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# DNA-Protein Interactions in f1 DNA Replication

David I. Greenstein

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## **DNA-PROTEIN INTERACTIONS**

### **IN $\phi$ 1 DNA REPLICATION**

A thesis submitted to the Faculty of the Rockefeller University  
in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy

by  
David I. Greenstein

January 6, 1989  
The Rockefeller University  
New York







To Jennifer

This is why I was late to dinner sometimes



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## Abbreviations

attP	Phage $\lambda$ attachment site
bp	Base pairs
BSA	Bovine serum albumin
CAP	Catabolite activator protein
CIAP	Calf intestinal alkaline phosphatase
DTT	Dithiothreitol
EtBr	Ethidium bromide
gpII	Gene II protein
gpX	Gene X protein
IC <sub>0</sub>	Infective centers at time zero
IG	Intergenic space
IHF	Integration host factor
IPTG	Isopropyl- $\beta$ -D-thiogalactopyranoside
m.o.i.	Multiplicity of infection
O.D.	Optical density
p.f.u.	Plaque-forming units
polIII	DNA polymerase III holoenzyme
REP	Rep helicase
RF	Replicative-form DNA
E $\sigma$ <sup>70</sup>	RNA polymerase holoenzyme
SDS	Sodium dodecyl sulfate
SS	Single-stranded DNA
SSB	Single-stranded DNA binding protein
TCA	Trichloroacetic acid



### Abstract

This thesis analyzes the interaction of two DNA-binding proteins with the plus-strand replication origin of bacteriophage f1. The origin has a bipartite structure consisting of a required core origin region and an adjacent A+T-rich enhancer sequence that potentiates replication approximately 100-fold. The core origin binds the initiator protein, and the enhancer contains three binding sites for the *E. coli* integration host factor (IHF). Both activator proteins bend the DNA sequence to which they bind, implying that together they wrap the origin DNA into a higher order structure that is active in initiation.

The replication initiator protein of bacteriophage f1 (gene II protein) is a multifunctional protein that participates in DNA replication at a number of levels. The gene II protein binds to the core origin in a novel two-step fashion. The first binding step involves interaction of two gene II protein molecules with an inverted repeat ( $\beta\gamma$ ) at the center of the core origin to form a binding intermediate, complex I. The second binding step involves addition of two protein molecules to complex I, resulting in formation of the functional complex, complex II. Of these two protein molecules, one binds to and contacts repeat  $\delta$ , the other gene II protein molecule protects the nicking site in a sequence-independent fashion. The sequence protected in complex II corresponds to the core origin sequence as determined previously by *in vivo* analyses. The enhancer-independent mutation mp1 in gene II protein (met<sup>40</sup>→ile) increases the cooperativity with which the protein binds to the core origin to form complex II. A model is presented for the binding reaction involving both protein-DNA and protein-protein interactions.

A major finding of this thesis is that IHF activates f1 DNA replication by binding to three sites within the replication enhancer. The growth defect of f1 in IHF mutants was shown to be at the level of DNA replication. This growth defect of f1 in IHF mutants is suppressed by an initiator mutation (mp1) that also suppresses the lack of the replication enhancer, indicating that the enhancer is the genetic site of action of IHF.





## Chapter 1

### **Introduction**

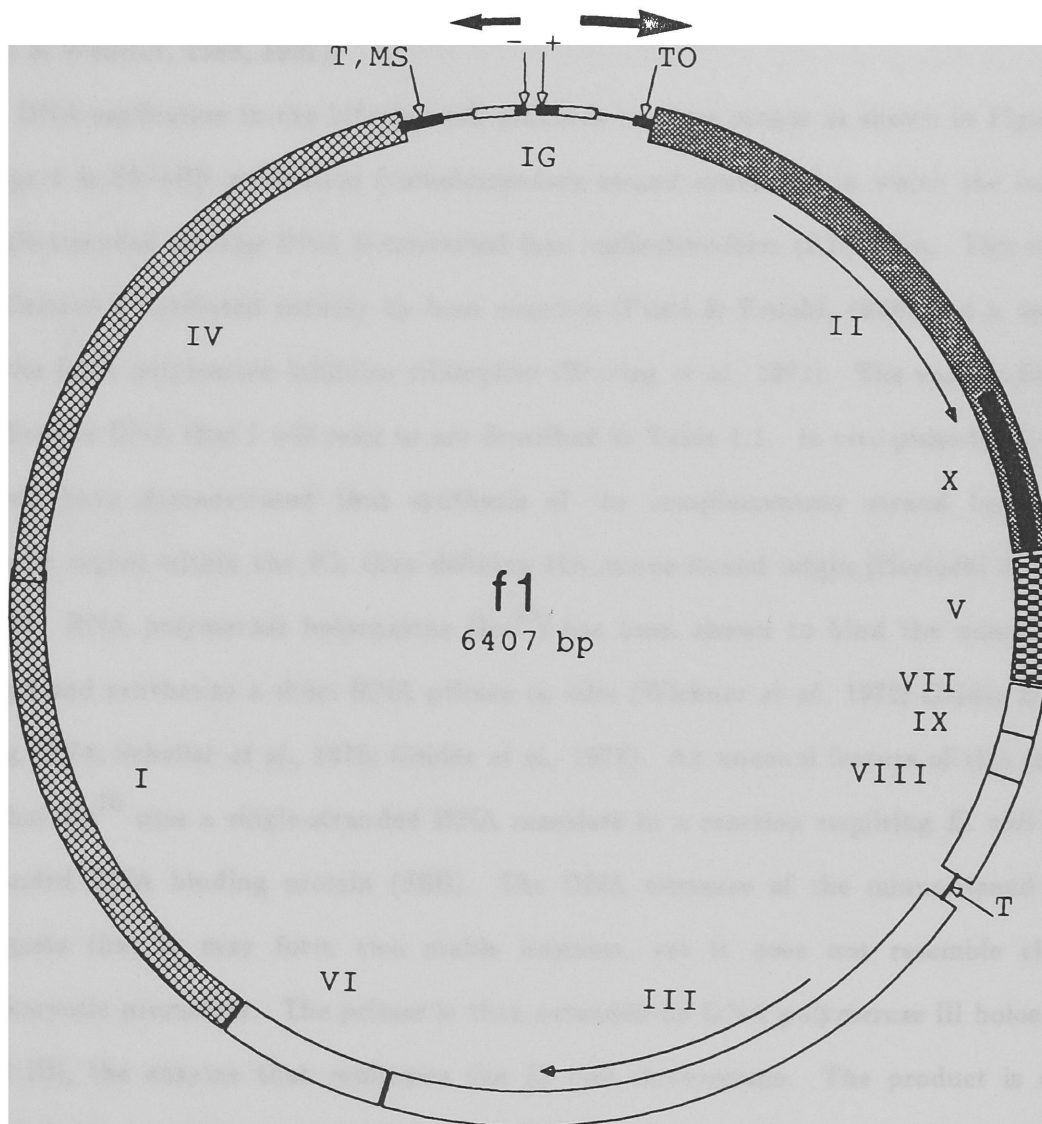


This thesis explores the interaction between DNA replication proteins and the plus-strand replication origin of the filamentous phage f1. The goal of this study is to better understand the mechanism of f1 DNA replication and its regulation. Since many aspects of DNA replication are conserved in both prokaryotes and eukaryotes, it is likely that certain features of other replication systems will mimic those utilized by bacteriophage f1. The advantage of choosing bacteriophage f1 as the object of study is that the phage and its host *E. coli* provide a system which is both genetically and biochemically tractable.

### Phage life cycle

The filamentous phages (f1, M13, and fd) are single-stranded DNA phages that infect male *E. coli* expressing a pilus encoded by the F-episome (Loeb, 1960; Marvin & Hoffman-Berling, 1963; Zinder *et al.*, 1963). Each of these three phages are essentially independent isolates of the same phage, differing only by several nucleotide substitutions (Beck *et al.*, 1978; Van Wezenbeek *et al.*, 1980; Beck & Zink, 1981; Hill & Petersen, 1982). Figure 1.1 shows the genetic map. The genome is small (6407 nucleotides) and contains nine genes coding for ten proteins. In addition, there is an intergenic space (IG) that contains the origins for plus- and minus-strand DNA synthesis (Horiuchi & Zinder, 1976), a packaging signal (Dotto & Zinder, 1983), and a rho-dependent terminator (Moses & Model, 1984). The single-stranded circular genomic DNA is encased in filamentous particles about 900 nm long and about 7 nm in diameter (Marvin & Hoffman-Berling, 1963). The phage particle contains of approximately 2700 copies of the major coat protein, the product of gene VIII (Marvin, 1978). In addition, there are several minor coat proteins at the ends. One of the minor coat proteins, the product of gene III, attaches to the receptor (Goldsmith & Konigsberg, 1977; Woolford *et al.*, 1977) at the tip of the pilus (Caro & Schnos, 1966). The pilus is thought to retract,

Figure 1.1. Genetic map of bacteriophage f1. The genes are indicated by roman numerals. Genes II and X are in frame and overlapping. IG designates the intergenic space as described in the text. The direction of the plus-strand and minus-strand origins are indicated by thick and thin arrows, respectively. The rho-independent transcriptional terminator is indicated by a T after gene VIII. The rho-dependent terminator and packaging (morphogenetic) signal is indicated by T, MS. The translational control operator in the 5'-untranslated portion of the gene II messenger RNA is indicated by TO.



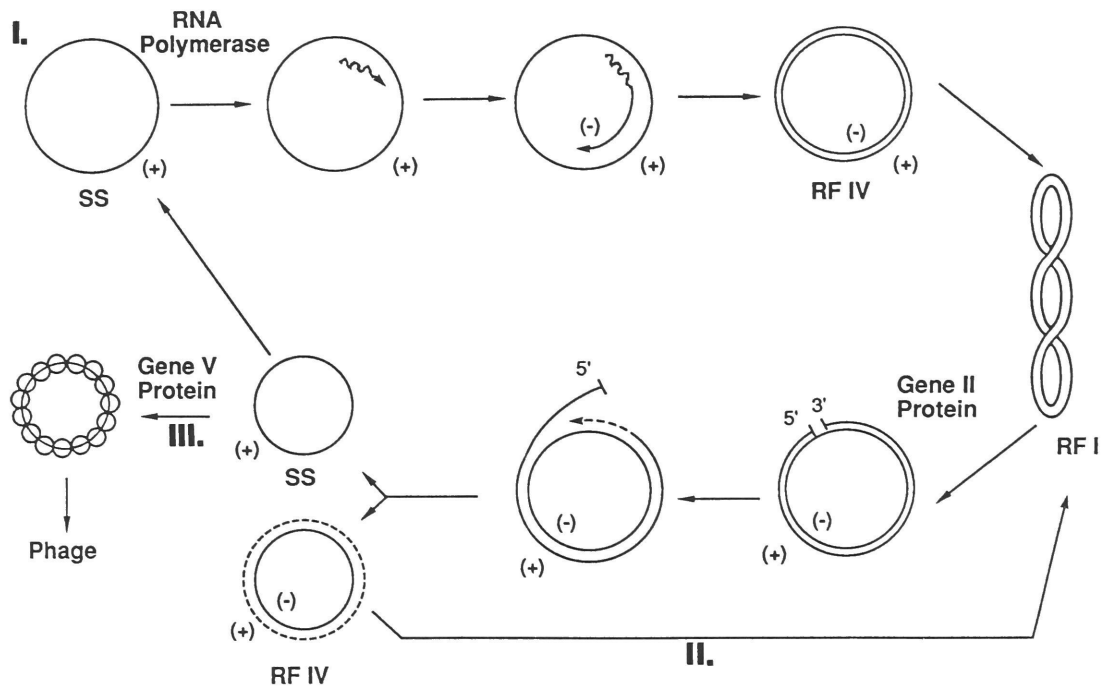


bringing the phage in contact with the bacterial cell surface (Marvin & Hohn, 1969; Jacobson, 1972). After uncoating of the virion particle (Smilowitz, 1974a), the infecting circular single-stranded DNA is brought into the cytoplasm in a process that remains unclear but appears to involve the *tolA*, *tolQ*, and *tolR* gene products (Smilowitz, 1974b; Sun & Webster, 1986, 1987).

DNA replication in the infected cell proceeds in three stages as shown in Figure 1.2. Stage 1 is SS→RF replication (complementary strand synthesis) in which the infecting single-stranded circular DNA is converted into replicative-form (RF) DNA. This stage of replication is mediated entirely by host enzymes (Pratt & Erdahl, 1968) and is sensitive to the RNA polymerase inhibitor rifampicin (Brutlag *et al.*, 1971). The various forms of replicative DNA that I will refer to are described in Table 1.1. *In vivo* pulse-label experiments have demonstrated that synthesis of the complementary strand begins in a specific region within the IG, thus defining the minus-strand origin (Horiuchi & Zinder, 1976). RNA polymerase holoenzyme ( $E\sigma^{70}$ ) has been shown to bind the minus-strand origin and synthesize a short RNA primer *in vitro* (Wickner *et al.*, 1972; Geider & Kornberg, 1974; Schaller *et al.*, 1975; Geider *et al.*, 1978). An unusual feature of this reaction is that  $E\sigma^{70}$  uses a single-stranded DNA template in a reaction requiring *E. coli* single-stranded DNA binding protein (SSB). The DNA sequence of the minus-strand origin suggests that it may form two stable hairpins, yet it does not resemble classical prokaryotic promoters. The primer is then extended by DNA polymerase III holoenzyme (pol III), the enzyme that replicates the *E. coli* chromosome. The product is duplex DNA having a single-stranded gap and still bearing the RNA primer (Tabak *et al.*, 1974). The RNA primer is removed using the 5'→3' exonuclease activity of DNA polymerase I (Chen & Ray, 1976). Surprisingly, mutant phage have been constructed bearing deletions of the minus-strand origin (Kim *et al.*, 1981; A. Roth & K. Horiuchi, unpublished results). These phage grow poorly, make tiny plaques, and show defects in the formation of replicative form DNA. Possibly these phage employ cryptic minus-strand

Figure 1.2. f1 DNA replication. The cycle of phage DNA synthesis in the infected cell is shown as described in the text. Stage I is SS→RF synthesis in which the infecting single-stranded circular DNA is converted to replicative form DNA. Stage II is RF→RF synthesis in which the intracellular pool of replicative form DNA is expanded. Stage II results from the concerted action of the two replication origins. Rolling-circle replication from the plus-strand origin produces single strands, which are then converted to replicative form DNA by the action of the minus-strand origin. Newly synthesized DNA is indicated by dotted lines. Stage III is RF→SS synthesis. The single-stranded products of rolling-circle replication form a complex with gene V protein and are packaged into phage particles for export.







origins.

The second stage of replication is RF $\rightarrow$ RF replication in which the intracellular pool of replicative-form DNA is expanded. This stage is brought about by the concerted action of the two replication origins: rolling-circle type replication (Gilbert & Dressler, 1968) from the plus-strand origin followed by conversion of the progeny single-stranded circles to double strands by action of the minus-strand origin (Horiuchi & Zinder, 1976). The resulting replicative-form DNA molecules are both intermediates in DNA synthesis and templates for transcription for the synthesis of the phage-encoded proteins. All of the transcription units of the phage have the same polarity as the genomic single-stranded DNA (Jacob & Hofschneider, 1969; Smits *et al.*, 1978; Pieczenik *et al.*, 1975). Therefore, transcription proceeds in the same direction as plus-strand rolling-circle replication. It is suspected that transcription into the replication fork is inhibitory, as proposed for the replicaton of the *E. coli* chromosome (Brewer, 1988), though the idea has not been rigorously tested.

Plus-strand rolling-circle replication has been carried out *in vitro*. The requirements are the phage-encoded gene II protein (initiator protein), the *E. coli* Rep helicase, pol III, SSB, a supercoiled replicative-form DNA template (RFI), ATP, and precursor deoxynucleotides (Meyer & Geider, 1982; Geider *et al.*, 1982). The involvement of these proteins in plus-strand replication *in vivo* is well documented. Gene II protein is absolutely required for plus-strand synthesis (Pratt & Erdahl, 1968). It binds to the plus-strand origin (Horiuchi, 1986) and introduces a specific nick in the plus-strand of RFI (Meyer *et al.*, 1979). The 3'-hydroxyl end of the nick serves as the primer for plus-strand rolling-circle replication. I will describe gene II protein and its activities in more detail below. Plus-strand DNA synthesis also requires the *E. coli* Rep helicase (Denhardt *et al.*, 1967, 1972), a single-strand DNA-dependent ATPase that unwinds duplex DNA in advance of the replication fork (Kornberg *et al.*, 1978). The products of a round of rolling-circle synthesis are a single-stranded circle and a relaxed double-stranded circle (RFIV).

**Table 1.1. Forms of Duplex DNA Referred To**

Form	Description
RFI	negatively supercoiled circle
RFII	nicked circle
RFIII	unit length linear
RFIV	relaxed covalently-closed circle

Unit length linear DNA is not a replicative intermediate for f1, but is referred to as RFIII according to the designation of Roulland-Dussoix & Boyer (1969).

The RFIV DNA must be supercoiled by *E. coli* DNA gyrase for another round of replication to ensue (Horiuchi *et al.*, 1979) because the gene II protein requires a supercoiled substrate for nicking (Meyer & Geider, 1979b). Inhibitors of DNA gyrase inhibit plus-strand DNA replication *in vivo* (Fidanian & Ray, 1972, 1974; Horiuchi *et al.*, 1979).

In addition, there are reports that *dnaA*, *dnaB*, and *dnaG* are required for phage DNA replication *in vivo* (Olsen *et al.*, 1972; Bouvier & Zinder, 1974; Ray *et al.*, 1975; Dasgupta & Mitra, 1976). In light of the establishment of *in vitro* activities for these proteins (Kornberg, 1980), it is difficult to incorporate them into current models of f1 replication. Moreover, these proteins are not required in the *in vitro* replication system of Meyer & Geider (1982). Consequently, the effect of these mutations (*dnaA*, *dnaB*, and *dnaG*) on f1 DNA replication is thought to be indirect.

The third stage of replication (RF→SS) occurs late in infection. DNA replication is asymmetric; the single-stranded circles are packaged into phage particles for export instead of being converted into replicative-form DNA (Horiuchi & Zinder, 1976). An important feature of f1 DNA replication is that replication of the two strands occurs one at a time. The native form of pol III has been found to be a dimer (reviewed by McHenry, 1985). This observation is consistent with the "trombone" model for coupling between leading and lagging strand synthesis (see Alberts, 1984). According to this model, one subunit of pol III would participate in leading strand synthesis, while the other would participate in lagging strand synthesis. This model would not apply to f1 replication, allowing for asymmetric synthesis of single strands late in infection. Possibly the location of the minus-strand origin is important for the uncoupling of plus- and minus-strand DNA synthesis. The minus-strand origin is located immediately upstream of the plus-strand origin and is replicated last during plus strand synthesis.

DNA replication in the infected cell is tightly regulated. Unlike virulent phage, which kill their host, the filamentous phage persistently infect their host. Ordinarily, infection with f1 is not lethal, though the growth rate of the host slows about twofold. In



the f1 steady state, the replicative-form DNA is maintained as a plasmid with a copy-number of approximately 20-40, and phage are continuously exported at a rate of 100-200 per hour (Lerner & Model, 1981). The infected state is quite stable with the frequency of curing being approximately  $10^{-3}$ . The mechanism whereby this regulation is achieved is not completely understood, though the phage-encoded single-stranded DNA binding protein (gene V protein) appears to play a crucial role. Gene V protein binds cooperatively to single strands, forming rod-shaped complexes (gene V complex) (Alberts *et al.*, 1972; Oey & Knippers, 1972; Webster & Cashman, 1973; Pratt *et al.*, 1974). The formation of gene V complex inhibits minus-strand DNA synthesis, presumably by virtue of the cooperative binding of gene V protein, thereby occluding the minus-strand origin (Pratt & Erdahl, 1968; Van Dorp *et al.*, 1979, Fulford & Model, 1988b). Gene V complex is thought to facilitate packaging. The packaging signal, a very stable hairpin ( $\Delta G \simeq 60$  kcal/mol), is thought not to be bound by gene V protein, and has recently been shown to be located at one end of the gene V complex (G. Smith, personal communication). Single-stranded DNA fails to accumulate in gene V mutants (Pratt & Erdahl, 1968). Gene V protein has also been shown to repress the synthesis of the gene II protein. Genetic and biochemical evidence suggests that gene V protein inhibits the translation of gene II protein by binding to the 5'-untranslated region of the gene II messenger RNA (Model *et al.*, 1982; Yen & Webster, 1982; Dotto & Zinder, 1984ab; B. Michel, unpublished results). Recently, Fulford and Model (1988ab) have proposed that gene II protein can also "encourage" minus-strand DNA synthesis based on *in vivo* pulse-labeling experiments in which the gene II protein was overexpressed beyond the amount normally found during the course of an infection. Such an amount of gene II protein appears to relieve the gene V protein mediated repression of minus-strand synthesis. A regulatory role has also been proposed for the gene X protein. Gene II and gene X overlap in frame, with gpX being identical to the C-terminal 30% of gpII (Yen & Webster, 1981). Gene X protein is not required for DNA replication (Fulford & Model,





1984). It appears to modulate the accumulation of single strands for phage export. The biochemical basis of gpX action remains unclear. In this thesis, I have focused on analyzing the mechanism of action of gene II protein in its established role, plus-strand synthesis.

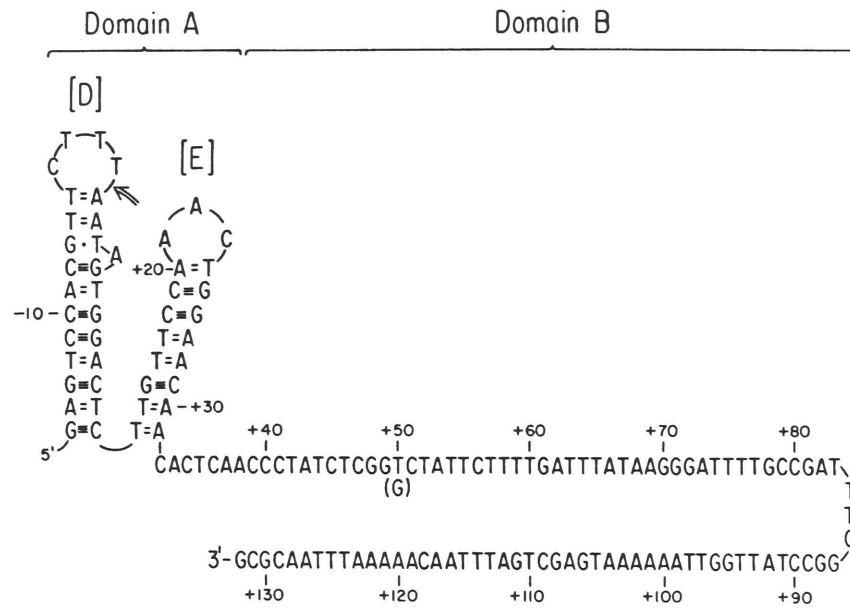
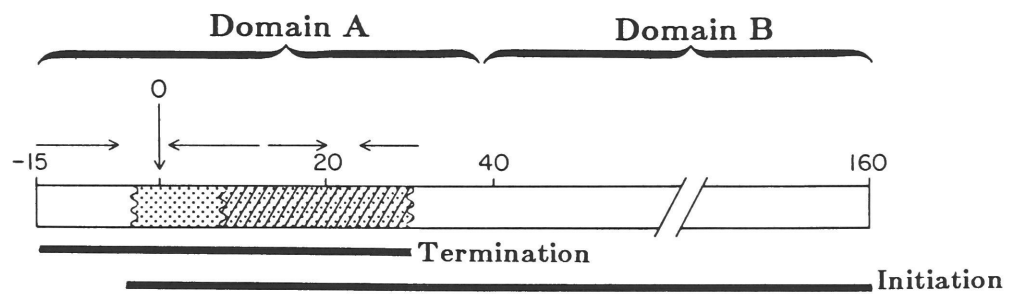
### **The plus-strand replication origin**

Considerable effort has been spent in characterizing the origin of plus-strand replication. Figure 1.3 diagrams the plus-strand origin, with its domains and signals. The plus-strand origin contains signals for both the initiation and termination of DNA synthesis (Horiuchi, 1980; Dotto & Horiuchi, 1981; Dotto *et al.*, 1982b). It can be divided into two domains, A (core origin sequence) and B (replication enhancer sequence) (Dotto *et al.*, 1984; Johnston & Ray, 1984). This division of the origin into core and enhancer is general to replication origins from both prokaryotes and eukaryotes. The signals for termination and *in vitro* nicking by the gene II protein are contained entirely within the core origin (Dotto *et al.*, 1984). Signals for initiation extend from four nucleotides before the nicking site (-4) to more than 100 nucleotides downstream, including most of the core origin and all of the enhancer (Cleary & Ray, 1980, 1981; Dotto *et al.*, 1982ab, 1984). Phage with lesions in the enhancer grow poorly and frequently acquire compensatory mutations that restore efficient replication (Dotto & Zinder, 1984ab; Kim & Ray, 1985). These compensatory mutations are of two types: mutations that lead to an overproduction of the gene II protein, and mutations in gene II that lead to production of an altered protein. The compensatory mutations restore efficient replication by  $A^+B^-$  origins (wild-type domain A, defective domain B), but not by  $A^-B^+$  or  $A^-B^-$  origins. In Chapter 8, I establish a molecular phenotype for the enhancer-independent compensatory mutations that result in altered gene II proteins.

Figure 1.3. The plus-strand replication origin of bacteriophage f1. (a). Nucleotide sequence. This sequence is necessary and sufficient for efficient origin function (Cleary & Ray, 1980, 1981; Dotto *et al.*, 1982a, 1984). Only the sequence of the plus strand is shown. Domains A and B refer respectively to the core origin sequence and the replication enhancer sequence, as described in the text. The site of nicking by the gene II protein is indicated by an arrow. This site is defined as 0. Position +1 corresponds to nucleotide 5781 on the f1 sequence of Hill & Petersen (1982). [D] and [E] indicate two palindromic sequences found within domain A. These palindromic sequences are drawn as hairpins for illustrative purposes, and this does not mean that such structures actually form. A base substitution at position +50, found in the closely related phage M13, is indicated. The sequence data are from Beck & Zink (1981), Hill & Petersen (1982), and van Wezenbeek *et al.* (1980). The complete extent of domain B is not shown in (a) but is shown in (b).

(b). Signals contained within the plus-strand origin. Brackets show the extent of domains A and B as indicated. The site of nicking by the gene II protein (0) is indicated by a vertical arrow. Palindromic sequences found within domain A, corresponding to hairpins D and E, are indicated by horizontal arrows. Horizontal lines indicate the sequence requirements for initiation and termination. The crosshatched region and the dotted region show the sequence requirements for gene II protein binding and nicking, respectively.

A

**B**



### The initiator protein--the gene II protein

Gene II protein ( $M_r=46,000$  daltons) is absolutely required for plus-strand rolling-circle DNA replication (Pratt & Erdahl, 1968). Properties of gene II mutants *in vivo* suggested that the encoded protein was an endonuclease (Fidanian & Ray, 1972; Lin & Pratt, 1972). Gene II protein is a multifunctional protein that plays central roles in phage DNA replication. First, it introduces a single-strand break at a specific site on the plus strand of supercoiled-replicative form DNA (Meyer *et al.*, 1979). The 3'-hydroxyl end of the nick serves as the primer for initiation of plus-strand DNA synthesis. The gene II protein also functions at a step beyond nicking: DNA molecules that have been nicked by gene II protein still require the gene II protein for their unwinding and replication (Geider *et al.*, 1982). Upon completion of a round of synthesis, gene II protein cleaves and circularizes the displaced single strand (Harth *et al.*, 1981). The gene II protein also has site-specific topoisomerase activity (Meyer & Geider, 1979b).

A large portion of this thesis (Chapters 3, 4, 5, 7, and 8) deals with the interaction between gene II protein and the replication origin. Geider *et al.* (1982) demonstrated that the gene II protein could remain associated with RFII after nicking, thereby protecting the specifically nicked DNA molecule from *Bal31* nuclease digestion. Filter-binding experiments have shown that the gene II protein binds specifically to either restriction fragments or superhelical DNA containing the *f1* origin of replication (Horiuchi, 1986). Sequences required for binding supercoiled DNA and restriction fragments were identical and did not include the nicking site. The effects of salt concentration, temperature, and time of incubation on the binding were also the same for restriction fragments and supercoiled DNA. Thus, the binding of gene II protein to the origin occurs regardless of superhelicity, while the subsequent hydrolysis reaction requires superhelicity.

In Chapter 3, I localize more precisely the binding site of the gene II protein, employing DNase I protection (Galas & Schmitz, 1978), methylation interference (Siebenlist &



Gilbert, 1980), and gel retardation (Garner & Revzin, 1981; Fried & Crothers, 1981) experiments. I show that gene II protein binds to the core origin in two steps involving more than one gene II protein molecule. The stoichiometry of the binding intermediate and the functional complex are determined using a double-label gel-binding assay. In Chapter 7, I present evidence indicating that gene II protein bends the replication origin. A model is presented for the binding reaction involving both protein-DNA and protein-protein interactions.

In Chapter 4, I analyze the double-strand cleavage activity of gene II protein in the presence of  $Mn^{2+}$ . I show that the cleavage product has an unusual structure in which the plus and minus strands are linked. I suggest a model in which superhelicity favors nicking by stabilizing an unusual DNA structure at the nicking site.

In Chapter 5, I develop a direct assay for Rep helicase mediated unwinding of the DNA template. I use this assay to examine the DNA sequence requirements for the unwinding reaction. An initiation-defective origin is shown not to be a substrate for *in vitro* unwinding. This defect correlates with abnormal binding by the gene II protein to the mutant origin.

A major difficulty in the genetic analysis of DNA replication is that mutations in the replication origin or in replication proteins are often lethal. In the Appendix, I present a system for the genetic selection of origin and gene II protein mutants. This type of selection may be applicable to other replication systems.

### **Integration host factor**

In this thesis (Chapter 6), I propose that the *E. coli* integration host factor (IHF) plays an accessory or enhancing role in plus-strand DNA synthesis. IHF is a heterodimer consisting of two nonidentical proteins, IHF- $\alpha$  ( $M_r=11,200$  daltons) and IHF- $\beta$  ( $M_r=10,600$  daltons) (Nash & Robertson, 1981), that are the products of the *E. coli* genes *himA* and *himD* (or *hip*), respectively (Miller & Friedman, 1980; Miller & Nash,





1981; Kikuchi *et al.*, 1985). IHF functions in a number of processes (for a comprehensive review see Drlica & Rouviere-Yaniv, 1987; or Friedman, 1988), the best characterized of which is  $\lambda$  site-specific recombination. IHF is a DNA-binding protein (Nash & Robertson, 1981) that binds to three sites within the phage  $\lambda$  attachment site (attP) (Craig & Nash, 1984). This binding is required for formation of a higher order structure called the attP-intasome (Pollock & Nash, 1983; Griffith & Nash, 1985). IHF plays a role in a number of cellular processes including site-specific recombination, transcription, translation, transposition, phage morphogenesis, and DNA replication. I have catalogued the known functions of IHF in Table 1.2. It appears that IHF acts in all these processes as an accessory or enhancing factor rather than as an absolute requirement. IHF has been shown to induce DNA bending upon binding (Stenzel *et al.*, 1987; Prentki *et al.*, 1987; Robertson & Nash, 1988). In Chapter 7, I present evidence that IHF bends the f1 replication origin. In Chapter 6, I propose that IHF is an activator of f1 replication and that IHF binds to multiple sites within the replication enhancer. Evidence is presented that IHF functions in plus-strand replication *in vivo*. Furthermore, I show that gene II mutations that restore efficient replication from origins with a defective replication enhancer (enhancer-independent mutations) also suppress the effect of IHF mutations on f1 replication.

Table 1.2. Functions of IHF

System	Reference
<b>Site-specific Recombination</b>	
$\lambda$	Miller & Friedman, 1980; Nash & Robertson, 1981; Miller & Nash, 1981
$\phi 80$	Leong <i>et al.</i> , 1985
P22	Leong <i>et al.</i> , 1985
type 1 fimbriae phase variation	Eisenstein <i>et al.</i> , 1987
<b>Transcription</b>	
Mu early genes (A,B)	Goosen <i>et al.</i> , 1984; Krause & Higgins, 1986
<i>ilvGMEDA</i>	Friden <i>et al.</i> , 1984
IHF	Miller <i>et al.</i> , 1981
<i>gyrA</i>	Friedman <i>et al.</i> , 1984
<b>Translation</b>	
$\lambda$ cII	Hoyt <i>et al.</i> , 1982; Mahajna <i>et al.</i> , 1986
<b>Transposition</b>	
Tn5	J. Makris, personal communication
Tn10	Morisato & Kleckner, 1987
IS1	Gamas <i>et al.</i> , 1987b; Prentki <i>et al.</i> , 1987
<b>Replication</b>	
pSC101	Gamas <i>et al.</i> , 1986
f1	Greenstein <i>et al.</i> , 1988; this work
$\lambda$	L. Hensel, personal communication
P1 plasmid partitioning	Funnell, 1988
<b>Packaging</b>	
$\phi 21$	Feiss <i>et al.</i> , 1985
$\lambda$ cos-154	Bear <i>et al.</i> , 1984
<b>Antitermination</b>	
<i>ilvBN</i>	P. Tsui, B. Stevenson, & Freundlich, personal communication

Table 1.2 catalogues the functions of IHF in various cellular processes. IHF represses its own transcription. IHF is an activator of the rest of the processes listed here.

## Chapter 2

### **Materials and Methods**



## Bacteria, phage, and media

### Bacteria

Table 2.1 lists the bacterial strains (*E. coli* K) that were used or constructed during the course of this work. Our standard *E. coli* HfrC strain K38, K91, and a *recA56* derivative, K902, were used for plasmid growth and phage f1 propagation. IHF mutant strains were constructed by P1(vir) transduction (Miller, 1972).

### Phage

f1 phage and its derivatives were from the collection of N. Zinder. R209 and R218 (Boeke *et al.*, 1979) are f1 derivatives that have 4 bp inserted (resulting in formation of an *EcoRI* site) in domain B at the *HaeIII* G/D border (for an f1 restriction map see Horiuchi *et al.*, 1979). R218 also has a missense mutation in gene V (*arg*<sup>21</sup>→*cys*) that results in over-production of the gene II protein (Dotto & Zinder, 1984a). M13mp1 was described (Messing *et al.*, 1977; Dotto & Zinder, 1984b). R348 which contains an amber mutation at the mp1 mutation site (codon 40 of gene II) on the wild-type genetic background, was constructed by mutagenesis using the oligonucleotide Ken-0 (5'-GATAAATTCTAGCCGGAAGA-3'). R350 was constructed by marker rescue of R348, using the M13mp1 *TaqI* C fragment containing the mp1 mutation (*met*<sup>40</sup>→*ile*). The presence of the mp1 mutation in R350 was verified by dideoxy sequencing (Sanger *et al.*, 1977). R348 and R350 were constructed by A. Roth and K. Horiuchi. Phage differing in the number of bp inserted at the *HaeIII* G/D border were prepared as follows. R<sup>+8</sup> (insertion of 8 bp) was prepared by treatment of R209 with *EcoRI*, followed by end-filling with DNA polymerase I Klenow fragment, and ligation using T4 DNA ligase. R<sup>+10</sup> (insertion of 10 bp), R<sup>+16</sup> (insertion of 16 bp), and R<sup>+22</sup> (insertion of 22 bp), were constructed by insertion of two, four, and six copies, respectively, of the oligonucleotide Ken-13 (5'-AATTGC-3') at the *EcoRI* site of R209. R209 was digested with *EcoRI* and ligated with a 10-fold molar excess of kinased Ken-13 oligonucleotide using T4 DNA

Table 2.1. Bacterial Strains Used

Name	Genotype	Source
K38	HfrC ( $\lambda$ ) <i>phoA6 tonA22 garB10 ompF627 relA1 pit-10 spoT1 T2<sup>r</sup> PO2A</i>	1
K91	K38 $\lambda$ cured	2
K361	W3110 <i>strep<sup>r</sup>=F<sup>-</sup> (<math>\lambda^-</math>)thyA36 deoC2 strep<sup>r</sup></i>	3
K561	K38 <i>lacI<sup>q</sup></i>	4
K840	<i>F<sup>-</sup> his871 relA1 rpsL181 gal-3 ilvY::Tn10 rep-71</i>	5
K902	<i>K98 su2 recA56 srl900::Tn10</i>	6
K924	<i>HfrC (<math>\lambda</math>) polA1 argH</i>	7
K1018	K38 <i>ilvy864::Tn10 rep-71</i>	8
K1019	K561 <i>ilvy864::Tn10 rep-71</i>	9
K1032	<i>F<sup>-</sup> his<sup>-</sup> ilv<sup>-</sup> bio<sup>-</sup> thi<sup>-</sup> (<math>\lambda</math> lysogen deleted for all genes except <i>N</i> and <i>cI857</i>)</i>	10
K1067	K924 <i>ilvy864::Tn10 rep-71</i>	11
K1117	<i>HfrH strep<sup>r</sup> <math>\Delta</math>592[gal-bio] hip/<math>\Delta</math>1::cam<sup>r</sup></i>	12
K1120	K91 <i>hip/<math>\Delta</math>1::cam<sup>r</sup></i>	13
K1131	K361 <i>hip/<math>\Delta</math>1::cam<sup>r</sup></i>	14
K1132	K924 <i>hip/<math>\Delta</math>1::cam<sup>r</sup></i>	15
K1133	<i>F<sup>-</sup> MudI1734(kan<sup>r</sup>) ara::Muc<sup>ts9</sup> <math>\Delta</math>(<i>proAB-argF-lacIPOZYA</i>) <i>xIII rpsL</i></i>	16
K1134	LE392 <i>himA/<math>\Delta</math>82::Tn10/ hip/<math>\Delta</math>3::cam<sup>r</sup></i>	17
K1135	N5271 containing the plasmid pPL <i>hip.himA-5</i>	18
K1141	K91 <i>himA/<math>\Delta</math>82::Tn10/</i>	19
K1142	K361 <i>himA/<math>\Delta</math>82::Tn10/</i>	20
K1143	K924 <i>himA/<math>\Delta</math>82::Tn10/</i>	21
K1150	K361 <i>hip/<math>\Delta</math>3::cam<sup>r</sup></i>	22
K1151	K91 <i>hip/<math>\Delta</math>3::cam<sup>r</sup></i>	23
K1152	K924 <i>hip/<math>\Delta</math>3::cam<sup>r</sup></i>	24
K1170	K361 <i>himA/<math>\Delta</math>82::Tn10/ hip/<math>\Delta</math>1::cam<sup>r</sup></i>	25
K1171	K91 <i>himA/<math>\Delta</math>82::Tn10/ hip/<math>\Delta</math>1::cam<sup>r</sup></i>	26
K1172	K924 <i>himA/<math>\Delta</math>82::Tn10/ hip/<math>\Delta</math>1::cam<sup>r</sup></i>	27
K1173	K361 <i>himA/<math>\Delta</math>82::Tn10/ hip/<math>\Delta</math>3::cam<sup>r</sup></i>	28
K1174	K91 <i>himA/<math>\Delta</math>82::Tn10/ hip/<math>\Delta</math>3::cam<sup>r</sup></i>	29
K1175	K924 <i>himA/<math>\Delta</math>82::Tn10/ hip/<math>\Delta</math>3::cam<sup>r</sup></i>	30
K1225	<i>F<sup>-</sup> endA1 hsdR17(rk-mk-) supE44 thi-1 recA1 gyrA96 relA1 <math>\phi</math>80dlacZ<math>\Delta</math>M15</i>	31
K1244	K561 <i>hip/<math>\Delta</math>1::cam<sup>r</sup></i>	32

All strains and their designations (K numbers) are from the collection of N. Zinder. The source of each strain is listed in detail on the following page.

### Source of Strains

1. A. Garen (S26).
2. N. Zinder.
3. M. Nomura. For a pedigree of W3110 consult Bachman (1987).
4. Davis *et al.*, (1985).
5. I. Tessman (IT1101).
6. Fulford & Model (1984).
7. C. Hill (CH1330).
8. P1(cm c<sup>ts</sup>) transduction using K840 as donor.
9. See K1018.
10. D. Court [MZ-1 (through D. Denhardt)].
11. See K1018.
12. R. Weisberg (RW1717). The *hip*/Δ1::cam<sup>r</sup>/ mutation lacks 2 bp of *hip* coding sequences near the middle of the gene and contains in their place a 1.3 kb fragment from the plasmid pBR325 that confers chloramphenicol resistance (Kikuchi *et al.*, 1985).
13. Transduction with P1(vir) using K1117 as the donor and K91 as the recipient. P1(vir) transductions will be abbreviated by P1 donor→recipient.
14. P1 K1117→K361.
15. P1 K1117→K924.
16. M. Casadaban. POI1734 (phage on induction) used to produce phage Mu. IHF mutants fail to plate phage Mu. This criteria was employed in characterizing IHF<sup>-</sup> transductants. POI1734=MAL103 MudI1734[kan<sup>r</sup> (Castilho *et al.*, 1984)].
17. R. Weisberg (E582). LE392=hsdR514 supE44 supF58 lacYi galK2 galT22 metR55. The *hip*/Δ3::cam<sup>r</sup>/ mutation lacks 132 bp that encode amino acids 3-46 of the *hip* gene and contain the 1.3 kb fragment described above that codes for chloramphenicol resistance (Kikuchi *et al.*, 1985). The *himA*/Δ82::Tn10/ mutation was described by Friedman *et al.* (1984).
18. H. Nash (HN880) (Nash *et al.*, 1987). N5271=*galk ilv his* (λ *cIts857 N7 N53 ΔBam ΔH1*) was described by Flamm & Weisberg (1985).
19. P1 K1134→K91.
20. P1 K1134→K361.
21. P1 K1134→K924.
22. P1 K1134→K361.
23. P1 K1134→K91.
24. P1 K1134→K924.
25. P1 K1117→K1142.
26. P1 K1117→K1141.
27. P1 K1117→K1143.
28. P1 K1134→K1142.
29. P1 K1134→K1141.
30. P1 K1134→K1143.
31. BRL (DH5α).
32. P1 K1117→K561.





ligase. The identity of the insertion phages was confirmed by sequencing the origin region by the method of Maxam and Gilbert (1977).

Phage growth following transfection was analyzed as follows. Phage supercoiled replicative-form DNA (RFI) was prepared as described (Zinder & Boeke, 1982) and further purified on CsCl-ethidium bromide density gradients (Radloff *et al.*, 1967). Competent cells of female strains (K361 and K1173) were prepared by the standard high efficiency method of Hanahan and were transfected as described (Hanahan, 1983). Following transfection, the infective-centers at time zero ( $IC_0$ ) were determined by diluting the culture and titering on an indicator lawn of K91. A 0.2 ml aliquot of transfected cells (10 ng of f1 RFI) was diluted into 50 ml of SOC medium (Hanahan, 1983) and grown with shaking at 37°C. At the indicated times, 1 ml of culture was withdrawn and centrifuged at 8000 x g for 30 seconds in an Eppendorf microcentrifuge. The supernatant was heated at 65°C for five minutes, and the phage were titered.

### **Media**

Fortified broth was described (Zinder & Boeke, 1982). DO minimal medium was described (Vogel & Bonner, 1956). Nutrients (bactotryptone, yeast extract, agar) were from Difco. L-amino acids were from Calbiochem. Glycine was from Sigma. Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was from Boehringer Mannheim Biochemicals. Antibiotics (generally from Sigma) were used at the concentrations recommended by Maniatis *et al.* (1982) for maintenance of plasmids coding for antibiotic resistance.

### **Plasmids and cloning procedures**

Restriction enzymes, T4 DNA ligase, and synthetic linkers were from New England Biolabs and Collaborative Research and used as recommended. T4 polynucleotide kinase was from P-L Biochemicals. DNA polymerase I Klenow fragment was from Bethesda Research Laboratories. Calf intestinal alkaline phosphatase (CIAP) was from Boehringer Mannheim Biochemicals. Plasmid and phage RF DNA were prepared according to Mani-



atis *et al.* (1982) or Zinder & Boeke (1982).

