Transcription of the Bacteriophage T4 Template. Detailed Comparison of *in Vitro* and *in Vivo* Transcripts*

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ABSTRACT: The ribonucleic acid (RNA) that is synthesized asymmetrically on mature T4 deoxyribonucleic acid (DNA) template by *Escherichia coli* RNA polymerase has been compared in detail with *in vivo* prereplicative T4 RNA by hybridization and hybridization–competition. The *in vitro* synthesis comprises two major classes of early T4 *in vivo* transcripts—those that become less and those that become more abundant after DNA synthesis. Marked similarities of abundance distributions of early RNA species synthesized *in vivo* and *in vitro* are demonstrated. This suggests that the interaction of DNA and RNA polymerase alone can properly determine quantitative as well as qualitative aspects of T4 RNA production. *In vitro* transcription is estimated to correspond (subject to certain stated assumptions) to approximately 45% of one template strand. This transcription includes delayed early RNA, the synthesis of which is known to be inhibited *in vivo* by chloramphenicol. Transcription of denatured DNA is abnormal with respect to all applicable criteria—symmetry, abundance distribution, and late RNA transcription. We discuss the implications of these results for the analysis of control elements of transcription that are active during viral development.

In the preceding paper (Brody *et al.*, 1970) we have explored the experimental parameters determining the selective transcription of T4 prereplicative RNA *in vitro*. We now turn our attention to a detailed analysis of *in vitro* synthesized T4 RNA, comparing the *in vitro* transcription product of *Escherichia coli* RNA polymerase on mature viral DNA with T4 prereplicative RNA, according to criteria that have been developed in studying the program of T4 RNA synthesis during viral development (Bolle *et al.*, 1968a; Salser *et al.*, 1970).

The results of this analysis provide a wider view of the correspondence between *in vitro* and *in vivo* T4 RNA. Evidently it is possible to generate RNA abundance distributions *in vitro* that resemble *in vivo* prereplicative RNA in many ways. A minor part of the *in vitro* synthesized RNA is abnormal in the sense that corresponding *in vivo* RNA species or polynucleotide sequences are difficult to assign. This small fraction is analyzed further. We also present some results on the relation between symmetry and selectivity of transcription.

Materials and Methods

Methods of preparing RNA polymerase and DNA, reaction conditions, preparation of RNA, and hybridization conditions have been described in the preceding paper (Brody *et al.*, 1970). Certain procedures pertinent only to these experiments are described below.

Simultaneous Analysis of RNA–RNA and RNA–DNA Complexes. Hybridization was at 60° for 6 hr in 2X SSC (0.3 M NaCl–0.03 M Na-citrate) as previously described, followed by digestion with 12.5 μg/ml of pancreatic and 1.25 μg/ml of T1 RNase at 37° for 30 min. Samples were then: (a) precipitated with 8.3% trichloroacetic acid in the presence of 50 μg of denatured DNA and filtered on nitrocellulose membranes to detect RNA–RNA and RNA–DNA complexes (RT; Figure 1) or (b) slowly filtered through nitrocellulose membranes in 0.5 M KCl–0.01 M Tris-Cl, pH 7.5, for detection of DNA–RNA hybrids (RH; Figure 1). In either case, filters were dried and digested with 1 ml of 0.5 M NH_4OH overnight to dissolve the nucleic acid complexes and degrade the labeled RNA. Freshly prepared Bray's scintillation fluid (13 ml) (60 g of naphthalene, 4 g of 2,5-diphenyloxazole, 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, and 100 ml of methanol added to 1 l. of 1,4-dioxane) was then added, and samples were counted with external standardization to determine counting efficiency.

Comparison of RNA–RNA and RNA–DNA Hybridization. Interpretation of hybridization–competition experiments of labeled and unlabeled symmetric RNA is complicated by the need to consider the properties of labeled antimessenger RNA species. Their competition at the DNA template occurs only as the result of RNA–RNA duplex formation and is dependent on the presence of complementary RNA in the unlabeled competitor. The effectiveness of competition depends on the relative extent of concurrent RNA–DNA and RNA–RNA duplex formation. In the following control experiment, RNA–DNA and RNA–RNA duplex formation have been concurrently measured, taking advantage of the fact that RNA–DNA and RNA–RNA duplexes are both resistant to RNase but that only RNA–DNA duplexes are retained by nitrocellulose membranes in neutral salt solution. The details of the analysis for radioactivity have been arranged so that there are no variations of counting efficiency (see above). The ^3H-labeled RNA for this experiment has been synthesized *in vitro* on a denatured T4 DNA template and is symmetric

* From the Department of Biophysics, University of Chicago, Chicago, Illinois. *Received August 27, 1969.* E. N. B. was a postdoctoral fellow of the National Science Foundation and E. P. G. holds a U. S. Public Health Service Research Career Development Award. This research was supported by grants of the National Institute of Child Health and Human Development (HD 01257) and the National Institute of General Medical Sciences (GM 15880).

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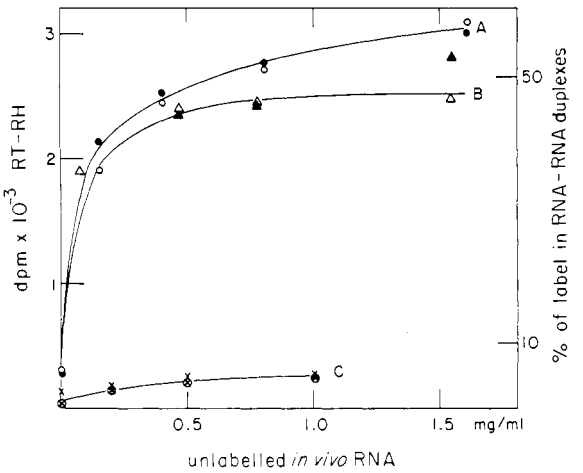

FIGURE 1: Formation of RNA–RNA duplexes. Symmetric [^{3}H]RNA, synthesized on denatured T4 DNA template, is hybridized with varying quantities of *in vivo* prereplicative and late T4 RNA in the presence or absence of 20 μg/ml of denatured T4 DNA. RNA–DNA duplexes are detected by retention on nitrocellulose membranes (RH). The sum of RNA–DNA and RNA–RNA duplexes is detected as trichloroacetic acid precipitable radioactivity after pancreatic and T1 RNase digestion (RT). The difference between RT and RH is plotted along the abscissa as a fraction of the input radioactivity. Samples are counted as described in the text. (A) Reaction of 0.14 μg/ml of *in vitro* [^{3}H]RNA with unlabeled 20-min *in vivo* RNA: (○) 20 μg/ml of denatured DNA present; (●) no DNA. (B) Reaction of 0.14 μg/ml of ^{3}H *in vitro* RNA with unlabeled 5-min *in vivo* RNA: (△) 20 μg/ml of denatured DNA present; (▲) no DNA. (C) Control reaction of *in vivo* [^{3}H]RNA with unlabeled 20-min *in vivo* RNA: (⊗) Reaction of 1.0 μg/ml of RNA labeled 0–5 min after infection; (×) reaction of 4 μg/ml of RNA labeled 1–20 min after infection.

