A single amino acid substitution reduces the superhelicity requirement of a replication initiator protein

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ABSTRACT

The origin of rolling circle replication in filamentous coliphage consists of a core origin that is absolutely required and an adjacent replication enhancer sequence that increases *in vivo* replication 30 to 100-fold. The core origin binds the initiator protein (gpII) which either nicks or relaxes negatively superhelical replicative form DNA (RFI). Nicking at the origin, but not relaxation, leads to initiation of DNA replication. Our results indicate that the ratio of nicking to relaxation (nicking-closing) *in vitro* depends on the superhelical density of the substrate. We have studied the effect of a single amino acid substitution in gpII, which allows wild-type levels of replication in the absence of the enhancer, on origin nicking and binding. The enhancer-independent mutation yields more nicking and less relaxation of RFI, compared to the wild-type protein. The mutant gpII also shows a reduced requirement for superhelicity of the substrate in the nicking reaction. At the same time, the mutant gpII increases the co-operativity of protein-protein interactions in origin binding. We propose that the relaxation activity of gpII negatively regulates replication initiation, and that both increase in the negative superhelicity of the substrate and action of the replication enhancer may antagonize the relaxation activity.

INTRODUCTION

The replication initiator protein (gene II protein or gpII) of filamentous coliphage (f1, M13, and fd) is a multifunctional protein that plays central roles in phage DNA replication at a number of levels. It introduces a single-strand break at a specific site on the plus strand of negatively supercoiled replicative form DNA (RFI) (1). The 3'-hydroxyl end of the nick serves as the primer for initiation of plus strand rolling-circle replication (2). gpII functions also at a step beyond nicking; DNA molecules that have been nicked by gpII still require the protein for their

unwinding and replication (3). Upon completion of a round of replication, gpII functions in termination by cleaving and circularizing the displaced single strand (4). gpII also has a sequence-specific topoisomerase activity: when incubated with gpII *in vitro*, approximately 60% of RFI molecules are nicked to yield RFII, while the other 40% are converted to relaxed, closed form (RFIV) as a result of a nicking and joining reaction (5). RFIV has been found to be produced *in vivo* and require supercoiling by DNA gyrase in order to serve as a substrate for replication (6).

The plus strand replication origin consists of two adjacent domains, core origin sequence and replication enhancer sequence (7, 8). The core origin sequence (about 50bp) is absolutely required for replication and contains three repeats (β , γ and δ) which serve as the binding site for gpII (9, 10). The replication enhancer (about 100bp) stimulates *in vivo* replication 30 to 100-fold (7, 8) and contains three binding sites for the *Escherichia coli* integration host factor (IHF; 11). *In vivo* studies have shown that IHF stimulates filamentous phage DNA replication approximately 30-fold through its action on the replication enhancer sequence (11).

Several enhancer-independent mutations of gpII have been isolated, which allow wild-type levels of replication in the absence of either the enhancer sequence or IHF (12, 13). These mutations map to three positions within the N-terminal portion (codon 40 (12), 41 (this study), and 73 (13)) of gene II (410 codons). The mutation M40I (Met40→Ile; previously called mp-1) has been shown to increase the co-operativity with which the protein binds the origin to form a functional complex for the nicking reaction (14).

In this paper, we further characterize the enhancer-independent mutants, and the nicking activity of an enhancer-independent G73A (Gly73→Ala) mutant gpII. We report that the G73A mutant gpII has a remarkably reduced requirement for superhelicity of the substrate DNA, and propose that the nicking-closing (relaxation) activity of the initiator protein negatively regulates DNA replication.

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MATERIALS AND METHODS

Bacteria, phage and plasmids

Our standard *Escherichia coli* HfrC strain K38, a *supD* derivative K37 (15), and a *recA56* derivative K902 (16) have been described. Competent cells of *E. coli* DH5 were purchased from Bethesda Research Lab.

Two mutants of bacteriophage f1, R209 (17) and R485 (18), have been described.

