ANALYSIS OF IN VITRO REPLICATION OF DIFFERENT DNAs

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INTRODUCTION

Though DNA replication in *Escherichia coli* has been intensively studied for nearly two decades, biochemical events involved in this procese are only now beginning to be unraveled.[1] This progress has come from the use of thermosensitive mutants[2] of *E. coli* conditionally blocked in DNA replication in in vitro complementation systems.[3] These point mutations have been mapped on the *E. coli* chromosome and have been named *dna*A, *dna*B, *dna*C etc. There are two classes of genetic mutants which are arbitrarily separated based on their phenotypic effect on DNA synthesis in vivo. One class is involved in initiation (and/or termination) of DNA replication (*dna*A, *dna*C, *dna*H, *dna*I), while the second class is involved in elongation of DNA chains (*dna*B, *dna*G, *dna*E, *dna*Z). The two classes of mutants are distinguished by their ability to finish rounds of DNA synthesis (initiation mutants) or to immediately cease DNA synthesis at nonpermissive temperatures (elongation mutants).[4] The large number of *dna* mutants of *E. coli* clearly indicated the complexity of DNA replication. Though most biochemists were aware of this, attempts to isolate replication proteins were severely hampered by the presence of repair enzymes, in particular, the DNA polymerizing activity of DNA polymerase I. This difficulty was partly eliminated when DeLucia and Cairns[5] isolated an *E. coli* mutant deficient in polymerase activity due to DNA polymerase I. This mutant, which was made available to all biochemists, was rapidly exploited and lead to the isolation of two additional DNA polymerizing activities, DNA polymerase II and DNA polymerase III.[6] The latter DNA polymerase was identified as the *dna*E gene product.[7,8] These observations emphasized that biochemical studies of in vitro DNA synthesis required the use of *E. coli dna* mutants in order to distinguish DNA repair from DNA replication.

A number of partially in vitro/in vivo systems have been developed and studied. These included studies with toluenized *E. coli*,[9] *E. coli* cells gently lysed and deposited on membrane filters[10] and plasmolyzed *E9 coli* cells.[11] These systems advanced our information on replication. They clearly demonstrated that DNA elongation required ATP and was thermolabile with various *dna* ts mutants. The Bonhoeffer technique[10] was used to isolate the *dna*E gene product[8] which was shown to be DNA polymerase III. These systems are of limited usefulness because they are not soluble and responsive to exogenous DNA. These difficulties, however, did not interfere with the isolation and identification of at least six different proteins involved in DNA replication which were coded for by the T4 phage genome.[12,13] The combination of these six different protein fractions replicates a number of different DNAs. A number of highly original mechanisms have been proposed for the role of a number of these proteins in DNA replication. Thus, for example, the complex of T4 DNA coded proteins 44 and 62 catalyze a DNA-dependent ATPase which appears to play an important role in the movement of replication forks during dNMP polymerization. In addition, a number of these proteins play an important role in converting T4 DNA polymerase from a nonprocessive enzyme to a processive one.

The T7 DNA-directed replication system is simpler than the T4 phage system. The isolation of at least four different proteins required for DNA replication was carried out by complementation assays (except for the T7 DNA polymerase). In this system, exogenous T7 DNA is required.[14,15] At present, this system also replicates a number of different DNAs (single-stranded and denatured T7 DNA) as does the T4 system. It is likely that additional factors are required to make these systems more specific. In the T7 system, the priming enzyme has been isolated and characterized. The gene 4 protein acts as a primase as well as a protein which stabilizes small ribooligonucleotides to single-stranded DNA. These are properties similar to those found with the *dna* G protein described below.

Soluble systems from *E. coli* involved in the replication of single-stranded circular DNA and primed DNA templates have been isolated.[16,17,18] The complexity of these systems, which are described in more detail below, varies considerably. Since these systems are derived from *E. coli*, it is assumed that the most complicated system (ϕX174 DNA conversion to ϕX RFII) reflects more realistically the enzymes involved in *E. coli* DNA synthesis at the replication fork.

Nature of Elongation of Primed DNA Templates

The discovery that DNA polymerase III[7,8] (*dna*E gene product) was responsible for DNA synthesis prompted us to examine the different DNA polymerases of *E. coli* for their ability to elongate RNA-primed DNA templates generated *in situ* with *E. coli* RNA polymerase.[19] Under the conditions used, DNA polymerase I and DNA polymerase II readily elongated primed templates (the latter in the presence of *E. coli* binding protein), while DNA polymerase III did not; the addition of small amounts of crude extract from *E. coli* permitted DNApolymerase III to catalyze DNA synthesis. This effect was used as an assay for the isolation of proteins which permitted DNA polymerase III to catalyze dNMP incorporation. Three separate and distinct proteins were required in conjunction with DNA polymerase III for the elongation of primed DNA templates. These were DNA-elongation factor I (EFI), DNA-elongation factor III (EFIII) and the *dna*Z protein.[20] All four proteins have been isolated free of one another; these proteins are required for elongation of a circular DNA template (5,500 to 6,000 nucleotides) containing a small (~60 nucleotides long) RNA primer formed with RNA polymerase (Table 1). The elongation reaction, depending upon the DNA polymerase utilized, required four or five proteins. When poly $dA_{2,000}$ · oligo dT_{12-18} was used in place of ϕX RNA · DNA hybrid, no incorporation of dTMP occurred unless ATP or dATP was present (Table 2).[21,22] The latter requirement was observed with the synthetic polynucleotides because the only dNTP required for DNA synthesis under these conditions is dTTP. In contrast, when ϕX DNA · RNA hybrids were used, all four dNTPs were required masking the role of ATP or dATP in the elongation reaction. No evidence of incorporation of ATP or dATP into acid-insoluble material was found with the synthetic polynucleotide system (Table 2). The *E. coli* binding protein was not required for DNA polymerase III catalyzed DNA synthesis; it was essential, however, for DNA polymerase II activity. Spermidine, at the concentration indicated, inhibited reactions catalyzed by both DNA polyoerases.

The mechanism of the elongation reaction has been examined by Wickner.[22] As summarized in Figure 1, the *dna*Z protein interacts with DNA EFIII to form a complex. The *dna*Z · EFIII complex in the presence of ATP (or dATP), DNA EFI and a primed template catalyzes the formation of a DNA EFI-primed DNA template complex. This isolated DNA complex interacts with DNA polymerase III (in the absence of ATP) to form a complex containing DNA polymerase III and DNA EFI which, in the presence of dTTP, elongates the oligo dT primer yielding long poly dT chains. This series of

TABLE 1

Requirements for DNA Elongation of Primed SS DNA

Additions	DNA polymerase used	
	III	II
	dTMP incorporated pmol/20 min	
Complete	28.3	37.4
−DNA elongation factor I	0.3	<0.2
−DNA elongation factor III	0.3	<0.2
− *dna* Z gene product	0.3	0.2
−DNA polymerase	0.2	<0.2
− *E. coli* binding protein	—	0.7
+ Spermidine (3 m*M*)	9.4	7.9

Note: In the above experiments, *E. coli* binding protein was omitted in the complete system used for DNA polymerase III since it inhibited dNMP incorporation in contrast to its effect on DNA polymerase II.

From Wickner, S. and Hurwitz, J., *Proc. Natl. Acad. Sci. U.S.A.*, 73, 1053, 1976. With permission.

TABLE 2

Requirement for ATP or dATP with Poly $dA_{2000} \cdot dT_{12-16}$

Additions	dTMP incorporated (pmol/20 min)
Complete	22.7
Omit oligo dT_{16}	0.8
Omit ATP	0.7
Omit ATP + dATP	24.2
Omit EFI, or *dna* Z, or EFIII	1.2—2.7
Omit DNA polymerase III	0.3

Note: The conditions used here were as described[20-21] with the exception that poly $dA_{2000} \cdot$ oligo dT_{12-16} was used in place of $\phi X \cdot$ DNA $\cdot$ RNA hybrid and 10 μM ATP or dATP was added; [^{3}H]dTTP (480 cpm/pmol) was the only other nucleotide added.

reactions occurs only in the presence of a primed template; when oligo dT is omitted, no complex is formed.

How ATP (or dATP) participates in the *dnaZ*·DNA EFIII catalyzed transfer of DNA EFI to primed DNA template is not clear. Unfortunately, the purified proteins required in this system contained DNA-dependent ATPase activity. The sum of ATP hydrolyzed by each enzyme fraction alone was equivalent to the amount of ATP hydrolyzed when all proteins were combined. No labeled ATP, ADP, or AMP was detected in any of the complexes isolated as described in Figure 1.

Studies in Kornberg's laboratory [23] suggest that DNA polymerase III is present in a complex consisting of at least four different subunits. They have called these subunits α, β, γ, and δ which have molecular weights in sodium dodecyl sulfate polyacrylamide electrophoresis of 140,000, 40,000, 52,000, and 32,000 daltons, respectively. The molecular weights of the above subunits agree with those we have observed; DNA pol-

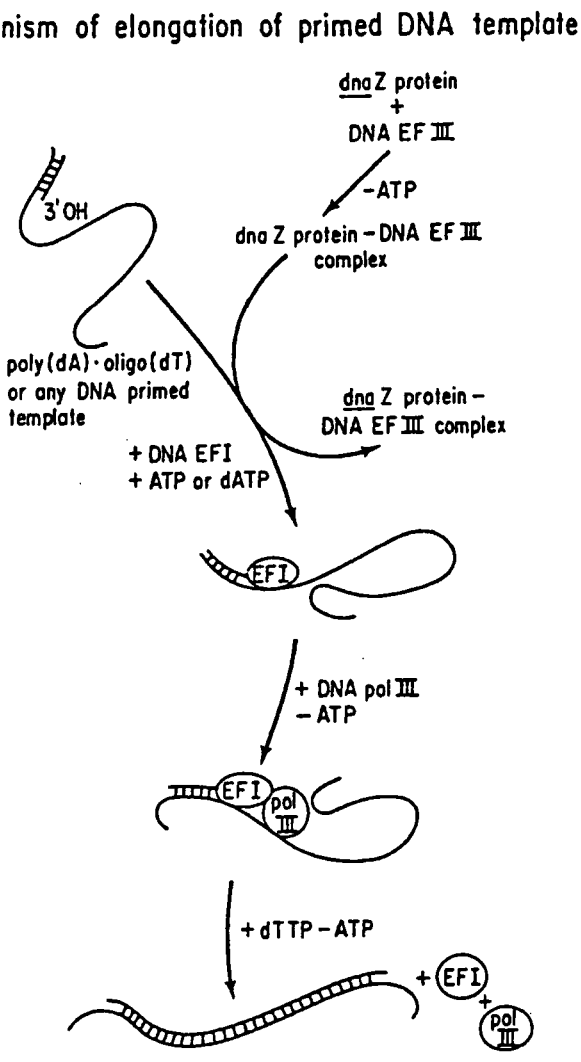

FIGURE 1. Mechanism of elongation of primed DNA template. All products described here were isolated and characterized. (From Wickner, S., *Proc. Natl. Acad. Sci. U.S.A.*, 73, 3511, 1976.)

ymerase III, DNA EFI, and *dnaZ*[42] appear to be similar to the subunits α, β, and γ. The subunit structure of DNA EFIII is unknown. The molecular weight of the native protein is 63,000 daltons.[22] It is possible that the subunit structure of DNA EFIII is 32,000 daltons, a value similar to the subunit δ of the DNA polymerase III holoenzyme.