(Figure 1, curves A and B): hybridization with 1500 μg/ml of late T4 RNA converts 64% of the label into RNase resistance. Correction for self-hybridization (no unlabeled RNA) and for the RNase resistance of *in vivo* RNA (curve C) yields an estimate of 55% antimessenger in this RNA. The extent of RNA–RNA duplex formation of labeled antimessenger with 800 μg/ml of unlabeled late T4 RNA is not sensibly decreased by the presence of up to 40 μg/ml of denatured DNA (data not shown). RNA extracted from cells late in T4 infection is estimated to contain 3–5% of its nucleotides in T4 transcripts (Landy and Spiegelman, 1968; and below) so that 40 μg/ml of T4 DNA competes relatively ineffectively with a comparable concentration of T4 messenger (24–40 μg/ml) RNA in complexing these antimessenger species. The same effect can be shown in another way: the presence of 20 μg/ml of DNA does not appreciably affect the determination of antimessenger content of the symmetric RNA (Figure 1; compare open and closed symbols in curves A and B). Therefore, under our annealing conditions, if a certain *in vitro* synthesized antimessenger RNA cannot be competitively prevented from DNA–RNA hybridization, it is because both the identical and the complementary sequence are lacking from (or not sufficiently abundant in) the unlabeled RNA competitor.

DNA. For the titration shown in Figure 5, DNA was examined as follows: 95% of the absorbance at 260 mμ sedimented and was precipitable by 7% $HClO_4$. Concentration was determined assuming ε (P) 6600.

Efficiency of Counting DNA–RNA Hybrids on Nitrocellulose Filters (Figure 5). Six identical samples of DNA–RNA hybrid on nitrocellulose membrane were prepared. Three of these were digested overnight with 1 ml of 0.5 M NH_4OH and counted in Bray's scintillation fluid (as described above). The other three samples served as controls for the normal, inhomogeneous counting in toluene scintillation fluid. Our particular counting configuration on membranes with ^{14}C-labeled RNA gave 73% counting efficiency as determined against the external standardization and a set of ^{14}C standards supplied by Packard Instrument Co.

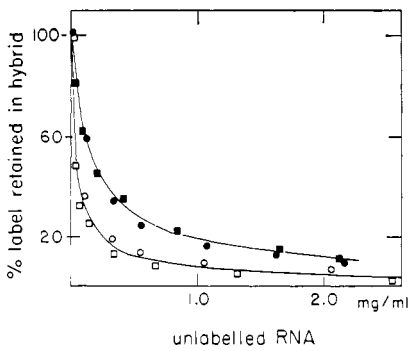

FIGURE 2: Hybridization–competition of labeled *in vitro* and prereplicative T4 RNA. The hybridization mixture contains 10 μg/ml of denatured T4 DNA, 1.4 μg/ml of ^{3}H-labeled (1–5 min) *in vivo* RNA, 0.6 μg/ml of [^{14}C]ATP-labeled *in vitro* RNA and varying quantities of unlabeled 5- and 20-min RNA. The denatured DNA is in excess of the ^{3}H-labeled *in vivo* RNA (Bolle *et al.*, 1968a), but the ^{14}C *in vitro* RNA is already saturating some sites on the DNA (Figure 5): 100% hybridization: 739 cpm of ^{3}H, 945 cpm of ^{14}C, which is 13 and 22%, respectively, of these input radioacitivities. Maximum background in the absence of DNA: 5 and 8 cpm of ^{3}H and ^{14}C, respectively. The [^{14}C]RNA has been synthesized at DNA excess. Analysis is by method II of the preceding paper. [^{14}C]RNA competition: (○) 5-min unlabeled *in vivo* RNA; (●) 20-min unlabeled *in vivo* RNA; [^{3}H]RNA competition: (□) 5-min unlabeled *in vivo* RNA; (■) 20-min unlabeled *in vivo* RNA.

Results

Further Characterization of the Selective Transcription of the T4 Template. The experiments described in this section deal with the application of three criteria to the comparison of *in vivo* and *in vitro* synthesized RNA: (1) relative synthesis of two classes of early RNA—prereplicative and postreplicative early (classes I and II; for definitions see preceding paper); (2) abundance distribution of different early RNA species; and (3) antimessenger content. The last of these criteria has been applied before; the two others arise from recent studies of the program of transcription during T4 development (Bolle *et al.*, 1968a; Salser *et al.*, 1970).

Criterion 1. TWO CLASSES OF EARLY RNA. Two classes of T4 RNA are postulated not to be under the direct control of the viral maturation genes (such as gene 55) in *E. coli* B. Species of prereplicative early RNA (class I; Brody *et al.*, 1970) decrease in abundance during the late part of the latent period and constitute the major part of the RNA made just before DNA synthesis is initiated (5 min at 30°; Bolle *et al.*, 1968a).

Postreplicative early RNA (II) species begin to be synthesized before DNA replication is initiated but become more abundant after DNA synthesis starts. At the end of the eclipse