Plasmid and phage RF were prepared according to Maniatis *et al.* (19). Plasmid pD16, a derivative of pBR322 (20), which carries two copies of the wild-type f1 origin, has been described (7; see Fig. 1). Plasmid pD10.Δ40,56 (7) is a derivative of pBR322, and carries a wild-type phage origin at the *EcoRI* site and another origin with a deletion in the replication enhancer at the *Bam*HI site. Plasmid pMBS1 which carries a 15 bp sequence corresponding to the center of the core origin (repeats β and γ), has been described (10).

pDG117IIA, a plasmid for overproduction of gpII, has been described (10). Its derivatives carrying M40I or G73A mutation in gene II were constructed by ligating the larger *Cla*I-*Hpa*I fragment of pDG117IIA and the smaller *Cla*I-*Hpa*I fragment of φM40I or φG73A RF (see below). Plasmid placI^qAH3 was constructed by ligating an *Eco*RI fragment of pUC4K (Pharmacia Co., Ltd.), a *Hind*III-*Eco*RI fragment carrying the P15A origin from pACYC184 (21), and a *Hind*III-*Eco*RI fragment containing *lac*P gene from pMJR1560 (22). placI^qAH3 was co-transformed with pDG117IIA or its derivatives into *E. coli* DH5.

Chemical reagents, and Enzymes

Restriction enzymes, phage T4 DNA ligase, T4 polynucleotide kinase, DNA polymerase I klenow fragment, and calf thymus topoisomerase I were obtained from Takara Shuzo Co., Ltd. or from New England Biolabs. Procedures for restriction enzyme digestion, and end-labeling of restriction fragments, were as described by Maniatis *et al.* (19). ³²P labeled nucleoside triphosphates were from Du Pont—New England Nuclear.

Construction of enhancer-independent gpII mutants

f1 amber mutants H1 (amber at codon 73 of gene II) and H2 (amber at codon 40) were obtained by hybridizing synthetic oligonucleotides Kn-18 (5'-TATTTTAAATGCAATCTATG-AGTAATGTGTAGG-3') and Kn-25 (5'-TAGCTGATAAATTCTAGCCGAGAGGGT-3'), respectively, to single stranded DNA of wild-type f1 phage and by screening turbid plaque formers for amber mutants after transfection of K37 (*supD*).

Spontaneous mutations that overcome a defective replication enhancer were obtained as clear plaque formers from a mutant phage R485 as described by Michel and Zinder (18), and were identified as M40I (Met40→Ile), N41S (Asn41→Ser), G73A (Gly73→Ala), and G73C (Gly73→Cys) by nucleotide sequencing (23). The mutants were transferred to the genetic background of wild-type phage by hybridizing a *Hpa*I-*Cla*I restriction fragment of mutant RF to single-stranded DNA of H1 or H2, and by subsequent transfection of a *sup*⁺ strain K38. The phages thus obtained were named φM40I, φN41S, φG73A, and φG73C, respectively.

Other amino acid substitutions at codon 73 or codon 40 were isolated from H1 or H2, respectively, either spontaneously by plating on K38, or by hybridizing synthetic oligonucleotides Kn-21 (5'-TTTAAATGCAATXXXGTAGTAATGTGT-3', where X refers to an equal mixture of the four nucleotides) or

Figure 1. Initiation of DNA replication from a phage origin that is defective in the replication enhancer upon infection with enhancer-independent gene II mutants. (A) Double origin assay of DNA replication. The plasmid pD16 contains two wild-type origins (indicated as oriI and oriII) in direct repeats, while pD10.Δ40,56 contains both a wild-type origin (at oriI site) and a defective origin with a deletion in the replication enhancer sequence (at oriII) in direct repeats. Upon superinfection with helper phage, initiation at oriI and termination at oriII result in production of the small plasmid I', and initiation at oriII and termination at oriI produce the larger plasmid II'. (B) Cells (K902) containing pD10.Δ40,56 (lanes 1–6), or pD16 which carries two wild-type origins (lanes 7–10), were infected with wild-type f1 (lanes 2 and 7), M40I mutant (lanes 3 and 9), G73A mutant (lanes 4 and 10), G73C mutant (lane 5), and N41S mutant phages (lane 6). At 35 minutes after infection, the cells were harvested and DNA was extracted and analyzed on a 1.0% agarose gel containing ethidium bromide at 0.5 μg/ml. The position of the superhelical form of the parental plasmid (p) and its resolution products I' and II' are indicated. The yield of II' reflects the enhancer-independent activity of each mutant gpII produced by mutant helper phages. Single stranded DNAs of I' and II' are marked I' ss and II' ss, respectively. The band just above I' has not been characterized and might be the open circle of I'.