A plasmid containing domain B, pUCB, was constructed by subcloning the 170 base pair (bp) *Bgl*II-*Bam*HI fragment (from nucleotide +46 to nucleotide +217) from pD39 (Dotto *et al.*, 1984) into the *Bam*HI site of pUC19 (Norrandar *et al.*, 1983). pDG117, containing the wild-type domain A region and part of domain B, was constructed by inserting a *Bam*HI linker (5'-CGGGATCCCG-3') at the unique *Asu*I site of R218, and cloning the resulting 151 bp *Eco*RI-*Bam*HI fragment into pBR322 (Bolivar *et al.*, 1977) between the *Eco*RI and *Bam*HI sites. The defective origins  $\Delta$ 83 (a deletion from -14 to +3), pD30 (a 13 bp insertion at +8), pD29 (a deletion from the left end to +5), and  $\Delta$ +29 (a deletion from +29 to the right end) were previously described by Dotto *et al.* (1982a, 1984) and are shown in Figure 3.2. In order to conveniently end-label the defective origins for the footprinting experiments, some of them were subcloned. For  $\Delta$ 83 and pD30, an *Eco*RI site was constructed at position -57 (*Asu*I site) by insertion of an 8 bp *Eco*RI linker (5'-GGAATTCC-3'). The *Eco*RI-*Bam*HI fragment (the *Bam*HI site was at the *Hpa*II L/H border) containing each defective origin was cloned into pBR322 between the *Eco*RI site and the *Bam*HI site. The deletion  $\Delta$ +29 extends from position +29 (f1 sequence) into pBR322 to nucleotide 1568 (Sutcliffe, 1978).  $\Delta$ +29 was subcloned by insertion of an *Eco*RI linker at the *Asu*I site at position -57 as above. The 182 bp *Eco*RI-*Xho*II fragment (*Xho*II site at position 1667 of pBR322) containing the  $\Delta$ +29 defective origin was cloned between the *Eco*RI and *Bam*HI sites of pBR322. pMBS1, containing a 15 bp element corresponding to the center of domain A (positions +6 to +20), was constructed as follows. The two 17-mers DAGR1 (5'-AGCTGGACTCTTGTTC-3') and DAGR2 (5'-AGCTGGAACAAGAGTCC-3') were synthesized on an Applied Biosystems model 380A DNA synthesizer and purified (after de-protection) by gel filtration on a G-75 column (90 cm x 1.2 cm diameter) with 10 mM tri-ethylamine bicarbonate (pH 8.5) as buffer. The purified 17-mers were annealed and cloned into the *Hind*III site of pUC19. The plasmid pMBS2 was constructed by annealing DAGR3 (5'-



AGCTCTTTAATAGTGGACTCTTGTTCCACTATTAAAG-3') and DAGR4 (5'-AGCTCTTTAATAGTGGACAAGAGTCCACTATTAAAG-3'), and cloning them into the *Hind*III site of pUC19. The plasmids pD10-wt (pD16) and pD10- $\Delta$ 40.56 were described (Dotto *et al.*, 1984). The mutant origin  $\Delta$ 40.56 has a deletion of 17 bp from position +40 to +56, counting from the gpII nicking site. A double-origin assay was performed to analyze origin function as described previously (Dotto & Horiuchi, 1981). The plasmid pD38 is a pBR322 derivative that contains the M13 origin-containing *Hpa*II H fragment at the *Bam*HI site (Dotto *et al.*, 1984). A plasmid containing a tandem duplication of the origin, pJ1, was constructed by ligation of the origin *Hpa*II H fragment from pD38 (having *Bam*HI ends) into the *Bam*HI site of pBR322. A clone having a head to tail duplication of the origin was designated pJ1. pJ1 was provided by J. Benn and K. Horiuchi. The plasmid pD2 was described by Dotto *et al.* (1981). The plasmid pAR1, a derivative of pD2 that produces the mp1 mutant gene II protein (met<sup>40</sup>→ile), was constructed and provided by A. Roth and K. Horiuchi. The f1 origin-containing plasmid pD10 was described (Dotto *et al.*, 1982).

### End-labeling of DNA fragments

To label the plus strand of the origin, 10  $\mu$ g of f1 RFI were digested with *Asu*I, treated with 15 units of CIAP, and phenol extracted and ethanol precipitated twice. 5'-end-labeling was carried out with [ $\gamma$ -<sup>32</sup>P]-ATP (Amersham) and T4 polynucleotide kinase, essentially as described by Maniatis *et al.* (1982). After the kinase reaction, the DNA was precipitated with ethanol and digested with *Cla*I. The 315 bp origin fragment (containing all of domains A and B) was isolated from a 2% agarose gel. The fragment was eluted electrophoretically, extracted with phenol, and precipitated with ethanol twice. To label the minus strand, *Asu*I-digested RFI DNA was labeled at the 3'-end with [ $\alpha$ -<sup>32</sup>P]-dGTP and DNA polymerase I Klenow fragment. The end-labeled origin fragment was isolated as above. The defective origins ( $\Delta$ 83, pD30, and  $\Delta$ +29) were la-



beled at the *EcoRI* site placed at position -57. Again, labeling of the plus strand was by kinase reaction at the *EcoRI* site, and labeling of the minus strand was by end-filling. The defective origin pD29 was labeled at the *EcoRI* site of pBR322. pDG117 was labeled at the *BamHI* site which was introduced at position -57. pUCB and pMBS1 were labeled at the *EcoRI* site from the polylinker region of pUC19. A diagram of the end-labeled fragments used in the binding experiments appears in Figure 3.2.

For the electrophoretic analysis of gene II protein-origin complexes, plasmids were digested with restriction enzymes and treated with CIAP, after which the origin fragments were isolated on 2% agarose gels. Following electroelution and ethanol precipitation, the origin fragments were end-labeled with [ $\gamma$ - $^{32}\text{P}$ ]-ATP and T4 polynucleotide kinase. Unincorporated label was removed by a G-50 spun column procedure (Maniatis *et al.*, 1982). The pDG117 origin fragment was the 192 bp *EcoRI*-*BanI* fragment, similar to the 151 bp fragment used in the footprinting experiments except that it contained an additional 38 bp of pBR322 sequence (from the *BamHI* site to the *BanI* site at position 413 of pBR322). The 190 bp  $\Delta$ +29 origin fragment was the *EcoRI*-*BamHI* fragment used in the footprinting experiments (described above and in Figure 3.2). The 208 bp fragment containing the 15 bp element from the center of domain A (DAGR1-DAGR2 oligonucleotides) was prepared by digestion of pMBS1 with *BstNI*. The control pBR322 fragment was the 187 bp *EcoRI*-*EcoRV* fragment.

### Purification of gene II protein

The gene II protein (MW=46 kDal) used in the experiments shown in Figures 3.3(b) and 3.4 was purified from K38 containing pD2 (Dotto *et al.*, 1981) by the procedure of Meyer & Geider (1979a), and was approximately 90% pure. The gene II protein used in the rest of the experiments (except for those in Chapter 8) was purified to homogeneity by a new method involving a novel runaway expression vector and a purification procedure that relied on the ability of the gene II protein to be renatured after treatment



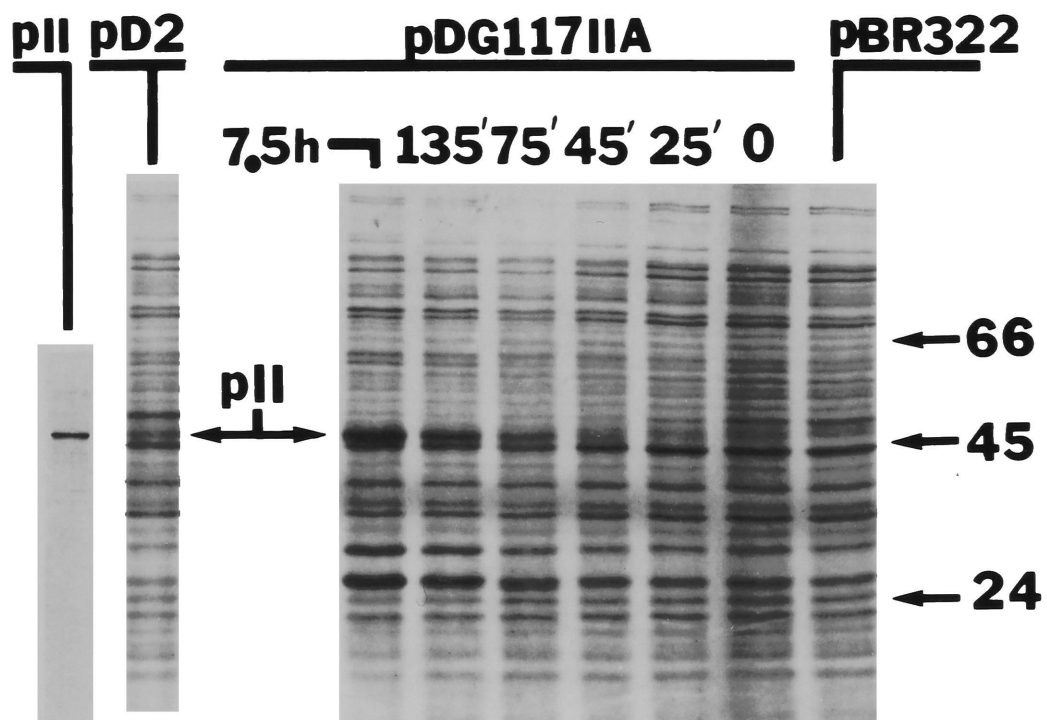


with guanidine hydrochloride. Briefly, an *EcoRI* restriction fragment (Fulford & Model, 1988a) containing gene II under control of the *tacI* promoter (de Boer *et al.*, 1983) was cloned into pDG117 at the *EcoRI* site. The resulting plasmid, pDG117IIA, contains an  $f1 A^+B^-$  origin and an IPTG-inducible gene II protein. Upon induction, both gene II and gene X proteins are greatly overproduced (approximately 30 mg/liter culture) due to positive feedback--the gene II protein produced acts at the *f1* origin, stimulating DNA replication and leading to amplification of the available templates for transcription. Figure 2.1 shows a time course of induction of gene II protein from K561 bearing pDG117IIA grown in rich medium. Gene II protein is maximally induced at late times (5-7.5 hr) after induction with IPTG (Figure 2.1 and unpublished data). The plasmid pDG117IIA produces approximately 5-10-fold more gene II protein than does pD2 which constitutively produces gene II protein using its own promoter. Induction in minimal medium is more rapid (maximal at 35 minutes after induction) but the maximal amount of gene II protein produced is about 5-fold lower due to the lethal effect of the induction (data not shown). K561 bearing pDG117IIA was grown in 4 l of fortified broth containing 100  $\mu$ g/ml ampicillin at 37°C to an O.D.<sup>660 nm</sup> of 0.3 (approximately  $1.5-1.8 \times 10^8$  cells/ml), at which time IPTG was added to 2 mM. The cells were harvested 5 hours later (17.5 g wet weight), and broken using a carver press. The broken cells were resuspended in 100 ml of 100 mM maleic acid-NH<sub>4</sub><sup>+</sup> (pH 6.8 at 23°C), 10% glycerol, 1 mM EDTA, and 5 mM  $\beta$ -mercaptoethanol (buffer A). The broken cell suspension was centrifuged at 100,000 x g for 1 hr at 4°C in a Type 60 Ti rotor (31.5 Krpm). The gene II protein was located in the pellet, which was washed in buffer A and resuspended in 80 ml of buffer A. 7 M guanidine hydrochloride (Heico extreme purity) was added to a final concentration of 1 M, with stirring for 1 hr at 4°C, followed by centrifugation at 100,000 x g for 1 hr at 4°C. The gene II protein was located in the pellet, which was resuspended in 25 ml of buffer A. Solid guanidine hydrochloride was added to a final concentration of 7 M, with stirring for 1 hr at 4°C, followed by centrifugation at 100,000



x g at 4°C for 1 hr. The supernatant fraction was dialyzed against 2 l of buffer A with four changes of the buffer. The precipitate that developed was collected by centrifugation at 10,000 rpm in a Sorvall SS-34 rotor at 4°C for 10 minutes. The precipitate was resuspended in 20 ml of Buffer A containing 7 M guanidine hydrochloride, and dissolved by stirring for 1 hr at 4°C, followed by centrifugation at 10,000 rpm for 10 minutes at 4°C. A 10 ml portion of the supernatant fraction containing the gene II protein was loaded on a Sephacryl S200 column (90 cm x 2.5 cm diameter) equilibrated with 7 M guanidine hydrochloride, 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 50 mM  $\beta$ -mercaptoethanol. The column was run at room temperature (23°C) and 7 ml fractions were collected. The gene II protein was located very close to the void volume. 10 ml of the peak Sephacryl S200 fraction was loaded on a Sephacryl S400 column (90 cm x 2.5 cm diameter) which was prepared and run as above. These gel filtration columns containing 7 M guanidine hydrochloride cannot be stored for more than several months, because eventually guanidine hydrochloride precipitates in the tubing and in the column. Perhaps a lower concentration of guanidine hydrochloride (4 M) would permit storage of the columns. The peak fractions were diluted with four volumes (1:5 dilution) of the column buffer and renatured by dialysis against 25 mM imidazole-HCl (pH 6.8 at 23°C), 10% glycerol, 400 mM KCl, 1 mM EDTA, and 5 mM  $\beta$ -mercaptoethanol (buffer B) containing 1 M guanidine hydrochloride, for 2 hr at 4°C, followed by dialysis against buffer B for 24 hr with four changes of the buffer. Renaturation of gene II protein was dependent on the protein concentration at which the protein was renatured. Best results were obtained when gene II protein was renatured in the concentration range of 10-100  $\mu$ g/ml. The gene II protein obtained (2 mg) had a specific activity of  $2.5-5.0 \times 10^5$  units/mg, which is comparable to the specific activity obtained by Meyer & Geider (1979a). Both methods of protein preparation gave the same footprinting and gel retardation results. One unit is the amount needed for the relaxation of 0.25  $\mu$ g of f1 RFI to RFII and RFIV

Figure 2.1. Induction of gene II protein. 100 ml cultures of K561 bearing pDG117IIA, pD2, and pBR322, respectively, were grown at 37°C. At an  $O.D._{660nm}=0.3$ , 0.2 ml of each culture was taken for analysis of total protein by precipitation with 5% trichloroacetic acid (TCA). Also, IPTG was added (to the culture of K561 bearing pDG117IIA) at this time to a final concentration of 2 mM, and a 0.2 ml aliquot was treated with 5% TCA at various times after induction for precipitation of total protein. The figure shows the analysis of these induced and un-induced protein samples on a 12% polyacrylamide Laemmli gel stained with Coomassie blue. The plasmids are indicated above the gel lanes containing the respective protein samples. The time after which IPTG was added is indicated for the K561/pDG117IIA culture. Equal cell equivalents were added to each lane, corresponding to 0.2 ml of a culture at an  $O.D._{660nm}=0.3$ . The positions of protein molecular weight markers (kdal) are indicated at the right side of the gel. The leftmost lane shows the analysis of purified gene II protein (100 ng) on a silver-stained 15% polyacrylamide Laemmli gel.





in 30 minutes at 30°C in 20  $\mu$ l of 20 mM Tris-Cl (pH 8.0), 5 mM  $\text{MgCl}_2$ , 5 mM dithiothreitol (DTT), and 80 mM KCl. The amount of gene II protein was estimated from the intensity of the band on silver-stained (Wray *et al.*, 1981) 12% Laemmli (1970) gels, and by the Bradford (1976) dye-binding protein assay. Figure 2.1 shows a typical sample of gene II protein (100 ng) visualized by silver-staining of a 15% polyacrylamide Laemmli gel.

### **Binding of gene II protein to the origin**

#### **DNase I protection experiments**

DNase protection experiments (Galas & Schmitz, 1978) were performed in a 10  $\mu$ l reaction containing the following: 10-60 fmol end-labeled restriction fragment, 5-40 ng (100-800 fmol) gene II protein, 100 ng pBR322 DNA, 80 mM KCl, 70 mM imidazole-Cl (pH 6.8) [or 50 mM Hepes (pH 7.0)], 5 mM  $\text{MgCl}_2$ , 5 mM DTT, and 4% glycerol. After addition of gene II protein, the mixture was incubated for 10 minutes at room temperature (23°C), after which 30 ng of DNase I (Boehringer Mannheim Biochemicals) in a volume of 1  $\mu$ l was added. Thirty seconds later, the digestion was stopped by addition of 90  $\mu$ l of stop mix: 0.3 M NaOAc (pH 5.1), 66 mM  $\text{NH}_4\text{OAc}$ , 6 mM EDTA, 0.1% sodium dodecyl sulfate (SDS). The DNA was then extracted with phenol (in cases where purified gene II protein was used, identical results were obtained if the phenol extraction step was omitted and the samples were precipitated with ethanol directly), precipitated with ethanol, and dissolved in 2  $\mu$ l of 80% formamide containing bromphenol blue and xylene cyanol tracking dyes. The samples were electrophoresed on standard 8% polyacrylamide sequencing gels (Maxam & Gilbert, 1977). Each gel lane typically contained approximately 5,000-20,000 Cherenkov cpm. The gels were dried, and autoradiography was carried out at -70°C on Fuji RX50 film with an intensification screen.





### **Methylation interference experiment**

A methylation interference experiment was performed according to the method of Siebenlist and Gilbert (1980). End-labeled restriction fragments were methylated with dimethyl sulfate (Aldrich) as described (Maxam & Gilbert, 1977). The binding reaction contained 50-100 fmol of end-labeled origin-containing fragment and 10 ng (200 fmol) of gene II protein in 50  $\mu$ l of binding buffer (20 mM Tris-HCl, pH 8.0, 5 mM  $MgCl_2$ , 5 mM DTT, 200  $\mu$ g/ml Pentex bovine serum albumin [BSA], and 80 mM KCl). The binding reaction was carried out on ice for 2 minutes. The reaction mixture was then filtered through Millipore filter HA (0.45  $\mu$ m pore size), which had been presoaked in the binding buffer without BSA. The filter was washed 5 times with 0.5 ml of binding buffer without BSA at room temperature. For elution of the bound DNA, the filters were placed in siliconized glass vials, and soaked in 1 ml of eluting solution (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.2% SDS) at 37°C for 30 minutes with gentle agitation. The eluate was precipitated with ethanol, dried briefly, and resuspended in 100  $\mu$ l of 1 M piperidine. The cleavage reaction was carried out at 90°C for 30 minutes. The samples were then lyophilized, resuspended in 10  $\mu$ l of  $H_2O$ , and lyophilized. The latter two steps were repeated 5 times. The samples containing equal amounts of radioactivity were analyzed by electrophoresis on standard 8% polyacrylamide sequencing gels. Autoradiography was carried out as described above.

### **Polyacrylamide gel electrophoresis of gene II protein-origin complexes**

Gene II protein-origin complexes were analyzed on 5% polyacrylamide gels (4.94% acrylamide 0.06% bisacrylamide) as described (Fried & Crothers, 1981), except that the gel buffer was 20 mM Tris-Cl (pH 8.0), 0.1 mM EDTA, 1 mM DTT. Electrophoresis was carried out at room temperature (23°C) at 6.5  $Vcm^{-1}$  with recirculation of the buffer. Sharper bands were obtained on the gel if 1xTBE buffer (0.09 M Tris/0.09 M boric acid/2.8 mM EDTA) containing 1 mM DTT was used, in which case recirculation of the



buffer was not necessary. The binding reactions typically contained 15 fmol of origin-containing restriction fragment and 0-100 fmol of gene II protein in 20  $\mu$ l of buffer containing 20 mM Tris-Cl (pH 8.0), 6 mM  $MgCl_2$ , 5 mM DTT, 200  $\mu$ g/ml BSA, 80 mM KCl, and 5% glycerol. The reaction was carried out at room temperature for 5 minutes before gently loading on the gel, which had been pre-run for 1 hr. Marker lanes contained radioactive DNA molecular weight markers and tracking dyes. The gels were dried, and autoradiography was carried out at room temperature.

### **Footprinting of isolated complexes**

The binding reactions were carried out as described in the previous section, except that 50 fmol of end-labeled origin-containing restriction fragment (the 151 bp *EcoRI*-*Bam*HI fragment from pDG117) and 5-40 ng (100-800 fmol) of gene II protein were used. Following a 10 minute incubation at room temperature, 30 ng of DNase I in a volume of 1  $\mu$ l was added for 30 seconds. Immediately after digestion, the samples were loaded onto the gel (5% polyacrylamide as described above) while it was running at 2  $Vcm^{-1}$ . When all the samples were loaded, the gel was run at 6.5  $Vcm^{-1}$ . Complex I, complex II, and unbound DNA were visualized by autoradiography at room temperature, and the corresponding gel bands were excised. The DNA was eluted electrophoretically, precipitated with ethanol, and dissolved in 4  $\mu$ l of 90% formamide plus tracking dyes. The samples were electrophoresed on standard 8% polyacrylamide sequencing gels as described above. Autoradiography was carried out at  $-70^{\circ}C$  on Kodak XAR-5 film with an intensification screen.

### **Methylation interference with formation of the individual complexes**

The gene II protein-origin complexes were isolated from a non-denaturing 5% polyacrylamide gel, and methylation interference was studied for each species. 50 fmol of end-labeled, origin-containing restriction fragment was first methylated with dimethyl sulfate as described by Maxam & Gilbert (1977), and then incubated with 0-400 fmol of



gene II protein for 5 minutes at room temperature. Complex I, complex II, and unbound DNA were separated on a 5% polyacrylamide gel and visualized by autoradiography. The DNA fragments were excised from the gel, eluted electrophoretically, and recovered by ethanol precipitation. The methylated DNA was then cleaved with 1M piperidine at 90°C for 30 minutes. Following lyophilization, the samples were electrophoresed on standard 8% polyacrylamide sequencing gels, and autoradiography was carried out at -70°C on Kodak XAR-5 film with an intensification screen.

### **Stoichiometry of gpII binding**

Radiolabeled gpII was prepared as follows. K561 bearing pDG117IIA was grown at 37°C in 100 ml of DO minimal media supplemented with 200 µg/ml of each amino acid except methionine and with 100 µg/ml ampicillin. At O.D.<sup>660nm</sup>=0.5, IPTG was added to a final concentration of 2 mM. Five minutes after induction, the cells were pulse-labeled for two minutes with 10 mCi of <sup>35</sup>S-methionine (1128 Ci/mmol from New England Nuclear). The cells were chilled on ice and pelleted by spinning at 7000 rpm for 10 minutes at 4°C. After washing in ice-cold calcium saline (0.14 M NaCl, 2 mM CaCl<sub>2</sub>), the cells were resuspended in 1 ml of 10% sucrose, 0.1 M NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM DTT, and 1 mM EDTA. The resuspended cells were frozen on dry ice, put on ice for two minutes, and placed at room temperature until melting was complete. Then 0.1 ml of 2 mg/ml lysozyme in 0.25 M Tris-HCl (pH 8.0) was added, followed by incubation on ice for 45 minutes. The lysozyme-digested cells were heat shocked at 37°C for two minutes and then centrifuged at 25,000 rpm for 20 minutes at 4°C in a Type 40 rotor and the supernatant was collected (Fxn I). All subsequent purification steps were carried out at 4°C unless otherwise noted. Ammonium sulphate (0.24 grams) was added to 0.8 ml of Fxn I with stirring for 1 hour, followed by centrifugation at 8000 x g in an Eppendorf microcentrifuge for 20 minutes. The pellet was resuspended in 1 ml of 50 mM Tris-HCl (pH8.0), 10 mM KCl, 10% glycerol, 5 mM β-mercaptoethanol, and 1 mM



EDTA, and dialyzed against two liters of 25 mM Tris-HCl (pH 8.0), 10% glycerol, 5 mM  $\beta$ -mercaptoethanol, 1 mM EDTA (buffer C) containing 50 mM KCl (C-50) for 18 hours with two changes of buffer (Fxn II). Fxn II was loaded onto a 1 ml DE52 column equilibrated with C-50. The column was washed with 5 ml of the same buffer. The  $^{35}\text{S}$ -gpII was eluted with 3 ml of C-250 and 0.2 ml fractions were collected. The protein peaks were located by liquid scintillation counting, and the gpII activity was located using the  $\text{Mn}^{2+}$  cleavage assay (see below). The three peak fractions were pooled and dialyzed against 1 liter of C-50 for four hours with one change of buffer (Fxn III). Fxn III was loaded onto a 1 ml heparin sepharose (Pharmacia) column equilibrated with C-50. The column was washed with 3 ml of C-100, followed by 3 ml of C-250. The  $^{35}\text{S}$ -gpII was eluted with 3 ml of C-500, and 0.3 ml fractions were collected.

The  $^{35}\text{S}$ -gpII thus obtained had a concentration of  $1.6 \pm 0.1 \mu\text{g gpII/ml}$  and was approximately 75% pure. The concentration of  $^{35}\text{S}$ -gpII was determined by densitometry of a photographic negative (diapositive) of a silver-stained 12% SDS-PAGE gel using purified gpII as standard. The concentration of the gpII standard was determined by amino acid analysis on an Applied Biosystems amino-acid analyzer using pre-column derivitization (performed by Dr. D. Atherton of the Rockefeller University protein sequencing facility). The  $^{35}\text{S}$ -gpII obtained had a specific activity of  $18,700 \pm 2,100 \text{ cpm/pmol}$ . The gpII sample was counted by excising the gpII protein band located by autoradiography of a dried 12% SDS-PAGE gel that had been fixed in 50% methanol and 10% acetic acid for 30 minutes. The counting efficiency of the  $^{35}\text{S}$  in the 12% SDS-PAGE gel was lower than that from the gel used to analyze gpII binding to the origin (described below). To determine the correction factor, a DNA fragment that had been end-labeled with  $[\alpha\text{-}^{35}\text{S}]\text{-dATP}$  was loaded onto a 12% SDS-PAGE gel and a 5% binding gel, the radioactive band was located by autoradiography of the fixed and dried gels, and the radioactivity in the excised bands was determined by liquid scintillation counting in an Intertechnique SL 36 liquid scintillation spectrometer. The correction factor was





found to be  $3.0 \pm 0.1$ .

For the binding analysis, the 151 bp *EcoRI-BamHI* origin restriction fragment from pDG117IIA was used. The fragment was end-labeled by treatment with T4 polynucleotide kinase and  $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$  subsequent to treatment with CIAP. The amount of the origin restriction fragment was quantitated by densitometry of a photographic negative (Polaroid 665 Professional positive/negative instant pack film) of an ethidium-bromide stained 2% agarose gel using known amounts of a f1 *HaeIII* digest as standard. The concentration of f1 standard was determined by absorbance at 260 nm using a value of  $1.0 \text{ A}^{260} \text{ unit} = 50 \text{ } \mu\text{g/ml}$  for double-stranded DNA. The amount of radioactivity in the origin fragment was quantitated by liquid scintillation counting of the excised band from a fixed and dried binding gel. The specific activity of the restriction fragment was adjusted to  $23,000 \pm 1,800 \text{ cpm/pmol}$  by addition of nonradioactive origin restriction fragment. The binding of the  $^{35}\text{S}$ -gpII to the  $^{32}\text{P}$ -origin was analyzed essentially as described. The gel (22.5 cm long x 20 cm wide x 1 mm thick) was a 5% polyacrylamide (4.94% acrylamide 0.06% bisacrylamide) gel and the buffer was 1X TBE (89 mM Tris-borate (pH8.3) and 2.3 mM EDTA). The complete binding reaction contained 75.1 fmol origin restriction fragment, and 417.4 fmol  $^{35}\text{S}$ -gpII in 60  $\mu\text{l}$  of 25 mM Tris-HCl (pH 8.0), 100 mM KCl, 5% glycerol, 6 mM  $\text{MgCl}_2$ , 5 mM DTT, 1 mM  $\beta$ -mercaptoethanol, 0.2 mM EDTA, 200  $\mu\text{g/ml}$  Pentex BSA, and 5  $\mu\text{g/ml}$  pBR322. Electrophoresis was at 125 volts for 6 hours. Following electrophoresis, the gel was fixed in 50% methanol and 10% acetic acid for 30 minutes and dried. The complexes were visualized by autoradiography at  $-70^\circ\text{C}$  on Kodak XAR-5 film with an intensification screen. The complexes were excised from the dried gel and liquid scintillation counting was performed. Channel 1 (0-100) counted the total counts  $= ^{32}\text{P} + ^{35}\text{S}$ . Channel 2 (70-100) counted  $45.4 \pm 1.4\%$  of the  $^{32}\text{P}$  counts and  $\leq 0.2\%$  of the  $^{35}\text{S}$  counts. The  $^{32}\text{P}$  counts were calculated by channel 2-background/0.454, and the  $^{35}\text{S}$  counts were calculated by channel 1-background=total cpm= $^{35}\text{S} + ^{32}\text{P}$ . The  $^{35}\text{S}$  in each complex was also counted in the



absence of  $^{32}\text{P}$  by adding  $^{35}\text{S}$ -gpII to 75.1 fmol of non-radioactive origin restriction fragment, locating the complexes by autoradiography as above, and counting directly. Both methods of determination of the  $^{35}\text{S}$  counts were in agreement to within 5%. The background levels of radioactivity were determined by excising the corresponding region of the gel from a lane in which the  $^{35}\text{S}$ -gpII preparation had been electrophoresed without an origin fragment.

### **Binding of IHF to the origin.**

#### **Polyacrylamide gel electrophoresis of IHF bound to the origin**

Procedures for DNA manipulations and end-labeling of restriction fragments were essentially as described by Maniatis *et al.* (1982). Binding to a *HpaII-Sau96I* digest of f1 RFI was analyzed by gel retardation (Garner & Revzin, 1981; Fried & Crothers, 1981). Purified IHF (Nash *et al.*, 1987b) (kindly provided by H. Nash and C. Robertson) was added (0-0.55 ng) to 5.5 fmol of labeled *HpaII-Sau96I* digest of f1 RFI on ice in a 20  $\mu\text{l}$  reaction volume containing 30 mM Tris-HCl (pH 7.5), 80 mM KCl, 5% glycerol, 6 mM  $\text{MgCl}_2$ , 5 mM DTT, and 220  $\mu\text{g/ml}$  Pentex BSA. After a five minute incubation at room temperature, the binding reactions were gently loaded onto a 5% polyacrylamide gel (4.94% acrylamide, 0.06% bisacrylamide) with TBE buffer (89 mM Tris-borate [pH 8.3], 2.3 mM EDTA) and electrophoresed at 6 V/cm for 3 hr at room temperature.

#### **DNase I protection of the origin by IHF**

Binding of IHF to the origin was also analyzed by DNase I footprinting (Galas & Schmitz, 1978) using the f1 *Sau96I-ClaI* fragment containing the origin that was end-labeled as described above. 10-20 fmol of end-labeled origin restriction fragment was incubated in a 9  $\mu\text{l}$  reaction volume containing 56 mM Tris-HCl (pH 7.5), 4.4% glycerol, 89 mM KCl, 5.6 mM  $\text{MgCl}_2$ , 5.6 mM DTT, and IHF (0-0.5  $\mu\text{M}$  as indicated). After incubation at room temperature for 10 minutes, 30 ng of DNase I was added for 30 seconds, after which 90  $\mu\text{l}$  of stop mix (0.3 M NaOAc [pH 5.1], 67 mM  $\text{NH}_4\text{OAc}$ , 0.1% SDS, and



25 mM EDTA) was added. The DNA was precipitated with ethanol, resuspended in 4  $\mu$ l of 80% formamide containing bromphenol blue and xylene cyanol tracking dyes, boiled for 4 minutes, and electrophoresed on standard 8% polyacrylamide sequencing gels (Maxam & Gilbert, 1977). Footprinting to the separated IHF binding sites was analyzed on 20% polyacrylamide sequencing gels. Site 1 was separated from site 2' and site 2'' by digestion of R209 RFI at the *EcoRI* site, which also served as the site of labeling. Maxam-Gilbert sequencing reactions of the end-labeled origin fragments served as markers. Autoradiography was carried out at  $-70^{\circ}\text{C}$  on Fuji RX50 film with an intensification screen.

### Analysis of DNA bending

DNA bending was analyzed by the method of Wu & Crothers (1984). A series of circularly permuted origin restriction fragments was produced by digestion of pJ1 with *Bam*HI, *Taq*I, *Sau*96I, *Ava*I, *Tha*I, and *Mbo*II, individually. The 393 bp origin fragments were isolated on a 2% agarose gel as described above. The *Tha*I and *Taq*I fragments were found to be slightly contaminated with other restriction fragments from the plasmid vector. Therefore, the *Taq*I and *Tha*I fragments were further purified on a preparative 4% polyacrylamide gel. Binding of IHF and gpII to the end-labeled 393 bp restriction fragments was analyzed as described on a 4% polyacrylamide (3.95% acrylamide, 0.05% bisacrylamide) gel with 1X TBE buffer.

### Purification of the mp1 gene II protein

The mp1 mutant gene II protein (met<sup>40</sup>→ile) was purified from DH5 $\alpha$  (K1225) bearing pAR1 by a modification of the method of Meyer & Geider (1979a) as follows. A 4 liter culture of DH5 $\alpha$  bearing pAR1 was grown to O.D.<sup>660nm</sup>=6.65 in a Lab-Line/S.M.S. Hi-Density Fermenter (No. 29500) at 37 $^{\circ}\text{C}$  using O<sub>2</sub> aeration at a flow rate of 5-7 liters/min. The cells were harvested by centrifugation at 7000 rpm for 20 minutes at



4°C in a GSA rotor. The cells (49.7 g wet weight) were resuspended in 50 ml of 10% sucrose, 50 mM Tris-Cl (pH 7.5), 20 mM spermidine-trihydrochloride, 5 mM DTT, 1 mM EDTA, and 0.1 M NaCl. Lysozyme was added to a final concentration of 300 µg/ml and the suspension was incubated for 45 minutes on ice. The suspension was frozen in a dry ice-ethanol bath and thawed rapidly at 37°C with gentle shaking. The lysed cell suspension was centrifuged at 20,000 rpm in a Type 21 rotor for 2 hr at 4°C (all subsequent steps were also carried out at 4°C). The supernatant was collected (Fxn I) and ammonium sulfate (0.3 grams per ml of Fxn I) was added over a 30 minute period with stirring. The slurry was centrifuged at 20,000 rpm in a Type 21 rotor for 30 minutes. The pellet was resuspended in 50 ml of 50 mM Tris-Cl (pH 8.0), 10% glycerol, 1 mM EDTA, 10 mM KCl, and 5 mM DTT. The resuspended pellet was dialyzed against 2 liters of 25 mM Tris-Cl (pH 8.0), 10% glycerol, 5 mM DTT, 1 mM EDTA (Buffer D) containing 50 mM KCl (D-50). Dialysis was carried out for 6 hours with 3 changes of the buffer. The sample was frozen quickly in a dry ice-ethanol bath and stored at -70°C (FxnII). Fxn II was loaded onto a 230 ml DE52 column (47 cm x 2.5 cm diameter) equilibrated with D-50. The column was washed with 5 column volumes of D-50, and the mp1 gene II protein was eluted with a 1 liter linear gradient formed using D-50 and D-1000. The peak fractions were pooled and dialyzed against D-50 (Fxn III). Fxn III was loaded onto a 22 ml Heparin-Sepharose CL-6B (Pharmacia) column (28 cm x 1 cm diameter) equilibrated with D-50. The column was washed with 3 column volumes of D-50, followed by 5 column volumes of D-250. The mp1 gene II protein was eluted with D-500 and dialyzed against 25 mM Hepes (pH 7.0), 10% glycerol, 5 mM DTT, 1mM EDTA (buffer E) containing 50 mM KCl (E-50)--(Fxn IV). Fxn IV was dialyzed against E-50 and loaded onto a 2 ml Biorex 70 column equilibrated with E-50. The column was washed with 10 column volumes of E-50 followed by 3 column volumes of E-250. The mp1 gene II protein was eluted using a 20 ml linear gradient of E-250 to E-2000. The mp1 gene II protein obtained (50 µg) was dialyzed against 50% glycerol, 25 mM Hepes (pH 7.0), 100 mM





KCl, 5 mM DTT, 1 mM EDTA and stored at  $-20^{\circ}\text{C}$ . Wild-type gene II protein was isolated from DH5 $\alpha$  containing pD2 in parallel. Both the wild-type and mutant proteins were approximately 30% pure.

### **Mn<sup>2+</sup>-dependent double strand cleavage activity**

#### **Cleavage reaction**

Cleavage reactions were carried out as described by Meyer & Geider (1979b). Purified gene II protein (5-20 ng) was incubated with f1 RFI (0.1 ng-1.0  $\mu\text{g}$ ) in 20  $\mu\text{l}$  containing 20 mM Tris-Cl (pH 8.0), 80 mM KCl, 5 mM DTT, and 5 mM MnCl<sub>2</sub> for 30 minutes at  $30^{\circ}\text{C}$ . The reactions were terminated by addition of 1  $\mu\text{l}$  of stop mix (0.2 M EDTA (pH 8.0), 20% sucrose, 1% SDS, and 0.01% bromphenol blue) and analyzed either on a 0.6% agarose gel, an 8% polyacrylamide gel, or an alkaline agarose gel (in which case the stop mix was not added) as described (Maniatis *et al.*, 1982).

#### **Mapping of cleavage sites**

The origin-containing plasmid pDG117 (20  $\mu\text{g}$ ) was cleaved with gene II protein in the presence of Mn<sup>2+</sup> (described above), and the cleavage product (RFIII-Mn<sup>2+</sup>) was isolated on a 0.6% agarose gel. The origin-containing plasmid (pDG117) was also nicked by gene II protein in the presence of Mn<sup>2+</sup> after a brief incubation (1 minute), and the nicked product (RFII-Mn<sup>2+</sup>) was isolated on a 0.6% agarose gel. The gel-purified DNA samples were eluted electrophoretically, precipitated with ethanol, and further purified by extraction with phenol and precipitation with ethanol. Purified RFII-Mn<sup>2+</sup> (10  $\mu\text{g}$ ) and RFIII-Mn<sup>2+</sup> (10  $\mu\text{g}$ ) were each divided into two portions. One portion was digested with *Bam*HI (set B) and the other portion was digested with *Eco*RI (set E). Sets B and E were each divided in half. Half of each set was end-labeled by treatment with T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]-ATP following treatment with CIAP. The other half of each set was end-labeled using treatment with the Klenow fragment of DNA polymerase



I and [ $\alpha$ - $^{32}\text{P}$ ]-dATP. The conditions used for end-labelling were described above. Following end-labeling, set B was digested with *Eco*RI and set E was digested with *Bam*HI. The end-labeled restriction fragments were analyzed on a 8% polyacrylamide 50% urea DNA sequencing gel or a 8% polyacrylamide-70% formamide sequencing gel. Maxam-Gilbert sequencing reactions performed on the 151 bp *Bam*HI-*Eco*RI fragment served as markers. The identity of the individual bands in Figure 4.2 was confirmed by Maxam-Gilbert sequencing following their isolation on preparative DNA sequencing gels. The radioactive DNA fragments were eluted from the preparative DNA sequencing gel by suspension of the gel slice in 400  $\mu\text{l}$  of N buffer (0.1 M Tris-Cl [pH 7.9], 0.6 M NaOAc, and 2.5 mM EDTA) at 37°C overnight, followed by precipitation with ethanol.

### Sequencing of the cleavage product

RFIII-Mn $^{2+}$  was sequenced by the method of Maxam & Gilbert (1977) using the *Bam*HI site of pDG117 as the site of labeling. The *Bam*HI end-labeled fragment that extended to the site of cleavage by gene II protein was isolated on a 70% formamide 8% polyacrylamide gel and purified by electroelution. Following the base-specific cleavage reactions, the sample was loaded onto a 70% formamide 8% polyacrylamide DNA sequencing gel.

## Unwinding reactions

### Source of proteins

Gene II protein was purified as described above. SSB was provided by K. Horiuchi or was purchased from US Biochemical Corp. The Rep helicase was purified from the *E. coli* strain MZ-1 bearing pRep0 (kindly provided by D. Denhardt) by a modification of the method of Scott & Kornberg (1978). FxnIIIb (described by Scott & Kornberg, 1978) was dialyzed against 20% glycerol, 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM DTT (Buffer F) containing 25 mM NaCl (F-25). Following dialysis, the sample was loaded



onto a 4 ml column of ATP agarose (AGATP Type 4 from Pharmacia) which had been equilibrated with buffer F-25. The column was washed with 5 column volumes of buffer F-250, and the Rep helicase was eluted with 2 column volumes of buffer F-1000. The Rep helicase obtained (approximately 400  $\mu\text{g}$ ) had a specific activity of approximately  $1 \times 10^7$  units/mg and was approximately 80% pure as judged from SDS-PAGE gel electrophoresis and silver-staining.

### **Assay of Rep helicase**

The Rep helicase was assayed as follows. Rep helicase was added to 20  $\mu\text{l}$  reactions containing 50 mM Tris-Cl (pH 7.5), 10 mM DTT, 100  $\mu\text{g}/\text{ml}$  BSA, 5 mM  $\text{MgCl}_2$ , 6% sucrose, 50  $\mu\text{M}$  ATP, 0.7  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]-ATP, and 20 ng f1 single-stranded DNA. Following incubation at 30°C for 20 minutes, 50  $\mu\text{l}$  of ice cold 1.25 N  $\text{HClO}_4$ /1 mM  $\text{NaH}_2\text{PO}_4$  was added, followed by 180  $\mu\text{l}$  of  $\text{H}_2\text{O}$  saturated with benzene:isobutanol (1:1). Then 24  $\mu\text{l}$  of a 5% solution of ammonium molybdate was added, and the samples were vortexed for 30 seconds and centrifuged for 10 seconds in a table top Eppendorf microfuge. Then 500  $\mu\text{l}$  of benzene:isobutanol (1:1) was added, and the samples were vortexed for 30 seconds and centrifuged for 1 minute. 200  $\mu\text{l}$  of the organic layer (upper phase) was counted for Cherenkov cpm. Duplicate reactions were performed, except that the f1 single-stranded DNA was omitted. The Rep helicase activity was the single-stranded DNA-dependent ATPase activity, with the unit definition being the amount required to produce 1 pmol of ADP per minute of incubation. Where necessary, the Rep helicase was diluted in 10% sucrose, 50 mM Tris-Cl (pH 7.4) for assay.

### **Unwinding assays**

Unwinding reactions were carried out in 20  $\mu\text{l}$  reactions containing 100 ng of the origin-containing plasmid pD10, gene II protein (10-40 ng), Rep helicase (250 ng), SSB (600 ng), 20 mM Tris-Cl (pH 8.0), 80 mM KCl, 5 mM DTT, 6 mM  $\text{MgCl}_2$ , 5% glycerol, and 1 mM ATP. Following the incubation (20 minutes at 30°C), 2  $\mu\text{l}$  of stop mix (0.23



M EDTA [pH 8.0], 7% sucrose, 7% SDS, and 0.03% bromphenol blue) was added, the samples were vigorously vortexed, and loaded onto a 0.6% agarose gel containing 0.5  $\mu\text{g}/\text{ml}$  ethidium bromide and 1X TBE buffer.

For the identification of the strand specificity of the unwinding products, the unwound DNA was transferred to a nitrocellulose membrane (Southern, 1975) and hybridized with the radioactive probes cw=(5'-GTATCACGAGGCCCTT-3') and ccw=(5'-AAGCTGTCAAACATGA-3') which had been labeled by treatment with T4 polynucleotide kinase and [ $\gamma$ - $^{32}\text{P}$ ]-ATP.





## Chapter 3

### **Binding of Gene II Protein to the Replication Origin**



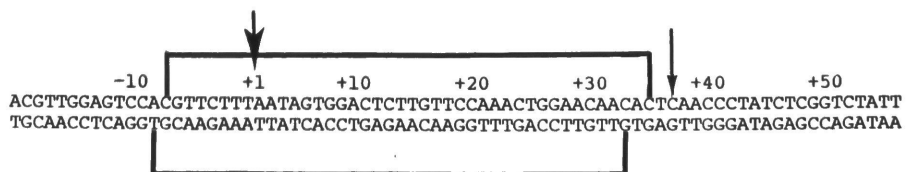
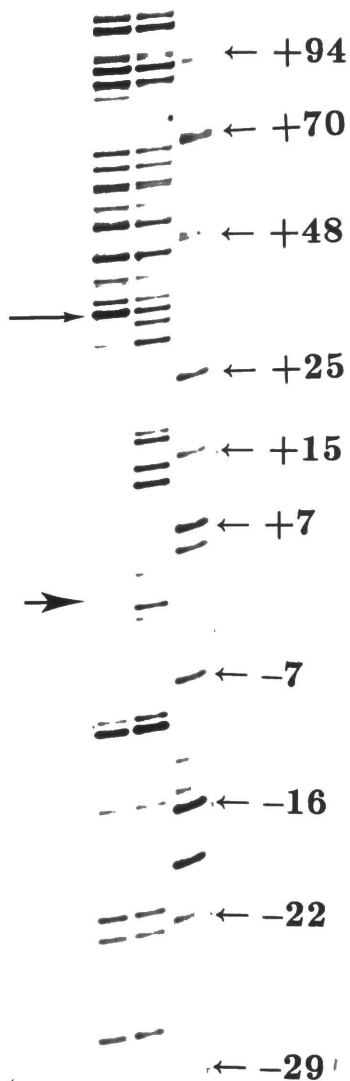
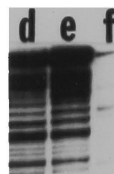
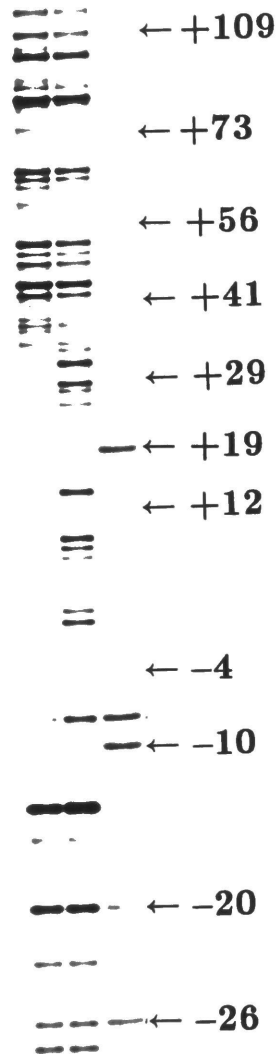
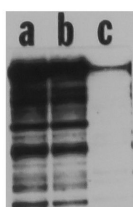
Before I undertook this work, the enzymology (Meyer & Geider, 1982) and the DNA sequence requirements (Zinder & Horiuchi, 1985) of f1 DNA replication were well established. What seemed to be missing was a synthesis: a detailed understanding of the DNA-protein and protein-protein interactions that mediated replication. I chose the gene II protein-origin interaction as a starting point because of the central importance of the initiator protein in f1 DNA replication. Here I show that gene II protein binds to the core origin in two steps involving DNA-protein and protein-protein interactions. Methylation interference experiments indicate that gene II protein makes homologous contacts with three repeat elements within the core origin. Surprisingly, these essential contacts are not within the nicking site.