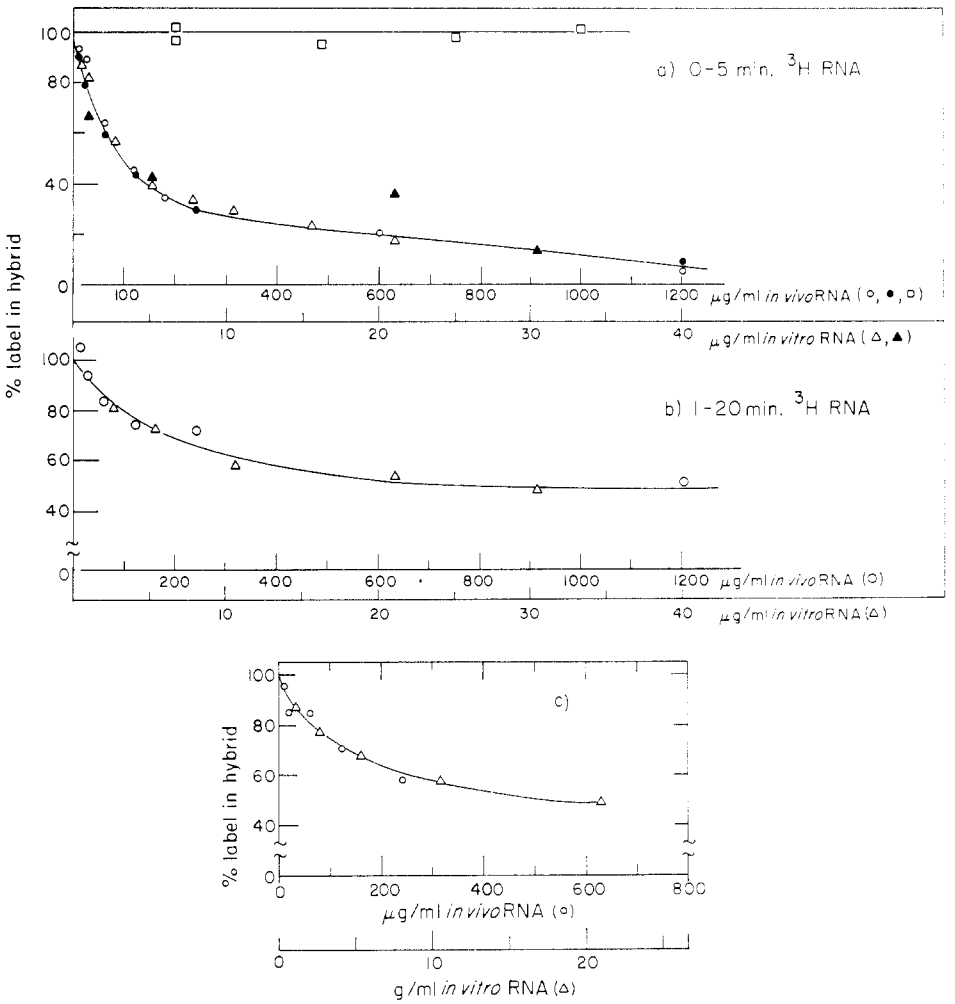

FIGURE 3: Competing ability of *in vitro* RNA. The *in vitro* RNA, which was unlabeled, was synthesized at enzyme excess (20 units of enzyme/μg of DNA, 45-min reaction at 37° in standard reaction medium). The *in vivo* competitor was unlabeled 5-min RNA. All hybridizations are with 10 μg/ml of denatured T4 DNA. Analysis is by method II of the preceding paper. (a) Competition of ^{3}H-labeled 0–5 min *in vivo* RNA (1.4 μg/ml) by unlabeled *in vitro* T4 RNA (△, ▲; two experiments are shown), unlabeled 5 min *in vivo* RNA (○, ●; two experiments are shown), and unlabeled RNA from uninfected *E. coli* B (□); 100% hybridization is 1011 and 954 cmp in the two experiments, which are 18 and 17% respectively, of the input radioactivity. The backgrounds are 40 and 10 cpm, respectively. (b) Competition of ^{3}H-labeled 1–20-min *in vivo* late T4 RNA (5.4 μg/ml); 100% hybridization = 82 cpm, which is 15% of the trichloroacetic acid precipitable input radioactivity. The background is 11 cpm. Symbols as above. (c) Competition of ^{3}H-labeled very late (48–53 min after infection) T_4 RNA (6.0 μg/ml); 100% hybridization = 1672 cpm, which is 42% of the input radioactivity. The background is 18 cpm. Symbols as in A.

period and thereafter they constitute the major part of the RNA species that overlap with (compete with) prereplicative RNA (Salser *et al.*, 1970). However, they are distinct from late RNA (III) species with regard to dependence on DNA synthesis and the action of T4 gene 55 (Bolle *et al.*, 1968b).

Criterion 2. VARIATIONS IN THE ABUNDANCE OF DIFFERENT EARLY RNA SPECIES. Bolle and coworkers (1968a) have suggested an interpretation of "DNA excess" hybridization–competition between *in vivo* T4 RNA (for example, labeled and unlabeled prereplicative RNA) in terms of the existence of more and less abundant species in the viral RNA population at any one time after infection. Such a distribution of abundances should provide a criterion for *in vivo–in vitro* RNA comparison. In fact, completeness of competition of *in vivo* by *in vitro* RNA indicates not only the qualitative correspondence of *in vivo* and *in vitro* transcription products but also some correspondence of relative abundances. Consider, as an extreme contrary example, a DNA template that is transcribed along identical segments *in vitro* and *in vivo* but with different distributions of transcripts. Let the template be transcribed uniformly *in vitro* so that the yield of any nucleotide sequence in RNA is proportional to the length of its complementary coding sequence in the genome. Let the corresponding *in vivo* transcription proceed nonuniformly to yield, in the latter case, very abundant and extremely rare RNA species. *In vivo* RNA should be an excellent competitor for certain *in vitro* species and an extremely poor competitor for others. Conversely, *in vitro* RNA should be a potent competitor for the rare *in vivo* species and poor competitor for the abundant ones. When DNA is in excess with respect to labeled RNA both of the above competitions should therefore be ineffective and incomplete.

These criteria will now be applied to a comparison of T4 early *in vivo* RNA and T4 RNA synthesized *in vitro* with mature viral DNA and *E. coli* RNA polymerase.

Criteria 1 and 2. RNA I AND II SYNTHESIS *in vitro* AND THE COMPARISON OF ABUNDANCES. A hybridization–competition of labeled 5-min and *in vitro* RNA with unlabeled 5- and 20-min *in vivo* RNA is shown in Figure 2. The characteristically better competition of labeled early RNA with 5-min than with 20-min unlabeled RNA is paralleled by the behavior of these competitors with labeled *in vitro* RNA. The result suggests that the major part of the *in vitro* synthesized RNA corresponds to the prereplicative early RNA species. The exact quantitative details of this comparison which has been made with many different samples of *in vitro* RNA vary to some extent but the principal features apply to RNA synthesized with limiting amounts of DNA *or* RNA polymerase.

The converse experiment, comparison of the competing power of unlabeled *in vitro* and early *in vivo* RNA, is shown in Figure 3a,b,c. Three different labeled *in vivo* RNA "probes" are used: RNA labeled 1–5 min (prereplicative), 17–20 min (late), and 48–53 min (very late) after infection of *E. coli* B^E by T4 am^+ at 30°. All three experiments are at excess of DNA over labeled RNA and in all three cases the competition curves due to unlabeled *in vivo* and *in vitro* RNA can be brought to (or near to) coincidence by suitably adjusting the concentration scale. The *in vitro* RNA is approximately 30 times as effective a competitor as 5-min *in vivo* RNA for all three labeled RNAs. This scale factor arises because total *in vivo* RNA contains little T4 RNA; Landy and Spiegelman (1968) found 3.5% T4 messenger in *E. coli* B RNA 3.5 min after infection at 37° and we observe a scale factor close to the reciprocal of this number. In six other "DNA excess" experiments which were less extensive than that shown in Figure 3, the observed scaling factors correspond with 3.3–5% T4 transcripts in *in vivo* 5-min RNA. More importantly, the ratio does not change drastically when the labeled probes change in content from the prereplicative to the postreplicative early species. The fact that *in vitro* RNA and *in vivo* 5-min RNA have equal competing power for 17–20-min and 48–53-min *in vivo* RNA suggests that the postreplicative early RNA species are synthesized *in vitro*. The relative ability of 5-min and 20-min *in vivo* RNA to compete with labeled *in vitro* RNA (Figure 2) suggests that prereplicative early RNA is the major *in vitro* transcription product. The invariance of the scaling factors of Figure 3 to the nature of the labeled *in vivo* RNA probe appears to mean that the relative abundance of these two major classes of transcription products in the *in vitro* RNA is the same as for *in vivo* RNA at 5 min after infection. The real situation is more complicated as the experiments described below will show.

The conditions of synthesis for the *in vitro* RNA of Figures 2 and 3 are, as a matter of convenience, somewhat different, the ^3H-labeled RNA having been synthesized under conditions of limiting enzyme while the unlabeled RNA used for the experiment shown in Figure 3 was synthesized at enzyme excess (details in the captions of Figures 2 and 3). This confirms what we have already stated (Brody *et al.*, 1970): the observed selectivity and preferential synthesis are *not* dependent on the DNA:enzyme ratio.

