IN VIVO AND *IN VITRO* TRANSCRIPTION OF T_7 EARLY GENES BY T_7 RNA POLYMERASE

J.R. LILLEHAUG, D. HELLAND and N.O. SJÖBERG
Department of Biochemistry, University of Bergen, Årstadveien 19, 5000 Bergen, Norway

Received 2 April 1973

1. Introduction

In contrast to the T-even phages, coliphages T_7 and T_3 produce their own RNA polymerase [1, 2]. T_7 RNA polymerase, isolated from the soluble fraction of disrupted infected bacteria has been claimed by Summers and Siegel [3] to transcribe only late T_7 genes *in vitro*. The data of Studier [4] indicate that early phage proteins are produced also late in the infection period. The question arises at which time and by which of the two polymerases the mRNA's coding for these early proteins are transcribed. The activity of T_7 RNA polymerase can be distinguished from *E. coli* RNA polymerase since only the latter is inhibited by rifampicin [1].

The stability of T_7 mRNA was studied by Summers [5], who determined the mRNA half-life to be approx. 15 min. One of us showed that early mRNA was labelled during a 3 min pulse 14 to 17 min after infection at 30°C [6]. It is therefore plausible that early phage proteins synthesized at late times are coded by mRNA synthesized both at early and late times after infection.

Recently Scherzinger et al. [7] showed that early phage proteins (ligase and lysozyme) are synthesized *in vitro* in a system where T_7 RNA polymerase was the only RNA polymerase present. This suggests that early mRNA's are transcribed by T_7 RNA polymerase *in vivo*. In this paper we show that *in vitro* mRNA synthesized by T_7 RNA polymerase is partially competed against by class I mRNA in hybridization–competition experiments, thus confirming the results of Scherzinger et al. [7]. Furthermore, we found that T_7 RNA polymerase is responsible for at least part of the early T_7 mRNA synthesis taking place in the late period.

2. Materials and methods

2.1. Chemicals

All chemicals used were of highest commercial grade. Unlabelled triphosphates and Trizma buffer were from Sigma Chemical Co. [5-^{3}H]Uracil and [5-^{3}H]uridine-5'-triphosphate were from The Radiochemical Centre, Amersham. Rifampicin was a gift from CIBA.

2.2. Bacterial strains and phages

E. coli B, *E. coli* 011 (permissive host for T_7 amber mutants), T_7 wild type, and T_7 am 23 were gifts from Prof. W. Studier. *E. coli* B^E rs 30 was from Prof. R.H. Epstein.

2.3. Growth conditions and isolation of phages

The bacteria were grown at 30°C on a slightly modified casamino acid–glycerol medium [8]. The same medium was used for phage propagation. Phage was purified according to the method of Yamamoto [9]. When T_7 was purified for strand separation, the method described by Bövre [10] was employed. Membranes from phage infected cells were isolated as described by Haarr [11]. The temperature for all phage infections was 30°C.

2.4. Hybridization techniques

Hybridization and hybridization competition experiments were performed according to the methods

of Hall et al. [12]. The hybrids were collected on nitrocellulose filters, and the radioactivity was determined in the nuclear Chicago scintillation counter, using toluene–PPO–POPOP scintillation mixture (10 ml/vial).

2.5. DNA preparation

Native T_7 DNA was isolated from purified phage by phenol extraction [13]. Isolated DNA preparations had A_{260}/A_{280} ratios of 1.88 to 1.95. The absorbance was measured in a Zeiss PMQ II spectrophotometer. The separation of T_7 DNA strands was carried out according to Bövre [10].

2.6. RNA polymerase assay

The T_7 RNA polymerase specific assay (0.25 ml) contained 5.5×10^{-4} M ATP; 3.5×10^{-4} M CTP and GTP; 1.2×10^{-4} M [^{3}H]UTP (1.4 Ci/mM); 5 mM β-mercaptoethanol; 40 mM Tris-HCl pH 7.9; 20 mM $MgCl_2$; 25 μg/ml native T_7 DNA; 20 μg/ml rifampicin and membrane suspension (containing the RNA-polymerase) to a final conc. of 150 μg/ml protein. The incubation temperature was 30°C. When assaying the *E. coli* RNA polymerase rifampicin was omitted, the $MgCl_2$ concentration was changed to 8 mM, and 0.5 M KCl was added.

