Bull. Inst. Chem. Res., Kyoto Univ., Vol. 46, No. 3, 1968

Transfer of the Genetic Information of Bacteriophage ϕX-174 in a Cell-free System

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Received February 28, 1968

A cell-free system having high RNA and protein synthesizing activities was prepared from *Escherichia coli* Q 13. When the replicative form DNA of bacteriophage ϕX-174 was added to the system, remarkable stimulation of the RNA and protein synthesis was observed. The synthesized RNA was only hybridized with the "minus" strand of the replicative form DNA, showing that the nucleotide sequence of the minus strand was transcribed by RNA-polymerase. The protein synthesized in this system was identified by various methods, and it was demonstrated that the major product was the phage-coat protein. The significance of the above observations is discussed in relation to the mechanism of expression of the genetic information encoded in the phage DNA.

INTRODUCTION

It has been generally accepted that the following three principal processes are involved in transfer of the genetic information (the central dogma) : 1. Self-replication of DNA. 2. Transcription of the genetic information of DNA into the nucleotide sequences of RNA. 3. Translation of the genetic code of RNA into the amino acid sequences of protein. One of the promising ways to understand the mechanism of transcription and translation at the molecular level is to investigate the reactions involved in each step with well-defined cell-free systems. Thus, efforts to establish a cell-free system suitable for this study have been made by Hayashi and his co-workers, including the author. As the result, the replicative form DNA (abbreviated as RF-DNA) of bacteriophage ϕX-174** and a purified RNA and protein synthesizing system from *Escherichia coli* cells were chosen for the following reasons: 1. The RF-DNA is one of the smallest DNA molecules known at present (molecular weight$=3.4\times10^6$)[1]. 2. The method for the preparation of the sizable amount of intact RF-DNA has been developed[2]. 3. The ϕX-174 DNA genome contains six[3] or at most seven[4] complementation groups which limit the maximum number of proteins which may be synthesized *in vitro*. 4. The methods for preparing the RNA polymerase[5,6] and protein synthesizing system[7] from *E. coli* cells have been established. Accordingly, a series of experiments to elucidate the mechanism of transfer of the genetic information encoded in the ϕX-174 RF-DNA

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** Bacteriophage ϕX-174 has a single-stranded DNA with ring structure. Upon infection, the single-stranded DNA ("+" strand) is converted into a double-stranded complementary form ("+" and "−" strands), called "replicative form", and multiplies manyfold. This "replicative form" DNA then serves as template for both the replication of progeny single-stranded DNA and the formation of messenger RNA.

was started. In this paper, the main results obtained from these experiments are summerized.

EXPERIMENTAL

(1) Synthesis of ϕX-174-specific RNA from RF-DNA

The first attempt was to elucidate the mechanism of transcription of RNA upon ϕX-174 FR-DNA. For this purpose, a purified RF-DNA preparation which was shown to contain more than 90% of intact double-stranded circles under the electron microscope[8,9] and a purified DNA-dependent RNA polymerase from *E. coli* C (host of ϕX-174) free from both contaminating DNase and RNase as tested with a sensitive assay method[5], have been prepared. The RNA synthesized in this system was characterized by nearest neighbor analysis and by hybridization-tests[10]. The frequences of nearest neighbor nucleotides adjacent to guanosine in the synthesized RNA showed a good agreement with the values expected from RNA complementary to the minus strand of RF-DNA. As seen in Table 1, the synthesized RNA hybridized well with denatured RF-DNA (a mixture of "+" and "−" strands) but not with mature single-stranded DNA ("+" strand). These data clearly indicated that the intact circular RF-DNA generated the synthsis of RNA complementary to only one strand, the complement ("−" strand) of mature phage DNA, in accordance with the case of *in vivo* transcription (Table 1)[11]. When RF-DNA circles were broken, however, RNA's complementary to both strands were produced (Table 1). It is, therefore, clear that the strand selection mechanism is a unique feature of normal genetic transcription upon intact double-stranded circular DNA. This asymmetric transcription was also observed *in vitro* systems with phage α DNA[12] and phage T_4 DNA[13,14].