Criterion 3. ASYMMETRY AND TRANSCRIPTS COMPLEMENTARY TO *in vivo* PREREPLICATIVE RNA. The synthesis of T4 RNA *in vitro* is symmetric when the template is denatured DNA and asymmetric when native DNA templates are employed (Covill *et al.*, 1965; Geiduschek *et al.*, 1968; Brody *et al.*, 1970). Figures 1 and 4 show the RNase resistance acquired by annealing low concentrations of labeled *in vitro* RNA with unlabeled *in vivo* RNA. The *in vitro* RNA is synthesized under the following conditions: (a) on native DNA templates at either limiting enzyme or DNA concentrations and in the presence of Mg^{2+} or Mn^{2+} at different total ionic strengths (Figure 4); (b) on denatured DNA (curves A and B of Figure 1). A control experiment with labeled *in vivo* prereplicative RNA is shown for comparison. Assuming that the *in vivo* RNA is negligibly self-complementary, its RNase resistance (Figure 4; ▽) is subtracted from the other data to yield the RNase resistance contributed by *in vitro* synthesized complement of prereplicative RNA. For ten preparations of native T4 DNA made with a number of different enzyme preparations, at varying enzyme:DNA ratios and with varying durations of synthesis, the content of anti 5-min T4 RNA in the *in vitro* product is between less than 1% and 8%; on sheared and sonically degraded DNA templates the proportion of antimessenger synthesis is somewhat higher.

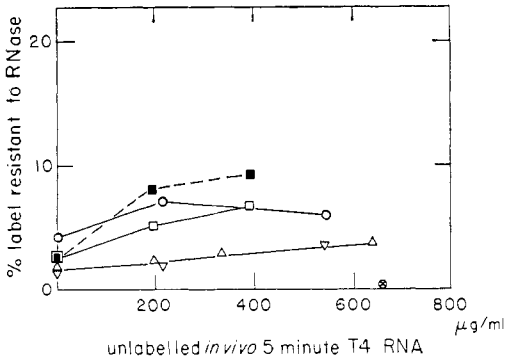

FIGURE 4: Antimessenger content: RNA–RNA complex formation of labeled *in vitro* RNA with unlabeled prereplicative (5-min) RNA: (△) 0.25 μg/ml of *in vitro* RNA ([^{14}C]ATP) synthesized in 45 min in standard reaction medium at 30° (0.39 μmole of RNA synthesized/μmole of DNA template). Self-complementarity: 8.2 μg/ml of RNA annealed to give 3.7% resistance to T1 and pancreatic RNase. This is less than 2% in excess of the resistance of *in vivo* prereplicative RNA to digestion under the same condition. (○) 0.63 μg/ml of RNA ([^{32}P]GPP) synthesized nearer enzyme excess over DNA (standard reaction medium; reaction 45 min at 30°; 1.50 μmoles of RNA synthesized/μmole of DNA template); (■) 0.097 μg/ml of RNA ([^{3}H]UTP) synthesized at low ionic strength in enzyme excess over DNA (Brody *et al.*, 1970); (□) 0.12 μg/ml of RNA ([^{3}H]UTP) synthesized at low ionic strength in DNA excess over enzyme (Brody *et al.*, 1970); (▽) 1.40 μg/ml of [^{3}H]RNA labeled *in vivo* 0–5 min after infection.

Extent of Transcription of the T4 Template. A titration of T4 DNA with RNA synthesized *in vitro* has been carried out over a 10^4-fold range of RNA to DNA (Figure 5). The RNA for this titration had been made for 60 min at 30°. In the titration of DNA with this RNA, conditions of DNA excess are defined by slope unity for the log–log plot; for example, with 10 μg/ml of DNA, deviations from linearity first become apparent at 0.2 μg/ml of RNA. At high RNA:DNA ratio the curve for 0.4 μg/ml of DNA approaches a plateau from which the extent of transcription of the template can be calculated, using the following additional considerations: (1) the hybridized RNA is corrected for the background of radioactivity