Radioactivity of TCA precipitable material was determined. For *in vitro* RNA production the assay mixture was scaled up to 4 ml, and the reaction was allowed to proceed for 30 min.

2.7. RNA isolation

2.7.1. *In vivo* RNA

At the time desired, the infected cells were poured on frozen medium (40 g/100 ml culture) containing 10^{-2} M NaN_3. Further RNA isolation was performed as described by Hall and Crouch [14]. The pulse-labelled RNA was isolated similarly except the ethanol precipitation was omitted.

Class I RNA was obtained in two ways: i) *E. coli* B cells growing in the log phase (about 5×10^8 cells/ml) were infected by T_7 phage at an m.o.i. of 17 to 20 at 5 min after the addition of 400 μg chloramphenicol per ml. At 8 min after infection, the culture was poured on 0.4 vol crushed, frozen medium. Further isolation was as described for *in vivo* RNA. ii) Class I RNA was also isolated from *E. coli* B cells infected with T_7 am 23. In this case class I RNA is the only phage specific RNA synthesized [5].

2.7.2. Isolation of RNA synthesized *in vitro*

At the end of the *in vitro* RNA synthesis, DNAase (final conc. 20 μg/ml) was added and the solution was incubated at 37°C for 10 min. Then 25% SDS to a final conc. of 0.5% and 1 vol of freshly distilled phenol saturated with water was added. This mixture was shaken for 3 min at 60°C and then for 30 min in the cold. The extraction was repeated twice. Finally, the RNA solution was dialyzed against 3×1 ℓ of 0.5 M KCl, 0.01 M Tris-HCl pH 7.3 for 24 hr at 4°C. The labelled RNA was always tested for absorption on nitrocellulose filters. The amount of radioactivity on the filter did not exceed 5% of the total acid precipitable label.

3. Results and discussion

3.1. In vivo experiments

Summers and Siegel [3] have shown that the development of T_7 phage becomes resistant to rifampicin when the drug is added later than 3 to 4.5 min after T_7 infection at 30°C. From these observations they concluded that all class I RNA synthesis by host RNA polymerase has been initiated by that time. Their results do not exclude the synthesis of class I RNA late in the infection period. To investigate this, mRNA was pulse-labelled *in vivo* between 14 and 17 min after infection at 30°C. The RNA was isolated as described in Methods, and characterized by hybridization competition experiments [12]. Unlabelled class I RNA should not compete with the labelled *in vivo* RNA if class I RNA was not synthesized during the pulse. In fig. 1 the hybridization competition curve is shown. Approx. 30% competition is found using class I RNA as competitor. This observation shows that class I RNA is produced also late in the infection period. Thus, the question arises whether the host RNA polymerase is still active, or if it is the phage induced RNA polymerase which transcribes the early T_7 genes. When *E. coli* cells sensitive to rifampicin are infected with T_7 in the presence of the drug, the host RNA polymerase