Kn-22 (5'-TAGCTGATAAATTXXXGCCGAGAGGGT-3') to single-stranded DNA of H1 or H2, respectively, and subsequent transfection of K38.

Each mutant phage was tested for its ability to suppress a defective replication enhancer by infecting K902 harboring pD10.Δ40,56 (7) and by measuring amount of II' DNA produced (see legend to Fig. 1).

Protein purification

To overproduce gpII and its mutant protein, *E. coli* DH5 bearing both placI^qAH3 and pDG117IIA or its mutant derivative was grown at 37°C in 400ml of LB-broth containing 50μg/ml

Table 1. Amino acid substitutions at codons 40, 41, and 73 of gene II.

Codon	40	41	73
Enhancer-dependent	<u>Met</u>	<u>Asn</u>	<u>Gly</u>
	Asn		Gln
	Gln		Leu
	Leu		Thr
	Lys		Val
	Phe		
	Ser		
Enhancer-independent	Arg	Ser	Ala
	Cys		Arg
	Ile		Asn
	Thr		Cys
	Val		Lys
			Ser

The mutant phages were isolated as described in *Materials and Methods*. They were then tested for their ability to initiate DNA replication from a phage origin that is defective in the replication enhancer function by superinfecting cells (K902) harboring pD10.Δ40,56 (see *Materials and Methods*). The amino acid residues in the wild type are underlined.

ampicillin to an O.D. 660nm of 0.3. IPTG was added to final concentration of 2mM, and 5 hours later cells were harvested and broken using French-press. Purification of gpII was carried out as described previously (10).

IHF was prepared from K5746, an IHF overproducing strain, kindly provided by H.Nash, using the published procedure (24).

Preparation of f1 RFI topoisomers with various levels of supercoiling

f1 DNA of different superhelical densities was prepared by relaxation of supercoiled RFI with calf thymus topoisomerase I in the presence of various concentrations of ethidium bromide (25). The relaxation was carried out at 30°C for 60 minutes in a buffer containing 35mM Tris-HCl (pH 8.0), 5mM MgCl₂, 5mM DTT, and 20μg/ml BSA. The relaxed DNA was extracted twice with phenol, and was precipitated with ethanol. Their linking numbers were measured by the band-counting method (26) after electrophoresis on agarose gels in 2×TAE (80mM Tris-acetate, 2mM EDTA, pH8.0) containing various concentrations of chloroquine (0–10.0μg/ml). The superhelical density of each preparation was then calculated based on a value of 10.5 base pairs/helical turn. The superhelical densities thus determined of topoisomers which were prepared with topoisomerase I at various concentrations of ethidium bromide were in agreement with the values reported by Singleton and Wells (25), who measured the superhelical density by ethidium bromide fluorescence.

Nicking reaction and separation of RFI, RFII, and RFIV molecules

For nicking reactions, f1 origin-containing DNA (0.1–1.0μg) was incubated with purified gpII protein (5–20ng) in a 20μl reaction mixture containing 20mM Tris-HCl (pH8.0), 80mM KCl, 5mM DTT, and 5mM MgCl₂ at 30°C for 30min. The reaction was terminated by addition of 1μl of stop mixture (0.2M EDTA (pH8.0), 20% sucrose, 1% sodium dodecyl sulfate, and 0.01% bromophenol blue), and the products were analyzed by gel electrophoresis. To separate RFI, RFII, and RFIV molecules, the sample was electrophoresed on a 0.7% agarose gel in 2×TAE buffer in the absence of ethidium bromide for 4 hours at 3V/cm, stained with 0.5 μg/ml ethidium bromide, and then electrophoresed for an additional hour under the same conditions