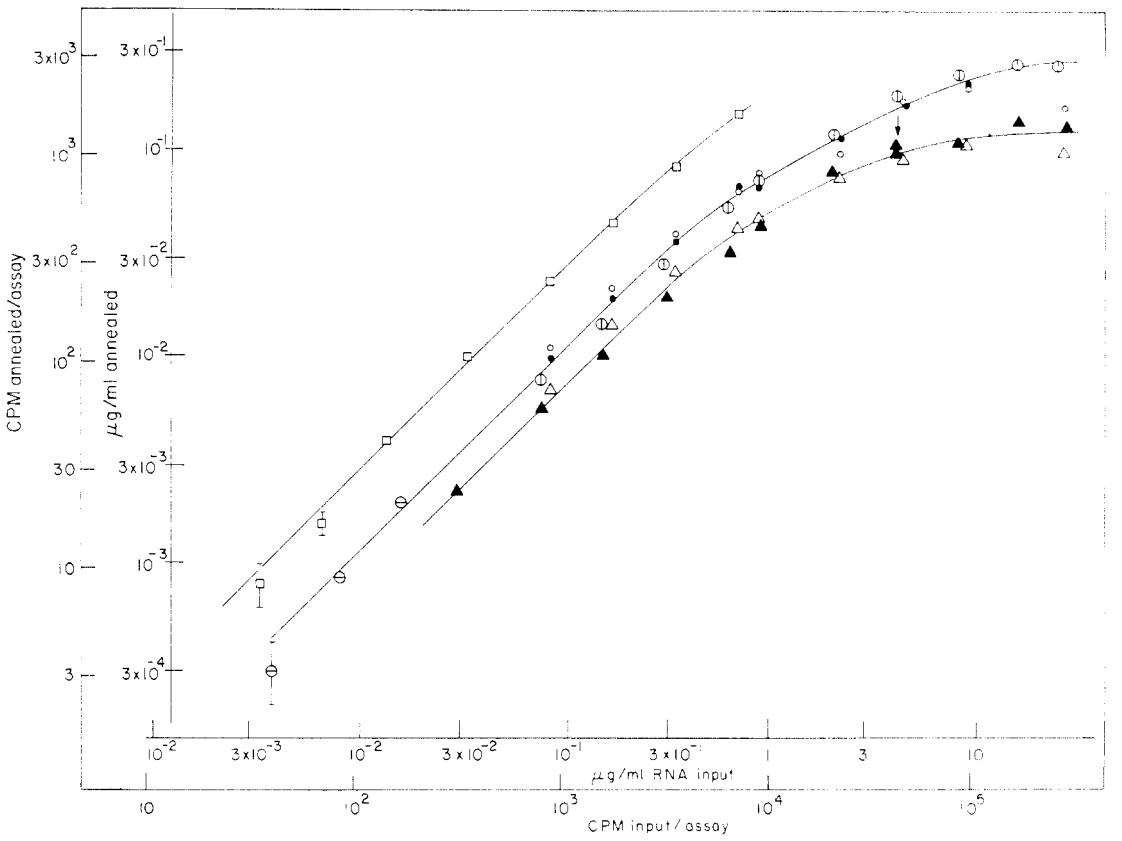

FIGURE 5: Titration of DNA with *in vitro* T4 RNA. The ^{14}C-labeled ATP RNA was synthesised with 1.3 units of enzyme/μg of DNA for 60 min at 30° in the standard reaction medium to yield 0.7 μmole of RNA synthesized per μmole of DNA template. The RNA is asymmetric: there is less than 1% antiearly RNA present but the assay described in the text (*cf.* Figure 6) gives an estimate of 5.5% antilate RNA. Hybridization: Hybrids were digested with pancreatic and T1 RNase before filtration. Backgrounds: with 7.6 μg/ml of ^{14}C *in vitro* RNA added (2.11×10^5 cpm/ml) the background in the absence of DNA was 85 cpm/ml (average of two determinations) or 0.04% of input. This is only 3% of the radioactivity annealing to the lowest concentration of DNA used, 0.4 μg/ml. Titration curves are drawn with an initial slope of one. At the very lowest concentrations used (*ca.* 1–5 ng of RNA/assay) there may be deviations from linearity due to adsorption of RNA to the glassware. Error lines indicate counting (statistical) error of 1–2 cpm at the lower limit. Average hybridization efficiencies at DNA excess (linear portion of the titration) are 7.5, 11.5, and 28%, respectively, at 0.4, 1, and 10 μg/ml of DNA, respectively. (△, ▲) 0.4 μg/ml of DNA; (○, ●, ⊖, ⓘ) 1 μg/ml of DNA; (□) 10 μg/ml of DNA.

bound in the absence of DNA ($\leq$8% of the total); (2) the counting efficiency of ^{14}C is measured to be 73% on nitrocellulose filters in this experiment and the specific activity of the ATP is 41.4×10^6 dpm/μmole; (3) the A content of T4 DNA is taken to be 32.3 mole %. At the plateau 99.4 μμmoles of AMP contained in RNA anneal to 1 ml of T4 DNA containing 0.08 A_{260} unit of DNA or 392 μμmoles of dAMP in DNA. If the adenine content of the synthesized RNA can be assumed equal to that of the DNA template this corresponds to 25.(3)% saturation of the DNA by RNA or 50.(6)% of one strand.

Error Level of the in Vitro Transcription. A small fraction of the *in vitro* synthesized T4 RNA is antimessenger. Its synthesis occurs at enzyme or DNA excess (Figure 6 and Brody *et al.*, 1970). In order to understand how this RNA arises, it is useful to summarize what we know about synthesis of antimessenger RNA *in vivo:* It has been found that a substantial fraction of labeled T4 late (20 min) RNA forms RNase resistant RNA–RNA complexes with unlabeled early (5 min) RNA. The mutually interacting RNA species appear to be much more abundant at 20 min than at 5 min after infection; they are never concurrently abundant since mixtures of labeled and unlabeled RNA collected at different times of infection form more RNA–RNA complexes than labeled and unlabeled RNA collected at any one time (Geiduschek and Grau, 1969). It seems likely that one class of the mutually interacting species is transcribed in the antimessage sense. This would imply some abnormality of termination of transcription *in vivo.* Convergent transcription of the T4 chromosome is known to occur but the number of such convergent sets of transcription units is not established.

That *in vitro* transcription also yields antimessenger is shown in Figure 6 where the formation of RNase resistant complexes by labeled *in vitro* and 5-min *in vivo* RNA is compared. The conversion of labeled *in vivo* 5-min RNA to RNase resistance by unlabeled *in vivo* 20-min RNA is small. Relative to this control, 3% of labeled ^{32}P *in vitro* RNA complexes with 300–500 μg/ml of 5-min unlabeled *in vivo* RNA while 10% of the label complexes with unlabeled 20-min *in vivo* RNA. The 3% of "antiearly" sequences found in this experiment correspond to an RNA synthesized at or near polymerase excess. We have already shown that RNA synthesized with limiting amounts of RNA polymerase frequently contains even less antiprereplicative RNA (Figure 4). The

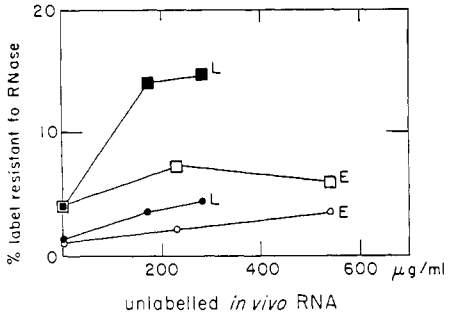

FIGURE 6: Antimessenger content: comparison of RNA–RNA duplex formation with prereplicative and late RNA: (○, ●) 1.4 μg/ml of [^{3}H]RNA labeled *in vivo* 0–5 min after infection (○, same as Figure 4); (□,■) 0.09 μg/ml of [^{32}P]GTP labeled *in vitro* RNA synthesized near enzyme excess, described in caption of Figure 4; (○,□) RNase resistance after interaction witn prereplicative (5 min) *in vivo* RNA; (●,■) RNase resistance after interaction with late (20-min) *in vivo* RNA. The same preparations of *in vivo* 5- and 20-min RNA were used for both parts of this experiment.

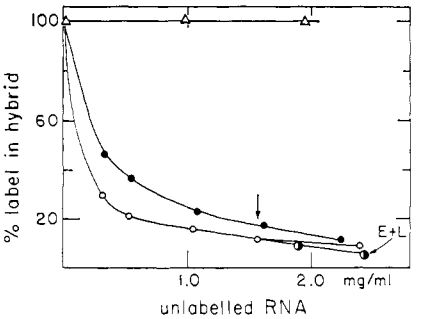

FIGURE 7: Hybridization–competition at RNA excess. The *in vitro* RNA is that used for the titration of Figure 5. Competition with (○) 5-min and (●) 20-min T4 RNA; competition with 1560 μg/ml of 5-min RNA (↓) + 20-min RNA to give the total concentration shown (◑); competition with uninfected *E. coli* RNA (Δ); 3.8 μg/ml of *in vitro* RNA, 0.4 μg/ml of denatured DNA; 100% hybridization is 1068 cpm which is 2.5% of the input radioactivity.

proportion of labeled *in vitro* RNA forming RNase resistant complexes with *in vivo* 20-min RNA, but not with *in vivo* 5-min RNA, is consistently observed whether syntheses are with limiting or excess quantities of enzyme. Clearly, the ratio of antilate:antiearly RNA is very different from the ratio of late:early RNA.

The synthesis of anti 20-min RNA *in vitro* probably arises from continuation of transcription across early–late junctions of the template. The prereplicative early (class I) and late (class III) cistrons probably have predominantly opposite polarities (Nakata and Stahl, 1967; Stahl *et al.*, 1966; Guha *et al.*, 1968). The postreplicative early cistrons may have both polarities (Guha *et al.*, 1968). It might seem paradoxical that antilate *in vitro* transcripts do not appear as plateaux in DNA–RNA hybridization–competition mixed competitor assays. The experiments referred to at the beginning of this section suggest, rather surprisingly, that anti 20-min transcripts of *in vitro* RNA may also be represented *in vivo*, albeit at a lower relative concentration.

