TRANSCRIPTIVE COMPLEX: ISOLATION BY CESIUM SULFATE-CENTRIFUGATION

Shigetaka Naito and Akira Ishihama

Department of Biochemistry, Institute for Virus Research
Kyoto University, Kyoto, Japan

Received January 30, 1973

SUMMARY

Formation of the "CsCl complex" (an RNA polymerase-DNA complex isolatable by equilibrium centrifugation in CsCl) was further shown to require the presence of more than two species of nucleoside triphosphate substrates and double-stranded DNA suggesting that the formation at least of a single phosphodiester bond renders the RNA polymerase-promoter DNA complex resistant to dissociation by such high concentration of CsCl. Growing RNA chains were still attached to RNA polymerase-DNA complex even after centrifugation whereas completed chains were recovered as released from the complex and banded at the position of free RNA in Cs_2SO_4 equilibrium centrifugation.

INTRODUCTION

The early genes (18-20 % of the total genome) of T7 DNA are transcribed by host RNA polymerase, whereas the late genes are transcribed by the phage-speciefied RNA polymerase, one of the products of the early genes (1, 2). Based on *in vivo* studies, a single unique initiation site for *E. coli* RNA polymerase has been proposed for transcription of these early genes. Early *in vitro* works (3-6), however, suggested multiple sites on T7 DNA for binding and initiation of RNA synthesis by the DNA-dependent RNA polymerase from *E. coli*.

Recently, Davis and Hyman (7) observed by electronmicroscopy that RNA synthesis is initiated on a single site near one end of the T7 DNA and then proceeds towards the other end. In the previous report (8), we provided supporting evidence that the T7 DNA bearing only one RNA polymerase could be isolated by the CsCl equilibrium centrifugation. The present report provides details on the mechanism of the "CsCl complex" formation and, in addition, demonstrates that the RNA polymerase-DNA complex bearing growing RNA chains can also be isolated free from completed RNA chains by equilibrium centrifugation in Cs_2SO_4.

MATERIALS AND METHODS

RNA polymerase: Labelled and unlabelled RNA polymerases of E. coli K12 W3350 were prepared and assayed as described previously (8, 9, 10). The standard reaction mixture contained in 0.25 ml: Tris-HCl (pH 7.8 at 37°C), 120 mM; Mg acetate, 5 mM; Mn sulfate, 2 mM; dithiothreitol (DDT), 0.1 mM; GTP, CTP, UTP, 0.16 mM; ^{3}H-ATP (specific radioactivity of 5 to 10×10^{3} cpm/nmole), 0.16 mM; T7 DNA and RNA polymerase.

CsCl and Cs_2SO_4 equilibrium centrifugation: As described previously (8) solid CsCl or Cs_2SO_4 was directly added to the reaction mixture for RNA synthesis followed by addition of glycerol to make a 10 % glycerol solution containing 10 mM Tris-HCl (pH 7.8 at 4°C), 10 mM $MgCl_2$, 0.1 mM ethylenediamine tetraacetate (EDTA), and 0.1 mM DTT. The resulting solution was centrifuged at 4°C in Beckman SW 50.1 rotor at 40,000 or 44,000 rpm for 40 hrs.

Sucrose gradient centrifugation: Fractions of Cs_2SO_4 equilibrium centrifugation were treated with 0.2 % sodium dodecyl sulfate (SDS) followed by dialysing Cs_2SO_4 against 0.05 M Na acetate buffer (pH 5.0) containing 0.01 % Salcosyl NL 97, 0.1 M NaCl and 1 mM EDTA, and applied to centrifugation in 5 to 20 % (w/v) sucrose in Na acetate buffer. E. coli ribosomal RNA was run in a parallel tube as a reference.

