"Distinguishable Steps in the Enzymatic Synthesis of Bacteriophage ϕX174 Replicative Form In Vitro"

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Received March 12,1976

SUMMARY

On the basis of kinetic evidence bacteriophage ϕX174 replicative form synthesis occurs in at least two stages. The first stage reaction converts the DNA to an activated form in the absence of DNA synthesis. This "activated" form may be isolated and can be shown to have different properties than unreacted DNA. The second stage comprises the DNA synthetic reaction but also requires the dnaG protein.

INTRODUCTION

The conversion in vitro of bacteriophage ϕX174 single-stranded DNA to its double-stranded, replicative form requires the activity of at least ten proteins (1,2). Aside from the process of DNA synthesiswhich requires four of these proteins (DNA polymerase III, elongation factors and DNA binding protein) (1,3,4), many proteins appear to be needed for the "initiation" of DNA synthesis. Although it is postulated that one of the "initiation" proteins (dnaG protein) synthesizes the primer (5), the role of the other remaining proteins remains obscure.

We will present evidence that bacteriophage ϕX174 replicative form synthesis takes place in two stages. The first stage, whichis protein and ATP dependent, alters the DNA in such a way that the DNA may be re-isolated and subsequently converted to a double stranded form efficiently. This second stage reaction requires less than ten proteins but more than just DNA polymerase III and elongation factors.

MATERIALS AND METHODS

Cells (Escherichia coli H562) were grown in broth at 30°C, harvested, washed and frozen in a solution of 50mM Tris-HCl, pH 7.5 containing 10% sucrose as previously described (6). Cell-free extracts were prepared as described by Wickner, Wright and Hurwitz (7). Routinely cell extracts contained 9-13 mg per ml protein.

To render cell extracts stable and free of detectable deoxy or ribotriphosphates they were freed of nucleic acid and concentrated by ammonium sulfate precipitation. For each 1.0 ml of extract, 74 µl of neutralized saturated ammonium

sulfate was added. The mixture was passed over a DEAE-cellulose column (equal in bed volume to the cell extract volume) which was previously equilibrated with S E D buffer (50 mM Tris-HCl, pH 7.5, 1 mM E D T A , 2 mM dithiothreitol and 10% sucrose) which also contained the same concentration of ammonium sulfate as the extract. The protein was collected (as judged by the yellow color) after passage through the column. The eluate was adjusted to 40% saturation with neutralized, saturated ammonium sulfate. After 10 minutes at 0°C, the precipitate was collected by centrifugation and dissolved in the original volume of S E D buffer. The precipitation was repeated and the precipitate was washed with SED buffer containing ammonium sulfate (50% saturation), re-collected by centrifugation and finally dissolved in 1/30 of the original extract volume of S E D buffer. This preparation (10-12 mg/ml of protein) was used for the preparation of the "initiation complex" (see below) and also served as the starting extract for further purification of second-stage active components.

Purification of "second-stage active" components was achieved by ammonium sulfate fractionation and phosphocellulose chromatography of the above fraction. Following precipitation of the activity at 40% saturated ammonium sulfate, the precipitate was extracted with S E D buffer (original fraction volume) containing ammonium sulfate at 35% saturation and then again with S E D at 25% saturation. The insoluble matter was finally dissolved in S E D but containing 20% glycerol and 20 mM NaCl in place of sucrose (1/5 original fraction volume). This preparation contained 50-80% of the original second-stage activity. Following dialysis, the active fraction was applied to a column of phosphocellulose equilibrated with the above buffer; 20-40% of the original activity is eluted with the same buffer containing 50 mM NaCl.

Assays. For synthesis of ϕX174 RFII, the reactive mixture (50 µl) was 20 mM Tris-HCl pH 7.5, 12 mM $MgCl_2$, 4 mM dithiothreitol, 2 mM in ATP and contained ϕX174 DNA, 100 p moles nucleotide; 2 n moles each dATP, dGTP and dCTP; 0.2 n moles of [^{3}H] TTP (1.0 x 10^3 cpm/p mole); 1.5 µg of rifampicin and cell extract, 5 µl. The incubation was at 30°C for varying amounts of time. Reactions were stopped by the addition of trichloroacetic acid (5%). Acid insoluble radioactivity was determined by liquid scintillation counting.