### **Binding of gene II protein to the replication origin**

Filter-binding studies (Horiuchi, 1986) demonstrated that the gene II protein binds specifically to the origin of replication, requiring sequences around palindrome E (see Fig. 1.3), though the precise location of the binding site was not determined. Both superhelical and linear DNA bound the protein. To determine the location of binding sites, I examined the binding of the gene II protein for each strand of the replication origin using the DNase I footprinting technique (Galas & Schmitz, 1978). The origin fragment used in this analysis was end-labeled at the unique *AsuI* site (position -56) using either T4 polynucleotide kinase following CIAP treatment, or DNA polymerase I Klenow fragment (see Materials and Methods). The results shown in Figure 3.1(a) indicate that the gene II protein protects 41 bases of the minus strand from DNase I, from position -9 to position +32 (lane a), and 46 bases of the plus strand, from position -12 to +34 (lane d). A site of enhanced cleavage is present on the plus strand at position +36 (thin arrow in Figure 3.1(a)), while no such hypersensitive site was found on the minus strand. The data are summarized in Figure 3.1(b). The protected region resides entirely within

Figure 3.1. Binding of the gene II protein to the origin. (a). DNase I protection experiment. Lane a, footprint to the minus strand of the origin (40 ng gene II protein). Lane b, minus strand DNase I control (no gene II protein added). Lane c, Maxam-Gilbert G reaction for the minus strand. Lane d, footprint to the plus strand of the origin (40 ng gene II protein). Lane e, plus strand DNase I control (no gene II protein added). Lane f, Maxam-Gilbert G reaction for the plus strand. The 315 bp origin-containing *AsuI*-*ClaI* fragment was labeled by 3'-end-filling with [ $\alpha$ - $^{32}$ P]-dGTP and DNA Polymerase I Klenow fragment at the *AsuI* site for the analysis of the minus strand. The plus strand was labeled by treatment with T4 polynucleotide kinase and [ $\gamma$ - $^{32}$ P]-ATP at the *AsuI* site, following treatment with CIAP. The thick arrow indicates the site of nicking by the gene II protein on the plus strand. The gene II protein nicks the origin-containing fragment at a low efficiency (0.5%). The nicking is strand-specific and occurs at the correct site (data not shown). The thin arrow indicates the site of enhanced cleavage by DNase I on the plus strand.

(b). Summary of results. The region protected by the gene II protein is underlined; weak protection is indicated by broken lines.





domain A. No binding to domain B was observed in the presence (Figure 3.1) or absence (data not shown) of domain A.

### Binding of gene II protein to defective origins

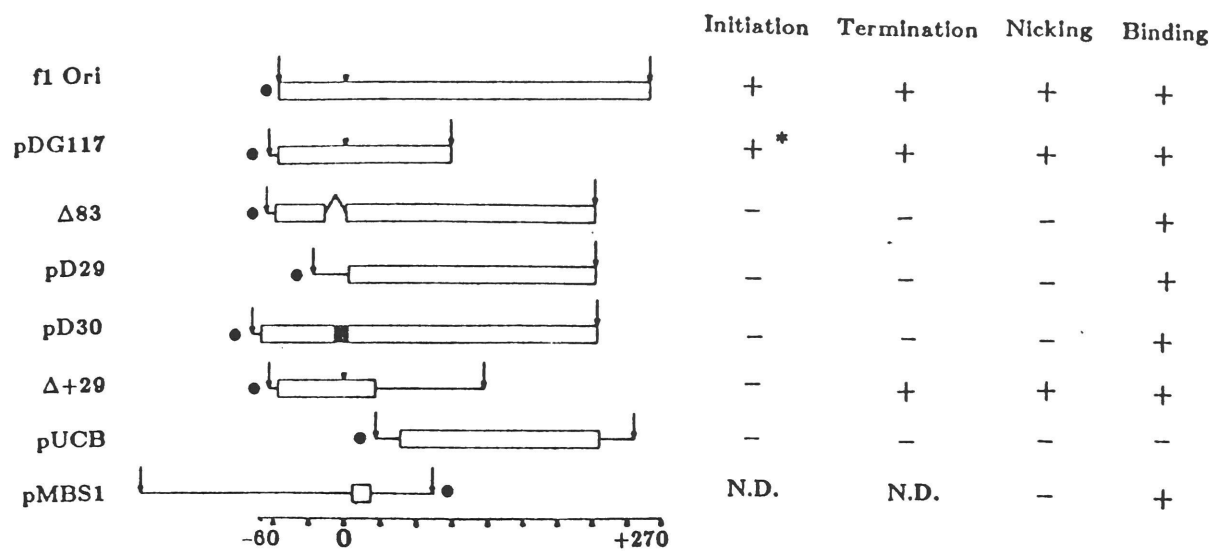
A series of defective origins had previously been constructed and characterized, leading to an understanding of the overlapping signals within the origin (Dotto *et al.*, 1982a, 1984). Figure 3.2 lists the properties of the defective origins used in the present study. Filter-binding studies (Horiuchi, 1986) using these deletion mutants have led to the following conclusions: 1) a number of the defective origins could bind the gene II protein; 2) the nicking site was dispensable for binding; 3) sequences required for binding were located around palindrome E. DNase I footprints of gene II protein to the defective origins are shown in Figure 3.3. I investigated the binding of the gene II protein to an  $A^+B^-$  (wild-type domain A and defective domain B) origin, pDG117 (Figure 3.3(a) and 3.3(b), panel 1). The gene II protein protected essentially all of domain A in the absence of a functional domain B sequence. The extent of the protected region, from position -12 to position +34, and the enhanced cleavage at position +36 on the plus strand (data summarized in Figure 3.3(c)), were identical to those observed in the presence of a functional domain B region (see Figure 3.1). In addition, the  $A^+B^-$  origin showed footprints over the same range of protein concentrations as the wild-type origin (Figure 3.3(a), panel 1).

In the case of two deletions that extend from the left past the nicking site ( $\Delta 83$ , panel 2; and pD29, panel 3 of Figure 3.3), gene II protein protected approximately 44 bp, a result similar to that for the wild-type origin. In both cases, gene II protein protected the nonspecific sequences, corresponding to where the nicking site would have been, had it not been deleted. Both  $\Delta 83$  and pD29, exhibit the enhanced cleavage at position +36, which occurs in the wild-type footprint (see Figure 3.3(c)).

pD30 was constructed by insertion of a 10 bp *Hind*III linker (5'-CCAAGCTTGG-3') at the filled-in *Hin*fI site at position +8. (Dotto *et al.*, 1984). As a consequence of the

Figure 3.2. Properties of wild-type and defective origins. The wild-type and defective origins used in the present study are listed at left. Beside each is a diagram of its DNA sequence. The open bars are f1 sequence. Thin lines are nonspecific sequence. pDG117 contains the f1 origin sequence from the left end of the origin to position +88 (*Hae*III G/D border) relative to the nicking site. The defective origin  $\Delta$ 83 has an internal deletion (indicated by a chevron) of the f1 origin from position -14 to position +3. pD29 contains a deletion of the f1 origin from the left end to position +5. pD30 has a 10 bp insertion at position +8 (indicated by a black bar). As a consequence of the insertion, the wild-type nicking site is positioned 13 bp upstream of its normal position.  $\Delta$ +29 has a deletion from position +29 to the right end. pUCB contains only the domain B region of the f1 origin from position +46 to the right end. pMBS1 contains a 15 bp element corresponding to the center of domain A (positions +6 to +20). The nicking site is indicated by a small arrow head for those origins that are nicked by the gene II protein. The vertical arrows indicate convenient restriction sites described in Materials and Methods. The filled circles show the sites used for end-labeling as described in Materials and Methods. The properties of each origin are listed at right. The asterisk indicates that pDG117 requires qualitative or quantitative changes in gene II protein production for efficient initiation (as described in text). N.D. indicates that these properties were not determined.







insertion, the wild-type nicking site is positioned 13 bp upstream of its normal position. pD30 RFI cannot be nicked by gene II protein *in vitro* (see Figure 3.2). As shown in panel 4 of Figure 3.3(a) and (b), gene II protein bound to pD30 and protected 46 bp from DNase I digestion. The pattern of protection closely resembles that of the wild-type origin (summarized in Figure 3.3(c)). The inserted linker is protected, whereas the displaced nicking site is not. The result indicates that the left side sequence of domain A (up to +11) alone cannot bind gene II protein. Again, pD30 showed footprints over the same range of protein concentrations as the wild-type origin (Figure 3.3(a), panel 4).

The sequence requirements for binding and nicking at the right end of domain A were determined by the deletion mutants  $\Delta+11$  and  $\Delta+29$  (Dotto *et al.*, 1984). The deletion  $\Delta+11$  removes all of palindrome E and fails to bind the gene II protein (Horiuchi, 1986). The right end deletion to position +29,  $\Delta+29$ , can bind gene II protein, be nicked by gene II protein, and terminate replication, but cannot initiate DNA synthesis. The results of the DNase I protection experiment for  $\Delta+29$  are shown in Figure 3.3(a) and (b), panel 5. Gene II protein protects 31 bp on the plus strand, from position -7 to position +24 (Figure 3.3(a), panel 5). On the minus strand, gene II protein protects 33 bp, from position -9 to position +24 (Figure 3.3(b), panel 5). The results are summarized in Figure 3.3(c). The left boundary of the footprint is similar to that of the wild-type origin; however, the protected region is about 10 bp shorter on the right side and the enhanced cleavage at position +36 is absent. Nevertheless,  $\Delta+29$  showed footprints over the same range of protein concentrations as the wild-type origin (Figure 3.3(a), panel 5).

Figure 3.3. Binding of gene II protein to defective origins. (a). DNase I protection experiments with the plus strands labeled. Panels 1-5, footprinting to pDG117,  $\Delta 83$ , pD29, pD30, and  $\Delta +29$ , respectively. Lane a, Maxam-Gilbert G-reaction. Lane b, DNase I control (no gene II protein). Lanes c-f, footprints with 40, 20, 10, and 5 ng of gene II protein, respectively. The lanes marked by + and 0 are the pDG117 origin fragment with and without added gene II protein, showing the nick at the origin marked by an arrow at the side of the panel.

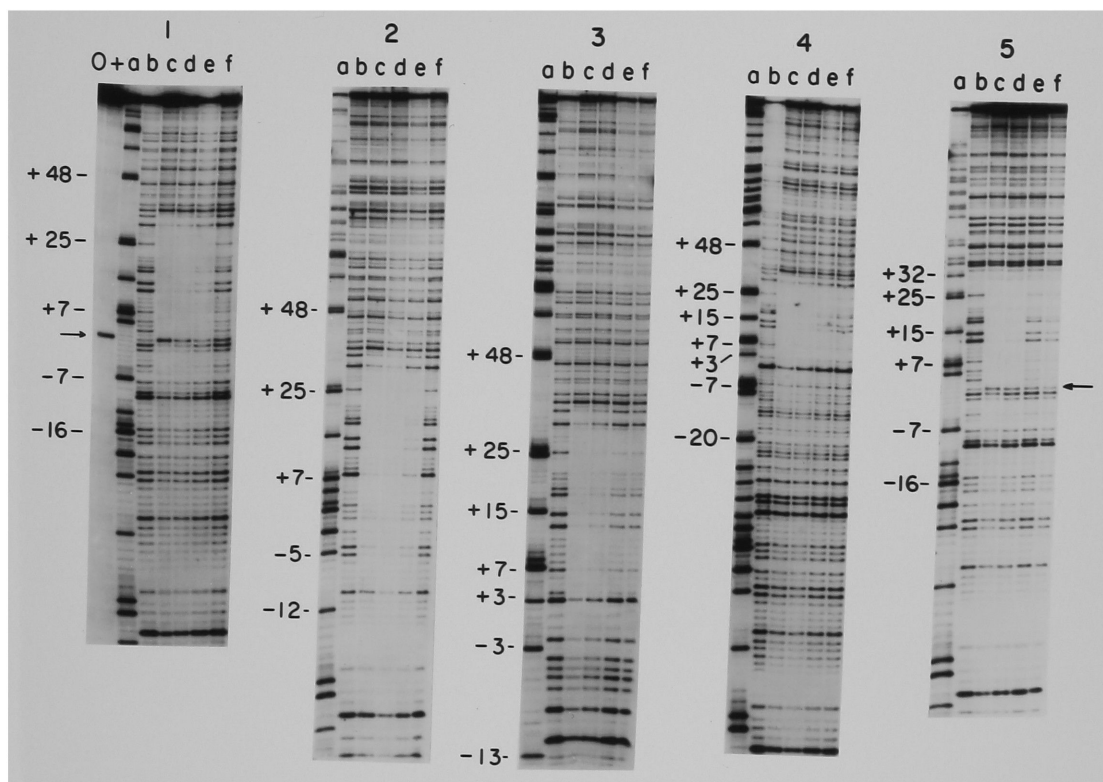






Figure 3.3(b). DNase I protection experiments with the minus strands labeled. The panels are labeled as in (a), except lane c is the footprint with 20 ng of gene II protein.



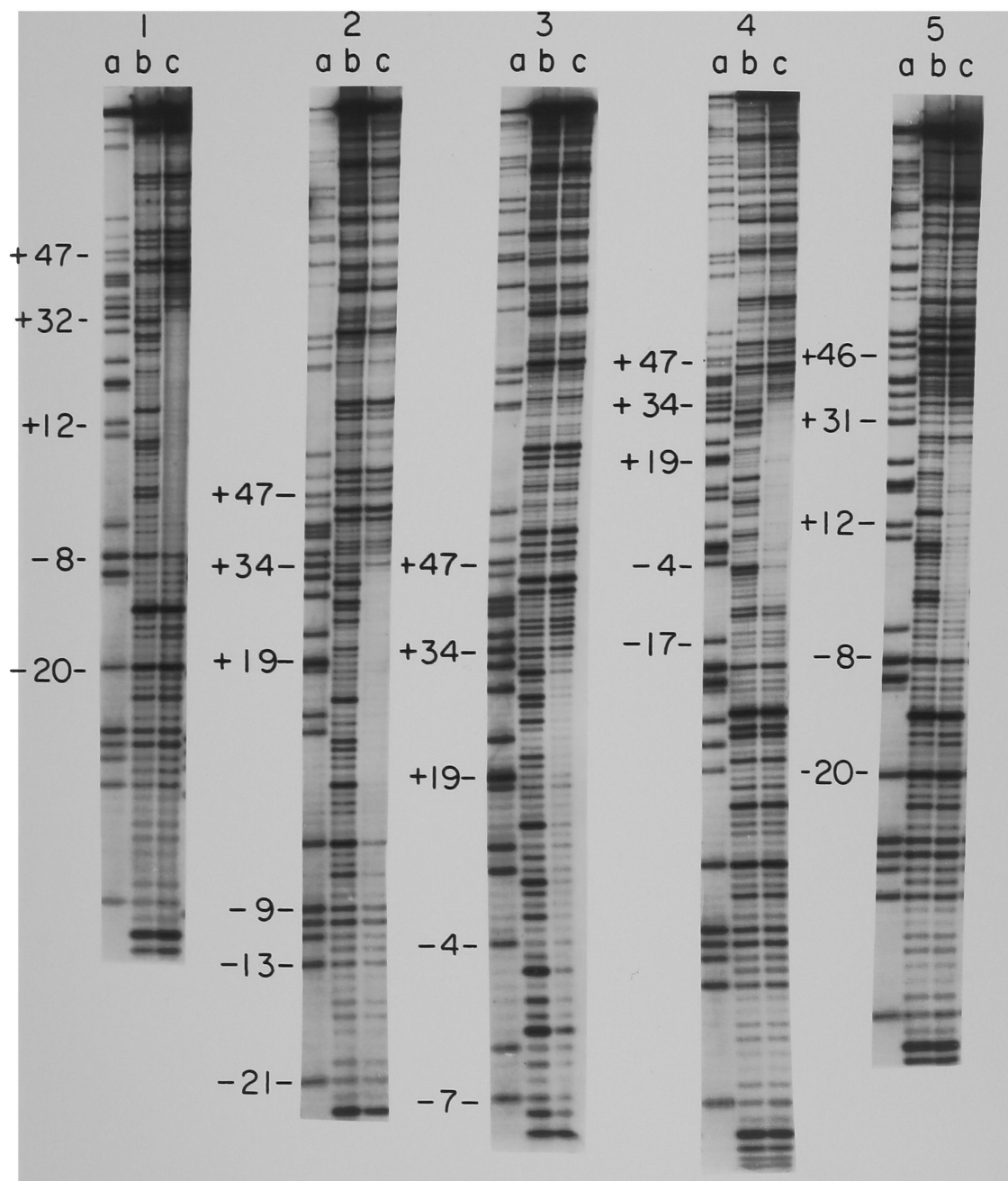






Figure 3.3(c). Summary of footprinting results to the defective origins. Line 1, pDG117. Line 2,  $\Delta 83$ . Line 3, pD29. Line 4, pD30. Line 5,  $\Delta +29$ . The nucleotides that are different from the wild-type sequence are written in lowercase. The arrow indicates the nicking site. The protected regions are underlined as in Figure 3.1

↓  
 -10      +1      +10      +20      +30      +40  
 |      |      |      |      |      |

1 5'-ACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATT-3'  
 3'-TGCAACCTCAGGTGCAAGAAATTATCACCTGAGAACAAGGTTTGACCTTGTTGTGAGTTGGGATAGAGCCAGATAA-5'

2 5'-cgGTTtttctgCCcttTgacgTtggAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATT-3'  
 3'-gcCAaaaagcGGgaaActgcAaccTCACCTGAGAACAAGGTTTGACCTTGTTGTGAGTTGGGATAGAGCCAGATAA-5'

3 5'-gttTgacAGcttAtcaTCgaTAAgctTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATT-3'  
 3'-caaActgTCgaaTagtAGctATTcgaACCTGAGAACAAGGTTTGACCTTGTTGTGAGTTGGGATAGAGCCAGATAA-5'

4 5'-cgtTctttaaatagtGgaCTccAAgctTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATT-3'  
 3'-gcaAgaattatcaCctGaggTTcgaACCTGAGAACAAGGTTTGACCTTGTTGTGAGTTGGGATAGAGCCAGATAA-5'

5 5'-ACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAaAaAcgtCTgcgaCCTgagcaacaacATg-3'  
 3'-TGCAACCTCAGGTGCAAGAAATTATCACCTGAGAACAAGGTTTGACCTTtTgcaGAcgtGGActcgttggttgTAc-5'



### Essential contacts between gene II protein and the origin

Figure 3.4 shows the results of a methylation interference experiment performed on the plus strand and the minus strand of the wild-type and various defective origins. In this experiment, origin-containing restriction fragments were first methylated with dimethyl sulfate. The complex formed with gene II protein was isolated on a nitrocellulose filter and subsequently cleaved with piperidine for gel electrophoretic analysis. Therefore, fragments that result from cleavages at G-residues that are essential for the binding should be reduced in intensity compared to the control. On the plus strand (see Figure 3.4 (a)), there are three G-residues (positions +7, +8, and +15) which, when methylated, interfere with binding. There are three G-residues on the minus strand (see Figure 3.4(b)) whose methylation interferes with binding (positions +10, +18, and +19). Methylation of other G-residues, including one at position +12 of the minus strand, did not interfere with binding. The defective origins exhibit the same pattern of methylation interference.

### The core binding sequence

The G-residues whose methylation interfered with binding (Figure 3.4) were located in the partially symmetric sequence 5'-TGGACTCTTGTTCCA-3', between positions +6 and +20 at the center of the protected region. I therefore tried to test if this sequence was sufficient to bind the gene II protein. The 15 bp sequence from the center of domain A was synthesized and cloned into the *Hind*III site of pUC19 (see Materials and Methods). Figure 3.5(a) shows the result of a DNase I footprinting experiment on a fragment containing the 15 bp element. Gene II protein binds to the fragment containing the 15 bp element and protects approximately 25 bp on both strands from DNase I digestion. The protected region extends from position +3 to position +29 on the plus strand (lane d), and from position +1 to position +24 on the minus strand (lane a).

Figure 3.4. Methylation interference experiment. A methylation interference was performed on the plus strand (a) and the minus strand (b) (data shown on page 50) of wild-type (pDG117) and mutant fragments. The origins are pDG117 (lanes a-b),  $\Delta 83$  (lanes c-d), pD29 (lanes e-f), pD30 (lanes g-h), and  $\Delta +29$  (lanes i-j). End-labeled DNA fragments were methylated with dimethyl sulfate, and subsequently incubated with gene II protein. The complex formed was trapped by and then eluted from a nitrocellulose membrane, as described in Materials and Methods. The recovered DNA was cleaved with piperidine and analyzed by gel electrophoresis (lanes b, d, f, h, and j). Lanes a, c, e, g, and i are untreated controls: the methylated fragments were cleaved with piperidine without treatment with gene II protein. The G-residues at positions +15, +8, and +7 (on the plus strand) are reduced in intensity in the samples isolated from the DNA-protein complex as compared to the controls. For pD30 (lanes g-h), the effect is not as great as for the other origins, but it is clear in the original autoradiogram. The G-residues at positions +19, +18, and +10 (on the minus strand) are reduced in intensity in the samples isolated from the DNA-protein complex. Some of the smaller DNA fragments have slightly reduced intensities in the treated as compared to the untreated samples. This is an artifact of gel electrophoresis, probably caused by contaminating SDS in the sample.



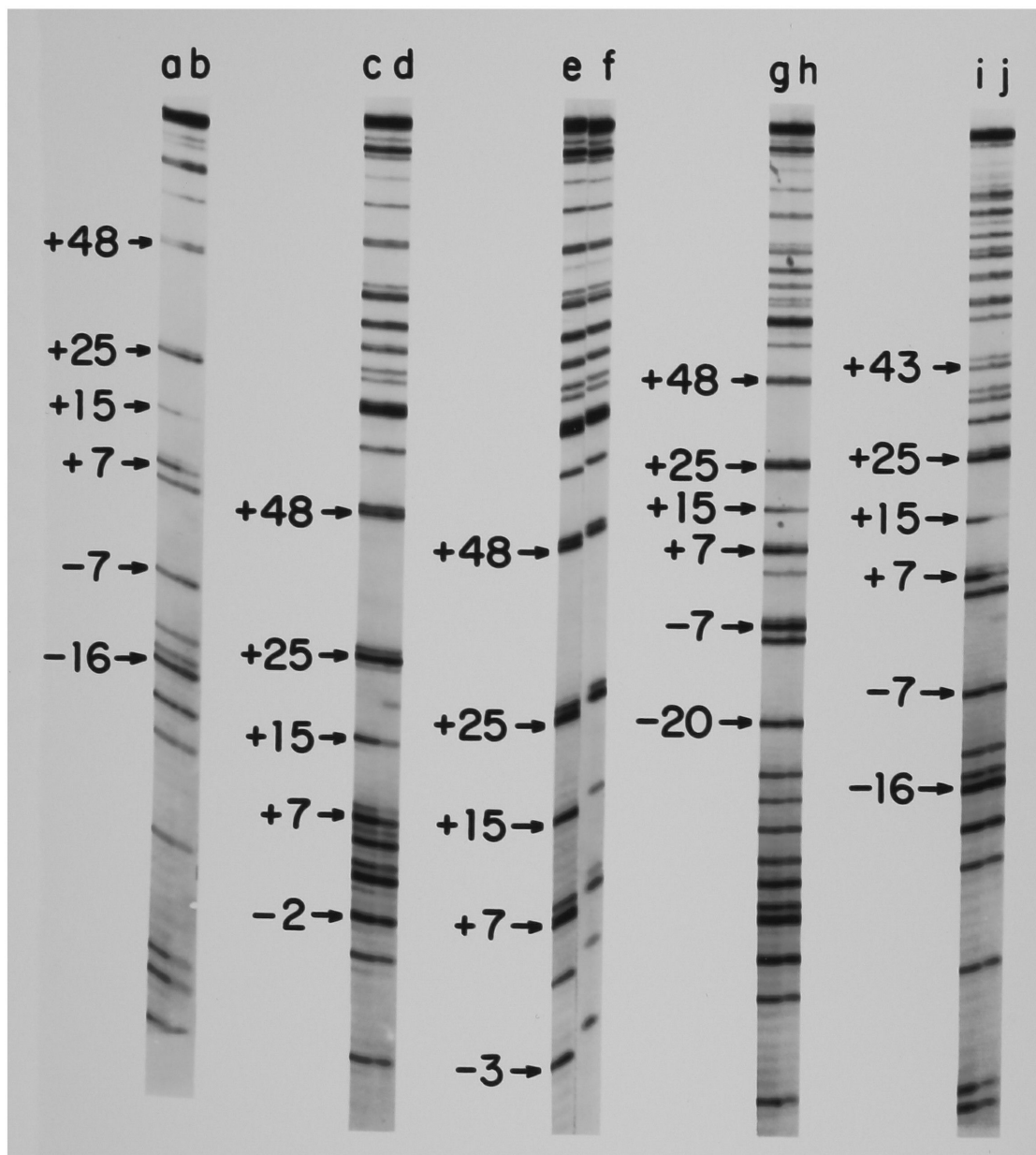
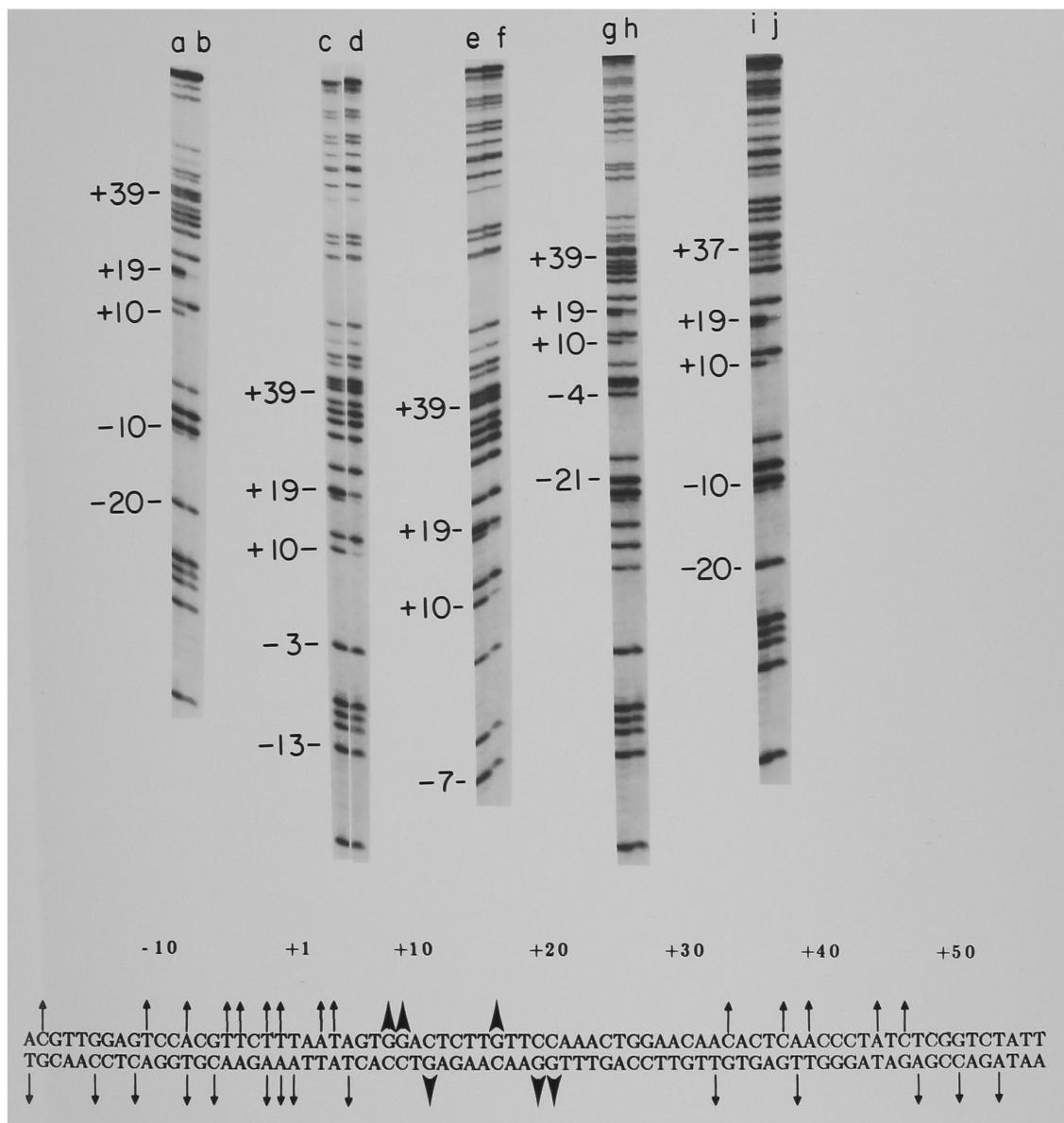






Figure 3.4(b). Methylation interference experiment with the minus stand labeled. See the previous page for an explanation of the experiment and a description of the gel lanes.

Figure 3.4(c). Summary of the results showing the wild-type sequence (pDG117). The six G-residues identified by methylation-interference are indicated by the large arrowheads. The small arrows indicate positions where G-residues have been substituted without affecting the pattern of methylation interference (i.e., G-residues at these sites do not cause methylation interference).





There are two sites of enhanced cleavage, one located at position -1 on the plus strand, the other located at position -3 on the minus strand. The data are summarized in Figure 3.5(b). I call this 15 bp sequence the core binding sequence. Although the core binding sequence is able to bind the gene II protein, it yields a protection pattern that is shorter on both ends than the wild-type pattern.

To compare the binding efficiency of the wild-type replication origin and the 15 bp core binding sequence, I performed a quantitative footprinting experiment. Figure 3.6 shows the results of the experiment performed on the minus strand of the wild-type origin (panel 1), and the minus strand of the *EcoRI-PvuII* fragment containing the 15 bp element (panel 2). The quantitation of the results is complicated by the observation that the two fragments differ in their sensitivities to DNase I in the absence of the gene II protein (lane b). Nonetheless, the fragment containing the 15 bp element appears to bind gene II protein slightly less well (approximately fourfold) than the wild-type origin.

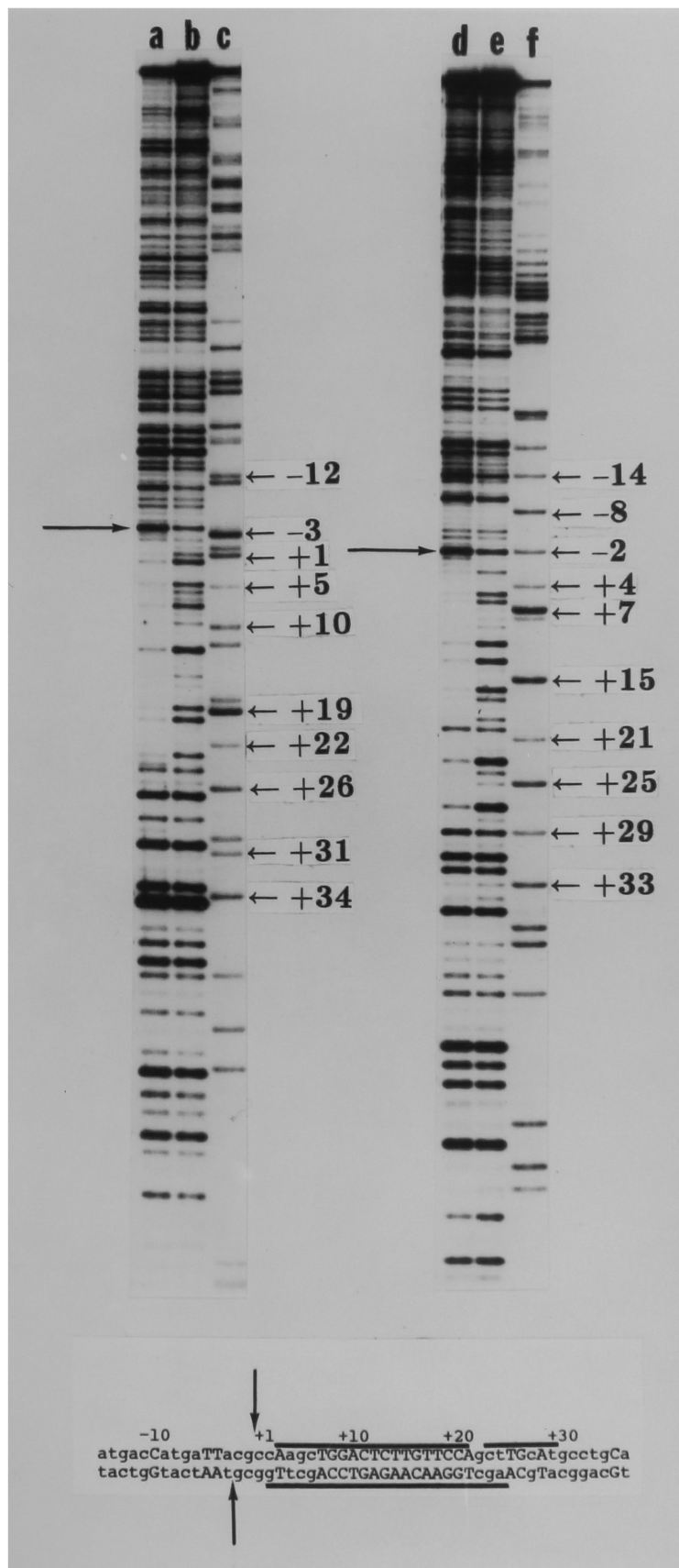
Both halves of the core binding sequence are necessary for binding. In footprinting experiments on a fragment containing the sequence from +12 to +32, only very weak protection by gene II protein was found (data not shown). In addition, the deletion mutant  $\Delta+11$ , which deletes the right end of the origin to position +11, does not bind gene II protein (Horiuchi, 1986).

### **Analysis of gene II protein-origin complexes**

Gene II protein-origin complexes were analyzed by polyacrylamide gel electrophoresis by the procedure described by Fried & Crothers (1981). Figure 3.7 (lanes a-d) shows the results obtained with an f1 origin ( $A^+B^-$ ) fragment ( $MW=1.3 \times 10^5$  daltons). Gene II protein forms two discrete complexes with the f1 origin. At lower gene II protein concentrations (lane b), what I call complex I forms ( $MW_r$  corresponding to  $3.5 \times 10^5$  daltons using DNA molecular weight markers). At higher concentrations of gene II protein (lanes c and d), complex II ( $MW_r$  corresponding to  $5.6 \times 10^5$  daltons) forms. Identical

Figure 3.5. Binding of gene II protein to the core binding sequence. (a). The 15 bp element, corresponding to the center of domain A, was chemically synthesized and cloned into the *Hind*III site of pUC19 (Materials and Methods). Binding to the *Eco*RI-*Pvu*II fragment containing the 15 bp element was analyzed by DNase I footprinting. Lane a, footprint with 40 ng of gene II protein, minus strand labeled. Lane b, DNase I control for the minus strand (no gene II protein). Lane c, Maxam-Gilbert G reaction for the minus strand. Lane d, footprint with 40 ng of gene II protein, with the plus strand labeled. Lane e, DNase I control (no gene II protein) for the plus strand. Lane f, Maxam-Gilbert G reaction for the plus strand. (b). Summary of results. The protected region is underlined. Nucleotides that differ from the wild-type sequence are written in lower-case.







results were obtained with the wild-type ( $A^+B^+$ ), pD29, and  $\Delta 83$  origins (data not shown). Gene II protein did not bind to a control fragment from pBR322 (Figure 3.7, lanes l-n).

Both complexes I and II were formed with the initiation defective origin  $\Delta+29$  (Figure 3.7, lanes e-g). However, the formation of complex II required a higher concentration (about four times--compare Figure 3.7, lanes g and b) of the protein than for the wild-type origin. The  $\Delta+29$  complexes migrate slightly faster than the wild-type ( $A^+B^-$ ) complexes, but this may be due to the position of the binding site within the fragments: in the case of the wild-type origin, the binding site is in the middle of the fragment, whereas for  $\Delta+29$ , it is closer to one end. The 15 bp core binding sequence binds the gene II protein in one step, forming only complex I (Figure 3.7, lanes h-k) and requiring about fourfold more gene II protein (compare Figure 3.7, lanes k and b).

### Footprinting of isolated gene II protein-origin complexes

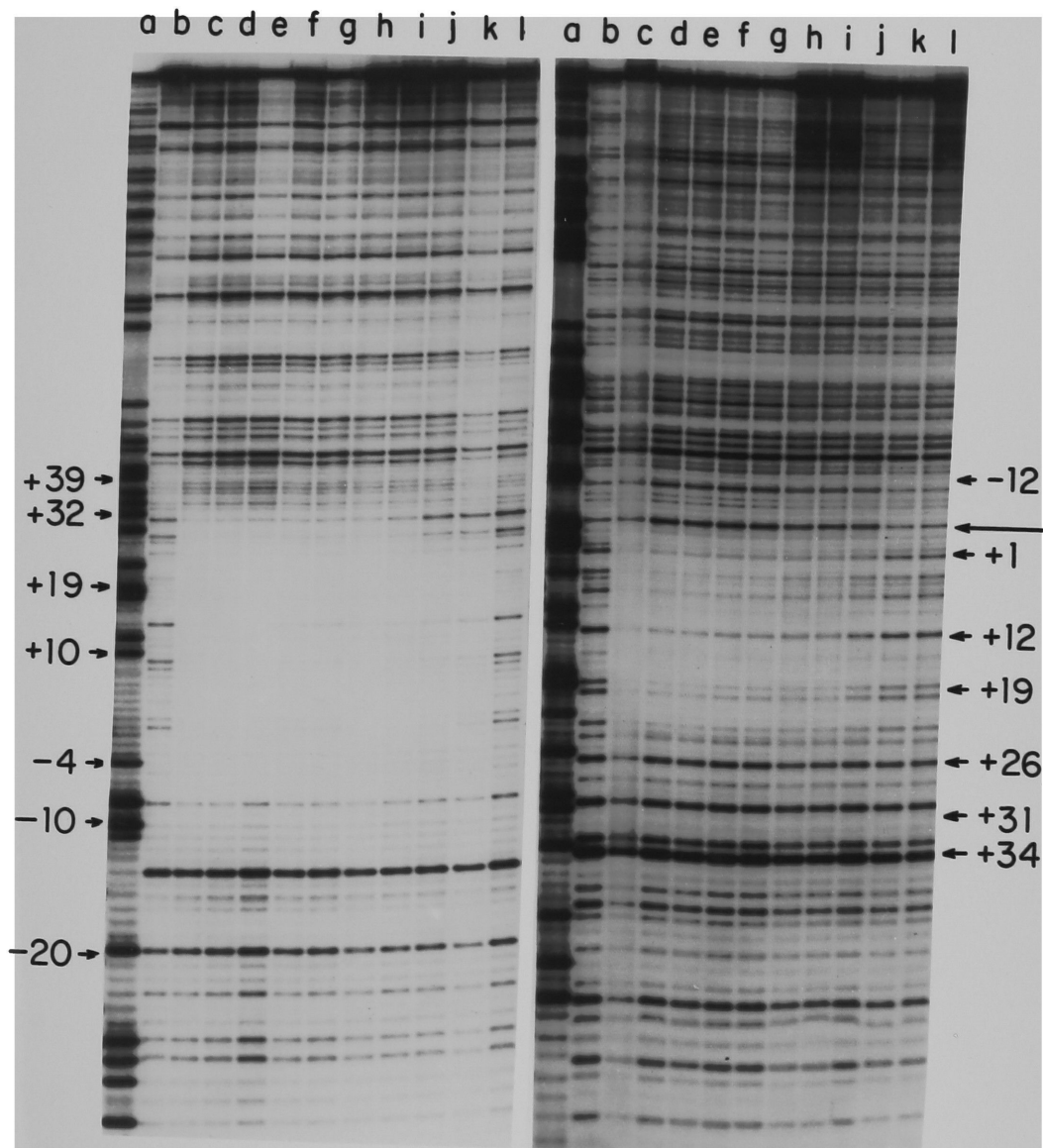
DNase I footprinting experiments were performed on the isolated gene II protein-origin complexes I and II. An origin-containing restriction fragment labeled on the plus strand was incubated with gene II protein and briefly treated with DNase I. Separation of the complexes followed by analysis on a DNA sequencing gel permitted the detection of each respective DNA-protein interaction. A similar analysis was performed by Andrews *et. al.* (1987) to analyze the intermediates in binding of the yeast FLP recombinase to its target site.

In complex I (Figure 3.8, lane b) gene II protein protected 27 bp extending from position +2 to position +28. The protection pattern of complex I closely resembles that obtained with the core binding sequence (compare Figures 3.5 and 3.8). The protection pattern of complex II (Figure 3.8, lane c) extends 40 bp from position -7 to position +33. Thus the protection pattern of complex II corresponds to complete protection of the wild-type origin sequence (compare Figures 3.1 and 8). The level of protection observed

Figure 3.6. Quantitative footprinting experiment. The relative binding strengths of the wild-type origin (panel 1) and the fragment containing the 15 bp core binding sequence (panel 2) were evaluated by quantitative footprinting. The respective fragments (100 fmol) were labeled on the minus strand. Lanes a and b are the Maxam-Gilbert G-reactions and the DNase I controls (no gene II protein), respectively. Lanes c-l are footprints with 40, 36, 32, 30, 25, 20, 16, 12, 10, and 5 ng of gene II protein, respectively.

1

2





in complex II (Figure 3.8, lane c) is greater than that observed in complex I (Figure 3.8, lane b). Probably, complex II results from a more stable gene II protein-origin interaction than complex I.

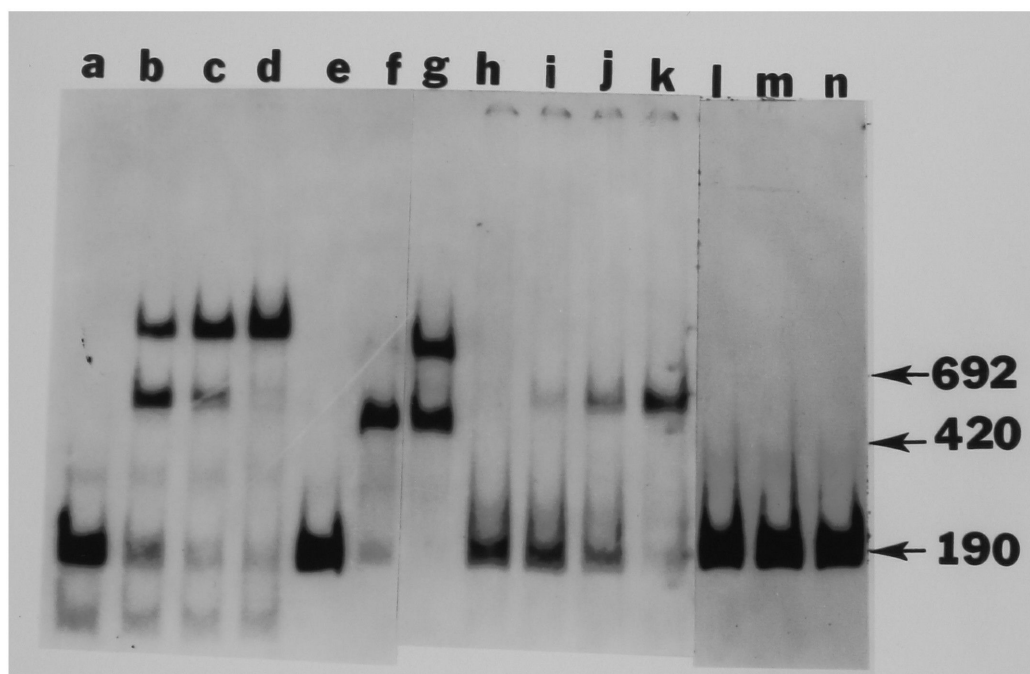
### **Methylation interference with formation of the individual complexes**

I reasoned that the methylation interference experiment performed by filter-binding (Figure 3.4) may have failed to identify essential contacts unique to complex II because both complexes would be retained on the filter. In order to determine the essential contacts for formation of the two complexes, I performed a methylation interference experiment for separately isolated complexes. In this experiment, a methylated origin-containing restriction fragment was incubated with gene II protein, and complex I, complex II, and unbound DNA were separately isolated by polyacrylamide gel electrophoresis. Following cleavage by piperidine at methylated G-residues, the products were analyzed on a DNA sequencing gel. Fragments that result from cleavages at G-residues that are essential for formation of the respective complexes should be reduced in intensity compared to the control. Nine G-residues were found whose methylation inhibited the formation of complex II (Figure 3.9(a), panels 1 and 2, lane b). Five of these G-residues are on the plus strand at positions +7, +8, +15, +25, and +26 (Figure 3.9(a), panel 1, lane b), while four of these G-residues are located on the minus strand at positions +10, +18, +19, and +29 (Figure 3.9(a), panel 2, lane b). The nine G-residues required for complex II formation are located in three clusters (as summarized in Figure 3.9(b)): the first between positions +7 and +10; the second between positions +15 and +19; and the third between positions +25 and +29. These G-residues include the six essential G-residues in the core binding sequence as well as a homologous set at the right end of domain A.