The existence of a hybrid between nascent RNA and double-stranded RF-DNA during transcription in the cell-free system has been shown[15]. The DNA-RNA complex isolated from the system was RNase-resistant. The complex was dis-

Table 1. Hybridization of RNA with RF- and single-stranded DNA.

radioactive RNA	hybridized amount (cpm)	
	on denatured RF-DNA	on single-stranded DNA
RNA formed *in vivo* (a)	3473	160
RNA formed *in vitro* (b)		
on intact RF-DNA	6800	700
on sonicated RF-DNA	7100	5000
RNA formed in the "coupled system" (c)		
ribosome region	214	2
soluble region	187	0

(a) Isolated from ϕX-174 infected *E. coli* which was pulse-labelled with P^{32} in 50-51.5 min after infection (from table 1 of ref. 11).

(b) Synthesized by RNA polymerase with either intact or sonicated RF-DNA as template (estimated from Fig. 4 and 5 of ref. 10).

(c) Synthesized in the "coupled system"[28].

sociated either by heating or formamide treatment, but not by deproteinization, suggesting that this material was a DNA-RNA hybrid through base pairings. Further analysis revealed that the hybridized region held a constant size (3-3.5 S) during the RNA synthesis and was moving along the DNA strand, enclosing the terminus of the growing RNA chain. As the similar complex was detected in *E. coli* cells infected with ϕX-174[16], it was assumed that this DNA-RNA hybrid was an intermediate in the process of genetic transcription. Very similar DNA-RNA complexes were found in other organisms; T_2-infected *E. coli*[17], *Neurospora*[18] and *Drosophila*[19]. Bremer and Konrad, however, reported a DNA-RNA complex held together by protein[20].

In a cell-free RNA synthesizing system where the initiation of transcription was synchronized, five distinct RNA fractions could be recognized by sucrose density-gradient centrifugation at the end of the reaction (Fig. 1)[15]. The largest RNA was assumed to be the result of the transcription of almost entire DNA molecule, suggesting that some of the RNA molecules synthesized were polycistronic.

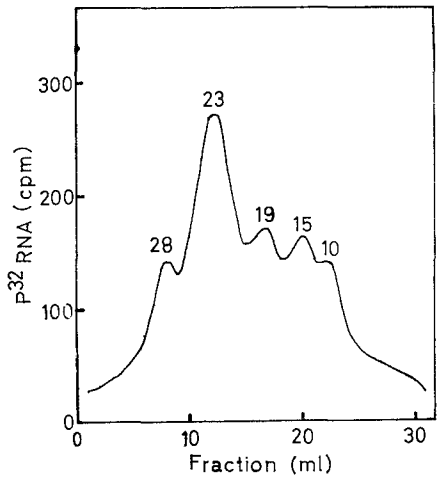

Fig. 1. Sedimentation analysis of ϕX-RNA synthesized *in vitro*. Numeral above each peak is approximate sedimentation coefficient (from Fig. 1 of ref. 15).

Using the above synchronized system, it was possible to prepare RNA which contained different radioisotopes at the both ends of the molecules[21]. In this experiment, the RNA synthesis was started in the presence of H^3-labelled CTP and UTP. Thereafter, ^{32}P-labelled GTP and excess amount of non-labelled CTP and UTP to dilute ^{3}H-labelled ones were added to the reaction mixture and the reaction was continued. When the RNA thus prepared was treated with venom phosphodiesterase, which can sequentially hydrolyze polynucleotides from their 3' ends, ^{32}P-GMP was preferentially released. The result suggested that RNA synthesis proceeded from 5' to 3' end of the molecule, in accordance with other's observations[22,23,24].

