Luria (1965) and Green (1964) carried out RNA synthesis in the presence of both Mn²⁺ and Mg²⁺, whereas Colvill *et al.* (1965) used Mg²⁺ only, and here we use Mn²⁺ only. This suggested that the choice of bivalent cation influences the symmetry of transcription of bacteriophage T4 DNA by *E. coli* polymerase. To test this possibility RNA was synthesized in the presence of Mg²⁺ as the only bivalent cation, and its self-complementarity measured as before (Table 1). The highest degree of ribonuclease-resistance observed after self-annealing was close to 5%. The same degree of resistance was found when the RNA was heat-denatured just before the ribonuclease was added. Thus we find no evidence of self-complementary RNA synthesis when bacteriophage T4 DNA is transcribed by *E. coli* RNA polymerase in the presence of Mg²⁺. As shown in Table 1 the use of Mg²⁺ instead of Mn²⁺ also decreased the self-complementarity of the RNA synthesized by

Fig. 2. Analysis of the hybridization of bacteriophage T4 DNA with *Micrococcus* cRNA and *E. coli* cRNA. The ordinate, h/r , shows the ratio of cRNA concentration to hybridization. The abscissa, h , shows the cRNA concentration. $\circ$, *Micrococcus* cRNA, slope 2.63 ± 0.03 ; $\bullet$, *E. coli* cRNA, slope 3.69 ± 0.09 .

Micrococcus RNA polymerase, but to a much smaller extent. It is noteworthy that the ATP/GTP incorporation ratio at the 5'-terminus of the RNA chains was considerably influenced by substituting Mn²⁺ for Mg²⁺ in the transcription of bacteriophage T4 DNA by *E. coli* RNA polymerase (Maitra, Nakata & Hurwitz, 1967). Also, homopolymer-primed ribohomopolymer synthesis by *Micrococcus* RNA polymerase is greatly affected by the choice of bivalent cation (Steck, Caicuts & Wilson, 1968). An alteration in the type of initiation site on the DNA that the enzyme will recognize could clearly have profound effects on the self-complementarity of the RNA product, such as those we have observed.

Direct comparison of cRNA synthesized by E. coli polymerase and Micrococcus polymerase. The cRNA used in these experiments was synthesized in the presence of Mn²⁺ as the only bivalent cation. The proportion of the DNA template able to hybridize with the two types of cRNA was measured directly by annealing bacteriophage T4 DNA with different concentrations of cRNA. The saturation value was determined from the slope of the plot of h/r against h (Fig. 2) as described above. The saturation value of *Micrococcus* cRNA was 0.39, that of *E. coli* cRNA, 0.27. The results of a series of separate experiments are shown in Table 2. The average saturation values are 0.38 for *Micrococcus* cRNA and 0.27 for *E. coli* cRNA.

Competition experiments were carried out between *Micrococcus* cRNA and *E. coli* cRNA. In one experiment the concentration of labelled *E. coli*

Table 2. Saturation values of *Micrococcus* cRNA and *E. coli* cRNA.

In each experiment bacteriophage T4 DNA was annealed with a series of concentrations of cRNA. The saturation value (r_s) was obtained from the slope ($1/r_s$) of the plot of h/r against h , determined by regression analysis. Expts. 3 and 5 are shown in detail in Fig. 2. The significance of the ratio ($r_{\max.}/r_s$) is discussed in the Appendix (Bishop *et al.* 1969).

Type of cRNA	Expt. no.	No. of cRNA concentrations	Slope of plot ($1/r_s \pm \text{s.e.m.}$)	Saturation value (r_s)	Highest observed hybridization ($r_{\max.}$)	$r_{\max.}/r_s$
<i>Micrococcus</i>	1	5	2.71 ± 0.18	0.37	0.31	0.84
	2	8	2.85 ± 0.14	0.35	0.29	0.83
	3	6	2.63 ± 0.03	0.39	0.33	0.84
	4	5	2.52 ± 0.12	0.40	0.33	0.82
<i>E. coli</i>	5	6	3.69 ± 0.09	0.27	0.23	0.85
	6	5	3.84 ± 0.35	0.26	0.18	0.69

