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In Vivo Identification of Intermediate Stages of the DNA Inversion Reaction Catalyzed by the Salmonella Hin Recombinase

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ABSTRACT

The Hin recombinase catalyzes a site-specific recombination reaction that results in the reversible inversion of a 1-kbp segment of the Salmonella chromosome. The DNA inversion reaction catalyzed by the Salmonella Hin recombinase is a dynamic process proceeding through many intermediate stages, requiring multiple DNA sites and the Fis accessory protein. Biochemical analysis of this reaction has identified intermediate steps in the inversion reaction but has not yet revealed the process by which transition from one step to another occurs. Because transition from one reaction step to another proceeds through interactions between specific amino acids, and between amino acids and DNA bases, it is possible to study these transitions through mutational analysis of the proteins involved. We isolated a large number of mutants in the Hin recombinase that failed to carry out the DNA exchange reaction. We generated genetic tools that allowed the assignment of these mutants to specific transition steps in the recombination reaction. This genetic analysis, combined with further biochemical analysis, allowed us to define contributions by specific amino acids to individual steps in the DNA inversion reaction. Evidence is also presented in support of a model that Fis protein enhances the binding of Hin to the *hixR* recombination site. These studies identified regions within the Hin recombinase involved in specific transition steps of the reaction and provided new insights into the molecular details of the reaction mechanism.

THE DNA strand exchange reaction is a fundamental process in nature. Such reactions are used to generate genetic diversity through allelic exchange via homologous recombination or by transposition. Furthermore, DNA rearrangements catalyzed by site-specific recombinases occur by a mechanism used by various organisms to affect gene expression. The Hin recombinase catalyzes a reversible, site-specific recombination reaction within the chromosome of *Salmonella typhimurium* and other closely related Salmonella spp. Strand exchange occurs between two homologous chromosomal sites, *hixL* and *hixR*, that flank an invertible promoter (Johnson and Simon 1985). It is the reversible inversion of this promoter segment that results in the alternate expression of two antigenically distinct flagellin proteins, a phenomenon known as flagellar phase variation (Figure 1; Stocker 1949).

The study of site-specific recombination in prokaryotes has yielded many discoveries, including the involvement of recombinational enhancer (RE) elements, the importance of the minor groove DNA-binding domain for protein-DNA interactions, homology of the minor groove-binding domain to minor groove recognition regions in eukaryotic homeodomain proteins, and mechanistic similarities between prokaryotic site-specific recombination and V(D)J recombination (*v*ariable (and sometimes *d*iverse) gene segment *j*oining) used to generate antibody diversity (Tonegawa 1983). Similarities between what is known about the mechanisms of Hin-mediated, site-directed recombination and V(D)J recombination used in the generation of antibody diversity has led to speculation that V(D)J recombination may have originated, in part, from a prokaryotic system related to the Hin system (Simon *et al.* 1980; Lewis and Wu 1997).

The Hin-mediated DNA inversion reaction, diagrammed in Figure 1, requires the activity of Hin recombinase to catalyze cleavage of pairs of phosophodiester bonds in both *hix* sites and is stimulated by the binding of an accessory protein, Fis, to a third, recombinational enhancer site (Johnson and Simon 1985). A third, nonspecific DNA-binding factor, HU, facilitates the DNA looping between *hixL* and the enhancer, and it is required for high rates of recombination (Johnson *et al.* 1986). The interaction between Hin, Fis, HU, and their cognate binding sites creates a nucleoprotein complex called the invertasome (Johnson *et al.* 1987). This complex holds the invertible DNA fragment in a specific topological configuration, allowing DNA strand exchange to occur.

Hin binds to each of the two *hix* sites as a dimer, making contacts in both the minor and major grooves of the substrate DNA (Hughes *et al.* 1992; Feng *et al.* 1994b; Glasgow *et al.* 1989). The 26-bp *hix* sites are composed of two symmetrically related 12-bp half sites

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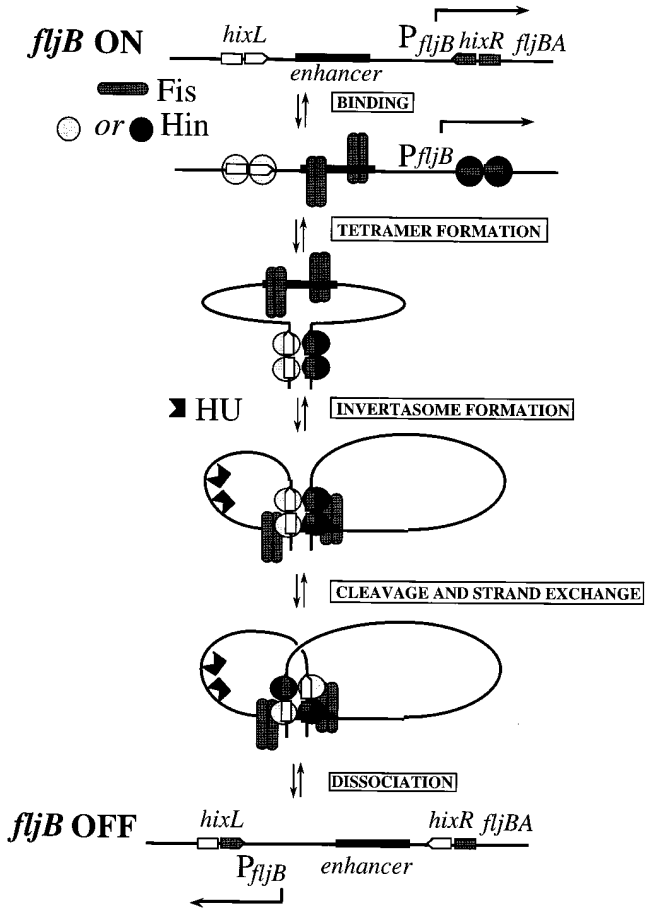