Another way of detecting minor species of the *in vitro* RNA is by hybridization–competition at excess of labeled *in vitro* RNA over DNA (*cf.* Bolle *et al.*, 1968b, especially Figure 4 caption). In view of the RNA–RNA complexation that we have just discussed, this competition (Figure 7) only exhibits species whose complements *and* identical transcripts are *both* absent from prereplicative RNA. The *in vitro* labeled RNA for this experiment is the one on which the titration of Figure 5 was done. The comparable competition at DNA excess fails to show any competing ability in late RNA that is absent from early RNA (data not presented). However, the RNA excess competition shows a small fraction (3–4% of the hybridized label) which is not competed for by 2400 μg/ml of 5-min RNA but competed for by a mixture of 1560 μg/ml of 5-min and 860 μg/ml of 20-min RNA. Considerations analogous to those described by Bolle *et al.* (1968b) lead to the conclusion that 0.7–0.9% late transcript in the labeled *in vitro* RNA would produce this result.

This experiment also places a lower limit on the fraction of the template which codes for the early species of the *in vitro* RNA. Comparison with the titration of Figure 5 shows that for the RNA excess competition of Figure 7, binding in the absence of competitor corresponds to 89% of the DNA saturation plateau [after background correction; the curve of Figure 5 at that point (↓) is drawn through a coordinate that corresponds to 95% of the plateau value] or 22.6% of the template (adenine) saturated; of this, only 0.7–0.9% is accounted for by species that are in late transcripts. Accordingly, at least 43.4% of one strand of the template codes for the early species of the *in vitro* RNA. (This is the value that would obtain if the only species that annealed at RNA:DNA ratios greater than 10 were rare, late transcripts.)

Comparison of in Vitro RNA with T4 in Vivo Chloramphenicol RNA. When *E. coli* are infected with T4 in the presence of chloramphenicol, not only do they fail to synthesize DNA and the late transcripts, the synthesis of early T4 RNA is also incomplete. The RNA polynucleotide sequences that are missing are the delayed prereplicative early and some or all postreplicative early species. The experimental evidence for this effect of chloramphenicol on early transcription has been presented in detail by Salser *et al.* (1970) and R. Sederoff, A. Bolle, and R. H. Epstein (unpublished data, 1970). Ennis and Cohen (1968) have shown a similar effect on early transcription by inhibiting protein synthesis through K^+ depletion. On the other hand, RNA synthesized on native DNA templates *in vitro* has been shown to contain all the prereplicative species present 5 min after infection. One would therefore expect it to contain species that are not present in chloramphenicol *in vivo* RNA; Figure 8 shows that this expectation is realized. It has subsequently been shown that the transcripts that are blocked by chloramphenicol *in vivo* appear in the *in vitro* synthesis as a result of propagation from immediate early transcription units whose *in vivo* reading does *not* require protein synthesis in the infected cell (A. J. E. Colvill, submitted for publication; Milanesi *etal.*, 1970).

Denatured T4 DNA Templates. The RNA synthesized on denatured T4 DNA templates is symmetric (Colvill *et al.*, 1965; Geiduschek *et al.*, 1968; Brody *et al.*, 1970). Figure 9 shows the detailed hybridization–competition of such an RNA, done as a double label experiment with asymmetric early *in vitro* synthesized RNA. Clearly the symmetric RNA made on denatured DNA templates contains some fraction (*ca.* 14%) of its radioactivity in species that are more effectively

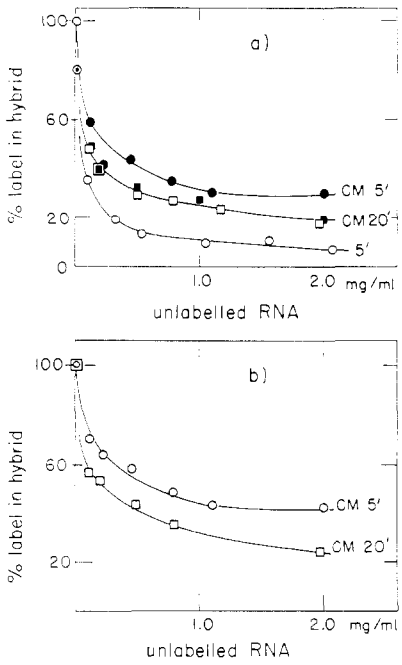

FIGURE 8: Competition of *in vitro* RNA and normal prereplicative *in vivo* RNA with chloramphenicol RNA. (a) Competition of *in vitro* RNA; 10 μg/ml of denatured DNA, 0.078 μg/ml of [^{14}C]RNA (identical with RNA of Figures 5 and 7); 26% of label bound to DNA in absence of competitor (223 cpm). (b) Competition of 3.5 μg/ml of 1–5 min *in vivo* RNA, 10 μg/ml of denatured DNA; 21% of label bound to DNA in the absence of competitor (336 cpm); (○) unlabeled 5-min RNA; (●) unlabeled 5-min CM RNA (200 μg/ml of CM added 3 min before infection); (□,■) unlabeled 20-min CM RNA (two samples; 100 or 200 μg/ml of CM added 3 min before infection).

competed for by 20-min than by 5-min RNA. This RNA is in sense *and* antisense sequences (Figure 9 caption). This is not the only alteration of transcription that has taken place: (1) one cannot reconstruct the competition curves A and B of Figure 9 by a linear combination of competition curves such as Figures 2 or curves C and D of Figure 9. The transcription product of denatured DNA must, accordingly, represent an alteration of the abundance distribution of early species relative to each other, or else the competition properties for antimessenger are completely different from those of messenger. That the first of these alternatives must be at least part of the whole explanation is indicated by the fact that somewhat greater than 5% of the label is poorly competed for by either 5- or 20-min RNA (compare Figure 9, curves B and C). This is in spite of the fact that more than 50% of the label in the transcription product of denatured DNA is exceedingly effectively competed for by 200 μg/ml of either early or late *in vivo* RNA. Thus the RNA synthesized on the denatured DNA template really does follow the expectation that it differs from RNA synthesized on native DNA with respect to all the criteria stated at the beginning of Results.

A Correlation between Late and Symmetric Transcription. A variety of procedures described in this and the preceding paper have led to detectable late RNA synthesis *in vitro*. We have reasoned that if any of these procedures were to provide a model for late *in vivo* transcription, then they should give late transcripts without giving symmetric RNA. We have constructed graphs of late RNA *vs.* symmetry of transcription for syntheses at elevated temperatures, with denatured and sonicated templates, in Me_2SO–water mixtures, and with repeated additions of enzyme. Figure 10 shows the difference in competition by 5- and 20-min *in vivo* RNA as a function of the fraction of RNA complementary to unlabeled early *in vivo* RNA. Deviations from the competition pattern of *in vivo* prereplicative RNA arise either because of the synthesis of late RNA or because the distribution of abundances among early RNA species is altered. For the *in vitro* synthesized RNA, such deviations are always accompanied by concomitant increases of antimessenger content. In fact, beyond a certain point, increases in symmetry apparently are not matched by further increases in the synthesis of nonearly species. By way of comparison, Figure 10 also includes the properties of *in vivo* late (20-min labeled) RNA and of late RNA synthesized *in vitro* in a complementation that constitutes an *in vitro* assay of a product of T4 gene 55 (Snyder and Geiduschek, 1968). Extensive additional data on many other RNA preparations (data not shown) establish an identical correlation with self-complementarity of RNA as the measure of symmetric transcription. This way of representing the results of numerous experiments therefore emphasizes that none of the manipulations involving T4 DNA and purified *E. coli* RNA polymerase that have been described in this and the preceding paper are adequate models for the switch to late transcription. The graph also provides a criterion for judging the adequacy of any *in vitro* system as a model for this *in vivo* switch.

