

Loma Linda University

## TheScholarsRepository@LLU: Digital Archive of Research, Scholarship & Creative Works

---

Loma Linda University Electronic Theses, Dissertations & Projects

---

9-1985

### Assay for Rifampicin Resistant M13 RF Synthesis

Carol Bauer Wheeler

Follow this and additional works at: <https://scholarsrepository.llu.edu/etd>



Part of the [Biology Commons](#), and the [Microbiology Commons](#)

---

#### Recommended Citation

Wheeler, Carol Bauer, "Assay for Rifampicin Resistant M13 RF Synthesis" (1985). *Loma Linda University Electronic Theses, Dissertations & Projects*. 732.

<https://scholarsrepository.llu.edu/etd/732>

This Thesis is brought to you for free and open access by TheScholarsRepository@LLU: Digital Archive of Research, Scholarship & Creative Works. It has been accepted for inclusion in Loma Linda University Electronic Theses, Dissertations & Projects by an authorized administrator of TheScholarsRepository@LLU: Digital Archive of Research, Scholarship & Creative Works. For more information, please contact [scholarsrepository@llu.edu](mailto:scholarsrepository@llu.edu).

## ABSTRACT

### Assay for Rifampicin Resistant M13 RF Synthesis

by

Carol Bauer Wheeler

We have developed a rapid assay which can detect the function of the origin of  $\phi$ X174 complementary strand DNA in M13 by virtue of its ability to initiate the synthesis of M13 complementary strands in the presence of the antibiotic rifampicin. A segment of DNA carrying the sequence recognized by the replication protein n' has been isolated from bacteriophage  $\phi$ X174 and cloned into an M13 bacteriophage vector. Protein n' is a component of the bacterial primosome, a multi-protein assembly which initiates the synthesis of primers during DNA replication in the bacterium Escherichia coli. The recognition site for n' on the  $\phi$ X174 viral strand DNA constitutes the origin of replication for the  $\phi$ X174 complementary strand.

On  $\phi$ X174 viral single-stranded DNA, synthesis of the complementary strand is initiated by the primosome. RNA polymerase is not involved. By contrast, the formation of primers on M13 viral DNA is accomplished by the direct action of RNA polymerase. Rifampicin inhibits this enzyme and, therefore, prevents the synthesis of M13 complementary strands. M13 clones which include the  $\phi$ X174 n' recognition site acquire the rifampicin-insensitive characteristic of the  $\phi$ X174 replication origin.

We have designed an assay in which small cultures of Escherichia coli, incubated with chloramphenicol and rifampicin are infected with M13,  $\phi$ X174, or M13 phage hybrids which contain a fragment of DNA bearing the  $\phi$ X174 n' recognition site. Rapid lysates were applied to agarose gels to detect the presence of double-stranded replicative form DNA in the cells. Numerous phage isolates could be tested simultaneously in one day. We will use the assay to screen isolates which have been mutagenized in the region of the  $\phi$ X174 insert.

LOMA LINDA UNIVERSITY

Graduate School

---

ASSAY FOR RIFAMPICIN RESISTANT M13 RF SYNTHESIS

by

Carol Bauer Wheeler

---

A Thesis in Partial Fulfillment of the  
Requirements for the Degree Master of Arts  
in Biology

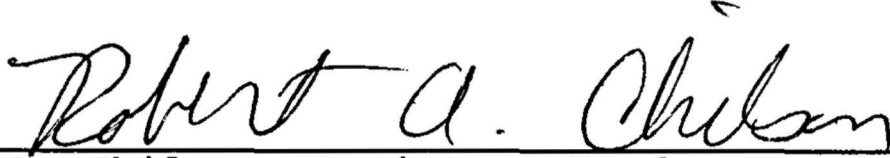
---

September 1985

Each person whose signature appears below certifies that this thesis in his opinion is adequate, in scope and quality, as a thesis for the degree Master of Arts.

  
\_\_\_\_\_, Chairman  
Anthony J. Zuccarelli, Associate Professor of Microbiology

  
\_\_\_\_\_  
Robert L. Nutter, Professor of Microbiology

  
\_\_\_\_\_  
Robert A. Chilson, Assistant Professor of Biology

## ACKNOWLEDGEMENTS

My sincere thanks and appreciation to the following people:

- The faculty and staff of the Biology and Microbiology Departments of Loma Linda University for their friendship and support during my educational endeavors.

- Committee members, Dr. Robert Nutter, and Dr. Robert Chilson for their welcomed criticism and helpful suggestions for the preparation of this manuscript.

- Dr. Anthony Zuccarelli, for his time, patience, and guidance, and for sharing his knowledge and fascination with science.

## TABLE OF CONTENTS

INTRODUCTION.....	1
MATERIALS AND METHODS.....	14
(a) Bacterial strains.....	14
(b) Bacteriophage strains.....	14
(c) Enzymes and chemicals.....	14
(d) Media and solutions.....	15
(e) M13 phage preparations.....	16
(f) Phage DNA.....	17
(g) Assay for rifampicin resistant M13 RF synthesis..	17
(h) Agarose gel electrophoresis.....	19
RESULTS.....	21
DISCUSSION.....	27
LITERATURE CITED.....	35
APPENDIX.....	41

## LIST OF FIGURES

- Figure 1      The cistron map of  $\phi$ X174
- Figure 2      The n' recognition site and neighboring regions of  $\phi$ X174
- Figure 3      Stages of DNA synthesis in  $\phi$ X174
- Figure 4      Chain growth at the replicating fork of E. coli
- Figure 5      Replication proteins of E. coli
- Figure 6      Priming systems of bacteriophages M13 and  $\phi$ X174
- Figure 7      The effect of multiplicity of infection on the yield of parental RF
- Figure 8      Effects of NaCl and ethidium bromide on the migration of RF and single-stranded DNA
- Figure 9      Effect of rifampicin on RF production in M13mp7 and the clone, M13mp7-M9
- Figure 10     Relative amounts of RF DNA produced with increasing amounts of rifampicin
- Figure 11     Effect of chloramphenicol on parental RF production
- Figure 12     Effect of rifampicin on parental RF production by M13mp7 and the clone, M13mp7-M9, in the presence of chloramphenicol
- Figure 13     Relative amounts of RF DNA produced with increasing concentrations of rifampicin and 150  $\mu$ g/ml chloramphenicol
- Figure 14     Effect of rifampicin on the parental RF synthesis of  $\phi$ X174



## INTRODUCTION

Protein and nucleic acid interactions are vital to the function of the cell. Enzymes interact with nucleic acids in numerous ways and yet surprisingly little is known about the specific mechanisms of such interactions. The elucidation of specific rules or patterns of recognition in interaction of proteins with nucleic acids would prove to be of great import and have medical and research applications. One such interaction which is particularly amenable to investigation is one involved in the replication of bacteriophage  $\phi$ X174 in its bacterial host, Escherichia coli C.

$\phi$ X174 is a bacteriophage which contains a circular single-stranded DNA molecule (Sinsheimer, 1959) of 5386 nucleotides packaged in an isometric capsid composed primarily of three proteins. This virus is well suited for protein/DNA studies as its genome has been sequenced and is genetically well-characterized (Sanger et al., 1978).

The  $\phi$ X174 genome codes for 11 proteins (Figure 1). Genes F, G, and H are responsible for the protein capsid and spikes. The product of cistron J is probably associated internally with the viral DNA (Fiddes and Godson, 1978). The products of five other genes, A, B, C, D, and E appear in the infected host cells but are not part of the mature virus particle (Denhardt, 1975). Protein K has also been

detected in viral infections (Shaw et al., 1978; Pollock et al., 1978; Tessman et al., 1980).

ØX174 is particularly interesting because of its genetic economy. The viral DNA with 5386 nucleotides would be expected to code for a maximum of 201,000 daltons of protein if each three nucleotides specified an amino acid of 112 daltons (the mean molecular weight of amino acids in the viral coat proteins). In actual fact, the ten unique ØX174 proteins total 223,000 daltons. This apparent discrepancy is attributed to the translation of some DNA regions in more than one frame. As a result, two proteins with different amino acid sequences are generated from a single nucleotide sequence. We refer to this phenomenon as "overlapping genes" (Figure 1).

Surprisingly, there exist four small DNA segments flanking the F, G, and H genes which are not used to produce proteins (Figure 1). In view of the compact genetic arrangement represented by the overlapping genes, one would speculate that there must be some indispensable role associated with these non-coding regions (hereafter called "intercistrons"). These suspicions are substantiated. Each of the intercistrons has a ribosome binding site (Shine and Dalgarno, 1974). The H-A intercistron contains one of the three RNA polymerase promoter sequences and also a major mRNA termination site. The J-F intercistron contains a hairpin loop marking the location of a minor mRNA terminator

(Godson, 1978). The F-G intercistron, largest of the four with 110 nucleotides (nucleotide positions 2285 to 2394), contains a recognition site for protein n' (or factor Y), a DNA replication protein of E. coli. Protein n' triggers the formation of a complex called the primosome which, in turn, initiates replication of DNA (Kornberg, 1978; Meyer et al., 1978; Shlomai and Kornberg, 1980c; Wickner and Hurwitz, 1975).

Shlomai and Kornberg (1980c) identified a segment of the F-G intercistron which appears to be active in recognition of the protein n'. Analysis of restriction fragments placed the recognition element between nucleotides 2301-2354 on the published nucleotide sequence (Sanger et al., 1978). Calculation of the free energy contributions to nucleotide pairing as well as resistance to destabilization by single-stranded binding protein (SSB) and resistance to digestion by exonuclease VII suggest that a hairpin secondary structure occupies most of the putative recognition site (Figure 2).

The synthesis of  $\phi$ X174 DNA during infection of the host cell can be divided into three stages (Figure 3). In the first stage the single-stranded viral DNA serves as a template for the production of a complementary strand resulting in parental replicative form or "parental RF". This takes only a few seconds. In the second stage the parental RF becomes the template for the production of 10 to

20 more double-stranded rings. The resultant double-stranded molecules, called progeny RF, initially contain interruptions in the new strand (RFII) which are later sealed to produce covalently closed circles (RFI). About 12 minutes after infection the third stage begins in which single-stranded DNA is produced from RF via a rolling circle mechanism. The single-stranded DNA is packaged into phage coats which are released upon lysis.

The conversion of an infective single-stranded DNA molecule into a duplex replicative form is achieved through a series of four enzymatic steps: prepriming, priming, chain elongation and termination. This thesis addresses the details of the prepriming stage and how the prepriming complex forms at a specific site on the  $\phi$ X174 single-stranded DNA. The six proteins involved in prepriming generate a complex on which the dnaG protein transcribes a short primer. Chain elongation is effected by DNA polymerase III holoenzyme. Termination includes excision of the primer, filling of the gap by DNA polymerase I, and sealing by polynucleotide ligase. The infecting viral DNA circle, coated with single-stranded DNA binding protein (SSB), appropriates the system used by the cell to initiate synthesis of DNA during discontinuous replication of the cellular genome (Figure 4). Assembly of a prepriming complex or "preprimosome" is accomplished by the interaction of six proteins:  $n$ ,  $n'$ ,  $n''$ ,  $i$ , dnaB, and dnaC (Figure 5)

(Wickner, 1978; McMacken and Kornberg, 1978; Shlomai et al., 1981).

Protein n', identified as factor Y by Wickner and Hurwitz (1975), is a single polypeptide chain with a native molecular weight of 76,000 daltons. There are approximately 70 molecules of n' per E. coli cell. It is resistant to N-ethylmaleinide and is inactivated by heat (Shlomai and Kornberg, 1980a). A unique 55-nucleotide fragment from the F-G intercistron of  $\phi$ X174 DNA contains an n' recognition site which can be regarded as the starting signal for complementary strand DNA replication in the virus (Shlomai and Kornberg, 1980a). This DNA segment stimulates an ATPase activity of n' even when the single-strand DNA fragment is coated with SSB (Sholomai and Kornberg, 1980c). In fact, approximately 30 of the 180 molecules of SSB bound to  $\phi$ X174 DNA are displaced by protein n' in an ATP-dependent reaction which initiates assembly of the primosome (Shlomai and Kornberg, 1980b). As well as having a role in the replication of E. coli and  $\phi$ X174, protein n' also displays ATPase and primosome assembly functions at two sites on the ColE1 and pBR322 plasmids (Zipursky and Marians, 1980; Nomura and Ray, 1980; Nomura et al., 1982; Boldicke et al., 1981).

Protein n is heat and acid-resistant and N-ethylmaleimide sensitive. It is a dimer in purified solution with a molecular weight of about 12,000 daltons per polypeptide

chain. It occurs at a frequency of about eighty molecules per cell. Binding depends on a direct interaction with SSB. About 30 *n* monomers can be bound to an SSB-coated circle; however, in primosome formation an input of only 2-3 *n* monomers is required and only one monomer is bound per circle. Retention of *n* on the circle is dependent on *n'*. Since protein *n'* alone can recognize the assembly site in the F-G intercistron (Shlomai and Kornberg, 1980c) protein *n* probably promotes the interactions that are required for *i*, *n''*, *dnaC* and *dnaB* to assemble (Low et al., 1982). Proteins *n* and *n'* on SSB coated DNA sustain assembly when supplemented with *i*, *n''*, *dnaC* and *dnaB*.

