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RNA SYNTHESIS IN VITRO DIRECTED BY LAMBDA PHAGE DNA

1) ANALYSIS OF PREFERENTIAL RNA SYNTHESIS IN VITRO

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Abstract—— RNA synthesized *in vitro* by DNA dependent RNA polymerase of *E. coli* with λ phage DNA as a primer was analyzed by the hybridization technique in relation to two active forms of the enzyme and to time course of the reaction. Hybridization with two different λ-φ80 hybrid phage DNA's, enabled us to analyze which region of λ DNA was transcribed *in vitro*.

The results showed as follows. 1) RNA hybridized to the right arm of λ DNA was synthesized with preference at early time of the reaction. The ratio of the right-arm RNA to the left is 5 to 7. This value indicates a probable distribution of initiation sites on λ DNA. 2) The preferential RNA synthesis took place only when F-II(22S) enzyme was used, while by 15-S enzyme RNA was synthesized equally in both arm of λ DNA. This result strongly supports the argument that FII(22S) is the true transcriptase of *E. coli*. 3) The preferential synthesis of right-arm RNA *in vitro* was decreased in the course of time. After 20 min the left-arm RNA became relatively excess in amount. For the synthesis of the left-arm RNA, escape from a normal termination under the condition is suggested. 4) The assay system used here has great advantages to analyze quantitatively what region of λ DNA is to be transcribed *in vitro*. By using two types of hybrid phage DNA's, each half of λ DNA is easily prepared in a large scale and the homogeneity of each half is genetically guaranteed.

INTRODUCTION

Since Jacob and Monod proposed "operon theory" and two steps of gene expression in 1961[1], the essential role of transcription in the regulation of gene expression has come to be convinced in general. Operon reading and successive processes in phage expression *in vivo*[2] have been confirmed as the regulation at the step of synthesis

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of mRNA by certain evidences.

On the one hand the entity responsible for transcription was shown by Hurwitz et al[3]. and Weiss et al[4]. They found DNA dependent RNA polymerase (EC,2.7.7.6. Nucleosidetriphosphate:RNA nucleotidyl transferase) in *E. coli* and *M. lysodeiticus* which could synthesize RNA in the presence of four ribonucleoside triphosphates and DNA as primer. Wood and Berg[5] showed that RNA synthesized by the enzyme of *E. coli* could stimulate the incorporation of amino acid into acid insoluble fraction. It comes now certain that DNA dependent RNA polymerase is the entity responsible for mRNA synthesis *in vivo*. Recently strepotovaricin which is the new type inhibitor of RNA synthesis *in vitro* was discovered, and the resistant mutants of *E.coli* were isolated. Some of these mutants were temperature sensitive and lack of RNA synthesis at high temperature *in vivo*. In addition, RNA polymerase isolated from these mutants were also temperature sensitive *in vitro*.[6] These results distinctly support the argument that the entity concerned with synthesis of mRNA is DNA dependent RNA polymerase.

New method for preparation of RNA polymerase from *E.coli* was established in our laboratory. Using this method, two different active fractions of enzyme can be obtained. One (FI) is stepwisely eluted from protamine precipitates at 0.05-0.10M KCl, the other (FII) at 0.10-0.20M. The property of each fraction, processed by further purification, in many respects as reported previously[7].

In relation to the regulation of transcription, heterogeneity of transcriptase and the specificity of initiation signal on DNA are considered to be focus at present. Two alternatives on this aspect can be proposed.

1) Correspondence between RNA polymerase and signals on DNA recognized by enzyme molecule are universal in all units of transcription. By this assumption the regulation of transcription demands a) gene masking or b) conformational change of template DNA. For these cases, specific site for initiation is necessary but heterogeneity of initiation signal is not required for principle. The type of regulation of gene masking may be consistent with that by repressor[8,9] and expect to play an essential role in higher order organism[10]. No direct evidence of the template modification has been reported but this has been suggested because conformational change of DNA certainly takes place and it may be closely related to the successive mRNA synthesis in phage infected cells[11].

2) Correspondence between heterogeneity of RNA polymerase and variety of initiation signal is so specific that a certain type of enzeme can only recognize corresponding sites of units of transcription. In bacteria no direct evidence of heterogeneity of enzyme was reported, though in animal cells two kinds of RNA polymerase were detected[12]. However, the modification of RNA polymerase after infection was reported by Walter *et al*[13]. In addition RNA corresponding to the late part is synthesized *in vitro* by enzyme prepared in infected cells[14],[15]. On the other hand our previous experiments suggested the heterogeneity of RNA polymerase in recognizing function[7].

These two alternatives give us two patterns on the aspect of regulation of trans-

cription. The mode of action categolized as 1-a) gives negative control and that as 2, gives positive control of gene expression.

To analyze the molecular mechanism of the regulation *in vitro*, λ DNA gives many advantages derived from its structural properties and improved genetical information about λ phage. Molecular weight of λ^{wild} DNA is 3.1 x 10^7 daltons and thus the amount of genetic information is relatively small and the DNA is prepared easily as an intact molecule. DNA regions corresponding to gene cluster of early funtion and late function are fractionated by several method[16,17,18]. Two strands of DNA are separable by using technique of sedimentation equilibrium in a medium containing synthetic ribopolymer[19].

Accordingly, the determination of RNA synthesized *in vitro* whether it corresponds to early or late gene is quantitatively possible using λ DNA as template. The main interest of this paper will be concentrated on preferential reading of early region by using RNA polymerase prepared by the new method and clarifying condition necessary to preferential reading.

MATERIALS AND METHODS

1) Chemicals and isotopes

8-^{14}C-ATP (specific activity, 4-11.3 C/mM), ^{3}H-GTP (specific activity, 1.3 C/mM) and ^{3}H-CTP (specific activity, 9.8 C/mM) were purchased from Schwarz Bioresearch Company, U.S.A. and other non-labeled ribonucleoside triphosphates were obtained from Pabst Laboratories and Sigma Biochemical Corporation, U.S.A.. RNase, DNase and electrophoretically pure DNase were purchased from Worthington Biochemical Corporation, U.S.A.. Nitrocellulose membrane filter (MF30) was purchased from Sartorious Membrane-filter Corporation, Germany. Sodium Dextran sulfate-500 was purchased from Pharmacia, Uppsala, Sweden.