Figure 1.—The reversible, Hin-mediated inversion of a 996-bp segment of the *S. typhimurium* chromosome results in inversion of a promoter driving the expression of *fljB* flagellin gene and *fljA*, which encodes a repressor of the alternative flagellin gene *fliC*. A model that DNA strand exchange occurs with Hin subunit exchange is depicted here. Note the *hixL* designation for the leftward *hix* site and the *hixR* designation for the rightward *hix* site, which indicate differences in sequence between the outer half-sites that are not part of the invertible element.

separated by a 2-bp core, and are symmetrically oriented (Glasgow *et al.* 1989). The Hin dimers can synapse to bring the *hix* sites into local proximity, forming a tetramer (Heichman and Johnson 1990). After formation of the invertasome and Hin-catalyzed scission of four phosphodiester bonds (Johnson and Bruist 1989), a topological rearrangement occurs within the invertasome, and Hin rejoins pairs of exchanged DNA ends.

In this study, we describe the isolation of Hin mutants that are defective in recombination. The catalytically inactive point mutants of Hin that retain their ability to bind DNA ($B^+ R^-$) were first separated from recombination-deficient mutants using the P22 challenge phage system (Hughes *et al.* 1988, 1992). Up to now, there has been no convenient way to distinguish different classes of Hin $B^+ R^-$ mutants with respect to particular steps in the inversion reaction that they may affect. Because the inversion reaction requires the interaction of Hin with its *hix* recombination sites and with Fis protein, these Hin mutants may result from blocks in any of several distinct steps in the recombination pathway. To distinguish among classes of mutants blocked at different steps in invertasome assembly and DNA strand exchange that follows invertasome assembly, we constructed genetic vectors for the detection of Hin- and Fis-dependent recombination intermediates *in vivo*. Using the genetic tools developed, we were able to define blocks in specific reaction steps resulting from single amino acid changes in Hin. These included subsets of Hin mutants blocked in distinct steps in the invertasome assembly pathway defined using only biochemical studies *in vitro* (Johnson and Bruist 1989).

MATERIALS AND METHODS

Bacteria and bacteriophage strains: All *S. typhimurium* strains are derived from strain LT2. Isogenic strains MS1883 (*leuA414 hsdSB supE40* Fels$^-$) and MS1868 (*leuA414 hsdSB* Fels$^-$) were used for phage growth and selections for the products of crosses between phage and plasmids (Graña 1985). Strain TH2128 is *hsdLT6 hsdSA29 hsdSB metA22 metE551 trpD2 ilv-452*. Challenge phage assays were performed with isogenic strains LT2 and TH2285 (*fis-3*::Cam) (Numrych *et al.* 1991) carrying the Hin expression plasmid pKH66 (Hughes *et al.* 1988) and *hin* mutant derivatives of pKH66. Constructions of the *hixL(I)*, *hixL(II)*, *hixR(I)*, *hixR(II)*, *hixC*, and 10G challenge phages have been described previously (Hughes *et al.* 1988, 1992).