Protein *n''* is a 17,000 dalton polypeptide and is not abundant. Its function is still obscure.

Individual subunits of protein *i* have a molecular weight of about 22,000 daltons. It occurs as a nonglobular oligomer of three identical subunits and there are about 50 molecules per cell. About 1.7 molecules are associated with the intermediate (Arai et al., 1981b). The trimeric protein is incorporated into the preprimosome at a stage requiring participation of *dnaB* and *dnaC* proteins following the actions of proteins *n*, *n'*, and *n''*. Protein *i* (alone or in cooperation with *dnaC*) is important in the reaction which later incorporates *dnaB* protein into the preprimosome. Later still, after DNA elongation, the third enzymatic step, has begun, protein *i* is probably released from the complex

since antibodies directed against protein *i* no longer inhibit priming. This suggests it might not be required once the prepriming intermediate is formed (McMacken and Kornberg, 1978).

Protein dnaC is a polypeptide with a native molecular weight of 29,000 daltons (Kobori and Kornberg, 1982a). It is known to form a tight complex with dnaB protein in the presence of ATP (Wickner and Hurwitz, 1975). Based on gel densitometry measurements, the complex contains six dnaC protein monomers per dnaB protein hexamer (30,000 daltons) and has a total molecular weight in the vicinity of 480,000 daltons (Kobori and Kornberg, 1982b).

Protein dnaB is a 50,000 dalton polypeptide which is a DNA-dependent ATPase or rNTPase (Reah-Krantz and Hurwitz, 1978; Arai and Kornberg, 1981a) and judged to be a hexamer (Arai *et al.*, 1981c). In vitro dnaB protein forms a binary complex with ATP that has a strong affinity for single-stranded DNA. If bare single-stranded DNA is available a ternary complex of DNA, dnaB protein and ATP forms creating a unique secondary structure on the single-stranded DNA that is recognized by primase. In its interaction with single-stranded DNA, the dnaB protein hexamer covers about 80 nucleotide residues (Arai and Kornberg, 1981b). In the absence of SSB and other factors, priming in this simple three component system lacks the specificity observed in vivo (Arai and Kornberg, 1979). For specific priming on

SSB-coated DNA, dnaB protein must be part of the primosome complex. When DNA polymerase III holoenzyme is not available to catalyze the elongation step, multiple RNA primers, 10 to 60 nucleotides long, are synthesized on the single-stranded  $\phi$ X174 template.

Initiation of fragments in discontinuous replication of the E. coli chromosome as well as conversion of  $\phi$ X174 single-stranded DNA to RF are insensitive to rifampicin, an antibiotic which specifically inhibits the enzyme RNA polymerase. This observation is attributed to a priming mechanism which does not use RNA polymerase. Other replication systems such as in the phage M13 use RNA polymerase for the priming function and are inhibited by rifampicin.

The protein coded for by the dnaG gene is responsible for the priming function in discontinuous replication of E. coli and  $\phi$ X174. Because its function is priming rather than transcription and because dNTPs can be substituted for rNTPs in all but the first and perhaps the second position, it is called primase (Rowen and Kornberg, 1978). Primase is a single polypeptide of 60,000 daltons with 50 to 100 copies per cell. The locomotive-like preprimosome, when augmented by primase, becomes a primosome. In E. coli replication the primosome moves progressively with the replicating fork to prime the repeated initiations of nascent strands (Arai and Kornberg, 1981c; McMacken et al., 1977). When primase



action is coupled directly to replication, the primer is only a few nucleotides long due to early extension by DNA polymerase. Upon addition of DNA polymerase and dNTPs, this RNA chain is covalently extended by DNA synthesis (Bouche et al., 1978).

Once assembled, the primosome remains bound even after the circle becomes a covalently closed, supercoiled duplex (RFI). Conservation of the primosome facilitates the next stage of RF replication by directing initiation of the rolling circle to the unique site of gene A protein cleavage, priming complementary strand synthesis at the replication fork and serving as a helicase. The parental RF bearing the primosome may be the sole template for replication, while the numerous supercoiled progeny RF are produced for transcription.

The conversion of single-stranded DNA to RF in each of three small phages (M13,  $\phi$ X174, and G4) provides models for distinctive initiation mechanisms used by E. coli. After initiation of the complementary strand, elongation and termination are identical in all three cases. The enzymatic requirements for these three different priming pathways may be summarized as follows. Site-specific initiation on M13 DNA coated with SSB depends only upon transcription by RNA polymerase holoenzyme with a functional sigma subunit (Kaguni and Kornberg, 1982). With SSB-coated G4 DNA, primer synthesis is effected by primase alone (Geider et al.,

1978). SSB-coated DNA of  $\phi$ X174 requires primase and all the preprimosome proteins. In the absence of SSB protein, dnaB protein forms a complex with virtually any single-stranded DNA in the presence of ATP and primase to produce multiple short primers along the entire length of the DNA (Figure 6) (Arai and Kornberg, 1979). The specificity of initiation sites on M13 and G4 is due to a stretch of DNA which is not coated by SSB. This area exhibits a secondary hairpin configuration (Arai and Kornberg, 1979) similar to that of  $\phi$ X174 yet the primosome assembles only on the  $\phi$ X174 hairpin and not on those in the DNA of G4 or M13 (Kornberg, 1978; Meyer et al., 1978). In each case the hairpin structure seems to insure that initiation commences at a singular specific location. However, it is likely that the n' protein recognizes finer features such as a distinctive nucleotide sequence, the pattern of mismatched regions in the hairpin, the size and sequence of the terminal loop, or particular nucleotides in the single-stranded regions flanking the hairpin.

Two basic approaches lend themselves to probe this interaction. One is the protection of nucleotides from chemical or enzymatic attack by bound protein in order to discover points of contact between amino acids and nucleotides. The other strategy is to isolate viral mutants with debilitating alterations in the recognition site. We hope to incorporate both methods in ensuing research.

To carry on such research a supply of the DNA fragment containing the recognition site will be necessary. The ligation of DNA fragments into cloning vectors is a powerful method for amplification. Dr. Joachim Messing and coworkers have constructed a cloning vehicle using the bacteriophage M13 (Messing et al., 1977; Groneborn and Messing, 1978; Messing et al., 1981) which lends itself particularly well to this task. A number of other features also recommend M13 vectors for our work.

(a) Because M13 is a filamentous phage, a relatively large amount of DNA may be inserted into the molecule without disturbing the virus coat packaging process.

(b) M13 does not kill its host cell but allows it to grow at a slower rate while extruding new M13 virus particles through its wall into the growth medium. The yields are very high and purification is simple.

(c) M13-directed DNA replication is easily distinguishable from that of  $\phi$ X174 because it is primed by the rifampicin-sensitive enzyme RNA polymerase rather than by primase.

(d) As a design feature of the M13mp7 cloning vector, a portion of the E. coli lactose operon was added to the virus genome (Messing et al., 1977). In appropriate host cells the  $\beta$ -galactosidase enzyme of the viral lac operon is

expressed and can be detected by the blue color which appears around the virus plaques when they are grown on a special agar medium. A synthetic 42 base-pair "cloning region" which was subsequently inserted near the amino terminus of the viral  $\beta$ -galactosidase gene, does not disrupt its function. The cloning region, however, allows the virus DNA to be cut open into a linear form by any of the five different restriction enzymes. If a DNA fragment from another source is added to the virus at any of the restriction sites in the cloning region and closed again, the function of the surrounding  $\beta$ -galactosidase gene is destroyed. Successful clones, those which have additional DNA within the cloning region, are easily identified by the fact that they no longer produce blue plaques on the screening agar plates.

(e) Messing's laboratory has created a variety of M13 vectors which differ primarily in the sequence of the cloning region and the restriction enzyme sites it contains. The M13mp7 vector is distinctive in that the cloning site is a perfect palindrome. Even in the single-stranded state found in the virus particles, the DNA of the cloning region is expected to form a perfectly base-paired hairpin. If the restriction enzyme which cuts at the point of the hairpin is used to clone foreign DNA fragments, single-stranded DNA of the resulting clone will have a small double-stranded

section between the single-stranded loops of M13 and foreign DNA. The beauty of this arrangement lies in the fact that it allows the two portions of the molecules to be separated from each other using a restriction enzyme which cuts in the double-stranded portion. Restriction enzymes do not usually operate on single-stranded DNA. Since n' protein interacts with its recognition element on  $\phi$ X174 DNA only when the DNA is single-stranded, the characteristics of the M13mp7 vector permit us to amplify and isolate large amounts of the n' recognition element in the single-stranded condition.

## MATERIALS AND METHODS

### A. Bacterial Strains

(i) Escherichia coli K12 JM103 ( $\Delta$ lacpro, thi-, strA<sup>R</sup>, supE, endA<sup>-</sup>, sbcB15, F'traD36, proA<sup>+</sup>B<sup>+</sup>, lacI<sup>q</sup>, lacZ  $\Delta$  M15) (Pratt et al., 1966; Messing et al. 1981). According to recent findings (Felton, 1983) it is a P-1 lysogen and hsdR<sup>+</sup> rather than hsdR<sup>-</sup>. This strain also carries both a proline operon and an incomplete lac operon on the F' plasmid. Other genetic modifications exist which make the cell an ideal host for cloning procedures using M13mp7.

(ii) Escherichia coli C is the wild type host strain for bacteriophage  $\phi$ X174 (Sinsheimer, 1959).

### B. Bacteriophage Strains

(i) M13mp7 (Messing, 1977; Gronenborn and Messing, 1978; Messing, 1981) was used as the cloning vehicle.

(ii)  $\phi$ X174 wt is the wild type virus (Sinsheimer, 1959).

### C. Enzymes and Chemicals

(i) Rifampicin, tris hydroxymethyl aminomethane hydrochloride (Tris-HCL), tris hydroxymethyl aminomethane hydroxide (Tris-OH), agarose (type 1), bromphenol blue, lysozyme (eggwhite, 3X crystallized, grade I), were from the Sigma Chemical Co., St. Louis, MO.

(ii) Ethidium bromide (grade B) and chloramphenicol (grade

B) were purchased from Calbiochem-Behring Co., La Jolla, CA.

(iii) Bacto-tryptone and yeast extract were purchased from Difco Laboratories, Detroit, MI.

(iv) Dimethylsulfoxide (DMSO), phenol, disodium dihydrogen ethylenediamine tetraacetate dihydrate (EDTA), glycerol, polyethylene glycol (PEG 8000), ether, sodium acetate, potassium cyanide, sodium borate 10-hydrate, 2-propanol and other reagent grade chemicals were purchased from J. T. Baker Chemical Co., Phillipsburg, NJ.

(v) Sucrose (ultrapure) was purchased from the Schwarz/Mann Inc., Spring Valley, NY.

(vi) Triton X-100 was purchased from Packard Inst. Co., Inc., Downers Grove, IL.

(vii) S1 endonuclease from Aspergillus oryzae was purchased from Miles Laboratories, Elkhart, IN.

(viii) Ribonuclease A (bovine pancreatic) was purchased from Worthington Biochemicals, Freehold, NJ.

#### D. Media and Solutions

(i) YT broth contains 5.0 g NaCl, 8.0 g tryptone, and 5.0 g yeast extract dissolved in sufficient deionized water to make one liter and adjusted to a pH of 7.0.

(ii) Tris-EDTA is 10 mM Tris-OH, 0.1 mM EDTA. The pH was adjusted to 7.4 (25°C) with HCl.

(iii) STET buffer (Holmes and Quigley, 1981) contains 50 mM Tris-HCl, 8.0% (w/v) sucrose, 5.0% (v/v) triton X-100, and

50 mM EDTA. The pH was 8.0 (25°C).

(iv) Tris-acetate gel buffer (1X) is 40 mM Tris-OH, 5 mM sodium acetate, 1 mM EDTA, 10 mM NaCl, 0.1  $\mu\text{g/ml}$  ethidium bromide. The pH was adjusted to 8.3 (25°C) with glacial acetic acid.

(v) TES buffer is 20 mM Tris-HCl, 1.0 mM EDTA, and 20 mM NaCl. The pH was adjusted to 7.5 (25°C).

(vi) Borate buffer is 50 mM sodium borate, 1.0 mM EDTA, and 10 mM KCN.

(vii) TEPCN buffer is 0.1 M NaCl, 1.0 mM EDTA, 10 mM Tris-OH and 10 mM KCN, adjusted to pH 7.4 (25°C) with HCl.

(viii) S1 endonuclease buffer is 200 mM NaCl, 30 mM sodium acetate, and 5 mM  $\text{ZnSO}_4$ . The pH was adjusted to 5.0.

#### E. M13 Phage Preparations

E. coli JM103 was grown to about  $5 \times 10^8$  cells/ml in YT broth, infected with about two phage per cell and allowed to grow for six hours at 37°C with aeration. Cells were collected by centrifugation at 7,000 revs/min for 15 minutes in a Sorvall GSA rotor at 5°C. Dry PEG 8000 and NaCl were added to the supernatant to final concentrations of 3% (w/v) and 0.5 M, respectively, and the mixture was kept on ice overnight. The precipitate was collected again by centrifugation at 7,000 revs/min for 15 minutes in a Sorvall GSA rotor at 5°C. Phage were eluted from the precipitate by resuspending it three times in 5 ml volumes of TES buffer.