Our studies,[24] as well as those of Scheckman et al.,[17] indicate that the elongation reaction, as described above, is a general reaction and occurs with a wide variety of primed DNA templates. This is true when the concentration of primer ends is low and the template strand long. The general conditions used to isolate DNA polymerases involve the use of high concentrations of primer ends generated with pancreatic DNAse. With this type of DNA substrate, the DNA elongation proteins marginally stimulate the *E. coli* DNA polymerases (I, II and III).

In contrast to the general role of the DNA elongation proteins, synthesis of a primer terminus is complex and varies depending on the DNA used. To date, three different

mechanisms have been observed in generating primer ends.[17,22,25] These three different mechanisms of forming primed DNA templates do not depend on any virus-coded proteins. All the enzymes involved in these systems are coded for by the genome of *E. coli.*

fd Pathway of RFII DNA Synthesis

The synthesis of fd RFII DNA from single-stranded circular fd (or M13) DNA required DNA-dependent RNA polymerase activity for the generation of a primed fd DNA template. This observation can be made in vivo as well as in vitro.[19,26,27] The primed single-stranded circular DNA is converted to the RFII form by action of the DNA elongation proteins — DNA polymerase III, *dnaZ*, DNA EFI, and DNA EFIII. There is a high degree of specificity in the site at which the RNA primer is formed. This specificity is maintained by the action of a number of DNA binding proteins as discussed below (discrimination reaction).

The G4 (ST-1 or α3) Pathway of RFII DNA Synthesis

Another pathway for RFII formation is observed with G4 DNA[25] as well as other single-stranded circular DNAs isolated from viruses such as ϕX + b, ST-1[28] and α3.[29,30] In this pathway, the specific viral DNA, the *dnaG* protein, *E. coli* binding protein, DNA polymerase III, *dnaZ*, DNA EFI, and DNA EFIII are required. The nucleotide requirement for DNA RFII formation depends upon the DNA used. Zechel et al.[25] and Rowen et al.[30] showed that the *E. coli dnaG* protein (which they have called primase) catalyzed a rifampicin-resistant formation of a small polyribonucleotide using G4 DNA as a template. Wickner[28] confirmed these observations and showed that the *dnaG* protein catalyzes the incorporation of ribo- and deoxynucleotides *de novo* yielding short primer strands. In this system rNTPs and dNTPs could be used interchangeably (Table 3). Surprisingly, with G4 DNA as the template, a specific requirement for ADP was found; this requirement could be satisfied by high concentrations of ATP (∼ 100-fold greater than ADP). Unequivocal evidence that the nucleotide incorporating activity is associated with the *dnaG* complementing activity was obtained. The *dnaG* protein isolated from a thermolabile *dnaG* mutant was more thermolabile than the wildtype *dnaG*protein in its ability to incorporate nucleotides, as well as its ability to act in the ϕX RFII complementation assay.[28] In addition, the ribo- and deoxynucleotide incorporating activity copurified with the role of the *dnaG* protein in ϕX RFII formation.[30] The *dnaG* protein catalyzed synthesis of deoxyoligonucleotides or ribooligonucleotides on single-stranded circular DNA could serve as primers for the elongation reactions catalyzed by DNA polymerase I and T4 DNA polymerase in the absence of DNA EFI, DNA EFIII, and *dnaZ*. The mechanism of generation of the oligonucleotide primer on G4 or ST-1 DNA was studied[28,30] and is summarized in Figure 2. The first requirement is the attachment of *E. coli* binding protein to G4 DNA. This presumably accentuates a specific site on the DNA protein complex to which *dnaG* binds; this complex is capable of synthesizing an oligonucleotide in a reaction requiring ADP, TTP (or UTP), and dGTP (or GTP). The oligonucleotide is then elongated by DNA polymerase III (or II) in combination with elongation proteins, *dnaZ* protein, DNA EFI, and DNA EFIII. The DNA elongation proteins can be replaced by DNA polymerase I or T4 DNA polymerase. As shown in Figure 2, the protein-DNA complexes have been isolated. Additional evidence suggests that *dnaG* is probably not required after priming; the elongation reactions occurs in the absence of added *dnaG* protein.

ϕX174 DNA Pathway of RFII Formation

A more complicated pathway occurs in forming ϕX RFII from ϕX174 DNA. The requirements in this system include the proteins coded for by the *dna*B, *dna*C(D), *dna*E (DNA polymerase III), *dna*G, and the *dna*Z loci; proteins also essential (lacking genetic mutants) include *E. coli* binding protein, replication factors X, Y, and Z, and DNA EFI, and DNA EFII. In our laboratory, ATP and the four dNTPs are required,[24] while the studies by Scheckman et al.[17] suggested that all four rNTP are required. The requirements reported by the Kornberg group for the same reaction include the proteins coded for by *dna*B, *dna*C, *dna*G, DNA polymerase holoenzyme (*dna*Z, DNA EFI, DNA EFIII, and DNA polymerase III), the *E. coli* binding protein, and two proteins which they have called proteins i and n.[17] It is not clear whether these two proteins correspond to the three replication factors isolated by Wickner and Hurwitz[24] which also lack genetic definition. The temporal order of involvement of the proteins required in the ϕX pathway has been carried out by Wickner and Hurwitz[24] and by Scheckman et al.[17] (Figure 3). For reasons presently not clear, a large number of proteins are required before the *dna*G protein can act on ϕX174 DNA. The interaction between DNA and proteins as well as protein·protein interactions have been observed by direct filtration on bio-gel columns; proteins complexed to DNA were isolated in the excluded volume and measured. The initial reaction involves the binding of some of the proteins to ϕX174 DNA.[24] It has been found that the *E. coli* binding protein and replication factors Y and Z bind to DNA. The binding of these proteins to ϕX174 DNA is nonspecific since fd DNA can also bind these components (Wickner, unpublished results)[34] even though replication factors Y and Z play no role in formation of fd RFII DNA. Chronologically, the next step involves the addition of the *dna*B protein to the complex. This reaction is dependent on the *dna*C protein, replication factor X, and ATP. A complex contaning equimolar amounts of the *dna*B protein and *dna*C protein is formed in the presence of ATP^{32} (and no other nucleotide). Presumably, the *dna* B·*dna*C protein complex in the presence of ATP and replication factor X results in a transfer of the *dna*B protein to the ϕX DNA protein complex. The proteins *dna*C and replication factor X were not detected in the complex[32,33] and were recovered in the included volume. The binding of the *dna*B protein to the ϕX DNA protein complex is specific; neither fd DNA or ST-1 DNA binds the *dna*B protein in the presence of the above-indicated proteins. The ϕX174 protein complex containing the *dna*B protein supports DNA synthesis when added to the *dna*G protein, the elongation proteins, and dNTPs. All of the complex products described in Figure 3 have been isolated except for those bracketed. It is likely that the *dna*G protein acts as a specific chain initiator recognizing the protein·ϕX174 DNA complex as it does in the less complicated systems, such as G4 or α3 DNA. Once a short oligonucleotide is formed, the DNA elongation system catalyzes extensive DNA synthesis.

A summary of proteins and nucleotides required in the four different systems described above is presented in Table 4. As emphasized above, the minimal requirements for nucleotides and proteins leading to primed DNA templates differ. In contrast, the elongation reaction is identical in all systems examined to date.

Discrimination Reaction

In vivo and in vitro it was shown that DNA-dependent RNA polymerase was essential for the replication of fd or M13 DNA. In contrast to this, ϕX174 and G4 DNAs are replicated by pathways independent of DNA-dependent RNA polymerase. With purified proteins (RNA polymerase, DNA polymerase III, *dna*Z, DNA EFI, and DNA EFIII), fd and ϕX174 single stranded circular DNAs readily supported RFII formation.[34] Thus, on purification, the RNA polymerase-coupled DNA synthesis system had

TABLE 3

Requirements for G4 and ST-1 DNA-Dependent DNA Synthesis

Additions	TMP incorporated (pmol/20 min) G4 DNA	ST-1 DNA
Complete	25.2	25.0
−*dna*G protein	<0.1	<0.1
−DNA binding protein	0.3	0.3
−DNA EFI	0.5	0.6
−DNA EFIII	0.6	0.2
−*dna*Z protein	0.5	0.2
−DNA polymerase III	0.4	0.4
−ADP	1.9	5.0
−ADP + other nucleotides	7.7—3.1	—

Note: Reaction mixtures were as described by Wickner.[28] Where indicated, the nucleotides substituted for ADP at a concentration of 20 μM were: all rNTPs, 3 other rNDPs, 4 dNTPs, 4 dNDPs, AMP, and α,β-methylene-ADP.

lost its specificity (Table 5). However, addition of crude extracts obtained from a *dna*C mutant strain of *E. coli* to the purified system resulted in a marked inhibition of ϕX174 DNA synthesis without affecting fd RFII formation.[35,36] These results indicated that factors essential for the discrimination between fd and ϕX viral DNAs were lost during purification of the elongation enzymes.[34,37] The discrimination reaction (selective inhibition of ϕX RFII formation in an RNA polymerase-dependent system) was used as an assay for the isolation of protein factors.[35,36] As shown in Table 6, in addition to RNA polymerase, the DNA elongation system, *E. coli* binding protein and RNase H, two other proteins, discriminatory factors α and β, were required for selective conversion of fd viral DNA to RFII without ϕX RFII formation from ϕX174 DNA. The combination of these proteins leads to the formation of RNA hydrogen-bonded to fd viral DNA; in contrast, no ϕX174 DNA·RNA hybrid was formed. The fd RFII formed under discriminatory conditions contains a unique gap. The site of initiation of fd DNA synthesis in vivo is similar to the site of DNA synthesis in vitro.[38]

In the presence of RNase H, *E. coli* binding protein, RNA polymerase, and the DNA elongation system, both fd and ϕX174 DNA do not support DNA synthesis; it was shown that under these conditions, RNA polymerase does not lead to detectable RNA synthesis. The addition of the two discriminatory proteins α and β in the presence of RNAse H and *E. coli* binding protein selectively leads to RNA hybridized to DNA only with fd DNA and not with ϕX174 DNA. Thus, the discriminatory proteins are positive effectors since they turn on the synthesis of fd RFII.

As shown in Figure 4, RNA polymerase (plus four rNTPs) forms small RNA chains on fd and ϕX single-stranded circular DNA even in the presence of *E. coli* binding protein. Such primed templates yield DNA FII structures heterogeneous in size. The addition of RNAse H (which only attacks RNA in RNA·DNA hybrid structures) prior to the addition of the DNA elongation proteins does not lead to DNA synthesis due to the removal of 3′-hydroxyl ends. These reactions show no specificity since ϕX and fd DNA behave similarly. In the presence of RNA polymerase, RNAse H, discriminatory factors α and β, RNA-primed DNA is only formed with fd DNA and not with

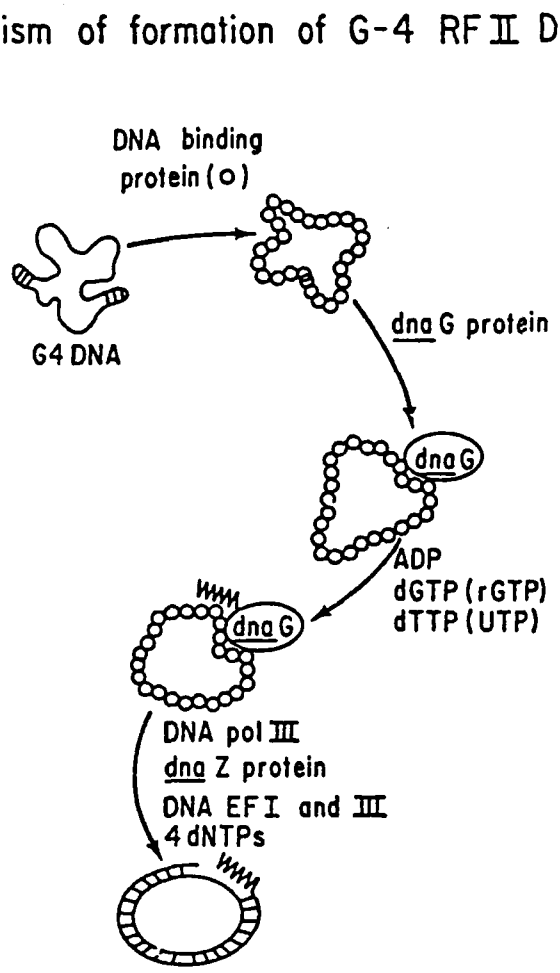

FIGURE 2. Mechanism of synthesis of G-4 RFII DNA. All products presented here were isolated by gel filtration. (From Wickner, S., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 2815, 1977.)