The constructions of phages P22 *hixL Kn6 arcH1605* and P22 *RE-hixL Kn6 arcH1605* were as follows: plasmid pMS284 (Youderian *et al.* 1983) is a pBR322-based plasmid (Bolivar *et al.* 1977) containing a kanamycin resistance cassette replacement of the *mnt* gene of phage P22 in a clone of the *sieA - mnt - arc - ant* region of P22 to yield *sieA - neo - arc - ant* such that the *neo* promoter is proximal to *sieA* and the entire *neo* cassette is flanked by *Pst*I sites, and it also carries the *arcH1605* (am) mutation. Digestion of pMS284 with *Pst*I, followed by ligation, yielded pMS361 (Graña *et al.* 1988; Wu *et al.* 1987), in which the *neo* gene has been inverted such that *neo* transcription is reversed to yield the *Kn6* allele. Plasmid pMS361 was partially digested with *Pst*I, and a double-stranded oligonucleotide containing *hixL* flanked by *Pst*I sites was ligated to this partially digested DNA. Plasmid pKH419 resulted from insertion of *hixL* into the *Pst*I site of pMS361 between the *neo* and *sieA* genes, as verified by restriction analysis. A 232-bp *Sfa*NI-*Hpa*II fragment from pES201 (Bruist and Simon 1984), which includes the *hixL* and RE, was filled in with a fragment of DNA polymerase I (Klenow) and subsequently ligated into *Sma*I-digested pBlueScript SK$^-$ (Stratagene, La Jolla, CA) to yield plasmid pJK105. Plasmid pMS361 was partially digested with *Pst*I, filled in with a fragment of DNA polymerase I (Klenow), and religated. Resulting transformants were screened for the presence of a plasmid in which the *Pst*I site proximal to the *ant* gene was removed to yield pJK108. A 248-bp fragment flanked by *Bam*HI and *Pst*I restriction sites from pJK105 was blunted by treatment with a fragment of DNA polymerase I (Klenow) and cloned into a similarly blunted *Pst*I site located at the P_{ant} distal end of the kanamycin resistance gene in pJK108. This construct, pJK107B, was verified by restriction and DNA sequence analysis and was subsequently used to construct P22 *RE-hixL Kn6 arcH1605* essentially as described below for P22 *hixL Kn6 arcH1605*.

Plasmid pKH419 was introduced into strain TH1901, which is lysogenic for phage P22 Ap521. P22 Ap521 is a derivative of P22 with a Tn*1* insertion in the *mnt* gene. The presence of the Tn*1* insertion in the *mnt* gene results in a phage that is too large to be packaged into a single phage particle. Induction of P22 Ap521 in the presence of pKH419 yielded viable phage because of recombination between pKH419 and P22 Ap521, in which the Tn*1* insertion was removed by homologous recombination with pKH419 and replaced by the *sieA - hixL - Kn6 - arcH1605 - ant* segment from pKH419 to yield phage P22 *hixL - Kn6 arcH1605*. To screen for the presence of the *arcH1605* mutation, recombinant phages were plated on MS1883, permissive for the *arcH1605* (am) mutation because of the *supE40* mutation in MS1883, and screened for restricted growth on MS1868. The *arcH1605*(am) mutation is not suppressed in MS1868, resulting in full derepression of the *ant* promoter, which is detrimental to phage growth. A single plaque was isolated and used to grow a high-titer lysate. DNA was isolated from this lysate, and both the *hixL - Kn6* insert and the *arcH1605* allele were verified by restriction analysis.

Challenge phages Tet-10G (P22 *hixL Kn6 10G arcH1605*) and Inv-10G (P22 *RE-hixL Kn6 10G arcH1605*) were constructed by crosses between either P22 *hixL Kn6 arcH1605* or P22 *RE-hixL Kn6 arcH1605*, respectively, and plasmid pPY190, in which the symmetrically mutant *hixC* 10G site had been cloned in the place of the *ant* operator as described (Hughes *et al.* 1992).

Media: Media conditions, concentrations of antibiotics and lactose indicators, transductional crosses, and transformations were as reported previously (Gillen and Hughes 1991; Adams *et al.* 1997).

Localized mutagenesis of the *S. typhimurium* chromosome and isolation of *hin* mutants: A Mu*d-lac* insertion in the *fliC* flagellin gene of *S. typhimurium* alternates between a Lac^+ and Lac^- state because of Hin-mediated inversion of a segment of the chromosome (Gillen and Hughes 1991). Strain TH560 carries a Tn*10d*Tc insertion linked to the *hin* region of the chromosome. TH560 was mutagenized by diethylsulfate (Roth 1970), and a transducing lysate was grown on the pooled mutagenized cells. This lysate was subsequently used to transduce TH1237 (*fliC5050*::Mu*dJ* (Lac^-, Kn^r)) to Tc^r and was screened for inheritance of chromosomal *hin* mutant alleles, as scored by the inability to switch from a Lac^- $fliC^{off}$ orientation to a Lac^+ $fliC^{on}$ orientation. Putative *hin* mutants were screened for complementation *in trans* by pKH66 induced for Hin expression, to screen for potential mutations in the *hix* sequences that may result in an inability to switch from a Lac^- $fliC^{off}$ orientation to a Lac^+ $fliC^{on}$ orientation. One of these chromosomal *hin* mutants, *hin-101*, which could be complemented *in trans* by pKH66 and originally isolated from a diethylsulfate-mutagenized pool, was used during the isolation of plasmid-encoded *hin* mutants.