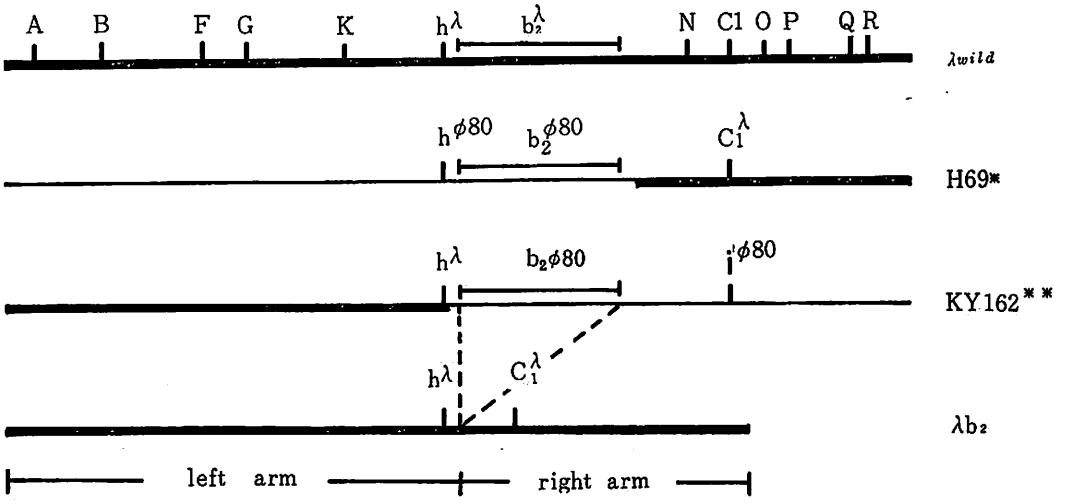
2) Media

Peptone broth contained polypeptone (Wako Drug Co.) 10 g, yeast extract 2 g, NaCl 5 g, per liter. Fluid for phage washing contained 10mM Tris-HCl pH 7.8 at 4C, 10mM $MgCl_2$, 0.1% of NaCl and 0.01% of gelatin. Diluent 2 for phage stock and dilution of phage lysate contained 10mM Tris, 10mM $MgCl_2$, 5% of NaCl and 0.001% of gelatin. λ agar contained polypeptone 10 g, NaCl 5 g, and bactoagar 12 g, (hard agar) or 7 g, (soft agar) per liter. EMB_0 plate is according to Lederberg[20].

3) Phages and bacterial strains

Escherichia coli K12 strain W3110 as the host of phages and W3110(λ) as a source of λ^{wild} phage were kindly gifted from Dr. Y. Yuan. Strain W3102(φ80) as a source of φ80 phage was kindly gifted from Dr. A. Matsushiro. Two lysogens of λ-φ80 hybrid phage were kindly supplied from Dr. T. Yura. W1485 (KY162) was originally isolated by N. Franklin et al[21] and W3350 (H69) was isolated by Y.Takeda[22] λ^{vir} phage was obtained from Dr. Y. Yuan and λb_2 phage was kindly gifted from Dr. J. Tomizawa.

Fig. 1. Maps of λ and λ-ϕ80 hydridphage DNA



$$\text{Possible region ratio} = \frac{\text{right arm}}{\text{left arm}} \fallingdotseq \frac{7}{11}$$

* : Takeda & Yura, 1968

** : Franklin et al, 1965

Capital letters on line (A……R) represent genetic markers characterized by Campbell[40]. Other markers represent : h= host range marker, CI= marker of immunity substance, b_2= b_2 region concerned with integration[23]. Wide line represents DNA derived from λ and narrow line from ϕ80. Region of A to h and R correspond to the part of late function, region of N to Q corresponds to early function and middle part including b_2 is the part of integration. Possible ratio of right arm to left is calculated.

Bacteria taken from a single colony on λ agar plate was grown in peptone broth by shaking at 37C. First phage lysate (small scale) was prepared by UV induction from each lysogen. Irradiation condition: Lysogens were grown in peptone broth at 1-2 x 10^8 cells/ml and 10 ml of culture in plate was irradiated. Irradiation at 120 erg/cm^2 distance from germicidal lamp (ca. 35 cm) was carried out. W3102(ϕ80) and W1485 (KY162) were irradiated for 40 sec and W3110(λ) and W3350(H69) were irradiated for 30 sec. Irradiated lysogens in peptone broth were shaken at 37C for 3 hr and then chloroformed. λb_2 and λ^{vir} phage were prepared firstly by infection of phage stock taken from single plaque isolation.

As shown in Table 1, the characters of each phage and its purities were checked by many points. By plating on EMB_0, λb_2 was further checked for its unstable lysogenization[23].

4) **Preparation of phage stocks**

Large scale preparations of each phage were carried out as follows. 5 ml of overnight culture of W3110 was inoculated in 100 ml of peptone broth and shaken at 37C

Table 1. **Characterization of each phages**

lysate of phages	dilution	No. of plaque on indicator					
		W3110	W3110(λ)	W3102(φ80)	CR63	W3350(H69)	W1485(KY162)
1. λ*wild*	10^{-3}	confl.	0	confl.	0	0	confl.
	10^{-7}	103	0	—	—	—	—
	10^{-9}	28	—	—	—	—	—
2. λb_2	10^{-3}	confl.	0	confl.	0	0	confl.
	10^{-7}	confl.	0	—	0	—	—
	10^{-9}	65	—	—	—	—	—
3. φ80	10^{-8}	confl.	confl.	0	confl.	confl.	0
	10^{-9}	17	—	—	—	—	—
4. H69	10^{-3}	confl.	0	confl.	0	7	confl.
	10^{-7}	confl.	—	—	—	0	confl.
	10^{-9}	42	—	—	—	—	—
5. KY162	10^{-3}	confl.	confl.	4	confl.	confl.	32
	10^{-7}	326	343	0	—	407	0
	10^{-9}	6	—	—	—	—	—
6. λ*vir*	10^{-7}	confl.	confl.	confl.	0	confl.	confl.
	10^{-9}	126	—	—	0	—	—