Figure 2. Nicking of linear DNA fragment by the mutant gpII. f1 RF digested with restriction endonuclease *AsuI* (*Cfr13I*) was terminally labeled with ³²P by T4 polynucleotide kinase (19), and subsequently digested with *Clal*. 0.01 pmol of DNA fragments thus obtained were used as the substrate for nicking reaction by 0.1 pmol of the wild-type or mutant gpIIs as described in *Materials and Methods*. After the reaction, the samples were boiled for 2 minutes in the presence of 80% formamide, chilled in ice-water, and electrophoresed in a 10% polyacrylamide gel containing 8 M urea. The arrowhead indicates the nicked DNA strand produced by gpII. Upper two bands are denatured *Clal/AsuI* fragments of f1 RF.

except that the running buffer contained 0.5 μg/ml ethidium bromide. The gel was visualized and photographed under a UV transilluminator. Under these conditions, RFI DNA was separated from RFII and RFIV by the first step electrophoresis, and RFIV migrated faster than RFII in the second step. The amount of DNA in the bands was quantified by densitometric scanning of a Polaroid 665 negative film. The measurement was always carried out within a range of DNA concentrations which gave a linear relationship between the amount of DNA and the densitometric value. Under the conditions we used, the densitometric value per unit amount of linear or nicked DNA was 1.4 ± 0.1 times higher than that of covalently closed circles, and therefore the amount of DNA in each band was normalized by this factor. Densitometric difference between RFI (negatively supercoiled) and RFIV (relaxed and closed) was within experimental errors.

Polyacrylamide gel electrophoresis of gene II protein-origin complexes

The binding reactions of gpII to the origin and polyacrylamide gel electrophoresis were carried out as described previously (14).

RESULTS

Gene II mutants that overcome defects in the replication enhancer

Phage with a defective replication enhancer, such as R209, which carries a 4 base-insertion within the enhancer, form very turbid plaques and tend to acquire suppressor mutations that give clear plaques (12, 13, 27). The suppressor mutations, which restore efficient replication in the absence of a functional enhancer, result in either amino acid substitution in or overproduction of gpII. Michel and Zinder (18) isolated 16 independent suppressor mutants from phage R485, a derivative of R209 that carried a gene IV amber mutation which facilitated marker rescue mapping of the suppressors. Nine of the mutants were overproducers. We have now studied the other seven mutations that mapped within the coding region of gene II. Nucleotide sequencing experiments

Figure 3. Nicking of RF-topoisomers with different negative superhelical densities by gpII. (A) Electropherogram. Each reaction contained 0.05 pmol (200 ng) f1 RF with different superhelical densities: $\sigma = -0.00$ (lanes 1, 8, and 15), -0.003 (lanes 2, 9, and 16), -0.016 (lanes 3, 10, and 17), -0.024 (lanes 4, 11, and 18), -0.033 (lanes 5, 12, and 19), -0.042 (lanes 6, 13, 20), and -0.057 (lanes 7, 14, and 21). Lanes 1–7, no gpII; lanes 8–14, 1 pmol of wild-type gpII; lanes 15–21, 1 pmol of G73A mutant gpII. Conditions for the nicking reaction and for agarose gel electrophoresis to separate RFI, RFII, and RFIV are described in *Materials and Methods*. The position of RFII (nicked molecules) and closed circles are indicated by an arrowhead and a bracket, respectively. (B) Quantification of RFII produced. The amounts of DNA in the bands shown in panel (A) were quantified by densitometric scanning of Polaroid 665 negative film as described in *Materials and Methods*. The amount of RFII pre-existed in the substrate was subtracted.

indicated that two mutants had Gly→Ala change at codon 73 (G73A), two had Gly→Cys at the same codon (G73C), two had Met→Ile at codon 40 (M40I), and the other had Asn→Ser at codon 41 (N41S).

The ability of the mutants to initiate DNA replication from an enhancer-defective origin was tested *in vivo* using a double origin plasmid pD10.Δ40,56 (28). This plasmid is a derivative of pBR322, which carries a wild-type f1 phage origin inserted at the *EcoRI* site (ori I) (see Fig. 1(A)) and an f1 origin with a deletion (Δ40,56) in the replication enhancer inserted in the same orientation at the *BamHI* site (ori II). pD16, a similar plasmid carrying two wild-type f1 origins, served as a control. Since the phage origin contains signals for both initiation and termination of replication (29, 30), helper phage infection of cells harboring the plasmid results in resolution of the plasmid into two components I' and II' as shown in Fig. 1, where production of II' depends on initiation from the origin at the ori II site by the action of the helper phage gpII.