(2) Synthesis of proteins by ϕX-174-specific RNA

The next question is to know whether RNA synthesized upon ϕX-174 RF-DNA in the cell-free system (abbreviated as ϕX-RNA) can function as messenger RNA in a cell-free system of protein synthesis. For this purpose, "S30"-fraction was prepared from *E. coli* Q 13, according to the procedure developed by Nirenberg and Matthai[7]. *E. coli* Q 13 is a mutant deficient in RNase I and polynucleotide phosphorylase (isolated at Dr. J. D. Watson's laboratory), so the cell-free system from this particular strain contained much less RNase-activity.

The first experiment was designed to know the binding ability of ϕX-RNA to ribosomes, as this is the essential process for protein synthesis[25]. A mixture of ϕX-RNA and ribosomes was incubated for 10 min at 37°C in the presence of 10 mM Mg^{++}. Sedimentation analysis clearly showed that ϕX-RNA, at least in part, had ability to form complexes with ribosomes[26].

When ϕX-RNA was added to the "S 30"-fraction, a significant incorporation of amino acids into polypeptide chains was observed[26]. Fig. 2 shows that the incorporation of ^{14}C-phenylalanine was linear for the first 2 hr and then reached a plateau. The rate of the incorporation increased, though it was not proportional, with the increasing amount of the RNA added at least to 2.3μg under the conditions given. When the system containing H^3-labelled ϕX-RNA was subjected to sucrose density-gradient centrifugation immediately after brief incubation, the sedimentation profile indicated clearly the formation of polysomes (Fig. 3).

Although it is clear that the ϕX-RNA promoted a significant amino acid incorporation in the test tube, this amino acid incorporation does not supply evidence for the synthesis of specific and complete protein molecules. It is required to

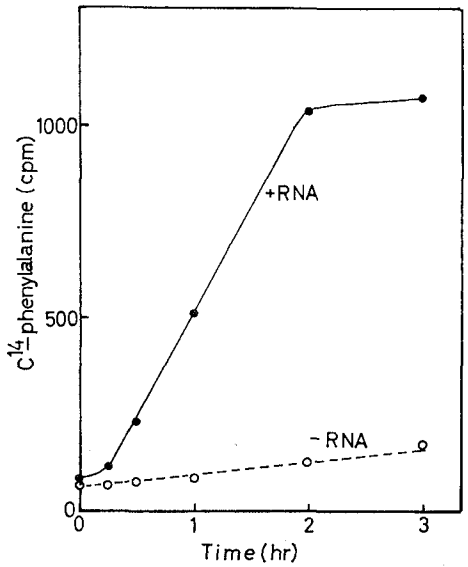

Fig. 2. Stimulation of the amino acid incorporation by ϕX-RNA in the cell-free protein synthesizing system[26].

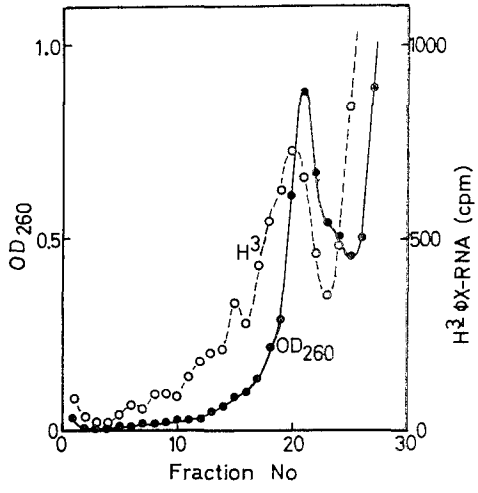

Fig. 3. Sedimentation analysis of the cell-free protein synthesizing system containing ^{3}H-labelled ϕX-RNA[26].

identify ϕX-specific proteins in the reaction products. Accordingly, the next attempt was made to provide a good protein synthesizing system and to identify the reaction products synthesized in this system under the direction of RF-DNA. According to Wood & Berg[27], when a protein synthesis system was combined with a RNA polymerase system, the incorporation of amino acids was much higher than the case where RNA synthesized by RNA polymerase was first isolated and then added to it. As their result was confirmed when ϕX RF-DNA was used as template[26], the former system (abbreviated as a "coupled system") was chosen for all the subsequent experiments.

