BBA 93166

The direction of *in vitro* genetic transcription on circular DNA

When circular, ΦX-174 replicative DNA (RF-DNA) is used as a template for RNA synthesis *in vitro* by a DNA-dependent RNA polymerase (EC 2.7.7.6) purified from *Escherichia coli*, the synthesized RNA has heterogeneous sedimentation constants ranging from 30 S to 10 S (ref. 1). As synthesis proceeds, progressively heavier molecules of RNA are obtained. Pulse and chase experiments show that some of the label initially incorporated into RNA with low S values is transferred to more rapidly sedimenting fractions indicating the growth of the polynucleotide chains in this system. Using this system it is possible to prepare RNA labeled with a different radioisotope at each end of the molecule. This differentially labeled RNA can be used to determine the direction of polynucleotide chain growth. This communication will present evidence which indicates that RNA synthesis by DNA-dependent RNA polymerase proceeds from the 5' to the 3' end of the molecule.

Purified *E. coli* C-122 DNA-dependent RNA polymerase and ΦX RF-DNA were prepared as described previously[1]. To synchronize the initiation of the reaction, the reaction mixture (see legend of Fig. 1) was preincubated at 25° for 10 min. The reaction was initiated by adding a mixture of four nucleoside 5'-triphosphates to a final concentration of: ^{3}H-ATP (2.48 C/mmole, Schwartz Bioresearch, Inc.) 25 mμmole/ml, ^{3}H-UTP (1.63 C/mmole, Schwartz Bioresearch, Inc.) 25 mμmole/ml, CTP 50 mμmole/ml and GTP 50 mμmole/ml. After 7 min ^{3}H-ATP and ^{3}H-UTP were chased with a 100-fold excess of unlabeled ATP and UTP. Simultaneously 50 mμmole/ml of ^{32}P-GTP (250 mC/mmole, prepared according to HARUNA *et al.*[2]) was added in order to label the RNA at the site of synthesis (the head). Fig. 1 illustrates the kinetics of incorporation of the nucleoside triphosphates into an acid-insoluble fraction.

The incubation mixture was sampled prior to chasing (at 7 min); the remainder was incubated for an additional 48 min. The reaction mixture was chilled at 0° and the nucleic acids were purified by the phenol method described previously[1]. The two samples were subjected to sucrose gradient analysis. A profile of the sample taken at 7 min is shown in Fig. 2A. It has been shown[1] that the heavier peak coinciding with RF-DNA is a hybrid complex composed of double-strand RF-DNA and newly synthesized RNA. It was concluded that this hybrid complex is an intermediate in genetic transcription. It has also been shown that the RNA involved in this complex can be freed from the template RF-DNA by treatment with formamide or heat. As illustrated in Fig. 2A, the hybridized RNA is completely released from RF-DNA upon incubation with 70% formamide for 20 min at 37°. This released RNA now sediments with the originally free RNA of 6 to 7 S. It is clear from Fig. 2A and Fig. 2B (formamide-treated 55-min sample) that more than 40% of the 6 S to 7 S ^{3}H-RNA synthesized during the first 7 min of incubation is transferred to RNA species with S values of more than 20 during the additional 48-min incubation with ^{32}P-GTP and 100-fold excess of unlabeled ATP and UTP. While the 7-min sample gives a profile with a homogeneous size distribution, subsequent