Figure 4. Binding of G73A mutant gpII to the origin. (A) Gel retardation analysis of the gpII-origin complexes. Binding of the wild-type gpII and G73A mutant proteins to an end-labeled restriction fragment (marked with an arrowhead; 315bp *AsuI/ClaI* fragment) containing the f1 origin was analyzed as described previously (14). Each lane contained 1 fmol of the DNA fragment. In addition, lanes 2–5 contained 1 fmol, 2 fmol, 4 fmol, and 8 fmol of G73A gpII, and lanes 6–9 contained 1 fmol, 2 fmol, 4 fmol, and 8 fmol of wild-type gpII, respectively. (B) Quantification of the origin binding. The binding data shown in (A) were quantified by excising the radioactive bands and measuring their radioactivity by liquid scintillation counting. The ordinate represents the percentage of DNA found in each complex. The values for binding of M40I gpII (previously called mpI) were taken from Greenstein and Horiuchi (14).

The results shown in Fig. 1(B) indicated that all the seven mutants efficiently initiated DNA replication from a phage origin that lacked the functional enhancer, except that the result obtained with the N41S mutant phage was less distinctive.

All the enhancer-independent gpII mutations previously described map at codons either 40 or 73 (12, 13). To obtain different amino acid substitutions at these codons, we first constructed phage carrying an amber mutation at codon 40 or 73 by hybridizing synthetic oligonucleotides Kn-25 or Kn-18 (see *Materials and Methods*), respectively, to single-stranded DNA of the wild type phage. From the resulting amber mutants H2 (amber at codon 40) and H1 (amber at codon 73), many revertants were isolated either spontaneously or by use of oligonucleotides Kn-22 or Kn-21 (see *Materials and Methods*). Each mutant was tested for its ability to initiate DNA replication from an enhancer-defective phage origin using the double origin plasmid system described above (data not shown). The results summarized in Table 1 indicate that substitution of Met at codon 40 by Arg, Cys, Ile,

Figure 5. Interaction of the G73A gpII with the minimal binding sequence. Binding of wild-type and G73A gpIIs to the *Bst*NI restriction digest of non-radioactive plasmid pMBS1 was analyzed by gel retardation assay. DNA in the gel was stained with ethidium bromide after electrophoresis, and photographed with Polaroid 665 negative film. Each reaction contained 0.125 pmol of pMBS1 DNA and different amounts of gpII. Lane 1 was a control without gpII; lanes 2–4 contained 0.5 pmol, 1 pmol, and 2 pmol of G73A gpII; and lanes 5–7 contained 0.5 pmol, 1 pmol, and 2 pmol of wild-type gpII, respectively. gpIIs specifically bound the 208bp fragment (marked with an arrowhead) which contains the minimal binding sequence (repeats β and γ).

Thr or Val resulted in enhancer-independent gpII, while that by Asn, Gln, Leu, Lys, Phe or Ser resulted in enhancer-dependent gpII. For codon 73, substitution of Gly by Ala, Arg, Asn, Cys, Lys or Ser yielded enhancer-independent gpII, while that by Gln, Leu, Thr or Val yielded enhancer-dependent gpII. Both amber mutants H1 and H2 grew on *supD*, *supE*, and *supP* strains, but did not plaque on *supF* strains, which suggested that substitution with tyrosine at either site produces non-functional gpII.

Reduced superhelicity requirement in the nicking activity of the mutant gpII

A linear DNA fragment containing the complete *f1* plus strand origin was terminally labeled with ^{32}P and used as the substrate for the nicking reaction by the wild-type and an enhancer-independent mutant G73A (Gly73→Ala) gpII. The result shown in Fig. 2 indicates that gpII of G73A mutant showed about 5-fold higher nicking activity than the wild-type gpII on this substrate. Measurement of radioactivity in the gel bands indicated that G73A gpII nicked approximately 20% of the substrate, while the wild-type gpII nicked about 4%. Use of increased amounts of gpIIs did not change the product distribution (data not shown).