Isolation of plasmid-encoded $B^+ R^-$ mutants of *hin*: Purified plasmid pKH66 was mutagenized as described previously (Adams *et al.* 1997) and introduced into strain TH1134 (*hin-101 fliC5050*::$MudJ^{off}$). Plasmids carrying mutations in the *hin* gene in pKH66 were identified by the inability to complement the *hin-101* mutation for recombination (R^-). Alternatively, plasmid pKH66 was mutagenized by passage through an *Escherichia coli mutD* strain, TH713 (*E. coli* strain LE30 obtained from S. Emr), which has a mutant form of the proofreading epsilon subunit of DNA polymerase III (Echols *et al.* 1983). Mutagenized pools of pKH66 were introduced into an *E. coli* strain TH613 that is used to assay *in vivo* inversion, in which expression of the *lac* operon is dependent on the ability of pKH66 to complement a *hin* mutation located on a lambda prophage (Bruist and Simon 1984). Colonies that remained Lac^- after two successive streakings for purification were grown overnight in LB supplemented with antibiotics, and plasmid DNA was isolated. Purified plasmids encoding putative defective *hin* alleles (R^-) were then retested for *in vivo* recombination activity by transforming them into TH613 and screening for the Lac^- phenotype in the transformants after growth at 37° for up to 48 hr. Plasmid preparations from those strains exhibiting the R^- phenotype after retesting were electroporated into *S. typhimurium* strains for challenge phage assays.

Strain LT2 or MS1883 carrying pKH66 *hin* mutant (R^-) alleles were screened for the ability of the mutant Hin protein to be stably expressed and bind to the various wild-type (WT) and consensus *hix* sites using the *hixC, hixL(I), hixL(II),* and *hixR(I)* challenge phages whose constructions have been described elsewhere (Hughes *et al.* 1988, 1992). The *(I)* and *(II)* designations for *hixL* and *hixR* refer to the two possible orientations of the *hix* site with respect to the *ant* gene promoter. Wild-type Hin protein expressed from pKH66 (after addition of 1 mm IPTG) binds to the *hixC* site (or to any of the other wild-type *hix* site configurations), resulting in repression of *ant* gene expression and phage lysogenization (Hughes *et al.* 1988). The ability of the *hixC* challenge phage to lysogenize results in turbid plaque formation. The inability of Hin mutant alleles to bind the *hixC* site in the *hixC* challenge phage results in lytic phage growth, and the *hixC* challenge phage forms clear plaques. Plasmid pKH66-encoded *hin* mutant alleles that are defective in the ability to complement a chromosomal *hin* mutation for recombination but are able to bind *hixC* in the challenge phage assay are referred to as binding-proficient, recombination-deficient ($B^+ R^-$) alleles.

DNA sequence analysis of $B^+ R^-$ *hin* mutants: Sequencing of $B^+ R^-$ *hin* mutants was performed on plasmid DNA purified by CsCl gradient purification (Sambrook *et al.* 1989) using the method of Sanger *et al.* (1977) or Applied Biosystems Inc. (Foster City, CA) automated sequencing per the manufacturer's instructions using the following primers, which were obtained commercially (Macromolecular Resources): HinD - TGGAAATTAGACAGACTG, HinE - TTATATCCATCCTGT TGT, and Hin2 - TACTGGTATCAATACTAT.

P22 challenge phage assays: Quantitative challenge phage assays were carried out in the TH437 (LT2) and TH2285 (*fis-3*::Cam) strains as described previously (Hughes *et al.* 1988), except those on the A131V and R8Q mutants, which were done in TH2128 and TH2128 containing additional *lac* repressor from a plasmid pTrc99A (Pharmacia Fine Chemicals, Piscataway, NJ), respectively, because of toxicity of these mutants in the LT2 background. MS1868 was not used because a mutation specific to the MS1868 genetic background that exhibited a slow-growth phenotype in *fis* strains was uncovered in the lab (Osuna *et al.* 1995).

Crude lysates containing overexpressed *hin* mutant proteins: The following *recA1 endA1 E. coli* strains were used to overexpress Hin mutant and wild-type proteins: DH1, JM109, DH5α, and XL-1 Blue (Stratagene). The level of Fis protein in *E. coli* increases more than 500-fold during the initial lag phase that follows subculturing, reaching a peak as the cells enter the exponential growth phase (Ball *et al.* 1992). The growth phase of the cells at the time of induction was chosen to maximize the intracellular levels of Fis, and the activity of the Hin mutant or wild-type protein, based on pilot experiments with WT Hin in these specific backgrounds. However, at times, the levels of Fis present in the crude extracts may have limited the observed *in vitro* activities, rendering the cleavage and inversion assays qualitative. At times, lysates expressing wild-type Hin were supplemented with 50 ng of purified Fis protein.