(3) Synthesis and identification of ϕX-174-specific proteins

(a) Preparation of a protein synthesizing system which responds to exogenous DNA

According to consideration described in the previous section, a protein synthesizing system which strikingly responds to exogenous DNA was prepared from *E. coli* Q 13 cells by the procedures based on Nirenberg & Matthai[7] and Wood & Berg[27] with modifications as follows[28] : Conditions for centrifugation of the soluble fraction was increased to remove DNA of host cells and the preincubation period of the ribosome-soluble fraction was prolonged in order to exhaust endogenous messenger RNA. The reaction system was constructed in such a way that the capacity of translation was excess over that of transcription ; so all the RNA synthesized was involved in the translation. In this system the rate of ^{3}H-UMP incorporation directed by RF-DNA was constant up to about 1 hr and then declined sharply (Fig. 4). On the other hand, the incorporation of ^{14}C-valine increased linearly for 1 hr after about 20 min lag-period and then reached a plateau. It was clearly demonstrated that the synthesis of ϕX-RNA as messenger was essential for the amino acid incorporation. Any treatment which suppresses RNA synthesis

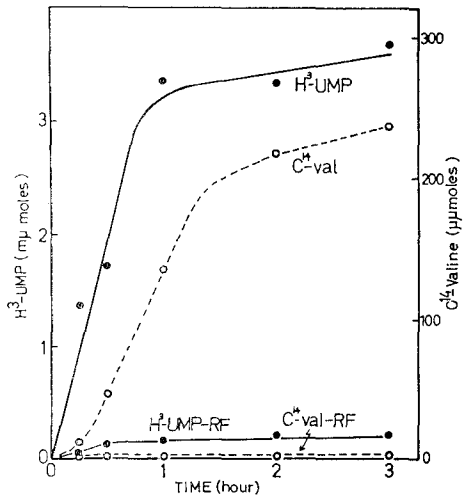

Fig. 4. Stimulation of nucleotide and amino acid incorporation by RF-DNA in the "coupled system"[28].

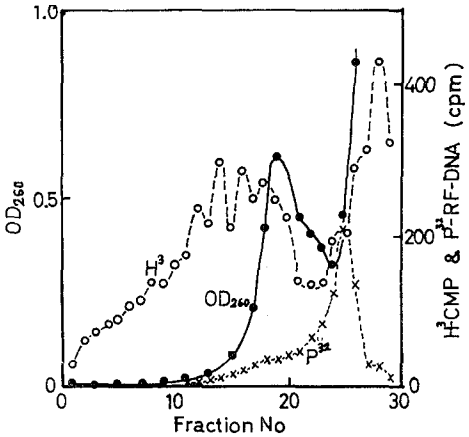

Fig. 5. Sedimentation analysis of the "coupled system" incubated with ^{3}H-CTP and ^{32}P-RF-DNA[28].

led to the inhibition of not only nucleotide but also amino acid incorporation into macromolecules. On the contrary, any treatment which affects protein synthesis inhibited only amino acid incorporation but not nucleotide incorporation.

The formation of polysomes was also identified in the "coupled system". The reaction mixture containing either ^{3}H-CTP or ^{14}C-valine was incubated and subjected immediately to sucrose density-gradient centrifugation. Fig. 5 shows that over 40% of RNA newly synthesized and labelled with ^{3}H-CMP distributed in regions heavier than the 80 S monosome. Similarly, approximately 45% of nascent polypeptides labelled with ^{14}C-valine were sedimented with the rate faster than that of the monosome. It was obvious that the RF-DNA directed protein synthesis took place on polysomes.

As seen in Fig. 5, some of the RF-DNA added into the system was also sedimented together with ribosomes, indicating the presence of complexes composed of RF-DNA, nascent RNA, nascent polypetptide, ribosomes and possibly RNA polymerase and sRNA in the incubation mixture. The complex appears to be similar to the phage T_4 DNA-RNA-ribosome complex described by Byrne *et al*[29].