A series of topoisomers with different negative-superhelical densities was prepared from RFI (see *Materials and Methods*) and used as substrates for the nicking reaction by wild-type and G73A gpII. When RFI was incubated with wild-type gpII, it was converted to a mixture of RFII (nicked molecules) and RFIV (relaxed, closed molecules). The results shown in Fig. 3 indicate that the higher the negative superhelicity of the substrate, the greater the ratio of RFII to RFIV in the product. When the substrate DNA had an average negative superhelical density of 0.016, the wild-type gpII converted 38% of the substrate to RFII and 62% to RFIV (see Fig. 3(B)). When the negative superhelical density was 0.057, 56% of DNA were nicked and 44% became RFIV. Within this range of superhelicity of substrate, no

Figure 6. Inability of IHF to affect the *in vitro* nicking reaction by gpII. (A) Nicking of RFI. Nicking reactions were carried out using 0.05 pmol (200ng) of *f1* RFI as described in *Materials and Methods*. Lane 1 was the control with no gpII. Lanes 2–4 contained 0.1 pmol, lanes 5–9 contained 0.2 pmol, and lanes 10–12 contained 0.3 pmol, respectively, of the wild-type gpII. Amounts of purified IHF added to the reaction were 0.05 pmol (lane 6), 0.1 pmol (lanes 3, 7, and 11), 0.2 pmol (lane 8), and 0.4 pmol (lanes 4, 9, and 12). (B) Nicking of linear DNA. The substrate was the end-labeled linear DNA fragment described in the legend to Fig. 2. 0.01 pmol of the fragment was incubated with 0.1 pmol of wild-type (lanes 2–4) or G73A (lanes 5–7) gpII. Lane 1 contained no gpII. Amounts of IHF added were none (lanes 1, 2, and 5), 0.02 pmol (lanes 3 and 6), or 0.06 pmol (lanes 4 and 7). Conditions for the reaction and for electrophoresis were the same as in Fig. 2. The arrowhead indicates the nicked product.

detectable amount of DNA remained unchanged after the reaction.

The results shown in Fig. 3 also demonstrate that the enhancer-independent mutant G73A does not require as much superhelicity for nicking as the wild-type protein. When the substrate DNA has a negative-superhelical density of 0.025, which corresponds to the value estimated for the *in vivo* superhelical density in *E. coli* (31), the wild-type gpII converted about 45% of the substrate DNA to RFII and 55% to RFIV. The G73A mutant gpII converted this substrate to a mixture of 90% RFII and 10% RFIV under the same experimental conditions. Thus, G73A gpII gives higher RFII:RFIV ratios than wild-type gpII. When the substrate was completely relaxed circles (RFIV), G73A gpII still converted about 30% of the molecules to RFII. The wild-type gpII converted only 8% of this substrate to RFII (Fig. 3). All these nicking activities were origin-specific; pBR322 or other supercoiled plasmids that did not contain the *f1* origin were not active as substrate (data not shown).

In vitro binding of enhancer-independent mutant G73A gpII to the origin

We have previously shown that gpII binds the core origin in two steps, first forming a binding intermediate (complex I) that

contains two gpII molecules per origin, and then a functional complex for nicking (complex II) that contains four gpII molecules. M40I mutation has been shown to increase the co-operativity of gpII in the formation of complex II (14). In order to study the effect of the enhancer-independent G73A mutation on origin binding, we purified the G73A mutant and wild-type protein in parallel and quantitated origin-binding using gel retardation. The G73A gpII showed increased co-operativity in the formation of complex II, compared to the wild-type gpII, though to a lesser extent than the M40I protein (Fig. 4). In addition, the G73A gpII, like the M40I gpII (14), formed both complexes I and II with a DNA fragment containing only two (β and γ) of the three repeats from the core origin sequence (Fig. 5). The wild-type protein formed only complex I with this fragment. These results indicate that both M40I and G73A mutations increase the co-operativity of protein-protein interaction necessary to form complex II and allow its formation in the absence of DNA sequences which are normally required (the binding repeat δ).