ϕX DNA. Under these conditions no DNA synthesis is observed in the presence of the DNA elongation system with ϕX174 DNA; in contrast, fd primed DNA readily supports DNA synthesis yielding a homogeneous negative strand containing a unique gap.

Similar studies were carried out with ST-1 DNA. In contrast to the requirements observed for selective synthesis of fd RFII, *E. coli* binding protein quantitatively prevented RNA polymerase from forming stable RNA · DNA hybrids with ST-1 DNA.

Nature of Reactions Catalyzed by Various Proteins

The complementation assay selects for protein required for DNA synthesis but does not define how these proteins act. In addition to this shortcoming, the complementation assay, if carried out properly, selects for the thermosensitive protein. If this is a subunit of an enzyme containing multiple subunits which differ, the protein isolated could lack enzymatic activity due to the loss of thermoresistant subunits during purification. Cognizant of these limitations, we have attempted to define enzymatic activities for each of the proteins isolated which are independent of DNA synthesis. If indeed a particular activity is detected, further verification is necessary to relate the mutiple activities. It is essential, if feasible, to compare activities of proteins isolated

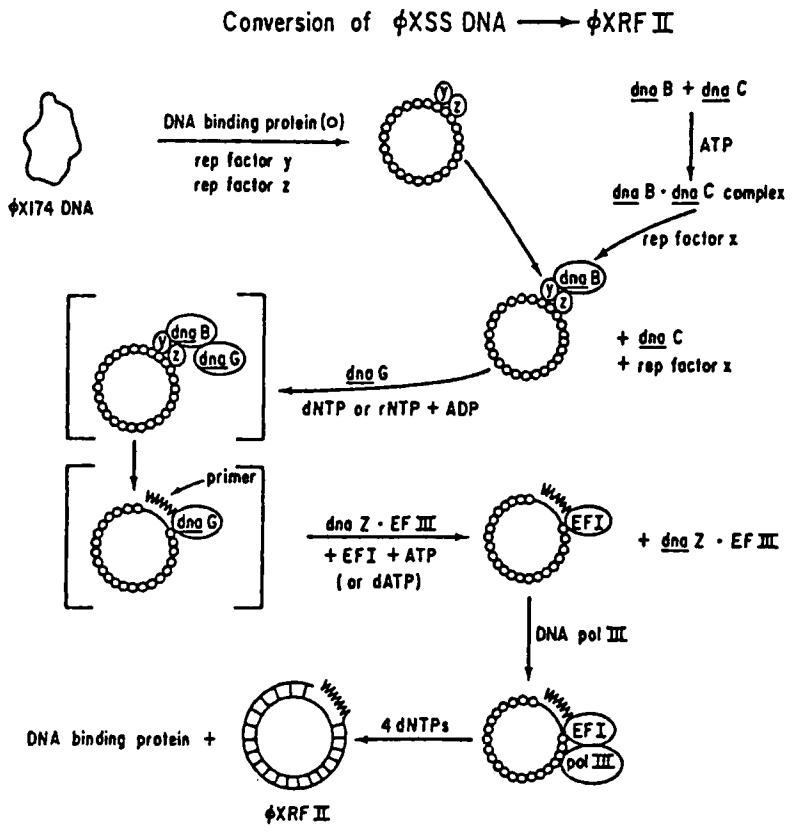

FIGURE 3. DNA protein complexes formed in the conversion of ϕX174 DNA to ϕX RFII DNA. The complexes bracketed have not been isolated; the others were isolated by gel filtration. These results have been summarized previously.[24,33]

from wild type extracts and thermosensitive mutants. The thermosensitive protein must be more thermolabile than the wild type protein. This we have done with a number of isolated proteins for which specific mutants are available. A number of the proteins have been shown to catalyze or participate in reactions independent of their role in DNA synthesis. A summary of these properties is presented in Table 7. A summary of the role a number of these proteins play in replication of single-stranded DNAs is presented in Figure 5.

STUDIES ON THE REPLICATION OF ϕX RFI DNA

The replication of ϕX174 DNA has been extensively studied in vivo. The cycle can be divided into three steps: (1) the conversion of the entering viral single-stranded circular DNA to a duplex circular replicative form (SS → RF), (2) duplication of the RF form to produce progeny RF molecules (RF → RF), and (3) the asymmetric synthesis of viral progeny single-stranded DNA (RF → SS).[39]

As described above, during the first stage, SS → RF, no new proteins other than those coded for by the host are required, and this system has been reconstructed in vitro with purified proteins.

The second step in the replicative cycle, RF → RF is dependent on only one viral gene product, the gene A protein which was shown in vivo[40,41] to introduce a site-specific cleavage of ϕX RFI. In vivo, in the absence of the ϕX gene A protein, ϕX174

TABLE 4

Requirements for in Vitro DNA-Dependent DNA Synthesizing Systems

Factors required	ϕX174 ϕXahb	G4 ST-1 ϕXtb α-3	fd M1 3	Primed SS DNA
Nucleotides				
Specific DNA	+	+	+	−
ADP	?	+	−	−
ATP	+	?	+	ATP or dATP
UTP, CTP, GTP	−	−	+	−
dATP, dCTP, dGTP, dTTP	+	+	+	+
Initiation reaction				
*dna*B, *dna*C(D) gene products, replication factors X, Y, and Z	+	−	−	−
*dna*G gene product	+	+	−	−
RNA polymerase	−	−	+	−
DNA binding protein	+	+	+	−
Elongation reaction				
DNA polymerase III	+	+	+	+
DNA elongation factor I	+	+	+	+
dna Z gene product	+	+	+	+
DNA elongation factor III	+	+	+	+

Note: +, yes; −, no; ?, unsure.

TABLE 5

fd and ϕX174 Viral DNA-dependent DNA Synthesis with Purified Proteins: Lack of Discrimination

Additions	dTMP incorporated with fd viral DNA (pmol/15 min)	dTMP incorporated with ϕX174 DNA (pmol/15 min)
Complete system	24.6	19.6
Omit RNA polymerase, or DNA EFI, or *dna*Z + DNA EFIII, or Pol III	<0.5	<0.5
Omit *E. coli* binding protein	10.2	9.9
Omit spermidine and *E. coli* binding protein	9.4	7.9
Complete + rifampicin (10 μg/m*l*)	1.8	1.1
Complete + crude fraction of *E. coli dna*C ts cells (3 μg)	21.4	18.3
Complete + crude fraction of *E. coli dna*C ts cells (30 μg)	31.0	1.6

Note: Reaction mixtures were as described previously.[35,36] The complete system included the proteins *E. coli* binding protein, DNA elongation factor I, DNA elongation factor II preparation (a mixture of DNA EFIII and *dna*Z), DNA polymerase III, and RNA polymerase. In the above experiments crude fractions from *dna*C ts mutants were used. Such fractions are inactive in supporting ϕX RFII synthesis in the absence of *dna*C gene product.

single-stranded DNA is converted to the closed supercoiled RFI which then accumulates.[42,45] In addition, Denhardt et al.[46] identified a host gene (*rep* gene) essential for the further replication of RF structures. In its absence, ϕX DNA replication proceeds no further than the first stage, SS → RF.

TABLE 6

Factors Required for Discrimination between fd Viral and ϕX174 DNA-Dependent DNA Synthesis

Additions	dTMP incorporated with fd viral DNA (pmol/15 min)	dTMP incorporated with ϕX174 DNA (pmol/15 min)
Complete	22.4	1.3
Complete omit RNA polymerase	0.2	<0.2
Complete omit *E. coli* binding protein	6.3	5.8
Complete omit RNAse H	36.5	18.6
Complete omit discriminatory factor α	4.8	<0.2
Complete omit discriminatory factor β	18.1	7.6
Complete omit discriminatory factors α and β	1.8	<0.2
Complete omit RNAse H, discriminatory factor α and discriminatory factor β	21.3	22.0
Complete + rifampicin (10 μg/ml)	0.8	0.7

Note: The complete mixture[35,36] included *E. coli* DNA binding protein, DNA elongation factor I, DNA elongation factor II preparation, DNA polymerase III, RNase H, discriminatory factor α, discriminatory factor β (Fraction VII), *E. coli* tRNA, RNA polymerase, and either fd viral DNA, or ϕX174 DNA, as indicated. The DNA was added last in all experiments and acid-insoluble material was measured.

The final stage of ϕX replication, the synthesis of progeny viral strands, occurs via a mechanism dependent on both the genes coded for by the ϕX genome and on the other proteins essential for generating RF structures.

We[47,48] and others[44,49,50] have isolated cell-free extracts from *E. coli* which catalyzes RF → RF and RF → SS DNA synthesis.

Studies on RF → RF Replication in Vitro

From in vivo studies (for a general review, see Denhardt[51]), it was known that progeny RF and/or progeny ϕX174 DNA synthesis depended upon the proteins coded by the *E. coli dna*B, *dna*C(D), *dna*E, *dna*G, *dna*Z and the *rep* gene loci in addition to the ϕX174 gene A product. Cell-free preparations of uninfected *E. coli* which catalyzed the conversion of single-stranded ϕX174 DNA to RFII contained a number of the above proteins. Extracts derived from ϕX174-infected *E. coli* (Fraction II) were used to supply additional proteins required for RF → RF replication as well as the ϕX coded gene A protein. In the presence of these two fractions, externally added ϕX RFI readily supported DNA synthesis (Table 8). Similar observations have been reported by Eisenberg et al.[44] The reaction was dependent on the two protein fractions, ATP, Mg^{++}, and the 4 dNTPs, and under suitable conditions net synthesis of ϕXRF products was obtained. ϕX RFI DNA-dependent synthesis was inhibited by *N*-ethylmaleimide, nalidixic acid, and novobiochin and was insensitive to rifampicin. A large number of other duplex DNA templates could not replace ϕX RFI in this system (colicin E, DNA, PM2 DNA, ϕX RFII DNA isolated from preparations of ϕX RFI DNA, and T3 DNA).