Conditions for formation of the "initiation complex" were the same as above only all four deoxytriphosphates were omitted. Incubations were for 10 minutes at 30°C. The complex was isolated by filtration through a column of Biogel A5M (0.5 x 10 cm) developed in buffer A (20 mM Tris-HCl pH 7.5, 1 mM E D T A , 12 mM $MgCl_2$, 4 mM dithiothreotol, 0.5 mM ATP and 2.5% sucrose). Complex was detected by adding 10 µl of deoxytriphosphates (same concentration as the assay conditions) to each fraction (50 µl) and 1 µl of original extract. Incubations were for 20 minutes at 30°C. The reaction was stopped by the addition of trichloroacetic acid (5%) and 100 µg of bovine serum albumin was added as carrier. For preparation of large amounts of complex, the reaction volume and column dimensions were scaled up twenty-fold.

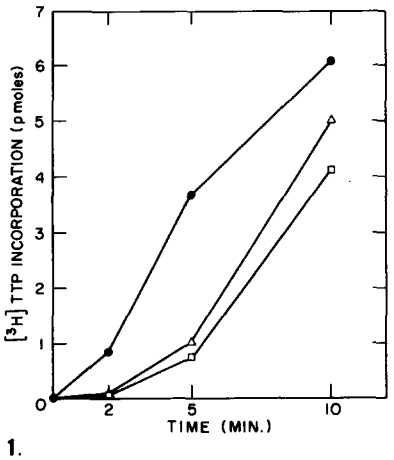
Purified dnaG protein (880 units/ml, reference 7) was kindly provided by Dr. Sue Wickner.

Analysis of the product of the reactions were made by alkaline sucrose density gradient centrifugation as previously described (6).

E. coli DNA polymerase III* and co-polymerase III* were purified through the phosphocellulose stage as described by Wickner et al. (8).

RESULTS

The partially purified extract, prepared as described in Methods, was capable of converting single-stranded bacteriophage ϕX174 DNA to a double-stranded (RFII)



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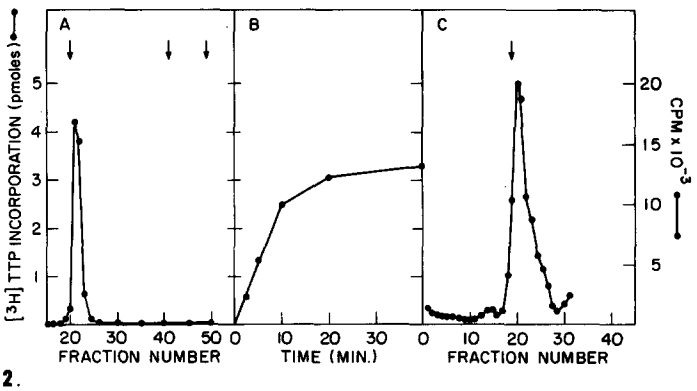

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Figure 1. Bacteriophage ϕX174-dependent DNA synthesis. The components of the reaction mixture are given in the methods. Incubation was for 7 minutes at 30°C in the absence of dNTPs. Deoxytriphosphates are added at time zero (o--o--o). In the first incubation ATP (Δ--Δ--Δ) or DNA (□--□--□) was omitted and then added along with dNTPs in the second incubation.

Figure 2 . Bacteriophage ϕX174 DNA synthesis in two stages. Following the first incubation (figure 1) reaction mixture was filtered through a Biogel A5M column (2A). To each fraction was added dNTP and extract and incubation was as in figure 1. The arrows (left to right) represent the position of elution of ϕX174 Marker DNA, hemoglobin and dNTP respectively. Panel B represents the kinetics of incorporation in the second stage. Panel C represents an alkaline sucrose density gradient analysis of the product of the second stage reaction. The arrow marks the position of circular fd DNA marker. The details are in the methods section.

form in a rifampicin-resistant reaction. In addition, extracts prepared in this way from dnaE and dnaG mutants were temperature-sensitive for DNA synthesis compared to extracts prepared from dna^+ cells.