Inability of IHF to affect the nicking reaction *in vitro*

Since the results described above indicated that replication enhancer-independent mutations in gpII affect the nicking reaction, and since *in vivo* experiments have shown that the replication enhancer functions through its binding to IHF (11), we tested whether IHF affects the *in vitro* nicking reaction by gpII. The result shown in Fig. 6(A) indicates that neither nicking nor relaxation of RFI was affected by addition of IHF. In addition, a terminally labeled DNA fragment containing the complete phage origin was tested for nicking by the wild-type and the mutant gpII in the presence or absence of IHF (Fig. 6(B)). No effect of IHF was observed in either case. Furthermore, f1 RF DNA of various superhelical densities was prepared and tested for nicking in the presence of IHF. No effect of IHF was observed (data not shown).

DISCUSSION

In vivo studies have demonstrated that filamentous phage DNA replication is enhanced some 30-fold by the presence of the replication enhancer sequence and that this enhancement depends on IHF (11). Mutant phage can be isolated that overcome a defective replication enhancer. Such mutations result in either overproduction of, or amino acid substitution in, gpII (12, 13, 27). Mutations of the first type have been found either in gene V, the translational repressor of gene II, or in the leader sequence of the gene II mRNA, the translational operator. (18). Mutations of the second type have been found only in the amino-terminal region of gpII, at codon 40, 41, or 73 (gene II has 410 codons) (Table 1). For the mutant M40I, it has previously been shown that the mutation increases the co-operativity with which gpII binds the origin to form a functional complex for the nicking reaction (complex II) (14). In the present work we found that the G73A mutation was similar to M40I in that it affected the gpII-origin interaction by increasing the co-operativity with which gpII forms complex II, though to a lesser extent than M40I (Fig. 4). In addition, both mutant gpIIs efficiently formed complex II with a DNA fragment that contained only the minimal binding sequence, repeats β and γ in inverted orientation. The wild-type protein formed only complex I with this DNA fragment (Fig. 5; 14). Therefore, the site in N-terminus of gpII defined by the enhancer-independent mutations is likely to mediate

protein-protein interactions that are important for co-operative binding to form a functional complex (complex II).

Besides its nicking activity for replication initiation, gpII possesses nicking and closing activity. Upon incubation with gpII *in vitro*, substrate RFI molecules are converted to a mixture of RFII and RFIV. Thus, gpII can be regarded as a sequence-specific topoisomerase. When RFI purified from *E. coli* which had an average negative superhelical density of 0.057 was used as substrate, 56% of RFI was nicked and the remaining 44% was relaxed by the wild-type gpII. On the other hand, 45% of RFI molecules that have an average negative superhelical density of 0.025, the value estimated for the *in vivo* level of supercoiling (31), were converted to RFII by gpII, and 55% became RFIV. When the negative superhelical density was 0.016, only 38% were converted to RFII, and 62% became RFIV (see Fig. 3). Furthermore, substrate DNA with a negative superhelical density of about 0.10, which was obtained by topoisomerase treatment in the presence of 6.0 μ g/ml ethidium bromide, was converted by gpII to a mixture of 70% RFII and 30% RFIV (data not shown). Thus, the higher the negative superhelicity of the substrate, the greater the ratio of RFII to RFIV in the reaction product. While RFII molecules are ready to enter a replication cycle *in vivo*, RFIV molecules produced by gpII must be converted back to RFI by the action of DNA gyrase in order to replicate (6). Thus, the nicking-closing activity of gpII would regulate replication initiation. Negative superhelicity of the substrate would control it by affecting the ratio of nicking to nicking-closing reactions by gpII. The mechanism by which superhelicity affects the ratio of RFII to RFIV in the reaction product is unclear at present. Energy carried in the negatively superhelical structures may affect the outcome of the reaction, possibly by modulating the structure of the DNA in its bent complex with gpII.