Strains containing pKH66 or derivatives with $B^+ R^-$ mutations were subcultured at a 1:100 dilution from a fresh 5-ml overnight culture into 250 ml LB broth supplemented with

antibiotic at 37° until reaching an OD_{600} between 0.2 and 0.4. For induction, the culture was diluted 1:4 with room temperature LB + antibiotic + IPTG at a final concentration of 1 mm, and was subcultured for 30 min at 30°. The final volume of these cultures was usually 1 liter. Cells were placed on ice for at least 20 min, pelleted at 4° at 8000 *g* in a GSA rotor, washed with 250 ml of ice-cold sterile ddH_2O and pelleted as before, and resuspended in ~2.5 ml of ice-cold sterile 20 mm Tris-HCl, pH 7.5. The cell suspension was then lysed by two passages through a French press at 20,000 p.s.i., and the resulting lysate was centrifuged at 16,000 × *g* in a tabletop centrifuge (Eppendorf) at 4° for 30 min. The resulting supernatants were used directly after quantification of protein content (Assay Kit; Bio-Rad Laboratories, Richmond, CA). Typically, the crude lysates contained ~10 mg ml^{-1} total protein. At times, aliquots of these extracts were quick-frozen in liquid nitrogen and stored at −80° in the presence or absence of 50% glycerol. For WT Hin, the presence or absence of glycerol does not seem to qualitatively affect inversion activity after one freeze-thaw (at 4°) cycle. Frozen extracts were only used for subsequent DNA-binding studies and/or Western blot analyses.

***In vitro* Hin activity assays:** DNA cleavage and inversion reaction conditions were described previously (Johnson and Bruist 1989; Haykinson *et al.* 1996) with the following modifications: 350–600 ng of pMS551 (Johnson and Simon 1985) were used as a substrate. Reactions were typically initiated by addition of 20 μg of crude lysate in a 25-μl total reaction volume. Cleavage reactions were incubated at 37° for 180 min and stopped by addition of 2 μl of 10% (w/v) sodium dodecyl sulfate (rapid quenching important) and 2 μl of 2 mg ml^{-1} proteinase K (Boehringer Mannheim, Indianapolis, IN) followed by incubation at 37° for 30 min and 65° for 10 min. Inversion reactions were quenched after 120 min of incubation at 37° by phenol:chloroform (1:1) extraction, followed by two chloroform extractions and precipitation of the DNA. The *in vitro* binding studies were as described previously (Adams *et al.* 1997) with the following modifications: CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate) was used at 20 mm, and a 120-bp fragment from a plasmid pJK110, which includes the *hixR* site, was used to make the *hixR* probe. For gel shift studies with *hin* mutants overexpressed from the pKH66 parent vector, 20–40 μg of crude lysate was used as the source of Hin wild-type or mutant proteins to detect binding to various *hix* sites.

RESULTS

New challenge phage substrates to detect Hin recombination intermediates—Tet-10G and Inv-10G: The intermediate steps in the Hin recombination reaction revealed by molecular and biochemical characterization include (1) Hin dimer formation and Fis dimer formation; (2) Hin dimers bound to *hix* DNA sites and Fis dimers bound to the recombinational enhancer; (3) invertasome formation, which includes Hin tetramer formation and possibly Hin/Fis interactions; (4) DNA cleavage by Hin, resulting in covalent attachment of Hin to *hix* DNA; (5) DNA strand rotation, resulting in the loss of four negative supercoils; and (6) religation and release (Johnson 1991). We sought to isolate Hin mutants that are specific to steps of the reaction after DNA binding (steps 3–6).

We previously used the *in vivo* challenge phage assay (Benson *et al.* 1986) to study the binding of Hin to the *hixL* and *hixR* recombination sites (Hughes *et al.* 1988). The fundamental utility of this system is that it provides a simple method to detect binding of Hin to the *hix* DNA sites *in vivo*. This way, amino acid substitution mutants in Hin that are defective in the DNA-binding step can be immediately distinguished from those that are binding proficient but defective in later stages of the recombination reaction. In the challenge phage system, a *hix* site is placed at the normal operator site for the *ant* gene promoter of bacteriophage P22 (Figure 2A). The *ant* gene encodes antirepressor, which if expressed, results in lytic growth of the phage. Binding of Hin to the *hix* sequence placed at the *ant* operator results in *ant* repression and lysogeny. The *hix* sites are symmetric 26-bp sequences and are labeled from position −13 on the left to position +13 on the right. Specific mutations in the consensus *hixC* site, such as the symmetric −10T changed to G and the corresponding +10A changed to C, referred to here as 10G (Figure 2A), render this site defective for Hin binding and result in increased *ant* expression and a lower frequency of lysogeny in strains expressing Hin (Hughes *et al.* 1992). The frequency of lysogeny is an indirect measure of Hin binding to a *hix* site, *in vivo*, and can be measured because the temperate phage confers kanamycin resistance to the cell. A consensus *hix* site, *hixC*, binds Hin in the *in vivo* challenge phage assay, as well as the wild-type *hixL* and *hixR* sites (Hughes *et al.* 1988). Hin mutants that are stable but incapable of lysogeny for the *hixC* challenge phage are presumably defective in DNA binding. We hypothesized that Hin mutants that are proficient in binding but defective in recombination could be isolated and identified as being defective in the recombination reaction *in vivo* but still able to bind the *hixC* challenge phage. This class of mutants has been described for the γδ resolvase site-specific recombination system (Hughes *et al.* 1990).