Although more than 80% of the input DNA is converted to a double-stranded form by 30 minutes of incubation, the reaction does not proceed linearly with time. There is a lag in incorporation for 5 minutes and then the reaction proceeds linearly for 25 minutes. It was found that the lag could be abolished if all of the components of the reaction except deoxytriphosphates were first incubated for five minutes. Following addition of deoxytriphosphates, the reaction proceeds linearly. If ATP or DNA is omitted in the first incubation and then added after five minutes, there is still a lag in incorporation. The results, summarized in figure 1, indicate that there are two reactions taking place that lead to the formation of double

stranded DNA. The first, slow reaction requires proteins, ATP and DNA but not deoxytriphosphates. Following the first reaction, addition of deoxytriphosphates to the system promotes immediate and rapid synthesis of DNA.

These results suggested that the DNA had to be acted upon prior to the requirement for deoxytriphosphates. In order to examine this reaction, the DNA following the first incubation was separated from the bulk proteins and small molecules by gel filtration (figure 2A) and the requirements for its subsequent conversion to the replicative form were studied. If the DNA is isolated after the initial reaction and then used as a substrate for RF formation, it promotes immediate DNA synthesis (figure 2B). There is no lag in incorporation seen. The product of the reaction is a full length, complementary strand of the template (figure 2C). In addition to the absence of a lag in the incorporation rate, the requirements for RF formation are such that a much reduced amount of extract (1/10) is sufficient to convert the re-isolated DNA to a double stranded form. The amount of extract capable of converting essentially all of the "treated" DNA to RF is sufficient to catalyze the formation of only 2.5% of that amount on untreated DNA (table 1). Furthermore, the activity of the extract on untreated DNA shows an exponential dependence with concentration whereas the extract activity on "treated" DNA shows a linear dependence. Thus it appears that the second reaction is considerably simpler in its requirements than the overall reaction is.

The conversion of the isolated DNA into a double-stranded form requires all four deoxytriphosphates and is not stimulated by the addition of ribotriphosphates. The amount of DNA synthesized is directly proportional to the amount of extract added in the second step. This property allowed for the partial purification of the activity required in the second stage* (see methods). The "second stage" activity could not be replaced by the addition of DNA polymerase III* and co-polymerase III* (units added were equal to that amount present in the

*The "second stage" activity fraction was found to contain the dnaG protein (> 42 units/ml, units defined in Ref. (7)) in addition to DNA polymerase III, E. coli unwinding protein and elongation factors. There was no detectable dnaB or dnaC(D) proteins (these assays were kindly performed by Dr. S. Wickner, National Institutes of Health).

TABLE I†

System		Activity (percent)
Complex	+ extract + dNTP	100%*
Complex	- extract + dNTP	0.1
Complex	+ extract + dATP, dGTP, -dCTP	6.7
Complex	+ extract + dNTP + rNTP	91.0
Complex	+ partially purified extract + dNTP	65.0
- Complex	+ extract + ϕX174 DNA + dNTP	2.5

†In all two stage reactions the final concentrations of DNA, ATP and dNTPs are always the same as the standard incubation mixture (see methods).

*100% activity represents 7-10 p moles of ^{3}H TTP incorporated. The amount of activity recovered in the two stage reaction was usually about 50% of the total activity possible for the given amount of DNA and extract.

TABLE II

1st Additions	2nd Additions	Activity (percent)
complete	complete	100%
complete - 10′ at 30°C + 10′ at 4°C	complete	32.0
complete - ϕX174 DNA + fd DNA	complete	<0.1
complete - ϕX174 DNA	complete + ϕX174 DNA	<0.1
complete - ATP	complete +ATP	15.0

The first incubation (7 min. at 30°C) contains all of the necessary components (see methods) for DNA synthesis except dNTPs. After gel filtration, dNTPs and extract are added and incubation is for 20 min. at 30°C. "First Additions" are to the first stage reaction. "Second Additions" are to the second stage reaction.