Fig. 3. Hybridization competition between labelled *Micrococcus* cRNA and unlabelled *E. coli* and *Micrococcus* cRNA. ○, Unlabelled *Micrococcus* cRNA, slope 1.032 ± 0.037 ; ●, unlabelled *E. coli* cRNA, regression coefficient 1.372 ± 0.12 . The concentration of labelled *Micrococcus* cRNA was $2.4 \mu\text{g./ml.}$, giving $r_{\max.} = 0.2$, $r_{\max.}/r_s = 0.51$.

cRNA was $2.6 \mu\text{g./ml.}$, which gave hybridization equal to 55% of the saturation value. The slope of the reciprocal plot gave $1/p_c = 1.07 \pm 0.11$. Thus all of the hybridizing *E. coli* cRNA sequences were competed against by *Micrococcus* cRNA. The competition of labelled *Micrococcus* cRNA by unlabelled *E. coli* cRNA is shown in Fig. 3, together with the control competition of labelled by homologous unlabelled *Micrococcus* cRNA. In the latter case the slope of the reciprocal plot (1.032 ± 0.037) does not differ significantly from 1. For the competition of labelled *Micrococcus* cRNA by unlabelled *E. coli* cRNA, the slope of the reciprocal plot gives $1/p_c = 1.37 \pm 0.12$, or $p_c \sim 0.73$. We conclude that either about 27% of the hybridizing *Micrococcus* cRNA sequences are absent altogether from *E. coli* cRNA, or about half of the *Micrococcus* cRNA

sequences are present in *E. coli* cRNA at a relative concentration between 10- and 100-fold lower, or that the distribution of sequences in *E. coli* cRNA is intermediate between these extremes. This conclusion is discussed in the Appendix (Bishop *et al.* 1969). Both conclusions are encompassed by the statement that the compositions of the sequences of bacteriophage T4 DNA transcribed by the two types of RNA polymerase are very significantly different.

The agreement between the saturation and competition experiments is remarkably good. Taking the simplest interpretation, the competition experiments suggest that 73% of the total DNA sequence transcribed by *Micrococcus* polymerase is also transcribed by *E. coli* polymerase, the remaining 27% being uniquely transcribed by *Micrococcus* polymerase. The saturation experiment shows that RNA transcribed by *E. coli* polymerase hybridizes with 71% as much DNA as the *Micrococcus* cRNA.

Competition experiments with mRNA. (1) Competition between bacteriophage T4 mRNA species. As a test of the method of analysis described in the Appendix (Bishop *et al.* 1969), we have applied it to the much-studied case of 'early' and 'late' bacteriophage T4 mRNA (Hall *et al.* 1964; Green, 1964; Geiduschek *et al.* 1966; Bolle *et al.* 1968). It was first demonstrated by Hall *et al.* (1964) that RNA isolated 20 min. after infection of *E. coli* B with bacteriophage T4 at 30° ('20 min. mRNA') competed completely with RNA labelled in the first few minutes of infection ('0-4 min. mRNA'). RNA isolated at 4 min. failed to compete completely with RNA labelled between 16 and 20 min. after infection. Thus two main classes of mRNA, early and late, were identified. Only the early mRNA is synthesized early in infection, but both types are synthesized at later times. Reciprocal plots of competition experiments between various types of mRNA are shown in Fig. 4. These closely conform with a linear relationship. The numerical results are summarized in Table 3. In competition between homologous

Fig. 4. Competition between early and late bacteriophage T4 mRNA: reciprocal plots. The ordinate, g/C , shows the ratio of unlabelled RNA concentration to competition. The abscissa, g , shows the unlabelled RNA concentration. (a) Competition between labelled 0–4 min. mRNA and unlabelled 4 min. (○) and 20 min. (●) mRNA. Each sample contained 63 μg . of labelled RNA/ml., which gave $c_0 = 720$ c.p.m./ μg . of DNA, $c_0/c_s = 0.59$. (b) Competition between labelled 16–20 min. mRNA and unlabelled 4 min. (○) and 20 min. (●) mRNA. Each sample contained 63 μg . of labelled RNA/ml., which gave $c_0 = 1190$ c.p.m./ μg . of DNA, $c_0/c_s = 0.44$.

Table 3. Competition between early and late bacteriophage T4 mRNA

The data are plotted in Fig. 4. The apparent competition (p_c) of labelled RNA sequences is taken from the slope ($1/p_c$) of the plot of g/C against g , which is obtained by regression analysis. t_3 refers to Student's test for the significance of the deviation of the regression coefficient from unity.