#### F. Phage DNA

One ml of phage preparation was extracted twice with 1 ml volumes of phenol (double-distilled and preserved with 0.1% 8-hydroxyquinoline) saturated with TES buffer. Residual phenol was removed from the remaining aqueous phase by shaking it vigorously twice with 3 ml volumes of ether. Any ether that could not be removed with a Pasteur pipette was evaporated under a gentle stream of N<sub>2</sub> gas. The DNA was precipitated by adding 1/10 volume of 3 M sodium acetate and three volumes of 2-propanol. After eight hours at -15°C, The DNA was collected by centrifugation at 10,000 revs/min for 30 minutes in a Sorvall SS34 rotor at -20°C. The pellets were drained, dried with N<sub>2</sub> and dissolved in 2 ml of Tris-EDTA.

#### G. Assay for Rifampicin-Resistant M13 RF synthesis

A culture of E. coli K12 JM103 was grown overnight in 10 ml of YT broth at 37°C with aeration. The overnight culture was diluted 1:20 in fresh YT broth and incubated at 37°C with aeration. The culture was monitored on a Coulter counter until it reached a concentration of  $5 \times 10^8$  cells/ml. Chloramphenicol (60 mg/ml in ethanol) was added to a final concentration of 150 µg/ml. Ten ml of cells were then placed in sterile bubbler tubes containing premeasured amounts of rifampicin and DMSO. Rifampicin (25 mg/ml in DMSO) had been dispensed into the tubes to provide final

concentrations ranging from 0 to 400  $\mu\text{g/ml}$  after the cells were added. Cell cultures with rifampicin were protected from light. The final concentration of DMSO was 1.0% (v/v). After 15 minutes additional incubation, the cells were infected with phage at a multiplicity of infection (MOI) of about 25. The cells were incubated with the phage for 15 minutes, stopped with equal volumes of ice cold TEPCN buffer and centrifuged for 5 minutes at 7,000 revs/min at  $4^{\circ}\text{C}$  in a Sorvall SS34 rotor. The supernatants were decanted and the pellets were washed twice with 10 ml volumes of TEPCN buffer, collecting cells by centrifugation after each washing. After the last wash the buffer was decanted and droplets on the walls of the centrifuge tubes were wiped away. Each pellet received 0.7 ml of STET buffer and was vortexed to resuspend the cells. A lysozyme solution at 10 mg/ml in Tris-EDTA was added to a final concentration of 667  $\mu\text{g/ml}$ . After waiting one minute, the samples were placed in a boiling water bath for 40 seconds and then centrifuged for 60 minutes at 10,000 revs/min at  $4^{\circ}\text{C}$  in the Sorvall rotor. After transferring the supernatants to clean 15 ml centrifuge tubes, 1/10 volume of 3 M sodium acetate and 3 volumes of 2-propanol were added and the mixture kept at  $-70^{\circ}\text{C}$  for 20 minutes. The samples were then centrifuged for 20 minutes at 10,000 revs/min at  $-20^{\circ}\text{C}$  in the Sorvall SS34 rotor. Supernatants were decanted and the pellets were dissolved in 0.3 ml of Tris-EDTA again adding 1/10 volumes

of sodium acetate and 3 volumes of 2-propanol. After 10 min at  $-70^{\circ}\text{C}$ , samples were centrifuged 30 minutes at 10,000 revs/min at  $-20^{\circ}\text{C}$  in a Sorvall rotor. The pellets were resuspended in 30  $\mu\text{l}$  of S1 endonuclease buffer containing 10  $\mu\text{g}/\text{ml}$  RNase A and 200,000 units/ml of S1 endonuclease. The mixture was incubated at  $37^{\circ}\text{C}$  for 15 min. Glycerol was added to a final concentration of 10% (v/v) and bromphenol blue to a concentration of 0.025% (v/v) before applying the material to agarose gels for electrophoreses. The above procedure was followed for  $\phi\text{X174}$  phage infections as well. However, the cell pellets were washed three times with borate buffer before adding the STET buffer.

#### H. Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed as described by Johnson and Grossman (1977). Slabs of 0.8% (w/v) agarose with 0.1  $\mu\text{g}/\text{ml}$  ethidium bromide and 10 mM NaCl (Keller, 1975; Johnson and Grossman, 1977), 13.5 x 28 cm and 1.5 mm thick in 1X gel buffer were cast in a Hoefer SE 620 Vertical Slab Gel electrophoresis unit. A teflon comb with 10 or 15 teeth was imbedded in the agarose to form the wells. The gel was cooled to approximately  $5^{\circ}\text{C}$  by putting the apparatus under refrigeration. After the gel had solidified, the comb was removed from the gel and the wells were washed with 1X gel buffer.

Samples, described in Methods Section G., approximately

20 1 in volume containing 10% glycerol and 0.025% bromphenol blue were placed in the wells. Electrophoresis took 14 to 24 hours at a constant potential of 100 volts (3.29 v/cm) using a Beckman Duostat regulated D.C. power supply. After electrophoresis the gels were stained in 1X gel buffer containing 0.5  $\mu\text{g}/\text{ml}$  ethidium bromide for at least 30 minutes and then destained in 1X gel buffer for 30 minutes.

In later stages of this work gels were made on a BioRad Laboratories DNA Sub-Cell Horizontal gel electrophoresis unit. These gels were 14.7 x 16.9 x 0.4 cm and were run for two to three hours at a constant 150 mA (about 100 volts).

Stained gels were placed on a Chromato-vue Transilluminator (Model C-61, Ultraviolet Products Inc.) and photographed with a Poloroid CU-5 Industrial Camera equipped with a 590 nm cut-off orange filter (Ealing Corp.) and Poloroid Type 665 positive/negative pack film. Exposures were one and a half to two and a half minutes. Film was developed for 2 minutes, rinsed, and fixed. The negatives were scanned and integrated on a Beckman DU-8 spectrophotometer.

## RESULTS

One of the aims of this work was to design an assay for  $\phi$ X174-directed complementary strand DNA synthesis which could be applied to a large number of isolates (e.g. from cloning or mutagenesis experiments). This goal required that we minimize the time and materials needed to accomplish each assay. In order to determine the minimum amount of phage necessary to adequately infect the host cells we infected E. coli JM103 with varying amounts of M13mp7 phage. The experiment was performed essentially as described in Materials and Methods (section G). Ten ml aliquots of exponentially growing cells were treated with chloramphenicol and infected with phage for 15 minutes. After washing the cells several times, they were lysed and centrifuged. The supernatants were precipitated twice with alcohol, treated with ribonuclease and then applied to 0.8% agarose gels. Figure 7a shows the gel after electrophoresis. Lanes 2 through 9 contain the RF DNA from cells infected with 0, 5, 10, 20, 30, 50, 75, and 100 phage per cell. The intensity of the RF bands increases with the MOI up to about 20 and then remains constant. The RF bands in each lane can be identified by their co-migration with RF DNA marker (lane 1). Each well also contains an intense, slowly-migrating band. Since this band appears in samples which were not infected with phage, we presume that it is the F' plasmid in the host strain (see Materials and

Methods, section A).

Similar results were obtained with E. coli C cells infected with bacteriophage  $\phi$ X174 (Figure 7b). Again lanes 2 through 9 represent the products of cells infected with increasing numbers of phage. The amount of RF produced did not increase appreciably at MOIs greater than 20. An MOI of 25 was used for all subsequent experiments.

It is difficult, especially in M13 infections, to completely remove phage particles and single-stranded DNA from the newly infected cells. In order to avoid confusing single-stranded molecules with RF DNA, we sought electrophoresis conditions which would provide optimal resolution between the two types of molecules. When 10 mM NaCl was added to the normal constituents of an 0.8% agarose gel and gel buffer (see Materials and Methods, section H) single-stranded M13 DNA ran slightly slower than RF I molecules. Figure 8 compares the migration rates of single-stranded DNA (lane 1) and RF DNA (lane 2). Most RF samples contained a small amount of single stranded DNA which could be identified by its co-migration with the single-stranded DNA marker in the adjacent lane. When the gel and buffer contained 0.1  $\mu$ g/ml ethidium bromide and only 1 mM NaCl, the single-stranded band migrated just ahead of RF I. However, discrimination between the two was still difficult (Figure 8b). Better separation was achieved when the gel and gel buffer contained both 10 mM NaCl and 0.1  $\mu$ g/ml ethidium

bromide (Figure 8c). Under these conditions the single-stranded band (lane 1) migrated almost 30% farther than the RF band (lane 2). We also found that resolution was affected by the electrical field strength. At higher voltages the individual bands were less sharp and the separation between single-stranded and RF molecules was less (data not shown). All subsequent gels contained 10 mM NaCl, 0.1  $\mu\text{g/ml}$  ethidium and were run at 70 volts (typically 140 mA) for 2 to 5 hours.

Rifampicin inhibits the enzyme RNA polymerase. It was necessary to determine the concentration of rifampicin which was sufficient to prevent the conversion of M13 single-stranded DNA to parental RF under our experimental conditions. E. coli JM103 cells were infected with M13mp7 phage in the presence of increasing amounts of rifampicin. Rifampicin at 25 mg/ml was dissolved in DMSO. The best results were obtained when 1.0% (v/v) DMSO was added to aid penetration of rifampicin into the cells (data not shown). After the infection the cells were washed twice with TEPCN buffer. The rapid boiling method of Holmes and Quigley (1981) was employed to release the RF in order to minimize the length of time required for the assay. After lysis the samples were centrifuged and the supernatants, after alcohol precipitation and RNase digestion, were applied to 0.8% agarose gels. Figure 9a shows a series of samples from infections performed with 0, 0.5, 1, 2, 4, 6, 8, 10, 20, 40,

70, and 100  $\mu\text{g/ml}$  rifampicin (lanes 3 through 14). Without rifampicin (lane 3) a bright band of RF DNA appeared in the lysate. The band was undetectable at levels of rifampicin above 40  $\mu\text{g/ml}$  (lanes 12-14). 1% DMSO was present in all the cultures.

The effect of the antibiotic on M13mp7 was compared to its effect on a clone of the virus which contained a fragment of  $\phi\text{X174}$  DNA that included the n' recognition element. The recombinant virus (designated M13mp7-M9) was used to infect portions of the same JM103 culture used in the previous experiment with rifampicin at the same concentrations. The lysates were processed simultaneously with those of the M13mp7-infected cultures described in the previous paragraph and were applied to a separate agarose gel. A photograph of the gel (Figure 9b) reveals a band of RF DNA (lane 3) which decreases in intensity as the concentration of rifampicin is increased, but remains visible even at the highest concentration (100  $\mu\text{g/ml}$ , lane 14).

The RF bands in the photographic negatives of Figure 9 were quantitated by densitometer scanning and integration in a Beckman DU-8 spectrophotometer. The graph in Figure 10 portrays the relative amounts of RF DNA in the wells as a function of the rifampicin concentration for both M13mp7 and the recombinant clone M13mp7-M9. M13mp7 RF disappears at 40  $\mu\text{g/ml}$ . However, the apparent 85% decrease in the amount of



RF from M13mp7-M9 relative to the rifampicin-free condition was unexpected and somewhat suspicious. We considered it possible that without rifampicin the assay detected not only parental RF made directly from infecting phage DNA but also progeny RF synthesized by subsequent replication of the parental molecules.

Chloramphenicol has been used to prevent RF replication in infections with the single-stranded DNA phages (Tessman, 1966). At concentrations above 150  $\mu\text{g}/\text{ml}$  protein synthesis in the cells is abolished. Since RF replication requires the action of phage-coded proteins produced after infection, this stage of viral replication is prevented by chloramphenicol. Parental RF synthesis, however, is not affected by the antibiotic. The relative amounts of RF produced in the presence or absence of chloramphenicol can be seen in Figure 11. With the addition of 150  $\mu\text{g}/\text{ml}$  of chloramphenicol (lane 4) the RF band is much fainter than the band representing RF produced in the absence of chloramphenicol (lane 2).

We repeated the experiments described above with the addition of chloramphenicol (and rifampicin, where indicated) to the cells 15 minutes before infection. Samples were incubated with S1 nuclease prior to application to the gel to prevent single-stranded DNA from interfering in the visualization of RF bands. Lysates of cells infected with M13mp7 in the presence of chloramphenicol and 0, 20,

50, 100, 200, 300, 400  $\mu\text{g}/\text{ml}$  rifampicin are shown after electrophoresis in Figure 12a (lanes 3-9). The RF bands quickly decrease in intensity and are not visible at the higher rifampicin concentrations. A graph of the relative amounts of DNA in these bands obtained by densitometry of the negative indicated that RF production approaches zero at less than 100  $\mu\text{g}/\text{ml}$  of rifampicin (Figure 13).

The results of the corresponding experiment with M13mp7-M9, performed under the same condition, are shown in Figure 12b. The recombinant phage carrying the  $\phi\text{X174}$  replication signal is distinctly resistant to rifampicin and is converted to RF even at the highest level tested (400  $\mu\text{g}/\text{ml}$ , Figure 12b, lane 9). On a quantitative basis (Figure 13) the synthesis of parental RF from this virus clone is not significantly diminished by rifampicin up to 300  $\mu\text{g}/\text{ml}$ .