TABLE III

System		Activity (percent)
Complex	+ *dna*$^{+}$ extract + dNTP at 30°C or 37°C	100%
Complex	+ *dnaG*^{ts} extract* + dNTP at 30°C	34
Complex	+ *dnaG*^{ts} extract + dNTP at 37°C	<0.1
Complex	+ *dnaG*^{ts} extract + 0.18 units *dnaG* protein + dNTP at 37°C	112

* Prepared as described in methods from *Escherichia coli* NY73.

extract added) indicating that the first stage reaction did not just lead to the synthesis of a primer on the ϕX174 DNA. Further evidence that a primer was not synthesized is that the ability of the treated DNA to remain active decreased with a half time at 0°C of one hour.

The formation of "active" DNA in the first stage reaction required incubation of the DNA with ATP and extract (Table II). Furthermore the ability of the input DNA to become active in the second stage was specific for ϕX174 DNA in that substitution of bacteriophage fd DNA in the first stage did not yield an "active" DNA in the second reaction. This result makes unlikely the possibility that a non-specific "activation" (priming, digestion, etc.) was occuring.

The second stage reaction has an absolute requirement for the *dnaG* protein. As shown in Table III, the activity of *dnaG*^{ts} extract in the second stage is temperature sensitive in function when compared to extract prepared from *dna*$^{+}$ cells. All activity can be restored to the *dnaG*^{ts} extract at the non-permissive temperature by the addition of purified *dnaG* protein.

The requirement for the *dnaG* protein is seen only in the second stage, in that formation of "active" DNA is not temperature sensitive when prepared with extracts from *dnaG*^{ts} cells (data not shown).

DISCUSSION

Our results suggest that the conversion of single-stranded ϕX174 DNA to its replicative form requires three distinct reactions. The first involves the association of proteins with the DNA specifically; this complex is labile and decays even at 0°C. The second stage leads to initiation of a new DNA chain (perhaps requiring the dnaG protein) and the third stage is synthesis of a full length complementary strand. Resolution of the system into its components will be required before this hypothesis can be proven.

It has already been shown that the ϕX174 RF promotion can be separated into two stages on the basis of kinetic analysis (1). This study showed that to abolish the lag for DNA synthesis, the dnaB, dnaC(D), X, Y, and Z proteins and DNA binding protein had to be added to the ϕX174 DNA in the presence of ATP. The dnaG protein, DNA polymerase III and factors I and II were not required initially. Thus the first stage proteins appear to "prepare" the DNA to be acted upon by the dnaG protein (perhaps for primer generation) and then the elongation proteins can act. It is not obviously apparent that if the dnaG protein were to "simply" synthesize an RNA primer as reported (5), why it had not acted in the first stage even though it was present. Perhaps the addition of deoxynucleotides are required before priming can take place. These questions will be addressed in subsequent studies.

REFERENCES

1. Wickner, S. and Hurwitz, J. (1974). Proc. Nat. Acad. Sci. USA 71, 4120-4124.
2. Schekman, R., Weiner, J., Weiner, A. and Kornberg, A. (1975). J. Biol. Chem. 250, 5859-5865.
3. Zechel, K., Bouche, J.P. and Kornberg, A. (1975). J. Biol. Chem. 250, 4684-4689.
4. Sherman, L., Ph.D. thesis, Massachusetts Institute of Technology (1976).
5. Bouche, J.P., Zechel, K. and Kornberg, A. (1975). J. Biol. Chem. 250, 5995-6001.
6. Molineux, I.J., Friedman, S. and Gefter, M.L. (1974). J. Biol. Chem. 249, 6090-6098.
7. Wickner, S., Wright, M. and Hurwitz, J. (1973). Proc. Nat. Acad. Sci. USA 70, 1613-1618.
8. Wickner, W., Schekman. R. Geider, K. and Kornberg, A. (1973). Proc. Nat. Acad. Sci. USA 70, 1764-1767.