Involvement of *E. coli* Proteins and the ϕX Gene A Protein in RF → RF Replication

It has been established in vivo that ϕX RF replication depends upon a number of the *E. coli dna* gene products in addition to the *rep* gene product and the ϕX A gene product. Utilizing extracts of *E. coli dna* ts mutants as a source of enzyme fractions, it was possible to demonstrate the requirement for a number of these proteins in the

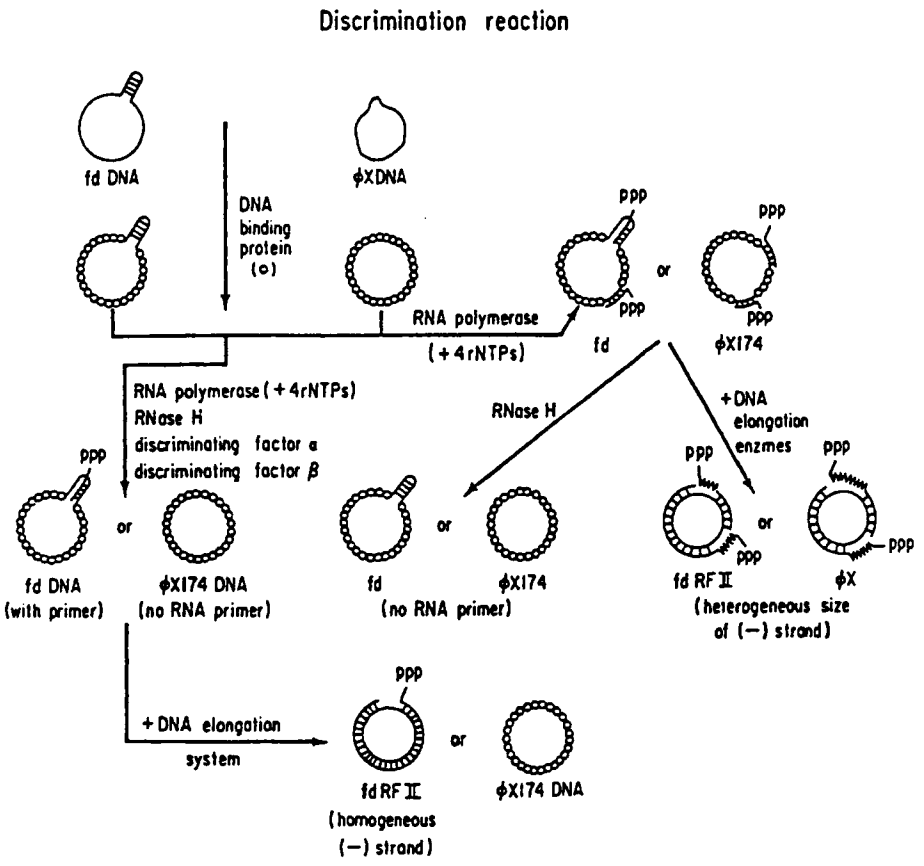

FIGURE 4. Reactions leading to the discrimination of ØX174 DNA and fd DNA. All the products described above have been isolated.

in vitro RF → RF system (Table 9). Mutants thermosensitive for *dna*C(D) gene product were unable to support RF replication even without being heat-treated. This is due to the marked lability of the *dna*C(D) protein. The addition of purified *dna*C(D) protein readily restored RF replication; in contrast, the addition of other purified *E. coli* gene products to the *dna*C ts extract (*dna*B or *dna*G) did not support RF replication. The requirements for *dna*B and *dna*G were only observed after heat treatment of their respective extracts prepared from thermosensitive mutants which inactivated the thermolabile protein. The addition of purified proteins *dna*B and *dna*G to heat-inactivated extracts stimulated RF replication approximately fivefold. For unknown reasons, the requirements for these components in the RF replication system were substantially lower than those required for conversion of ϕX174 DNA to RFII (except for the *dna*C(D) protein). The requirement for the *dna*E gene product (DNA polymerase III) was marginal. Additional of purified DNA polymerase III to heat-treated extracts from two different *dna*E ts mutants (*E. coli* BT 1026 and BT 1040) maximally stimulated RF replication only twofold. Similar difficulties were observed with the use of such extracts for the conversion of ϕX174 DNA to RFII.

Protein fractions derived from the *E. coli* strain D92 (*rep*⁻) were inactive in RF replication but were fully active in catalyzing ϕX174 DNA conversion to RFII. The combination of extracts derived from *rep*⁺ strains with those from *rep*⁻ strains readily stimulated RF replication. Protein fractions containing the ϕX gene A product were essential for any ϕX RFI-dependent dNMP incorporation. Infection of *E. coli* with

TABLE 7

Summary of Properties of Proteins Involved in SS to RFII Systems

Proteins involved in DNA synthesis	Molecular weight	Subunit structure (if known)	NEM sensitivity	Reaction independent of DNA replication
dna B	280,000	6-48,000	Resistant	DNA-dependent rNTPase
dna C	30,000	1-30,000	Sensitive	Interacts with *dna* B in presence of ATP
dna G	64,000	1-64,000	Resistant	Catalyzes DNA-dependent incorporation of nucleotides
dna E (Pol III)	180,000	1-140,000 1-$5,000 (?)	Sensitive	Polymerization of deoxynucleotide
dna Z	120,000	2-55,000	Sensitive	Interacts with DNA EFIII
DNA EFI	40,000	1-40,000	Resistant	Transferred to primed-template in DNA elongation reaction in presence of ATP (dATP) and *dna* Z·EFIII complex
DNA EFIII	63,000	—	Sensitive	Reacts with *dna* Z to form *dna* Z·EFIII complex
E. coli binding protein	80,000	4-20,000	Resistant	1. Binds to single-stranded DNA 2. Required for *dna* G catalyzed ribo- or deoxyoligonucleotide formation 3. Discrimination reaction
Replication factor X	45,000	1-45,000	Resistant	Involved in transfer *dna* B to ϕX174 DNA protein complex
Replication factor Y	55,000	?	Sensitive	DNA-dependent ATP or dATPase; works best with DNAs which require this protein for DNA synthesis; binds to DNA (nonspecific)
Replication factor Z	25,000	?	Sensitive	Binds to DNA (nonspecific)
E. coli RNA polymerase	480,000	$\alpha_2\beta,\beta',\sigma$	Sensitive	Selective formation of fd DNA - RNA hybrid
RNAse H	35,000	?	Sensitive	Removes RNA from RNA DNA hybrid; involved in discrimination
Discriminatory factor α	50,000	?	Sensitive	Involved in selective formation of fd DNA·RNA hybrid
Discriminatory factor β	20,000	1-20,000	Resistant	Involved in selective formation of fd DNA·RNA hybrid

phage mutants which were ϕX A⁻ (ϕX H90 or ϕX N14) yielded extracts unable to support RF replication. The addition of purified ϕX A protein to such reaction mixtures caused a rapid synthesis of DNA.

Characterization of the ϕX Progeny RF

The DNA synthesized in vitro was analyzed by zonal sedimentation in neutral and alkaline sucrose. In neutral gradients, the product sedimented as expected of ϕX RFI and ϕX RFII DNA but the amount of each varied. Early in the reaction, RFII predominated while late in the reaction RFI predominated. Similar results were observed in alkaline sucrose gradient centrifugation. These observations suggest that the initial product of the reaction is ϕX RFII which is then convered to ϕX RFI. The newly formed ϕX RFI possessed the same superhelical density as that of template ϕX RFI as measured by isopycnic banding in propidium diiodide-neutral CsCl.

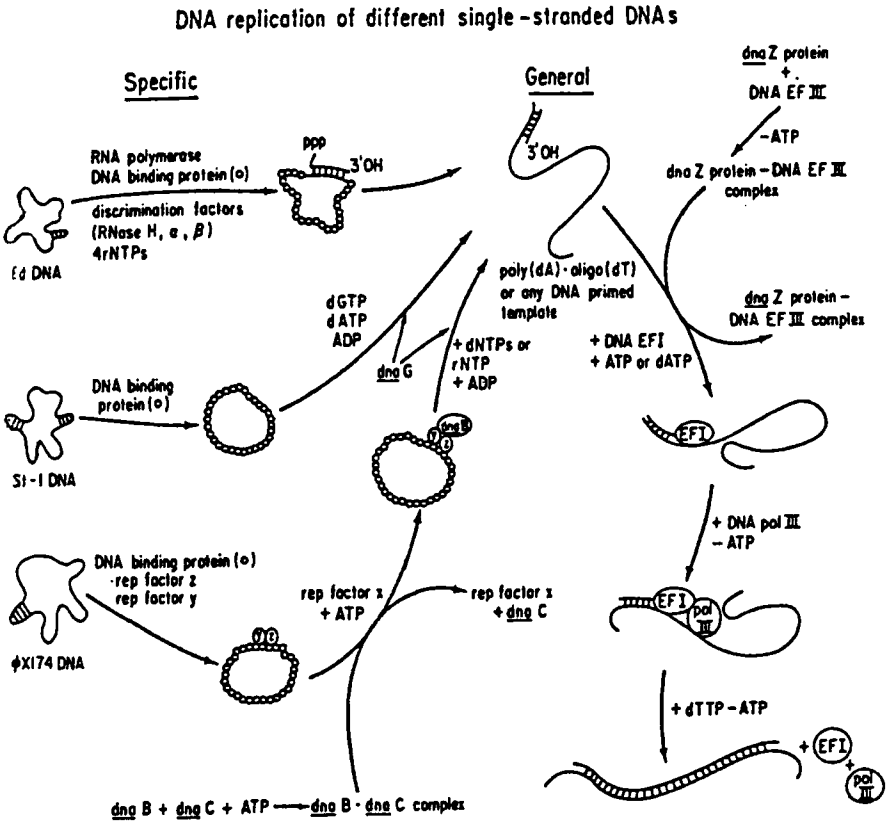

FIGURE 5. General summary of reactions leading to priming of SS DNAs and the elongation of med DNA complexes.

TABLE 8

Requirements for Synthesis of φX174 Progeny RF in Vitro

Additions	Incorporation of dTMP (pmol/40 min)
Complete	374
Omit φX RFI	21
Omit ammonium sulfate fraction	6
Omit ATP, or omit Mg^{++}, or omit dATP, dGTP, dCTP	2—4
Complete + *N*-ethylmaleimide (4 m*M*)	3
Complete + rifampicin (10 μg/m*l*)	400
Complete + nalidixic acid (250 μg/m*l*)	41
Complete + novobiocin (240 μg/m*l*)	38

Note: The conditions for the synthesis of DNA were as described by Sumida-Yasumoto et al.[47]

It was possible to show that RFI replication occurred semiconservatively. For this purpose, DNA was synthesized in vitro with [α^{32}P] dATP and 5-bromodeoxyuridine 5′-triphosphate (BrdUTP) in place of dTTP and the product was subjected to neutral sucrose gradient and CsCl equilibrium gradient centrifugation (Figure 6A). Approximately 20 and 80% of the BrdU-labeled DNA sedimented in neutral sucrose as RFI

TABLE 9

Involvement of *E. coli* Proteins and ϕX Gene A Product in ϕX RF Synthesis

Source of *E. coli* extract			Incorporation of dTMP (pmol/40 min)	
Fraction II and phage used for infection	Ammonium sulfate fraction (uninfected0	Purified gene product added	Without heat	After heat treatment
*dna*B ts (ϕXwt)	*dna*B ts	None	106	41
*dna*B ts (ϕXwt)	*dna*B ts	*dna*B	192	219
wt_1 (ϕX*am*3)	*dna*C ts	None	28	—
wt_1 (ϕX*am*3)	*dna*C ts	*dna*C	568	—
*dna*G ts (ϕXwt)	*dna*G ts	None	461	57
*dna*G ts (ϕXwt)	*dna*G ts	*dna*G	460	250
rep⁻ (ϕXwt)	*rep*⁻	None	5	—
rep⁻ (ϕXwt)	*rep*⁺	None	207	—
wt_2 (ϕXwt)	wt_2	None	121	—
wt_2 (ϕXH90)	wt_2	None	9	—
wt_2 (ϕXH90)	wt_2	ϕX gene A	120	—