There are six G-residues whose methylation inhibits the formation of complex I (Figure 3.9(a), panels 1 and 2, lane c). These G-residues are identical to the essential G-

Figure 3.7. Gel electrophoretic analysis of gene II protein-origin complexes. Radioactively labeled DNA fragments were incubated with various concentrations of gene II protein, and analyzed by polyacrylamide gel electrophoresis (Fried & Crothers, 1981). The 192 bp *EcoRI-BanI* fragment from pDG117, containing all of domain A and part of domain B (lanes a-d), was incubated with 0 (lane a), 25 fmol (lane b), 50 fmol (lane c), or 100 fmol (lane d) of gene II protein, respectively. (This fragment was slightly contaminated with a 218 bp and a 114 bp fragment that derived from the pBR322 vector and were not affected by gene II protein.) The 190 bp  $\Delta+29$  origin-containing *EcoRI-BamHI* fragment (lanes e-g) was incubated with 0 (lane e), 25 fmol (lane f), or 100 fmol (lane g) of gene II protein. The 208 bp *BstNI* fragment from pMBS1, containing the core binding sequence (lanes h-k), was incubated with 0 (lane h), 25 fmol (lane i), 50 fmol (lane j), or 100 fmol (lane k) of gene II protein. The 187 bp *EcoRI-EcoRV* fragment from pBR322 (lanes l-n) was incubated with 0 (lane l), 25 fmol (lane m), or 100 fmol (lane n) of gene II protein.







residues in the core binding sequence (summarized in Figure 3.9(b)). The three G-residues on the plus strand are located at positions +7, +8, and +15 (Figure 3.9(a), panel 1, lane c). The three G-residues on the minus strand are located at positions +10, +18, and +19 (Figure 3.9(a), panel 2, lane c). These six G-residues were preferentially detected in unbound DNA under conditions where most of the DNA was bound to gene II protein (Figure 3.9(a), panels 1 and 2, lane d). This experiment indicates that complex I and complex II result from sequential DNA protein interactions and that complex II differs from complex I in having an extra set of homologous contacts. The DNA isolated from complex II (Figure 3.9(a), panel 1, lane b) has an extra cleavage located on the plus strand at the gene II protein nicking site. Since this band is observed only in complex II, nicking most likely requires formation of complex II.

### Stoichiometry of the initiator protein-origin interaction

To determine the binding stoichiometry, I performed a double-label gel-binding experiment using  $^{35}\text{S}$ -labeled gene II protein ( $^{35}\text{S}$ -gpII) and a  $^{32}\text{P}$ -labeled origin-containing restriction fragment ( $^{32}\text{P}$ -ori) (see Materials and Methods). In this method, the gpII-origin complexes are separated by non-denaturing gel electrophoresis (Figure 3.10), and the amount of  $^{35}\text{S}$  and  $^{32}\text{P}$  in each complex is quantitated (Table 3.1). Comparison of the ratio of  $^{35}\text{S}/^{32}\text{P}$  in complex II ( $r_{\text{II}}$ ) to that in complex I ( $r_{\text{I}}$ ), provides a relative measure of the constitution of each complex. By measuring the specific activity of the  $^{35}\text{S}$ -gpII and the  $^{32}\text{P}$ -ori, respectively, the stoichiometry of each complex can be determined. The advantage of using *in vivo* labeled gpII instead of *in vitro* iodinated protein is that iodination can affect the binding affinity of the protein so that labeled and un-labeled protein differ in affinity for the DNA. By comparing the results obtained when the experiment is performed using  $^{35}\text{S}$ -gpII and  $^{32}\text{P}$ -ori (Figure 3.10, lane b) to those obtained when the experiment is performed using  $^{35}\text{S}$ -gpII and an equivalent amount of non-radioactive origin DNA (Figure 3.10, lane c), the amount of  $^{35}\text{S}$  can be measured in

Figure 3.8. Footprint analysis of isolated gene II protein-origin complexes. (a). An end-labeled origin-containing restriction fragment from pDG117 was incubated with gene II protein, treated with DNase I, and electrophoresed on a 5% polyacrylamide gel. DNA in the separated complexes was extracted from the gel and analyzed by denaturing gel electrophoresis. For detailed procedures see Materials and Methods. Lane a, DNase I control: the DNA fragment was treated with DNase I in the absence of gene II protein and was isolated from the gel in parallel with the complexes. Lane b, footprint of complex I formed in the presence of 10 ng of gene II protein. Lane c, footprint of complex II formed in the presence of 20 ng of gene II protein. The lanes marked G are the Maxam-Gilbert G-reactions.

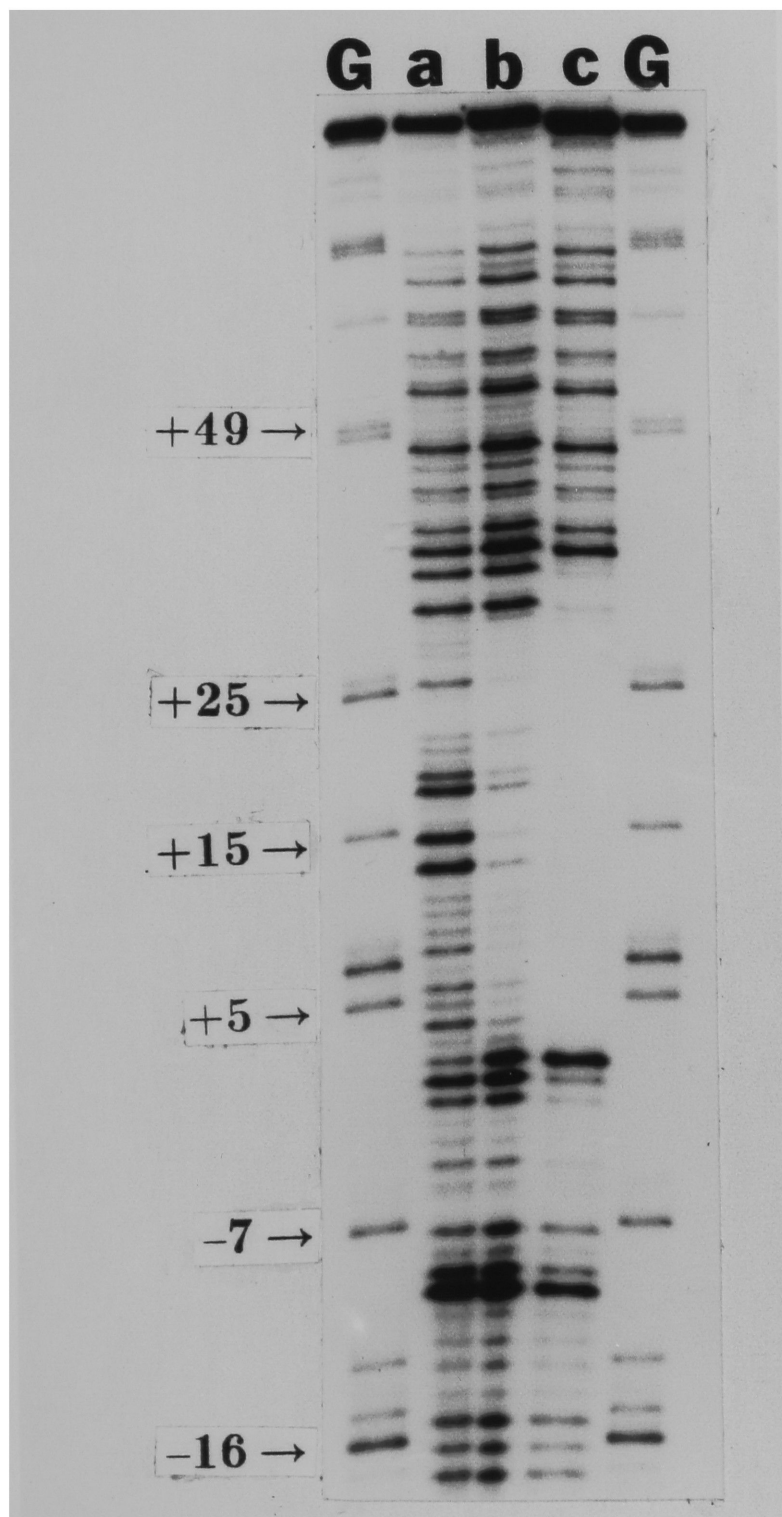
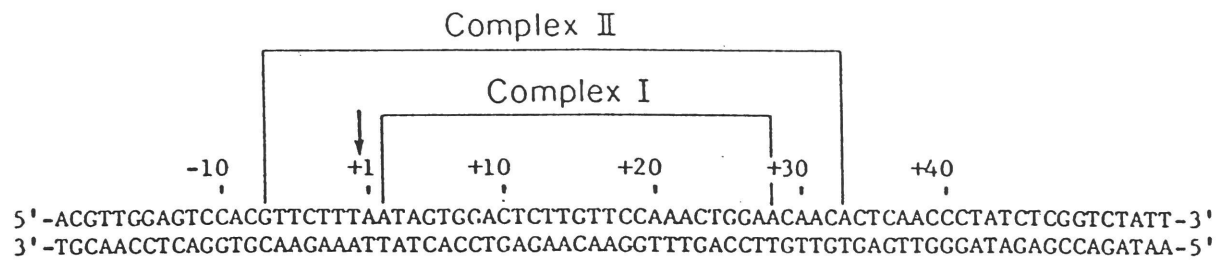






Figure 3.8(b). Summary of footprinting results. The protected region in the individual complexes is indicated above the DNA sequence.







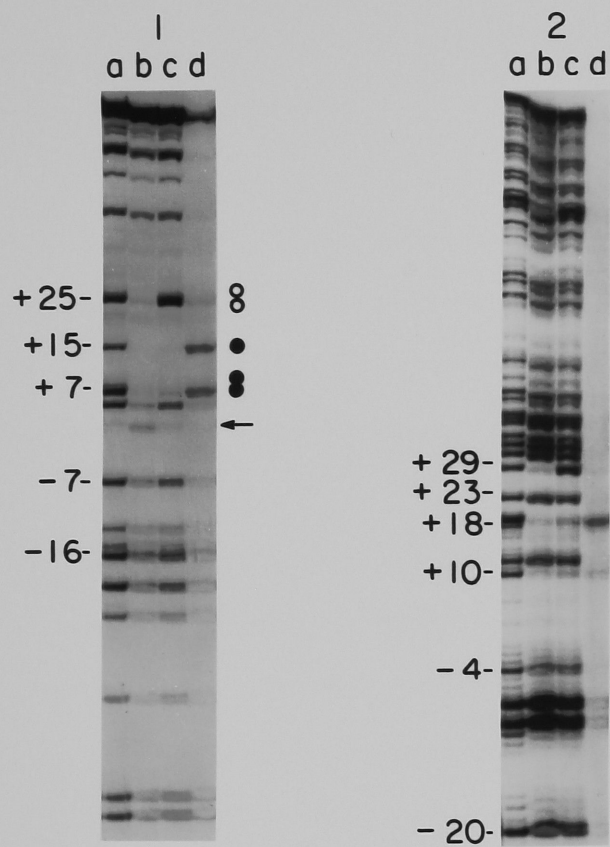
both the presence and absence of  $^{32}\text{P}$ . Such values were in agreement to within 5%. Background levels of radioactivity were determined by excising the regions of the gel corresponding to complex I and complex II from a gel lane in which the  $^{35}\text{S}$ -gpII preparation had been electrophoresed without an origin restriction fragment (lane d). Table 3.1 shows the amount of  $^{35}\text{S}$  and  $^{32}\text{P}$  and the ratio of  $^{35}\text{S}$  to  $^{32}\text{P}$  for each complex. The data indicate that complex II contains twice as much gpII per origin as does complex I ( $r_{\text{II}}/r_{\text{I}}=2.2\pm0.4$ ). I determined the ratio  $r_{\text{II}}/r_{\text{I}}$  in four independent experiments. The values were in agreement, the average being  $(r_{\text{II}}/r_{\text{I}})_{\text{av}}=2.1\pm0.5$ . I measured the specific activity of the  $^{35}\text{S}$ -gpII and the  $^{32}\text{P}$ -ori, respectively, (see Materials and Methods) in order to determine the absolute amount of gpII and origin in each complex. Table 3.2 shows the absolute amount of gpII and origin in each complex. The data indicate that complex II contains approximately four molecules of gpII per origin, and that complex I contains approximately two molecules of gpII per origin.

### Discussion

In this chapter, I have analyzed the interaction of the gene II protein with the fl origin of replication. The gene II protein binds to restriction fragments containing the origin and protects 40 base pairs from DNase I digestion. The protected region corresponds to domain A, the core origin sequence.

Gene II protein binds to the origin and forms two complexes that are separable by polyacrylamide gel electrophoresis (see Figure 3.7). Using DNA molecular weight markers, the increase in mass due to binding the first unit of gene II protein is equal to the increase due to binding the second unit ( $2 \times 10^5$  daltons). This was the first indication that complex II probably contains twice as many molecules of gene II protein as does complex I. A direct measurement of the stoichiometry (Figure 3.10 and Tables 3.1 and 3.2) confirmed this. The data indicate that complex I contains approximately two molecules of gene II protein per origin, whereas complex II contains approximately four

Figure 3.9. The essential contacts for formation of the two gene II protein-origin complexes were probed by methylation interference. (a). Origin-containing restriction fragments were methylated with dimethyl sulfate, incubated with gene II protein, and electrophoresed on a 5% polyacrylamide gel. DNA was isolated from the separated complexes, cleaved with piperidine, and electrophoresed on a standard sequencing gel. Panel 1, the DNA fragment (154 bp *BamRHI-EcoRI* fragment from pDG117) labeled on the plus strand. Panel 2, the DNA fragment (315 bp *AsuI-ClaI* fragment from f1 RFI) labeled on the minus strand. Lane a, unbound DNA without addition of gene II protein. Lane b, complex II formed with 5 ng of gene II protein. Lane c, complex I formed with 5 ng of gene II protein. Lane d, unbound fragment after incubation with 20 ng of gene II protein. An arrow at the side indicates a fragment formed by site-specific nicking by the gene II protein. (b). Summary of methylation interference results. The G-residues required for formation of both complexes (closed circles) and the G-residues required only for formation of complex II (open circles) are indicated on the DNA sequence. Repeated nucleotide sequences are indicated by horizontal arrows. A vertical arrow indicates the nicking site.



-10                      ↓                      +10                      +20                      +30                      +40                      +50  
 5'-ACGTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAAGTGAACAACACTCAACCCTATCTCGGTCTATT-3'  
 3'-TGCAACCTCAGGTGCAAGAAATTATCACCTGAGAACAAAGTTTGACCTTGTGTGAGTTGGGATAGAGCCAGATAA-5'

→                      ←                      →

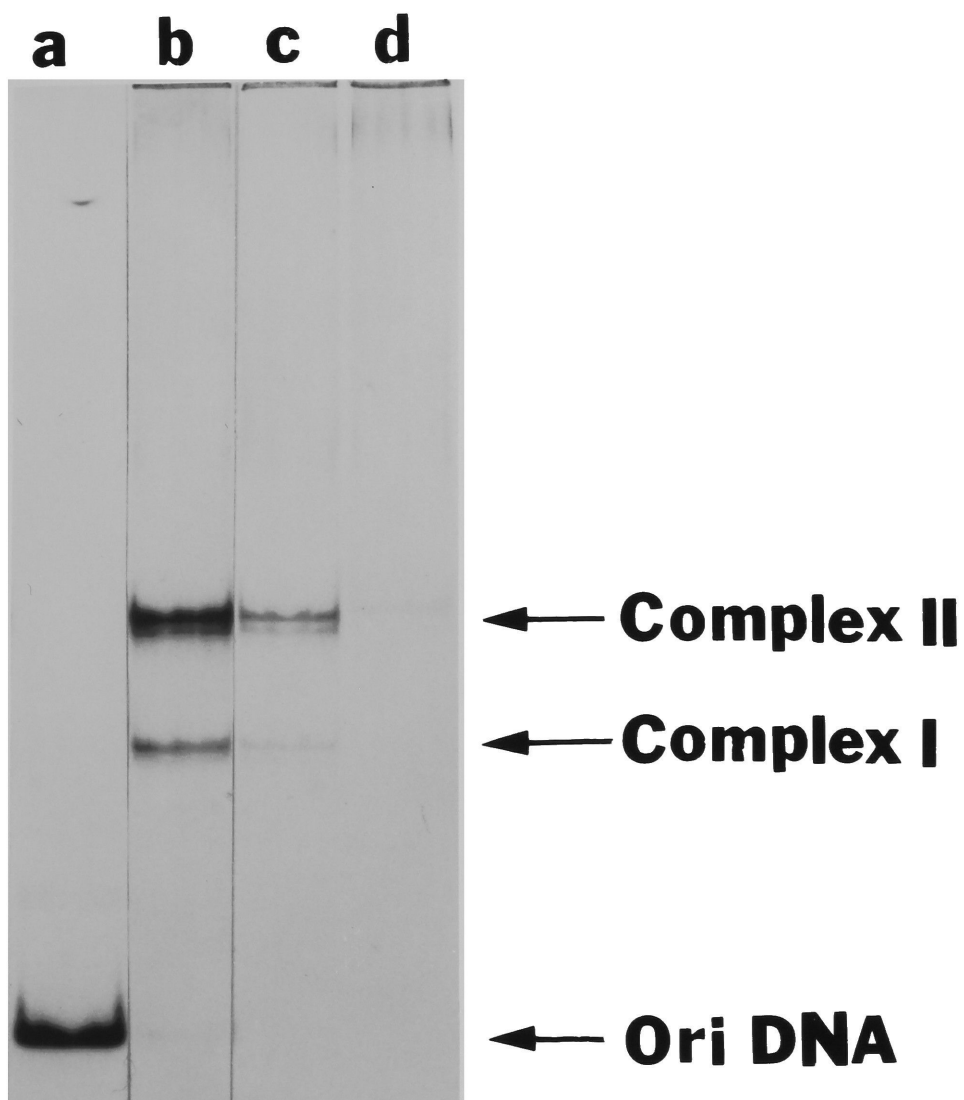


molecules of gene II protein per origin. The stoichiometry is determined by the constraint that complex II contains twice the amount of gpII per origin as does complex I (Table 3.1). This supports the previous binding data, which suggest that complex I contains an even number of gpII molecules since it requires an inverted repeat ( $\beta$ - $\gamma$ ) for its formation. Furthermore, gpII makes symmetric contacts with the inverted repeat ( $\beta$ - $\gamma$ ) as determined by methylation interference (Figures 3.4 and 3.9). The failure to detect two different binding patterns with the wild-type origin by DNase I footprinting may be due to interconversion or differential stability of the complexes during the footprinting procedure. The ability to detect binding intermediates by polyacrylamide gel electrophoresis but not by direct DNase I footprinting was reported by Andrews *et al.* (1987) for the interaction of the FLP recombinase with its target sequence.

From the footprints of the isolated complexes, I conclude that complex I results from interaction of gene II protein with the core binding sequence and gives a 27 bp protection pattern. The protection pattern of complex I (Figure 3.8, lane b) is identical to that obtained with a cloned copy of the core binding sequence (Figure 3.5, lane d). Direct footprinting of complex II shows the full 40 bp protection of domain A. Since the level of protection observed in complex II is greater than that observed in complex I (see Figure 3.8), complex II probably results from a more stable gene II protein-origin interaction. The three-dimensional structure of bovine pancreatic DNase I (Suck & Oefner, 1986) suggests that the DNase I footprint pattern overestimates the size of the actual DNA-protein interaction by approximately 5 bp per end because of the intrinsic DNA-binding surface of the DNase I molecule. If so, the region covered by gene II protein in complex I would be approximately 15 bp, the size of the core binding sequence. The covered region in complex II would correspond to roughly 30 bp, or twice the length of that in complex I. Therefore, the footprints I observe with complex I and complex II are reasonable protection patterns for one and two units of protein, respectively, binding to a contiguous stretch of DNA.

Figure 3.10. Stoichiometry of the gene II protein-origin interaction. Binding of gpII to the origin was analyzed on a non-denaturing 5% polyacrylamide gel as described in Materials and Methods. Lane a,  $^{32}\text{P}$ -labeled origin fragment. Lane b,  $^{32}\text{P}$ -labeled origin fragment and  $^{35}\text{S}$ -labeled gpII. Lane c, un-labeled origin fragment and  $^{35}\text{S}$ -labeled gpII. Lane d,  $^{35}\text{S}$ -labeled gpII. Complex II, complex I, and the origin restriction fragment are indicated by arrows.









**Table 3.1. Composition of GpII-Origin Complexes**

	$^{32}\text{P}$	$^{35}\text{S}$	$^{35}\text{S}/^{32}\text{P}$
Complex I	269±10 cpm	373±23 cpm	1.4±0.1
Complex II	945±30 cpm	2917±144 cpm	3.1±0.3

The amounts of  $^{32}\text{P}$  and  $^{35}\text{S}$  in each complex was determined as discussed in Materials and Methods. The values in Table 3.1 represent the average of two gel lanes b and one gel lane c (see Figure 3.10).

**Table 3.2. Stoichiometry of GpII-Origin Complexes**

	Ori DNA	GpII	GpII/Ori
Complex I	11.7±1.3 fmol	20.0±3.5 fmol	1.7±0.5
Complex II	41.1±4.5 fmol	156.0±25.3 fmol	3.8±1.0

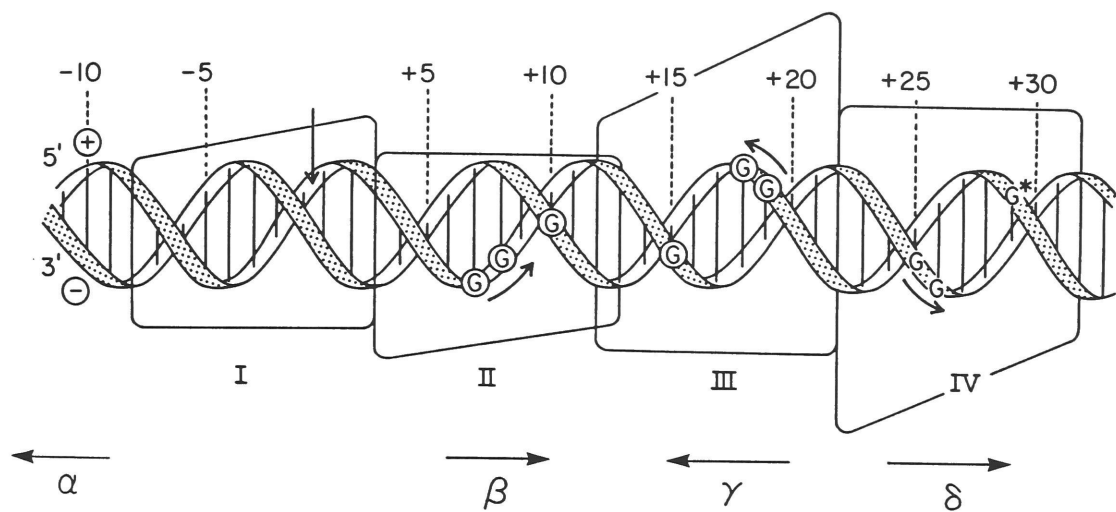
Stoichiometry of gpII-origin complexes. The composition of each complex was calculated using the values in Table 3.1 and the specific activities of the  $^{35}\text{S}$ -gpII (18,700±2100 cpm/pmol) and the  $^{32}\text{P}$ -origin (23,000±1800 cpm/pmol-see Materials and Methods).

I probed the contacts between gene II protein and the origin in the two complexes by methylation interference experiments. Methylation of any one of six G-residues (see Figure 3.9) in the core binding sequence inhibits formation of both complex I and complex II. Methylation of one of three G-residues located between positions +25 and +29 specifically inhibits the formation of complex II. This is consistent with the observation that the cloned core binding sequence can form only complex I (Figure 3.7). The nine G-residues are located in three symmetric clusters, suggesting that gene II protein interacts homologously with each repeat. Since the G-residues required for formation of complex I are a subset of those required for formation of complex II, complex I appears to be an intermediate in formation of complex II. Methylation interference experiments performed using filter-binding only identified the six G-residues in the core binding sequence as being essential contacts (see Figure 3.4). The most likely explanation is that methylation of G-residues located between positions +25 and +29 still permits formation of complex I and retention on the nitrocellulose filter.

Based on the results described above, I propose a model for gene II protein binding as schematically shown in Figure 3.11. There are four repeats of the sequence 5'-TGGAC-3' ( $\alpha$  &  $\beta$ ) or 5'-TGGAAC-3' ( $\gamma$  &  $\delta$ ) in alternate directions within domain A. The core binding sequence consists of repeats  $\beta$  and  $\gamma$  in inverted orientation. Full protection of 40 bp requires repeats  $\beta$ ,  $\gamma$ , and  $\delta$ . Repeat  $\alpha$  is dispensable for both binding and nicking, but required for termination of replication (Dotto *et. al.*, 1982b). Methylation interference experiments indicate that gene II protein makes homologous contacts with the repeats,  $\beta$ ,  $\gamma$ , and  $\delta$ .

Figure 3.11 depicts the B-form DNA structure of domain A, illustrating the protected region and the close contacts (i.e. nine sites of methylation interference). The points of methylation interference are located in repeats  $\beta$ ,  $\gamma$ , and  $\delta$  in the major groove. The symmetry of the core binding sequence, particularly that of guanine residues that interfere with formation of complex I upon methylation (see Figure 3.9), suggests that two

Figure 3.11. A model for binding of gene II protein to the replication origin. The structure of domain A is drawn as B-form DNA. Four subunits (marked I, II, III, and IV) of the gene II protein are assumed to bind to the origin. Subunits III and IV are located exactly on the same side of the DNA helix in a symmetrical configuration. The subunit II is located about  $70^\circ$  apart from III and IV. The subunit I is speculatively depicted as if the relative position of I to II is the same as that of III to IV. However, we do not have any data on the configuration of subunit I. The subunits are depicted by different shapes to indicate the rotation around the DNA helix. The points of methylation interference in the core binding sequence are circled. The G-residues that preferentially interfere with formation of complex II when methylated are shown uncircled. The G-residue at position +29 is marked by an asterisk to indicate that it is deleted in the mutant  $\Delta+29$ . The three repeats ( $\beta$ ,  $\gamma$ , and  $\delta$  as described in the text) required for binding are indicated. The repeat  $\alpha$  (position -13 to -9) is required for termination but not initiation. The plus and minus strands are labeled. The nicking site is indicated by a vertical arrow. The curved arrows show the 5'→3' direction on each strand.







gene II protein monomers (indicated as II and III in Figure 3.11) symmetrically bind to it. This notion is consistent with the symmetry between the repeats  $\gamma$  and  $\delta$ . Assuming that one gene II protein molecule binds to each of these sequence elements, the two molecules which bind to the right half of domain A (indicated as III and IV in Figure 3.11) should be located in a symmetrical configuration. In the deletion mutant  $\Delta+29$  the  $\delta$  repeat is partially destroyed and complex II is destabilized. The molecule that binds to the left half of the core binding sequence (indicated as II in Figure 3.11) should be located about  $70^\circ$  apart from IV. This model predicts two different sets of dimer contacts. Absence of symmetry in the sequences of the nicking site region reflects the non-symmetric nature of the nicking that occurs at a unique site on the plus strand. The configuration of the molecule bound at this region (indicated as I in Figure 3.11) is unclear. Its involvement is inferred from the footprint (Figure 3.8), the gel mobility (Figure 3.7), and the stoichiometry of complex II (Figure 3.10 and Tables 3.1 and 3.2).

It is remarkable that  $\Delta 83$ , pD29, and pD30 all bind gene II protein to yield full protection of approximately 40 bp. Note that the sequence of the protected region is different among the three mutants. Gene II protein must therefore recognize the sequence to the right of position +5, and thereby protect approximately 10 nucleotides to the left. In the wild-type origin, the nicking site is located within this 10 bp sequence. Obviously, only a specific sequence can be nicked, since  $\Delta 83$ , pD29, and pD30 are not nicked by gene II protein. However, when gene II protein is bound to the 15 bp core (position +6 to +20) it does not protect the 10 bp on the left. Evidently, repeat  $\delta$  is required for the gene II protein to properly bind and protect the 10 bp on the left. The interaction of monomer IV (see Figure 3.11) with repeat  $\delta$  may influence the binding of monomer I via protein-protein interactions. An alternative to this model is that binding of subunit IV causes a conformational change in the complex that results in protection of the leftmost 10 bp.

The initiation-defective origin  $\Delta+29$  can form complex II, but less efficiently (see Fig-



ure 3.7). Thus, the sequence to the right of position +28 affects the second binding step. Gene II protein bound to the  $\Delta+29$  origin, even though able to introduce the nick, fails to participate in the initiation of unwinding (see Chapter 5).

The requirement of sequences at the right end of domain A for the protection of the left end suggests that protein-protein interactions are involved in the binding. While gene II protein exists as a monomer in solution (Meyer & Geider, 1979a), protein-protein interactions could occur on the DNA or during binding. Protein-protein interactions are also implicated by the observation that the gene II protein binds only very weakly to repeats  $\gamma$  and  $\delta$  in the absence of repeat  $\beta$  (data not shown). Since binding of the first unit of gene II protein (interaction with  $\beta$ - $\gamma$ ) seems to aid the binding of the second (interaction with  $\delta$ ), the binding must be cooperative. Quantitation of the formation of complex II as a function of gene II protein concentration (see Chapter 8) confirms that the interaction is cooperative. Cooperative binding allows full binding to occur in an all-or-none fashion over a small range of protein concentrations. For instance, cooperative binding of the  $\lambda$  repressor protein cI is a crucial factor in regulating the  $\lambda$  lysis-lysogeny decision (for a review see Ptashne, 1986). The intracellular level of cI is tightly regulated by both positive and negative controls. The concentration of gene II protein in the infected cell is tightly controlled by the phage-encoded gene V protein, a translational repressor (Model *et al.*, 1982; Yen & Webster, 1982). Cooperative binding to the origin would cause the plus-strand initiation rate to be highly sensitive to the level of gene II protein when it is rate limiting for initiation.

In the case of the icosahedral single-stranded phage  $\Phi$ X174, the replication initiator protein, the phage-encoded gene A protein binds covalently at the 5'-end of the nick. The high stability of the complex, due to the covalent nature of the adduct, would allow DNA replication to be processive. For origins that form noncovalent complexes with their initiator proteins, multiple initiator protein molecules are often involved in the interaction. Notable examples include the oriC-dnaA protein complex (Fuller *et al.*, 1984)



and the  $\text{ori}\lambda\text{-O}$  protein complex (Tsurimoto & Matsubara, 1981). By this criterion, the  $\text{f1}$  origin would be a member of the latter class.



## Chapter 4

### **Mn<sup>2+</sup>-Dependent Double Strand Cleavage of the Origin**



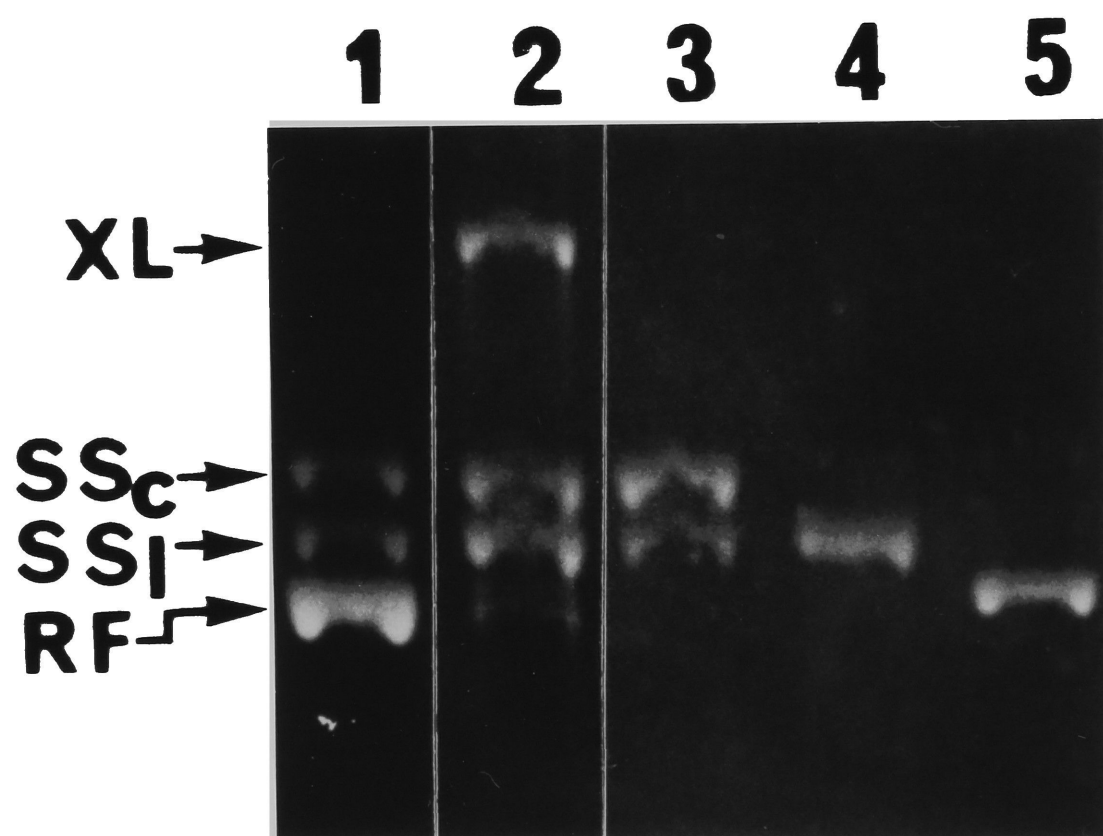


Gene II protein was purified and shown to nick the replication origin at a specific site in the presence of  $Mg^{2+}$  (Meyer *et al.*, 1979). When  $Mn^{2+}$  was substituted for  $Mg^{2+}$ , the enzyme produced a double-strand cleavage at the same site (Meyer & Geider, 1979b; Dotto *et al.*, 1981b). This activity of the enzyme has been a useful one to monitor during its purification. The  $Mn^{2+}$ -dependent double-strand cleavage activity has been interpreted to mean that the enzyme binds to its substrate as a dimer, and that the normally silent active site can become activated by addition of  $Mn^{2+}$  (Baas, 1985). Direct determination of the binding stoichiometry (see Chapter 3) would indicate that this view is at least partially incorrect. In this chapter, I have determined the structure of the cleavage product. The cleavage product has an unusual structure in which the plus and minus strands are linked to form a teleomeric hairpin. Formation of this product results from a two-stage reaction, the first stage of which is nicking at the origin, followed by the strand-joining activity. In contrast to nicking in the presence of  $Mg^{2+}$ , the  $Mn^{2+}$ -dependent double strand cleavage activity does not require a supercoiled substrate. Therefore, addition of  $Mn^{2+}$  removes the requirement of supercoiling for nicking, and results in an additional reaction-strand joining. The unusual structure of the cleavage product probably reflects distortion of the nicking site region as a consequence of its interaction with the protein.

### **Anomalous migration in alkaline agarose gels**

The first indication that RFIII formed by gpII in the presence of  $Mn^{2+}$  (RFIII- $Mn^{2+}$ ) had an abnormal structure was its anomalous migration in alkaline agarose gels (Figure 4.1). RFIII- $Mn^{2+}$  (indicated by XL in Figure 4.1) migrated slowly on alkaline agarose gels (Figure 4.1, lane 2), whereas RFIII produced by the restriction enzyme *Bam*HI comigrated with unit length phage linear single strands (Figure 4.1, lane 4). RFIII- $Mn^{2+}$  comigrated with linear single-stranded DNA having twice the chain length of phage

Figure 4.1. Migration of RFII-Mn<sup>2+</sup> on alkaline agarose gels. Purified gene II protein (approximately 20 ng) was incubated with f1 RFI (approximately 200 ng) in 20  $\mu$ l containing 20 mM Tris-Cl (pH 8.0), 80 mM KCl, 5 mM MnCl<sub>2</sub>, and 5 mM DTT for 30 minutes at 30°C (lane 2). No gene II protein was added to the sample in lane 1. Following the incubation, the samples were loaded on a 0.6 % alkaline agarose gel (Maniatis *et al.*, 1982). After electrophoresis, the DNA was stained with ethidium bromide. Lane 3 contained f1 single stranded phage DNA which contained both the circular (SS<sub>c</sub>) and linear forms (SS<sub>l</sub>). Lane 4 contained f1 RFI cleaved with the restriction enzyme *Bam*HI which cleaves f1 once. Lane 5 contained f1 RFI. XL (for cross-linked) designates the position of the anomalously migrating species which results from incubation of f1 RFI with gpII in the presence of Mn<sup>2+</sup>.



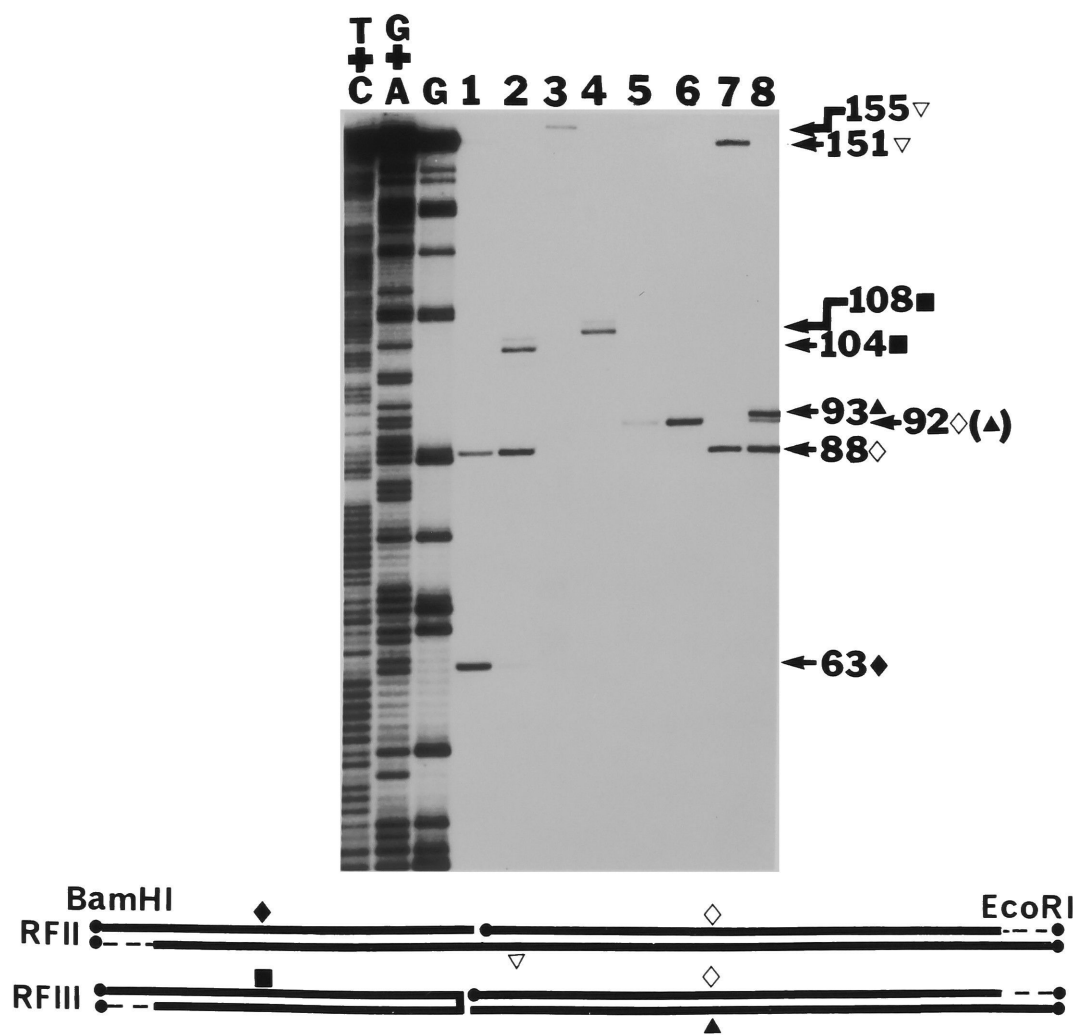


linear single-stranded DNA, as predicted using single stranded DNA markers. RFIII-Mn<sup>2+</sup> exhibited anomalous migration whether or not it was extracted with phenol or treated with pronase prior to electrophoresis (data not shown). The migration of RFIII-Mn<sup>2+</sup> on neutral agarose gels was normal even after treatment with alkali followed by neutralization. Thus, the anomalous migration is a property of the DNA and is not due to the formation of a covalent complex with gpII.

### **The plus and minus strands are joined**

In order to characterize the cleavage product, I mapped the cleavage endpoints and established the structure of both termini by chemical sequencing. As shown in Figure 4.2, the cleavage site in pDG117 is flanked by a *Bam*HI site and an *Eco*RI site. These sites were used to end-label fragments in order to determine the structure of the cleavage product. Labeling was carried out by 3'-end-filling with the Klenow fragment of DNA polymerase I (lanes 3-6) or 5'-end-labeling with T4 polynucleotide kinase following treatment with CIAP (lanes 1, 2, 7, 8). Following end-labeling, the DNA was digested with a restriction enzyme (*Eco*RI for DNA labeled at the *Bam*HI site, and *Bam*HI for DNA labeled at the *Eco*RI site) and subjected to electrophoresis on a 8M urea-8% polyacrylamide sequencing gel. The two fragments labeled at the *Bam*HI site that extend to the cleavage site migrate anomalously on the standard sequencing gel (104 and 108 base fragments indicated by filled squares in Figure 4.2): the fragments migrate as triplets on this gel. The two fragments with labels on the plus and minus strands, respectively, differ in size because the fragment labeled on the minus strand was end-filled, which adds 4 nucleotides. Initial attempts to chemically sequence these fragments using 8M urea-8% polyacrylamide sequencing gels were unsuccessful. The sequence could be read with base pair resolution to the nicking site, then the fragments began to migrate anomalously and base pair resolution was no longer apparent. In contrast, on a 70% formamide-8% polyacrylamide sequencing gel, the fragments migrate as single bands of

Figure 4.2. Mapping of cleavage sites. The cleavage sites were mapped as depicted at the bottom. RFII-Mn<sup>2+</sup> (lanes 1, 3, 5, and 7) and RFIII-Mn<sup>2+</sup> (lanes 2, 4, 6, and 8) were treated with *Bam*HI (lanes 1-4) or *Eco*RI (lanes 5-8) and end-labeled by reaction with the Klenow fragment of DNA polymerase I (lanes 3-6) or T4 polynucleotide kinase (lanes 1, 2, 7, and 8). The labeled DNA was then digested with *Eco*RI (lanes 1-4) or *Bam*HI (lanes 5-8), and analyzed by electrophoresis on an 8 M urea-8% polyacrylamide sequencing gel. Maxam-Gilbert reactions performed on the *Bam*HI-*Eco*RI fragment from pDG117 (5'-end-labeled at the *Bam*HI site) served as markers. The size of the various fragments in nucleotides is indicated at the side of the gel. Each fragment is given a symbol, which indicates the origin of the fragment on the picture at the bottom.







121 and 125 bases, respectively (Figure 4.3(a) and data not shown). These fragments (121 and 125 base fragments) were purified and sequenced chemically. The results of the sequencing analysis for characterization of the left side of the cleavage are shown in Figure 4.3(b). The result indicates that the plus and minus strands are joined. The left side of the cleavage has the same sequence whether it is labeled on the plus strand by treatment with T4 polynucleotide kinase (left panel in Figure 4.3 (b)), or end-filled on the minus strand by treatment with the Klenow fragment of DNA polymerase I (right panel in Figure 4.3 (b)). The sequencing information indicates that the T-residue at position -1 on the plus strand is linked to the A-residue at position -2 on the minus strand, as shown in Figure 4.3 (c).

The right side of the cleavage product has the structure of a one base 3'-overhang, though approximately 5% of the right-end products have blunt ends. The terminal base on the plus strand is the A-residue at position +1 (Figure 4.2, lanes 2, 6, and 8), and the terminal base on the minus strand is the A-residue at position -1 (Figure 4.2, lane 8). Following sizing to map the cleavage site, the fragments (each band in Figure 4.2) were gel-purified and chemically sequenced to verify their identities (data not shown).

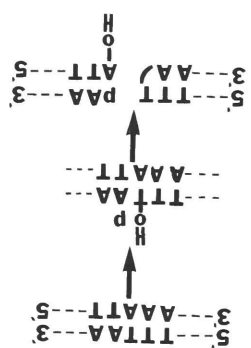
### **Kinetics of double strand cleavage**

The kinetics of double strand cleavage are shown in Figure 4.4(a). The origin-containing plasmid DNA is nicked quickly (within 1 minute of incubation--Figure 4.4(a), lane 3) and converted to the linear form more slowly (complete conversion after 1 hr--Figure 4.4(a), lane 7). These nicked molecules are true intermediates since they are converted to the linear form when they are purified and subsequently re-incubated with gpII (data not shown). Mapping of the nick (Figure 4.2, lanes 1, 3, 5, and 7) shows it to be at a unique site on the plus strand, the nicking site, between nucleotides -1 and +1. Only plasmids containing f1 origins are substrates. pBR322 is not a substrate for either

Figure 4.3. Structure of the cleavage product. (a). Denaturing gel analysis of the cleavage product. The sample from Figure 4.2, lane 2 (RFIII-Mn<sup>2+</sup> 5'-end-labeled at the *Bam*HI site) was analyzed on a 70% formamide-8% polyacrylamide gel (shown in the rightmost lane). The Maxam-Gilbert sequencing reactions that served as markers were described in Figure 4.2.

(b). Sequencing of the left side of the cleavage product. The 121 and 125 base fragments, 5'-end-labeled and 3'-end-labeled at the *Bam*HI site, respectively, were purified on a 70% formamide-8% polyacrylamide gel and subjected to chemical sequencing. The left and right panels show the sequencing reactions for the 5' and 3'-end-labeled fragments, respectively. Analysis was performed on a 70% formamide-8% polyacrylamide gel. The structure of the left end is diagramed between the two sequencing gel panels. An arrowhead shows the T residue at position -1.

(c). Mechanism of the Mn<sup>2+</sup>-dependent double-strand cleavage activity. The DNA sequence of the nicking site region is shown, with the plus strand being the top strand. The two steps of the reaction are shown: 1) nicking at the origin and 2) strand joining, as described in the text.





nicking or double-strand cleavage (data not shown). The enzyme cannot use a random nick. When f1 origin-containing plasmid DNA is nicked randomly with DNase I, it can be converted to the linear form upon incubation with gpII; however, the double-strand cleavage occurs at the origin, and the random nick is not used (data not shown).