RESULTS

Isolation of the initiation complex by CsCl centrifugation: The formation of the "CsCl complex" as measured by attachment of ^{35}S-labelled RNA polymerase to DNA (Fig. 1) was observed only when the mixture of RNA polymerase and DNA was incubated in the presence of substrates prior to centrifugation in CsCl. The number and combination of substrate required for the recovery of ^{35}S-labelled RNA polymerase as the CsCl complex are summarized in Table 1. ATP alone allowed no detectable binding of the polymerase to DNA while in the presence of two kinds of substrates 6 to 18 % of complex was observed as compared to that formed in the presence of ATP, GTP and UTP, suggesting that the formation at least of a single phosphodiester bond was required to make the complex resistant

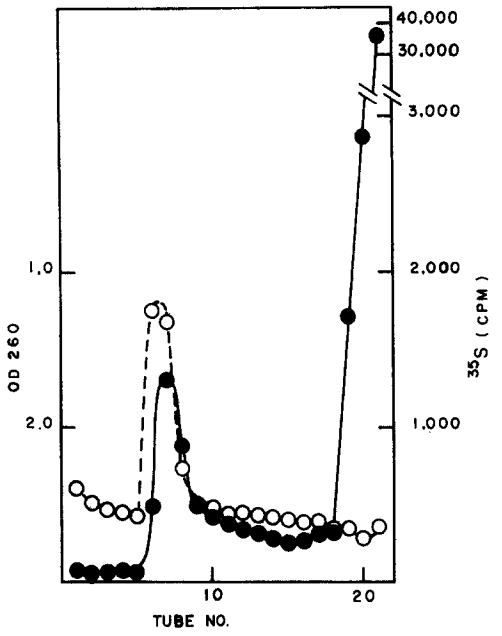

Fig. 1.

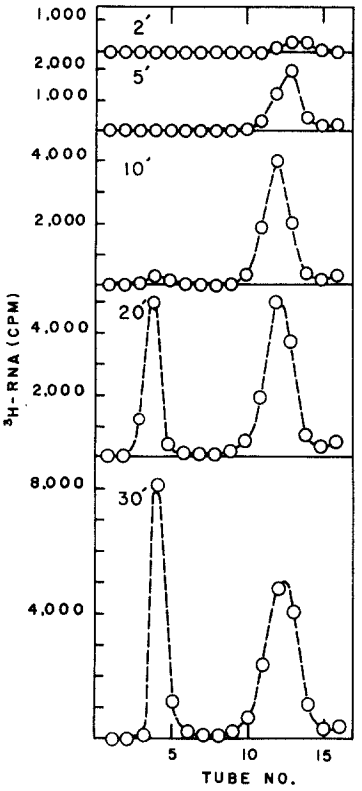

Fig. 2.

Figure 1 Isolation of the CsCl complex. A mixture of ^{35}S-labelled RNA polymerase (16.8 μg protein; approximately 4.4×10^4 cpm radioactivity) and T7 DNA (63 μg) was incubated for 5 min at 30°C in the standard reaction mixture lacking CTP, and centrifuged in CsCl. Of the input polymerase 7.7 % (1.2 μg) was found to be bound on 63 μg of T7 DNA; that is, 1.07 mole RNA polymerase per mole T7 DNA.

Figure 2 Isolation of the Cs_2SO_4 complex. RNA polymerase was mixed with T7 DNA at a input ratio of 5, and incubated at 30°C in the standard reaction mixture for RNA synthesis containing 0.15 M KCl. At the indicated time, aliquots were subjected to the Cs_2SO_4 equilibrium centrifugation and the distribution of product RNA was analyzed as described in MATERIALS AND METHODS.

---

to dissociation by such high concentration of CsCl. The antibiotics, rifampicin and streptolydigin which inhibit bacterial RNA synthesis by binding to the RNA polymerase, prevented the formation of the CsCl complex (Table 2). Moreover the enzyme bound on denatured DNA was still subject to dissociation by CsCl even after RNA synthesis was initiated. Thus, only the polymerase initiated RNA synthesis from double-stranded DNA appears to be isolated by this procedure.

Table 1
Effect of substrates on the formation of CsCl complex

	Substrate added	Complex formed (%)
Exp. 1	ATP	2.1
	ATP + GTP	17.9
	ATP + UTP	6.0
	ATP + CTP	17.4
	ATP + GTP + UTP	100
Exp. 2	GTP + CTP + UTP	100
	ATP + GTP + UTP	76.5
	ATP + GTP + CTP	82.7
	ATP + CTP + UTP	68.6

^{35}S-RNA polymerase and T7 DNA were mixed and incubated in the presence of substrates at the combinations indicated. The amounts of the enzyme recovered in the complex after centrifugation in CsCl were measured and represented as the relative values to the maximum: That is 3,965 cpm for Exp. 1 and 6,784 cpm for Exp. 2, respectively.