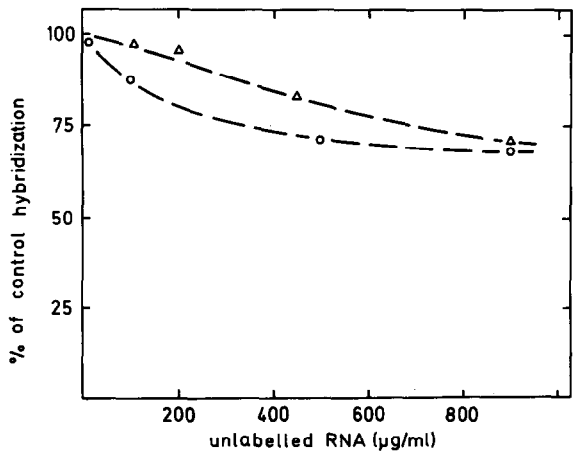

Fig. 1. Hybridization competition between *in vivo* class I RNA and *in vivo* 14 to 17 min labelled [^{3}H]uracil RNA. The class I RNA was isolated from *E. coli* B cells infected by T_7 phage at an m.o.i. of about 20 at 5 min after the addition of 400 μg/ml chloramphenicol. The cells were harvested 8 min after infection and the RNA isolated as described in Methods. The 14 to 17 min RNA was labelled with [5-^{3}H]uracil. The hybridization mixture in 0.5 ml, 0.5 M KCl, 0.01 M Tris-HCl, pH 7.3 contained 2.5 μg/ml denatured double stranded T_7 DNA, labelled RNA and the unlabelled competitor RNA at the amount indicated. (o—o—o) 14 to 17 min *in vivo* [^{3}H]RNA, produced in the presence of rifampicin, *E. coli* B^E rs 20 being the host. RNA concentration, 8.2 μg/ml. The specific activity of the labelled RNA was 1750 cpm/μg. (Δ—Δ—Δ) 14 to 17 min *in vivo* [^{3}H]RNA produced in absence of rifampicin, *E. coli* being the host. The reaction mixture contained 10 μg/ml of the labelled RNA (2500 cpm/μg). The reaction temperature was 67°C and the incubation time was 3 hr.

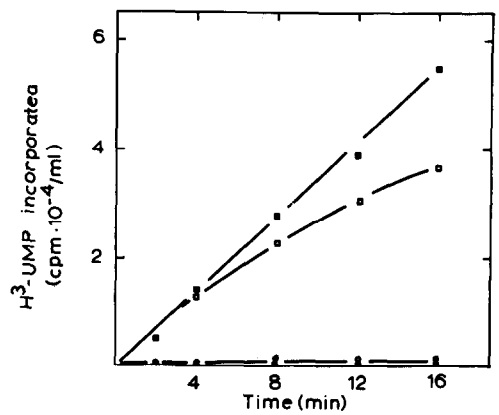

Fig. 2. *In vitro* assay of RNA polymerase in membrane preparations from *E. coli* B^E rs 30 cells infected with T_7 and harvested 12 min after infection. The isolation of the membrane and the assay conditions were as described in Methods. Assay conditions for the host RNA polymerase were altered so that the $MgCl_2$ and KCl concentrations were 8 mM and 0.5 M respectively. (■—■—■) T_7 RNA-polymerase activity. (□—□—□) T_7 RNA-polymerase activity in the presence of 50 μg/ml rifampicin. (o—o—o) Host RNA-polymerase activity. (●—●—●) Host RNA-polymerase activity in the presence of rifampicin (50 μg/ml).

should be blocked and the T_7 RNA polymerase should be responsible for the entire RNA synthesis [1]. The above experiment was repeated, using *E. coli* B^E rs 30, which is rifampicin sensitive. Rifampicin was added 10 min after infection, to a final conc. of 50 μg/ml. Control experiments with the sensitive bacteria showed normal phage production. Bacterial growth ceased immediately in the presence of the drug. The results of hybridization–competition experiments with *in vivo* RNA labelled in the presence of rifampicin are presented in fig. 1. These agree well with the results using RNA synthesized in the absence of the drug. It is thus concluded that T_7 RNA polymerase is capable of transcribing early T_7 genes *in vivo*.

3.2. In vitro experiments

To see whether or not *E. coli* RNA polymerase is active during the late period of T_7 infection, membrane preparations isolated according to Haarr [11] were used as a source of RNA polymerase for *in vitro* assays (fig. 2). A very low RNA polymerase activity was observed under the assay conditions optimal for host enzyme.