### **Double-strand cleavage does not require superhelicity**

Since nicked molecules (RFII) are substrates for  $\text{Mn}^{2+}$ -dependent double-strand cleavage, the reaction does not require superhelicity. This was confirmed further by digestion of a restriction fragment containing the f1 origin (Figure 4.4(b)). The 151 bp origin fragment from pDG117 was cleaved to an 88 bp and a 63 bp fragment. Fragments not containing f1 origins were not cleaved (data not shown). This means that the initial nicking reaction does not require superhelicity in the presence of  $\text{Mn}^{2+}$ .

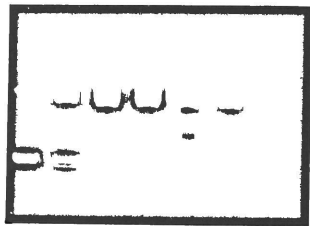
### **$\text{Mn}^{2+}$ reduces the DNA sequence requirement for nicking**

The DNA sequence requirements for nicking in the presence of  $\text{Mg}^{2+}$  were found to extend from four base pairs before the nicking site to 28 base pairs downstream (Dotto *et al.*, 1982ab; 1984). This sequence contains repeats  $\beta$ ,  $\gamma$ , and most of repeat  $\delta$ . The DNA sequence requirements for the  $\text{Mn}^{2+}$ -dependent double-strand cleavage reaction were not determined. Therefore, I decided to analyze the  $\text{Mn}^{2+}$ -dependent double strand cleavage reaction using mutant origins as substrates. Surprisingly, mutants that remove the nicking site are substrates for gpII in the presence of  $\text{Mn}^{2+}$ , though the product of the reaction is RFII instead of RFIII (Figure 4.5). The mutant pD30 was constructed by insertion of a 10 bp linker at the filled-in *Hin*I site at position +8 (Dotto *et al.*, 1984). As a consequence of the insertion, the wild-type nicking site is positioned 13 bp upstream from its normal position. The pD30 origin binds gene II protein to yield the wild-type 40 bp protection pattern (see Chapter 3): the inserted linker is protected whereas the displaced nicking site is not. This binding is nonfunctional because the

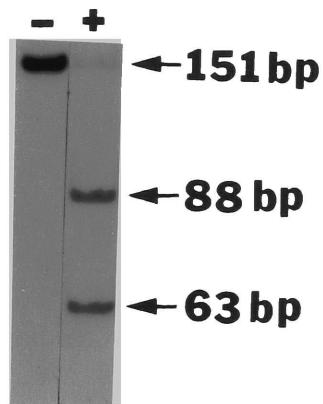
Figure 4.4.  $\text{Mn}^{2+}$ -dependent double-strand cleavage activity. (a). Time course of the cleavage reaction. Purified gene II protein (12.5 ng) was incubated with f1 RFI (250 ng) in 20  $\mu\text{l}$  containing 20 mM Tris-Cl [pH 8.0], 80 mM KCl, 5 mM  $\text{MnCl}_2$ , and 5 mM DTT. The reactions were incubated for various lengths of time at 30°C as follows: lane 1--no incubation, lane 2--30 seconds, lane 3--1 minute, lane 4--5 minutes, lane 5--10 minutes, lane 6--30 minutes, and lane 7--1 hour. Following the incubation, the reactions were stopped by addition of 1  $\mu\text{l}$  of stop mix (0.2 M EDTA (pH 8.0), 20% sucrose, 1% SDS, and 0.01% bromphenol blue), and the products were analyzed by electrophoresis on a 0.6% agarose gel containing ethidium bromide (0.5  $\mu\text{g}/\text{ml}$ ).

(b). Cleavage of a restriction fragment. The 151 bp *EcoRI-BamHI* origin-containing restriction fragment from pDG117 (approximately 10 fmol) was incubated with gene II protein (approximately 25 ng) under  $\text{Mn}^{2+}$  cleavage conditions (see above) for 1 hr. The restriction fragment had been 5'-end-labeled by treatment with  $[\gamma]\text{-}^{32}\text{P}\text{-ATP}$  and T4 polynucleotide kinase, following treatment with CIAP. The cleavage products were analyzed on an 8% polyacrylamide gel and were detected by autoradiography.

0    time →



← nicked  
← linear  
← circular







pD30 origin cannot be nicked by gene II protein and the origin is defective for initiation. As shown in Figure 4.5(a), the pD30 origin is nicked by gene II protein in the presence of  $\text{Mn}^{2+}$ . Since pD30 contains a wild-type nicking site, which is nevertheless displaced from its normal position with respect to the binding repeats, it was important to determine the position of the nick produced by gpII in the presence of  $\text{Mn}^{2+}$ . The nick was mapped essentially as depicted in Figure 4.2. The results (summarized in Figure 4.5 (b)) indicate that pD30 is nicked at a single site that corresponds to the normal position of the nick--the displaced nicking site is not nicked.

In the case of two deletions that extend from the left past the nicking site (pD29 and  $\Delta 83$ ), gpII nicked the defective origins in the presence of  $\text{Mn}^{2+}$  (Figure 4.5(a) and data not shown). For pD29, the RFII molecules were a mixture, containing one of four closely spaced nicks on the plus strand in the nicking site region (data summarized in Figure 4.5(b)). The major site of nicking was at the position corresponding to the wild-type nicking site. The nick for the mutant  $\Delta 83$  was not located.

The three binding repeats  $\beta$ ,  $\gamma$ , and  $\delta$  are needed for the nicking reaction in the presence of  $\text{Mn}^{2+}$  because plasmids containing only the core binding sequence (repeats  $\beta$  and  $\gamma$  in inverted orientation) are not nicked even if the region surrounding the nicking site is restored (as in the plasmid pMBS2--see Materials & Methods). The mutant  $\Delta +29$  (see Materials & Methods) contains repeats  $\beta$ ,  $\gamma$ , and most of repeat  $\delta$ , and is a substrate for double-strand cleavage by gpII in the presence of  $\text{Mn}^{2+}$  (data not shown).

## Discussion

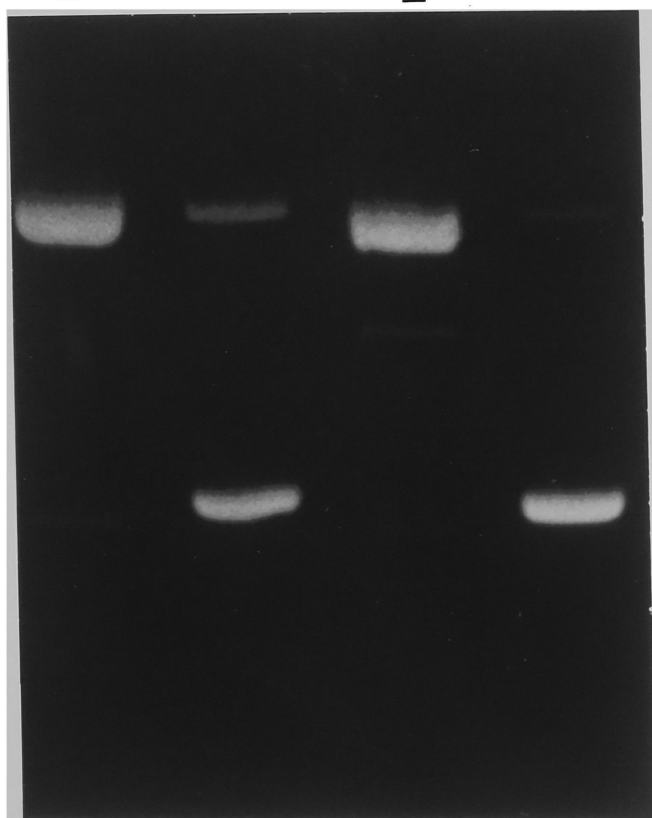
In this chapter, I have analyzed the  $\text{Mn}^{2+}$ -dependent double-strand cleavage activity of gpII in detail. The reaction occurs in two stages (see Figure 4.4): in the first stage, gpII introduces a nick at a specific site on the plus strand (wild-type nicking site); in the second stage, gpII makes a double-strand break by joining the T-residue (position -1) to the A-residue (position -2) one base 3' to it on the other strand (see Figure 4.3). Thus,

Figure 4.5.  $\text{Mn}^{2+}$  reduces the DNA sequence requirements for nicking. (a). Nicking of deletion mutants in the presence of  $\text{Mn}^{2+}$ . The defective origin plasmids pD30 and pD29 (see Materials & Methods) were incubated in the presence (+) or absence (-) of gpII under the  $\text{Mn}^{2+}$  cleavage conditions described in the legend to Figure 4.4. The products were analyzed by electrophoresis on a 0.6% agarose gel containing ethidium bromide (0.5  $\mu\text{g}/\text{ml}$ ).

**pD30** **pD29**

**+** **-**

**+** **-**



← **RFII**

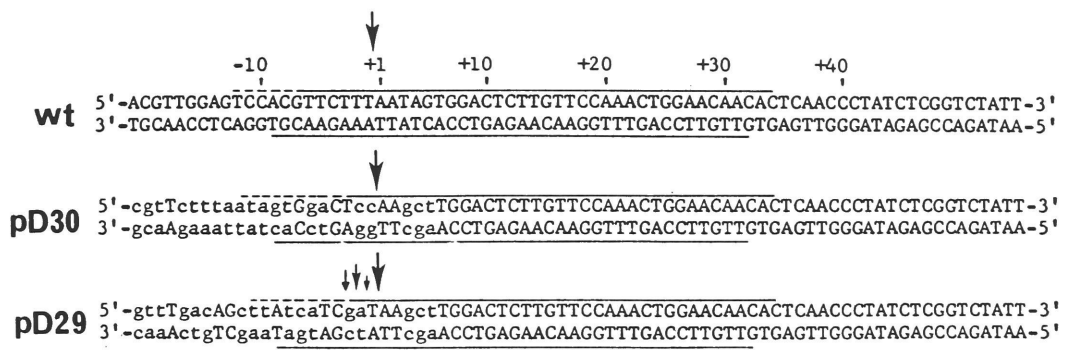
← **RFI**



the left side of the cleavage product has the structure of a telomeric hairpin in which the strands are joined. The right side of the product is a one base 3' overhang (see Figure 4.2). While a significant fraction of RFI is converted to RFIV by gpII in the presence of  $Mg^{2+}$ , no RFIV formation is detected in the presence of  $Mn^{2+}$ . Unlike reaction in the presence of  $Mg^{2+}$ , the  $Mn^{2+}$ -dependent cleavage activity does not require a supercoiled substrate: restriction fragments containing an *f1* origin are substrates (see Figure 4.4). Surprisingly, defective origins, having deletions which remove the nicking site, can be nicked by gpII in the presence of  $Mn^{2+}$  (see Figure 4.5) but not  $Mg^{2+}$  (Dotto *et al.*, 1982b, 1984). As shown in Chapter 3, these mutants bind gpII to give full 40 bp protection patterns. Protection of the nicking site is dependent on the presence of the binding repeats ( $\beta$ ,  $\gamma$ , and  $\delta$ ). Thus, addition of  $Mn^{2+}$  reduces the DNA sequence requirements for nicking by making the leftmost protected region cleavable regardless of its improper sequence. However, the DNA sequence of the nicking site region must be important for the double-strand cleavage to occur because the mutants pD30, pD29, and  $\Delta 83$  do not efficiently undergo double-strand cleavage and strand joining. Remarkably, the mutants pD30 and pD29 are nicked at the position corresponding to the wild-type nicking site. Therefore, gpII must be able to measure distance from the binding repeats. Perhaps this reflects the ability of subunits II, III, and IV (see Figure 3.11) to position subunit I which protects the nicking site independent of its DNA sequence.

How does  $Mn^{2+}$  change the course of the cleavage reaction as described above? A priori,  $Mn^{2+}$  could affect the DNA substrate, the enzyme, or both. While none of the data presented above bears on the role that  $Mn^{2+}$  plays in the cleavage reaction, certain features of the reaction suggest that  $Mn^{2+}$  acts at the level of the DNA.  $Mn^{2+}$  alters the gpII-origin interaction in two ways: first, it removes the requirement for a supercoiled substrate in order to nick at the origin; second, it leads to a further reaction in which the nicked molecule is a substrate for the strand joining reaction. Nevertheless, other features of the reaction retain specificity: only substrates containing the binding

Figure 4.5(b). Location of the nicks. The nicks were located essentially as depicted in Figure 4.2 and described in the text. The nucleotide sequence of the wild-type and the defective origins is shown. The top and bottom lines correspond to the plus and minus strands, respectively. Nucleotides that differ from the wild-type sequence are written in lowercase. The sites of nicking are indicated by arrows. For pD29, the size of the arrows indicates the relative efficiency of the various nicks. From left to right, the efficiencies of nicking were found to be in a ratio of 2:4:1:6.







repeats ( $\beta$ ,  $\gamma$ , and  $\delta$ ) participate in the nicking reaction, and only specifically nicked RFII molecules are substrates for the strand joining reaction. Thus  $\text{Mn}^{2+}$  alters the reaction only within a specific region, the nicking site. Recently, Laundon and Griffith (1987) have found that cationic metals, notably  $\text{Mn}^{2+}$ , promote sequence-directed bending of DNA. The nicking site is A+T-rich and might adopt a structure other than B-form DNA. The observation that the nicking site strongly binds the drug distamycin (data not shown) supports this notion. Distamycin is a basic oligopeptide antibiotic that binds strongly to certain DNA (Hogan *et al.*, 1979) sequences. Distamycin has been shown to bind to both intrinsically bent DNA (kinetoplast DNA) and DNA bent by the CAP protein (Wu & Crothers, 1984). Binding of distamycin has been shown to reduce the magnitude of the bending as determined by electrophoretic mobility measurements (Wu & Crothers, 1984) and by electron microscopy (Griffith *et al.*, 1986). I would like to speculate that  $\text{Mn}^{2+}$  and superhelicity favor nicking by stabilizing an altered DNA conformation at the nicking site. In the case of  $\text{Mn}^{2+}$ , the magnitude of the effect might be greater, considering that it favors an additional reaction--strand joining. The  $\text{Mn}^{2+}$ -dependent double-strand cleavage reaction must involve distortion of the DNA substrate because the product has an unusually distorted structure. Model building studies have indicated that the strand-joined product probably contains three unpaired bases in the loop. Perhaps this distortion of the nicking site is brought about by its DNA sequence and by bending induced by gpII (see Chapter 7).

What is the biological significance of this unusual double-strand cleavage reaction? This reaction is unlikely to occur *in vivo* to any significant extent for two reasons: first,  $\text{Mn}^{2+}$  is not present at 5 mM under physiological conditions; second, such a reaction would be disastrous to the initiation of DNA replication because the 3'-hydroxyl end of the nick, which serves as the primer, would be linked to the minus strand and hence unavailable for polymerization. One possibility is that the  $\text{Mn}^{2+}$ -dependent double-strand cleavage activity might reflect some aspects of the termination activity of gpII.



GpII functions in the termination of DNA replication by cleaving and circularizing the product single strand (Harth *et al.*, 1981). In this reaction, gpII must first introduce a nick between a T-residue (position -1) and the newly added A-residue (position +1), and then connect the 3'-hydroxyl of that T-residue (position -1) to the free 5'-phosphate that was generated by the nick that initiated replication. Thus, both the termination reaction and the  $\text{Mn}^{2+}$ -dependent double-strand cleavage reaction are comprised of cleavage and ligation between TTT and AA sequences. In the case of the  $\text{Mn}^{2+}$ -dependent cleavage, cleavage of the minus strand appears to be coupled with strand-joining because no products are found that have a one base 3'-overhang on both sides of the cleavage. A blunt-ended product is formed as a side product in low yield ( $\simeq 5\%$ ). Possibly, this less efficient cleavage of the minus strand is not compatible with strand-joining.

In this chapter, I have elucidated the mechanism of a double-strand cleavage and strand-joining reaction. This type of reaction is characteristic of several multifunctional proteins that mediate the initiation and termination of DNA replication or recombination. The A\* protein of  $\Phi\text{X174}$ , a truncated form of the larger A protein, has been found to cross-link the origin in the presence of  $\text{Mn}^{2+}$ , though the reaction mechanism was not determined (van der Ende *et al.*, 1981). These authors suggested that the strand-joining activity of the A\* protein was indicative of the manner in which the larger A protein mediates the termination of rolling-circle replication. In addition, a virion-associated DNase from Vaccinia virus has been found that catalyzes a strand-joining reaction (Lakritz *et al.*, 1985). Although the structure of the strand-joined product and the detailed mechanism of the reaction await elucidation, the Vaccinia virus nicking-joining enzyme is likely to be involved in the initiation or termination of viral DNA replication (Reddy & Bauer, 1989). A similar cleavage and strand-joining reaction is catalyzed by the  $\lambda$  integrase protein when the attachment site substrate is heteroduplex in the region of overlap between the bacterial and phage attachment sites (H. Nash unpublished data, cited by Nash *et al.*, 1987a; and by Richet *et al.*, 1988).



These enzymes, which break and rejoin DNA, do so without an exogenous energy source such as ATP. In order to do this, the energy of the phosphodiester bond must be conserved for use in the ligation step. For the phage  $\lambda$  integrase protein, as for most topoisomerases, the energy of the phosphodiester bond is conserved by formation of a covalent complex with the DNA substrate (Craig & Nash, 1983). In the case of gpII, it remains unclear how the energy of the phosphodiester bond is conserved because there is no evidence for the formation of a covalent complex with the DNA substrate. However, this remains an open question because it is difficult to exclude the possibility that a covalent complex forms, but is either unstable during isolation or transitory. The Mu A protein is another example of an enzyme that can break and rejoin DNA strands in the absence of an exogenous energy source, apparently without forming a covalent intermediate (reviewed by Mizuuchi & Craigie, 1986). However, in contrast to gpII, the Mu A protein has not been demonstrated to have topoisomerase activity (the ability to change the linking number of superhelical DNA). The Mu A protein catalyzes the strand-transfer reaction that forms the intermediate in Mu transposition. In doing this, the Mu A protein breaks four DNA strands and rejoins only two. Mizuuchi & Craigie (1986) have speculated that the strand-transfer reaction might therefore have a looser energy conservation requirement, obviating the need for a covalent enzyme-DNA complex. Perhaps the Mu A protein and gpII are examples of proteins which can conserve the energy of a phosphodiester bond without forming a covalent complex. Possibly both enzymes maintain an active-site environment which protects a non-covalent high energy phosphate bond from hydrolysis. These speculations await delineation of the respective active-sites.



## Chapter 5

### **Enzymatic Unwinding of the Origin**





Replication of f1 and  $\Phi$ X174 requires the product of the *rep* gene (Denhardt *et al.*, 1967, 1972), the Rep helicase, a single-stranded DNA-dependent ATPase that unwinds the DNA strands in advance of the replication fork (Eisenberg *et al.*, 1976; Scott *et al.*, 1977; Scott & Kornberg, 1978; Kornberg *et al.*, 1978). The Rep helicase is absolutely required for f1 plus-strand replication *in vitro* (Meyer & Geider, 1982). Geider *et al.* (1982) investigated intermediate stages in plus-strand DNA synthesis *in vitro* and found that in the presence of gene II protein, Rep helicase, SSB, and ATP, the DNA template was unwound to single-stranded products. The unwound DNA was sensitive to S1 nuclease and was visualized by electron microscopy. Gene II protein was required for unwinding at a step beyond nicking: f1 DNA molecules that had been nicked by gene II protein and purified, still required subsequent addition of gene II protein in order to be unwound by the Rep helicase. I wanted to test the simple hypothesis that the A+T-rich DNA sequence of the replication enhancer functioned in the *in vitro* unwinding reaction. To this end, I first developed a rapid unwinding assay that allowed the products of complete unwinding to be visualized by gel electrophoresis. The advantage of this assay is that the unwinding reactions are performed in the absence of S1 nuclease and the products are visualized by gel electrophoresis instead of by electron microscopy. In this chapter, I validate this assay for the unwinding reaction. I use this assay to explore the sequence requirements for *in vitro* unwinding. The A+T-rich DNA sequence of the replication enhancer is not required for the *in vitro* unwinding reaction described by Geider *et al.* (1982). The defective origin,  $\Delta$ +29, which can be nicked by the gene II protein, is not a substrate for *in vitro* unwinding. This unwinding defect correlates with this origin's truncated protection pattern with gene II protein (see Chapter 3), and with its initiation defect.



### Unwinding assay

Unwinding reactions were carried out essentially as described by Geider *et al.* (1982) (see Materials & Methods), except that the products were analyzed by agarose gel electrophoresis. The results are shown in Figure 5.1. Upon incubation with gene II protein, Rep helicase, SSB, and ATP, the f1 origin-containing substrate is converted into two faster-migrating DNA species (Figure 5.1, lane c). These were found to be single-strand circles and linear single strands, respectively (see Figures 5.2 and 5.3). In agreement with the results of Geider *et al.* (1982), unwinding required Rep helicase (lane d), SSB (lane e), gene II protein (lane f), and ATP (lane g). A non-hydrolyzable ATP-analogue, AMPPNP, cannot substitute for ATP (lane h).

### Parameters of the unwinding assay

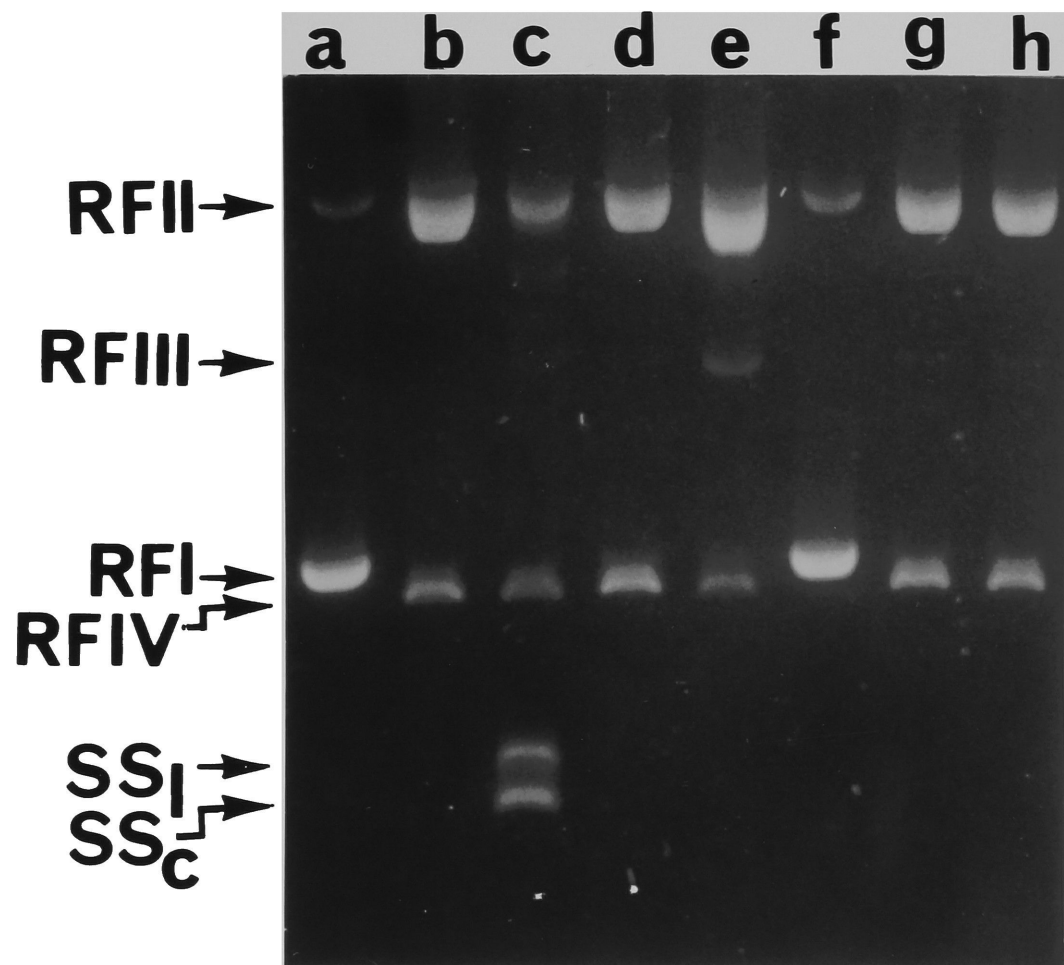
Figure 5.2 shows a time course of *in vitro* unwinding. Unwinding is rapid; products are visible after 1 minute (Figure 5.2, lane 4). This corresponds to a maximal rate of approximately 5000 base pairs per minute.

In agreement with Geider *et al.* (1982), gene II protein was found to be required for unwinding at a step beyond nicking. RFII produced by gene II protein in the presence of  $Mg^{2+}$  and subsequently purified (see the legend to Figure 5.2) was not a substrate for unwinding (Figure 5.2, lane 13) unless gene II protein was present in the incubation (Figure 5.2, lane 14).

### Products of unwinding

The nature of the two faster-migrating DNA species produced upon incubation of the origin-containing plasmid with gene II protein, Rep helicase, SSB, and ATP was analyzed. As shown in Figure 5.2, the upper band is sensitive to exonuclease VII, whereas the lower band is resistant. Therefore, the upper band is likely to be a linear

Figure 5.1. Requirements for unwinding. An origin-containing plasmid, pD10 (100 ng), was incubated in 20 mM Tris-Cl (pH 8.0), 80 mM KCl, 5 mM DTT, 6 mM  $\text{MgCl}_2$ , 5% glycerol, and 1 mM ATP with gene II protein (20 ng), Rep helicase (250 ng), and SSB (600 ng) for 20 minutes at 30°C--complete reaction. Following the incubation, 2  $\mu\text{l}$  of stop mix (0.23 M EDTA [pH 8.0], 7% sucrose, 7% SDS, and 0.03% bromphenol blue) was added, and the reactions were loaded on a 0.6% agarose gel containing 0.5  $\mu\text{g}/\text{ml}$  ethidium bromide and 1X TBE buffer. Lane a contained pD10 alone; Lane b contained pD10 incubated with gene II protein (20 ng); Lane c, the complete reaction; Lane d, the complete reaction minus Rep helicase; Lane e, the complete reaction minus SSB; Lane f, the complete reaction minus gene II protein; Lane g, the complete reaction minus ATP; Lane h, the complete reaction with AMPPNP substituted for ATP.





single strand, and the lower band is most likely single-stranded circular DNA. The lower band was purified and visualized by electron microscopy. It was found to be single-stranded circular DNA as expected (data not shown). The upper band was not visualized.

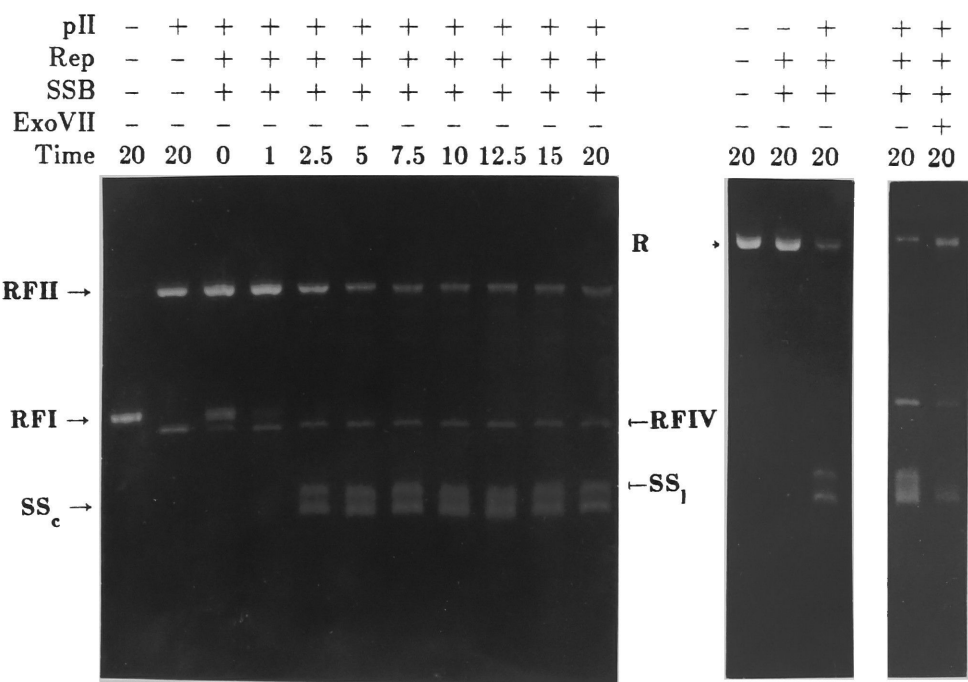
The substrate for unwinding, pD10, is a pBR322 derivative containing an *f1* origin that is positioned so that plus-strand replication would proceed 5'→3' in the clockwise direction on the standard map of pBR322. In order to further investigate the nature of the two reaction products, their strand specificity was tested. Reactions were performed and analyzed as described above. Following agarose gel electrophoresis, the DNA was transferred to a nitrocellulose membrane and analyzed by probing with strand-specific radioactive DNA probes. The results shown in Figure 5.3 indicate that the upper band contains the strand that runs 5'→3' in the clockwise direction, and the lower band contains the strand that runs 5'→3' in the counterclockwise direction. This indicates that the lower and upper bands represent the circular minus strand and the linear plus strand, respectively. Therefore, the reaction proceeds as diagramed at the top of Figure 5.3.

### Unwinding of defective origins

The DNA sequence signals for initiation were found to begin four bases before the nicking site and extend more than 100 base pairs downstream including both the core origin and the replication enhancer (Cleary & Ray, 1980, 1981; Dotto *et al.*, 1982ab, 1984). In order to determine whether sequences in the A+T-rich enhancer played a role in the *in vitro* unwinding reaction, I analyzed the unwinding of a series of defective origins (Figure 5.4). The defective origin, pD48 (Dotto *et al.*, 1984) has a deletion from the right that removes most of the replication enhancer sequence, but leaves the core origin intact. As shown in Figure 5.4, pD48 is a substrate for *in vitro* unwinding. The time course of appearance of unwinding products was similar for pD48 (panel 2) and pD10

Figure 5.2. Parameters of the unwinding assay. Left panel, time course of unwinding. Unwinding reactions were incubated for the indicated times (minutes) in 20 mM Tris-Cl (pH 8.0), 80 mM KCl, 5 mM DTT, 6 mM  $\text{MgCl}_2$ , 5% glycerol, 1 mM ATP, and analyzed by agarose gel electrophoresis. Center panel, requirement of gene II protein. RFII produced by gene II protein was purified after electrophoresis on a 0.6% agarose gel by electroelution, extraction with phenol, and ethanol precipitation. The specifically nicked RFII was incubated with the indicated enzymes, and the unwinding products were analyzed as described above. Right panel, nuclease sensitivity of unwinding products. Unwinding reactions were performed as described above, except lane 16 (counting from the left) contained 1 unit of exonuclease VII.







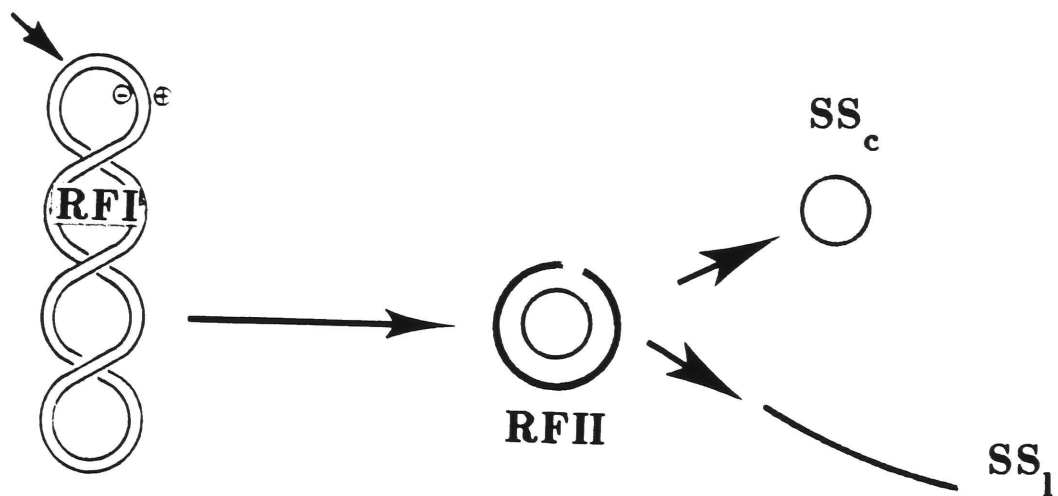
(panel 1) which contains the wild-type origin. In contrast, the defective origin,  $\Delta+29$  (Dotto *et al.*, 1984), has a deletion that removes the replication enhancer and extends into the core origin. The mutant  $\Delta+29$  is not a substrate for *in vitro* unwinding (Figure 5.4, panel 3). No unwinding products were visible for the mutant  $\Delta+29$ . In order to detect potential unwinding products for the mutant  $\Delta+29$  with greater sensitivity, the agarose gel was dried and probed *in situ* with radioactive DNA probes. This method is able to detect 0.5 fmol of unwinding products. With this greater sensitivity of detection, no unwinding products were apparent (data not shown).

### Discussion

In this chapter, I analyze the DNA sequence requirements for the *in vitro* unwinding reaction first reported by Geider *et al.* (1982), using a simple assay in which the products of complete unwinding are analyzed by agarose gel electrophoresis. In the presence of gene II protein, Rep helicase, SSB, and ATP, a plasmid containing an f1 origin (pD10) is unwound to a mixture of single-stranded circles and linear single strands. Each of these components is required for unwinding to occur (Figure 5.1). Gene II protein is required for unwinding at a step beyond nicking because RFII molecules produced by gene II and subsequently purified still require addition of gene II protein for their unwinding (Figure 5.2). The mutant pD48, which lacks most of the replication enhancer sequence, is still a substrate for the *in vitro* unwinding reaction (Figure 5.4). Therefore, the simple hypothesis that the A+T-rich replication enhancer sequence facilitates this Rep helicase mediated unwinding reaction was shown to be incorrect. Consequently, I began to favor the hypothesis that other proteins were responsible for the action of the replication enhancer sequence. These experiments eventually lead to the discovery that IHF enhances f1 DNA replication through interaction with the enhancer sequence (described in Chapter 6).

In contrast to pD48 ( $A^+B^-$ ), the mutant  $\Delta+29$  ( $A^-B^-$ ) was shown not to be a sub-

Figure 5.3. Identification of unwinding products. A complete unwinding reaction was carried out as described in the legend to Figure 5.1 and the text. Following electrophoresis, the DNA was transferred to a nitrocellulose membrane and hybridized with a radioactive 16-mer oligonucleotide probe (cw=5'-GTATCACGAGGCCCTT-3') that hybridizes to the counterclockwise strand of pD10. Following autoradiography, the nitrocellulose filter was washed at 65°C to remove the hybridized probe. The filter was then hybridized with a different radioactive 16-mer oligonucleotide probe (ccw=5'-AAGCTGTCAAACATGA-3') that hybridizes to the clockwise strand of pD10. The bottom panel shows autoradiographs. The diagram at the top portion illustrates the unwinding reaction and the products that are consistent with the hybridization results.



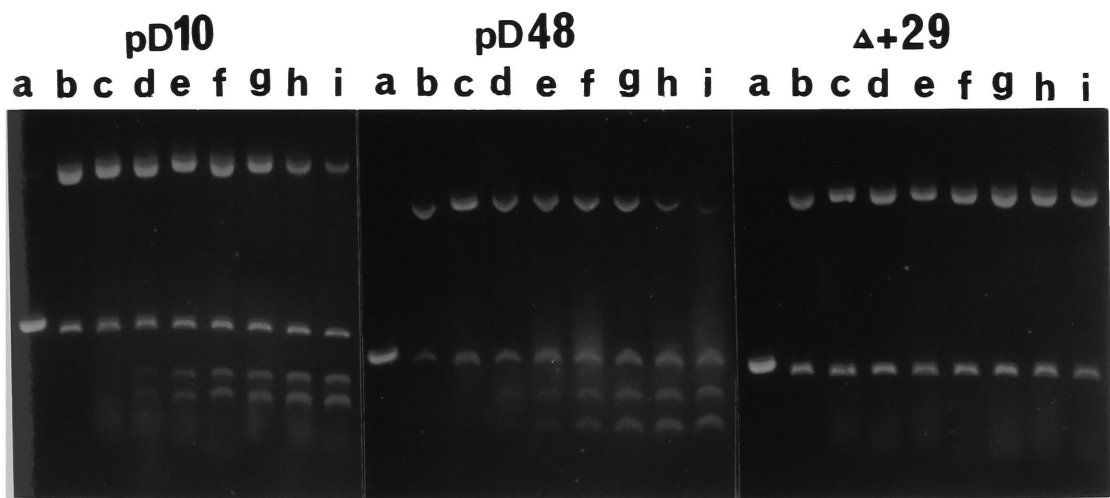
Probe cw ccw



strate for *in vitro* unwinding (Figure 5.4). This observation provides a possible basis for this mutant's initiation defect. The unwinding and replication defects of  $\Delta+29$  correlate with a truncated DNase I protection pattern with gene II protein (see Chapter 3). Possibly, a domain of gene II protein is improperly positioned when it binds to  $\Delta+29$  and cannot participate in the unwinding reaction.

Figure 5.4. Unwinding of defective origins. Unwinding reactions were carried out for various lengths of time using pD10 (wild-type origin), pD48 (a deletion from the right to position +48), or  $\Delta$ +29 (a deletion from the right to position +29) as substrates. Lanes a contained the substrate plasmid DNA alone. Lanes b contained the substrate incubated with gene II protein. Lanes c-i are complete unwinding reactions incubated at 30°C for 0, 30 seconds, 1, 1.5, 2, 5, and 20 minutes, respectively.







## Chapter 6

### **Integration Host Factor Interacts with the Replication Enhancer**



Since its identification, the mechanism of action of the replication enhancer sequence has remained elusive. In contrast to the core origin, the replication enhancer sequence (about 100 bp) is not absolutely required, but rather serves to potentiate DNA replication (Dotto *et al.*, 1984; Johnston & Ray, 1984). It is AT-rich and is located immediately downstream of the core origin sequence. Origins with deletions in the replication enhancer sequence replicate *in vivo* at about 1% of the wild-type level (Dotto *et al.*, 1984). Phage with lesions in the replication enhancer grow poorly, forming turbid plaques, and acquire compensatory mutations (Dotto & Zinder, 1984ab; Kim & Ray, 1985) that restore clear plaque morphology. Compensatory mutations that restore efficient replication by  $A^+B^-$  origins (wild-type domain A, defective domain B) alter (Dotto & Zinder, 1984b; Kim & Ray, 1985) or overproduce gpII (Dotto & Zinder, 1984ab). At first, I made no progress in understanding the mechanism of action of the enhancer. Then, I noticed sequences within the enhancer that resembled binding sites for the *E. coli* integration host factor (IHF). This observation prompted me to examine whether IHF plays a role in f1 DNA replication. In this chapter, I present the results of these experiments. I propose that IHF plays a role in f1 DNA replication via interaction with the replication enhancer sequence. IHF is a heterodimer consisting of two non-identical proteins IHF- $\alpha$  and IHF- $\beta$  (Nash & Robertson, 1981) that are the products of the *E. coli* genes *himA* and *himD* (or *hip*), respectively (Miller & Friedman, 1980; Miller & Nash, 1981; Kikuchi *et al.*, 1985). IHF is a DNA-binding protein that functions in a number of processes (for a review see Drlica & Rouviere-Yaniv, 1987; or Friedman, 1988) by binding to DNA and bending it, thereby stabilizing higher order DNA protein structures. From analogy with these other systems, I infer that higher order DNA protein structures mediate f1 DNA replication. The observation that f1 utilizes a host factor to potentiate its level of replication, provides a potential basis for coupling between the physiology of the host and the replication of a viral parasite.

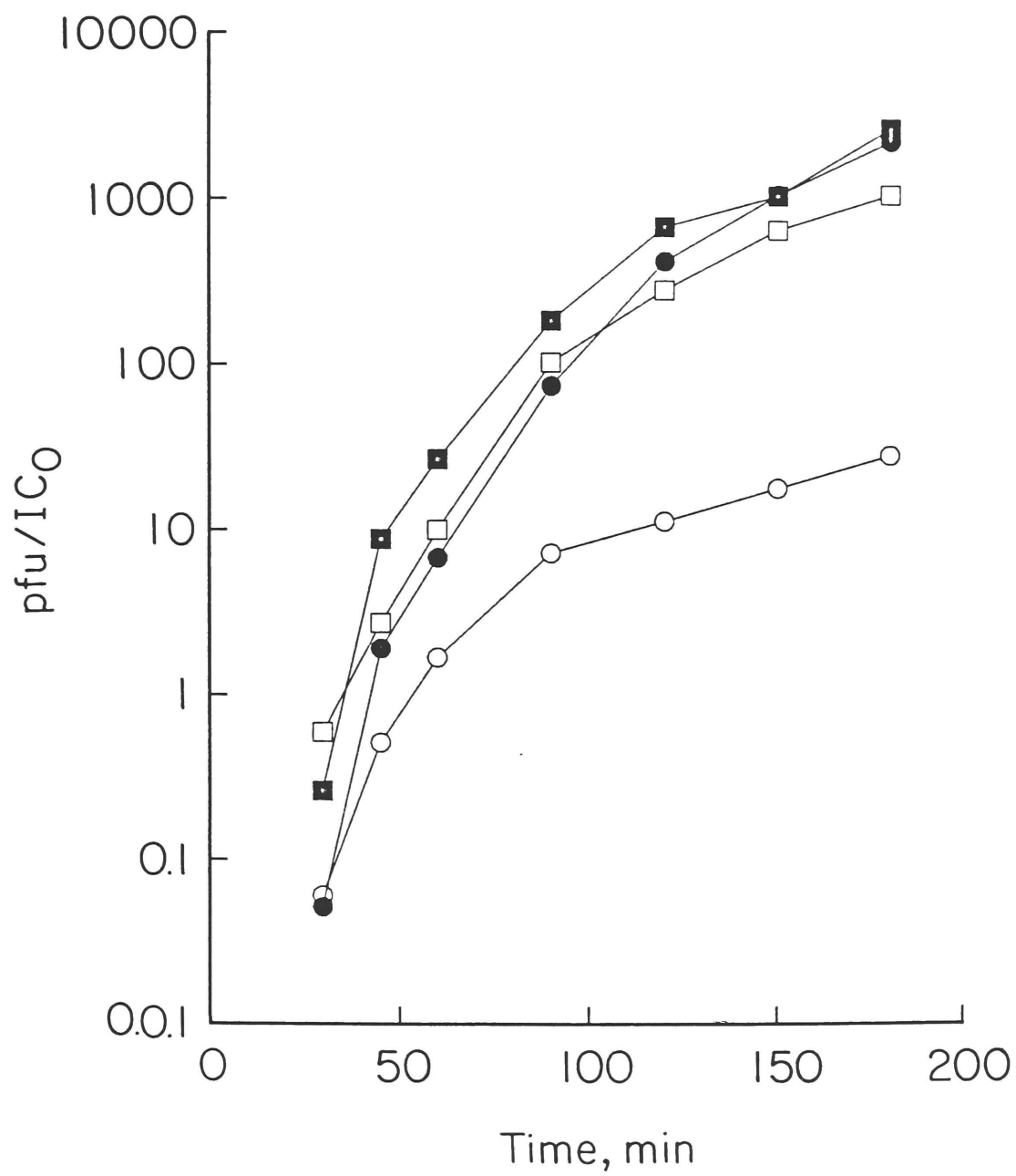


### IHF potentiates f1 growth

Initial attempts to determine whether f1 phage growth is affected by IHF mutations were inconclusive because IHF mutations depress the expression of the F-pili. For reasons that remain unclear, *himA* or *himD* mutant strains produce F-pili at a reduced level; hence they poorly participate in conjugal transfer (Gamas *et al.*, 1987a) and are inefficiently infected by the F-specific phages. Bacteriophage f1 infected IHF mutant strains with about 100-fold lesser efficiency as compared to wild-type strains. Experiments with radioactive f1 phage showed that the inefficient infection of IHF mutant strains could be explained by poor entry of the phage DNA into the cells (data not shown). To determine whether IHF plays a role in the intracellular growth of f1, I introduced f1 replicative-form DNA into IHF mutant and wild-type strains by transfection, and measured the kinetics of phage production during subsequent growth. Female strains ( $F^-$ ) were used as recipients to assure against reinfection. Figure 6.1 shows the results obtained using an isogenic pair of wild-type (K361-filled circles) and IHF mutant (K1173-open circles) strains. They indicate that upon transfection with the wild-type f1 DNA the IHF mutant strain produces phage at a reduced rate (approximately 3% of normal phage production at 2 hr). K1173 is a *himA himD* double mutant carrying a deletion and Tn10 insertion in the *himA* gene (*himA*[ $\Delta 82::Tn10$ ]) and a deletion and insertion in the *himD* gene (*himD*[ $\Delta 3::cat$ ]), so it is unlikely that K1173 produces any active IHF protein. Growth curves identical to that obtained for K1173 were obtained for single mutant strains carrying either the *himA*[ $\Delta 82::Tn10$ ], the *himD*[ $\Delta 3::cat$ ], or *himD*[ $\Delta 1::cat$ ] allele (data not shown). The reduced phage production of the IHF mutant strains was not due to differential cell growth following transfection because the number of infective centers increased at a rate similar to the wild type. Transfection efficiencies (infective center formation) for the IHF mutant strains were generally 50-100% of the wild-type values.

Figure 6.1. Effect of IHF on phage growth. Phage growth was analyzed after transfection of female ( $F^-$ ) strains as described in Materials and Methods. The growth curves show the plaque forming units (pfu) per infective center at time zero (ICo), for various times after transfection. The growth of f1 (circles) and R350 (squares) is shown in the wild-type (K361-filled symbols) and IHF mutant (K1173-open symbols) bacteria, respectively.