(b) Characterization of synthesized RNA

RNA synthesized in the "coupled system" could be divided into two classes; RNA attached to ribosomes and that in the soluble fraction (see Fig. 5). In order to examine whether any difference exists between them, RNA was isolated from respective fraction, and hybridization-test was applied. Both RNA's hybridized well with "heat-denatured" RF-DNA but little with mature single-stranded DNA (Table 1). This indicates that the RNA synthesized in the "coupled system" is also complementary to the complement ("−" strand) of mature phage DNA.

The approximate sedimentation coefficients of these RNA's estimated from the sedimentation profiles were around 9 S. The S values obtained here may be smaller than that inherent in those RNA's because of a possible degradation by RNases during the incubation and extraction processes. These RNA fractions were mixed with ribosomes in the presence of 10mM Mg^{++} and the mixtures were analyzed by sucrose density-gradient centrifugation. It was shown that they could attach to ribosomes with almost the same efficiency. No essential difference between them was, therefore, found.

(c) Characterization of synthesized proteins

As has been mentioned earlier, the genome of ϕX-174 is assumed to contain at least 6 genes[3]. Therefore, it is likely that several kinds of ϕX-174-specific proteins are synthesized in the cell-free system as well as in cells infected with the phage. Attention was focussed here on the coat protein since it was only the material available and has been well identified among the ϕX-174-gene products[30]. Three kinds of methods were applied for the characterization of the synthesized products; by DEAE-cellulose and Sephadex column chromatography and by disc electrophoresis. Analysis were done on the product of the "coupled system" in which the stimulation of the amino acid incorporation was 200-fold or more over the control (minus RF-DNA) so that polypeptides derived from the host DNA or

host messenger RNA were neglected.

Analysis by DEAE-cellulose column chromatography: Reaction products labelled with ^{3}H-leucine were prepared and co-chromatographed with ^{35}S-labelled authentic coat protein on a DEAE-cellulose column in the presence of 8 M urea[31]. As seen in Fig. 6, the authentic coat protein was eluted at around 0.3 M Tris-HCl forming a single sharp peak. About two thirds of the ^{3}H-counts applied to the column was eluted at the same region as the coat protein. Remaining radioactivity was eluted prior to the main peak. This product was assumed, therefore, to be more basic than the coat protein.

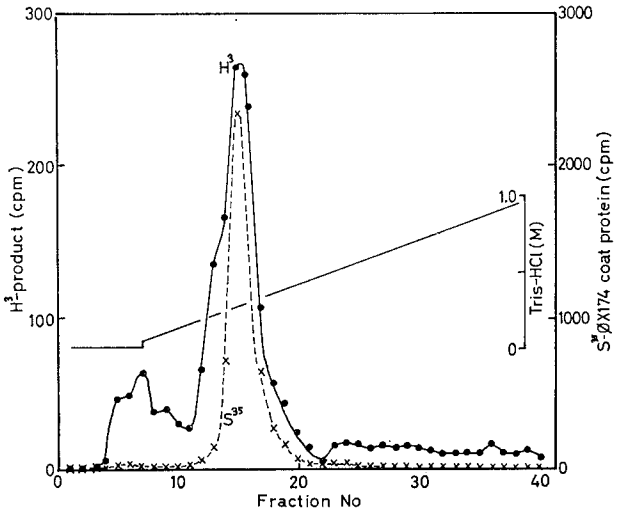

Fig. 6. DEAE-cellulose column chromatography of proteins synthesized in the "coupled system". ^{3}H-labelled product was chromatographed with ^{35}S-labelled authentic coat protein[31].

Analysis by Sephadex column chromatography: A mixture of the ^{3}H-labelled products and ^{35}S-labelled ϕX-174 coat protein was examined on Sephadex G 200 columns by using a procedure developed by Nathans[32]. Again, about two thirds of the ^{3}H-products applied to the column appeared at the position corresponding to ^{35}S-authentic coat protein which eluted just ahead of cytochrome C (molecular weight= 13,000) as marker.