Labelled mRNA	Unlabelled mRNA	Regression coefficient ($1/p_c$)	Apparent competition (p_c)	t_3
0–4 min.	4 min.	1.073 ± 0.041	0.93	1.78
0–4 min.	20 min.	1.113 ± 0.035	0.88	3.22
16–20 min.	4 min.	1.740 ± 0.130	0.57	5.69*
16–20 min.	20 min.	1.061 ± 0.017	0.94	3.58

* Significant at the 0.05 level of probability.

preparations (4 min. mRNA versus 0–4 min. mRNA; 20 min. mRNA versus 16–20 min. mRNA) the deviations of the slopes from unity are statistically insignificant. In the competition of 0–4 min. mRNA by 20 min. mRNA the deviation of the slope from unity falls well within the 0.05 probability level. Our results are therefore in agreement with the conclusion of Hall *et al.* (1964) and Bolle *et al.* (1968) that all or nearly all of the hybridizing mRNA sequences synthesized at early times are still present from 16–20 min. In the competition of 16–20 min. mRNA by 4 min. mRNA, $1/p_c = 1.74 \pm 0.13$, a clearly significant deviation from unity; $p_c \sim 0.57$. Either about 43% of the sequences labelled between 16 and 20 min. are completely absent at 4 min., or all are present at 4 min. but half of them are at a 100-fold lower relative concentration. This is in

substantial agreement with the conclusion of Hall *et al.* (1964) that 40% of the 16–20 min. mRNA sequences are absent at 4 min.

(2) Competition of *Micrococcus* cRNA by 4 min. mRNA and 20 min. mRNA. The competition of ^{14}C -labelled *Micrococcus* cRNA by unlabelled 4 min. mRNA and 20 min. mRNA is shown in Fig. 5. The slope of the reciprocal plot for 4 min. mRNA is 1.76 ± 0.10 and $p_c \sim 0.56$. This result is very similar to the competition of 16–20 min. mRNA by 4 min. mRNA, and the same conclusions apply. Either 44% of *Micrococcus* cRNA sequences are absent from 4 min. mRNA, or half of the *Micrococcus* cRNA sequences are present in 4 min. mRNA at 100-fold lower relative concentration. These sequences, or many of them, correspond to late mRNA. The competition of *Micrococcus* cRNA by 20 min. mRNA

Fig. 5. Hybridization competition between labelled *Micrococcus* cRNA and unlabelled mRNA. ○, Unlabelled 4 min. mRNA; ●, unlabelled 20 min. mRNA. The slopes of the reciprocal plots are: 4 min. mRNA, 1.76 ± 0.10 ; 20 min. mRNA, 1.11 ± 0.04 . The concentration of labelled cRNA was $1.8 \mu\text{g./ml.}$, which gave $r_{\text{max.}} = 0.19 \mu\text{g. of RNA}/\mu\text{g. of DNA}$, $r_{\text{max.}}/r_s = 0.50$.

Fig. 6. Hybridization competition between labelled mRNA and unlabelled *Micrococcus* cRNA. ○, Labelled 0-4 min. mRNA ($63 \mu\text{g./ml.}$) $c_0 = 720 \text{ c.p.m.}/\mu\text{g. of DNA}$, $c_0/c_s = 0.59$, slope of competition plot 0.985 ± 0.048 ; ●, labelled 16-20 min. mRNA ($63 \mu\text{g./ml.}$), $c_0 = 1190 \text{ c.p.m.}/\mu\text{g. of DNA}$, $c_0/c_s = 0.44$, slope of competition plot 1.27 ± 0.08 .

gives $1/p_c = 1.11 \pm 0.04$ and $p_c \sim 0.9$. Thus all or nearly all *Micrococcus* cRNA sequences are competed against by 20 min. mRNA.

E. coli cRNA is competed against completely by 4 min. mRNA, whether the cRNA is synthesized in the presence of Mg^{2+} or Mn^{2+} (Geiduschek *et al.* 1966). We have confirmed this result with *E. coli* cRNA synthesized in the presence of Mn^{2+} . Thus

the competition and saturation experiments we have carried out lead consistently to the conclusion that *Micrococcus* cRNA resembles 16-20 min. mRNA whereas *E. coli* cRNA resembles 4 min. mRNA.