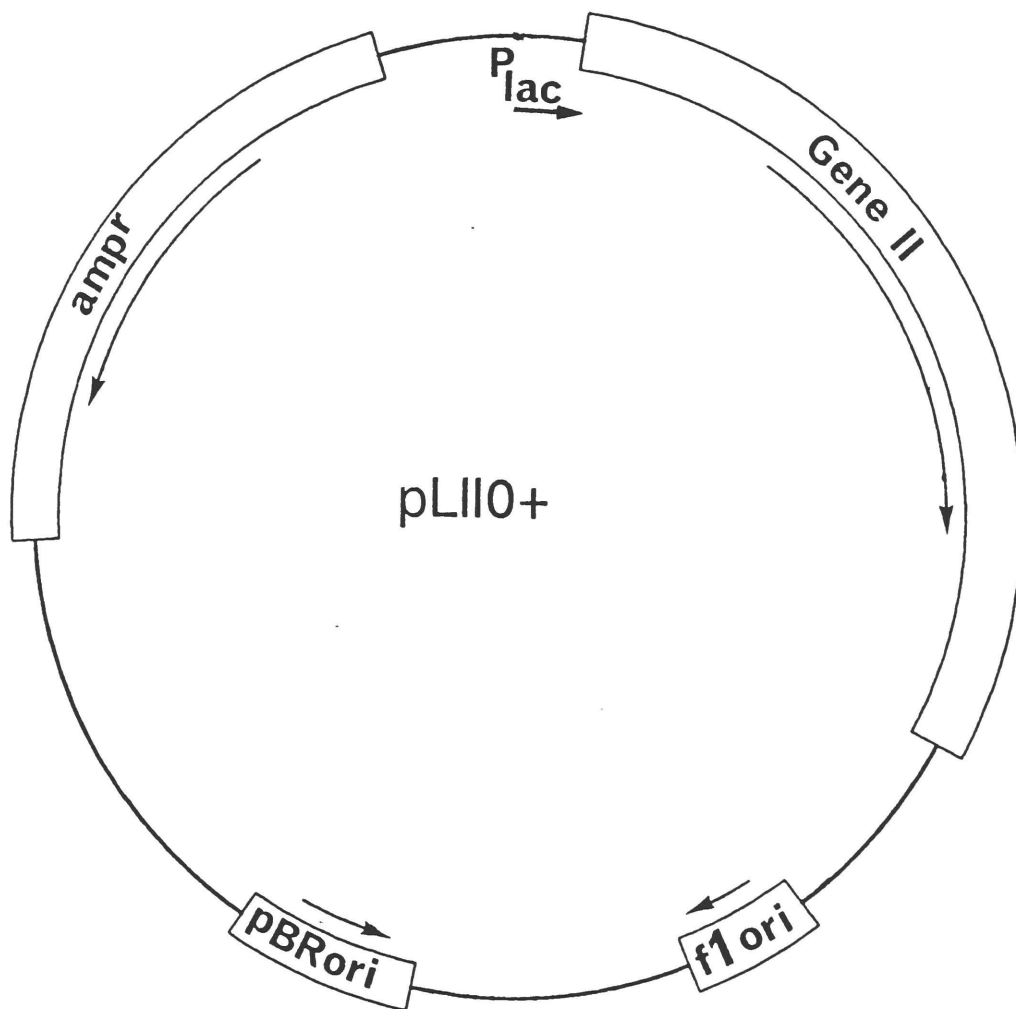
### IHF is required for transformation by f1-replicon plasmids

To establish whether IHF functions in f1 DNA replication, I analyzed the effect of IHF on transformation by an f1-origin-dependent plasmid, pLIIO+ (see Figure 6.2). pLIIO+ contains gene II under control of the *lac* *uv5* promoter, and both the f1 and pBR322 origins (Fulford, 1986). pLIIO+ can transform *polA* deficient bacteria (K924--see Table 6.1), because it can replicate in an f1-origin-dependent fashion in the presence of IPTG. Transformation of *polA* strains by pLIIO+ is dependent on the presence of IPTG and on the Rep helicase function of the recipient. The result shown in Table 6.1 indicates that pLIIO+ cannot transform K1175 (*polA1 himA himD*), whereas pFZY1, a plasmid dependent on the F-episome origin, can. Therefore, IHF acts at the level of DNA replication.

### IHF binds to the replication origin

In order to determine whether IHF has any binding sites in the f1 genome, I analyzed the binding of IHF to a *Sau96I-HpaII* digest of f1 DNA using gel retardation analysis (Garner & Revzin, 1981; Fried & Crothers, 1981). Figure 6.3 shows that IHF binds specifically to the 271 bp fragment (fragment H) that contains the origin of replication. As the concentration of IHF is increased (lanes a-d), the intensity of the free origin fragment diminishes and the intensity of the complex increases. The remaining DNA fragments are unaffected by the presence of IHF except for the 818 bp (fragment C) and the 176 bp (fragment I) fragments that slightly bind IHF at the highest concentration. Both of these fragments are from gene I and the significance of their weak binding is unknown.

Figure 6.2. Structure of an f1-replicon plasmid. pLIIO+ contains both the f1 and pBR322 origins, gene II under transcriptional control of the *lac* uv5 promoter, and a gene for ampicillin resistance.



**Table 6.1. Requirement of IHF For Transformation With An f1-Replicon Plasmid**

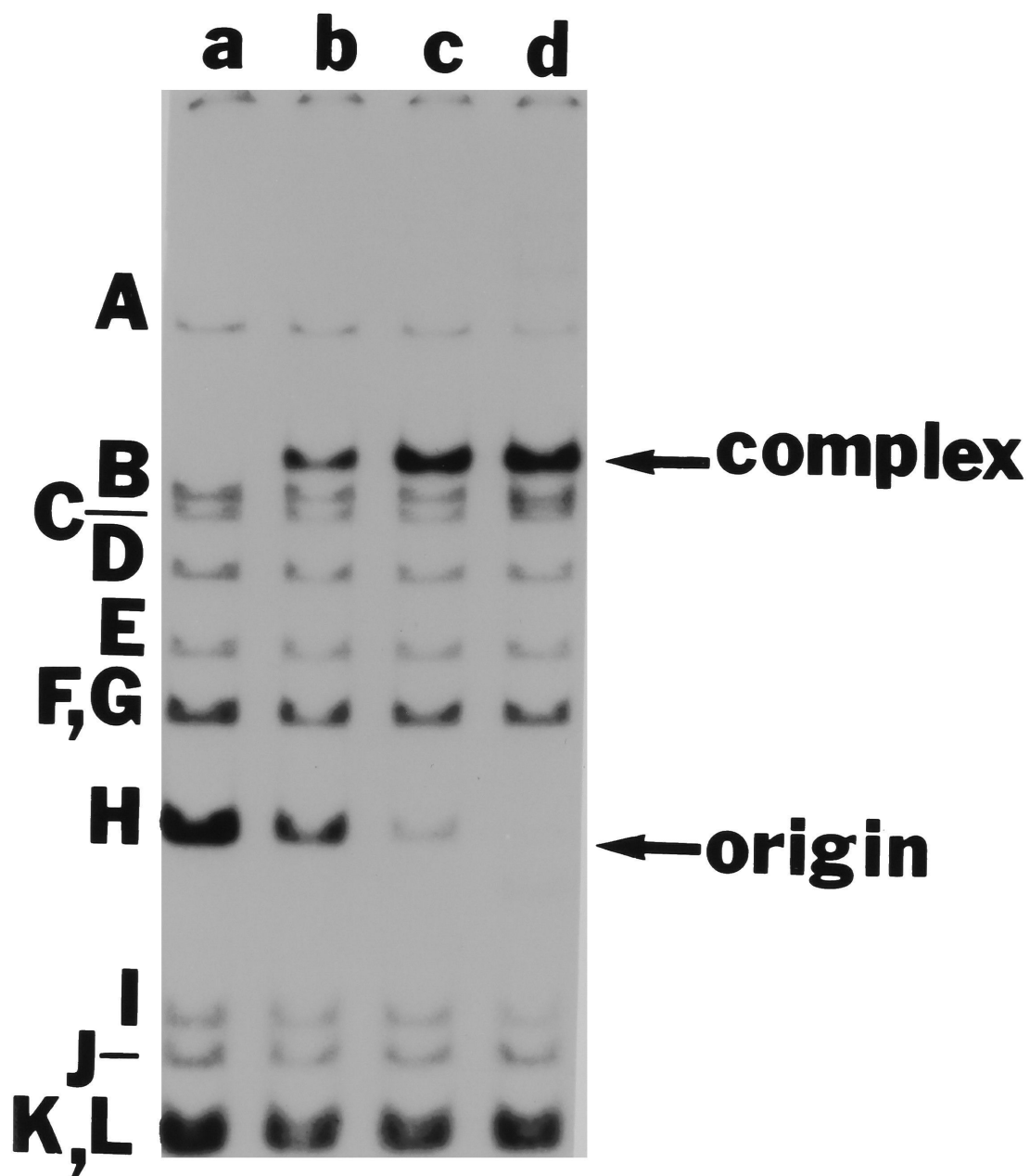
Strain	Plasmid		
	pLIIO+	pFZY1	pBR322
K934 ( <i>polA1</i> )	$6.0 \times 10^6$	$1.5 \times 10^6$	< 250
K1175 ( <i>polA1 himA himD</i> )	< 500	$4.0 \times 10^5$	< 250

The transformation efficiencies (ampicillin resistant colonies per  $\mu\text{g}$  of transforming DNA) of the plasmids pLIIO+ (Fulford, 1986), pFZY1 (Koop *et al.*, 1987), and pBR322 (Bolivar *et al.*, 1977), are listed for K924 (*polA1*) and for K1175 (*polA1 himA himD*). Preparation and transformation of competent cells were performed as described by Hanahan (1983). For transformation with pLIIO+, the media contained 2 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). For selection of transformants, 100  $\mu\text{g}/\text{ml}$  ampicillin was used for pLIIO+ and pBR322, and 50  $\mu\text{g}/\text{ml}$  was used for pFZY1. The values of <250 and <500 are given to indicate the limit of detection because in these cases no ampicillin resistant colonies were obtained.



Figure 6.3. Binding of IHF to the origin. The binding of IHF to a *Sau96I-HpaII* digest of f1 RFI was analyzed by gel retardation (Garner & Revzin, 1981; Fried & Crothers, 1981). Lanes a-d contained 0, 0.11, 0.22, and 0.55 ng of purified IHF, respectively, in addition to 23.3 ng of an end-labeled *Sau96I-HpaII* digest of f1 RFI (5.5 fmol of each fragment). Binding was analyzed on a non-denaturing 5% polyacrylamide gel with TBE buffer.







### The replication enhancer sequence has multiple IHF binding sites

To further characterize the interaction of IHF with the origin, DNase I footprinting experiments (Galas & Schmitz, 1978) were carried out. Figure 6.4(a) shows the results of this analysis. Lanes 1 and 4 are the DNase I controls (no IHF added) for the plus and minus strand, respectively. When IHF is added (lanes 2 and 5) a segment of DNA sequence from position +45 to position +77 (relative to the nicking site) is protected (site 1). As the concentration of IHF is increased (lanes 3 and 6) positions +106 to +162 are protected (site 2). Binding to site 2 is approximately 10-fold weaker than to site 1. These results are summarized in Figure 6.4(b). Site 1 contains one copy of a sequence that differs from the IHF consensus binding site at one position (the third base pair of the consensus sequence--see Figure 6.4(c)). Site 2 contains two copies of a sequence that differs from the consensus sequence at a single position (the eleventh base pair of the consensus sequence in each case--see Figure 6.4(c)). The relative orientation of the two sequences in site 2 (2' and 2'') are the same, but opposite from that of site 1.

In order to determine whether IHF binds to site 1 and site 2 in a cooperative or independent fashion, I footprinted the two sites individually. Figure 6.4(d) shows the results of DNase I footprinting to a fragment containing site 1 (lanes 7-9) and to a fragment containing site 2 (lanes 10-12). The two sites were separated from each other by cleavage at the R209 *EcoRI* site that is located between them (see Figure 6.4(b)). Comparison of Figure 6.4(a) with Figure 6.4(d) shows that the binding to either site is not affected by the presence of the other, and suggests that IHF binds the two sites independently. In contrast to the footprinting results, which indicate that IHF has multiple binding sites within the origin, the gel retardation results (Figure 6.3 and unpublished data) show the formation of only one species of IHF-bound complex even at high IHF concentrations and under a variety of conditions (data not shown). I am unable to detect binding of IHF to a fragment that contains only site 2 by gel retardation

Figure 6.4. Footprinting of IHF bound to the origin. The binding of IHF to the origin was analyzed by DNase I footprinting (see Materials and Methods for details). (a). Lanes 1-3, the reactions contained 1.6 nM f1 origin-containing restriction fragment (315 bp *Sau96I-ClaI* fragment) 5'-end-labeled on the plus strand, and 0, 40, and 160 nM IHF, respectively. Maxam-Gilbert sequencing reactions served as markers (lanes are marked to indicate the cleavage reaction). Lanes 4-6, the reactions contained 1.9 nM f1 origin-containing restriction fragment 3'-end-labeled on the minus strand, and 0, 61, and 243 nM IHF, respectively. The nucleotides are numbered relative to the nicking site (position 0).

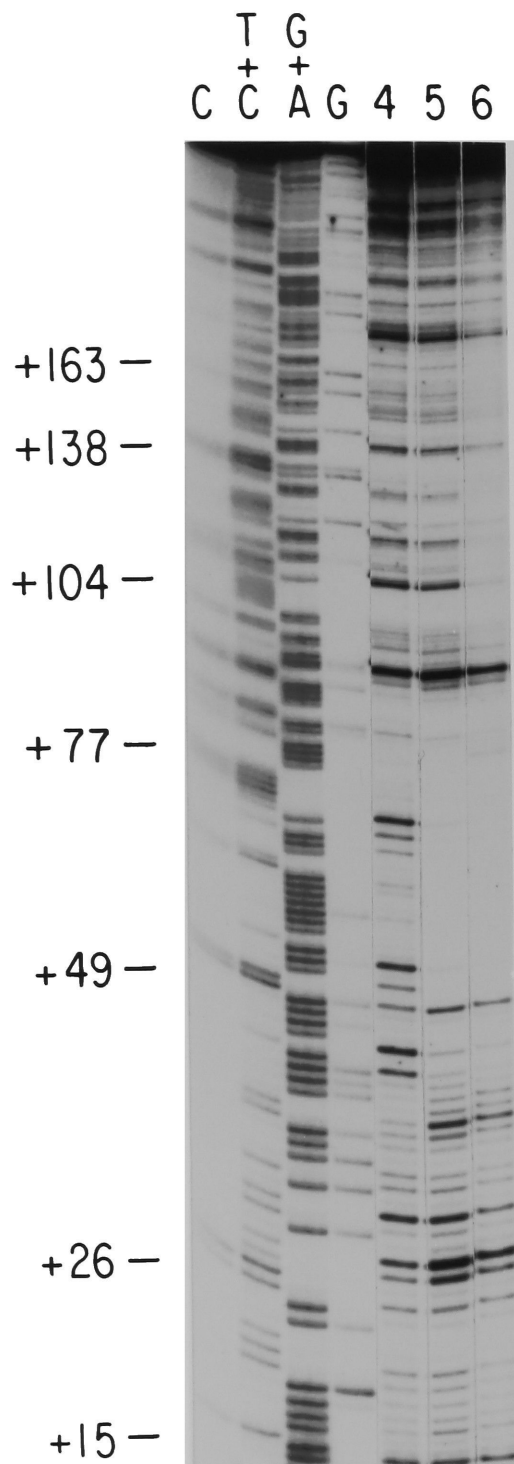
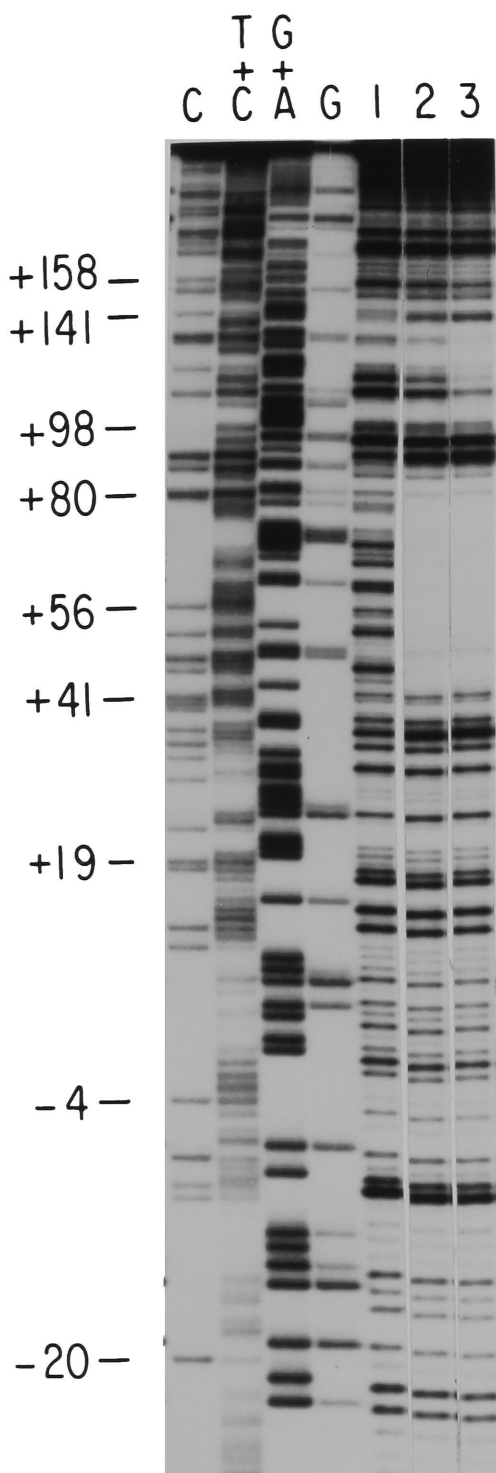






Figure 6.4(b). Summary of footprinting results. The origin region is shown as a horizontal line. A vertical arrow marks the site of nicking by gpII (0). DNA replication begins at the nick and proceeds rightward. The core origin sequence and the replication enhancer sequence (described in the text) are indicated. DNA sequences protected by IHF or gpII are shown by bars above (plus strand) and below (minus strand) the line. The leftward hatched bar shows the region strongly protected by IHF (site 1) and the rightward hatched bar shows the region weakly protected by IHF (site 2). The region protected by gpII is shown by the open bar. Repeated sequences within the core origin are indicated by arrows marked  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ . Arrows marked 1, 2', and 2'' are sequences within the replication enhancer that nearly match the consensus sequence for IHF binding (see below). The R209 *Eco*RI site is indicated. The deletion,  $\Delta 40.56$ , that disrupts site 1 is indicated by a bracket showing the extent of the deletion. Sequences required for initiation and termination of DNA replication, respectively, are shown.



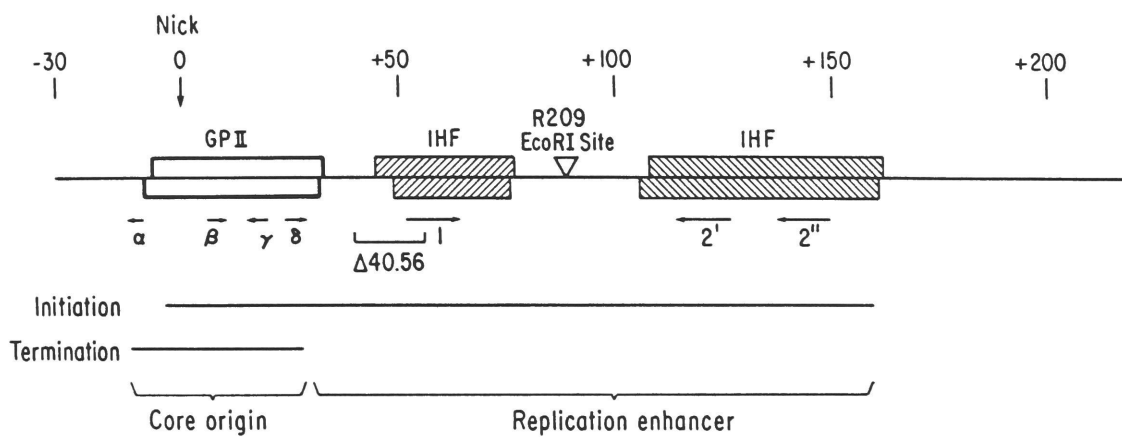
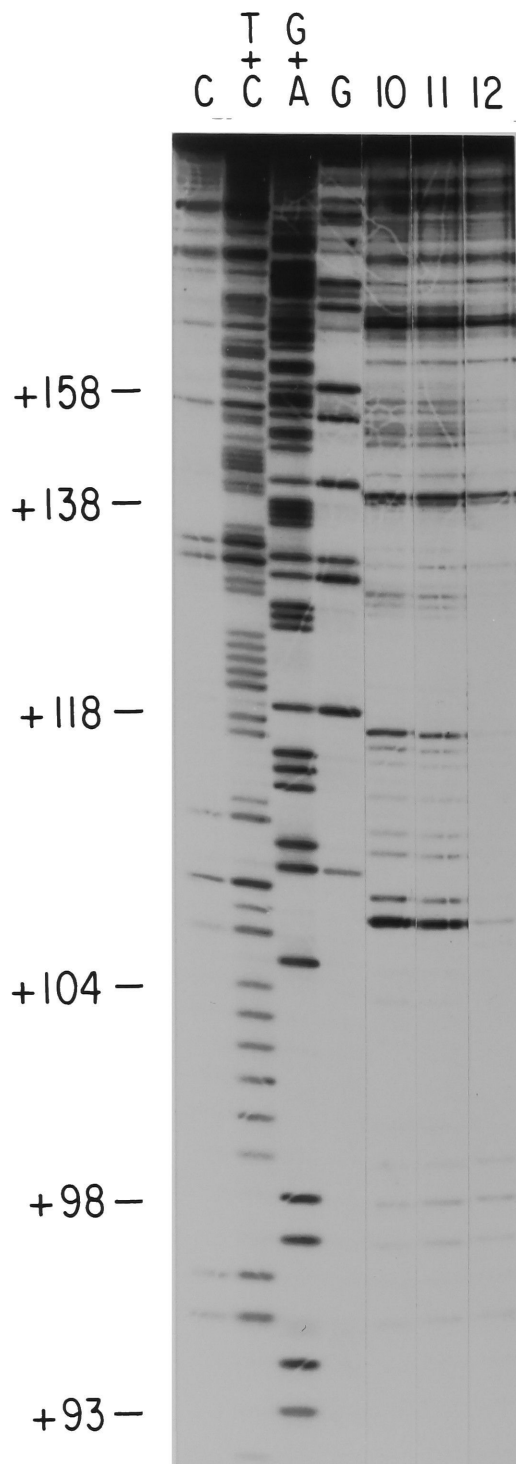
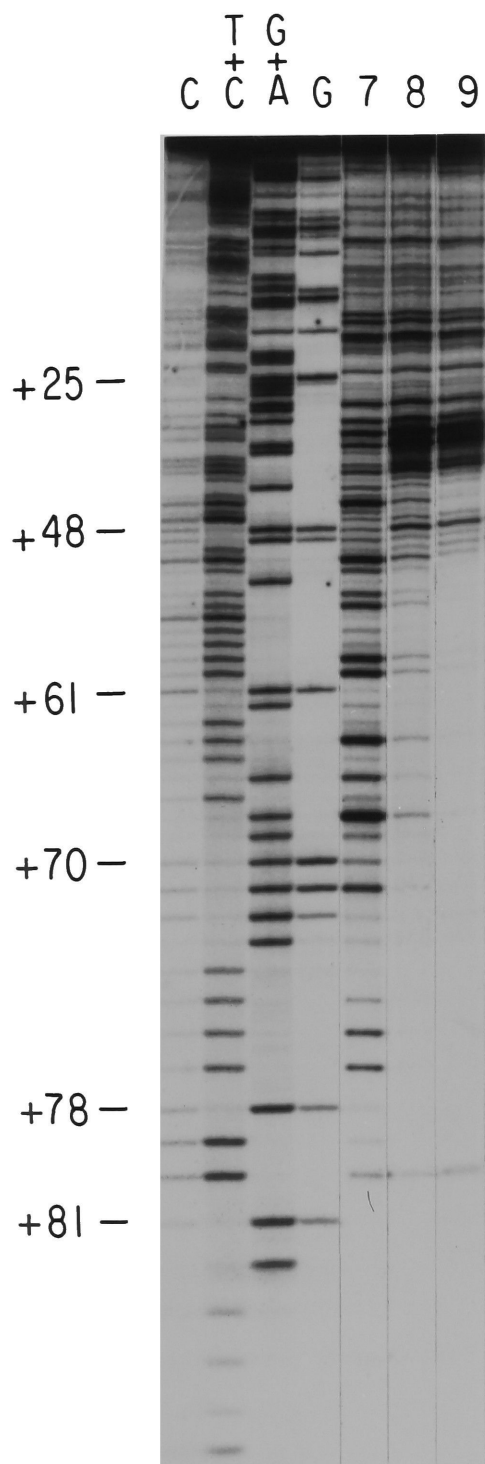


Figure 6.4(c). Nucleotide sequence of IHF binding elements. The sequences present in the f1 replication enhancer (1, 2', and 2'') are shown, as is the consensus sequence for IHF binding (Drlica & Rouviere-Yaniv, 1987; Craig & Nash, 1984; Leong *et al.*, 1985). Nucleotides that differ from the consensus sequence are shown in lowercase. Y denotes a pyrimidine, N denotes any base, and A/T denotes that this base may be either an A or a T. The nucleotides are numbered relative to the nicking site (position 0). The sequence for site 1 is from the plus strand, and the sequence for sites 2' and 2'' are from the minus strand. Site 2'' is not shown in Figure 1.3(a) on page 11.

Consensus 5'-YAANNMNTTGATA/T-3'

	+52		+64
1	TA <sub>t</sub> TC <sub>t</sub> TTT <sub>t</sub> TG <sub>t</sub> ATT		
	+127		+115
2'	TAAATTTT <sub>t</sub> TG <sub>t</sub> TA		
	+150		+138
2''	TAATATTT <sub>t</sub> TG <sub>t</sub> TA		

Figure 6.4(d). Footprinting to the separated IHF binding sites. Sites 1 and 2 were separated by cleavage of R209 RFI at the *Eco*RI site, which was used as the site of end-labeling. Lanes 7-9 contained the 145 bp *Eco*RI-*Sau*96I fragment 3'-end-labeled at the *Eco*RI site (plus-strand labeled). Lanes 10-12 contained the 170 bp *Eco*RI-*Cl*aI fragment 3'-end-labelled at the *Eco*RI site (minus-strand labeled). The reactions contained 1.3 nM origin-containing restriction fragment, and 0 (lanes 7 and 10), 42 (lanes 8 and 11), or 166 nM IHF (lanes 9 and 12).





analysis. Possibly, binding to site 2 may be unstable under the conditions of gel analysis.

### **Initiator protein mutations confer independence of IHF**

The mutation mp1 at codon 40 of gene II (met→ile) overcomes a defective replication enhancer sequence. This has been shown using indirect methods such as phage growth not being interfered with in the presence of an active f1 origin on plasmids and the ability of these phage to produce transducing particles from cells containing f1 origin plasmids (Dotto & Zinder, 1984b). The experiment shown in Figure 6.5 demonstrates this phenomenon more directly. Since the f1 origin contains both the signals for the initiation (Horiuchi & Zinder, 1976) and termination (Horiuchi, 1980) of DNA replication (see Figure 6.4(b)), a plasmid containing two wild-type origins in the same orientation (e.g., pD10-wt in Figure 6.5(a)) is resolved into two replicons when cells containing it are superinfected with f1 (Dotto & Horiuchi, 1981). Initiation at origin I and termination at origin II produce the small plasmid I', while initiation at origin II and termination at origin I produce the larger plasmid II' (Figure 6.5(b), lane 2). If, however, origin II has a deletion in the replication enhancer sequence (e.g., pD10-Δ40.56 in Figure 6.5(a)), then production of plasmid II' is markedly reduced (Figure 6.5(b), lane 5). The yield of plasmid I' is unaffected because while the origin II is initiation-defective, it is termination-proficient (Dotto *et al.*, 1984). When R350 carrying the mp1 mutation was used instead of the wild-type f1 phage, both plasmid I' and II' were produced from pD10-Δ40.56 (Figure 6.5(b), lane 6). This indicates that the gpII with the mp1 mutation can initiate replication from the enhancer-defective Δ40.56 origin, as well as from the wild-type origin.

It was therefore crucial to test if the mp1 mutation also confers IHF-independence. Growth of phage carrying the mp1 mutation was measured after transfection of IHF-deficient bacteria. The result shown in Figure 6.1 clearly indicates that R350 phage

Figure 6.5. Activation of a defective origin by an initiator mutation. (a). Double-origin assay of DNA replication. pD10-wt contains two wild-type f1 origins (indicated as ori I and ori II) in direct repeats. pD10- $\Delta$ 40.56 contains a wild-type f1 origin (indicated as ori I) and a defective origin (indicated as ori II) with a deletion in the replication enhancer sequence (indicated with an x). Upon superinfection with helper phage, initiation at ori I and termination at ori II result in production of the small plasmid I'. Initiation at ori II and termination at ori I produce the larger plasmid II'.



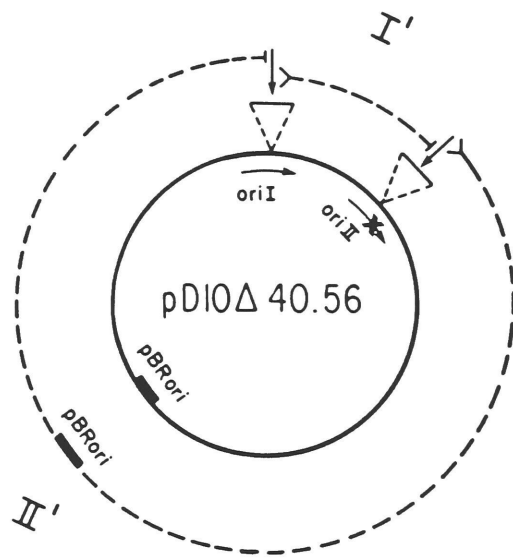
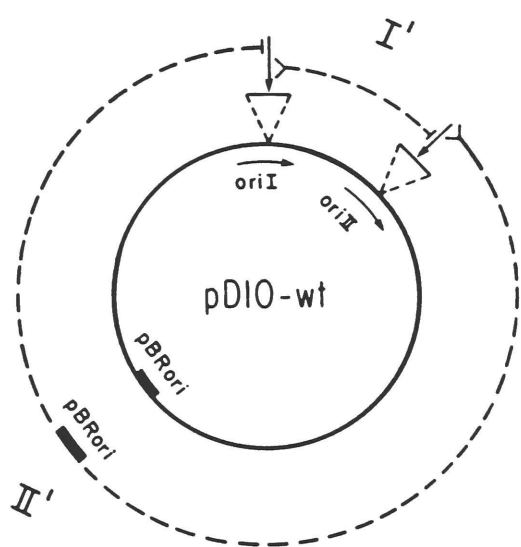
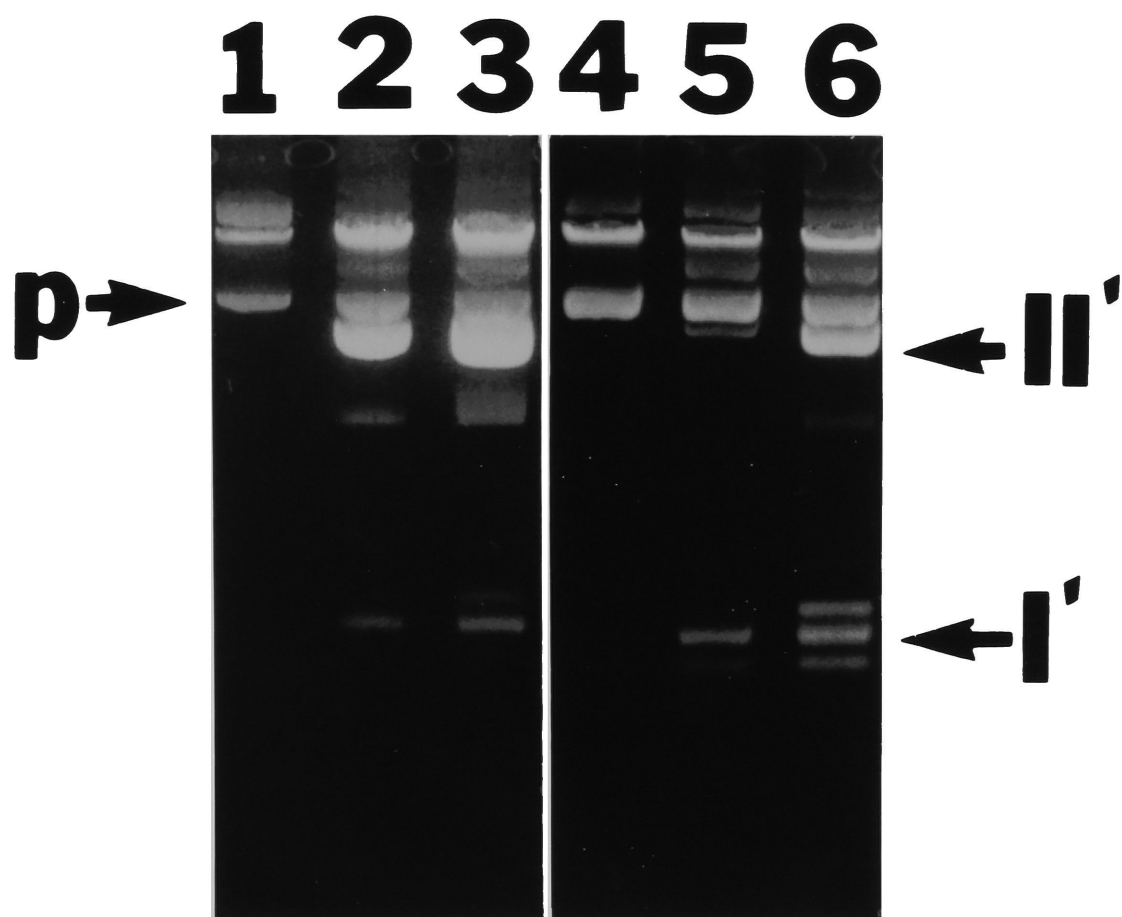


Figure 6.5(b). Results of the double-origin assay. Cells containing pD10-wt (lanes 1-3) or pD10- $\Delta$ 40.56 (lanes 4-6) were infected with f1 (lanes 2 and 5), R350 (lanes 3 and 6), or mock-infected (lanes 1 and 4). At 35 minutes after infection, the cells were harvested and DNA was prepared and analyzed on a 1.0% agarose gel containing 0.5  $\mu$ g/ml ethidium bromide. The position of the superhelical form of the parental plasmid (p) and its resolution products, I' and II', are indicated. Open circles and single-stranded circles of these plasmids are also seen.





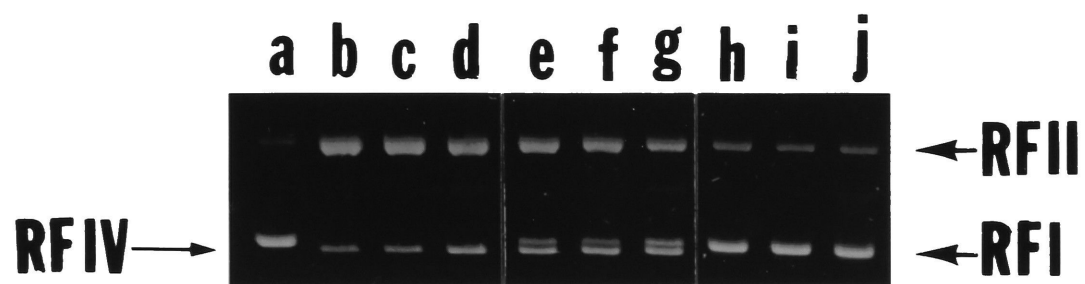
grows normally upon transfection of both the wild-type (K361, filled squares) and IHF mutant (K1173, open squares) bacteria, in contrast to the wild-type phage which requires IHF for optimal growth. This observation strongly suggests that IHF stimulates f1 DNA replication by interacting with the replication enhancer sequence. The result also indicates that the low growth rate of the wild-type phage in the IHF mutants is not due to an artifact of the transfection procedure, because R350 grows with normal kinetics in the IHF mutants.

Several gene II mutations other than *mp1* have also been shown to overcome defects in the replication enhancer sequence. They are at codon 73 of gene II (Kim & Ray, 1985; K. Horiuchi, unpublished data). Using the double-origin assay described above (Figure 6.5), phage carrying an alanine or a cysteine residue at codon 73 of gene II (glycine in the wild-type) are able to initiate replication from the  $\Delta 40.56$  origin as does R350 (K. Horiuchi, unpublished data). Furthermore, upon transfection, these codon 73 mutants grow normally on both wild-type (K361) and IHF mutant (K1173) bacteria (data not shown).

### **IHF does not affect *in vitro* nicking by gene II protein**

Since the IHF binding sites are adjacent to the gene II protein binding site (see Figure 6.4(b)), I was interested in determining whether IHF affects the *in vitro* nicking of the origin by gene II protein. Possibly, IHF might potentiate gene II protein nicking, thus explaining its role as an activator of replication. Figure 6.6 shows an analysis of *in vitro* nicking by gene II protein in the presence of different amounts of IHF. Upon incubation with gene II protein, the substrate RFI (lane a) is converted to a mixture of RFII and RFIV (lanes b and e). The nicking-closing activity was origin specific; pBR322, or other supercoiled plasmids not containing f1 origins, were not substrates (data not shown). Lanes b-d analyze the effect of IHF on nicking when the gene II protein is in excess (16 nM). The results indicate that addition of IHF at 2.4 nM (lane c) or at 24 nM (lane d) had no effect on the extent of the reaction. Similar results were obtained when

Figure 6.6. Effect of IHF on nicking by the gene II protein. Origin nicking assays were carried out in 20  $\mu$ l reactions containing 25 mM Tris-Cl (pH 8.0), 80 mM KCl, 5 mM  $\text{MgCl}_2$ , 5 mM DTT, and 100 ng f1 RFI (1.2 nM). Following incubation at 30°C for 30 minutes, 2  $\mu$ l of stop mix was added and the products were analyzed on a 0.6% agarose gel containing ethidium bromide (0.5  $\mu$ g/ml) using 1XTBE buffer. Arrows indicate the substrate (RFI) and the products (RFII and RFIV). RFIV migrates slightly faster than RFI under the conditions employed. Lane a contained no gene II protein or IHF. Lanes b-d contained gene II protein at 16 nM. Lanes e-g contained gene II protein at 2 nM. Lanes h-j contained gene II protein at 0.24 nM. Lanes a, b, e, and h contained no IHF protein. Lanes c, f, and i contained IHF at 2.4 nM. Lanes d, g, and j contained IHF at 24 nM.







gene II protein was added at an intermediate (2 nM, lanes e-g) or a limiting (0.25 nM, lanes h-j) amount. In these instances too, addition of IHF at 2.4 nM (lanes f and i) or at 24 nM (lanes g and j) did not appreciably change the extent of the reaction or the product distribution. Therefore, the simple hypothesis that IHF activates replication by stimulating gene II protein mediated nicking of the origin was shown not to be the case for the *in vitro* situation.

## Discussion

I have shown that IHF stimulates the intracellular replication of bacteriophage f1 (Figure 6.1). IHF is not absolutely required for f1 growth because *himA* or *himD* mutants can produce phage, albeit at a reduced rate (3% of the wild type). I propose that IHF functions at the level of DNA replication because an f1 replicon plasmid (pLIIO+) fails to transform IHF deficient bacteria (Table 6.1). In addition, a mutant phage, R350, containing a mutation (mp1) in gene II (met<sup>40</sup>→ile) grows normally on *himA* or *himD* strains. Since the mp1 mutation suppresses the effect of both the absence of IHF (Figure 6.1) and the absence of the replication enhancer sequence (Figure 6.5), IHF most likely functions through the replication enhancer. This notion is supported by footprinting experiments that show that IHF specifically binds to multiple sites within the replication enhancer sequence (Figure 6.4). Since the only known function of the replication enhancer sequence is the initiation of DNA replication, the results indicate that IHF enhances f1 DNA replication.

IHF plays a role in a number of cellular processes including site-specific recombination, transcription, translation, transposition, phage morphogenesis, and DNA replication. It appears that IHF acts in all these processes as an accessory or enhancing factor rather than as an absolute requirement. As described above, this also holds true for f1 replication. In its best characterized function, IHF plays a role in  $\lambda$  integrative recombination (Nash & Robertson, 1981) by binding to three sites within attP (Craig & Nash,



1984; Garner & Nash, 1986), leading to the formation of a higher order structure termed the attP-intasome (Pollock & Nash, 1983; Griffith & Nash, 1985). Interestingly, just as the lack of IHF in f1 replication is bypassed by certain gene II mutations (e.g., mp1), the lack of IHF in  $\lambda$  integrative recombination is mitigated by some  $\lambda$  integrase mutations (Miller & Friedman, 1980; Lange-Gustafson & Nash, 1984). I imagine that IHF functions in f1 DNA replication in an analogous manner. In Chapter 7, I show that both IHF and gpII bend the replication origin. Thus, higher order structures involving bent/wound DNA-protein complexes are likely to play a role in initiation of f1 DNA replication.

At which step in the initiation of DNA replication IHF functions is unknown, though it is likely to be at a step beyond nicking by the initiator protein gpII. IHF and gpII bind independently to the origin. In addition, IHF has no effect on the efficiency of nicking by gpII *in vitro* (see Figure 6.6). Since the lack of IHF is suppressed by the mp1 mutation in gene II, IHF probably functions in a step in which gpII participates. However, gpII is a multifunctional protein and is known to play several different key roles in f1 DNA replication. In particular, gpII is required for initiation of replication on a template-primer molecule that has already been nicked by gpII itself (Geider *et al.*, 1982). Thus, IHF may play a role in the initiation of unwinding, in the coupling of unwinding with synthesis of DNA, or in the entry of DNA polymerase III holoenzyme to the initiation complex.

How the mp1 mutation and certain mutations at codon 73 of gene II overcome the defective replication enhancer is not known. Other mutations that overcome the defective replication enhancer appear to involve the overproduction of gpII (Dotto & Zinder, 1984ab). These are the phage mutants R218 which carries a mutation in gene V ( $\text{arg}^{21} \rightarrow \text{cys}$ ) and R325 which carries a mutation in the leader sequence of gene II mRNA ( $\text{G}^{5977} \rightarrow \text{T}$ ) (Dotto & Zinder, 1984ab). When tested by the double-origin assay, however, both phages failed to stimulate initiation from the  $\Delta 40.56$  origin (K. Horiuchi, unpublished data). Possibly, in this assay, the phages failed to overproduce gpII because their



DNA replication was inhibited by the presence of plasmid molecules carrying complete phage origins. Suppression of defective enhancers by R218 and by R325 was originally based on phage interference measurements (Dotto & Zinder, 1984ab). The observed interference, however, appears to be due to the fact that the phages used in those studies carried a defective origin (K. Horiuchi, unpublished data). At any rate, the efficiency of suppression of the replication enhancer defect by overproduction of gpII by these phages must be less efficient than that by mp1 or codon 73 mutations. The R218 gene V mutation ( $\text{arg}^{21} \rightarrow \text{cys}$ ) does not compensate for the lack of IHF, either (data not shown).

IHF has been shown to function in the replication of pSC101 (Gamas *et al.*, 1986). It has recently been shown that IHF binds to a bent region at the pSC101 origin and bends it further (Stenzel *et al.*, 1987). Although the precise step at which IHF functions in pSC101 replication remains unclear, Stenzel *et al.* (1987) have proposed that IHF might facilitate the interaction of other replication proteins (dnaA, dnaB, and dnaC) with the origin or with each other. The observation that IHF promotes DNA replication from two different origins (pSC101 and f1) that employ different replication proteins, supports the idea that IHF acts by altering DNA conformation rather than directly interacting with other replication proteins. Better understanding of the function of IHF in the two replication systems will require *in vitro* analyses to determine at which step in initiation of replication IHF acts.



## Chapter 7

### **Protein-Induced DNA Bending at the Origin**





Since the discovery of Z-DNA (Wang *et al.*, 1979; Drew *et al.*, 1980), it has been suspected that DNA can exist in a variety of conformations and that these conformations might play active roles in the many functions of the genetic material (Dickerson *et al.*, 1982). The observation that eukaryotic chromosomal DNA is packed into nucleosomes (reviewed by McGhee & Felsenfeld, 1980) and that phage genomic DNA must be condensed in order to be packaged into phage heads, suggested mechanisms for conferring flexibility upon the ordinarily rigid DNA helix. Now it is generally accepted that DNA can be bent due to interactions with proteins or due to sequence-dependent alterations in the B-DNA helix. However, the mechanics underlying DNA bending is only beginning to be understood.