Bacteriophage  $\phi\text{X174}$  was grown in its host, E. coli C, and the experiment was repeated as above. The RF bands do not show a significant drop in intensity even at the highest rifampicin concentration tested (400  $\mu\text{g}/\text{ml}$ , Figure 14, lane 9). This is attributed to the fact that  $\phi\text{X174}$  uses primase to initiate replication and is not inhibited by rifampicin.

## DISCUSSION

The aim of this work was to develop a rapid assay by which numerous clones could be rapidly screened to determine whether a specific  $\phi$ X174 DNA segment was functioning in an M13 vector. The method we have developed takes advantage of the fact that the first step of phage M13 replication, conversion of phage single-stranded DNA to the parental replicative form (RF) DNA, required RNA polymerase for priming. When the conversion of infecting phage DNA to a duplex form is performed under conditions which inhibit RNA polymerase activity, synthesis of DNA, which is independent of RNA polymerase function, can be detected and measured by the efficiency of the conversion.

The replication system used by the vector M13 is quite different from that of  $\phi$ X174. Site-specific initiation and replication of M13 DNA coated with SSB depends on transcription by rifampicin-sensitive RNA polymerase holoenzyme with a functional sigma subunit. The specificity of initiation sites in M13 is due to a stretch of DNA not coated by SSB.

In  $\phi$ X174 the conversion to a prepriming intermediate is performed by six proteins:  $n$ ,  $n'$ ,  $n''$ ,  $i$ , dnaB, and dnaC (Fig. 1). Protein dnaB interacts with single-stranded DNA to form a unique secondary structure recognized by primase, the protein coded for by the dnaG gene.

n'', i, dnaC, and dnaB proteins to assemble. Protein i together with dnaC triggers a sequence of reactions which lead to the incorporation of dnaB protein and formation of the replication intermediate. Protein dnaB interacts with single-stranded DNA to form a unique secondary structure recognized by primase, the protein coded for by the dnaG gene.

Rifampicin, an antibiotic which specifically inhibits the E. coli RNA polymerase, blocks the conversion of single-stranded DNA of bacteriophage M13 to double-stranded RF as well as inhibiting the further multiplication of RF (Brutlag et al., 1971). The antibiotic binds firmly to the beta subunit of RNA polymerase and blocks initiation of RNA chains (Wehrli and Staehelin, 1971). The complex formed by RNA polymerase and rifampicin fails at the translocation step following the formation of the first phosphodiester bond (McClure and Cech, 1978) and, when bound at the promoter site, becomes a barrier to transcription by other RNA molecules initiated upstream (Kassavetis et al., 1978). Bacteriophages G4 and  $\phi$ X174 as well as the ColE1 plasmid depend on primase to perform the priming function and are therefore not affected by rifampicin. Likewise an M13 vector into which the initiation site from one of these molecules has been cloned will be able to replicate in the presence of rifampicin via the alternative initiation system.

Our first attempt to detect M13 clones with an inserted recognition site was to perform the standard phage plaque assay in the presence of rifampicin. We hoped that bacterial RNA synthesis would not be as sensitive to rifampicin as the initiation of M13 DNA synthesis. The experiment showed however, that any concentration of rifampicin in the medium which was low enough to allow the plating cells to grow (less than 2.5  $\mu\text{g/ml}$ ) also permitted the formation of plaques by the original M13mp7 vector virus. A more sophisticated assay which could detect the conversion of M13 single-stranded DNA into RF with rifampicin-inhibited cells was devised based upon the procedure of Nomura and Ray (1980). Other researchers working with the replication origin of phage G4 (Kaguni and Ray, 1979), the strand initiation sequence of ColE1 plasmid (Nomura and Ray 1980; 1982), mini-F plasmid (Imber et al., 1983), and  $\phi\text{X174}$  (Strathearn et al., 1983), have shown that rifampicin-resistant origins of replication inserted into bacteriophage M13 can be detected by in vivo assays.

The procedure is particularly well adapted to our needs as it allows for the testing of 20 to 30 samples during one day. The culture size of 10 ml is small enough to allow for easy handling and simultaneous processing of multiple samples.

Rifampicin and chloramphenicol were added to the cultures shortly before infection. By inhibiting protein

synthesis, chloramphenicol at 150  $\mu\text{g}/\text{ml}$  prevents the subsequent formation of progeny RF (Tessman, 1966).  $\phi\text{X174}$  and M13 RF replication require the action of a phage-coded protein, gene A and gene-II, respectively. Therefore, when chloramphenicol is present, only parental RF is made. In the absence of chloramphenicol the comparison between cultures infected with and without rifampicin is distorted by the fact that parental RF molecules are free to replicate further to create progeny RF molecules. This secondary replication occurs to varying degrees at different rifampicin concentrations.

Dimethylsulfoxide (DMSO) was included in the assay to enhance the absorption of rifampicin (Nomura and Ray, 1980). At a concentration of 1.0% (v/v) DMSO increased the effectiveness of the rifampicin, preventing the appearance of M13 RF. At the same concentration it had no effect on  $\phi\text{X174}$  RF formation at various rifampicin concentrations.

After allowing 10 minutes for the rifampicin and chloramphenicol to take effect, cells were infected with phage to a multiplicity of infection of 25 which we found to be close to the maximum capacity of E. coli host cells. Fifteen minutes after infection the cultures were poured into cold buffer containing KCN. The cells were washed several times in buffer in order to remove extracellular phage (Newbold and Sinsheimer, 1970). It was important to remove these phage since the single-stranded DNA which was

released from them in subsequent steps could be confused with RF DNA after gel electrophoresis.

The rapid boiling method of lysis developed by Holmes and Quigley (1981) was employed and proved to be faster and more convenient than the standard lysis procedure generally used for RF isolation (Godson and Boyer, 1974). The rapid boiling method requires a brief exposure of bacterial cells to 100°C in the presence of agents that weaken the cell wall and which help prevent DNA degradation by nucleases. Furthermore, the heating period irreversibly inhibits DNases. Presumably, the role of the lysozyme is to weaken the bacterial cell wall even though the reaction must take place in the brief period following addition of the enzyme and prior to the boiling. In addition, since lysozyme is positively charged, it will interact with the host DNA and may assist in the formation of the insoluble clot. Finally lysozyme may aid in releasing the phage RF from possible cell wall attachment sites (Holmes and Quigley, 1981). The precipitate which forms after cooling and centrifugation is presumably partially denatured genomic DNA and denatured proteins. The majority of the bacterial RNA and RF remain in solution and can be recovered by subsequent alcohol precipitation.

The samples were incubated with RNase and S1 nuclease just prior to electrophoresis. This prevented interference of RNA and single-stranded DNA with the visualization of the

RF bands. S1 nuclease is an endonuclease which cleaves single-stranded DNA (Vogt, 1973). In order to minimize cutting at transient single-stranded regions which exist in supercoiled and nicked double-stranded DNA, incubation was at 37°C for 15 minutes in 200 mM salt (Germond et al., 1974).

When samples were run on agarose gels using standard Tris-acetate gel buffer pH 8.3, the RF I band ran nearly the same distance as single-stranded DNA and it was difficult to determine the correct composition of a band based upon its location in the gel. In some cases the RF I band ran slightly faster than single-stranded DNA. Resolution between single-strand DNA and RF I on agarose gels was greatly improved when 10 mM NaCl and 0.1  $\mu\text{g/ml}$  ethidium bromide were added to the gel and gel buffer (Johnson and Grossman, 1977). Single-stranded DNA ran approximately 30% farther than the RF I band. Normally, closed circular duplex DNA (RF 1) contains super-helical turns which the physical-chemical forces of the Watson-Crick structure maintain in the molecule. Ethidium bromide works by binding to DNA at sites which appear to be saturated when one drug molecule is bound for every 4 or 5 nucleotides (Waring, 1965). This binding causes unwinding of the helix. With increasing amounts of ethidium bromide, the RF I superhelical DNA is first unwound to a relaxed condition and then becomes overwound in the opposite direction. This accounts for the



changes in its migration rate. At low levels of ethidium bromide, 0.1  $\mu\text{g/ml}$ , the RF I is unwound and does not migrate as rapidly as in the absence of ethidium bromide. This allows it to be distinguished from single-stranded DNA.

As shown in Figure 14,  $\phi\text{X174}$  parental RF formation occurs at rifampicin levels up to 400  $\text{mg/ml}$ . In comparison, M13mp7 parental RF synthesis decreases as the amount of rifampicin is increased and is completely abolished at levels above 200  $\mu\text{g/ml}$  (Figure 12a). When an M13mp7 clone carrying the  $\phi\text{X174}$  complementary strand replication origin (clone M9) was tested under the same conditions (Figure 12b) we observed the formation of replicative form DNA at concentrations of rifampicin up to 400  $\mu\text{g/ml}$ . This suggests that RF formation is occurring due to the incorporated n' recognition site from  $\phi\text{X174}$ .

Because this particular assay can differentiate between clones with functional and nonfunctional  $\phi\text{X174}$  replication sites, it can be used as a tool in further research. Should point mutations or deletions remove nucleotides vital to the n' recognition site, the clone could lose its ability to replicate under the conditions of the assay. Sequence analysis of these nonreplicating mutants would identify the specific nucleotides involved in the interaction. Identification of the specific nucleotides in the DNA and the amino acids in the protein which are directly involved

in the recognition process will lead to the construction of a conceptual and physical model of the interaction.

## LITERATURE CITED

- Arai, K., Kornberg, A. (1979) A general priming system employing only dnaB protein and primase for DNA replication. *Proc. Natl. Acad. Sci. USA* 76: 4308-4312
- Arai, K., Kornberg, A. (1981a) Mechanism of dnaB protein action. II. ATP hydrolysis by dnaB protein dependent on single- or double-stranded DNA. *J. Biol. Chem.* 256: 5253-5259
- Arai, K., Kornberg, A. (1981b) Mechanism of dnaB protein action. III. Allosteric role of ATP in the alteration of DNA structure by dnaB protein in priming replication. *J. Biol. Chem.* 256: 5260-5266
- Arai, K., Kornberg, A. (1981c) Unique primed start of phage  $\phi$ X174 DNA replication and mobility of the primosome in a direction opposite chain synthesis. *Proc. Natl. Acad. Sci. USA* 78: 69-73
- Arai, K., Low, R. L., Kornberg, A. (1981a) Movement and site selection for priming by the primosome in phage  $\phi$ X174 DNA replication. *Proc. Natl. Acad. Sci. USA* 78: 707-711
- Arai, K., McMacken, R., Yasuda, S., Kornberg, A. (1981b) Purification and properties of Escherichia coli protein i, a prepriming protein in  $\phi$ X174 DNA replication. *J. Biol. Chem.* 256: 5281-5286
- Arai, K., Yasuda, S., Kornberg, A. (1981c) Mechanism of dnaB protein action. I. Crystallization and properties of dnaB protein, an essential replication protein in Escherichia coli. *J. Biol. Chem.* 252: 5247-5252
- Boldicke, T. W., Hillenbrand, G., Lanka, E., Staudenbauer, W. L. (1981) Rifampicin-resistant initiation of DNA synthesis on the isolated strands of ColE plasmid DNA. *Nucleic Acids Res.* 9: 5215-5231
- Bouche, J. P., Rowen L., Kornberg, A. (1978) The RNA primer synthesized by primase to initiate phage G4 DNA replication. *J. Biol. Chem.* 252: 765
- Brutlag, D., Schekman, R., Kornberg, A. (1971) A possible role for RNA polymerase in the initiation of M13 DNA synthesis. *Proc. Natl. Acad. Sci. USA* 68: 2826-2829

- Denhardt, D. T., (1975). The single-stranded DNA phages. C. R. C. Crit. Rev. Microbiol. 4: 161-222
- Fiddes, J. C., Godson, G. M. (1978) Nucleotide sequence of the J gene and surrounding untranslated regions of phage G4 DNA: comparison with phage  $\phi$ X174. Cell 15: 1045-1053
- Geider, K., Beck, E., Schaller, H. (1978) An RNA transcribed from DNA at the origin of phage fd single strand to replicative form conversion. Proc. Natl. Acad. Sci. USA 75: 645-649
- Germond, J. E., Vogt, V. M., Hirt, B. (1974) Characterization of the single-strand-specific nuclease S1 activity on double-stranded supercoiled polyoma DNA. Eur. J. Biochem. 43: 591-600
- Godson, G. N., Barrell, B. G., Staden, R., Fiddes, J. C. (1978) Nucleotide sequence of bacteriophage G4 DNA. Nature 276: 236-247
- Godson, G. N., Boyer, H. (1974) Susceptibility of the  $\phi$ X-like phages G4 and G14 to R Eco RI endonuclease. Virol. 62: 270-275.
- Gronenborn, B., Messing, J. (1978) Methylation of single-stranded DNA in vitro introduces new restriction endonuclease cleavage sites. Nature 272: 375-377
- Holmes, D. S., Quigly, M. (1981) A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. 114: 193-197
- Imber, R., Low, R. L., Ray, D. S. (1983) Identification of a primosome assembly site in the region of the ori 2 replication origin of the Escherichia coli mini-F plasmid. Proc. Natl. Acad. Sci. USA 80: 7132-7136.
- Johnson, P. H., Grossman, L. I. (1977) Electrophoresis of DNA in agarose gels. Optimizing separations of conformational isomers of double- and single-stranded DNAs. Biochem. 16: 4217-4225
- Kaguni, J. M., Kornberg, A. (1982) The subunit of RNA polymerase holoenzyme confers specificity in priming M13 viral DNA replication. J. Biol. Chem. 257: 5437-5443
- Kaguni, J., Ray, D. S. (1979) Cloning of a functional replication origin of phage G4 into the genome of phage M13. J. Mol. Biol. 135: 863-878