Phage lysates of λ, φ80, H69, KY162 were prepared by UV induction. That of λ*vir* and λb_2 were prepared by infection of isolated plaque to W3110. Diluent 2 is used for dilution of phage lysate. Plating according to double layer method[41] was carried out and plaque count was carried out after overnight incubation at 37C.

for about 30 min. Infection of phage at multiplicity of infection 2-3 to cultured medium (ca. 3-4 x 10^8 cells/ml). After standing for without shaking for 5-10 min then shaken at 37C. After 3-4 hr, lysis occurred usually then chloroform was added and succeeded by shaking for about 20 min. The crude lysate was collected and passed through celite (hyflosupercel). At this step titration of phage was carried out and contamination of other phages was checked.

Phase separation by dextran and polyethylene glycol was applied as the next step. 2 gr/l of dextran sulfate-500, 17.4 g/l of NaCl and 69 g/l of polyethylene glycol-6000 were mixed well and added to the phage lysate. The mixed solution thus obtained was standing for 48 hr at 4C. After dextran layer was sedimented at bottom of flask, polyethylene glycol layer was discarded and dextran layer was collected by low centrifugation. Equal volume of 2M KCl was added and stirred by glass rod, then white viscous precipitates were occurred. After centrifugation (10 min x 12,000 rpm) the supernatant was collected. Elution by KCl solution from dextran precipitates was repeated once. Loss of plaque forming unit (PFU) of phage up to this step is less than 10%. The eluate containing phage was incubated in the presence of 20 μg/ml of DNase at 37C for 30 min. After DNase treatment differential centrifugation was carried out twice (10 min x 15,000 rpm and 90 min x 20,000 rpm). Washing fluid

was used for suspending phage pellets. Final suspension was stocked at 4C with small volume of chloroform as stock lysate. Phage purified at this step is used for the source of DNA preparation to hybridization.

To prepare DNA for template in RNA synthesis *in vitro*, stock lysate of phage (1.0-1.5 ml/tube) was applied onto stepwise cushion of CsCl solution (1.0 ml of $\rho=1.3$, 1.5 ml of $\rho=1.5$ and 0.8ml of $\rho=1.7$). Centrifugation at 35,000 rpm for 60 min by SW50 rotor in a L2 ultracentrifuge was carried out. After centrifugation two white bands were usually observed at about middle of tube. Lower layer was collected from bottom because PFU of phage was scarcely detected from the upper one. CsCl solution containing phage was stocked at -90C as CsCl-purified phage stock. Before phenol treatment phage stock was dialyzed against standard saline citrate (SSC) for 5 hr.

5) Preparation of DNA

DNA's for hybridization and for template were separately prepared from different phage stock as described above. But the method of extraction of DNA was the same. Sodium dodecyl sulfate was added to 0.2% in phage stock and extraction by SSC-saturated phenol was carried out twice. Shaking of DNA solution was done gently by hand for 20 min, then ether treatment to eliminate contamination of phenol was done three times and aqueous layer was collected. DNA preparation used for template was dialyzed against 0.15M NaCl in 30mM Tris-HCl pH 7.8 at 4C and DNA solution to hybridization was dialyzed against 1/100 SSC for 24 hr. Spectrum of optical density of DNA solution was checked. Intactness of DNA was checked by sucrose density gradient centrifugation and by an analytical ultracentrifugation in a Spinco E[24]. S value of λ DNA thus obtained was 30-34S. From this, intactness of DNA was preserved by these processes of preparation. Denaturation of DNA was done as flollows: shearing of DNA by passing through i.v. injective gauze for 15 times and DNA concentration adjusted to 100 μg/ml by 1/100 SSC. The sample was heated in a boiling water bath for 5 min. After heating, sample was rapidly chilled in an ice bath. Hyperchromicity of DNA was usually 1.3-1.35 by this method.

6) DNA dependent RNA polymerase

DNA dependent RNA polymerase was prepared as reported previously[25]. Determination of enzyme unit and reaction mixture are essentially the same as that described previously. Exception is cited in each legend of Figure.

7) Preparation of RNA synthesized in vitro

Two methods of preparation were applied.

1) Direct trapping of template DNA as a denatured state on a nitrocellulose membrane filter (MF30) at indicated time. This method is originated by Y. Iida[26]. Relatively high concentration of DNA at denaturation was avoided by addition of equal volume of water and the diluted sample was rapidly boiled for 3 min. After boiling, the sample was rapidly chilled in an ice bath then passed through a MF30 filter at 10-15 μg/filter. Filtration was repeated once. By this method, over 95% of

denatured DNA was removed. If necessary, RNA was concentrated by an ethanol precipitation. By this step yield of RNA was reduced some extent. Labeled substrate was still contained in RNA sample without ethanol precipitation, but it did not result in significant increase of background of hybridization. Heating of the sample caused degradation of RNA (4-8S), but decrease of efficiency in hybridization was not observed under the condition described below.

2) DNase digestion method. At time indicated acetate buffer was added (final 0.05M KCl, 0.01M $MgCl_2$, 0.02M acetate buffer pH 5.6). Extraction of nucleic acid was carried out by phenol treatment. Electrophoretically pure DNase was added to 50 μg/ml and incubated for 20 min at 37C. After DNase treatment phenol extraction was once carried out. Finally concentration of RNA was done by an ethanol precipitation.

Result of hybridization experiments showed no quantitative difference between these two methods described above.