Note: The *E. coli* strains used corresponding to the various ts mutants described above were the following: *E. coli* BT1029 (*dna*B ts); *E. coli* LD332 (*dna*C ts); *E. coli* NY73 ϕX' (*dna* G ts), *E. coli* H560 (wild-type (wt_1)), *E. coli* D92 (*rep*⁻), *E. coli* 514 (wild-type [wt_2]). The conditions used for the isolation of various extracts and additions were as described by Sumida-Yasumoto et al.[47]

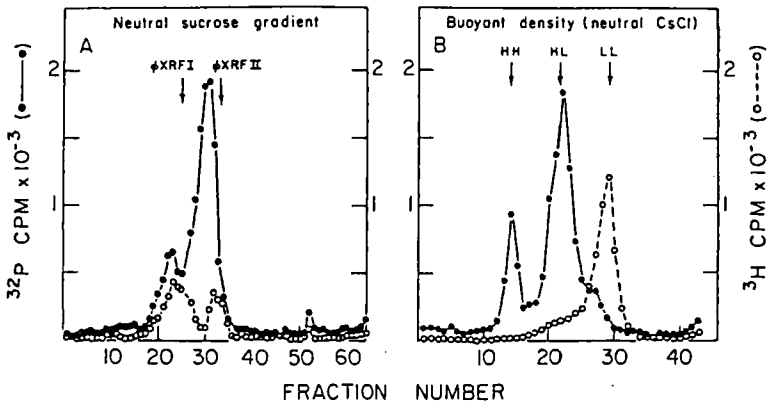

FIGURE 6. Neutral sucrose gradient and buoyant density sedimentation analyses of BrUrd-labeled ϕX DNA synthesized in vitro. DNA was synthesized with [α-^{32}] dATP (400 cpm/pmol), ϕX [^{3}H] RFI (6 cpm/pmol), 40μM BrdUTP (in place of dTTP), and fraction II (54 μg), prepared as described. Fractions were collected from the bottom of the tube, and acid-insoluble material was measured. Arrows indicate the positions expected for fully heavy (H·H), half-heavy (H·L), and completely light (L·L) ϕX RFI DNA. (From Sumida-Yasumoto, C., Yudelevich, A., and Hurwitz, J., *Proc. Natl. Acad. Sci. U.S.A.*, 73, 1887, 1976.)

and RFII forms, respecitvely. These products sedimented slightly faster than RFI and RFII containing only dTMP; in general, more RFII than RFI was always observed in products of density-labeled DNA compared to products formed with dTMP. When analyzed by neutral CsCl density centrifugation (Figure 6B) over 65% of the newly synthesized [^{32}P] DNA and 20% of the template [^{3}H] DNA banded at the density of

hybrid molecules (H·L). Approximately 20% of the ^{32}P-labeled product banded as expected of fully heavy (H·H) DNA molecules. In other experiments, products subjected to isopycnic banding in propidium diiodide-neutral CsCl, both RFI and RFII forms were mixtures of H·H and H·L forms.

ϕX RFI-Dependent Synthesis of ϕX174 Viral DNA in Vitro

The third step in the replication cycle of ϕX DNA in vivo has been shown to depend upon a number of *E. coli* host proteins plus all of the ϕX DNA coded proteins with the exception of the ϕX gene E protein.[45] In vitro, studies from Hayashi's laboratory[49] have shown that gently lysed *E. coli* cells infected with ϕX phage synthesized viral single-stranded DNA specifically without externally added ϕX RFI DNA. They demonstrated that the incorporation of radioactive dTTP yielded discrete DNA·protein complexes. No evidence for free single-stranded circular DNA formation was obtained; all progeny viral strand formation occurred via synthesis in protein-nucleic acid complexes. Similar results were obtained in vivo.[50]

In our studies, extracts freed of DNA by DEAE-cellulose treatment were prepared from *E. coli* infected with ϕX174 phage late in the infection cycle. The purpose of this was to accumulate large amounts of phage-coded proteins. These fractions were supplemented with ammonium sulfate fractions from uninfected *E. coli* as well as the purified *dnaC* gene product. As in the case of RF → RF replication, the synthesis of SS DNA (more correctly viral strand) depended on the presence of RFI, the two protein fractions, ATP, Mg^{++}, and the four dNTPs. It was inhibited by *N*-ethylmaleimide, nalidixic acid, and novobiocin but was unaffected by rifampicin (Table 10). The major difference between the Fraction II extracts used for RF → RF and RF → SS replication was the time of infection; the former was isolated from cells infected with wild type phage for 25 min, while the latter was isolated from cells with ϕX *am*3 for 50 min at 30°C.

A comparision of the *E. coli* gene products required for RF and those required for SS DNA synthesis is shown in Table 11. RF → RF synthesis was measured utilizing ammonium sulfate fractions from uninfected *E. coli dna* mutants supplemented with purified *dnaC* (as indicated) and ϕX A proteins. Where necessary, fractions were heated at nonpermissive temperatures and assayed in the presence or absence of purified gene products at 30°C. As shown, *dna* B, *dna* (C)D, *dna* G, *dna* Z, *rep*, and ϕX A proteins were required for maximal RF replication as well as SS DNA synthesis. In Experiment 13 (Table 11), fraction II derived from cells infected for 50 min with ϕX *am* H57 (F gene mutant) added to uninfected ammonium sulfate receptors supported RF replication but did not support SS DNA synthesis. When these extracts were mixed with extracts derived from cells infected for 50 min with ϕX *am* H90 (lacking the ϕX A protein, but containing the other ϕX coded proteins), RF → RF replication was not detected whereas SS formation occurred. These results suggest that the temporal switch in RF replication to SS DNA synthesis is due to viral proteins which are involved in viral DNA formation.

DNA Products Formed from RFI in the RF → RF and RF → SS Systems

The products formed in in vitro reactions primed with RFI depended upon the ϕX infected extract used (Figure 7). Fraction II prepared from cells infected for 25 min produced a mixture of ϕX RFII and ϕX RFI as determined by neutral sucrose gradient centrifugation. When the mixture of RFII and RFI was annealed to poly UG after denaturation and subjected to isopycnic banding, three labeled products were detected (Figure 7A), i.e., viral strand, complementary strand, and RFI. Viral and complementary strands coming from RFII were equally labeled. When RFI was converted to RFII

TABLE 10

Requirements for RF → SS DNA Synthesis in Vitro

Additions	dTMP incorporated (pmol/40 min)
Complete	64
Omit ϕX RFI DNA	3
Omit receptor ammonium sulfate fraction	2
Omit fraction II	4
Omit ATP, or omit Mg^{++}, or omit dATP, dGTP, dCTP	2—4
Complete + *N*-ethylmaleimide (4 m*M*)	4
Complete + rifampicin (10 μg/m*l*)	67
Complete + nalidixic acid (240 μg/m*l*)	6
Complete + novobiocin (240 μg/m*l*)	16

Note: Reaction mixtures were as previously described.[48] Viral strand formation was measured after hybridization to poly UG followed by CsCl isopycnic centrifugation for 70 hr as described by Baas et al.[52] The amount of viral strand which banded at the expected density is the value reported above. Under these conditions, > 90% of the incorporated ^{32}P was recovered in this fraction. Reference markers of ϕX174 [^{14}C] DNA, ϕX [^{3}H] RFI and ϕX [^{3}H] RFII were run in parallel gradients.

TABLE 11

Gene Products Required for RF Replication and SS Synthesis in Vitro

Experiment No.	Source of extract and phage used for fraction II	System studied (dTMP incorporated: pmol/40 min)	
		RF	SS DNA
1	BT1029 (*dna*B ts) (ϕX *am*3)	43	21
2	1 + *dna*B protein	210	68
3	LD332 (*dna*C ts) (ϕX *am*3)	1	5
4	3 + *dna*C protein	222	251
5	NY73 ϕX′ (*dna*G ts) (ϕX *am*3)	55	35
6	5 + *dna*G protein	218	109
7	AX729 (*dna*Z ts)	4	11
8	7 + *dna*Z protein	55	33
9	D92 (rep_{ts}) (ϕX *am*3)	3	4
10	9 + *rep* protein	248	102
11	BT1029 (ϕX *am* H90)	3	4
12	11 + ϕX A protein	153	99
13	BT1029 (ϕX *am*H57)	117	<1
14	11 + 13	<1	118

Note: Reaction mixtures (0.05 m*l*) were as described[47,48] with the exception that the RF system contained ammonium sulfate fractions from the *E. coli* strains indicated, purified ϕX A protein, except in reaction 11, and 0.2 unit of *dna* C protein except in reaction 3; fraction II was replaced by purified ϕX A protein. In the case of the SS DNA system, ammonium sulfate fractions used for the RF system supplemented with 0.2 unit of *dna* C protein was added. In reactions 13 and 14, extracts were prepared from cells infected for 50 min with ϕX amH57.[48]

by nicking with pancreatic DNAse and analyzed as above, both strands were also found to be equally labeled. Thus, both viral and complementary strands are equally labeled during RF replication.

Products formed during a 20-min incubation with fraction II isolated from *E. coli* infected for 50 min with ϕX *am*3 were shown by neutral sucrose gradient centrifugation

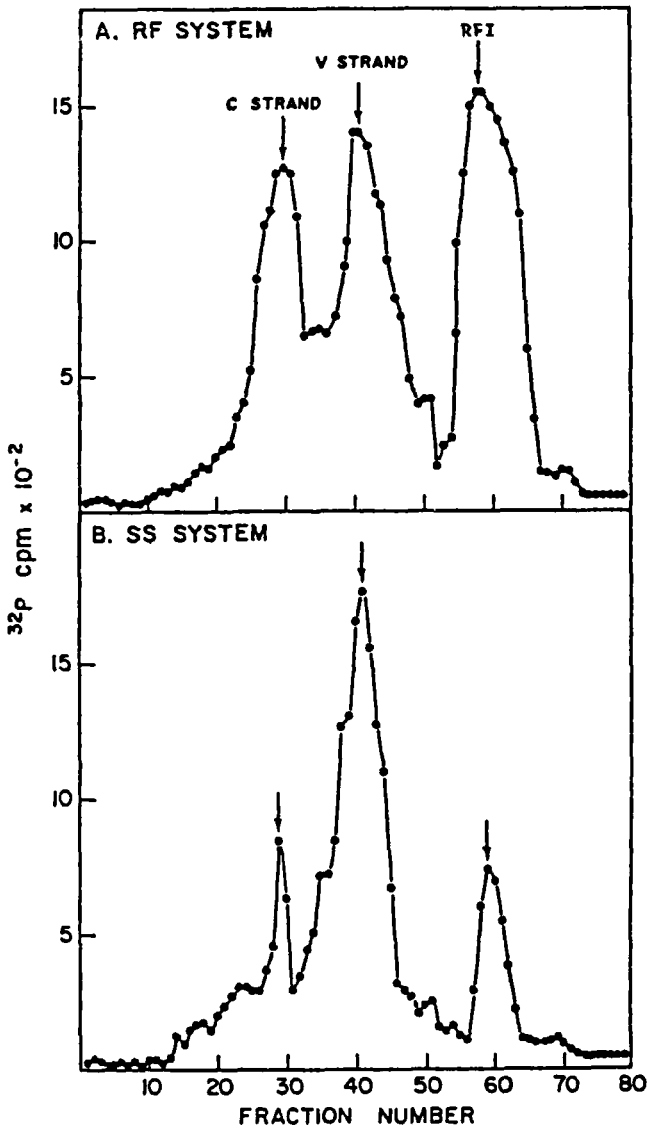

FIGURE 7. Poly (U,G)/CsCl gradients of DNA products formed in RF (A) and SS (B) synthesizing systems in vitro. Conditions were as described except that reaction mixtures were incubated at 30°C for 20 min. (From Sumida-Yasumoto, C. and Hurwitz, J., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 4195, 1977.)

to be a mixture consisting of 19% RFI, 61% RFII forms, and 20% SS DNA. The RFII products included a structure containing an extended single-strand viral tail (σ RFII). Approximately 75, 10, and 15% of the total label incorporated were recovered as viral strand, as complementary strand, and RFI, respectively, after poly UG annealing and CsCl isopycnic banding (Figure 7B). The label present in RFI was due to the selective synthesis of the viral strand.