The competition of *Micrococcus* cRNA by 20 min. mRNA is noteworthy, because the *Micrococcus* cRNA shows 40% self-complementarity (Fig. 1 and Table 1). At least 20% of the total RNA must be complementary to the anti-sense strand of the DNA. The 90% competition by 20 min. mRNA suggests that hybridization competition can be brought about by RNA-RNA hybridization between complementary sequences of the labelled and unlabelled RNA. Thus, where cRNA competes with mRNA, we cannot distinguish between transcription of the sense and anti-sense strands *in vitro*.

(3) Competition of 0-4 min. mRNA and 16-20 min. mRNA by *Micrococcus* cRNA. Reciprocal plots of competition experiments are shown in Fig. 6. In the competition of 0-4 min. mRNA by unlabelled *Micrococcus* cRNA $1/p_c = 0.985 \pm 0.084$ and $p_c \sim 1.01$. All of the hybridizing RNA sequences labelled from 0 to 4 min. are evidently competed against by the cRNA. In the competition of 16-20 min. mRNA, $1/p_c = 1.27 \pm 0.08$ and $p_c \sim 0.78$. The difference between the two RNA preparations is evidently real. However, this result would be produced if half of the 16-20 min. mRNA were present in the cRNA at only a 10-fold lower relative concentration [see the Appendix (Bishop *et al.* 1969)].

DISCUSSION

Our results consistently lead to the conclusion that *Micrococcus* RNA polymerase transcribes the bacteriophage T4 DNA template more completely than the *E. coli* polymerase does. *Micrococcus* cRNA hybridizes with more bacteriophage T4 DNA sequences than *E. coli* cRNA, and *E. coli* cRNA competes incompletely against *Micrococcus* cRNA. Most of the *Micrococcus* cRNA is competed against by 20 min. bacteriophage T4 mRNA, showing that the additional sequences transcribed by *Micrococcus* polymerase are largely late-mRNA sequences. The control mechanism that prevents the late sequences from transcription by *E. coli* polymerase clearly does not operate, or operates less well, against *Micrococcus* polymerase.

In the Appendix (Bishop *et al.* 1969) we show that the saturation value underestimates the actual complementarity between bacteriophage T4 DNA and cRNA isolated from the enzymically synthesized DNA-RNA hybrid by at least one-half. Since 38% of bacteriophage T4 DNA hybridizes with *Micrococcus* cRNA, we estimate that at least 76% of the bacteriophage T4 DNA sequences are represented by complementary RNA sequences in

this cRNA. Similarly, since 27% of bacteriophage T4 DNA hybridizes with *E. coli* cRNA, at least 54% of bacteriophage T4 DNA sequences are represented in *E. coli* cRNA. In making these estimates, we assume that cRNA made on the native and denatured templates hybridizes with DNA to the same proportion of its actual complementarity.

It is clear that *E. coli* cRNA transcribes some bacteriophage T4 DNA sequences symmetrically but transcribes the late sequences not at all or 100 times less frequently. Results of Cohen & Hurwitz (1967) on the hybridization of *E. coli* cRNA made on a template of bacteriophage λ DNA are consistent with the interpretation that the early sequences of bacteriophage λ DNA are symmetrically transcribed by purified *E. coli* RNA polymerase. In both cases, then, some restriction apparently prevents or greatly decreases the transcription of the late mRNA sequences, but both strands of the early sequences are available for transcription to a considerable extent. This might result if sense and anti-sense sequences alternate in each strand of the early region of the DNA, and boundaries between different cistrons or operons are not recognized *in vitro*. An alternative explanation is that the DNA primary structure determines transcription *in vitro*, A..T-rich regions being transcribed by the *E. coli* RNA polymerase (Cohen & Hurwitz, 1967), and almost any region, without restriction, by the *Micrococcus* enzyme.

The number of active molecules of RNA polymerase that can bind to native DNA is limited (Richardson, 1966; Jones & Berg, 1966). The number that can bind to denatured or single-stranded DNA is much less limited (Berg, Kornberg, Fancher & Dieckmann, 1965; Richardson, 1966; Bremer, Konrad & Bruner, 1966). In both cases the number of molecules that bind is less than the limit imposed by their physical dimensions. These results are consistent with the existence of a very limited number of sites in the native molecule, which would lead to the conclusion that *Micrococcus* RNA polymerase recognizes more or different sites than does *E. coli* polymerase. The same results are also open to the interpretation that a much larger number of potential binding sites exists in the native DNA molecule than is observed, perhaps all of those found in the denatured molecule. Secondary factors may prevent more than a proportion of these sites from being occupied. If, for example, a partial separation of the DNA duplex is required for binding, binding to adjacent sites may be prevented. According to this interpretation, RNA polymerase from both sources might recognize the same sites, but the *Micrococcus* enzyme would find it more easy to occupy them.

