Studies on the Functional Relationship between ϕ X174 and S13 Coded Proteins Using an *In Vitro* DNA Synthesis System

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Received April 6, 1981

The functional relationship between bacteriophage ϕ X174 and S13 gene products was investigated using an *in vitro* DNA synthesis system in two stages: (a) replication of the closed circular duplex form DNA (RF to RF) and (b) synthesis of single-stranded DNA from RF and formation of the intermediate 50S complex in phage morphogenesis (RF to 50S complex). Partially purified gene A products of ϕ X174 and S13 were able to function reciprocally in the stage from RF to RF replication. In the stage of RF to 50S complex, extracts from *Escherichia coli* infected with ϕ X174 catalyzed the formation of 50S complex from S13 RF DNA. Extracts from *E. coli* infected with S13 also had the ability to catalyze the formation of 50S complex from ϕ X174 RF DNA. Thus, all gene products required for DNA replication and phage morphogenesis were found to function reciprocally between ϕ X174 and S13.

Bacteriophages S13 and ϕ X174, small circular single-stranded DNA phages, were isolated independently and have been shown to be serologically related.^{1~3)} Their DNAs are almost identical in physical characteristics.⁴⁾ Genetic recombination can take place among some mutants,⁵⁾ and all but one of S13 or ϕ X174 gene products complement their ϕ X174 or S13 counterpart in *in vivo* complementation test.⁶⁾ The phage-coded proteins are found to be similar to each other in profiles of polyacrylamide gel electrophoretic patterns.^{7,8)}

Despite these similarities, S13 and ϕ X174 show rather high degree of divergency in base sequence. Godson reported that there was up to a 35% base mismatching under stringent denaturation conditions in heteroduplex analysis, and that only $4.7 \pm 1.9\%$ of the DNA of ϕ X174 is highly homologous with S13 DNA.⁹⁾ Recently, Compton and Sinsheimer observed less homology than did Godson under mild denaturation conditions and more homology under stringent conditions.¹⁰⁾ DNA fragments produced by many restriction en-

zymes are also slightly different.¹¹⁾ It is reported that the host dependency is different between these phages.¹²⁾ Therefore, it is assumed that the mechanism of DNA replication is different between the two phages. In terms of functional relationship in DNA synthesis, it is important to know how many phage-coded proteins can be exchangeable between the two phages.

In vitro systems for DNA synthesis of ϕ X174 have been developed and revealed the mechanism of DNA synthesis in three stages, (I) conversion of circular single-stranded parental DNA to the duplex replicative form (SS to RF), (II) replication of the closed circular duplex form (RF to RF) and (III) synthesis of single-stranded DNA from RF and formation of the intermediate 50S complex in the phage morphogenesis (RF to 50S complex).^{13~16)} In stage (II), only ϕ X174 gene A product is required in addition to host proteins, as was first shown by Tessman for the S13.^{14~16)} In stage (III), all phage-coded gene products except for gene E is required.¹⁷⁾ In this work the functional relationship of gene products of ϕ X174 and S13 was investigated using *in vitro* DNA synthesis system in stage (II) and (III). It

Abbreviations: RF, replicative form; RFI, covalently closed circular RF; RFII, open circular RF.

is shown that all gene products except for gene E of S13 and ϕ X174 are reciprocally functioning.

MATERIALS AND METHODS

Bacterial and phage strains. *E. coli* H560 (*polA*, *endA*, *thyA*) was used for the preparation of protein fractions. Phage ϕ X174 *am3*, a lysis-defective mutant ϕ X174, S13 wild type (obtained from Dr. Tessman) and fd wild type were used.

Nucleotides. Unlabeled deoxyribonucleotide triphosphates (dNTPs) and ribonucleotide triphosphates (rNTPs) were obtained from Boehringer Mannheim GmbH. [Methyl-³H] dTTP was purchased from New England Nuclear Corporation.