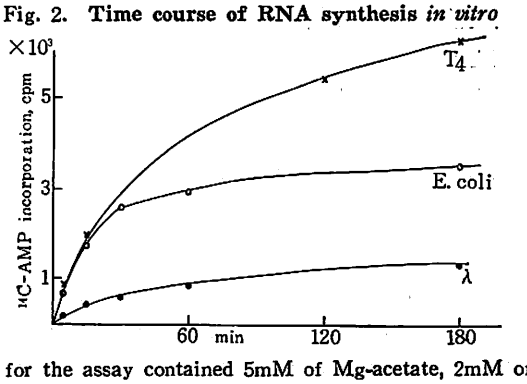
8) **Method of hybridization**

The procedure of Nygaard and Hall[27] was followed with minor modification. Hybridization mixture is 0.5M KCl in 10mM Tris-HCl pH 7.8 at 4C. Time of incubation at 65C was 3 hr then slowly cooled. RNase treatment was carried out at 10 μg/ml at 37C for 30 min. Then excess of 0.5M KCl buffer was added and filtration through a MF30 filter presoaked in 0.5M KCl buffer was carried out. Washing of membrane filter with 0.5M KCl buffer (about 50 ml/filter) is carried out. The membrane was dried under lamp and then taken into a vial containing 7 ml of toluen-PPO cocktail. Radioactivity of sample was counted by scintillation counter, Beckmann model LS-200B.

RESULTS

1) **Kinetics of RNA synthesis in vitro by λ DNA**

Fig. 2. **Time course of RNA synthesis *in vitro***

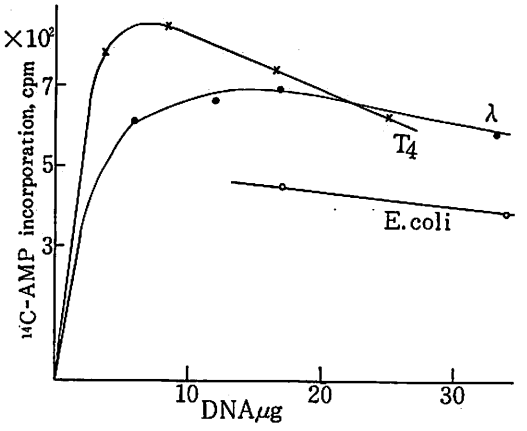


Reaction mixture for the assay contained 5mM of Mg-acetate, 2mM of Mn-sulfate, 120 mM of Tris-HCl pH 7.8 at 37C, 5mM of β-mercaptoethanol, 400μM of each GTP, UTP and CTP, 100μM of ^{14}C-ATP (1300 cpm/mμmole). Final volume of reaction mixture was 0.25 ml. In this experiment 5 unit of FII (22S) enzyme of DEAE fraction was used. Amount of DNA used were: 10μg of λ (—●—), 12μg of E. coli (—○—) and 23μg of T4 (—×—). Reaction was carried out at 37C and at time indicated TCA insoluble radioactivity was counted by gasflow counter.

Fig.2 shows the time course of RNA synthesis *in vitro* by FII (22S) enzyme of DEAE fraction. This indicates that within 60 min the reaction proceeded linearly and after 60 min the rate of synthesis was decreased with time. Kitano reported previously that the duration of the reaction was determined by ratio of DNA (μg)/enzyme (unit)[24]. This time course was given under the condition of DNA excess. Since the enzyme at DEAE fraction is contaminated with a trace RNase activity, TCA insoluble count in reaction mixture does not increase linearly after 60 min. Under the optimum condition (DNA excess, long primer and purified FII-22S) reaction proceeded over 9 hr[24]. Anyway within 60 min the reaction proceeded linearly under the condition described below.

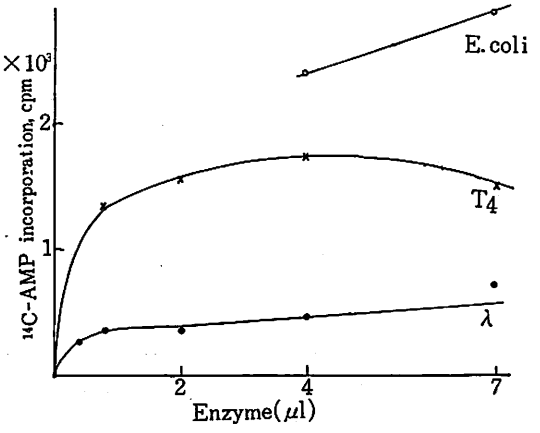
Analysis of initial rate of RNA synthesis was done by two ways.

Fig. 3. DNA saturation curve of FII(22S)



0.01 ml of FII(22S) of DEAE fraction and varied amount of λ DNA (—●—), E. coli DNA (—○—) and T4 DNA (—×—) were used. Reaction time was 5 min at 37C. Reaction mixture was as in Fig.2 except specific activity of ^{14}C-ATP is 1500 cpm/mμmole.

Fig. 4. Enzyme saturation curve of FII(22S)

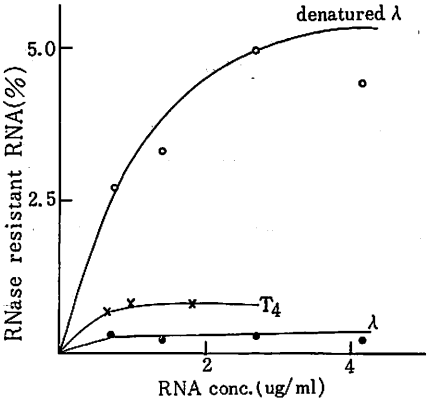


1μg of each DNA and varied volume of FII (22S) of DEAE fraction were used. Others are as in Fig. 3.