The replication enhancer-independent mutant gpII (G73A) gave reaction products that had much higher ratios of RFII to RFIV, and showed much higher nicking activity on relaxed DNA than the wild-type gpII (Figs. 2 and 3). In this respect, the mutant gpII is similar to Int-h, a mutant of λ integrase which leads to efficient site-specific recombination in the absence of IHF and which reduces requirement for negative superhelicity of recombination substrates (32). The reduced superhelicity requirement of the enhancer-independent mutant gpII suggests that similar mechanisms are operating for the action of IHF and for that of superhelicity on the initiation reaction of replication.

The nicking-closing reaction by gpII does not require any high energy cofactors. Based on studies of other topoisomerases and of cisA protein of phage ϕ X174 (33, 34), it is suggested that both nicking and nicking-closing reactions by gpII may proceed from a common step in which a phosphodiester bond at the nicking site is converted to a covalent bond with an amino acid residue in the polypeptide. In the case of gpII, such a covalent intermediate has not been detected. Recently, Mizuuchi and Adzuma (35) have demonstrated that in the Mu DNA strand transfer reaction mediated by the MuA protein, a chiral phosphate inverts its configuration, suggesting that the MuA protein carries out a one-step transesterification reaction without forming a covalent intermediate. It is tempting to think that this is also how gpII works in the nicking-closing reaction, since a covalently bound intermediate of gpII has not been detected. Then, the effect of the G73A mutation may be explained by assuming that the mutant protein holds onto the nicking site less well than the wild-type, in a sense that it only poorly excludes water from the active site, thus allowing hydrolysis of the phosphodiester bond before

ligation. Regardless of the mechanism, when the substrate has higher negative superhelicity, gpII must turn one strand around the other strand more times before completely relaxing the substrate. If there is a certain probability per turn with which the phosphodiester bond is hydrolyzed, then the higher the negative superhelicity of the substrate, the larger the amount of nicked form produced. This model by itself may not entirely explain the relation between the yield of RFII and the superhelicity of substrate, which is not exactly proportional (see Fig. 3(B)).

The apparent increment of nicking activity in the enhancer-independent G73A mutant might be due to impairment of the closing activity of gpII by this mutation. The observation that a number of different amino acid substitutions at positions 40, 41 and 73 result in enhancer-independence (Table 1) is consistent with these mutations resulting in the loss of a function, possibly the closing activity. Although the G73A mutation increases the co-operativity of protein-protein interactions in complex II formation (see Fig. 4), we cannot assume a simple correlation between increase of nicking activity and increase in the co-operativity of complex II formation. Another enhancer-independent mutation M40I, which shows a greater increase in the co-operativity of complex II formation (14), does not show any change in the product distribution of the nicking reaction (RFII:RFIV ratio, data not shown).

How do IHF and enhancer sequence affect DNA replication of filamentous phage *in vivo*? IHF functions in a number of processes including site-specific recombination, transcription, translation, transposition, phage morphogenesis and DNA replication (for review, see 36, 37). It appears that IHF acts in all these processes as an accessory or enhancing factor rather than as an absolute requirement. It has been well established that IHF induces DNA bending upon binding. It may be possible that IHF positively regulates the nicking activity of gpII by changing the conformation of the replication origin through binding to the enhancer sequence. Effects of negative superhelicity of the substrate on the nicking reaction may be caused through a similar mechanism. If so, IHF may reduce the requirement for negative superhelicity, as has been suggested for the case of the strand transfer reaction in phage Mu transposition (38). Our observation that an IHF-independent mutant gpII has reduced requirement for superhelicity is in accordance with this notion.

In our *in vitro* attempts, however, neither the nicking activity nor the nicking-closing activity of gpII on purified f1 RF was affected by addition of IHF, regardless the superhelical density of the substrate (Fig. 6). Moreover, the conventional *in vitro* replication system for the phage DNA (39) was not affected by the presence of either the enhancer sequence or IHF (unpublished data), even though stimulation of f1 DNA replication *in vivo* by the replication enhancer sequence and IHF has been well established (7, 8, 11). A possibility would be that there may be an additional factor(s) in the cell which is necessary for manifestation of the IHF effect and that this hypothetical factor(s) is missing in the *in vitro* system. Such a factor(s) may co-operate with IHF to stimulate replication, or may be an inhibitor of replication that can be counteracted by IHF. Whether such a factor(s) exists remains to be studied.

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