Analysis by acrylamide gel electrophoresis: The ^{3}H-labelled product was fractionated together with ^{35}S-labelled authentic coat protein by using a modified acrylamine gel electrophoresis technique[33,34]. As shown in Fig. 7, the authentic coat protein migrated forming a single peak. Analysis of the ^{3}H-distribution showed the presence of one major and a few minor components which migrated faster than the former. The major one was just at the position of ^{35}S-labelled authentic coat protein. The minor components may, therefore, correspond to those eluted prior to the main component from the DEAE-cellulose column.

These data indicate that the main product synthesized in the "coupled system" in the presence of ϕX-174 RF-DNA was its coat protein, or a protein closely

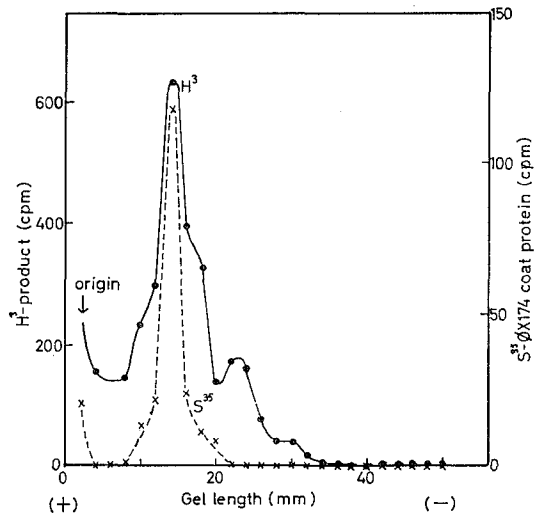

Fig. 7. Acrylamide gel electrophoresis of the proteins synthesized in the "coupled system". ^{3}H-labelled product was chromatographed with ^{35}S-labelled authentic coat protein[28].

related to this. This conclusion was also confirmed by preliminary applications of immunological assay and finger-printing technique for the synthetic products[31].

CONCLUSION

One of approaches to elucidate the mechanism of expression of genetic information encoded in DNA is to analyze the mechanism of transcription and translation in well-defined cell-free systems. For the purpose, bacteriophage ϕX-174 has been used as a source of genetic material because of its small size and availability of its RF-DNA.

Extensive studies have been made by Hayashi *et al.* to elucidate the mechanism of transcription by means of a DNA-dependent RNA polymerase system. They showed that when the circular RF-DNA was used as template only one of the strands was transcribed into RNA and that a DNA-RNA hybrid functioned as an intermediate in the transcription. It is also shown that RNA is synthesized from 5' to 3' end of the chain and that RNA thus made is, in part, polycistronic.

A remarkable stimulation of amino acid incorporation was obtained in the *E. coli* ribosome-soluble system when the ϕX-RNA synthesized in the cell-free transcription system was added. However, the efficiency of the protein synthesis became much higher when the RNA synthesis was coupled with the protein synthesis. Accordingly, a cell-free system which strikingly responded to exogenous DNA was prepared from *E. coli* Q 13. When RF-DNA was added to the system, remarkable and synchronized synthesis of RNA and protein was observed. The protein synthesis strictly depended on the RNA synthesis.

Hybridization-test showed that RNA made in the system was complementary to the "−" strand of RF-DNA (complement of the mature phage DNA), in ac-

cordance with the transcription *in vivo*. Sedimentation analysis of the reaction products provided evidence that the nascent RNA which was still bound to RF-DNA could attach to ribosomes and made them assemble into polysomes as soon as protein synthesis started. It is likely, therefore, that proteins were synthesized on such DNA-RNA-ribosome complexes.

Proteins synthesized in the system were analyzed by various methods and it was demonstrated that the major product was the coat protein or polypeptide closely related to the coat protein. Therefore, it is clear that the following reactions occurred in this "coupled system"; the nucleotide sequences of the "−" strand of RF-DNA was first transcribed into RNA by RNA polymerase and the messenger RNA thus synthesized was translated into specific proteins by the protein synthesizing assembly.