Preparation of protein fractions. Protein fractions capable of promoting the replication of RF to RF and RF to 50S complex were prepared by the method of Wickner *et al.* and of Yasumoto *et al.*, respectively, except for the following modifications.^{17,19)} (a) For preparation of uninfected ammonium sulfate fraction, *E. coli* H560 was grown at 37°C in L-broth medium supplemented with 50 μ g/ml thymine to a cell density of 0.6 absorbance unit at 610 nm. Cells were lysed and the lysate was centrifuged at 24,000 rpm for 45 min in a Hitachi RPS 25 rotor at 2°C. (b) For preparation of phage infected protein fractions (Fraction II), *E. coli* H560 was grown in TPG-CA medium supplemented with 50 μ g/ml thymine at 30°C to a cell density of 0.3 absorbance unit at 610 nm. Cells were infected with ϕ X174 or S13 for 25 min in order to prepare Fraction II, which is required for the replication of RF to RF, and for 50 min to prepare Fraction II, which is required for the formation of 50S complex. Cells were lysed and the lysate was centrifuged in a Hitachi RPS 40T rotor at 32,000 rpm for 30 min to obtain Fraction II for RF to RF synthesis system and for 90 min to prepare Fraction II for RF to 50S complex formation system.

Protein fractions prepared by the above method were frozen in small portions at -20°C.

Preparation of DNA. RFI DNAs of ϕ X174, S13 and fd were prepared by the procedure of Komano and Sinsheimer.²⁰⁾

Reaction mixture. The standard reaction mixture (50 μ l) contained 50 mM Tris·HCl (pH 7.5), 10 mM MgCl₂, 4 mM DTT, 1 mM ATP, 0.2 mM NAD⁺, 40 μ M each of dATP, dGTP, dCTP and [methyl-³H] dTTP (200 cpm/pmol), 0.1 mM each of CTP, UTP and GTP, 1 μ g of RFI DNA, 2 mM spermidine-3HCl, 240 μ g protein of uninfected ammonium sulfate fraction and 60 μ g protein of Fraction II. Spermidine-3HCl was omitted from the reaction mixture for RF to 50S complex analysis.

The reaction mixture was incubated at 30°C. The reaction was stopped by adding ice-cold trichloroacetic acid (TCA) to the final concentration of 10%. The precipitates formed were collected onto Whatman GF/C glass filter discs, and were washed with ice-cold 5% TCA and then with ice-cold ethanol. The discs were dried and counted for radioactivity by a Beckman liquid scintillation counter. When reaction products were characterized by sedimentation analysis, the reaction was stopped by adding EDTA and Sarkosyl or only EDTA as described by Yasumoto.¹⁷⁾

Sedimentation analysis. Products were analyzed by one of sucrose gradient centrifugation described as follows;

(I) A neutral 5 to 20% linear sucrose gradient containing 50 mM Tris·HCl (pH 7.4), 0.5 M NaCl and 3 mM EDTA was centrifuged in a Hitachi RPS 40T-2 rotor at 37,000 rpm for 4.5 hr at 2°C.

(II) An alkaline 5 to 20% linear sucrose gradient containing 0.5 M NaCl, 3 mM EDTA, 0.1% sarkosyl and 0.3 N NaOH on a cushion of 0.2 ml of 55% CsCl solution was centrifuged in a Hitachi RPS 40T-2 rotor at 39,000 rpm for 3 hr at 2°C.

(III) A neutral 5 to 30% linear sucrose gradient containing 20 mM Tris·HCl (pH 7.4), 5 mM EDTA, 0.1 M NaCl on a cushion of 0.5 ml of 55% CsCl solution was centrifuged in a Hitachi RPS 40T rotor at 39,000 rpm for 2.5 hr at 2°C.