Isolation of the elongation complex by Cs_2SO_4 centrifugation: Addition of the fourth substrate allows the bound polymerase to proceed the elongation of the initiated RNA segment. Distribution of product RNA was then analysed by Cs_2SO_4 equilibrium centrifugation which enables banding of both DNA and RNA in a same centrifugal tube. Shortly after addition of the fourth substrate almost all the product RNA labelled with ^{3}H-ATP was found to be attached to the enzyme-DNA complex while prolonged incubation over 5 min at 37°C (or 10 min at 30°C) in the T7 DNA-directed reaction gave a new peak in Cs_2SO_4 centrifugation which corresponds to the buoyant density of free RNA (Fig. 2). This observation suggested that only the growing RNA chains were resistant to high concentration of Cs_2SO_4 whereas the completed chains were recovered as released from the complex. Thus, this procedure appears to enable us to isolate the growing RNA-RNA polymerase-DNA complex (elongation complex) without completed RNA chain.

Supporting evidence was provided by experiments in which size distribution of the product RNA isolated by Cs_2SO_4 centrifugation was analyzed by sucrose

Table 2

Factors affecting the formation of CsCl complex

Substrate	None	-
	1 XTP	-
	2 XTP	+
	3 XTP	+
	4 XTP	+
Temperature	0°C	-
	30°C	+
Template	Native DNA	+
	Denatured DNA	-
Antibiotics	Rifampicin	-
	Streptolydigin	-
Ionic strength	No KCl	+
	0.1 M KCl	+

Factors which affect the formation of CsCl complex are qualitatively summarized. Details will be described elsewhere (Naito and Ishihama, in preparation)

sedimentation. RNA dissociated from the complex by SDS gave broad peaks and their sedimentation velocities increased concomitantly with proceeding the reaction whereas the RNA recovered as the released form in Cs_2SO_4 gradient showed a single and discrete peak with sedimentation coefficient of approximately 30S (molecular weight of approximately 2×10^6) irrespective of the incubation time (Fig. 3). This value is very close to that of the putative product of the entire early genes (18 to 20 % of T7 phage genome) of T7 DNA, and also to the summed value of the four or five spieces of the *in vivo* early mRNA (11, 12) suggesting that the purified host polymerase transcribes the entire early genes of T7 DNA into a single large RNA molecule. Based on the observation that the 30S free RNA appears for the first time after 5 min incubation at 37°C (or 10 min at 30°C) the rate of RNA chain growth or the rate of RNA polymerase movement along DNA can be calculated to be 24 nucleotides

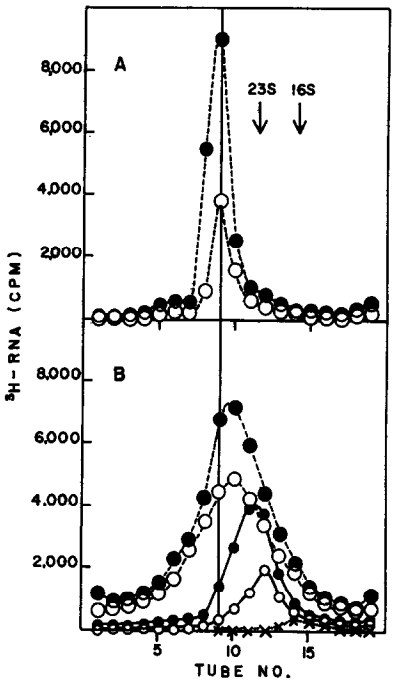

Figure 3 Analysis of the size of product RNA. Both free (A) and DNA-bound (B) product RNA isolated by Cs_2SO_4 centrifugation as shown in Fig. 2 were subjected to centrifugation in sucrose gradient following treatment with SDS. Centrifugation was carried out in Beckman SW 50.1 rotor for 2 hrs at 45,000 rpm. ×----×, 2 min; o—o, 5 min; ●—●, 10 min; ○---○, 20 min; ●---●, 30 min.

per second at 37°C (or 12 nucleotides per second at 30°C) under the condition employed.