The challenge phage designed to detect Hin tetramer formation, Tet-10G: In various prokaryotic promoter regions, dimers of repressors or activators interact with each other to create a DNA loop between the bound DNA sites (Schleif 1992). In the *lac* operon, cooperative interactions take place such that a defect in binding to a mutant *lac* operator is suppressed by a second WT *lac* operator placed at an upstream or downstream site (Besse *et al.* 1986). In some cases, such as in the regulation of the *ara* operon by AraC, it is only in the absence of one binding site that the binding defect is observed at a second site (Martin *et al.* 1986). Because Hin dimers bound to *hix* sites interact to form tetramers, we hypothesized that placement of a wild-type *hix* site upstream of a defective *hix* site in the challenge phage system would allow suppression of poor binding to the defective binding site and an increase in the frequency of lysogeny (Figure 2B). The frequency of lysogeny (FOL) for wild-type strain LT2 expressing Hin from pKH66 is more

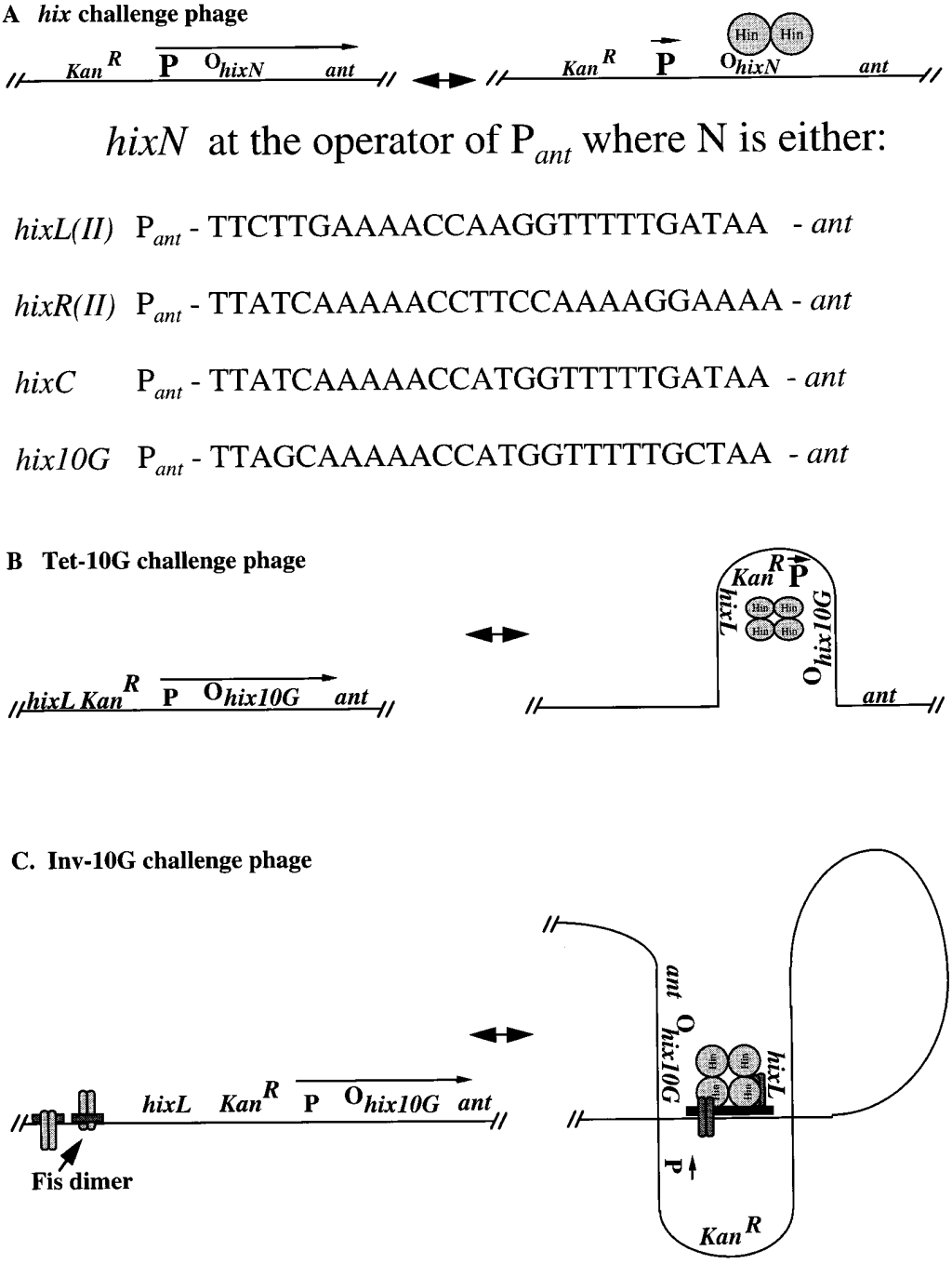

Figure 2.—Diagrammatic representation of challenge phage genomes. (A) The *hixN* construct in which the P_{ant} operator *hix* site is any one of the *hix* sequences shown below. Generally, data for only one of the two *hixL* and *hixR* site orientations (orientation II), with respect to the *ant* gene promoter, are presented in this paper. The *hixC* and *hix10G* constructs are dyads with inverted symmetry (palindromes). (B) The Tet-10G construct which, in addition to the 10G operator site, also has a wild-type *hixL* site inserted upstream of the kanamycin-resistance determinant (at the *Pst*I restriction site flanking the kanamycin resistance cassette) in the phage genome, was designed to measure Hin's ability to pair two *hix* sites, as diagrammed to the right. (C) The Inv-10G construct which, in addition to the 10G operator site, also has a wild-type *hixL* site as well as the recombinational enhancer inserted upstream of the kanamycin-resistance determinant in the phage