Isolation and characterization of product DNA. The reaction mixture for RF to 50S complex system was centrifuged in a neutral 5 to 30% sucrose gradient as described above. Fractions corresponding to 50S or 20S regions of the sucrose gradient were pooled, 10 mM EDTA and 0.2% SDS (final) were added to each pooled fraction and the mixture was heated at 60°C for 15 min followed by a 3.5 hr incubation at 37°C with auto-digested proteinase K (1 mg/ml). The mixtures were dialyzed against 50 mM Tris·HCl (pH 8.0) containing 5 mM EDTA for 12 hr. The purified DNA was analyzed by 5 to 20% sucrose gradient centrifugation as described above.

RESULTS

Replication of RF to RF

Fraction II prepared from *E. coli* infected with S13 incorporated dTMP into S13 DNA in the presence of uninfected ammonium sulfate fraction (normal reaction). This reaction depended on RFI DNA added exogenously and Fraction II (Table I). When RF DNA of phage fd was used as a template in place of S13 RF DNA, no incorporation of dTMP was observed. Omission of dNTPs, ATP or Mg²⁺ abolished the activity. CTP, GTP and UTP

TABLE I. REQUIREMENTS FOR RF TO RF SYSTEM

Omission or addition	dTMP Incorporation (%)		
	Fraction II: ϕ X174 DNA: S13	Fraction II: S13 DNA: ϕ X174	Fraction II: S13 DNA: S13
Complete	100 ^a	100 ^b	100 ^c
–DNA	15	11	12
–Fraction II	21	20	23
–Mg ²⁺	9	5	5
–rATP	35	28	33
–dATP, dCTP, dGTP	24	25	26
–rCTP, rGTP, rUTP	116	96	100
+rifampicin (100 μ g/ml)	131	101	108
+NEM ^d (10 mM)	13	16	15
+PCM ^e (10 mM)	—	—	14
+fd RF	14	14	15
–S13 or ϕ X RF			

Assay conditions were described in MATERIALS AND METHODS. The reaction mixture was incubated for 40 min at 30°C. One-hundred percent [³H] dTMP incorporation corresponds to: ^a 12.0 pmol; ^b 12.2 pmol; ^c 11.0 pmol;

^d *N*-Ethylmaleimide; ^e *p*-Chloromercuriphenylsulfonate.

FIG. 1. Kinetics of dTMP Incorporation and Product Analysis of the RF to RF System.

The complete reaction mixture (200 μ l) was incubated for 40 min at 30°C and the product was analyzed by sucrose gradient sedimentation. Reaction product of ϕ X174 RF DNA and Fraction II prepared from S13-infected cells were sedimented in a sucrose gradient I (a) or II (b) as described in MATERIALS AND METHODS. Reaction products of S13 RF DNA and Fraction II prepared from ϕ X174-infected cells were sedimented in a sucrose gradient I (c) or II (d). Reaction products of S13 RF DNA and Fraction II prepared from S13-infected cells were sedimented in a sucrose gradient I (e) or II (f). Fractions 41~48 were discarded because no acid-insoluble radioactivity was detected (a, c and e). The arrow indicates the position of ϕ X174 single-stranded circular DNA. Inserted figures represent kinetics of [³H] dTMP incorporation into acid-insoluble materials (g, h and i). O—O, complete reaction mixture; □—□, RF DNA was omitted; ×—×, fd RF DNA was used in place of S13 or ϕ X174 RF DNA.

were not required. DNA synthesis was abolished by *N*-ethylmaleimide (NEM) or *p*-chloromercuriphenylsulfonate (PCM) but not by rifampicin.

Fraction II prepared from *E. coli* infected with ϕ X174 incorporated dTMP into S13 RF DNA. Fraction II from S13-infected *E. coli* also incorporated dTMP into ϕ X174 RF DNA (exchanged reaction). These reactions showed the same characteristics as that of normal reaction (Table I).