With the RF → SS system the following was observed: (1) the rate of synthesis was linear from 20 to 25 min and then abruptly plateaued, (2) the extent of synthesis varied (in some experiments net synthesis was achieved [Table 11]), (3) prolonged incubation after viral DNA synthesis stopped resulted in formation of RFI and RFII, how-

ever, only the viral strand of both RF forms was labeled, (4) no net formation of RFII or RFI occurred. These results suggest that fractions which catalyzed SS DNA synthesis did not catalyze RF replication. Furthermore, RF replication was blocked by addition of Fraction II which catalyzed RF → SS DNA synthesis. Under these conditions DNA synthesis was restricted to viral strand formation.

Protein-DNA Complexes Formed in the RF → SS System

In vivo, ϕX 174 viral DNA synthesis is coupled to ϕX maturation. Fukisawa and Hayashi[50] have studied maturation of ϕX in vivo and demonstrated the formation of proteins complexed to DNA which sedimented with an S value of 50. This 50S complex contained σ RFII molecules. We have detected similar complexes during in vitro RF → SS DNA formation. Under the conditions used, although SS DNA synthesis stopped after 25 min, the formation of different protein-nucleic acid complexes continued over a period of 140 min (Figure 8). After incubation at 30°C for various times, reaction mixtures were subjected to neutral (nondenaturing) sucrose gradients above a dense CsCl shelf. In these experiments both [^{3}H]-labeled ϕX RFI template and newly labeled [^{32}P] DNA rapidly formed protein complexes which distributed with time between a large 50S peak and material with an S value of 132 (Figure 8A, B, and C). In a separate experiment (Figure 8D), a [^{32}P]-labeled peak was detected in fraction 6 corresponding to a complex of 132S; this fraction contained 3 10^8 plaque forming units (PFU)/m*l* while fraction 15 of the same gradient contained 10^8 PFU/m*l*. Infectious phage formation in vitro depended upon the presence of ϕX RFI DNA, ϕX A protein, ϕX F protein, *rep* protein, and DNA synthesis. When fraction II (as used in Figure 8D) was increased twofold, radioactivity from [^{3}H] RFI template was recovered in fractions 1 to 10; 10, 20, and 35% of the input [^{3}H] was recovered in the 114 to 132S region after 20, 40, and 140 min of incubation, respectively. Kinetic studies suggest that 30 to 50S DNA complexes are formed first and then converted to 60 to 70S complexes, and eventually to 132S and 114S complexes. These large protein·DNA complexes (50S and greater) were not observed with the RF → RF replication system.

Structure of DNA in 50S and 60 to 70S Protein·DNA Complexes

After centrifugation (as in Figure 8), material in the 50S region was pooled, deproteinized, and the DNA characterized by neutral sucrose gradient centrifugation (Figure 9A). The DNA present in this complex was a mixture of RFI and RFII forms; the former sedimented faster than RFII and contained RFII structures with extended SS tails (σ forms). The DNA in the 60 to 70S complex, analyzed in the same manner, contained a mixture of RFI, σ RFII, and circular and linear SS DNA (consisting of DNA of up to one unit in length). The nature of these DNA structures was also verified by electron microscopy. After deproteinization, the DNA in the 50S complex was analyzed by alkaline sucrose gradient centrifugation (Figure 9C); DNA products longer than one unit length were detected. These results resemble those obtained in vivo and suggest that σ RFII forms are intermediates in ϕX174 SS viral DNA formation.

Overall Pathway of RF Replication and SS DNA Synthesis in Vitro

At present the details of the specific reactions involved in RF → RF and RF → SS DNA synthesis remain to be elucidated. However, a number of important observations regarding the reactions catalyzed by some of the proteins involved in DNA replication have been determined and will be discussed in further detail below. The overall pathways of the ϕX replication cycle is clearer, however, and Figure 10 summarizes the two replication systems considered here.

The proteins required for the reactions (in part) include the *E. coli* coded proteins

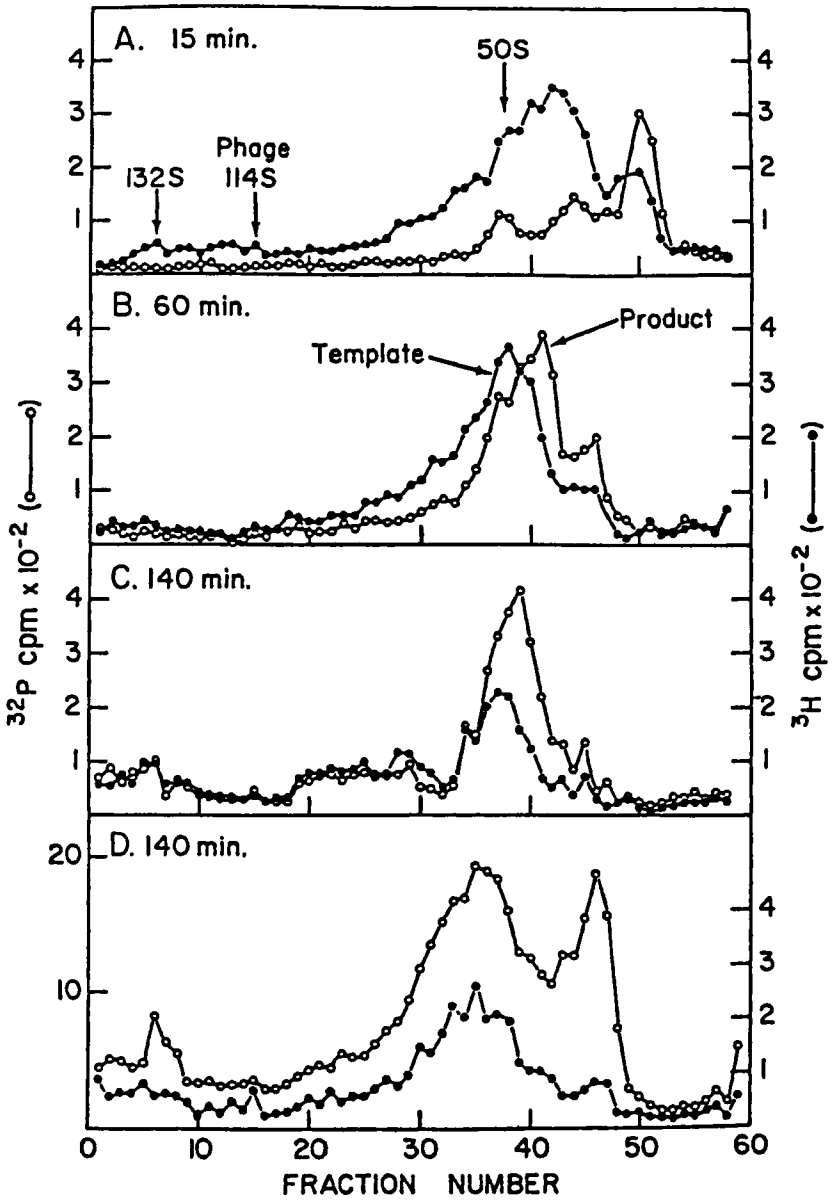

FIGURE 8. Neutral sucrose gradients analyses of DNA-protein complexes formed in vitro during SS DNA synthesis. Conditions were as described previously. (From Sumida-Yasumoto, C. and Hurwitz, J., *Proc. Natl. Acad. Sci. U.S.A.*, 74, 4195, 1977.)

dna B, *dna* C(D), *dna* G, *dna* Z, *rep*, *nal* A, novobiocin sensitive protein, *E. coli* binding protein, replication factors X, Y, and Z, elongation factors I, III, and *dna* E (DNA polymerase III). Requirements of the last six proteins are inferred from previous studies but have not been demonstrated. ϕX RFI (Figure 10(a)) is attacked by the ϕX A protein which acts at the A cistron of the viral strand yielding an RFII structure (Figure 10(b)) containing the ϕX A protein linked to the 5′-end. This intermediate can be replicated by fractions devoid of DNA and ϕX A protein activity. In the presence of the proteins listed above (the ϕX A protein, the only essential phage function, and other unknown host proteins), RFII is replicated by formation of a σ RF structure

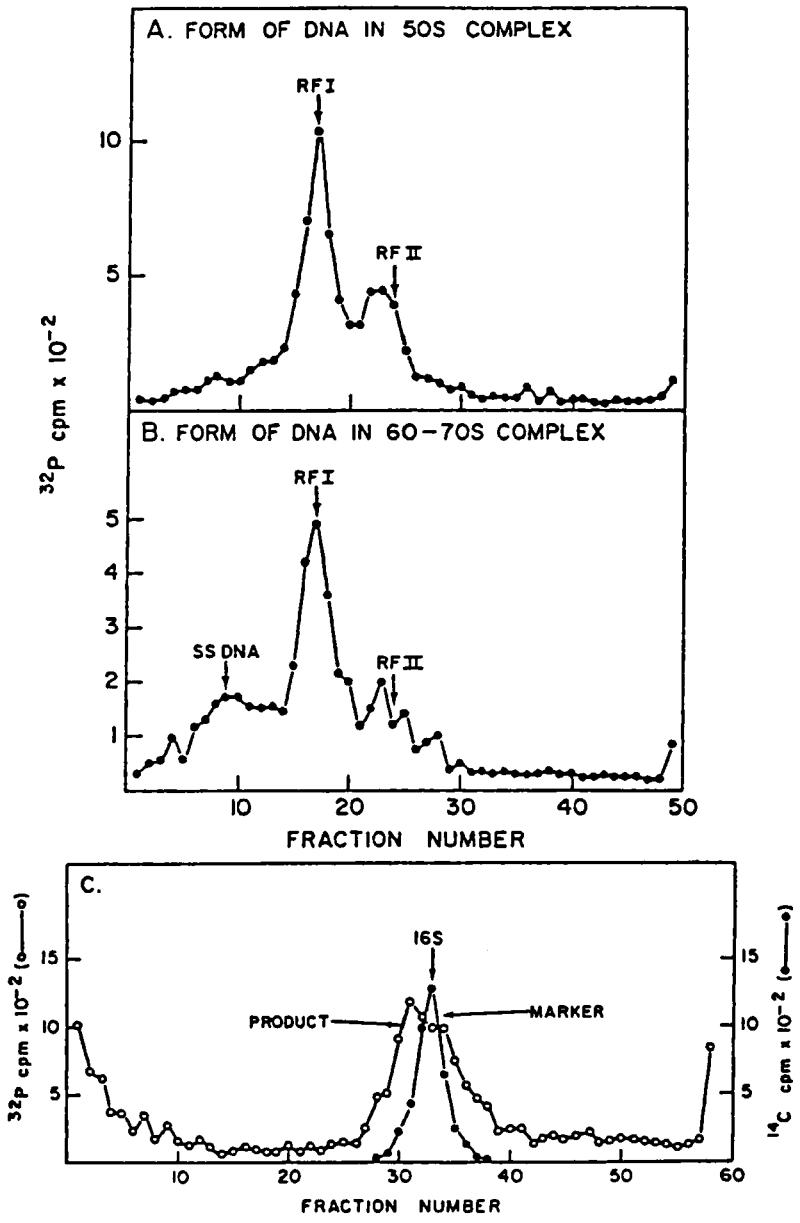

FIGURE 9. Sucrose gradient analysis of DNA from the 50S and 60 to 70S complexes. In this figure, neutral sucrose gradient of DNA isolated from 50S (Figure 9A) and 60 to 70S complexes (Figure 9B) are shown. Alkaline sucrose gradient of DNA from 50S complex is presented in Figure 9C. Conditions were as described previously.[48]

containing double-stranded tails which have been observed by electron microscopy; both viral and complementary strands in these structures are synthesized *de novo* as small pieces (Figure 10(c)) which can be chased into RFII and RFI forms.[47a] Synthesis continues until the A cistron of the viral strand appears as a single-stranded region; this site can then be cleaved by the ϕX A protein, probably generating structure d and possibly e. Structure e is an RFIII·ϕX A protein intermediate which could upon circularization yield structure *d*. Studies by Eisenberg et al.[53] suggest that the ϕX A pro-