genome, was designed to measure Hin's ability to form an invertasome-like structure with Fis as diagrammed to the right. The Tet-10G and Inv-10G phages with two *hix* sites incorporated into their genomes have a mismatch at the crossover sites to prevent recombination of the phage genome *in vivo* (Heichman *et al.* 1991).

than 100-fold lower with the mutant 10G site on a challenge phage (10G phage), compared to the FOL with the consensus *hixC* site phage (Figure 3A). A wild-type *hixL* sequence was placed ~1 kb upstream of the 10G mutant site to create the Tet-10G challenge phage, so called, based on the hypothesis that it may detect the formation of a Hin tetramer *in vivo*. This is roughly the same distance that exists between *hix* sites in the *S. typhimurium* chromosome (Johnson and Simon 1985). The idea was that binding of Hin to the *hixL* site could cooperate with a second Hin dimer in binding the defective 10G site, and repression would result only if the Hin dimers were proficient in tetramer formation. Placing the *hixL* sequence 1 kb upstream of the defective 10G operator site resulted in a 5- to 10-fold increase in the FOL compared to the 10G site alone (Figure 3A). Placement of *hixL* upstream of the *ant* operator region lacking a *hix* site had no effect on the FOL (data not shown). Thus, the Tet-10G phage was designed as an *in vivo* measure of Hin tetramer formation, and suppression of a defective *hix* site occurs in the presence of an upstream wild-type binding site.

The challenge phage designed for Hin-Fis invertasome formation, Inv-10G: When purified Hin and Fis proteins were

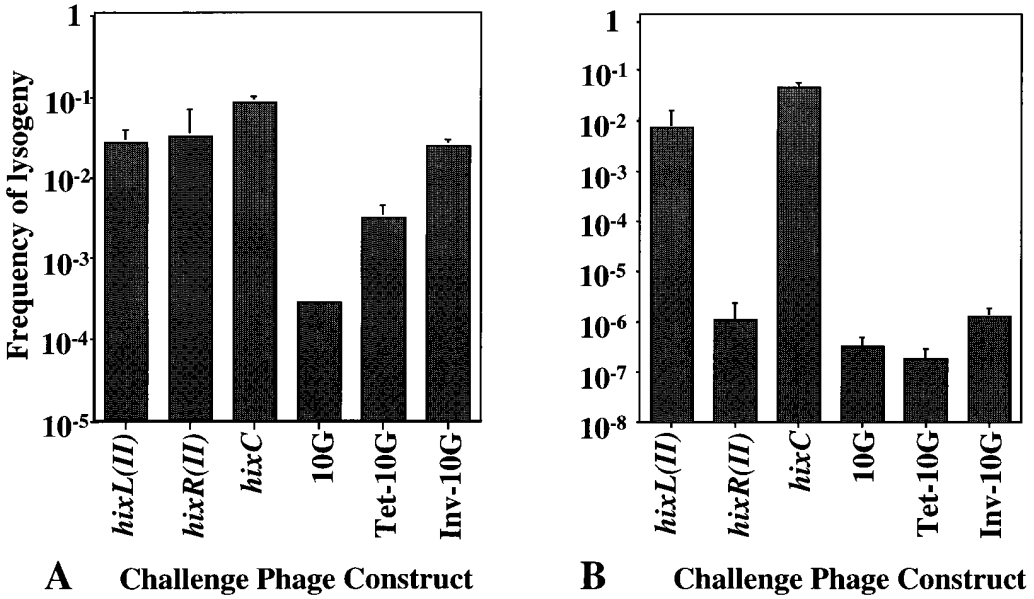

Figure 3.—The frequency of lysogeny of the different *hix* challenge phages used in this study in isogenic (A) TH437 *fis*$^+$ and (B) TH 2285*fis*$^-$ strains expressing wild-type Hin from plasmid pKH66. FOLs are the average of at least three independent assays with the estimated standard deviation based on a sample ($n - 1$) shown in the error bars. Note the differences in scales for the FOL values between A and B.