Firstly, in the reaction by constant amount of enzyme (22S) and varied amount of DNA the incorporation of ^{14}C-AMP into TCA insoluble form was examined. Result of DNA saturation (Fig.3) indicates that the constant amount of enzyme gives different maximum initial rate corresponding to each primer. This relative value differs when enzyme or DNA preparation is changed but by using λ DNA the enzyme activity is in the range of 1/2-1/4 of that by T4 DNA as standard. This may not be due to the existence of aggregation or dimer of DNA because this ratio is not largely changed by addition of salt or heating at 65C. The condition of DNA excess for the reaction was determined from DNA saturation curve in each experiment.

Secondly, in the reaction by constant amount of DNA and varied amount of enzyme (22S) the initial rate of RNA synthesis was analyzed (Fig.4). From this result the number of enzyme molecules initiated on DNA can be calculated after normalization by the rate of RNA synthesis on each DNA. Thus it can be estimated that one enzyme molecule started on each 1x10^6 daltons of T4 DNA in average (Fig. 4). As the rate of RNA synthesis by ϕ80 and λ DNA is identical with that by *E. coli* and two folds of that by T4 DNA, the relative number of molecules on a constant length of λ DNA to T4 DNA is about 1/9 based on the value obtained from this figure. Therefore it is calculated from this data that about 4 molecules of 22-S enzyme per one molecule of λ DNA started RNA synthesis. The number of initiation sites of λ DNA calculated as above was varied from experiment to experiment but within 10 molecules per one

Fig. 5. **Self-annealing test of RNA synthesized *in vitro***



^{3}H-GMP labeled RNA was prepared *in vitro* by FII (22S) of DEAE fraction. Reaction was at 37C for 35 min and in the condition of DNA excess. Preparation of RNA was carried out by DNase treatment (in Material and Methods). Reaction mixture of self annealing (final, 0.12 ml) contained 0.5M KCl, 10mM Tris-HCl pH 7.8 at 4C and varied amount of ^{3}H-RNA. After incubation at 67C for 2 hr RNase digestion was carried out (final 10μg/ml) at 37C for 30 min. After RNase digestion, 0.5 ml of 10% TCA and 0.2 ml of 2.5 mg/ml of bovine serum albumin was added. TCA insoluble precipitates were collected by low centrifugation and washed twice with 5% TCA. Final precipitates were dissolved in ca. 0.5 ml of 2N NH_4OH and samples were applied in planchets. Denatured DNA used as primer was prepared as described in Methods. RNase resistant cpm of RNA synthesized *in vitro* directed by native λ DNA (—●—) ,denatured λ DNA (—○—) and native T4 DNA (—×—) in relation to input ^{3}H-RNA were plotted.

molecule of λ DNA.

2) Asymmetric RNA synthesis in vitro primed by λ DNA

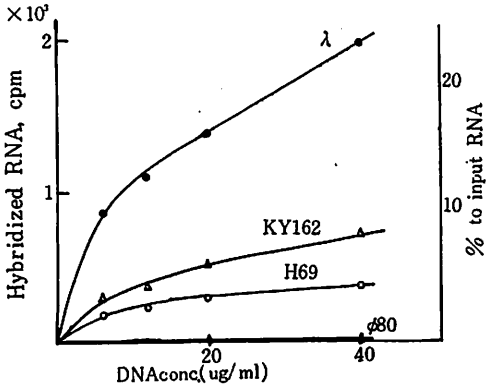
By the self-annealing method, the ratio of self annealed RNA to input RNA was determined. RNA synthesized *in vitro* directed by denatured λ DNA was examined as control. As can be seen in Fig.5, in the case of RNA primed by native λ DNA the amount of RNase resistant RNA after incubation is not increased regardless increase of input RNA. But RNA primed by denatured DNA was self annealed at constant rate and the ratio of self-annealed RNA to input RNA is about 5% by this figure. This value is relatively low as compared with that by reported by Naono and Gros[28]. This is mainly due to the low efficiency in counting ^{3}H-RNA sample with carrier bovine serum albumin by gas flow counter. RNase resistant part of RNA directed by native DNA is almost negligible and clearly different from that of control. From this experiment, asymmetric synthesis of RNA *in vitro* by using λ DNA as primer is concluded.

3) Analysis of RNA synthesized in vitro by hybridization technique

Fig. 6 represents DNA saturation curve of hybridization in the presence of the constant amount of ^{3}H-RNA. RNA was prepared at 20 min of the reaction by λ DNA as primer. Over 20% of input RNA was hybridized to denatured DNA in this experiment. Efficiencies of hybridization in experiments performed were 20-40%. Background checked by heterologous DNA was lower than 100 cpm.

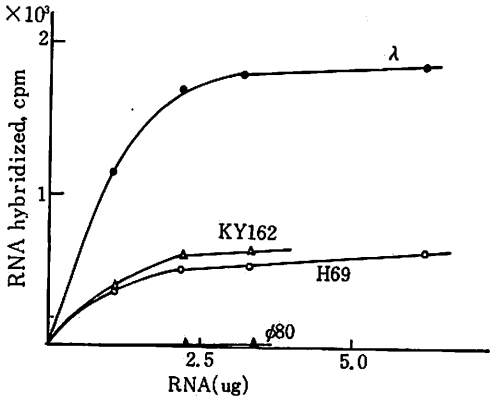
RNA saturation curve indicates that 18% of denatured DNA is covered by 20-min product RNA if complete asymmetric RNA synthesis is taken place in this system

Fig. 6. **Hybridization of ^{3}H-RNA synthesized *in vitro* driected by λ DNA**
(DNA saturation curve)



Each reaction mixture of RNA synthesis contained the same as described in Fig. 1, except using ^{3}H-GTP (4×10^4 cpm/mμmole) as radioactive XTP and the volume of reaction mixture containing 0.1M KCl is 1.2 ml. The ratio of DNA (μg)/enzyme (unit) is 4/3. FII (22S) of DEAE fraction was used. RNA was prepared at 40 min after reaction and membrane method described in Methods was applied. Reaction mixture of hybridization (0.5 ml) contained 8.6×10^3 cpm of ^{3}H-RNA and varied amount of denatured DNA of each species: λ^{wild} (—●—), H69 (—○—), KY162 (—△—) and φ80 DNA (—▲—).