A more general question raised by our results is whether the increased transcription of bacteriophage

T4 DNA by *Micrococcus* RNA polymerase is an artifact of transcription *in vitro*, or whether it reflects the properties of the two enzymes *in vivo*. Bacteriophage T4 DNA is transcribed *in vivo* by *E. coli* RNA polymerase, and restricted transcription may reflect a specific adaptation of the bacteriophage T4 DNA template to this enzyme. Colvill *et al.* (1965) have shown that the degree of symmetry of RNA synthesis *in vitro* is not related to the homology of template and enzyme. The restriction on the transcription of late mRNA regions may, however, be of a different nature.

We estimate [see the Appendix (Bishop *et al.* 1969)] that 27% of bacteriophage T4 DNA hybridizes with cRNA synthesized by *Micrococcus* polymerase on the denatured bacteriophage T4 DNA template, whereas 38% of the DNA hybridizes with cRNA synthesized on the native template. Denaturation of the template thus appears to restrict transcription in this case. Although denatured DNA binds a greater amount of *E. coli* RNA polymerase than does native DNA (Berg *et al.* 1965; Richardson, 1966; Bremer *et al.* 1966), nevertheless the rate of RNA synthesis is greater with the native template, with RNA polymerase from either *E. coli* (Hurwitz, Furth, Anders & Evans, 1962; Bremer *et al.* 1966) or *Micrococcus* (Fox & Weiss, 1964). This is explained by the finding that *E. coli* polymerase synthesizes smaller RNA molecules on the denatured template, showing that on average the rate of RNA chain growth is lower than it is with the native template (Bremer *et al.* 1966). Our finding that denaturation of the bacteriophage T4 DNA template restricts transcription by the *Micrococcus* enzyme, together with other evidence (Bishop, 1969a), suggests that many sequences are located at a sufficient distance from a binding site on the denatured molecule so that they are not transcribed *in vitro*, owing to the low rate of chain growth. This interpretation suggests that a specific part of the denatured template is transcribed, rather than that some copies of every sequence are transcribed.

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APPENDIX

Methods for the Analysis of Deoxyribonucleic Acid-Ribonucleic Acid Hybridization Data

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Our immediate aim is a qualitative characterization of cRNA*. To do this, we have asked two types of question. (1) What proportion of all the sequences of the DNA are represented by complementary RNA copies? (2) What proportion of the different RNA sequences in one RNA preparation are also present in a second? These depend on two types of experiment. (1) Saturation of the DNA by excess of RNA. If every DNA sequence that has been transcribed hybridizes fully with complementary RNA, the proportion of the DNA transcribed is obtained by measuring the amount of RNA hybridized to unit amount of DNA. In practice, the precondition is not fulfilled, and means have to be found of overcoming this. (2) Hybridization competition (Nygaard & Hall, 1964). A labelled RNA preparation is diluted by an unlabelled preparation. The common sequences are diluted out completely if a sufficiently high concentration

of the unlabelled sequences can be added. The labelled RNA hybrid at this point represents those sequences present in the labelled RNA and absent from the unlabelled RNA.

When the RNA contains a single sequence (e.g. 16S rRNA) or a uniform distribution of sequences (e.g. partially degraded rRNA) interpretation of the data is relatively straightforward. When the RNA is a complex mixture of many different sequences at different relative concentrations, several serious problems of interpretation arise. (1) If the number of sequences is very large, the attainable concentration of each may be restricted by the limited solubility or availability of RNA (Bishop, 1969b; Melli & Bishop, 1969). (2) The rarer species in a complex mixture may be overlooked because their relative concentration is very low. (3) These difficulties are increased if a large proportion of the RNA is inert, or hybridizes with a very small part of the DNA (e.g. rRNA in an mRNA preparation). (4) The presence of inert RNA or rRNA prevents measurement of the absolute concentration of the RNA sequences that are responsible for most of the

* Abbreviations: cRNA, RNA synthesized *in vitro* by using purified RNA polymerase and a DNA primer; mRNA, messenger RNA; rRNA, ribosomal RNA; tRNA, transfer RNA.