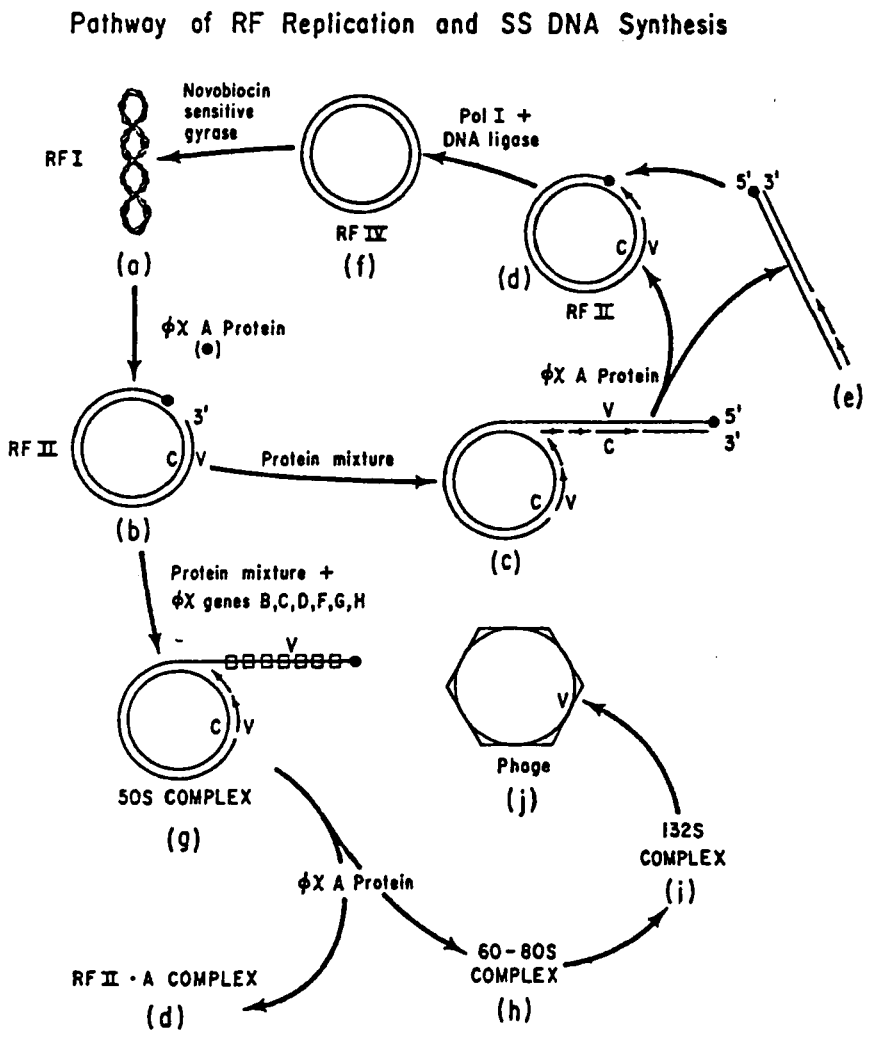

FIGURE 10. Postulated overall mechanism of RF and SS DNA synthesis. The protein mixture described above for the conversion of (b) → (c) and (b) → (g) includes all the proteins essential for SS → RFII. These proteins in combination with DNA gyrase do not catalyze synthesis of RF to RF. Thus, additional factors are required.

tein can ligate ends in an intramolecular reaction RFIII forms have been observed by electron microscopy but their role in RF replication is unknown. Conceivably, the φX A protein itself catalyzes circularization of RFIII structure and is then removed from the complex to yield RFII. RFII (d), after removal of the φX A protein, can be converted in vitro by DNA ligase and DNA polymerase I to an RFIV form (relaxed RF, f) which can be converted to the supertwisted RFI by DNA gyrase.[52,53,54-57] The conversion of gapped φX RFII to the RFIV form is the rate-limiting step in RF → RF synthesis both in vivo and in vitro.[47a]

The replication of RFI is blocked by φX coded proteins which are essential for phage maturation. This is detected by a cessation of complementary strand formation. In this way, RFI upon conversion to RFII·φX A protein complex can only support SS DNA synthesis. This pathway yields *σ* RFII forms containing single-stranded tails complexed to phage proteins in which only the viral strand is labeled (g). Viral strand DNA synthesis occurs by formation of short discontinuous fragments which are rapidly

chased into large fragments[34a] as in the case of RF replication. The 50S complex is attacked by the ϕX A protein leading to the reformation of a RFII·A complex (d) and a new 60 to 80S complex (h). These complexes vary in size due to changes in amount and composition of viral proteins associated with the DNA. Eventually, these intermediates are converted to a 132S structure (i) and then to phage (114S, [j]).

Characterization of the Proteins Involved in ϕX RFI DNA Replication

The requirements for the replication of ϕX RFI include the proteins essential for SS → RF formation as well as additional proteins. The additional proteins already isolated and partially characterized now include the ϕX A protein, the *rep* protein, the *nal* protein, and the protein involved in DNA gyrase activity. It is likely that these additional proteins are necessary for the utilization of duplex DNA structures and for this reason their characterization is of particular importance if we are to understand more complicated DNA replication systems. At present, attempts to reconstruct the RF → RF system with the 11 proteins already characterized (in the SS to RF system) plus the additional proteins involved in the RF → RF system have been unsuccessful and suggest the need for additional factors.

Some of the details of the properties of the proteins specifically involved in RF → RF replication have been studied and are presented below.

ϕX A protein

As described above, this protein coded for by the A cistron, is essential for the initial attack on the ϕX RFI DNA. It was first purified by Henry and Knippers[42] from extracts of *E. coli* infected with ϕX *am*3. Such preparations catalyzed a specific attack of ϕX RFI forming an RFII in which the discontinuity resided specifically in the viral strand. In our laboratory, we have purified the ϕX A protein using the complementation assay in which ϕX RFI-dependent DNA synthesis required the ϕX A protein.[43] Using this procedure as an assay, a homogeneous protein preparation of mol wt 59,000 was isolated. In accord with the results of Henry and Knippers,[42] the enzyme acted as an endonuclease and introduced a discontinuity in the viral strand in the A cistron of ϕX RFI DNA. The requirements for action of the ϕX A protein are exacting. In the presence of Mg^{++}, only superhelical ϕX RFI was attacked by the protein while the relaxed ϕX replicative form (RFIV) was inactive (Figure 11). No other superhelical DNA replaced ϕX RFI DNA.

The product of the action of the ϕX A protein was shown to be a DNA-protein complex, ϕX RFII·ϕX A protein. This complex was stable and could be isolated by sucrose gradient centrifugation (Figure 12). The complex could be used in place of ϕX RFI DNA and the ϕX A protein for RF → RF replication (Figure 13). In such structures, the ϕX A protein appears to be covalently linked to the 5′ end of the nicked viral strand. The isolated RFII·ϕX A protein complex was attacked by exonuclease III but not by DNA polymerase I, either in the presence or absence of the four dNTPs; these results suggest the presence of a free 3′-hydroxyl end and a blocked 5′-terminus. Similar results were obtained after proteinase K treatment and/or phenol treatment.

The ϕX A cistron also codes for the ϕX A* protein.[58] This protein (33,500 daltons) has been purifed to homogeneity and some of its properties studied (Ikeda and Yudelevich, unpublished observations).[43a] The ϕX A* protein does not substitute for the ϕX A protein in any of the reactions catalyzed by the latter enzyme, nor does this protein affect the rate of ϕX RFII formation by the ϕX A protein. Purified ϕX A* protein contained no detectable nuclease activity on duplex or single-stranded DNAs. The ϕX A* protein binds to duplex DNAs and inhibits the action of nucleases on these DNAs. Interestingly, in the presence of an excess of the ϕX A* protein, colicin EI, fd RFI,

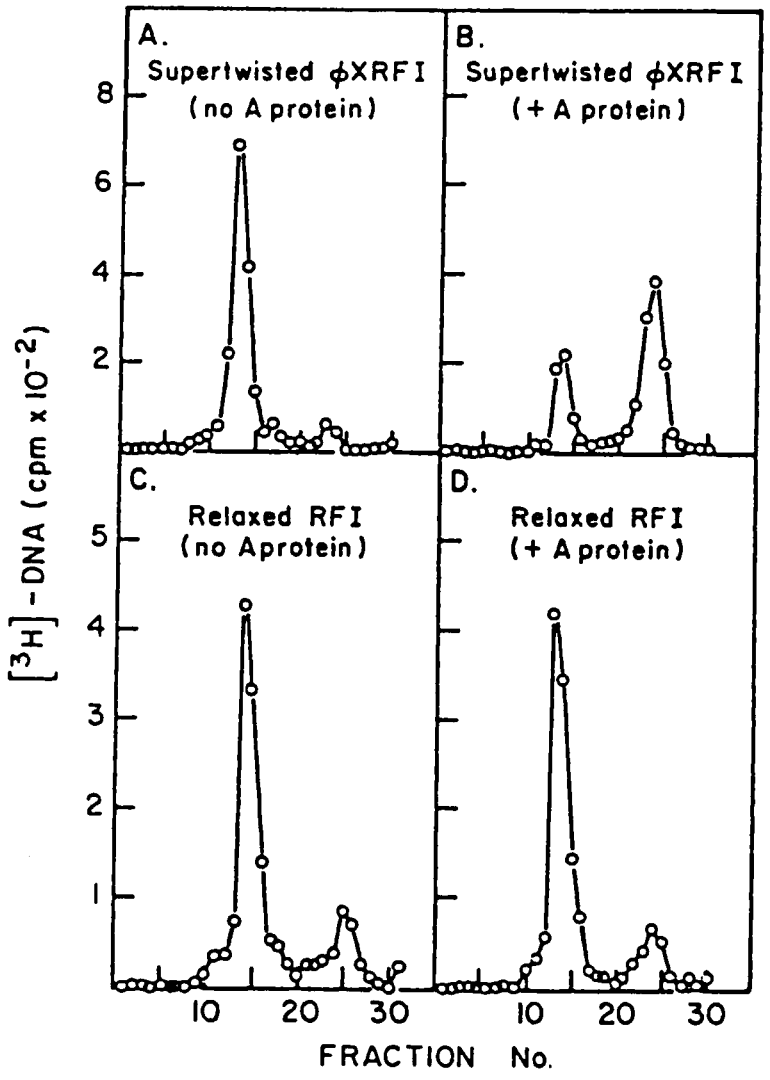

FIGURE 11. Endonuclease activity of the ϕX A protein on relaxed and supertwisted ϕX RFI DNA. Relaxed ϕX RFI DNA was prepared by treating ϕX RFI DNA with pancreatic DNAse I, followed by phage T4 DNA ligase treatment. This ϕX RFIV was isolated by centrifugation in $PrdI_2$-CsCl gradients. The DNA had the density of completely relaxed DNA. Endonuclease assays were carried out with 430 pmol of supertwisted ϕX RFI and relaxed ϕX RFI which were incubated with 0.15 μg of ϕX A protein (step VI for 30 min, and each reaction mixture was sedimented through a 5 to 20% alkaline sucrose gradient. (From Ikeda, J. E., Yudelevich, A., and Hurwitz, J., *Proc. Natl. Acad. Sci. U.S.A.*, 73, 2669, 1976.)

and PM2 DNAs are completely resistant to attack by a preparation of *E. coli* nuclease. In the absence of the ϕX A* protein, the DNAs are degraded to small molecular weight DNA fragments. With ϕX RFI DNA, an excess of the ϕX A* protein also inhibits degradation of the DNA to small molecular weight fragments but yields ϕX RFII and ϕX RFII forms. The site of breakage of the DNA is unknown.