Fig. 7. **Hybridization of ^{3}H-RNA synthesized *in vitro* directed by λ DNA**
(RNA saturationi curve)



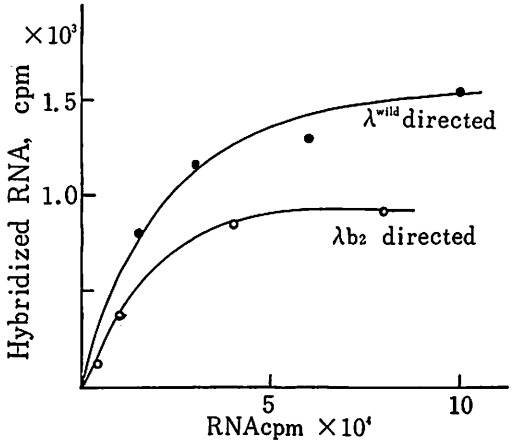
Reaction mixture of hybridization (0.5 ml) contained varied amounts of ^{3}H-RNA and 1 μg of denatured DNA of each species : λ^{wild} (—●—), H69 (—○—), KY162 (—△—) and φ80 DNA (—▲—). Others were the same as in Fig. 6.

(Fig. 7).

λb_2 in place of λ^{wild} was used as primer to analyze which arm was transcribed ***in vitro***. The reasons of using λb_2 DNA as primer are follows. The region of b_2 is deleted in λb_2 DNA as shown in Fig.1. The b_2 region located in the middle of linear molecule is about 16% of whole λ^{wild} DNA[23], possibly inert in transcription ***in vitro***[29] and lack of b_2 region does not affect lytic cycle of phage multiplication. Two hybrid phage DNA's using for trapping each arm RNA by hybridization have a common region in the middle part of DNA including b_2 region (or att region). Although this common region is derived from φ80, excluding b_2 region could lower the noise of hybridization. In Fig. 8 two different RNA's prepared by λ^{wild} and λb_2 DNA as primer exhibit different efficiencies of hybridization to an identical denatured λ^{wild} DNA. This suggests that b_2 region may be transcribed ***in vitro*** at 25 min of the reaction. But this difference of hybridization is larger than that of two DNA's. This is explained by assuming that not all region is transcribed at 25 min but b_2 region is transcribed at this time.

Efficiencies of hybridization vary with different preparations of DNA used. Therefore the values at plateau obtained from DNA saturation by two hybrid phage DNA's do not directly indicate the relative amount of RNA hybridizable to each arm in the sample, but indicate only actual values of RNA hybridized. Thus a normalization is necessary to each experiment. For the purpose, denatured λb_2 DNA was used as template and RNA prepared in the reaction was regarded as a standard, based on that random reading is occured when denatured DNA is used as primer[30]. As shown in Fig. 1, the ratio of DNA regions of two arms in length is calculated from the map of λb_2 and two hybrid phage DNA's. Therefore the relative amount of RNA hybridized to each arm of the standard RNA (denatured λb_2 DNA directed RNA) can give

Fig. 8. **Hybridization of ^{3}H-RNA directed by λ^{wild} and λb_2 DNA to denatured λ^{wild} DNA (RNA saturation curve)**



Reaction mixtures of RNA synthesis contained the same as described in Fig. 2 except containing 0.1M KCl and using 100μM of ^{3}H-CTP (5.6×10^4 cpm/mμmole) as radioactive XTP. Enzyme used was FII(22S) of DEAE fraction. Each reaction mixture contained: 76 μg of λ^{wild} DNA and 50 units of enzyme in 0.5 ml and 48 μg of λb_2 DNA and 50 units of enzyme in 0.5 ml. Reaction at 37C was stopped at 25 min then ^{3}H-RNA was purified by membrane method described in Methods. Reaction mixture of hybridization (0.5 ml) contained 0.4 μg of denatured λ^{wild} DNA and varied amounts of ^{3}H-RNA directed by λ^{wild} DNA and varied amounts of ^{3}H-RNA directed by λ^{wild} DNA (—●—) and λb_2 DNA (—○—).

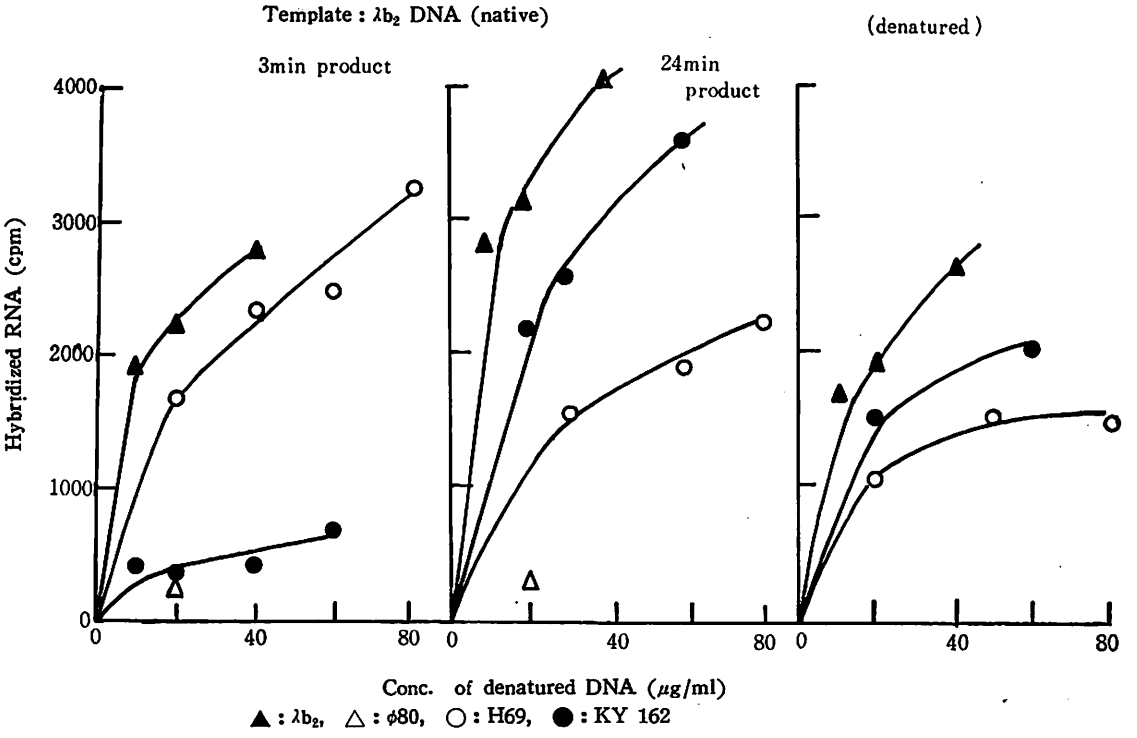
the relative efficiencies of hybridization of each of two hybrid-phage DNA's (as in Table 2).