Discussion

Fidelity of the in Vitro Transcription. The resemblance between *in vitro* transcription of mature T4 DNA by *E. coli* RNA polymerase and *in vivo* transcription of the T4 genome during the first minutes after viral infection extends to the following properties: (1) choice of the transcribing strand (Grau *et al.*, 1969); (2) selective synthesis of early RNA species with predominant synthesis of those species (prereplicative early) that become rare late in infection; in addition, there are these similarities in the timing of *in vivo* and *in vitro* transcription; (3) there is a correspondence of the initial transcription of the T4 template *in vitro* and *in vivo*, and delayed prereplicative RNA nucleotide sequences which appear *in vivo* only after a time lag are also delayed *in vitro* (Milanesi *et al.*, 1969, 1970); (4) the *in vitro* and *in vivo* sequential appearance of transcripts of the rII genes and an adjoining, nonessential region of the T4 chromosome can be scaled to each other (R. Sederoff, A. Bolle, and R. H. Epstein, to be published; E. N. Brody, to be published). Thus, the major transcriptive events of the prereplicative period of T4 viral development are capable of being mimicked qualitatively and perhaps quantitatively by mature viral DNA and host RNA polymerase *in vitro*.

Selective transcription of early RNA occurs at enzyme and DNA excess (as defined by kinetics of incorporation) and at low ionic strength in the presence of Mn^{2+}, where others have shown nonspecific binding of RNA polymerase to DNA. It is known that enzyme binding to DNA is much more rapid than initiation of RNA chains (Richardson, 1966a; Sternberger and Stevens, 1966). The fact that specificity of transcription is retained for wide variations of the relative proportions of DNA to enzyme must mean that enzyme

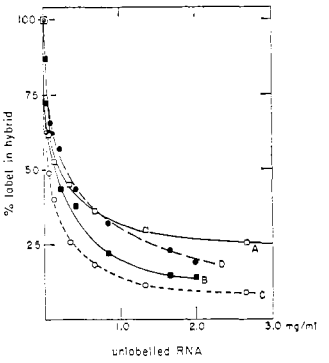

FIGURE 9: Hybridization–competition of T4 RNA synthesized on denatured T4 DNA template. The experiment is done as a double label competition experiment with 10 μg/ml of denatured T4 DNA, 0.13 μg/ml of [^{3}H]UTP-labeled RNA synthesized on denatured T4 DNA (standard medium, 45-min synthesis at 30°, 0.04 μmole of RNA synthesized/μmole of DNA), and 0.64 μg/ml of [^{14}C]ATP labeled RNA synthesized on native T4 DNA (same conditions). (A) Competition of [^{3}H]RNA by *in vivo* 5-min RNA. (B) Competition of [^{3}H]RNA by *in vivo* 20-min RNA. (C) Competition of [^{14}C]RNA by *in vivo* 5-min RNA. (D) Competition of [^{14}C]RNA by *in vivo* 20-min RNA. 100% hybridization: 258 cpm of ^{3}H and 1010 cpm ^{14}C, which is 10 and 25%, respectively, of these input radioactivities. Maximum background in the absence of DNA, 2 and 8 cpm of ^{3}H and ^{14}C, respectively. Antimessenger content: [^{14}C]RNA, see Figure 2 (△); [^{3}H]RNA: 0.13 μg/ml of [^{3}H]RNA was 41% resistant to pancreatic and T1 RNase after annealing with 660 μg/ml of unlabeled 5-min *in vivo* RNA and 40% resistant to pancreatic and T1 RNase after annealing with 210 μg/ml of unlabeled 20-min *in vivo* RNA.

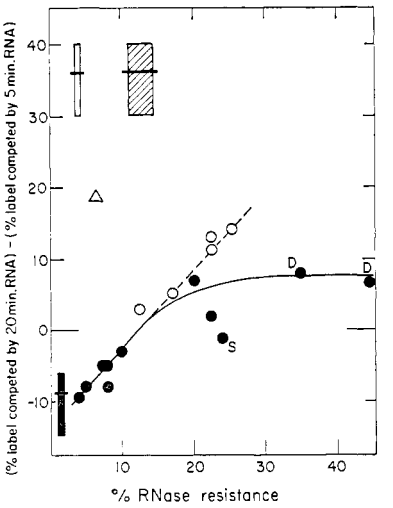

FIGURE 10. A correlation between late (class III) and symmetric T4 RNA synthesis with *E. coli* and *M. lysodeikticus* RNA polymerase. Ordinate: the difference in competition by *ca.* 1.5–2 mg/ml of unlabeled 5-min and 20-min RNA. Abscissa: RNase resistance of *in vitro* RNA in the presence of 300–600 μg/ml of unlabeled *in vivo* 5-min RNA (except as noted below): (●) *in vitro* RNA made with *E. coli* RNA polymerase; (○) *in vitro* RNA made with *M. lysodeikticus* RNA polymerase; (S) *in vitro* RNA made with sonicated DNA template; (D) *in vitro* RNA made with denatured DNA template. (▌) Control: Range and mean of four competition experiments with labeled *in vivo* prereplicative RNA and RNA–DNA hybridization analysis methods I or II. (▯) Control: Range and mean of experiments with labeled *in vivo* 20-min RNA (including 1–20-min and 17–20-min labeling); RNase resistance with unlabeled 20-min *in vivo* RNA is shown. (▨) As above but RNase resistance with unlabeled 5-min *in vivo* RNA. (△) T4 RNA III synthesis with *in vitro* complementation (Snyder and Geiduschek, 1968, Figure 3; the vertical coordinate comes from a mixed competitor assay and RNase resistance with unlabeled 20-min *in vivo* RNA is shown).

readily moves from DNA sites at which it binds reversibly to loci at which RNA chains are initiated. Even the RNA polymerase binding to discrete, well-separated sites on λ and T7 DNA occurs at many more sites than serve for the initiation of transcription by unmodified bacterial RNA polymerase *in vivo* (Richardson, 1966b; Jones and Berg, 1966; Stead and Jones, 1967; Kourilsky *et al.*, 1968; Summers and Siegel, 1969; Szybalski, 1969; Roberts, 1969). The significance of restricted polymerase–DNA binding is clearly not obvious. Yet it seems unlikely that this discrete binding is irrelevant. We suggest that binding occurs at sites which constitute a large part of, but not the entire, DNA sequence that signals RNA chain initiation; the polymerase binding sites are virtual sites for the initiation of RNA synthesis and are relatively numerous compared with actual promoters. Darlix *et al.* (1969) have shown that RNA polymerase "core" binds to DNA in the absence of initiation factors (σ); *E. coli* σ does not, by itself, bind tightly to DNA but does bind to RNA polymerase (Burgess *et al.*, 1969) and determines specificity of initiation (Travers and Burgess, 1969; Bautz *et al.*, 1969; Goff and Minkley, 1970). Corebound σ must *a priori* determine recognition of DNA sequences or signals directly or allosterically (more likely the former). It seems possible that only those *virtual* initiation sites which also fulfil the binding requirements of particular σ factors and the initiation–catalytic requirements for forming the first phosphodiester bond are *actual* initiation sites.