Products of these reactions were analyzed by centrifugation through neutral and alkaline sucrose gradients (Fig. 1). In neutral sucrose gradient centrifugation the incorporated radioactivity was distributed over three major components as shown in Fig. 1a, c and e. The faster sedimenting component corresponded to RFI, the component sedimented in the middle corresponded to sigma type DNA and the slower sedimenting component corresponded to RFII. A small amount of rapidly sedimenting components was also detected. When products were analyzed by alkaline sucrose gradient centrifugation, RFI, single-stranded DNA longer than unit length sedimenting slightly faster than marker DNA

(ϕ X174 single-stranded circular DNA) and single-stranded circular DNA were detected. Small DNA fragments of approximately 5 to 10S were also detected (Fig. 1b, d and f). In the exchanged reaction, relatively larger amount of small DNA fragments was detected than in the normal reaction in the alkaline sucrose gradient. These fragments were probably attached by hydrogen bonding to RF DNA, because they were not detected in the neutral sucrose gradient. The rate of incorporation in the exchanged reaction was constant for 40 min (Fig. 1g, h; inserted figures) but in the normal reaction the rate was levelled off within 15 min (Fig. 1i; inserted figure). It is reported that the gene A product of ϕ X174 as well as S13 is only phage-coded protein required in the stage of RF to RF replication.¹⁴⁻¹⁶ Thus, the above results show that the *in vitro* complementation of gene A products between ϕ X174 and S13 is possible.

Formation of RF to 50S complex

The formation of 50S complex depended on the presence of S13 RF DNA and Fraction II, which was prepared from the cells harvested at 50 min after phage S13 infection (normal re-

FIG. 2. Product Analysis of RF to 50S Complex System.

Reaction products of S13 RF DNA and Fraction II prepared from S13-infected cells were analyzed by a sucrose gradient III described in MATERIALS AND METHODS. The incubation times of reaction mixtures were 15 min (a), 60 min (b), 120 min (c) and 140 min (d), respectively. Fractions 1~2 represent the CsCl cushion. S values were estimated by using ϕ X174 mature phage and single-stranded circular DNA as a sedimentation marker.

FIG. 3. Characterization of DNA in 50S Region.

50S region in Fig. 2(b) was pooled and the DNA was deproteinized as described in MATERIALS AND METHODS. The purified DNA was then centrifuged in a sucrose gradient I as described in MATERIALS AND METHODS. Arrows indicate the positions of reference ϕ X174 single-stranded DNA, RFI DNA and RFII DNA run in parallel gradient.

FIG. 4. Product Analysis of Exchanged Reaction in the Stage of RF to 50S Complex.

(a) Reaction product of ϕ X174 RF DNA and Fraction II prepared from S13-infected cells.

(b) Reaction product of S13 RF DNA and Fraction II prepared from ϕ X174-infected cells. Both reaction mixtures were incubated for 60 min at 30°C and the products were sedimented as described in Fig. 2.

action; data not shown). NEM inhibited the incorporation of dTMP. RF DNA of phage fd did not serve as a template (data not shown). Incorporation of radioactivity into 50S complex ceased within 60 min, but synthesis of 20S molecules continued for over 120 min (Fig. 2a,

b, c). When the reaction mixture was incubated for 140 min, a small peak sedimenting faster than 50S appeared (Fig. 2d).

After centrifugation, fractions of 50S regions were pooled and deproteinized, and the DNA was characterized by neutral sucrose

gradient centrifugation (Fig. 3). The DNA sedimented at the position between RFI and RFII was the sigma type structure DNA. The DNA from 20S region, analyzed by the same way, sedimented as RFII (data not shown).

When 2 mM spermidine was added to the reaction mixture in the formation of RF to 50S complex, incorporation of dTMP into S13 RF DNA was observed. However, the products of this reaction were heterogeneous; 17~20S molecules were obtained and 50S complex was not formed (data not shown).