DISCUSSION

The CsCl equilibrium centrifugation technique has been shown to be a useful procedure for isolating stable RNA polymerase-DNA complexes (8). The experiments summarized in the present report revealed that the formation of the "CsCl complex" required the presence of more than two species of substrates including purine nucleotides during incubation at physiologic temperature. Thus, the formation at least of a single phosphodiester bond or the initial dinucleotide seems to be prerequisite for making the polymerase-DNA complex resistant to dissociation by CsCl. Similar finding has been provided with use of high concentration of ammonium sulfate by So and Downey (13). Maximal recovery of the enzyme in the complex was observed when a mixture of RNA poly-

merase and T7 DNA was incubated in the presence of three substrates, in particular, at the combination of GTP, CTP and UTP. Details of the observations on the effect of substrate combination will be published elsewhere (Naito and Ishihama, in preparation).

Surprisingly no CsCl complex was detected when the reaction was carried out with heat-denatured DNA as template. Both strands, therefore, appear to play certain role in keeping the polymerase attached on DNA though local unwinding has been proposed for DNA region being transcribed (14, 15).

Fate of growing RNA was then analysed by equilibrium centrifugation in Cs_2SO_4 which allows banding of DNA and RNA in a same centrifugal tube. As expected, the growing RNA was recovered as bound on the RNA polymerase-DNA complex and could be dissociated by treatment with SDS suggesting that the nascent RNA is linked to DNA through the RNA polymerase. Similar findings have been reported for the elongation complex isolated by sucrose density gradient centrifugation in isotonic buffer solution (16, 17), while direct pairing of RNA to DNA has been found in some transcriptive complexes (18, 19).

Besides the "Cs_2SO_4 complex", the product RNA was also found in a position with bouyant density of free RNA, which appeared later than 5 min of incubation at 37°C and exhibited the invariable sedimentation coefficient of 30S as analyzed by sucrose gradient centrifugation. The finding indicates that the entire early genes of T7 DNA is transcribed by E. coli RNA polymerase into a single RNA molecule leaving the mechanism unclear how these genes are transcribed into four or five small pieces of mRNA in infected cells.

ACKNOWLEDGEMENTS: The authors wish to thank Drs. R. Fukuda and K. Ito for valuabe suggestions during the course of this work. This work was supported in part by grants from the Ministry of Education of Japan and the Matsunaga Science Foundation.

REFERENCES

1. Summers, W. C. and Siegel, R. B., Nature, 228, 1160 (1970)
2. Chamberlin, M., McGrath, J. and Waskell, L., Nature, 228, 227 (1970)
3. Richardson, J. P., J. Mol. Biol., 21, 83 (1966)

4. Sentenac, A., Ruet, A. and Framageot, P., Europ. J. Biochem., 5, 385 (1968)
5. Bautz, E. K. F. and Bautz, F. A., Nature, 226, 1219 (1970)
7. Davis, R. W. and Hyman R. W., Cold Spring Harbor Symp. Quant. Biol., 35, 269 (1970)
8. Fukuda, R. and Ishihama, A., Biochem. Biophys. Res. Commun., 45, 1255 (1971)
9. Ishihama, A., Biochemistry, 11, 1250 (1972)
10. Ishihama, A. and Ito, K., J. Mol. Biol., 72, 111 (1972)
11. Siegel, R. B. and Summers, W. C., J. Mol. Biol., 49, 115 (1970)
12. Studier, F. W., Science, 176, 367 (1972)
13. So, A. G. and Downey, K. M., Biochemistry, 9, 4788 (1970)
14. Chamberlin, M. and Berg, P., J. Mol. Biol., 8, 297 (1964)
15. Bick, M. D., Lee, C. S. and Thomas, C. A. Jr., J. Mol. Biol., 71, 1 (1972)
16. Bremer, H. and Konrad, M. W., Proc. Natl. Acad. Sci., 51, 810 (1964)
17. Richardson, J. P., J. Mol. Biol., 21, 115 (1966)
18. Hayashi, M., Proc. Natl. Acad. Sci., 54, 1736 (1965)
19. Aloni, Y. and Attardi, G., J. Mol. Biol., 70, 363 (1972)