4) **Preferential RNA synthesis in vitro directed by λb_2 DNA**

The quantitative analysis was carried out to determine which part of λb_2 DNA was transcribed *in vitro*. Two hybrid phage DNA's were used for hybridization. H69 DNA has the region derived from the right arm of λ DNA (about 35% of whole DNA) and KY162 has the left arm of λ DNA (about 55%). Therefore the relative amount of RNA hybridized to each arm of λ DNA can be given by DNA saturation curve of RNA sample after the values are normalized by the efficiencies of hybridization to each denatured DNA.

RNA samples at the various times of the reaction were hybridized with H69, KY162 and λb_2 DNA. The condition of RNA synthesis is described in legends. As can be seen in Fig. 9, at the early time of the reaction RNA synthesized was hybridized to H69 DNA with preference. RNA hybridized to H69 DNA corresponds to the region of right arm of λ DNA that is the part of early function. This preference of RNA synthesis at 3 min is almost exclusive and the normalized index of relative amount of RNA (right/left) is 5 to 7 in the experiments. On the contrary at 24 min this preference is lost and reversely RNA corresponding to left arm is excess in amount, and the nomalized index is near 0.5.

Fig. 9. **Preferential RNA synthesis *in vitro* directed by λb_2 DNA**

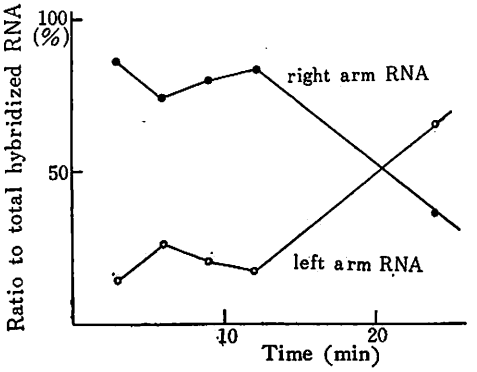


Reaction mixtures of RNA synthesis were the same as that of Fig. 8. Each contained: 380 μg of λb_2 DNA and 300 units of FII (22S) in 5.0 ml. and 50 μg of denatured λb_2 DNA and 200 units of enzyme in 1.5 ml. At this ratio, DNA was excess in reaction mixture. Time of sampling was 3 min and 24 min as for native λb_2 DNA and 25 min for denatured λb_2 DNA. Each reaction mixture of hybridization (0.5 ml) contained varied amounts of denatured DNA of each species : λb_2 (—▲—), $\phi 80$ (—△—), H69 (—○—) and KY162 DNA (—●—), and constant amount of ^{3}H-RNA 9×10^3 cpm of 3-min product, 1.2×10^4 cpm of 24-min product and 1.1×10^4 of denatured λb_2 directed RNA.

Temporal change of this preferential RNA synthesis is shown in Fig. 10 and Table 2. In Fig. 10, the ratios of hybridized RNA to each arm to total hybridized RNA are plotted in relation to the time of the reaction. From this it is concluded that left-arm RNA became excess in amount after around 20 min and the preference in RNA synthesis of right arm is conserved at least up to about 10 min. If net synthesis could be extraporated by these amount of RNA estimated at each time, the net RNA synthesis between 12 and 24 min might be exclusively accounted as left-arm RNA. Synopsis of these is listed in Table 2.

RNA synthesized by 15-S enzyme was tested similarly as above. As described in Discussion, FI or 15-S enzyme differes in many respects from FII (22S). As it can be seen in Table 2, no preferential RNA synthesis was observed when 15-S enzyme was used. This result means that RNA synthesized by 15-S enzyme contains almost equal amount of RNA hybridized to each arm.

Fig. 10. **The time course of preferential RNA synthesis**



Hybridization experiments were carried out as described in Fig. 9 and final level of cpm hybridized to H69 and KY162 were added (total hybridized cpm). Relative ratios (%) of ^{3}H-RNA hybridized to H69 DNA (—●—) and KY162 (—○—) to total hybridized cpm were plotted relating to the time of sampling.

Table 2. **Preferential synthesis of λ RNA *in vitro***

	Exp.	Template DNA	Enzyme	Time of sampling	RNA hybridized with a) H-69 cpm	b) KY-162 cpm	Relative ratio a/b	Calculated* R/L
A	—1)	native λb_2	22S	3min	737	110	6.6	5.1
				6min	2870	1550	1.5	1.2
				12min	412	521	0.8	0.6
	2)	native λb_2	22S	3min	2916	310	9.4	7.3
				6min	4617	1200	3.8	3.0
				9min	2361	551	4.3	3.3
				24min	1785	3248	0.55	0.43
	3)	denatured λb_2	22S		1478	1808	0.82	
B	—1)	native λb_2	15S	6min	360	153	2.3	1.0
				30min	390	172	2.2	1.0
	2)	denatured λb_2	22S		295	245	1.42	