Fraction II which was prepared from the cells harvested at 50 min after phage S13 infection catalyzed the formation of 50S complex from ϕ X174 RF DNA. Fraction II prepared from the cells infected with ϕ X174 also catalyzed the formation of 50S complex from S13 RF DNA (exchanged reaction). When the product was analyzed by neutral sucrose gradient centrifugation in the exchanged reaction, 50S complex was also detected (Fig. 4). The incorporation of radioactivity into 50S complex by using Fraction II prepared from ϕ X174 infected cells and S13 RF DNA was slightly lower than that by using Fraction II prepared from the cells infected with S13 and ϕ X174 RF DNA. Furthermore, heterogenous peaks of 17~20S region were detected in the former case (Fig. 4b). It was reported that the formation of 50S complex requires all phage-coded gene products except for gene E.¹⁸⁾ Thus all phage-coded gene products except for gene E of ϕ X174 can complement their S13 counterparts and *vice versa* in the *in vitro* 50S complex formation system.

DISCUSSION

It is known that gene A protein is the only phage-coded product required in the stage of RF to RF replication.^{14~16)} This protein nicks the viral strand in RFI DNA uniquely in the gene A region to form RFII.^{14,15)} In this report it was shown that the gene A proteins of bacteriophage ϕ X174 and S13 were able to function reciprocally in the *in vitro* RF to RF replication system. This result would indicate

that the DNA sequence recognized by respective gene A proteins is homologous between ϕ X174 and S13, or there is a tolerance in the sequence recognition by these proteins. However, the reaction rate and the amount of short DNA fragments in exchanged reaction were different from that in the normal reaction (Fig. 1). It is not clear why these discrepancies were observed between the two reactions. A possible reason is that the rate of reaction of gene A protein is slow as the conformation of DNA recognized by this protein is slightly different in exchanged reaction.

Recently Borrias *et al.* have reported that ϕ X174 is able to use the products of bacteriophage G4 genes E, F, G, and H, but only the gene H product of ϕ X174 is functional in G4 infection.²¹⁾ The functional relationship between ϕ X174 and G4 genes was determined by complementation experiments using amber mutants. However, Duguet *et al.* carried out complementation experiments *in vitro* between ϕ X174 and G4, and found that G4 FRI DNA could be cleaved in a functional manner by ϕ X174 gene A product.²²⁾ On the other hand, Tessman,¹⁶⁾ and Francke and Ray²⁴⁾ showed that the gene A product of ϕ X174 was *cis* acting *in vivo*. The result presented here indicates that this protein acts in *trans*. It is difficult to reconcile the difference of mode of action of the gene A products between *in vivo* and *in vitro*. However, Ikeda *et al.* also concluded that gene A protein is able to act in *trans in vitro*.²⁵⁾ They showed that gene A protein isolated from cells infected with ϕ X174 gene E mutant acts in RF DNA prepared from the cells infected with gene A mutant *in vitro*.

Jenz *et al.* performed the complementation test *in vivo* between suppressible mutants of ϕ X174 and S13.⁶⁾ They reported that all but one of S13 or ϕ X174 gene products complement their ϕ X174 or S13 counterpart. Since they had no mutant in gene C of ϕ X174 and in gene D of S13, they could not test the complementation activity of these genes between ϕ X174 and S13. On the other hand, all phage-coded gene products except for gene E were

required for the formation of 50S complex from RF.¹⁸⁾ In the present report it was shown that all phage-coded gene products except for gene E of ϕ X174 and S13 were functionally exchangeable with each other in the *in vitro* formation of 50S complex from RF.

Recently, we also found that S13 DNA was replicated by common machinery with ϕ X174 in the stage of SS to RF *in vitro*.²⁶⁾ However, heteroduplex analysis of the DNAs indicates that mismatches in base pairs distribute over the wide range along the phage genomes.^{9,10)} It is likely that the important structure or sequence of viral DNA to be recognized by phage-coded proteins and host cell proteins has not been changed in the process of evolutionary divergences.