- Kassavetis, G. A., Kaya, K. M., Chamberlin, M. J. (1978) Escherichia coli RNA polymerase-rifampicin complexes bound at promoter sites block RNA chain elongation by Escherichia coli RNA polymerase and T7-specific RNA polymerase. Biochem. 17: 5798-5804
- Kobori, J. A., Kornberg, A. (1982a) The Escherichia coli dnaC gene product II. Purification, physical properties, and role in replication. J. Biol. Chem. 257: 13763-13769
- Kobori, J. A., Kornberg, A. (1982b) The Escherichia coli dnaC gene product III. Properties of the dnaB-dnaC protein complex. J. Biol. Chem. 257: 13770-13775
- Kornberg, A. (1978) Aspects of DNA replication. Cold Spring Harbor Symp. Quant. Biol. 43: 1-9
- Kornberg, A. (1982) DNA Replication, 1982 supplement p. 5123 W. H. Freeman and Company, San Francisco, CA.
- Low, R. L., Arai, K., Kornberg, A. (1981) Conservation of the primosome in successive stages of  $\phi$ X174 DNA replication. Proc. Natl. Acad. Sci. USA 78: 1436-1440
- Low, T. L., Shlomai, J., Kornberg, A. (1982) Protein n, a primosomal DNA replication protein of Escherichia coli. Purification and characterization. J. Biol. Chem. 257: 6242-6250
- McClure, R., Cech, C. L. (1978) On the mechanism of rifampicin inhibition of RNA synthesis. J. Biol. Chem. 253: 8949-8956
- McMacken, R., Kornberg, A. (1978) A multienzyme system for priming the replication of  $\phi$ X174 viral DNA. J. Biol. Chem. 253: 3313
- McMacken, R., Ueda, K., Kornberg, A. (1977) Migration of Escherichia coli dnaB protein on the template DNA strand as a mechanism in initiating DNA replication. Proc. Natl. Acad. Sci. USA 74: 4294-4194
- Messing, J., Crea, R., Seeburg, P. H. (1981) A system for shotgun DNA sequencing. Nucleic Acids Res. 9: 309-321
- Messing, J., Gronenborn, B., Muller-Hill, B., Hofschneider, P. H. (1977) Filamentous coliphage M13 as a cloning vehicle: Insertion of a HindII fragment of lac regulatory region in M13 replicative form in vitro. Proc. Natl. Acad. Sci. USA 74: 3642-3646

- Meyer, R. R., Shlomai, J., Kobori, J., Bates, D. L., Rowen, L., McMacken, R., Ueda, K., Kornberg, A. (1978) Enzymatic conversion of single-stranded  $\phi$ X174 and G4 circles to duplex forms: discontinuous replication. Cold Spring Harbor Symp. Quant. Biol. 43: 289-293
- Newbold, J. E., Sinsheimer, R. L. (1970) The process of infection with bacteriophage  $\phi$ X174 XXXII. Early steps in the infection process: Attachment, eclipse and DNA penetration. J. Mol. Biol. 49: 49-66
- Nomura, N., Low, R. L., Ray, D. S. (1982) Identification of ColE1 DNA sequences that direct single-strand to double-strand conversion by a  $\phi$ X174 type mechanism. Proc. Natl. Acad. Sci. USA 79: 3153-3157
- Nomura, N., Ray, D. S. (1980) Expression of a DNA strand initiation sequence of ColE1 plasmid in a single-stranded DNA phage. Proc. Natl. Acad. Sci. USA 77: 6566-6570
- Pollock, T. J., Tessman, I., Tessman, E. S. (1978) Potential for variability through multiple gene products of bacteriophage  $\phi$ X174. Nature 274: 34-37
- Reha-Krantz, L. J., Hurwitz, J. (1978) The dnaB gene products of Escherichia coli I. Purification, homogeneity, and physical properties. J. Biol. Chem. 253: 4043-4050
- Rowen, L., Kornberg, A. (1978) Primase, the dnaG protein of Escherichia coli. An enzyme which starts DNA chains. J. Biol. Chem. 253: 758-764
- Sanger, F., Coulson, A. R., Friedmann, T., Air, G. M., Barrell, B. G., Brown, N. L., Fiddes, J. C., Hutchison, C. A. III., Slocumb, P. M., Smith, M. (1978) The nucleotide sequence of bacteriophage  $\phi$ X174. J. Mol. Biol. 125: 225-246
- Shaw, D. C., Walker, J. E., Northrop, F. D., Barrell, B. G., Godson, G. N., Fiddes, J. C. (1978) Gene K, a new overlapping gene in bacteriophage G4. Nature 272: 510-515
- Shine, J., Delgarno, L. (1974) The 3'-terminal sequence of Escherichia coli 16S ribosomal RNA: Complementary to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA 71: 1342-1346

- Shlomai, J., Kornberg, A. (1980a) A prepriming DNA replication enzyme of Escherichia coli. I. Purification of protein n': A sequence-specific, DNA-dependent ATPase. *J. Biol. Chem.* 255: 6789-6793
- Shlomai, J., Kornberg, A. (1980b) A prepriming DNA replication enzyme of Escherichia coli. II. Actions of protein n': A sequence-specific, DNA-dependent ATPase. *J. Biol. Chem.* 255: 6794-6798
- Shlomai, J., Kornberg, A. (1980c) An Escherichia coli replication protein that recognizes a unique sequence within a hairpin region in  $\phi$ X174 DNA. *Proc. Natl. Acad. Sci. USA* 77: 799-803
- Shlomai, J., Polder, L., Arai, K., Kornberg, A. (1981) Replication of  $\phi$ X174 DNA with purified enzymes. I. Conversion of viral DNA to a supercoiled, biologically active duplex. *J. Biol. Chem.* 256: 5233-5238
- Sinsheimer, R. L. (1959) Purification and properties of bacteriophage  $\phi$ X174. *J. Mol. Biol.* 1: 37-53
- Strathearn, M. D., Low, R. L., Ray, D. S. (1984) Selective cloning of a DNA single-strand initiation determinant from  $\phi$ X174 replicative form DNA. *J. Virol.* 49: 178-182
- Tessman, E. S. (1966) Mutants of bacteriophage M13 blocked in infections DNA synthesis. *J. Mol. Biol.* 49: 218-236
- Tessman, E. S., Tessman, I., Pollock, T. J. (1980) Gene K of bacteriophage  $\phi$ X174 codes for a nonessential protein. *J. Virol.* 33: 557-560
- Vogt, V. M. (1973) Purification and further properties of single-strand-specific nuclease from Aspergillus oryzae. *Eur. J. Biochem.* 33: 192-200
- Waring, M. J. (1965) Complex formation between ethidium bromide and nucleic acids. *J. Mol. Biol.* 13: 269-282
- Wehrli, W., Staehelin, M. (1971) Actions of rifamycins. *Bact. Rev.* 35: 290-209
- Wickner, S., Hurwitz, J. (1975) Association of  $\phi$ X174 DNA-dependent ATPase activity with an Escherichia coli protein, replication factor Y, required for in vitro synthesis of  $\phi$ X174 DNA. *Proc. Natl. Acad. Sci. USA* 72: 3342-3346

- Wickner, S. H., (1978) DNA replication proteins of Escherichia coli. Ann. Rev. Biochem. 47: 1163-1191
- Zipursky, S. L., Marians, K. J. (1980) Identification of two Escherichia coli factor Y effector sites near the origins of replication of the plasmids ColE1 and pBR322. Proc. Natl. Sci. USA 77: 6521-6525



APPENDIX

Figure 1. The cistron map of  $\phi$ X174. The positions of 10 cistrons (eleven if the protein fragment A\* is counted) and the functions of their protein products are shown. Flanking the genes H, G, and F, are four spaces indicating the intercistrons J-F, F-G, G-H, and H-A. Three mRNA initiation sites are also indicated.

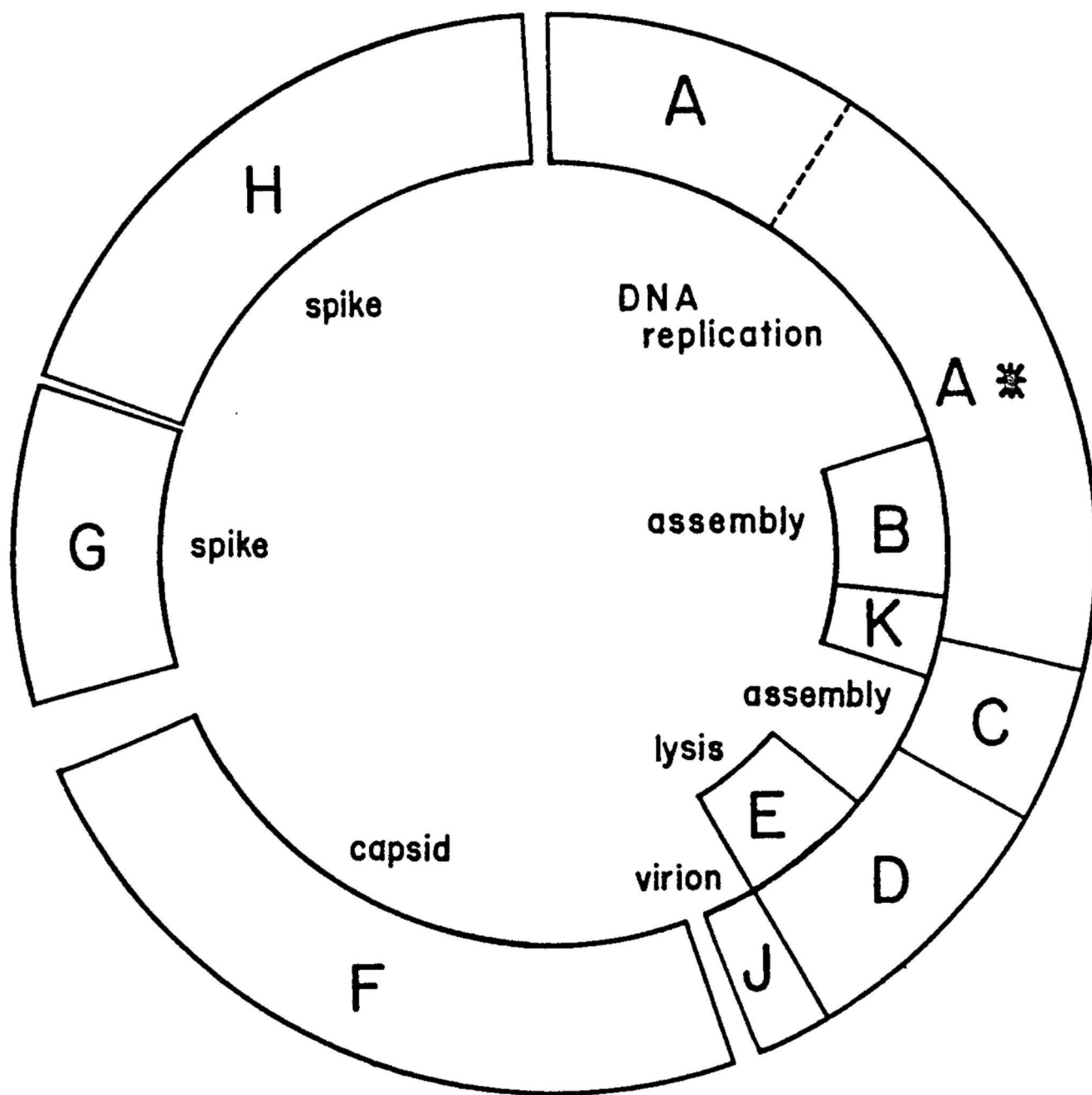


Figure 2. Nucleotide sequence and proposed secondary structure of a portion of the F-G intercistron of  $\phi$ X174.



Figure 3. Stages of DNA synthesis in  $\phi$ X174 infections. (a) Parental RF synthesis: Single-stranded viral DNA enters the cell and serves as a template for the formation of a complementary strand. The resultant molecule is a double-stranded parental replicative form (RF). (b) RF progeny molecules. (c) Single-stranded DNA synthesis: The progeny RF produce viral-type single-stranded DNA by an asymmetrical rolling circle mechanism. The DNA is packaged into phage coats and released upon lysis.