The existence of intrinsically bent DNA was predicted based on natural periodicities found in chromatin DNA sequences (Trifonov & Sussman, 1980). Subsequently, the abnormal gel mobility of DNA from the kinetoplast of *Leishmania tarentolae* was attributed to systematically bent DNA caused by periodicities in the DNA sequence (Marini *et al.*, 1982). Recently, sequence-directed DNA bending was shown to result from adenine:thymine tracts of at least 4 base pairs in length (Koo *et al.*, 1986). Macroscopic bending of the DNA occurs when the A:T tracts are repeated at distances that are multiples of 10.4 bp--the helical repeat of B-DNA (Ulanovsky *et al.*, 1986; Koo *et al.*, 1986). The existence of bent DNA in solution has been suggested based on measurements of electric dichroism (Marini *et al.*, 1982) and differential decay of birefringence (Hagerman, 1984). Bent kinetoplast DNA has recently been visualized by electron microscopy under a variety of fixation conditions (Griffith *et al.*, 1986; Ray *et al.*, 1986). Anomalous migration on polyacrylamide gels is predicted for bent DNA (sequence-dependent or protein-directed) by electrophoresis theory, which views the DNA as reptating through the gel pores and which calculates the gel mobility as being proportional to the mean square end-to-end distance of the DNA fragment (Lerman & Frisch, 1982; Lumkin &



Zimm, 1982). DNA bending reduces the end-to-end distance of the DNA fragment and results in a slower migration on polyacrylamide gels than would be predicted from the chain length of the fragment. Consequently, bent DNA fragments migrate more slowly when the bend is located in the center of the fragment as compared to when it is located at the end. Using this property, Wu & Crothers have developed a circular permutation assay to map the locus of sequence-directed or protein-induced bending (1984). Sequence-directed DNA bending has been found at some replication origins ( $\lambda$ --Zahn & Blattner, 1985a; pSC101--Stenzel *et al.*, 1987; pT181--Koepsel & Kahn, 1986; f1--K. Horiuchi, unpublished data), the phage  $\lambda$  attachment site (Ross & Landy, 1982), the SV40 T-antigen binding site (Ryder *et al.*, 1986), and a large number of promoters, to list a few examples. The precise role these sequences play in the various processes remains to be elucidated.

DNA bending appears to be a feature in the interaction of several initiator proteins with their respective origins. The replication initiator protein of phage  $\lambda$ , the O protein, binds to four iterons in the replication origin (Tsurimoto & Matsubara, 1981). The binding of the O protein results in bending of the origin as analyzed by electrophoretic mobility experiments (Zahn & Blattner, 1985b). Dodson *et al.* (1985) analyzed the interaction of the O protein with the origin by electron microscopy, and found that multiple O protein molecules bind to the origin to form a wrapped DNA protein complex called the O-some. Formation of the O-some results in DNA helix destabilization as detected by sensitivity to single-strand specific nucleases (Schnos *et al.*, 1988). Since the O protein mediated helix destabilization involves functional origin sequences, it is likely that the alteration in origin conformation is an important prepriming step for  $\lambda$  replication. Likewise, Mukherjee *et al.* (1985) have shown that the replication initiator protein of the drug resistance plasmid R6K induces conformational changes within the origin. At present, the functional role of protein-induced bending in R6K replication remains unclear. However, Mukherjee *et al.* (1985) have discussed the possibility that protein-



induced bending might facilitate assembly of a replication fork at the  $\gamma$ -origin. The R6K initiator protein bound to the  $\gamma$ -origin has recently been shown to promote binding to the  $\beta$ -origin via DNA-looping (Mukherjee *et al.*, 1988). Formation of the looped-DNA protein complex most likely involves protein-induced DNA structural changes. Finally, protein-induced DNA bending has been shown to be a feature of the initiator protein-origin interaction for the plasmid pT181 (Koepsel & Khan, 1986). Like bacteriophage f1, pT181 replicates by a rolling-circle mechanism. Thus, protein-induced conformational changes occur at a variety of origins that replicate by different mechanisms. Liu-Johnson *et al.* (1986) have proposed that the energy stored in DNA bending might be stored for utilization in later steps, such as DNA unwinding. While this idea was proposed for the function of the CAP protein in transcriptional activation, it should also be applicable to DNA replication.

In this chapter, I investigate protein-induced DNA bending at the f1 origin. I show that binding of gene II protein results in bending of the DNA. Both the binding intermediate (complex I) and the functional complex (complex II) are bent. Complex II is approximately twice as bent as complex I. I show that binding of IHF to its strong binding site (site 1) results in bending, in agreement with the data of others showing that IHF bends the DNA upon binding (Stenzel *et al.*, 1987; Prentki *et al.*, 1987; Robertson & Nash, 1988). Finally, I show that the phage growth rate is highly sensitive to the phasing between the IHF binding sites within the replication enhancer sequence. From these experiments, I infer that higher order DNA-protein structures play a role in the initiation of f1 DNA replication.



### The initiator protein bends the origin

One of the unusual features of the gpII-origin interaction is that binding to a downstream repeat ( $\delta$ ) is required for protection of an upstream functional site, the nicking site. If the DNA were bent, then the monomer bound to repeat  $\delta$  could influence binding to the nicking site by a direct protein-protein interaction. To determine whether the binding of gpII results in bending of the DNA, I analyzed the complexes formed by gpII with a series of circularly permuted origin fragments that differ in the placement of the binding site, according to the method of Wu & Crothers (1984). The results show that the mobilities of complex I and complex II are affected by the placement of the binding site within the DNA fragment (Figure 7.1(a)) which is indicative of protein-directed DNA bending. Figure 7.1(b) shows a plot of the mobility against the position of the end of the DNA fragment for each complex. Both complexes I and II have the same bend center at approximately position +6 in repeat  $\beta$ . A priori, the effect of bending could have switched the mobility of the two complexes so that complex I (two molecules of gpII/origin) had a lower mobility than complex II (four molecules of gpII/origin). I have shown that this is not the case by performing the bending experiment with different amounts of gpII. Since complex II is formed preferentially at higher gpII concentrations than complex I, the two complexes could be distinguished (data not shown). The electrophoretic mobility (apparent molecular weight) of the DNA-protein complexes is determined mainly by their structure (DNA bending and solution drag effects), molecular weight, and charge. Figure 7.1(c) shows a plot of the change in apparent molecular weight ( $\Delta MW_r$ ) upon binding of two gpII molecules versus position of the DNA end, for each complex. The change in apparent molecular weight for binding of two gpII molecules to the fragment ( $\Delta MW_{rI}$ --formation of complex I) is roughly equal to that for the binding of two gpII molecules to complex I ( $\Delta MW_{rII}$ --formation of complex II). If I assume that the binding of two gpII molecules has the same consequences on molecular

Figure 7.1. Bending of the origin by the gene II protein. (a). Binding of the gene II protein to circularly permuted origin fragments (393 bp). The binding of gpII to circularly permuted origin fragments was analyzed on a non-denaturing 4% polyacrylamide gel. Each binding reaction contained approximately 0.2 fmol of origin restriction fragment and approximately 4 fmol (0.2 ng) purified gpII. Lane 1, *Bam*HI (-173); Lane 2, *Taq*I (-98); Lane 3, *Sau*96I (-57); Lane 4, *Ava*I (+45); Lane 5, *Tha*I (+129); Lane 6, *Mbo*II (+182). The numbers in parentheses represent the position of the end of the origin fragment relative to the nicking site (0). Each origin fragment is designated by the name of the restriction enzyme with which it was generated.



**1 2 3 4 5 6**







Figure 7.1(b). Mobility of the gene II protein-origin complexes. A plot of mobility (cm) versus position of the DNA fragment end (bp) is shown for the set of circularly permuted origin fragments (circles), complex I (triangles), and complex II (squares). The figure also shows the extrapolation of the bend center for the two complexes.

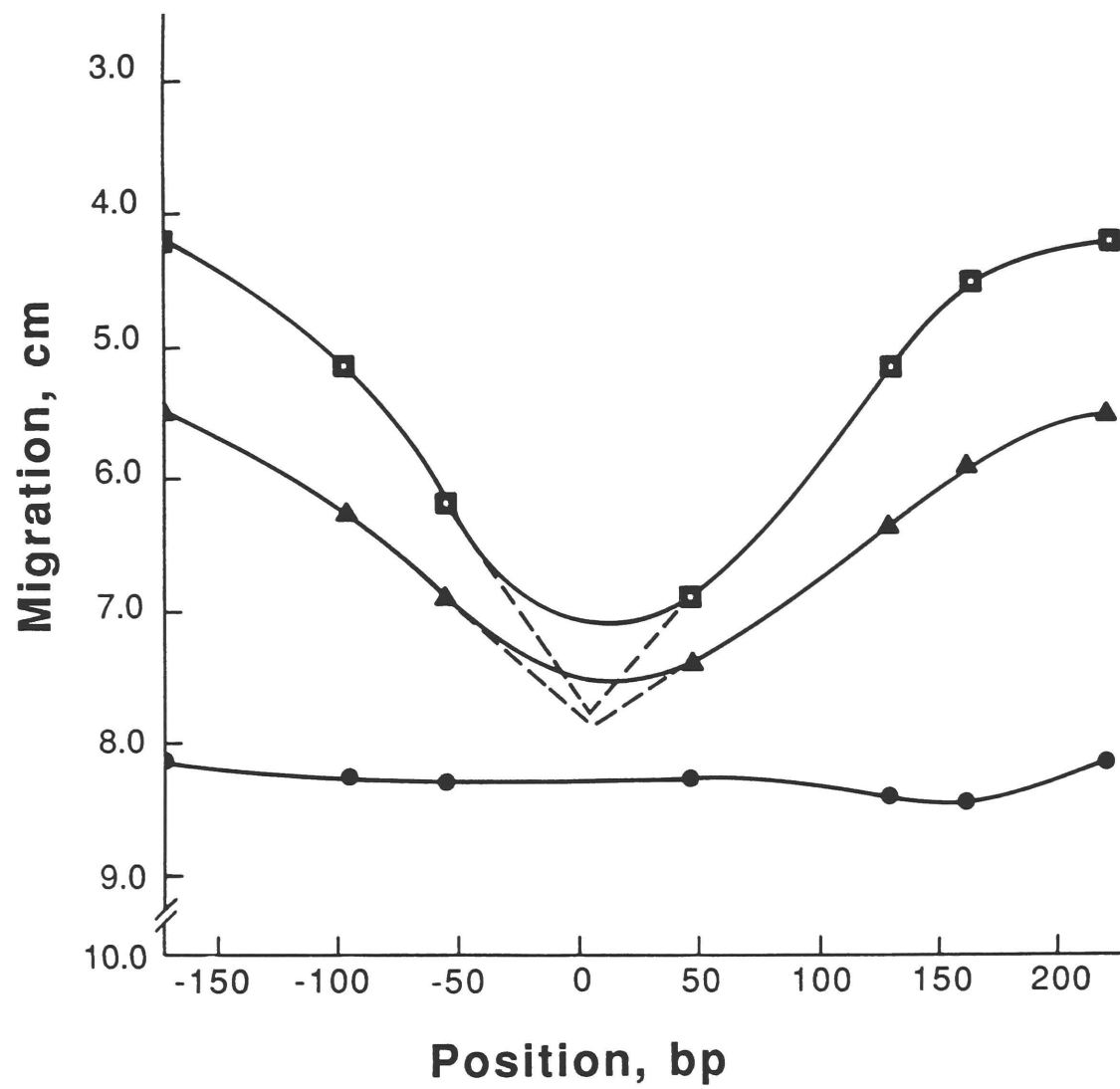
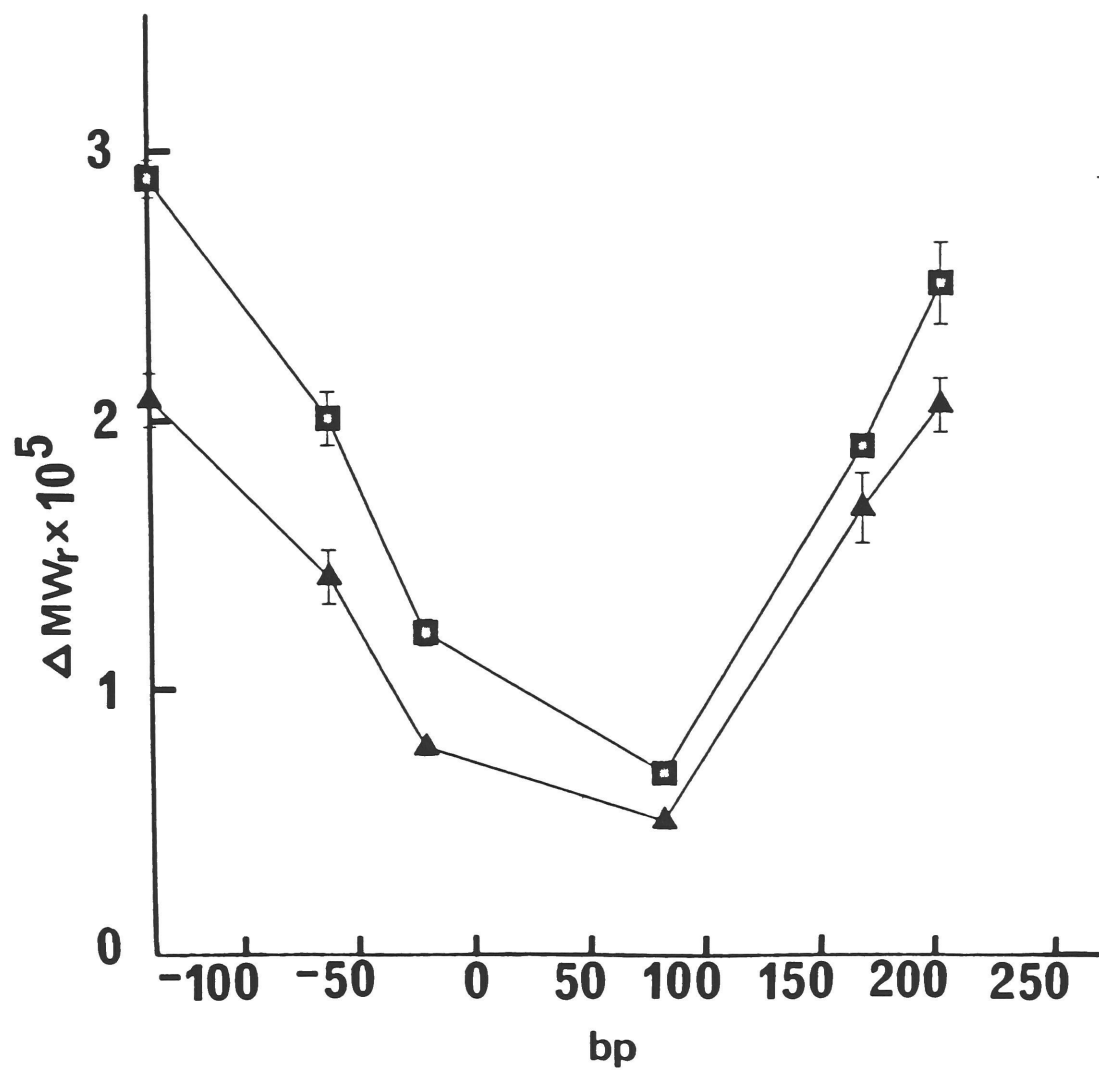


Figure 7.1(c). Change in apparent molecular weight upon binding of two gene II protein molecules. A plot of the change in apparent molecular weight versus the position of the DNA end.  $\Delta MW_{II}$  (triangles) is the apparent molecular weight of complex II minus the apparent molecular weight of complex I.  $\Delta MW_I$  (squares) is the apparent molecular weight of complex I minus the apparent molecular weight of the origin fragment.







weight, solution drag, and charge in either case, then the change in DNA bending must be approximately equivalent. Therefore, complex I should be bent by some angle  $\theta$ , and complex II should be bent by some angle  $\phi$ , and  $\theta < \phi \leq 2\theta$ . Most likely,  $2\theta$  is an upper-bound for the bending angle of complex II because the  $\Delta MW_{rI}$  is slightly larger than the  $\Delta MW_{rII}$  at all points (see Figure 7.1(c)).

### IHF bends the replication enhancer sequence

In Chapter 6, I showed that IHF activates f1 DNA replication, and that IHF binds to the replication enhancer sequence. Here I verify that IHF bends the replication enhancer sequence. I locate the bend center when IHF is bound to its strong binding site (site 1). Figure 7.2(a) shows the results obtained when IHF binds to the same set of circularly permuted origin fragments as used in Figure 7.1. The mobility of the complexes vary considerably with the position of the end of the fragment, indicating that IHF bends the replication enhancer sequence. Titration of IHF with each of the circularly permuted fragments shows that they all bind IHF with the same affinity of approximately  $5 \times 10^8 \text{ M}^{-1}$  (data not shown). Figure 7.2(b) shows a plot of mobility versus position of the DNA end for each of the complexes. The results indicate that the center of the protein-induced bends is close to the IHF binding site at position +79 with respect to the nicking site.

### Phasing within the replication enhancer

A turbid f1 phage mutant, R209, was constructed previously by insertion of 4 bp (GGCC→GGAATTCC) at the *Hae*III G/D border within the replication enhancer sequence to create an *Eco*RI site (see Figure 7.3(a)). This 4 bp insertion is not within one of the IHF binding sites, but rather between site 1 and site 2. DNase I footprinting studies have shown that the 4 bp insertion does not affect the binding of IHF (data not shown). I reasoned that the R209 insertion is deleterious because it either disturbs a

Figure 7.2. Bending of the origin by IHF. (a). Binding of IHF to circularly permuted origin fragments. The binding of IHF to circularly permuted origin fragments was analyzed on a non-denaturing 4% polyacrylamide gel. Each binding reaction contained approximately 0.2 fmol of origin restriction fragment and approximately 3.0 fmol (64 pg) purified IHF. Lane 1, *Bam*HI (−173); Lane 2, *Taq*I (−98); Lane 3, *Sau*96I (−57); Lane 4, *Ava*I (+45); Lane 5, *Tha*I (+129); Lane 6, *Mbo*II (+182). The number in parentheses is the position of the DNA end relative to the nicking site.

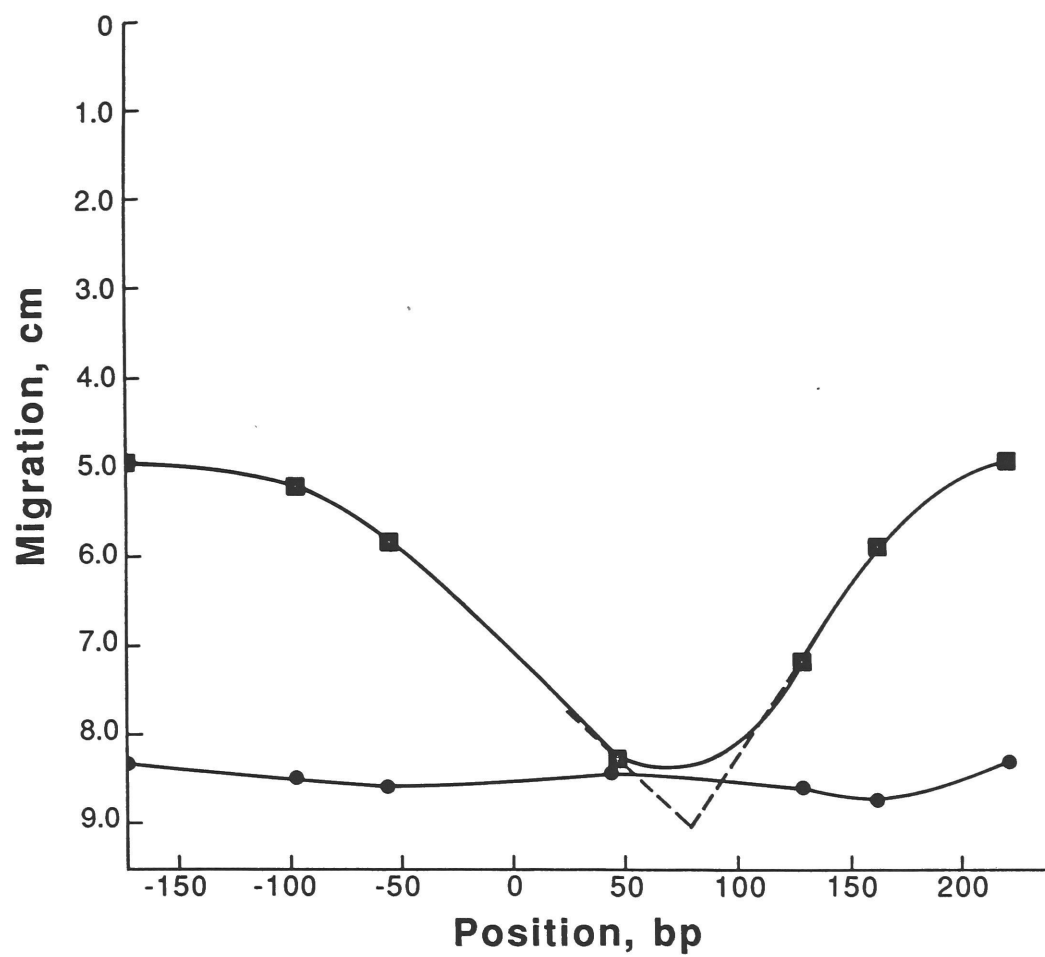
**1 2 3 4 5 6**







Figure 7.2(b). Mobility of the IHF-origin complex. A plot of mobility (cm) versus position of the DNA fragment end (bp) is shown for the set of circularly permuted origin fragments (circles), and the IHF-origin complex (squares). The figure also shows the extrapolation of the bend center for the complex.





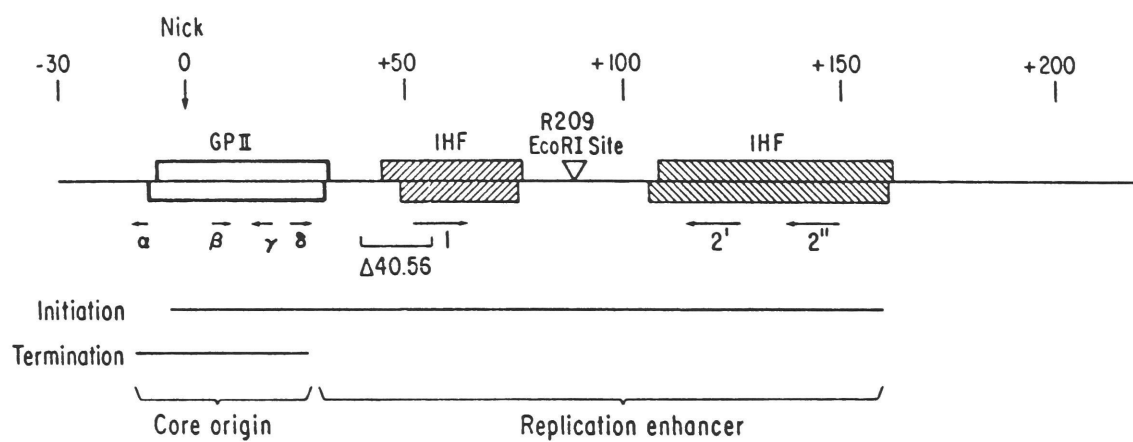


binding site for a replication protein other than IHF, or because it alters the phasing within the replication enhancer sequence. If the latter hypothesis is correct, then restoring the phasing within the replication enhancer sequence should improve the growth of the phage. Table 7.1 lists the phage that were constructed to test this hypothesis. The phage were constructed by making further insertions into the *EcoRI* site of R209 (see Materials and Methods). As shown in Table 7.1, restoration of the phasing results in formation of clear plaques. Figure 7.3(b) shows growth curves in liquid culture for the set of insertion phages. There is a strict correlation between the insertion and the growth characteristics. The wild-type phage, and the phage with 10 bp and 22 bp inserted between the strong and weak IHF binding sites, grow the best. The helical repeat of DNA in solution is 10.4 bp/turn for B-DNA (Wang, 1979). Therefore, insertion of integral numbers of helical turns (+10 bp, and +22 bp are close to one and two helical turns, respectively) restores optimal growth. Insertion of approximately half-integral numbers of helical turns (+4 bp and +16 bp) depresses the phage growth rate. Insertion of 8 bp ( $3/4$  turn) gives an intermediate result. These results indicate that the correct phasing within the replication enhancer is essential to its function and suggests that higher order DNA-protein structures play a functional role in f1 DNA replication.

## Discussion

The binding experiments presented in Chapter 3 demonstrated that the nicking site is protected regardless of its sequence, requiring instead the downstream repeat ( $\delta$ ) for its protection. In this chapter, I provide a possible basis for this observation. I show that gpII bends the origin upon binding, and that complex II is approximately twice as bent as complex I. Thus protein-directed DNA bending could facilitate a direct protein-protein interaction between monomer I and monomer IV, resulting in protection of the nicking site. Direct confirmation of this model awaits biochemical evidence characterizing the precise nature of the multiple protein-protein interactions.

Figure 7.3. Phasing within the replication enhancer. (a). Structure of the f1 origin. The origin DNA is shown as a horizontal bar. The minimal origin sequence and replication enhancer sequence are shown (described in text). A vertical arrow (designated 0) marks the site of gpII nicking. DNA replication begins at the nicking site and goes rightward by a rolling-circle mechanism. The sequence requirements for initiation and termination of DNA replication are shown by horizontal lines beneath the origin DNA. The horizontal arrows  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  are repeated sequences in the minimal origin. The IHF binding sites (site 1 and site 2) are shown, as are the IHF binding elements, 1, 2', and 2''. The region strongly protected by IHF (site 1) is shown by bars above and below the origin DNA, representing the protection of the plus and minus strands, respectively. The region weakly protected by IHF (site 2) is shown by a rightward hatched bar. The region in the core origin protected by gpII in complex II is shown by open bars. The site of insertion in R209 is marked by an *EcoRI* site.



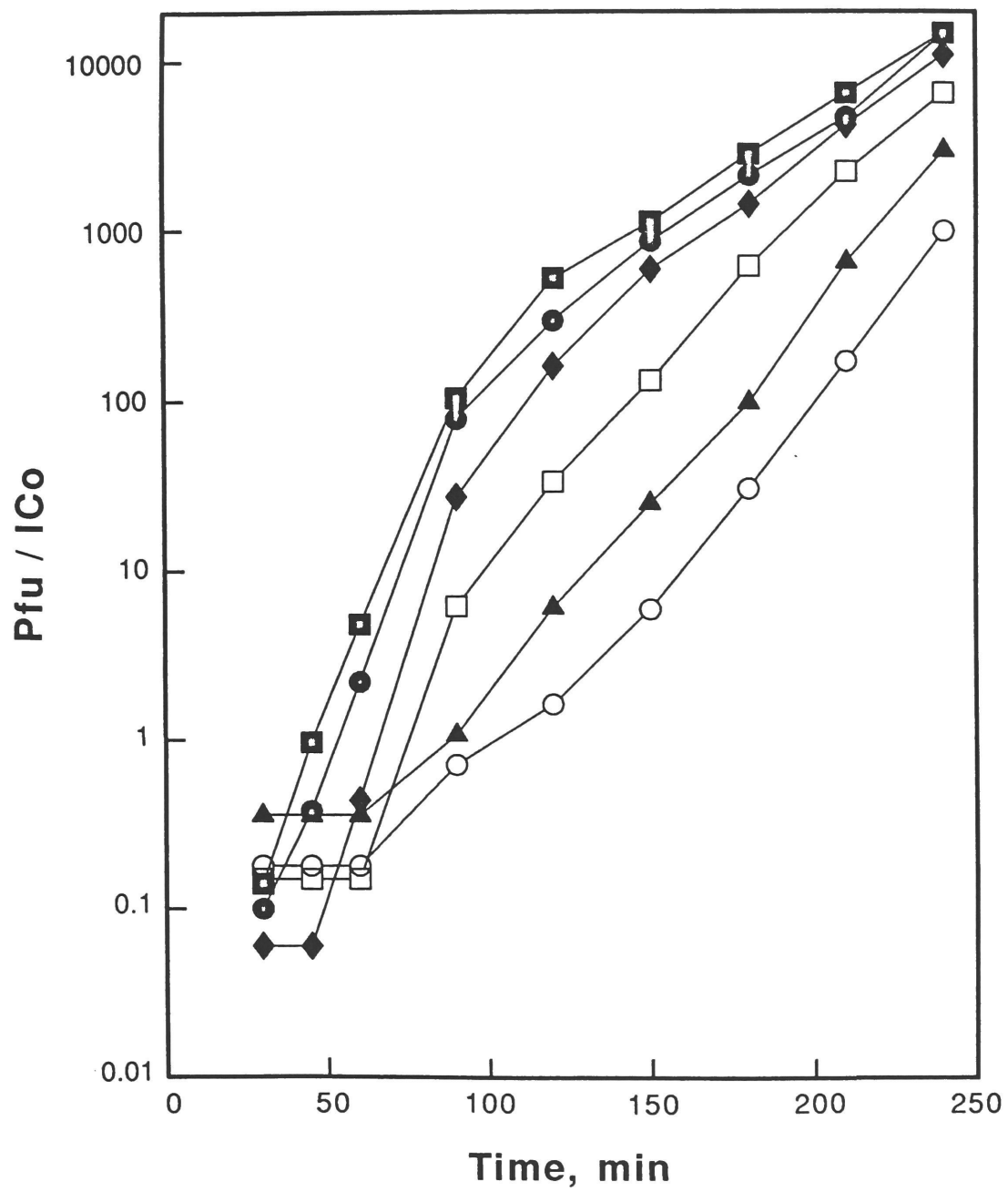
**Table 7.1. Insertions Within the Replication Enhancer**

Phage	Sequence	Plaque Morphology
f1	GGCC	clear
R <sup>+4</sup>	GGAATTCC	turbid
R <sup>+8</sup>	GGAATTAATTCC	intermediate
R <sup>+10</sup>	GGAATTGCAATTCC	clear
R <sup>+16</sup>	GGAATTGCAATTGCAATTCC	turbid
R <sup>+22</sup>	GGAATTGCAATTGCAATTGCAATTCC	intermediate

Sequence of the insertion phages. The sequence of the *Hae*III G/D border region in the replication enhancer is shown for the set of insertion phages. Their plaque morphology is listed to the right. The intermediate designation (clear to turbid) is used to indicate that the R<sup>+8</sup> and R<sup>+22</sup> plaques can be distinguished from the R<sup>+4</sup> (R209) and R<sup>+22</sup> plaques.



Figure 7.3(b). Growth of insertion phages. The growth of the series of insertion phages was analyzed after transfection of female ( $F^-$ ) *E. coli*. as described in Materials and Methods. The graph shows the plaque forming units (pfu) per infected center at time zero (pfu/ICo) at various times after transfection. The phage are f1 (filled squares),  $R^{+10}$  (filled circles),  $R^{+22}$  (diamonds),  $R^{+8}$  (open squares), R209 ( $R^{+4}$ --triangles), and  $R^{+16}$  (open circles).







I propose that initiation of f1 DNA replication involves bent DNA-protein structures. GpII bends the minimal origin sequence and IHF bends the replication enhancer sequence. The precise nature of the combined bent complex will require further structural analysis. The observation that the growth of f1 is sensitive to the phasing within the replication enhancer, strongly suggests that a higher order structure plays a functional role in f1 DNA replication. It is noteworthy that the site of linker insertion changes the phasing between the two IHF binding sites. Perhaps it is the correct phasing between the two IHF binding sites per se that is crucial to the function of the replication enhancer sequence. Regardless of the functional elements involved, there is a strict relationship between phage growth and helical periodicity within the replication enhancer sequence. Interestingly, the insertions in the replication enhancer primarily affect the initial growth rates. The slopes of the growth curves are similar at late times (3 hours). This supports the notion that IHF and the replication enhancer sequence are crucial for establishing the infection by promoting the initial rounds of replication.

In addition to protein-directed DNA bending, the f1 origin has DNA sequence elements that confer sequence-directed DNA bending. These kinetoplast-type elements are located within the replication enhancer sequence and the minus-strand origin. These elements result in abnormal migration of the origin-containing restriction fragment on polyacrylamide gels (K. Horiuchi, unpublished data). However, the f1 origin is not highly bent in a sequence-dependent manner because the kinetoplast-type elements are not all in phase with the helix screw. Whether these kinetoplast-type elements play a functional role remains to be determined.



## Chapter 8

### **An Enhancer-Independent Mutation Increases the Cooperativity of the Initiator Protein-Origin Interaction**



The mutation mp1 (met<sup>40</sup>→ile) in gene II protein suppresses both the lack of the replication enhancer and the lack of IHF (Dotto & Zinder, 1984b; Chapter 6). Since this mutation suppresses large deletions in the enhancer (Dotto & Zinder, 1984b; K. Horiuchi, unpublished results), I thought it most likely that the altered protein still acted through its normal binding site, the core origin. Consequently, I set out to characterize the effect of this activating mutation on the binding of gene II protein to the origin *in vitro*. In this chapter, I report that the mp1 mutation increases the cooperativity of the gene II protein-origin interaction. Probably, this site in the N-terminal region of gene II protein mediates protein-protein interactions that affect cooperative binding.

### Binding of the mp1 gene II protein to the origin

In order to characterize the effect of the mp1 mutation on the binding of the gene II protein to the origin, I purified the wild-type protein (gpII) and the mp1 mutant version (gpII<sup>mp1</sup>) in parallel, using a modification of the isolation procedure of Meyer & Geider (see Materials & Methods), and compared the interaction of both proteins with the origin using gel retardation analysis. Figure 8.1(a) shows the results of this analysis. When gpII is added to the origin, both complexes form, with complex I being predominant at low gpII concentrations, and complex II being predominant at higher gpII concentrations (as presented in Chapter 3). In contrast, gpII<sup>mp1</sup> forms complex II at a lower protein concentration than the wild-type protein, and the level of complex I is lower than that produced by equal amounts of the wild-type protein. The binding experiment shown in Figure 8.1(a) was quantitated by measuring the radioactivity in each band and the results are shown in Figure 8.1(b). The mp1 mutation alters the distribution between complex I and complex II. The binding curve for formation of complex II by gpII<sup>mp1</sup> is highly S-shaped, which is suggestive of an increased cooperativity of binding.

In order to quantitatively compare the cooperativity of gpII<sup>mp1</sup> with gpII, the binding



data from Figure 8.1(a) were analyzed using the Hill equation (discussed by Fersht, 1977). According to this formulation, if  $\log[Y/(1-Y)]$ , where  $Y$  is the degree of saturation of complex II, is plotted against the logarithm of the free gpII concentration ( $\log[\text{gpII}]_{\text{free}}$ ), then the slope of the line in the range of 50% saturation (Hill constant) is a measure of cooperativity. Hill constants ( $h$ ) that are greater than 1 are indicative of positive cooperativity. Figure 8.1(c) shows Hill plots for both proteins. Analysis of the data by linear regression indicates that  $h=1.9$  for gpII, and  $h=3.5$  for gpII<sup>mp1</sup>. This leads to the conclusion that the mp1 mutation increases the cooperativity with which the protein interacts with the origin to form complex II.

The binding of gpII<sup>mp1</sup> to the origin was also analyzed by DNase I footprinting. The results (shown in Figure 8.2) indicate that gpII<sup>mp1</sup> binds to the wild-type origin and gives the same 40 bp protection pattern produced by gpII. Both gpII and gpII<sup>mp1</sup> gave footprints over the same range of protein concentrations. Perhaps the difference between the gel retardation results (Figure 8.1) and the DNase I footprinting results (Figure 8.2) is that greater amounts of gene II protein are needed to produce a footprint than to form a bound complex. As a consequence of the cooperative binding, the footprints have an "all-or-none" character (see Chapter 3).

### **Binding of the mp1 gene II protein to the core binding sequence**

The above experiments indicated that gpII<sup>mp1</sup> is more active than gpII at forming complex II. One possibility is that the mutation increases the affinity of the protein for the repeat  $\delta$ , thus promoting formation of complex II. Another possibility is that the mutation strengthens a protein-protein interaction that participates in the formation of complex II. To attempt to discriminate between these possibilities, I decided to analyze the interaction of gpII<sup>mp1</sup> with a restriction fragment containing the core binding sequence (repeat  $\beta$  and  $\gamma$  in inverted orientation). The results (shown in Figure 8.3) indicate that gpII<sup>mp1</sup> can form both complex I and complex II with the core binding

Figure 8.1. Interaction of the mp1 gene II protein with the origin. (a). Gel retardation analysis of the gene II protein-origin interaction. Binding of the wild-type (gpII) and mp1 mutant (met<sup>40</sup>→ile) gene II proteins to an origin-containing restriction fragment (315 bp *AsuI*-*ClaI* fragment) was analyzed according to the method of Fried & Crothers (1981), as described in Chapter 3 and Materials & Methods. Each lane contained 1 fmol of origin-containing restriction fragment and the indicated amount (fmol) of the wild-type or mutant gene II protein, as indicated.



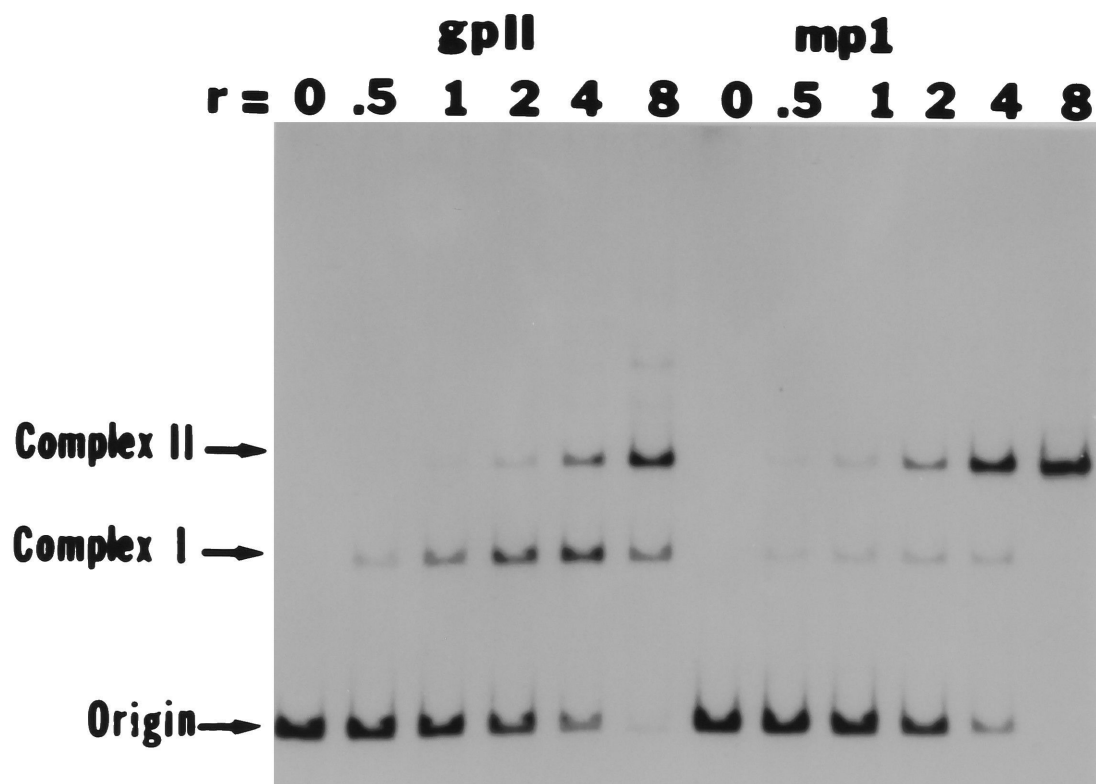






Figure 8.1(b). Quantitation of origin binding. The binding data shown in (a) were quantitated by excising the radioactive bands and measuring their radioactivity by liquid scintillation counting. The ordinate represents the percent of DNA found in each complex. The radioactivity in complex II and complex I are designated by the open and closed symbols, respectively. The square symbols show the binding data for the wild-type protein, and the triangular symbols show the data for the mp1 protein.

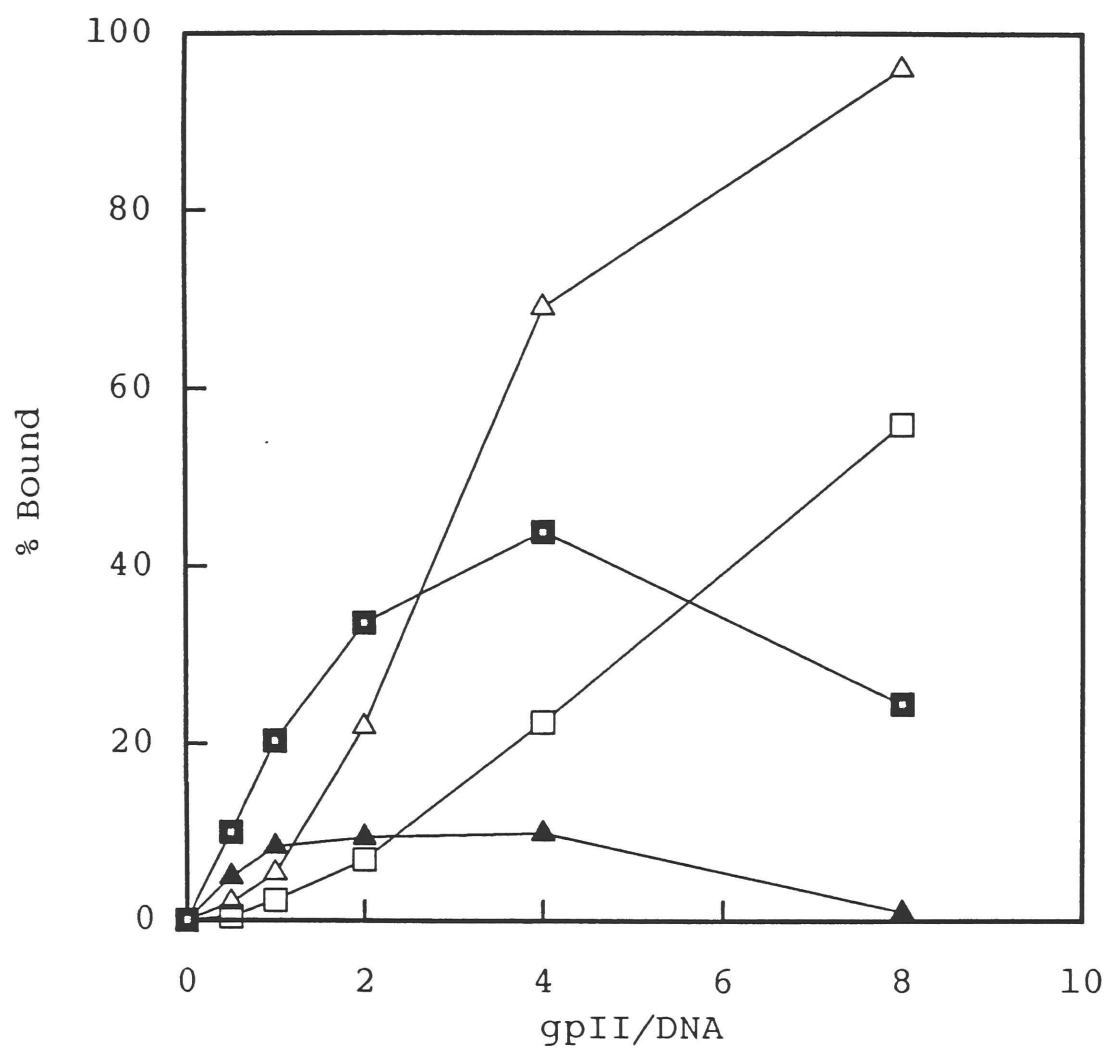
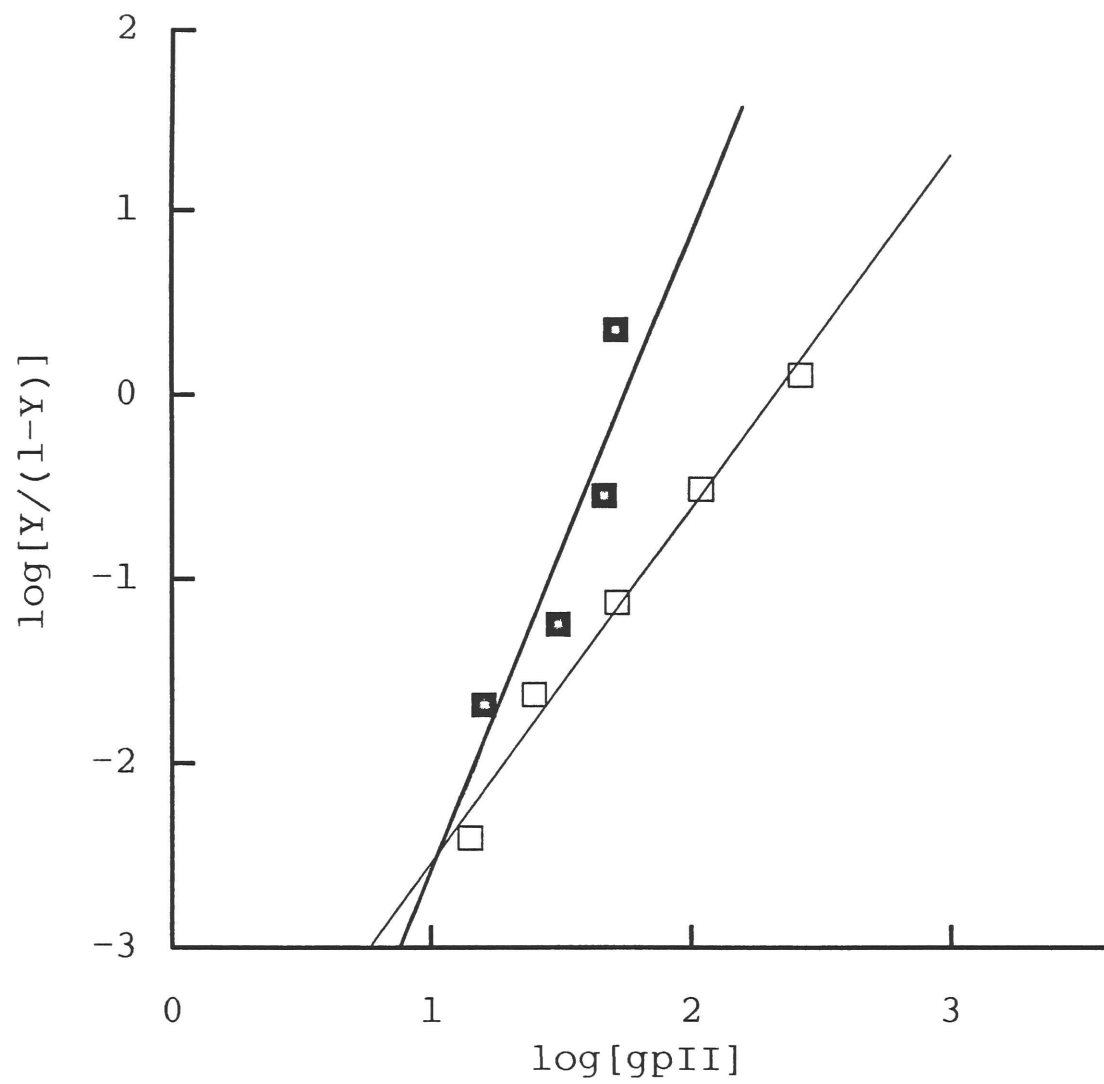


Figure 8.1(c). Hill plots for the binding of gpII<sup>mp1</sup> and gpII to the origin. The binding data from Figure 8.1(a) were analyzed using the Hill equation:

$$\log[Y/(1-Y)] = h \log[gpII]_{\text{free}} - \log K_{\text{IID}}$$

where Y is the fraction of DNA molecules that formed complex II,  $K_{\text{IID}}$  is the dissociation constant for complex II, and h is the Hill constant or cooperativity constant.  $\log[Y/(1-Y)]$  was plotted against  $\log[gpII]_{\text{free}}$  for gpII (open squares) and gpII<sup>mp1</sup> (closed squares). Only data with  $Y < 0.9$  were included. Lines were fit to the scatter points by linear regression.







sequence, in contrast to gpII which forms complex I only. Surprisingly, the binding of gpII<sup>mp1</sup> to the core binding sequence resembles the binding of gpII to the wild-type origin: complex I forms at low gene II protein concentrations, and complex II forms predominantly at a higher gene II protein concentration. Therefore, gpII<sup>mp1</sup> can form complex II independent of repeat  $\delta$ , and the mutation mp1 would appear to alter a protein-protein interaction.