The ϕX A* protein is found associated with mature viral protein-nucleic acid complexes.[58] This association fits well with our observation on the binding of this protein to DNA. However, the biological role of this protein in ϕX RF replication is still unclear.

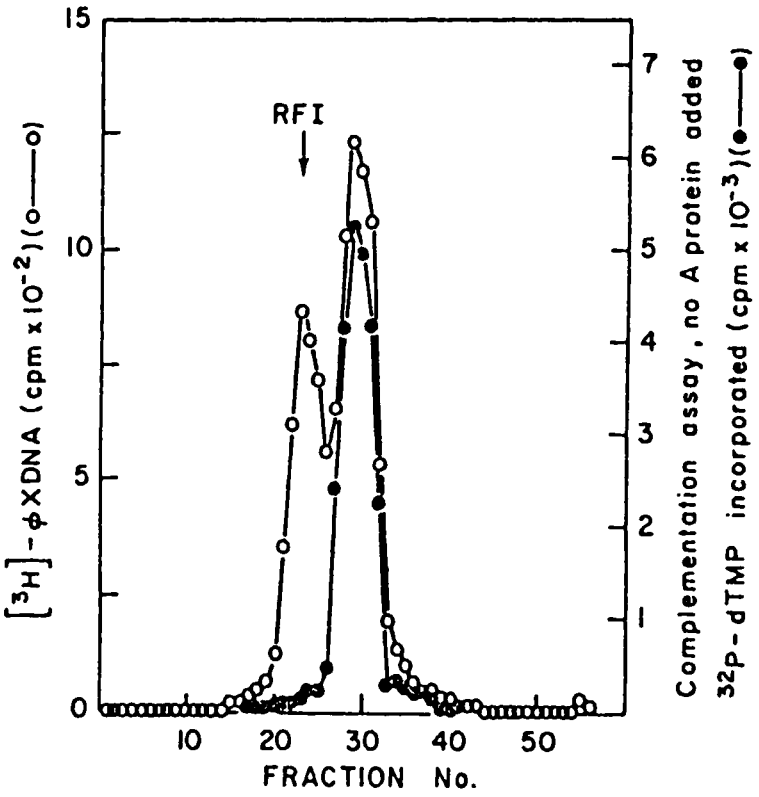

FIGURE 12. Isolation of complex formed between φX RFI DNA and φX A protein (RF·A complex). φX RFI [³H] DNA (105 nmol) was incubated with 7 μg of A protein (step VI), under standard conditions for endonuclease assay and then subjected to sucrose gradient centrifugation. (From Ikeda, J. E., Yudeleuich, A., and Hurwitz, J., *Proc. Natl. Acad. Sci. U.S.A.*, 73, 2669, 1976.)

DNA Gyrase Activity

The replication of duplex DNA in contrast to the conversion of SS → RFII was inhibited by nalidixic acid and novobiocin, two known inhibitors of DNA replication. In earlier studies on the replication of φX RFI DNA with limiting amounts of Fraction II, we observed that φX RFIV DNA (relaxed but covalently closed) was replicated, but with a prolonged lag in time. This contrasted the rapid rate of utilization of φX RFI. Furthermore, it was noted that under these conditions, the products formed during φX RFI replication (initially φX RFII which is then converted to φX RFI) varied in their degree of superhelicity, but eventually assumed the identical superhelical structure as the φX RFI formed in vivo. The finding that φX RFIV was not attacked by the φX A protein and that this DNA was replicated with a long lag suggested an active enzymatic process for introducing superhelicity. The system responsible for this reaction was discovered by Gellert et al.[54] who showed that the conversion of colicin EI RFIV DNA to RFI required ATP and Mg^{++} and was inhibited by low concentrations of novobiocin. In addition to the effect of novobiocin, it was shown that the DNA gyrase activity also includes the protein which is nalidixic acid sensitive.[55,56] This *nal* target protein has been purified using the DNA gyrase assay[56] as well as by the complementation assay[56] (and unpublished results of Sumida-Yasumoto).[47a] We have repeated these observations and have shown that the utilization of φX RFIV in φX A protein mediated replication is completely dependent upon DNA gyrase activity.[57] At present,

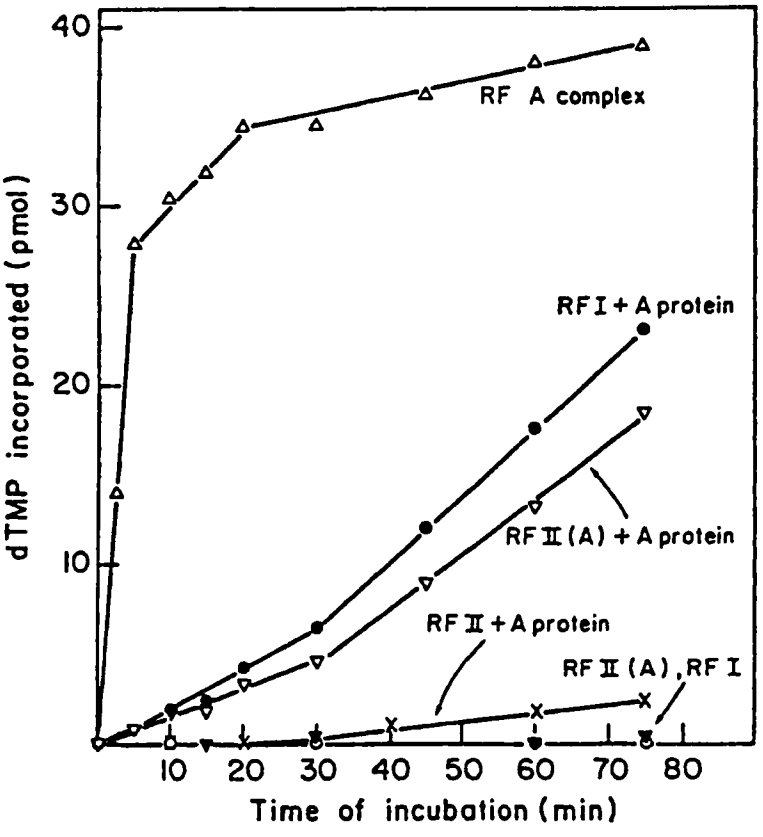

FIGURE 13. Rate of replication with RF·ØX A complex and other forms of ØX DNA. Complementation assays were carried out except that ØX RFI DNA was replaced with 340 pmol of RF·ØX A complex, and no ØX A protein was added (Δ); standard complementation assays contained 345 pmol of ØX RFI and 0.4 μg of ØX A protein (O). RF·A complex was treated with proteinase K (0.2 mg/mℓ) at 37°C for 20 min, extracted with phenol and ether, and the RFII(A) was isolated by PrdI$_2$-CsCl centrifugation and used as substrate (340 pmol) in the complementation assay to which 0.04 μg of A protein was added ( ). RFII was isolated from ØX-*am3*-infected cells and was used as a substrate (350 pmol) with 0.05 μg of A protein (X); 340 pmol RFII(A) was used as template with no A protein ( ), and 340 pmol of Ø X RFI as template with no A protein added (O).

the mechanism operating in the generation of superhelical DNA is unknown. Recent studies indicate an important role for supertwisted circular DNA structures in such biological reactions as integrative recombination of bacteriophage γ DNA,[59] replication of colicin EI[60] and ϕX DNA[57] and transcription.[61,62]

The exact role of the DNA gyrase system in ϕX RFI replication remains to be elucidated. It is clear that the replication of ϕX RFI is sensitive to novobiocin as in the replication of ϕX RFIV. It is possible that DNA gyrase or the novobiocin and *nal* target proteins play some role in the movement of the replicating fork. Interestingly, the rate of ϕX RFI replication is immediately halted upon addtion of nalidixic acid; similar experiments with novobiocin result in a gradual halt in DNA synthesis. Further studies are needed before the mechanism of action of these inhibitors will be defined.

Rep Gene Function

The *rep* protein has been purified to homogeneity in our laboratory.[47a] We have

utilized the ϕX RFI replication complementation assay for this purpose in which ammonium sulfate receptors derived from *rep* amber mutants of *E. coli* supplemented with the ϕX A protein or fraction II served as the assay system. This procedure has resulted in the isolation of a protein of 68,000 daltons which contains low levels of DNA-dependent ATPase activity. In contrast, *E. coli* extracts from a *rep* amber mutant carried through the same isolation procedure yielded fractions devoid of *rep* gene complementing activity, free of the 68,000 dalton protein but possessing the same low DNA-dependent ATPase activity. Eisenberg et al.[44] have also isolated the *rep* protein. In other studies, Scott et al.[63] utilized an assay in which the ϕX A protein, *E. coli* binding protein, and a DNA elongation system (holoenzyme) containing the DNA polymerase III system were used to measure *rep* protein activity. Their assay resulted in the synthesis of viral strand accompanied by formation of single-stranded viral DNA. They reported that the isolated *rep* protein is a DNA-dependent ATPase and suggest that the strand displacement reaction depends on the action of the *rep* protein, *E. coli* binding protein and the ϕX A protein. In this system, the *E. coli* binding protein is essential to bind to single-stranded DNA either in front or behind the moving replication site governed by the *rep* and ϕX A proteins. This is an attractive model for the action of the *rep* protein. In our laboratory, attempts to detect in vitro ϕX RFI DNA-dependent incorporation of dNMP in the presence of highly purified ϕX A protein, *E. coli* binding protein, *rep* protein, and the DNA elongation system (DNA polymerase III, *dna* Z, DNA EFI and III) have not been successful. It is possible that the DNA polymerase III holoenzyme preparation used by the Kornberg group[63,64] may contain additional factors necessary for this reaction. Our studies of RF replication with more crude fractions are in accord with in vivo observations which indicate a more complicated process in the synthesis of viral strand and complementary strands. However, the use of more simplified systems for strand displacement reactions and the role of ATP energized reactions in strand separation are now receiving close attention. There are now a large number of different proteins which may play a role in moving replication protein complexes. A number of these proteins contain DNA-dependent ATPase activity. Candidates for such reactions now include the *dna* B protein,[65] replication factor Y DNA-dependent ATPase,[66] an *E. coli* ATP-dependent enzyme which "unwinds" duplex DNA,[67] the T4 44/62 protein complex,[12] the T7 gene 4 protein[68,69] and the *rep* gene product.[63] How these proteins are coupled to priming and DNA elongation reactions will soon be elucidated.

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