## STAGES OF DNA SYNTHESIS IN ØX174 INFECTIONS

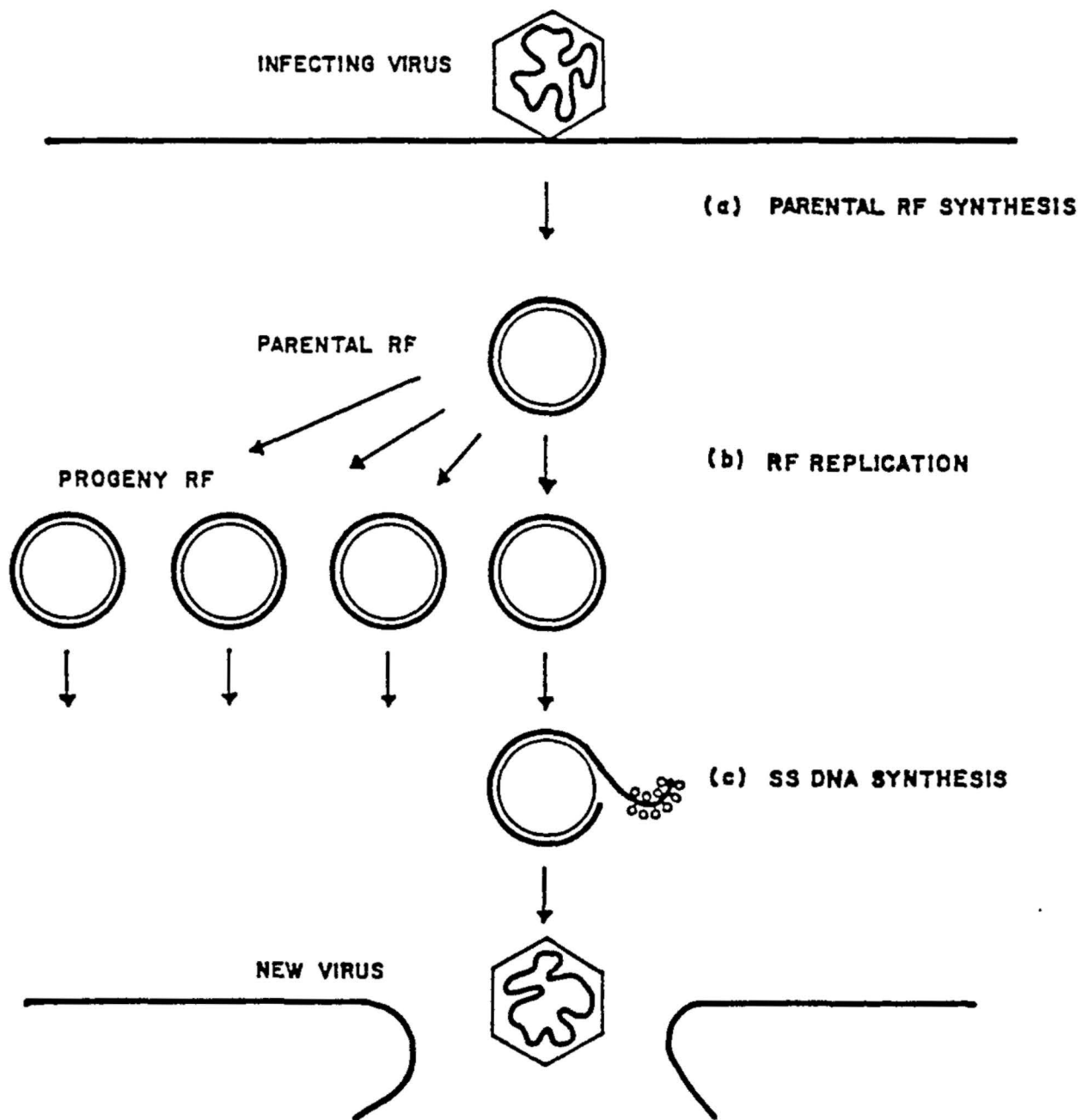


Figure 4. A model for DNA chain growth at one of the forks of a bidirectionally replicating E. coli chromosome showing the interactions of the formation and action of the primosome on the lagging strand.



## CHAIN GROWTH AT THE REPLICATING FORK OF E. COLI

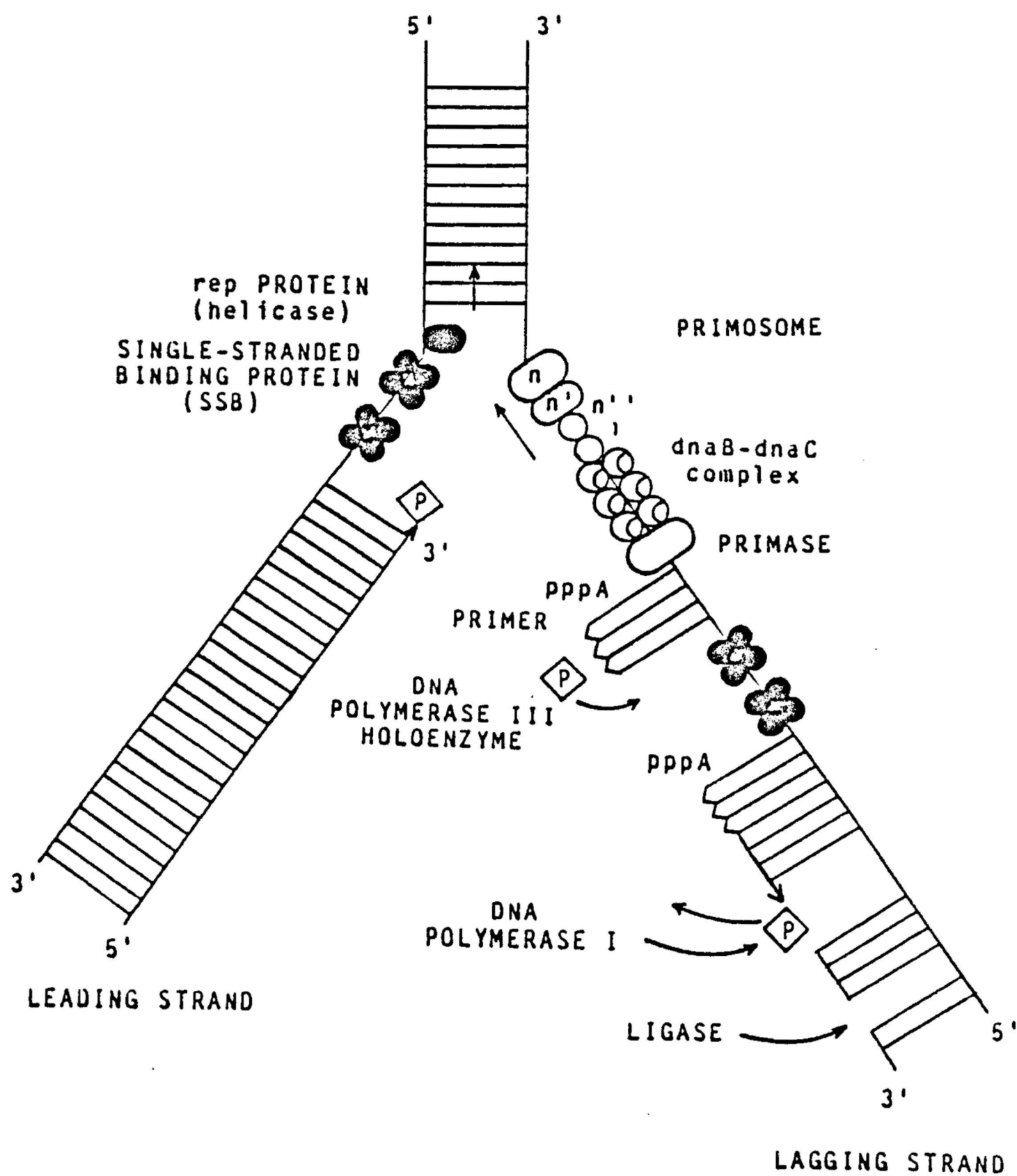


Figure 5. Replication proteins of E. coli involved in the conversion of  $\phi$ X174 single-stranded DNA into parental replicative form. The native molecular weight, number of subunits, functions and estimated number of molecules per cell are from Kornberg (1982).

## REPLICATION PROTEINS OF E. COLI

Protein	Native mass, kdal	Subunits	Function	Estimated molecules/cell
SSB	74	4	single-strand binding	300
protein i	66	3		50
protein n	28	2	primosome	80
protein n'	76	1	assembly	70
protein n''	17	1	and	--
dnaC	29	1	function	100
dnaB	300	6		20
primase	60	1	primer synthesis	50
pol III				
holoenzyme	760	2		20
	140	1		--
	25	1	processive	--
	10	1	chain	--
	37	1	x2 elongation	300
	52	1		20
	32	1		
	83	1		
pol I	102	1	gap filling	300
			primer excision	
ligase	74	1	ligation	300
gyrase	400	4	supercoiling	--
gyrA	210	2		250
gyrB	190	2		25
rep	65	1	helicase	50
helicase II	75	1	helicase	5000
dnaA	48		origin of replication	200

Figure 6. Priming systems of bacteriophages M13 and  $\phi$ X174. Bacteriophage M13 needs only RNA polymerase to initiate replication on an SSB Protein coated molecule. Recognition of a specific initiation site is signaled by a small region not coated with SSB. In contrast,  $\phi$ X174 requires six prepriming proteins as well as primase to initiate replication. No portion of the DNA molecule is left uncoated by SSB. Protein n' is assigned the function of initiation site recognition.

## INITIATION OF REPLICATION IN M13

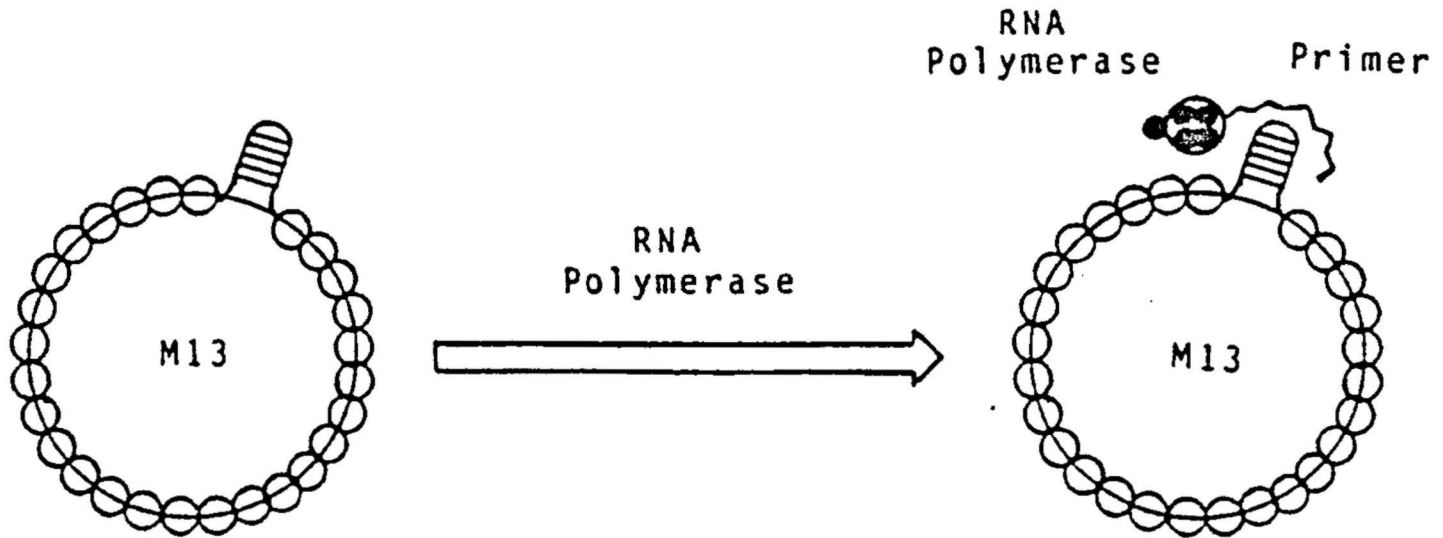
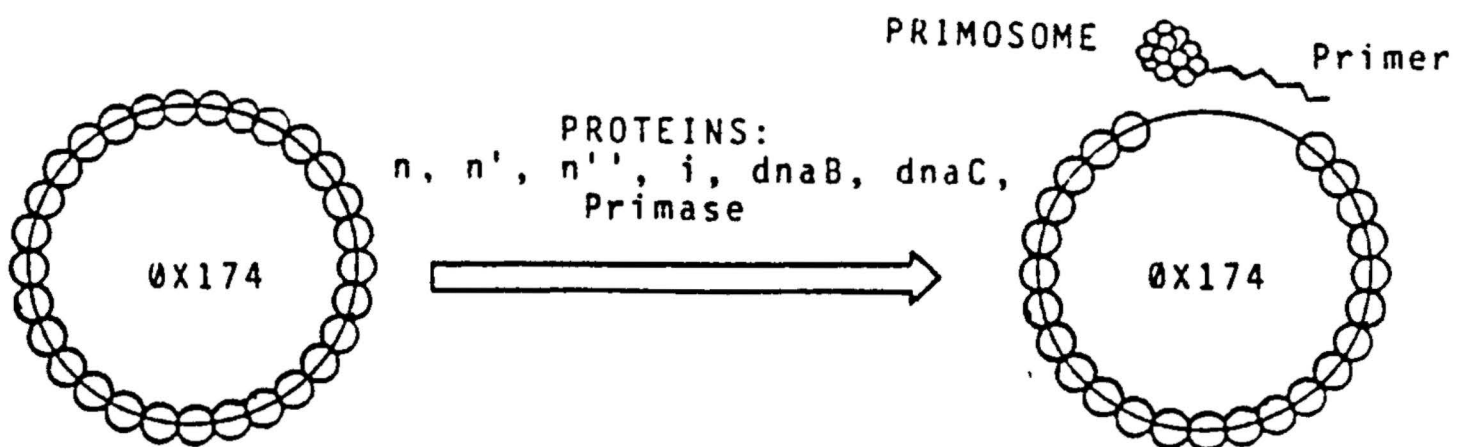
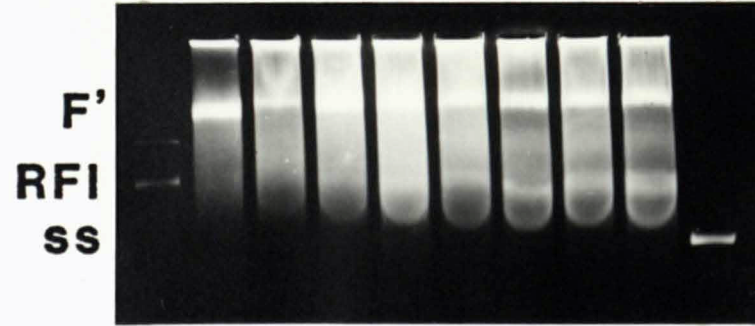
INITIATION OF REPLICATION IN  $\phi$ X174