Similar systems have been reported by others. Wood and Berg first combined cell-free protein synthesis system with RNA polymerase and obtained stimulation of amino acid incorporation when T_2 DNA was added as template, although they have not characterized the reaction products[27]. They also showed that single-stranded ϕX-DNA was completely inactive for stimulation of amino acid incorporation, but it was activated by conversion to a double-stranded form with DNA polymerase[35]. Imai *el al.* observed an increase in activity of tryptophan synthetase when DNA from derepressed wild-type *E. coli* cells was used as template for their RNA and protein synthesis systems[36]. The RNA synthesized above was proved to be specific for the tryptophan operon[37]. Cell-free synthesis of a part of the enzyme β-galactosidase has been reported to be carried out under the direction of ϕ80d*lac* DNA, where the cell-free system was prepared from a *E. coli* mutant containing a deletion of the entire *lac* operon[38].

Besides the coat protein, the synthesis of several other proteins was detected by DEAE-cellulose column chromatography and acrylamide gel electrophoresis. Those minor components may represent the products of other genes. It will be possible to identify or isolate the products of all genes by using this system if the technique to analyze those proteins is improved. One of the possible approaches to determine the gene products would be the procedure applied for RNA phage systems[39,40]; when ϕX-174 RF-DNA's containing *amber* mutations at the different cistrons are used as template in the presence or absence of suppressor t-RNA, the proteins synthesized in the presence of suppressor t-RNA would be the products of the corresponding genes. Many conditional lethal mutants of ϕX-174 have been isolated by Sinshemer's[41] and Hayashi's laboratories[3].

The predominance of the phage coat protein over other products in the cell-free system was observed. It is favorable to assume that this is a reflection of control mechanism which operates in phage replication in the infected cells, because such a differential translation has been observed during the process of RNA phage replication *in vivo*[40,42,43] and also in the cell-free protein synthesis directed by phage RNA[40,43,44,45,46,47]. Phage-specific proteins are supposed to be synthesized at different stages of the replication and with varying rates. This may be regulated at two levels; at the step of transcription and of translation. As described in section (1), the formation of five RNA fractions having characteristic sedimentation

coefficients in the synchronized transcription system, would suggest that some regulatory mechanism operates at the transcriptional level *in vitro*, resulting in the production of messenger RNA's with different numbers of cistrons. It is, thus, speculated that the population of the coat protein cistron among the messenger RNA molecules turns out to be very high compared with those of the others. If this is true, the DNA molecule must contain a device which dictates the number of times to transcribe a particular cistron and can operate even at cell-free systems. As the amount of coat protein synthesized *in vitro* under the direction of the messages from RNA phages was reported to be much greater than that of the other products, it is likely that the translation of ϕX polycistronic messenger RNA, if any, is also subjected to regulation in cell-free systems.

In addition to the problem of reading frequency, there is an another problem; the order of usage of each cistron encoded in genetic materials. The ordered translation of the polycistronic messages derived from RNA phages in cell-free system has been reported[44,48]. The fact that only "early" region of T_2 or T_4 phage DNA is transcribed by RNA polymerase *in vitro*[49] may be a reflection of the ordered translation occurring *in vivo*. In the case of ϕX-174, no clue is at present available to tell whether this phenomenon is controlled at transcriptional or translational level or both.

ACKNOWLEDGEMENTS

The author is grateful to Dr. Masaki Hayashi for his guidance throughout this study. His thanks are also due to Dr. Mituru Takanami for his help during the preparation of this manuscript.

ABBREVIATIONS

DNA : deoxyribonucleic acid, RNA : ribonucleic acid, ATP : adenosine triphosphate, GTP : guanosine triphosphate, UTP : uridine triphosphate, CTP : cytidine triphosphate, GMP : guanosine monophosphate, CMP : cytidine monophosphate, UMP : uridine monophosphate, Tris : tris (hydroxymethyl) aminomethane.

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