Reaction mixtures of RNA synthesis contained the same as described in Fig. 9. Series-A of experiments were identical with that described in Fig. 9. In series-B, 15-S enzyme derived from 22S of FII by aging was used. Reaction mixture of RNA synthesis contained 310 μg of λb_2 DNA and 300 units of 15-S enzyme in 1.8 ml. Preparation of RNA and the condition of hybridization were the same as that of Series-A. The standard RNA (denatured DNA directed RNA) was identical with that of Series-A.
Calculation index*: observed ratio a/b × relative efficiency of hybridization (normalyzed by the standard RNA)

DISCUSSION

1) In these experiments preferential RNA synthesis of right arm of λ DNA was observed in the early time of the reaction when FII (22S) enzyme was used. By 15-S enzyme preferential RNA synthesis did not take place. These results are well consist-

ent with the observations on the two entities of RNA polymerase reported previously[7]. By 15-S enzyme the reaction did not continued over 60 min and shearing of template DNA caused the increase of enzyme activity which was not observed by FII (22S)[31]. From these and the results reported here it is strongly suggested that FII (22S) is the entity resposible for transcription.

From the index of preference, the distribution of initiation sites can be calculated. In the condition applied in this experiment the rate of chain growth was about 10 nucleotides/sec as to λ DNA. Therefore at 3 min after the reaction started, RNA synthesized by one enzyme molecule only covers less than 4% of total RNA corresdonding to the whole λ DNA. Moreover the reaction is carried out under the condition of DNA excess, so the second cycle of the initiation on the same site on one DNA by the other enzyme molecule could be minimized as little as possible in the early time of the reaction. By these reasons the index calculated from data at 3 min reflects the distribution of sites to left and right arm of λ DNA (R/L: 5-7). The number of initiation sites on one molecule of λ^{wild} DNA was calculated by the kinetics of the initial rate (Fig. 4). The value obtained by this experiment was 4, though it varied within 10 per one molecule of DNA. On the other hand the calculation of number of binding sites is also possible by the isolation of DNA moieties complexed with RNA polymerase[32]. By Matsukage *et al*[33], the number of binding sites of λ DNA was supposed to be within 10. This is well coincides with the data above. On the other hand three initiation sites (perhaps corresponding to operators) were supposed to exsist[34,35]. If tentatively the number of initiation sites *in vitro* be 4 and the ratio of distribution of sites (R/L) be 6, the number of the sites on the left arm in average is below 1 per one molecule of λb_2 DNA. Furthermore intrinsic reasons, whereby synthesis of RNA hybridized to left arm takes place, are considered. i) The size of 3-min product RNA is corresponding to 4% of total length of λ DNA molecule as described above. Thus the enzyme molecule started at the site near the border of one arm can synthesize RNA of the other arm within 3 min. The length of RNA acts as an error in calculating the ratio of distribution of sites from the data of hybridization. ii) Contamination of 15-S enzyme in FII fraction causes synthesizing RNA from both arms equally. These possibilities incline to suggest that in this system no initiation site for FII (22S) exists in left arm.

2) Increase of synthesis of RNA hybridized to left arm was taken place in this system. After 20 min the amount of left-arm RNA was exceeded over that of right-arm RNA. The possibilities responsible for this phenomenon are as follows. a) No termination occurred in this system and some portion of enzyme molecules located near middle slide from right to left. Thus after 10 min, most of enzyme molecules are synthesizing RNA on the left arm. b) RNA polymerase itself is altered after reading. Thus the altered enzyme becomes to initiate its reaction on left arm. Following results make us suppose the possibility of escape from normal termination. Sedimentation behaviors indicates that the product RNA *in vitro* after 10 min is over 30S and has

single peak if enzyme purified by density gradient centrifugation is used in the reaction system of RNA synthesis[36]. Distribution of molecular size of RNA of induced λ-lysogen was reported by Kourilsky *et al*[35] and they suggested that at least three kinds of RNA was detected and two of them were not so large molecule like as 30S. RNA polymerase molecule started on one DNA molecule was supposed not to initiate RNA synthesis on the other DNA molecule[36]. By Fuchs *et al* RNA chain termination occurred only in a definite salt concentration, but at this condition the termination was taken place not at the genetically specific points but randomly[37]. Considering these facts, the escape of termination at the specific point is most probable but alteration of 22-S enzyme after reaction is also suggested. ^{35}S-labeled 22-S enzyme was incubated in complete reaction mixture for 45 min at 37C and then applied to glycerol density gradient centrifugation. The pattern of sedimentation profile indicated the presence of minor peak (about 3S) of radioactivity although the profile of the sample without template DNA did not show any minor peak[38].

All the possibilities are not to be denied by the results described above and in next step of experiment it is necessary to determine which is correct. The most important problem is the characterization of left-arm RNA synthesized *in vitro*. It must be examined which strand is transcribed and whether the large RNA molecule having both arms exists.

Decrease of right-arm RNA with time is contradict to the result of Maitra *et al*[39]. They showed that preference of RNA synthesis continued over 100 min without altering the ratio of RNA of each arm. Recently it may be probable that the termination is not occurred even by their condition and 0.2M KCl is necessary for terminaiton. If so, the result as a previously shown RNA synthesis at late time of the reaction may be only a reflection from the features at early time in the reaction.

3). The assay system applied in this experiments especially using hybrid-phage DNA for hybridization, enable us to analyze the molecular mechanism of transcription *in vitro* with less difficulties. The large scale preparation of each arms of λ DNA is available only by the method like this and the purities of each arms is genetically guaranteed. The normalization by RNA directed by denatured DNA is necessary because efficiency of DNA's in hybridization is varied not only by the method of the preparation of DNA but also among DNA's by the same method.

Finally the assumption that the existence of specific signal of initiation and specific RNA polymerase which can recognize corresponding signal of initiation is most probable explanation for the mechanism of preferential RNA synthesis. If it is the case, the process of switch off and on of transcription in phage infected cells must be consist of or partially consist of modification of RNA polymerase of *E. coli* that enable enzyme to recognize signal different from that of early initiation.

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