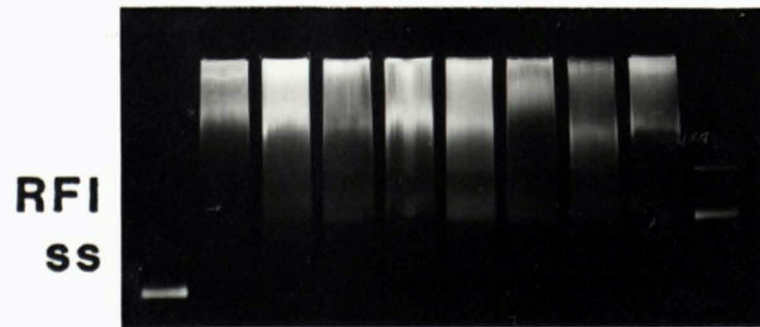
Figure 7. The effect of multiplicity of infection on the yield of parental RF. M13 RF synthesis was assayed as described in Materials and Methods (section G) except rifampicin and S1 nuclease were not used. The lysates were applied to an 0.8% agarose gel (Materials and Methods, section H) and run for two hours at 100v. (Panel a) Lanes 1 and 10 contain M13mp7 RF DNA and single-stranded DNA markers, respectively. Lane 2 contains a lysate of uninfected cells. Lanes 3 to 9 show the parental RF from cultures infected at MOI's of 5, 10, 20, 30, 50, 75, and 100 phage per cell. (Panel b) Lanes 1 and 10 contain  $\phi$ X174 single-stranded DNA and RF DNA, respectively. Lane 2 contains a lysate of uninfected cells. Lanes 3 to 9 show the parental RF from cultures infected at MOI's of 5, 10, 20, 30, 50, 75, 100.

Lane 1 2 3 4 5 6 7 8 9 10



(a)

Lane 1 2 3 4 5 6 7 8 9 10



(b)

Figure 8. Effects of NaCl and ethidium bromide on the migration of RF and single-stranded DNA. M13mp7 single-stranded DNA and RF DNA were applied to 0.8% agarose gels containing varying concentrations of NaCl and ethidium bromide. In each case the RF marker also contained a small amount of single-stranded DNA. Gels were run for 2 hours at 100v. (Panel a) 10 mM NaCl, no ethidium bromide. (Panel b) 1 mM NaCl, 0.1  $\mu\text{g/ml}$  ethidium bromide. (Panel c) 10 mM NaCl, 0.1  $\mu\text{g/ml}$  ethidium bromide.



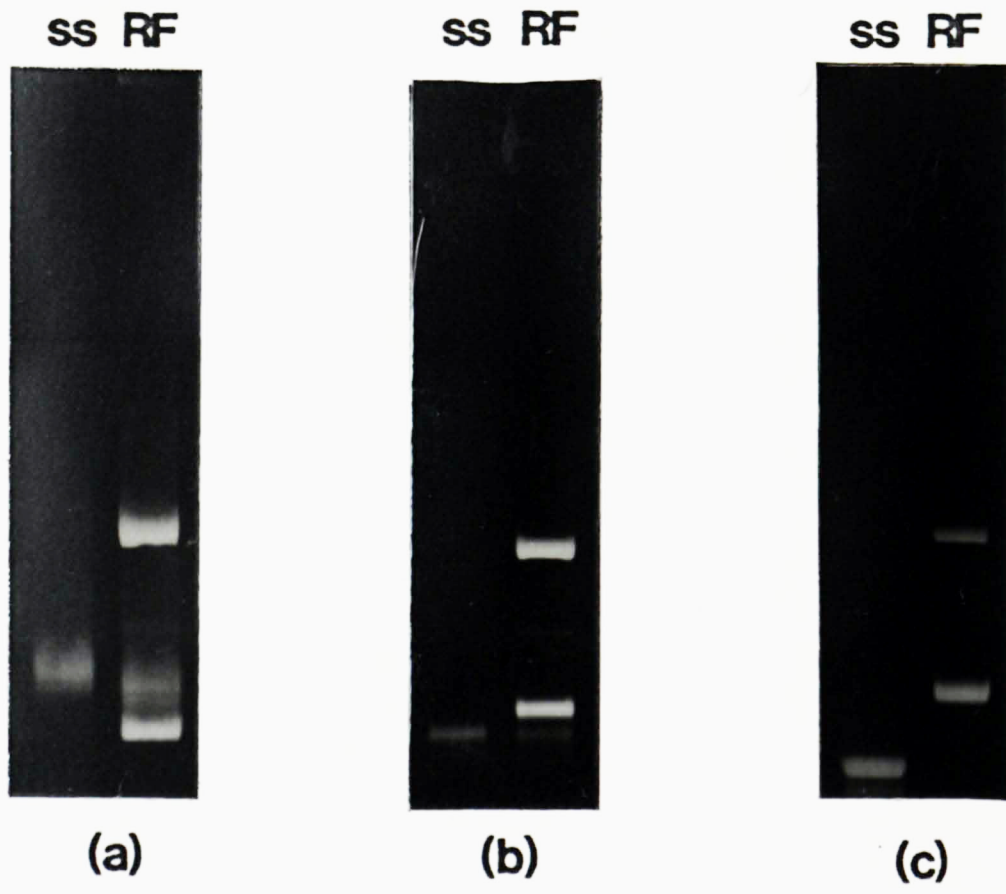
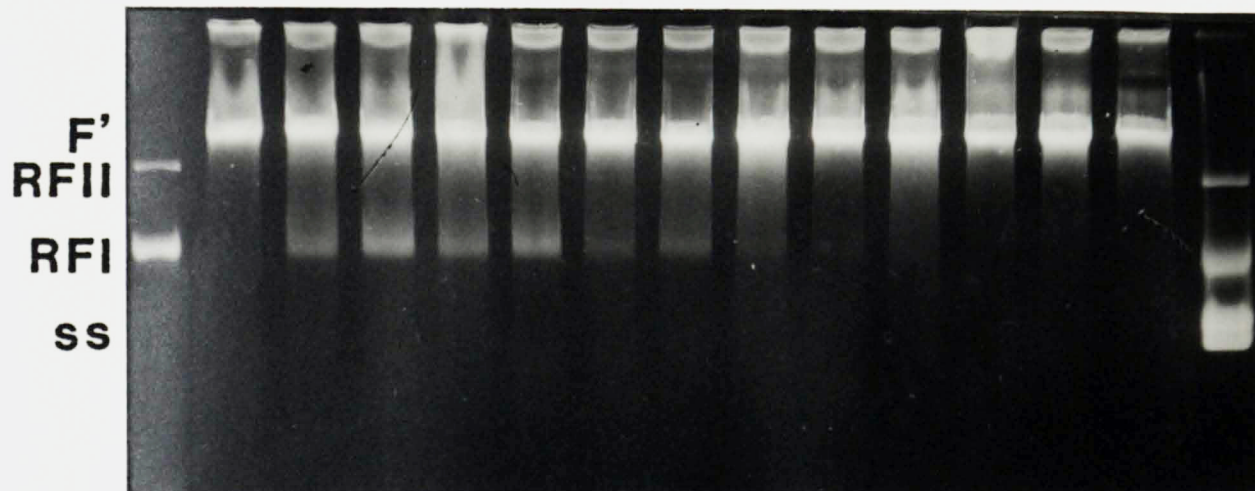


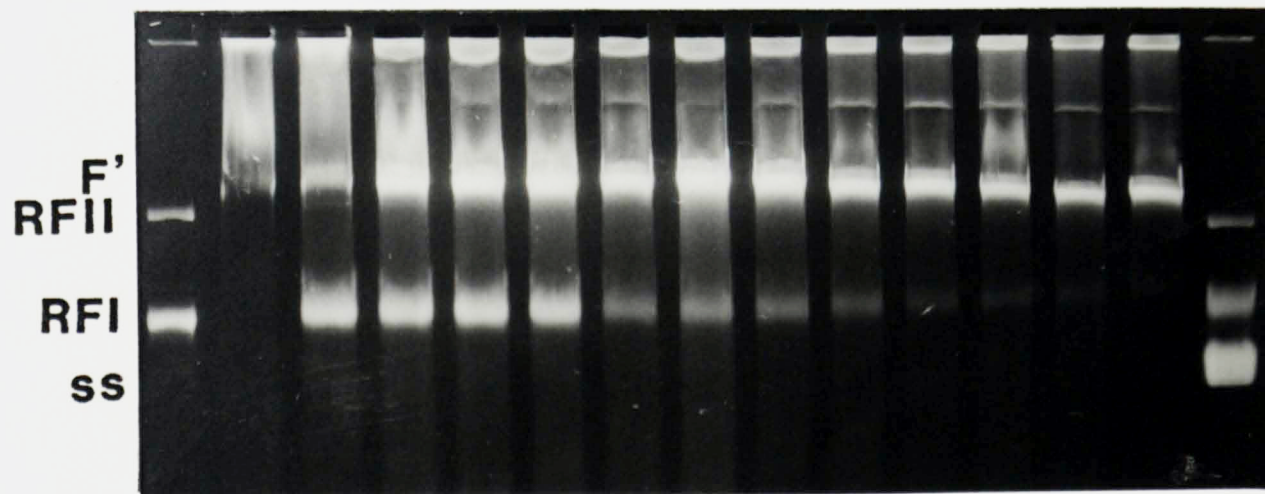
Figure 9. Effect of rifampicin on RF production in M13mp7 and the clone, M13mp7-m9. Rifampicin was added to exponentially growing *E. coli* JM103 cells 10 minutes before infections. Final concentrations of rifampicin were 0, 0.5, 1.0, 2, 4, 6, 8, 10, 20, 40, 70, 100  $\mu\text{g/ml}$ . No chloramphenicol was added. Bacteriophage were added at an MOI of 100. Cells were processed as described in Materials and Methods (section G) with the exception of incubation with S1 nuclease. Lysates from 1.67 ml of cells were applied to the wells. Marker RF DNA was applied to lane 1, a mixture of RF and single-stranded DNA was applied to lane 15 and a lysate from uninfected cells was applied to lane 2. Electrophoresis was for 2 hours at 100 volts. (Panel a) Cells were infected with M13mp7. (Panel b) Cells were infected with M13mp7-M9.

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



(a)

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



(b)

Figure 10. Relative amounts of RF DNA produced with increasing amounts of rifampicin. Photographic negatives of Figure 9 were quantitated by densitometer scanning and integration on a Beckman DU-8 spectrophotometer. Black circles represent relative amounts of M13mp7-M9 DNA. Relative amounts of M13mp7 DNA are represented by triangles.