The binding of gpII<sup>mp1</sup> to the core binding sequence was also analyzed by DNase I footprinting (shown in Figure 8.4(a)). GpII binds to the core binding sequence to give the 26 bp protection pattern described in Chapter 3 and summarized in Figure 8.4(b). In contrast, gpII<sup>mp1</sup> binds to the core binding sequence and protects 38 bp of the plus strand (Figure 8.4(a), lane 3) and 33 bp of the minus strand (Figure 8.4(a), lane 6). The protection pattern of gpII<sup>mp1</sup> bound to the core binding sequence (summarized in Figure 8.4(b)) resembles the protection pattern of the mutant  $\Delta+29$  bound to gpII (see Figure 3.3). The sequence that would correspond to the nicking site is protected, consistent with the observation (Figure 8.3) that gpII<sup>mp1</sup> can form complex II with the core binding sequence.

### Discussion

In this chapter, I have analyzed the binding of an enhancer-independent gene II protein mutant, mp1 (met<sup>40</sup>→ile), to the origin. Both the wild-type and mp1 mutant gene II proteins bind to the core origin sequence (Figure 8.2), forming two complexes that are separable by polyacrylamide gel electrophoresis (Figure 8.1(a)). The mp1 mutant gene II protein produces a greater amount of complex II relative to complex I than does the wild-type protein (Figure 8.1). Quantitation of the percentage of the DNA bound as complex II as a function of the mp1 gene II protein concentration, yields a curve with a pronounced S-shape (Figure 8.1(b)). The shape of the binding curve suggests that the mp1 mutation increases the cooperativity of the gene II protein origin interaction.

Figure 8.2. DNase I protection of the origin by the mp1 gene II protein. DNase I protection experiments were performed (see Materials & Methods and Chapter 3 for details) using the 315 bp *AsuI*-*ClaI* origin-containing restriction fragment (1.6 fmol) labelled on the minus strand by endfilling. Panels 1 and 2 show the results for gpII<sup>mp1</sup> and gpII, respectively. Lanes a-f contained gene II protein (or mp1 mutant protein) and origin DNA at a molar ratio (protein/origin) of 0, 32, 16, 8, 4, and 2, respectively. Maxam-Gilbert sequencing reactions served as markers and are not shown.

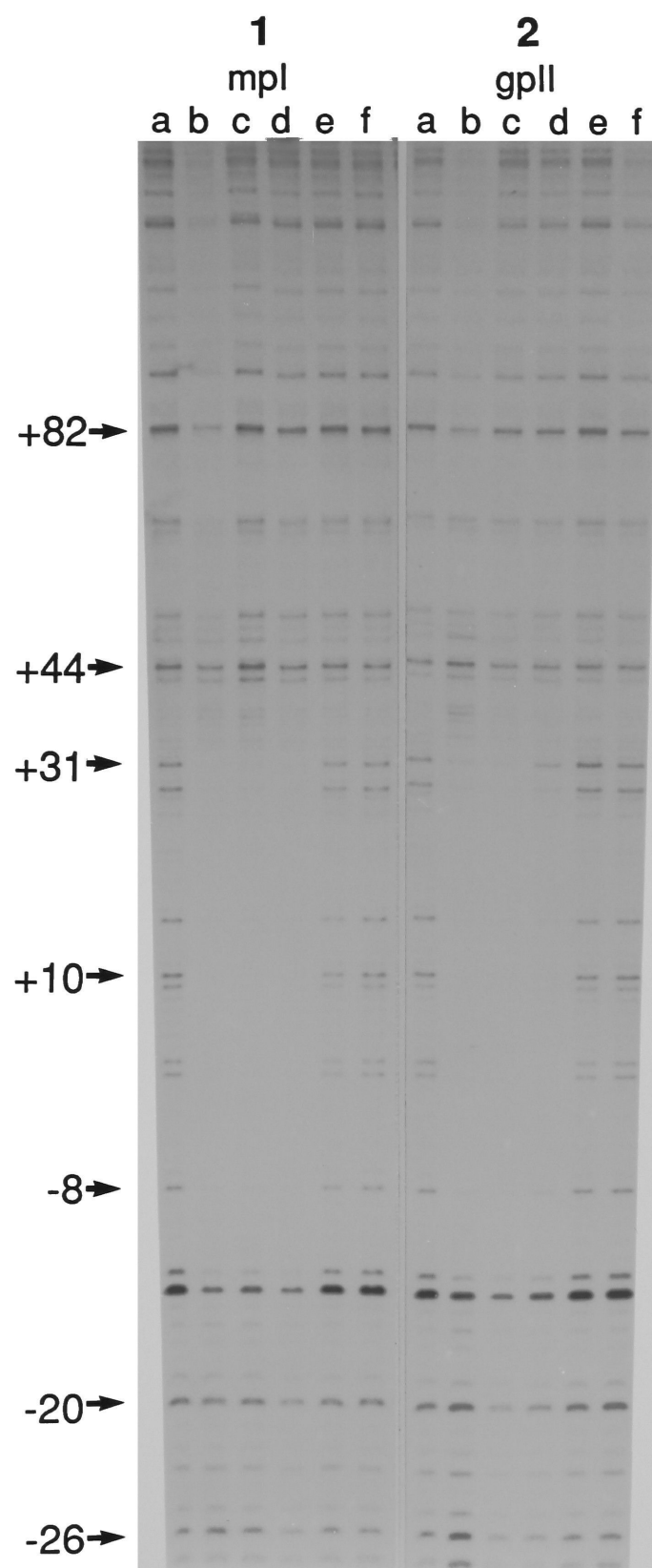
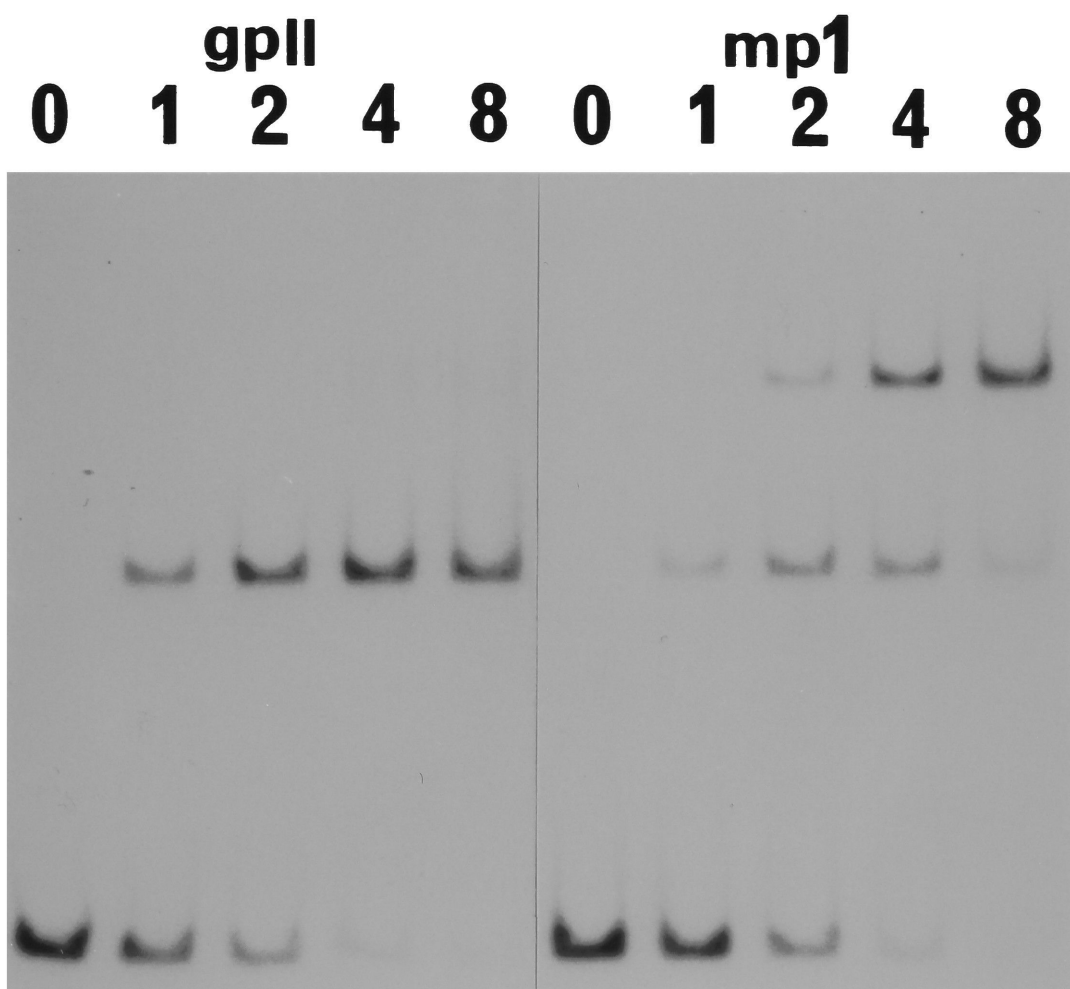






Figure 8.3. Interaction of the mp1 gene II protein with the core binding sequence. The binding of the wild-type and mp1 mutant proteins to the 208 bp *Bst*NI restriction fragment (5 fmol) from pMBS1, containing the core binding sequence (repeats  $\beta$  and  $\gamma$  in inverted orientation), was analyzed by gel retardation. Each reaction contained the indicated molar ratio of gene II protein (or mp1 mutant protein) to restriction fragment.







Quantitative analysis of the binding cooperativity using Hill plots (Figure 8.1(c)) indicates that the Hill constant for gpII<sup>mp1</sup> ( $h=3.5$ ) is significantly greater than that of gpII ( $h=1.9$ ). Therefore, the mp1 mutation increases the cooperativity with which complex II is formed. Since the complexes formed by the mp1 gene II protein comigrate with those formed by the wild-type protein (Figure 8.1(a)), both proteins are likely to bend the DNA to the same degree.

Analysis of the binding of gpII<sup>mp1</sup> to a fragment containing the core binding sequence (repeat  $\beta$  and  $\gamma$  in inverted orientation) indicates that the mutant protein can efficiently form complex II with a substrate containing only the inverted repeat, in contrast to the wild-type protein which forms complex I. Since two molecules of gene II protein bind to the core binding sequence, making symmetric contacts as identified by methylation interference experiments (Chapter 3), the additional binding by gpII<sup>mp1</sup> to form complex II must depend heavily on protein-protein interactions. DNase I footprinting experiments (Figure 8.4) indicate that gpII<sup>mp1</sup> protects an additional 8 bp of sequence, corresponding to the position of the nicking site, which is consistent with its ability to form complex II. It is unlikely that complex II formed on the core binding sequence by gpII<sup>mp1</sup> is functional, because when the nicking site sequence is restored in its proper location in front of the inverted repeat  $\beta$ - $\gamma$  (in the plasmid pMBS2), the mutant protein is still unable to nick the origin (data not shown). Therefore, in spite of the fact that gpII<sup>mp1</sup> can form complex II with just the core binding sequence, the repeat  $\delta$  is still needed for the proper positioning to allow nicking at the origin.

How does the mp1 mutation overcome the requirement of both the replication enhancer and IHF for optimum growth of the phage? A simple possibility is that by increasing the cooperativity of the gene II protein-origin interaction, the mp1 mutation favors formation of the functional complex, complex II, thereby compensating for the absence of the replication enhancer. This model would fit well with the observation that increased levels of gpII can compensate for deletions within the replication enhancer

Figure 8.4. DNase I protection of the core binding sequence by the mp1 gene II protein.

(a). DNase I protection experiments were performed using the 249 bp *EcoRI-PvuII* restriction fragment (approximately 5 fmol) containing the core binding sequence. The fragment was labeled on the plus strand in panel 1 and the minus strand in panel 2. Lanes a contained no gene II protein, lanes b contained 5 ng of gene II protein, and lanes c contained 5 ng of mp1 gene II protein. Maxam-Gilbert sequencing reactions of the respective fragments served as markers and are not shown.

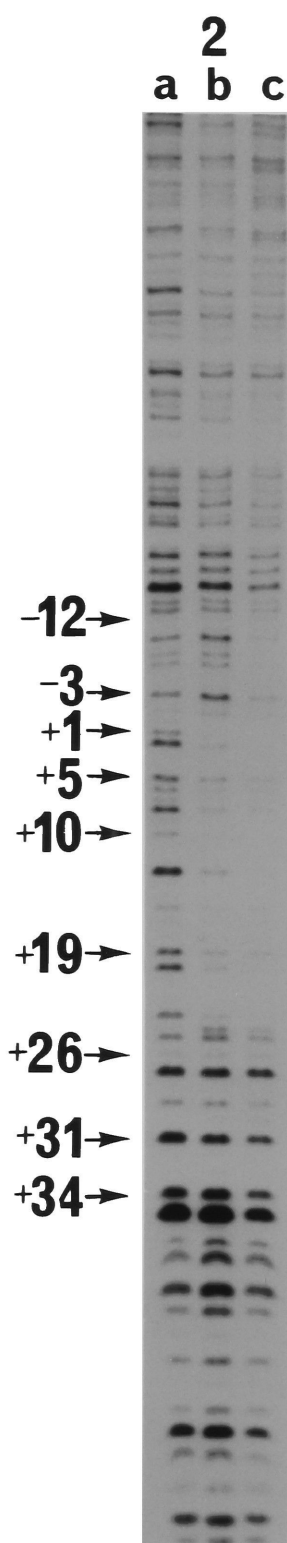
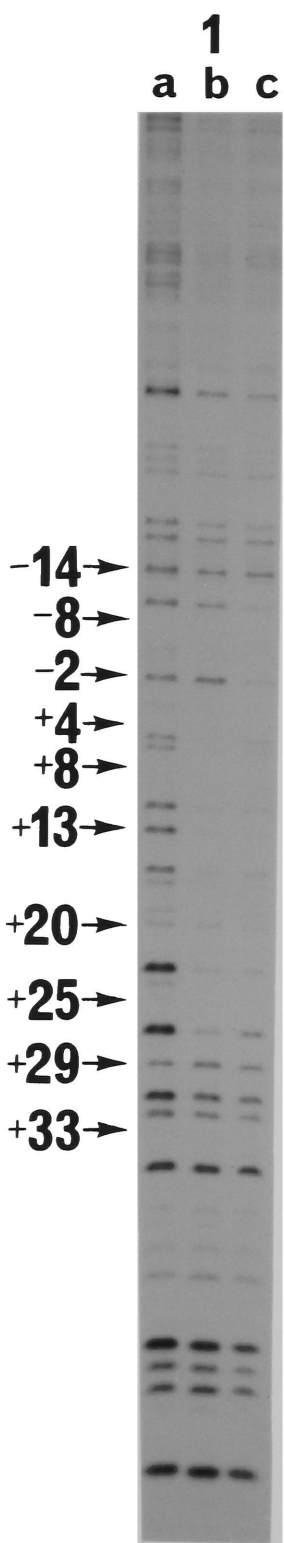






Figure 8.4(b). Summary of footprinting results. The nucleotide sequence of the restriction fragment containing the core binding sequence is shown. The region protected by gene II protein and the mp1 protein is underlined. The additional sequence protected only by the mp1 protein (corresponding to the location of the nicking site) is indicated by brackets. The repeats  $\beta$  and  $\gamma$  are indicated.

-10                  +1                  +10                  +20                  +30  
|                    |                    |                    |                    |

5'-atgacCatgaTTacgccAagcTGGACTCTTGTTCCAgctTGcAtgcctgCagggtGactctagaGgagggg-3'  
3'-tactgGtactAAtgcgGTtcgACCTGAGAACAAAGGTcgaACgTacggacGtccaGctgagatctCctcccc-5'

$\beta$                       ←  $\gamma$





(Dotto & Zinder, 1984a). Increasing either the cooperativity of the interaction, or the amount of gene II protein, should favor the formation of complex II *in vivo*. Recent evidence (K. Horiuchi, unpublished data, discussed in Chapter 6) suggests that increased levels of gene II protein are not as efficient as the coding changes in gene II (mp1 and codon 73 mutations) at overcoming the absence of the replication enhancer. The data shown in Figure 8.1(b) lead me to suspect that a change in binding cooperativity would be more potent than a change in the amount of gene II protein, because the binding curve (formation of complex II) for the mp1 protein lies above the one for the wild-type protein at all points. However, I cannot rule out the possibility that the mp1 mutation has an additional consequence besides increasing the cooperativity of binding to the origin.



## Chapter 9

## **Conclusion**



In this thesis, I have analyzed in detail the interaction of two DNA-binding proteins with the plus-strand replication origin of bacteriophage f1. The plus-strand replication origin has a bipartite structure consisting of a required core origin region and an adjacent A+T-rich enhancer sequence that potentiates replication approximately 100-fold. The function of each of these two elements depends on its interaction with a DNA-binding protein: the core origin contains a complex binding site for the initiator protein; the enhancer contains three binding sites for IHF. Both activator proteins bend the DNA upon binding to their recognition sequences, implying that together they wrap the origin DNA into a higher order structure that is active in initiation. This study was undertaken with the dual goals of better understanding the replication of bacteriophage f1 and gaining insight into the molecular mechanisms operative in the activation of DNA replication by an A+T-rich enhancer sequence. Since this division of replication origin into core and enhancer is shared by a number of replication origins from prokaryotes and eukaryotes, the underlying molecular mechanisms controlling function might be similar. Here, I briefly recapitulate some of the major findings of this thesis and speculate on future directions for this work.

### **Initiator protein-origin interaction**

The gene II protein binds to the core origin in a novel two-step fashion. The first binding step involves interaction with an inverted repeat ( $\beta$ - $\gamma$ ) at the center of the core origin. Two molecules of gene II protein bind in the first step to form complex I by making symmetric contacts with the inverted repeat as identified by methylation interference experiments. The second binding step involves addition of two protein molecules to complex I. Of these two protein molecules, one binds to and makes symmetric contacts with repeat  $\delta$  as identified by methylation interference experiments, and the other gene II protein molecule protects the nicking site and binds in a sequence-



independent fashion. Binding experiments performed on a series of defective origins established that the downstream repeat  $\delta$  is critical for gene II protein to bind to protect the upstream nicking site. Possibly, the gene II protein molecule bound to repeat  $\delta$  influences protection of the nicking site by transmitting protein-protein interactions via the neighboring gene II protein molecules. A more elegant possibility is that the gene II protein-induced bending of the origin and the concomitant DNA looping permits a direct interaction between the gene II protein molecule bound to repeat  $\delta$  and the one that protects the nicking site.

Gene II protein exists as a monomer in solution (Meyer & Geider, 1979a), so the protein-protein interactions might occur on the DNA or during binding. The simplest explanation is that the orientation of the binding repeats determines the disposition of the individual gene II protein molecules on the DNA. Therefore, there must be at least two different sets of dimer contacts because repeats  $\beta$  and  $\gamma$  are inverted head to head, whereas repeats  $\gamma$  and  $\delta$  are inverted tail to tail. A major unanswered question concerns the nature of the inferred protein-protein interactions. One goal for future studies would be to identify the domains of the gene II protein mediating the various interactions. One clue comes from the analysis of the enhancer-independent mutation (mp1) in gene II protein. This mutation of a methionine to an isoleucine in the N-terminal region of gene II protein increases the cooperativity with which the protein binds to the origin by strengthening protein-protein interactions that promote formation of complex II.

Gene II protein plays several key roles in plus-strand DNA replication. It binds to the replication origin (Horiuchi, 1986) and introduces a specific nick on the plus strand (Meyer *et al.*, 1979) that serves as the primer for plus-strand rolling-circle-type replication. Gene II protein promotes the establishment of the replication fork by initiating unwinding by the *E. coli* Rep helicase (Geider *et al.*, 1982). Following a round of replication, gene II protein functions in the reaction called termination by cleaving and circularizing the product single strand (Harth *et al.*, 1981). The genetic selection presented in





the Appendix should allow rapid isolation of gene II protein mutants. Characterization of the *in vitro* and *in vivo* properties of different mutants might enable the separation of the protein's various functions.

### Enhancement of DNA replication by IHF

A major finding of this thesis is that IHF activates f1 DNA replication by binding to three sites within the replication enhancer. The involvement of IHF in the intracellular growth of bacteriophage f1 was previously unsuspected. The growth defect of f1 in IHF mutants was shown to be at the level of DNA replication because IHF mutants do not support the replication of plasmids that depend on the f1 replicon. This growth defect of f1 in IHF mutants is suppressed by an initiator mutation (*mp1*) that also suppresses the effect of insertions and deletions within the replication enhancer, a *cis*-acting sequence whose only known role is to potentiate plus-strand DNA replication, implying that the enhancer is the genetic site of action of IHF. These *in vivo* findings are supported by the biochemical data that IHF binds to three sites within the replication enhancer. The exact molecular mechanism whereby IHF enhances f1 DNA replication was not directly addressed in this thesis and will no doubt serve as an important area for further work. IHF functions in a number of processes that occur in *E. coli* (see Table 1.2). IHF appears to function in each of these processes by binding to specific DNA sequences and bending them. Perhaps, protein-induced DNA bending *per se* is enough to account for the action of IHF in f1 DNA replication. It has recently been suggested that DNA bending could provide energy for the unwinding of the two DNA strands (Ramstein & Lavery, 1988), a necessary requirement for the establishment of the replication fork. If this were the case, then it might be possible to substitute the *E. coli* CAP protein (another DNA-binding/bending protein) for IHF, if the CAP binding sites were properly positioned within the replication enhancer sequence. Another possibility (similar to that suggested by Stenzel *et al.*, 1987, for the function of IHF in the replication of



pSC101) is that another protein is involved in f1 DNA replication, and that IHF, by bending the DNA, facilitates this protein's interaction with the gene II protein. Further experiments will be needed to elucidate the molecular mechanism of action of IHF in f1 DNA replication.

### **Higher order structures in f1 DNA replication**

The involvement of IHF in phage  $\lambda$  site-specific recombination, taken together with the observation that both gene II protein and IHF bend the replication origin, suggests that a higher order nucleoprotein structure mediates the initiation of DNA replication at the f1 origin. Support for this notion comes from the demonstration that insertion of half-integral numbers of turns between the strong and weak IHF binding sites is deleterious to phage growth, whereas insertion of integral numbers of turns is innocuous. Perhaps these half-integral-turn insertions adversely affect DNA replication by changing the relative orientations of the multiple protein-induced DNA bends. The involvement of a higher order structure is largely inferred, and no structural data is presented in this thesis concerning its nature. Electron microscopy of preinitiation complexes might provide information on the details of the higher order structure inferred to play a role in the initiation of f1 DNA replication.



## Appendix

### **Genetic Selection for Origin and Gene II Protein Mutants**



In this appendix, I briefly describe a simple plasmid-based genetic system for the selection of origin and gene II protein mutants. Although these results are preliminary, I believe they provide a good starting point for the isolation and characterization of new gene II protein and origin mutations. Severe origin and gene II protein mutants cannot be isolated directly from the phage because they are lethal. The genetic system described here permits the positive selection of these severe origin and gene II protein mutants. This genetic system permits selection both for and against origin function, so that loss-of-function mutants and revertants can be isolated. The principle behind this genetic selection is that plasmids that carry an f1 origin (in addition to a pBR origin) and gene II protein under control of the *tacI* promoter (e.g., pDG117IIA and pTII0+), kill the host upon induction (with IPTG), presumably because overreplication of the plasmid is lethal. Since f1 replicon plasmids can transform *pol A*-deficient bacteria, revertants (or pseudorevertants) of defective origins or gene II protein mutants may be obtained.

### Positive selection for origin and gene II protein mutants

In the course of studying the overproduction of gene II protein from the plasmid pDG117IIA (shown in Figure A-1.1 and described in Materials and Methods), I noticed that induction was lethal to the host. The plasmid pDG117IIA can only transform *lacI<sup>Q</sup>* bacteria (e.g., K561) under noninducing conditions (no added IPTG). The efficiency of plating of K561 bearing pDG117IIA on media containing IPTG was  $\simeq 10^{-4}$ . Surviving colonies contained plasmids that had undergone rearrangements (mostly deletions). Figure A-1.2 shows a growth curve of K561 bearing pDG117IIA in minimal medium. Upon induction with IPTG (2 mM), the cells undergo one division and then stop dividing. This lethal effect of induction was dependent on replication in the f1-mode, because induction was not lethal to K1019 (K561 *rep-71*), which cannot support the growth of f1

Figure A-1.1. Structure of pDG117IIA. The structure of pDG117IIA is shown. The construction of pDG117IIA is detailed in Materials and Methods. The checkered region (f1ori) shows the 151 bp  $A^+B^-$  f1 origin. The arrow beneath the f1 origin shows the direction of rolling-circle type replication. The black bar show the region of the plasmid coding for gene II protein under control of the *tacI* promoter, with the arrow in the direction of transcription. The open bar shows the gene for ampicillin resistance, and the stippled bar indicates the pBR origin.



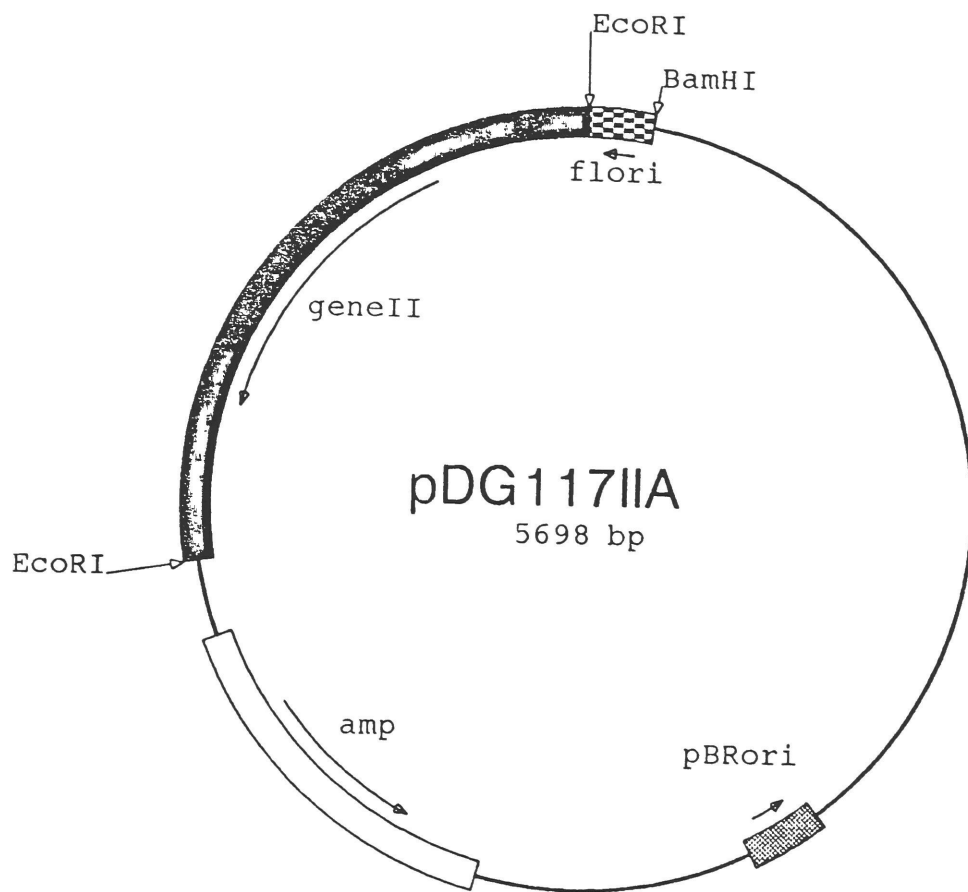
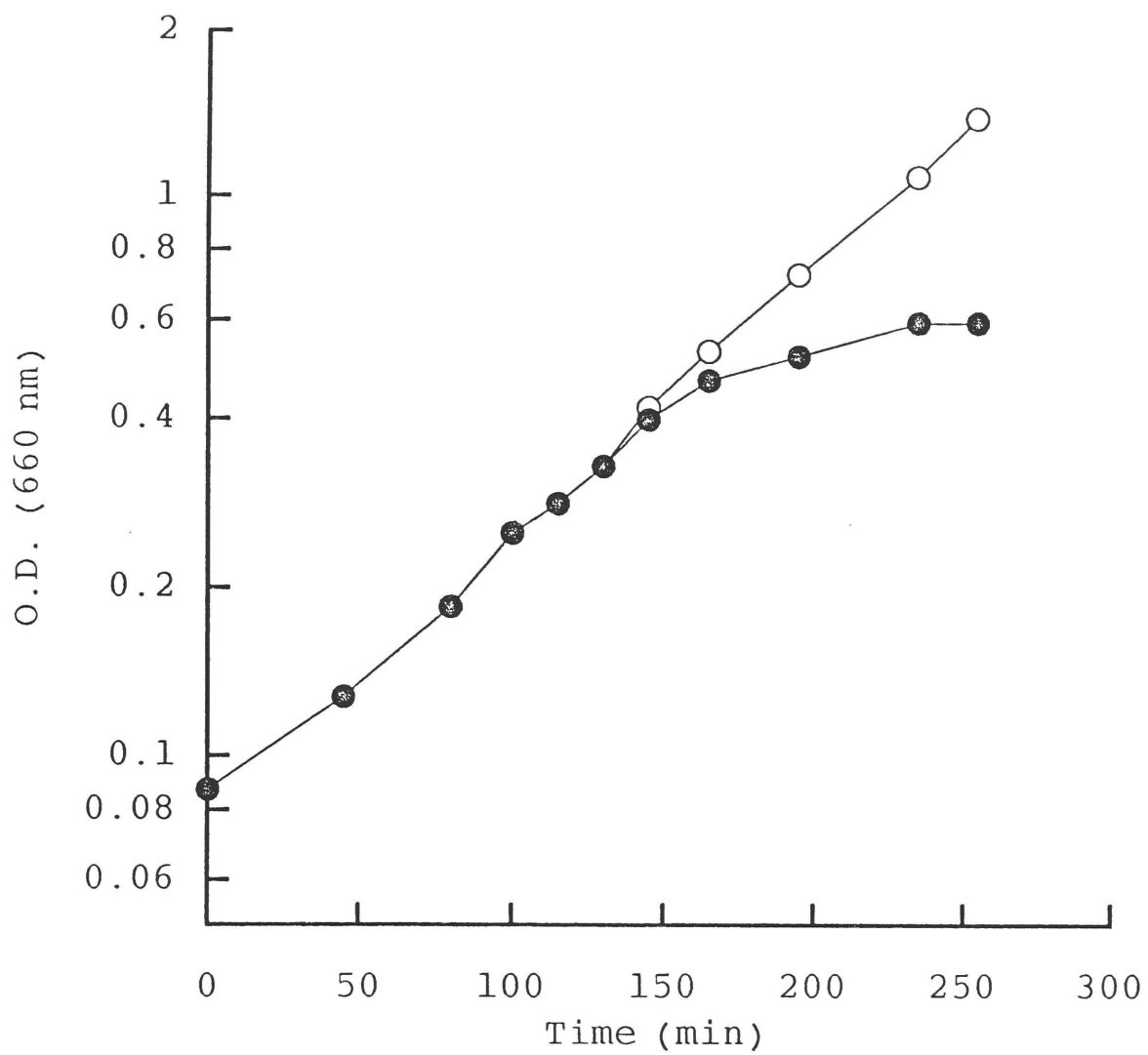


Figure A-1.2. Growth of K561 bearing pDG117IIA. Two identical 100 ml cultures of K561 bearing pDG117IIA were grown in D.O. minimal medium containing casamino acids (0.4%) and ampicillin (100  $\mu\text{g/ml}$ ). After 130 minutes of growth, IPTG (2 mM final concentration) was added to one culture (closed circles) and growth was allowed to continue. The figure plots the optical density (O.D.) at 660 nm during the course of growth for the uninduced culture (open circles) and the one induced at 130 minutes (closed circles).





because of a mutation in the Rep helicase. The lethal induction did not require the complete f1 origin because pDG117IIA contains an  $A^+B^-$  origin. The lethality was dependent on the particular plasmid construction used. Plasmid constructions that permitted transcription from the *tacI* promoter to enter the origin in the opposite direction of replication were not lethal. Presumably, transcription into the origin was inhibitory to replication. The lethality was also dependent on the use of the strong *tacI* promoter. Use of the *lac*<sup>uv5</sup> promoter was not lethal in the constructs employed.

To test whether this selection could be used to obtain origin and gene II protein mutants, the following pilot experiment was performed. A culture of K561 bearing pTIIO+ (Fulford, 1986--pTIIO+ was chosen because it contains a wild-type f1 origin in contrast to pDG117IIA which contains an  $A^+B^-$  origin) was mutagenized with nitrosoguanidine (Miller, 1972) and plasmid DNA was prepared. Mutagenized plasmid RFI was purified by preparative agarose gel electrophoresis, and used to transform K871 (K561*recA56*), and IPTG resistant mutants (IPTG<sup>r</sup>) were selected on minimal glycerol plates containing IPTG (2 mM) and ampicillin (100  $\mu$ g/ml). Plasmid DNA was isolated from the IPTG<sup>r</sup> mutants. The IPTG<sup>r</sup> mutations were shown to be plasmid linked by retransformation of K871 under inducing conditions.

The origin function of an initial set of IPTG<sup>r</sup> derivatives of pTIIO+ (pTIIO-1 to pTIIO-12) was tested by their ability to form ampicillin-resistant transducing particles when plasmid-containing cells were infected with f1. Table A-1.1 shows the results of this analysis. Two plasmids (pTIIO-5 and pTIIO-4) affect the formation of transducing particles relative to the phage yield. Cells harboring these two plasmids (individually) were able to plaque f1, indicating that the plasmid f1 origin was not interfering with the growth of the phage. The f1 origin region of these two plasmids was sequenced and found to contain the mutations indicated in Table A-1.1. The plasmid pTIIO-5 was found to have a deletion of 3 bp at the nicking site, which removed three T:A base pairs [ $\Delta$ [-3--10]]. The f1 origin in plasmid pTIIO-4 was found to result from a perfect tandem

Table A-1.1. Formation of Transducing Particles by IPTG<sup>r</sup>-Derivatives of pTIIO+

Plasmid	Amp <sup>r</sup> TP/ml	f1 pfu	TP/pfu	Mutation
pTIIO+	$6 \times 10^9$	$2.3 \times 10^{11}$	0.03	
pTIIO-1	$1.4 \times 10^7$	$2 \times 10^8$	0.07	N.D.
pTIIO-2	$3.6 \times 10^7$	$1 \times 10^9$	0.04	N.D.
pTIIO-3	$3.5 \times 10^9$	$2.8 \times 10^{10}$	0.13	N.D.
pTIIO-4	$2.5 \times 10^9$	$3.4 \times 10^{12}$	$7 \times 10^{-4}$	tandem duplication ( $\alpha\beta\gamma\beta\gamma\delta$ ) in the f1 origin
pTIIO-5	$2 \times 10^6$	$1.3 \times 10^{12}$	$2 \times 10^{-7}$	3 bp deletion [ $\Delta(-1--3)$ ] in the f1 origin
pTIIO-6	$2 \times 10^8$	$6.3 \times 10^9$	0.03	deletion in promoter--N-terminal region of gene II
pTIIO-7	$3 \times 10^7$	$1.2 \times 10^9$	0.02	N.D.
pTIIO-8	$4 \times 10^8$	$9.3 \times 10^9$	0.04	N.D.
pTIIO-9	$2 \times 10^8$	$1.6 \times 10^9$	0.13	N.D.
pTIIO-10	$9.3 \times 10^6$	$2 \times 10^8$	0.05	N.D.
pTIIO-11	$7.8 \times 10^6$	$7 \times 10^8$	0.01	N.D.
pTIIO-12	$3.7 \times 10^7$	$3 \times 10^8$	0.12	N.D.

As a screen for f1 origin function, IPTG<sup>r</sup>-derivatives of pTIIO+ were tested for their ability to form ampicillin-resistant transducing particles (Amp<sup>r</sup> TP), relative to their ability to interfere with the growth of superinfecting f1 phage. Individual 25 ml cultures of K871 bearing pTIIO+ or IPTG<sup>r</sup>-derivatives were grown in FB until a cell density of  $5 \times 10^7$  cells/ml, at which point f1 phage was added at an m.o.i. of 1, and growth was allowed to continue for 4.5 hours post-infection. The cells were then removed by centrifugation at 7,000 rpm for 10 minutes, and cells remaining in the supernatant were killed by heating at 65°C for 15 minutes. The supernatants were titered on K561 for Amp<sup>r</sup> TP (column 2) and for phage f1 (column 3 shows the f1 titer in pfu/ml). The fourth column of the table shows the number of transducing particles per phage (the values in column 2 divided by column 3). The transducing particle data suggested that two IPTG<sup>r</sup> mutants (pTIIO-4 and pTIIO-5) contained defective f1 origins. The f1 origin region of these two plasmids was sequenced and found to contain the indicated mutations. Restriction enzyme mapping experiments indicated that pTIIO-6 had a  $\approx 20$  bp deletion in the region of the plasmid containing the tac I promoter and the N-terminal coding region of gene II. Other IPTG<sup>r</sup> mutations were not determined (N.D.).

duplication of repeats  $\beta$  and  $\gamma$ .

The remainder of the IPTG<sup>r</sup> derivatives of pTIIO+ defined a class that still had functional f1 origins, as deduced from their ability to interfere with the growth of f1. Cells containing any of the plasmids in this class were not able to plaque phage f1. This inability to plaque f1 was most likely due to interference, because the cells were able to plaque phage f2, an F-specific RNA phage. One member of this class, pTIIO-6, was found to have a deletion in the region of the plasmid containing the *tacI* promoter and the N-terminal coding region of the gene II protein. Ostensibly then, this class defines gene II protein mutants.

Following this pilot experiment, mutants were isolated on a larger scale. The IPTG<sup>r</sup> mutants were divided into two classes. Class I mutations (putative origin mutations) were able to plaque f1. Class II mutations (putative gene II mutations) were unable to plaque f1, as a consequence of interference of the plasmid f1 origin with that of the phage. Members of both classes were able to plate f2. Table A-1.2 lists the members of both classes and any known mutations. The origin region of several members of class I was sequenced and found to contain mutations (see Table A-1.2).

Several members of class II were analyzed for gene II protein activity *in vitro*. Crude extracts were prepared from induced cultures and analyzed for Mn<sup>2+</sup>-dependent double-strand cleavage activity and for *in vitro* binding activity by gel retardation analysis. A large majority of the tested class II members had *in vitro* defects in gene II protein activity. These defects are summarized in Table A-1.3. I would like to emphasize that these results depend on analysis of crude extracts in a single experiment and should be viewed cautiously. These results are presented merely to demonstrate how mapping and sequencing of the class II mutations, coupled with more refined *in vitro* analyses, might help delineate the active domains involved in the various functions of gene II protein. Both pTIIO-125 and pTIIO-150 are candidates for proteins that are able to form complex II, yet unable to cleave the origin in the presence of Mn<sup>2+</sup>.

**Table A-1.2. IPTG<sup>r</sup> Mutants**

Plasmid	Mutation
<b>Class I</b>	
pTHO-4	tandem duplication ( $\alpha\beta\gamma\beta\gamma\delta$ ) in the f1 origin
pTHO-5	3 bp deletion [ $\Delta(-1--3)$ ] in the f1 origin
pTHO-17	origin sequenced-no change found
pTHO-18	large deletion in the plasmid
pTHO-30	origin sequenced-no change found
pTHO-38	large deletion in the plasmid
pTHO-41	large deletion in the plasmid
pTHO-42	1 bp deletion at nicking site [ $\Delta(-1)$ ]
pTHO-49	origin sequenced-no change found
pTHO-53	13 bp deletion [ $\Delta(+14+26)$ ] in the f1 origin
pTHO-54	large deletion in the plasmid
pTHO-55	3 bp deletion [ $\Delta(-1--3)$ ] in the f1 origin
pTHO-59	N.D.
pTHO-62	3 bp deletion [ $\Delta(-1--3)$ ] in the f1 origin
pTHO-81	large deletion in the plasmid
pTHO-84	large deletion in the plasmid
pTHO-86	3 bp deletion [ $\Delta(-1--3)$ ] in the f1 origin
pTHO-91	N.D.
pTHO-93	N.D.
pTHO-97	3 bp deletion [ $\Delta(-1--3)$ ] in the f1 origin
pTHO-101	N.D.
pTHO-112	N.D.
pTHO-132	tandem duplication ( $\alpha\beta\beta\gamma\delta$ ) in the f1 origin
pTHO-177	N.D.
pTHO-189	N.D.
pTHO-190	N.D.
pTHO-198	N.D.
pTHO-205	N.D.
pTHO-206	origin insertion
pTHO-210	N.D.
pTHO-215	$\simeq 20$ bp origin insertion
pTHO-222	N.D.



**Table A-1.2. IPTG<sup>r</sup> Mutants (continued)**

Plasmid	Mutation
<b>Class II</b>	
pTHIO-1 to pTHIO-3	N.D.
pTHIO-6	deletion in promoter--N-terminal region of gene II
pTHIO-7 to pTHIO-12	N.D.
pTHIO-13 to pTHIO-16	N.D.
pTHIO-19 to pTHIO-29	N.D.
pTHIO-31 to pTHIO-37	N.D.
pTHIO-39 to pTHIO-40	N.D.
pTHIO-43 to pTHIO-48	N.D.
pTHIO-50 to pTHIO-52	N.D.
pTHIO-56 to pTHIO-58	N.D.
pTHIO-60 to pTHIO-61	N.D.
pTHIO-63 to pTHIO-80	N.D.
pTHIO-82 to pTHIO-83	N.D.
pTHIO-85	N.D.
pTHIO-87 to pTHIO-90	N.D.
pTHIO-92	N.D.
pTHIO-94 to pTHIO-96	N.D.
pTHIO-98 to pTHIO-100	N.D.
pTHIO-102 to pTHIO-111	N.D.
pTHIO-113 to pTHIO-131	N.D.
pTHIO-133 to pTHIO-176	N.D.
pTHIO-178 to pTHIO-188	N.D.
pTHIO-191 to pTHIO-197	N.D.
pTHIO-199 to pTHIO-204	N.D.
pTHIO-207 to pTHIO-209	N.D.
pTHIO-211 to pTHIO-214	N.D.
pTHIO-216 to pTHIO-221	N.D.
pTHIO-223 to pTHIO-227	N.D.

Table A-1.2 catalogues the isolated IPTG<sup>r</sup>-derivatives of pTHIO+. The mutants were divided into two classes based on the ability of plasmid containing cells (K871) to be infected with f1. Cells containing class I mutant plasmids plaque f1, forming either clear or turbid plaques. Cells containing class II mutant plasmids were unable to plaque f1. Members of both classes were able to plaque f2. In some cases the nature of a mutation was determined by DNA sequencing or restriction mapping. The nature of most IPTG<sup>r</sup> mutations was not determined (N.D.) Plasmids pTHIO-5, pTHIO-62, and pTHIO-86, which all contain the same origin mutation, are not strictly independent because they were isolated from the same mutagenized culture (10 µg/ml nitrosoguanidine was used). Plasmid pTHIO-55 was isolated from a different mutagenized culture.

**Table A-1.3. Characterization of Possible Gene II Protein Mutants *In Vitro***

<b>Plasmid</b>	<b>Mn<sup>2+</sup>-cleavage Activity</b>	<b>Origin Binding</b>
pTHO+	+	Complexes I & II
pTHO-17	–	none
pTHO-30	+	Complexes I & II
pTHO-49	–	none
pTHO-56	–	none
pTHO-74	–	none
pTHO-95	–	none
pTHO-110	–	none
pTHO-125	–	Complexes I & II
pTHO-150	–	Complexes I & II
pTHO-180	–	N.D.

Individual 20 ml cultures were grown in D.O. minimal medium containing 0.4% casamino acids until O.D.<sub>660nm</sub> = 0.3 at which point gene II protein production was induced by addition of IPTG. After a further hour of growth, the cells were harvested and crude extracts were prepared as described in Materials and Methods. Mn<sup>2+</sup>-dependent double strand cleavage activity and origin binding were analyzed as described in Materials and Methods.

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**End**