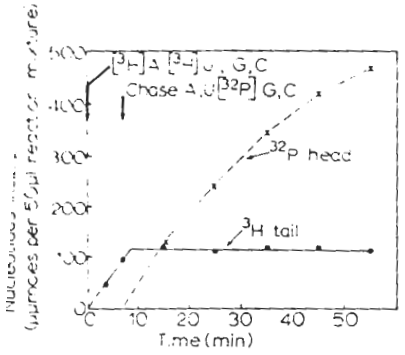

Fig. 1. Kinetics of the incorporation of [3H]ATP, [3H]UTP and [32P]GTP into RNA at 25°. The reaction mixture consisted of (per ml in order of addition): H_2O, 40 μmole Tris–HCl (pH 7.95), 2.0 μmole $MnCl_2$, 4.0 μmole $MgCl_2$, 10 μmole β-mercaptoethanol, 50 μmole NaCl, 15 μg ΦX RF DNA and 0.4 μg enzyme. After preincubation at 25° for 10 min, the reaction was initiated by adding a mixture of [3H]ATP, [3H]UTP, and unlabeled CTP and GTP. At specific times an aliquot of 50 μl before chase and 55 μl after chase was withdrawn and radioactivity in the 10 % cold trichloroacetic acid-insoluble fraction was determined. The number of μμmoles of nucleotides incorporated was calculated from the specific activities of the nucleoside triphosphates used.

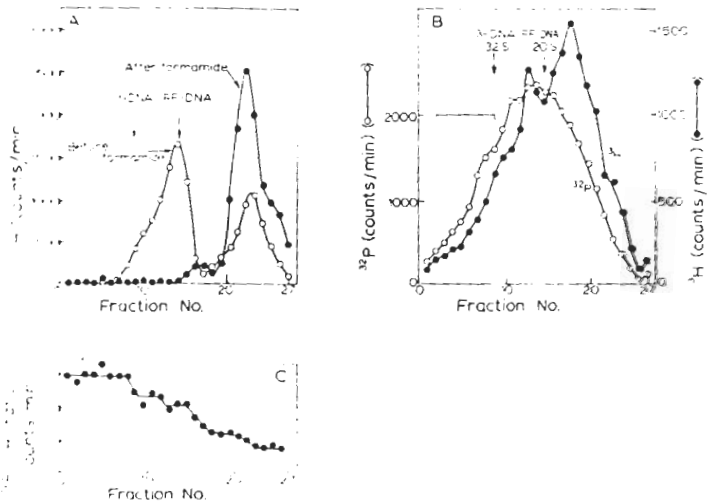

Fig. 2. Sucrose density-gradient profiles of the reaction products. RNA was purified by phenol extraction[1] from the 7th and 55th min samples of Fig. 1. After dialysing overnight against 0.03 M Tris·HCl buffer (pH 7.3) containing 0.1 M NaCl, half of the sample was layered on a 2.5 to 15 % linear sucrose gradient in the same buffer, and spun in a SW-25.1 rotor for 12 h at 5°. The other half of the sample was treated with 75 % formamide for 20 min at 37°, dialyzed against the above-mentioned buffer, and then subjected to sedimentation analysis as above. Radioactivity was determined as in Fig. 1. (A) 7-min sample, before (○ – ○) and after (● – ●) formamide treatment. (B) 55-min sample after formamide treatment. 32P-labeled λ DNA used as a marker was prepared from a lysogenic W3110 (λ) by a method similar to that of KAISER AND HOGNESS[8]. Purified λ DNA in 0.1 M NaCl (2 μg/ml was heated to 75° for 10 min and rapidly cooled in water in order to obtain a linear monomer form to which an S value of 32 has been assigned (HERSHEY, BURGI AND INGRAHAM[7]). 32P-labeled ΦX RF DNA was purified as reported[1]. The profile of these two marker DNA's was derived from a simultaneous run in a separate bucket. ● – ● 32P (head region) vs. 3H (tail region) was plotted as calculated from B.

RNA chain growth yields a heterogeneous size distribution with 5 or 6 reproducible peaks. Fig. 2C is a plot of the ratio of ^{32}P: ^{3}H (in counts/min) of each fraction as calculated from Fig. 2B. The ratio is highest for the largest RNA species and decreases stepwise as the size of the RNA decreases. This stepwise decrease in the ^{32}P: ^{3}H ratio implies that the initially synchronized polymerization stopped, or decreased in rate, at distinct sites on the template DNA.