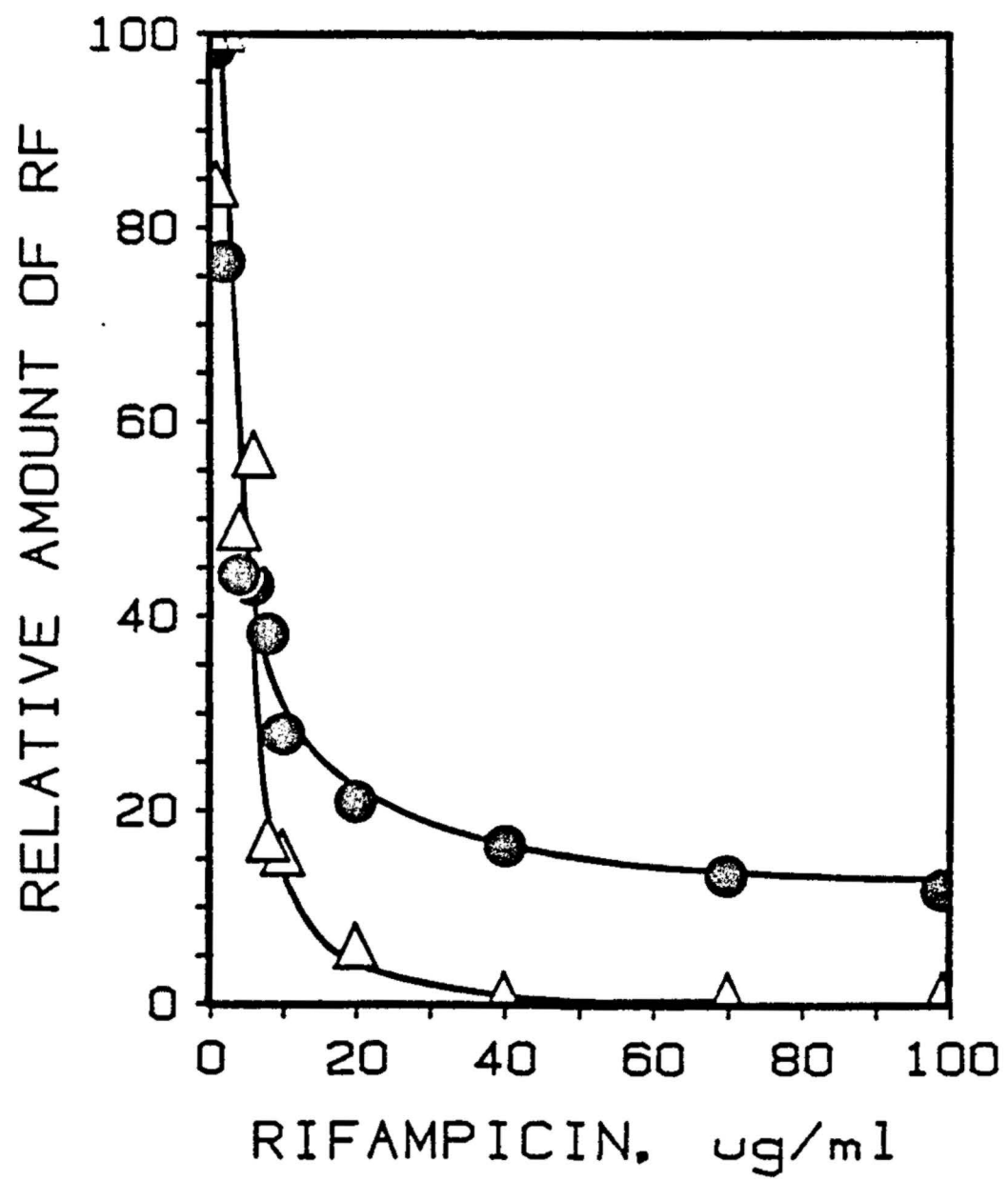


Figure 11. Effect of chloramphenicol on parental RF production. Ten ml of *E. coli* JM103 cells were grown to  $5 \times 10^8$  cells/ml. The samples in lanes 4 through 9 received 150  $\mu\text{g/ml}$  chloramphenicol. Rifampicin was added to samples 3 through 9 at concentrations of 100, 0, 10, 20, 50, 100, and 200  $\mu\text{g/ml}$ . After 10 minutes, the cells were infected with M13mp7 and the procedure in Materials and Methods (section G) was followed with the exception of incubation with S1 nuclease. Single-stranded DNA and RF DNA were applied to lanes 1 and 10, respectively. Lane 2 contains no rifampicin and no chloramphenicol. Lane 4 contains no rifampicin and 150  $\mu\text{g/ml}$  chloramphenicol. Electrophoresis was for 2 hours at 100 volts.

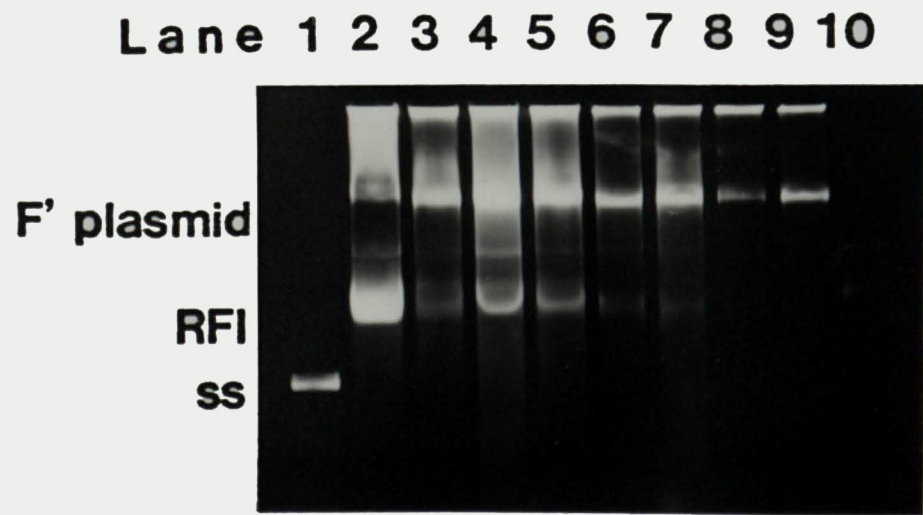
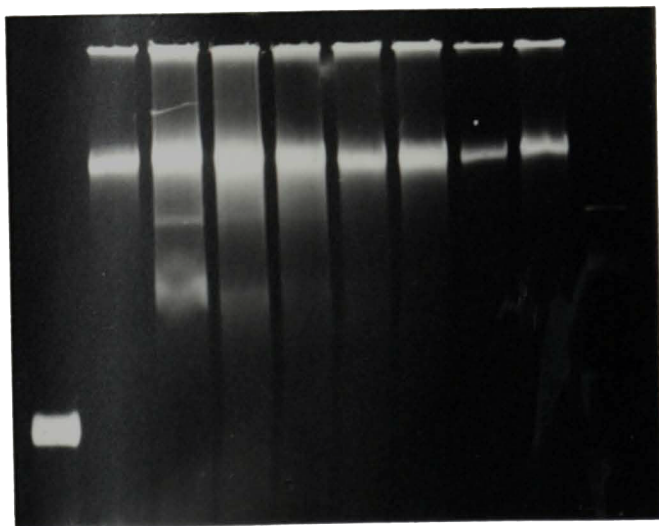


Figure 12. Effect of rifampicin on parental RF production by M13mp7 and the clone, M13mp7-M9, in the presence of chloramphenicol. Chloramphenicol at 150  $\mu\text{g/ml}$  and rifampicin at various concentrations were added to exponentially growing E. coli JM103 cells. After 15 minutes the cells were infected with 100 phage per cell. The assay was performed as described in Materials and Methods (section G). Lysates from 10 ml of cells were applied to each lane in the gel. Single-stranded DNA and RF DNA markers were added to lanes 1 and 10, respectively, and a lysate of uninfected cells was applied to lane 2. Electrophoresis was for 5 hours at 70 volts. (Panel a) The cells infected with bacteriophage M13mp7. Lanes 3 through 8 show the RF made at rifampicin concentrations of 0, 20, 50, 100, 200, 300, and 400  $\mu\text{g/ml}$ . (Panel b) Lanes 3 through 8 show the RF made from cells infected with bacteriophage M13mp7-M9 at rifampicin concentrations of 0, 20, 50, 100, 200, 300, and 400  $\mu\text{g/ml}$ .

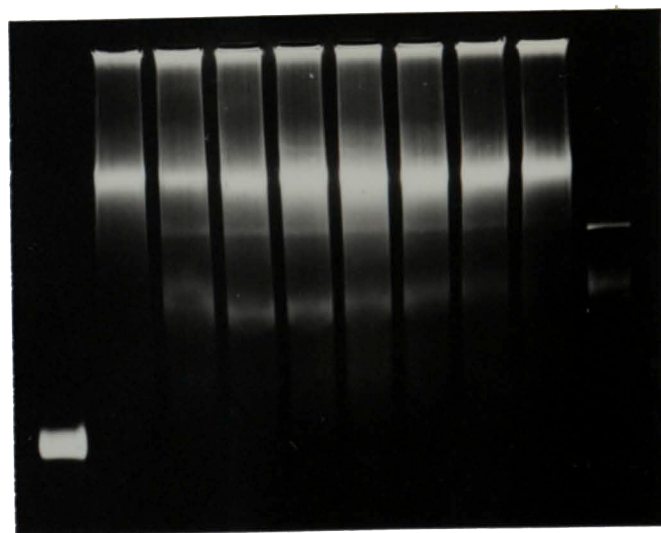


Lane 1 2 3 4 5 6 7 8 9 10



(a)

1 2 3 4 5 6 7 8 9 10



(b)

F  
RFII  
RFI  
SS

Figure 13. Relative amounts of RF DNA produced with increasing concentrations of rifampicin and 150  $\mu\text{g}/\text{ml}$  chloramphenicol. Photographic negatives of Figure 12 were quantitated by densitometer scanning and integration in a Beckman DU-8 spectrophotometer. The black circles indicate relative amounts of the clone, M13mp7-M9 DNA. Relative amounts of M13mp7 DNA are indicated by triangles.

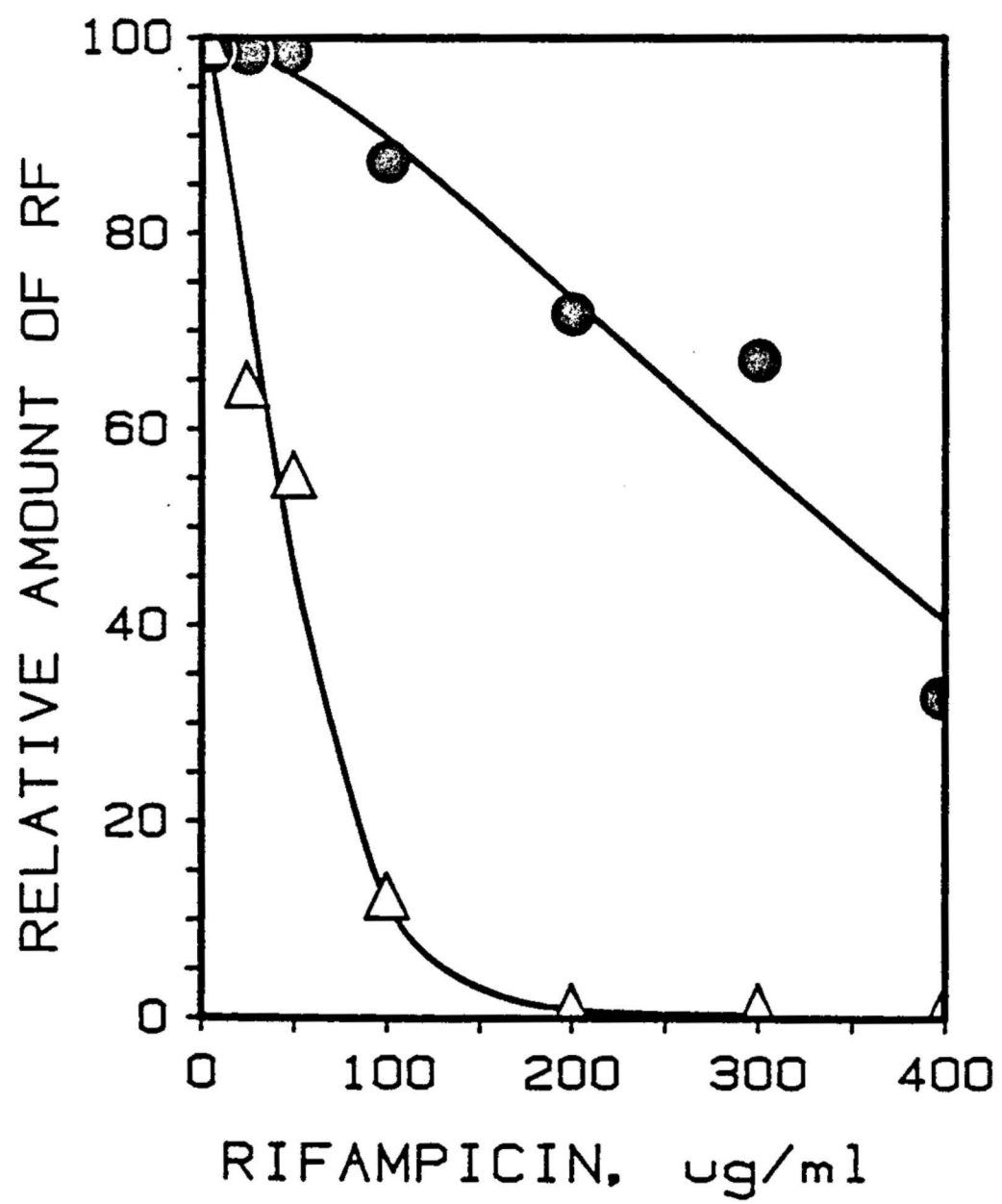


Figure 14. Effect of rifampicin on the parental RF synthesis of  $\phi$ X174. Exponentially growing E. coli C cells were treated as described in Materials and Methods (section G). Rifampicin was added to samples 3 through 9 at concentrations of 0, 20, 50, 100, 200, 300, and 400  $\mu\text{g}/\text{ml}$ . Chloramphenicol was added to all at a concentration of 150  $\mu\text{g}/\text{ml}$ . After 15 minutes the cells were infected with bacteriophage  $\phi$ X174 at a MOI of 100. Lysates from 20 ml of cells were applied to the gel. Lanes 1 and 10 are RF DNA and single-stranded DNA markers, respectively. Lysate from uninfected cells was applied to lane 2. Electrophoresis was for 5 hours at 70 volts.

Lane 1 2 3 4 5 6 7 8 9 10