Rifampicin did not influence the activity significantly. However, by increasing the isotopic concentration, we found a decrease in host RNA polymerase activity in the presence of rifampicin. The T_7 RNA polymerase, in contrast, is very active, and shows the normal insensitivity towards rifampicin. The RNA synthesized by cell membranes from *E. coli* B cells infected with T_7 and harvested 15 min after infection was characterized by hybridization–competition experiments (fig. 3). This *in vitro* RNA was found to hybridize with the r-strand of T_7 DNA only [10]. By a comparison of the *in vivo* results with those obtained *in*

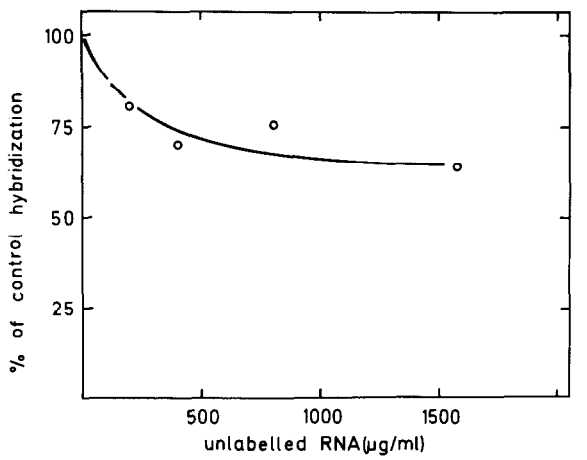

Fig. 3. Hybridization–competition between *in vivo* class I RNA and *in vitro* [^{3}H]RNA synthesized by membrane-bound T_7 RNA-polymerase. Membranes were isolated 15 min after phage infection at 30°C. The isolation of membranes, the *in vitro* RNA synthesis and RNA isolation were performed as described in Methods. The hybridization conditions were the same as described in the legend to fig. 1. The concentration of denatured double stranded T_7 DNA was 3 μg/ml and of the labelled RNA 3.5 μg/ml (specific activity 47 000 cpm/μg).

vitro, we conclude that the phage-specific RNA polymerase is capable of transcribing the complete T_7 genome *in vivo* as well as *in vitro*.

Further evidence suggesting that the phage-specific RNA polymerase transcribes class I RNA was obtained using RNA polymerase partially purified from isolated cell membranes [6]. This enzyme preparation was completely dependent on externally added T_7 DNA. By hibridization–competition experiments the RNA produced was found to contain approx. 40% class I RNA.

Acknowledgement

This work has been partly supported by a grant obtained by Professor A.P. Nygaard from Norges Almenvitenskapelige Forskningsråd, whom we wish to thank for their financial support.

References

[1] M. Chamberlin, J. McGrath and L. Waskell, Nature 288 (1970) 227.
[2] J.J. Dunn, F.A. Bautz and K.K.F. Bautz, Nature New Biology 230 (1971) 94.
[3] W.C. Summers and R.B. Siegel, Nature 228 (1970) 1160.
[4] F.W. Studier, Science 176 (1972) 367.
[5] W.C. Summers, J. Mol. Biol. 51 (1970) 671.
[6] J.R. Lillehaug, Cand. real thesis, University of Bergen (1971).
[7] E. Scherzinger, P. Herrlich and M. Schweiger, Molec. Gen. Genet. 118 (1972) 67.
[8] D. Frazer and E.A. Jerrel, J. Biol. Chem. 205 (1953) 291.
[9] K.R. Yamamoto and B.M. Alberts, Virology 40 (1970) 734.
[10] K. Bövre and W. Szybalski, in: Methods in enzymology, eds. L. Grossman and K. Moldave (Acad. Press, New York, London, 1971) Vol. 21, Part D, p. 350.
[11] L. Haarr, Biochim. Biophys. Acta 145 (1967) 202.
[12] B.D. Hall, A.P. Nygaard and M.H. Green, J. Mol. Biol. 9 (1964) 143.
[13] L. Grossman, S.S. Levine and W.S. Allison, J. Mol. Biol. 3 (1961) 47.
[14] B.S. Hall and R. Crouch, in: The biochemistry of virus replication, eds. S.G. Laland and L.O. Fröholm (Universitetsforlaget, Oslo, 1968) p. 49.