Acknowledgment. This work was supported in part by a scientific grant from the Ministry of Education, Japan.

REFERENCES

- 1) V. Sertic and N. Boulgakov, *C. R. Soc. Biol.*, **119**, 1270 (1935).
- 2) F. M. Burnet, *Brit. J. Exp. Path.*, **8**, 121 (1927).
- 3) S. A. Zahler, *J. Bact.*, **75**, 310 (1958).
- 4) J. H. Spencer, R. Cerny, E. Cerna and D. Delaney, *J. Virol.*, **10**, 134 (1972).
- 5) E. S. Tessman and R. Schleser, *Virology*, **19**, 239 (1963).
- 6) Y. Jenz, M. Gelfand, M. Hayashi, R. Schleser and E. S. Tessman, *J. Mol. Biol.*, **49**, 521 (1970).
- 7) A. B. Burgess and D. T. Denhardt, *J. Mol. Biol.*, **45**, 377 (1969).
- 8) D. H. Gelfand and M. Hayashi, *J. Mol. Biol.*, **44**, 501 (1969).
- 9) G. N. Godson, *J. Mol. Biol.*, **77**, 467 (1973).
- 10) J. L. Compton and R. L. Sinsheimer, *J. Mol. Biol.*, **109**, 207 (1977).
- 11) G. N. Godson and R. J. Roberts, *Virology*, **73**, 561 (1976).
- 12) A. Taketo, *Mol. Gen. Genet.*, **139**, 285 (1975).
- 13) R. R. Meyer, J. Shlomai, J. Kobori, D. L. Bates, L. Rowen, R. McMacken, K. Ueda and A. Kornberg, "Cold Spring Harbor Symposia on Quantitative Biology," Vol. XLIII, Cold Spring Harbor Laboratory, 1979, p. 289.
- 14) S. Eisenberg, J. G. Scott and A. Kornberg, "Cold Spring Harbor Symposia on Quantitative Biology," Vol. XLIII, Cold Spring Harbor Laboratory, 1979, p. 295.
- 15) C. S. Yasumoto, J.-E. Ikeda, E. Benz, K. J. Mariani, R. Vicuna, S. Sugrue, S. L. Zipursky and J. Hurwitz, "Cold Spring Harbor Symposia on Quantitative Biology," Vol. XLIII, Cold Spring Harbor Laboratory, 1979, p. 311.
- 16) E. S. Tessman, *J. Mol. Biol.*, **17**, 218 (1966).
- 17) C. S. Yasumoto and J. Hurwitz, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 4195 (1977).
- 18) M. Hayashi, "The Single-Stranded DNA Phages," ed. by D. T. Denhardt, D. Dressler and D. S. Ray, Cold Spring Harbor Laboratory, New York, 1978, p. 531.
- 19) S. Wickner, M. Wright, I. Berkower and J. Hurwitz, "Method in Molecular Biology," Vol. VII, ed. by R. B. Wickner, Marcel Dekker, Inc., New York, 1974, p. 195.
- 20) T. Komano and R. L. Sinsheimer, *Biochim. Biophys. Acta*, **155**, 295 (1968).
- 21) W. E. Borrias, M. Hagenaar, R. V. D. Brekel, C. Khlemeijer and P. J. Weisbeek, *J. Virol.*, **31**, 288 (1979).
- 22) M. Duguet, G. Yarranton and M. Gefter, "Cold Spring Harbor Symposia on Quantitative Biology," Vol. XLIII, Cold Spring Harbor Laboratory, 1979, p. 335.
- 23) B. Francke and D. S. Ray, *Virology*, **44**, 168 (1971).
- 24) J.-E. Ikeda, A. Yudelevich, N. Shimamoto and J. Hurwitz, *J. Biol. Chem.*, **254**, 9416 (1979).
- 25) K. Watabe, H. Sakai and T. Komano, *J. Biochem.*, **90**, (1981) in press.