The heaviest parts (specified by arrows in Fig. 2B) with a constant ^{32}P:^{3}H ratio were collected. After concentrating 10-fold by pressure dialysis, the sample was re-run on a sucrose gradient for further purification. The mean relative lengths of the head (^{32}P-labeled, most recently synthesized region) and the tail (^{3}H-labeled region, synthesized during the first 7 min) of these polynucleotide chains were calculated by assuming that the base compositions of both regions of the chain are identical to that of the ΦX-174 DNA (A:G:U:C = 25:23:33:19). The head is 89 %

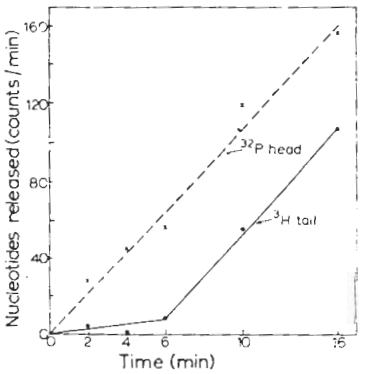

Fig. 3. Digestion of differentially labeled RNA by venom phosphodiesterase. The reaction mixture contained per ml: 35 μmole Tris-acetate (pH 8.8) 30 μmole magnesium acetate, 3.25 μg venom phosphodiesterase (Worthington Biochemical Corp.) and an appropriate amount of labeled RNA. The reaction was stopped by adding 500 μg of bovine serum albumin as a carrier, and 0.2 ml of 50 % trichloroacetic acid. After centrifugation at 10 000 rev./min for 10 min, trichloroacetic acid in the supernatant was extracted with ether 5 times and N_2 gas was used to remove a trace of ether. 0.8 ml of the supernatant was added to 20 ml of a dioxane scintillation fluid (BRUNO AND CHRISTIAN[8]) for assay in a Nuclear-Chicago liquid-scintillation spectrometer.

and the tail is 11 % of the molecule. If the tail has the 5' terminus and the head the 3' terminus, there should be a differential release of ^{32}P and ^{3}H into the acid-soluble fraction upon treatment with venom phosphodiesterase (EC 3.1.4.1). ^{32}P would appear without delay, whereas ^{3}H would appear only after an initial lag. On the other hand, if the tail has a 3' terminus, the situation would be reversed. Fig. 3 shows a 6-min lag in the release of ^{3}H into the acid-soluble fraction in contrast to the linear release of ^{32}P. Some 11 % of the input ^{32}P had been released into the acid-soluble fraction before the appearance of appreciable amounts of ^{3}H (6 min). ^{3}H is solubilized somewhat earlier than expected from the ratio of ^{3}H to ^{32}P in the sample. This may be due to endonucleases present as an impurity in the exonuclease preparation; a control RNA, prepared similarly but labeled oppositely (*i.e.* ^{32}P in the tail and ^{3}H in the head), gave the opposite result.

Considering the specificity of venom phosphodiesterase[3], these data prove that RNA is synthesized from the 5' to the 3' end. This conclusion is consistent with the data recently published by BREMER *et al.*[4] and MAITRA AND HURWITZ[5].

This work was supported by U.S.P.H.S. Grants GM 12934-01 and GM 12027-0251.

Department of Biology,
University of California,
San Diego, Calif. (U.S.A.)

SUSUMU TONEGAWA
MASAKI HAYASHI

1 M. HAYASHI, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 1736.
2 I. HARUNA, K. NOZU, Y. OHTAKA AND S. SPIEGELMAN, *Proc. Natl. Acad. Sci. U.S.*, 50 (1963) 905.
3 H. G. KHORANA, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 5, Academic Press, New York, 2nd edition, 1961, p. 79.
4 H. BREMER, M. W. KONRAD, K. GAINES AND G. S. STENT, *J. Mol. Biol.*, 13 (1965) 540.
5 U. MAITRA AND J. HURWITZ, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 815.
6 A. D. KAISER AND D. S. HOGNESS, *J. Mol. Biol.*, 2 (1960) 392.
7 A. D. HERSHEY, E. BURGI AND L. INGRAHAM, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 748.
8 G. A. BRUNO AND J. E. CHRISTIAN, *Anal. Chem.*, 33 (1961) 1216.

Received March 28th, 1966