Under the conditions of these experiments, there is little or no release of enzyme molecules or nascent RNA chains from the template (Maitra and Hurwitz, 1967; Bremer and Konrad, 1964; Chamberlin, 1968, personal communication). The synthesized chains of RNA can grow very long (Milanesi *et al.*, 1970) although synthesis slows down markedly as nascent RNA chains become longer. We have suggested that a small but detectable fraction of antilate RNA arises from such faulty termination and from the opposite polarity of early and late transcription units (Stahl *et al.*, 1966; Nakata and Stahl, 1967; Guha *et al.*, 1968). Nonetheless, termination-defective transcription is of some benefit in tracing connections between transcription units, *i.e.*, in studying the topography of transcription (Milanesi *et al.*, 1970).

The Program of T4 Prereplicative Transcription. The time sequence of prereplicative T4 transcription has been described recently. It is known that protein synthesis in the infected cell is required in order to complete this sequence (Salser *et al.*, 1970; Grasso and Buchanan, 1969; A. J. E. Colvill, submitted for publication). The early RNA that has been made after this entire sequence has run its course is characterized by a certain abundance distribution which subsequently changes (Salser *et al.*, 1970; Bolle *et al.*, 1968a), the changes commencing before late (class III) transcription starts (Haselkorn *et al.*, 1968; Salser *et al.*, 1970).

We interpret the observed resemblance between *in vitro* transcription of mature T4 DNA and prereplicative transcription of the infected cell in the following way: (1) The characteristic abundance distribution of T4 prereplicative and postreplicative early RNA (I and II) species in early RNA reflects the interaction of T4 promoters with host RNA

polymerase. (2) The nonrecognition of late transcription units is also a property of mature DNA and host RNA polymerase. (3) Changes in the relative proportions of RNA I and II occur after replication starts. Such changes also occur in gene 55 mutants, are therefore distinct from true late transcription, and may involve new recognition properties of the transcribing enzyme (Hall *et al.*, 1969). At least a part of the host RNA polymerase is used throughout T4 viral development (Haselkorn *et al.*, 1969). However a new initiation factor, σT4, appears after infection (Travers, 1969a) and changes in one of the core subunits in T4 infected cells have also been observed (Walter *et al.*, 1968). So there are two kinds of alterations which could account for these changed recognition properties.

The last question to consider is this: How are the delayed early transcripts generated *in vivo*? Almost all of these transcripts are synthesized on the T4 DNA *1* strand. It has been shown that they are synthesized *in vitro* by *E. coli* RNA polymerase from initiation sites for immediate early RNA by propagation (Milanesi *et al.*, 1969, 1970; Gold and Schweiger, 1970; E. T. Young, personal communication, 1969). Yet it is known that synthesis of delayed early T4 RNA *in vivo* is blocked by chloramphenicol (Ennis and Cohen, 1968; Salser *et al.*, 1970; Grasso and Buchanan, 1969). The following interpretations of this latter finding are possible. (1) A T4 protein is required for delayed early T4 RNA synthesis; this protein could be a modifier of RNA initiation or a new RNA initiation factor (1a) or it could be a modifier of RNA chain termination (1b). (2) The action of chloramphenicol is indirect. Two possibilities of this latter kind can be distinguished: (2a) chloramphenicol prevents the complete reading of T4 early transcription units [Alpers and Tomkins (1965) suggested the existence of such effects for the *lac* operon]; (2b) in the absence of protein synthesis, infection does not shut off host transcription (Okamoto *et al.*, 1962). Consequently promoter–polymerase relationships are very greatly dependent on protein synthesis after infection. The T4 transcription units normally turned on after several minutes *in vivo* compete very poorly for the available polymerase when host transcription is not shut off.

Hypothesis 2b appears less plausible because *Shigella dysenteriae* are not shut off completely by phage T4 and yet seem normal in their development of T4 phage. With regard to hypothesis 1b, Milanesi and coworkers (1970) have shown that prereplicative segments of the T4 chromosomes include units of transcription which have this structure: initiation site → immediate early → delayed early. The delayed early genes are apparently all contained in such units of transcription which are joined end to end. It seems likely that a chromosome whose structure is arranged for transcription by read-through would be transcribed that way in real life. We therefore favor the hypothesis that modified chain termination after T4 infection allows read-through of large early transcription units.

Travers (1969a) has recently made the extremely important observation that a new RNA initiation (σ) factor appears in T4 infected *E. coli*. This initiation factor predominantly promotes T4 DNA *1* strand transcription and yields much delayed early T4 RNA on short-term incubation (Travers, 1969b). If there is only one way of generating the delayed early transcripts *in vivo* then these observations would lead one to hypothesis 1a. The question of which of these mechanisms—altered initiation or altered termination—first yields the delayed early transcripts *in vivo* can be decided by direct examination of the size and contiguity of T4 *in vivo* synthesized T4 mRNA (R. Sederoff, A. Bolle, and R. H. Epstein, to be published). However, we believe that both these hypotheses of T4 transcription are valid and that both kinds of virus induced modifications of transcription apply to early RNA synthesis at *some* stage of T4 development. This implies that all or most delayed early transcripts can be generated *in vivo* in *two* different ways. If no prereplicative T4 transcription is exclusively dependent on a single viral effector of transcription, then one can predict that the structural gene for the presumptive T4 initiation factor is not among those whose function is essential for viral replication. In fact, amber mutants in the known T4 DO genes (these are the genes whose products are absolutely required for viral replication) have been screened for their transcription properties. None restricts its T4 transcription to immediate early RNA (C. Bordier and R. H. Epstein, personal communication; E. P. Geiduschek, unpublished observation).

The picture of T4 prereplicative transcription which we present is not the simplest possible and embodies some new ideas about the topology of transcription. It has been customary to think of the unique nucleotide sequence of a bacterial chromosome as defining a set of nonoverlapping transcription units. (The b_2 region of phage λ DNA appears to be an exception to this rule and exceptional chromosomes have been constructed; Bøvre and Szybalski, 1969; Levinthal and Nikaido, 1969). We believe that portions of the T4 chromosome contain overlapping transcription units and can be read in more than one mode. We do not know whether this is a very important aspect of the physiological and developmental genetics of viruses. In principle, such an arrangement allows a relatively small number of effectors of transcription to produce many variations in the duration and yield of gene action. It will be interesting to see whether this kind of strategy is widespread among relatively simple developmental processes.

Acknowledgment

We should like to thank A. Daniels for skillful technical assistance, G. Milanesi for valuable discussion, and R. H. Epstein, R. Sederoff, and R. Haselkorn for their helpful comments on the manuscript.

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