added to supercoiled DNA substrate, including the *hix* sites flanking the recombinational enhancer element, a stable protein-DNA complex called the invertasome was observed (Heichman and Johnson 1990). We hypothesized that if Hin could bind to a wild-type *hixL* site and suppress a defective *hix* site at the *ant* operator in the Tet-10G phage, then placement of both a wild-type *hixL* site and an RE upstream might allow invertasome formation in the challenge phage and even better suppression of the 10G-binding defect (Figure 2C). Such a phage was constructed and designated Inv-10G, so called, based on the hypothesis that it may detect the formation of an invertasome-like structure *in vivo.* A mismatch in the core sequences of the two *hix* sites on the Inv-10G phage was necessary to prevent recombination from occurring on the phage genome (Johnson and Simon 1985). Therefore, the connectivity between complex formation and subsequent steps that take place in the normal invertasome, including strand exchange, is not the same in the Inv-10G challenge phage, which was designed merely to mimic the invertasome. Results shown in Figure 3A demonstrate that the presence of both an upstream *hixL* site and the RE in the Inv-10G phage were able to suppress the 10G-binding defect, and the FOL obtained with wild-type Hin expressed in strain LT2 is nearly the same as the FOL observed with the *hixC* challenge phage. These data argue that more efficient repression of *ant* is supported by the formation of a synaptic complex between the two *hix* sites and the recombinational enhancer. This effect depends on the presence Hin, Fis, and the enhancer (Figure 3, A and B). Placement of both a wild-type *hixL* site and the RE upstream of the *ant* operator region lacking a *hix* site had no effect on the FOL (data not shown). These experiments suggest that the Inv-10G challenge phage can be used to assay the formation of an invertasome-like structure on the phage *in vivo*, and that challenge phages can be used to select for the assembly of not only looped structures, but also of higher-order protein–DNA structures such as the invertasome.

Effect of Fis on Hin repression of *ant* in the *hixL*, *hixR*, *hixC*, 10G, Tet-10G, and Inv-10G challenge phages: binding of Hin to *hixR*, 10G, Tet-10G, and Inv-10G requires Fis: We hypothesized that suppression of the defective *hix* site in the Tet-10G phage resulted from Hin dimers interacting to form tetramers, and suppression of the defective *hix* site in the Inv-10G phage resulted from interaction of Hin dimers as well as interactions between Hin and Fis to create the invertasome. Because tetramer formation can occur in the absence of Fis, but invertasome formation requires Fis (Heichman and Johnson 1990), suppression in the Tet-10G phage was expected to be independent of Fis, while suppression in the Inv-10G phage was expected to be Fis dependent. Binding to individual *hix* sequences, *hixC*, *hixL*, and *hixR*, as well as 10G, was also tested as a control: we expected that binding of Hin to the individual *hix* sites would be independent of Fis. As shown in Figure 3B, we did not obtain the expected results.

As expected, repression by Hin with either the *hixC* or *hixL* challenge phages were essentially the same in isogenic *fis*$^+$ and *fis*$^-$ strains expressing Hin from pKH66. Also as expected, suppression by the presence of upstream *hixL* and RE sequences in the Inv-10G phage was dependent on a functional *fis* gene. In the *fis* mutant strain, the FOL for the Inv-10G phage was reduced up to 10^4-fold, compared to the *fis*$^+$ strain, to the same FOL observed for the 10G phage in the *fis*$^-$ background.

Lysogeny by the 10G phage relative to *hixC* was down 10^5-fold in the *fis*$^-$ background compared to the more than 100-fold reduction in the isogenic *fis*$^+$ strain. The binding of Hin to individual *hix* sites is thought to be

independent of Fis (Glasgow *et al.* 1989). In addition, Hin repression in the *hixR* and Tet-10G challenge phages was also dependent on Fis, with a reduction of up to 10^4-fold without Fis. Somehow, Fis is able to interact with either Hin or unknown P22 DNA sequences, to enhance the ability of Hin to repress in the 10G, Tet-10G, and *hixR* challenge phages. We have not observed an effect of Fis on lysogeny of wild-type P22 phage (data not shown).

DNA binding-proficient, recombination-deficient (B^+ R^-) Hin mutants: A large number of *hin* mutants were isolated and subjected to genetic classification using the various challenge phage screens devised above. Plasmid pKH66, containing the *hin* gene under an inducible promoter, was mutagenized, and 450 independent *hin* mutants were obtained that were no longer able to catalyze recombination (R^-). The *in vivo* inversion assays that were used as a qualitative screen measured the ability of Hin expressed from pKH66 to invert a DNA fragment containing a promoter on the Salmonella chromosome in a *hin*$^-$ strain or on a lambda prophage in *E. coli* (see materials and methods). For either assay, inversion activity caused by Hin expressed from pKH66 in the tester strain results in a Lac^+ phenotype on MacConkey lactose plates after growth at 37° for at least 24 hr, whereas the R^- *hin* mutants yield only Lac^- colonies (tested up to 48 hr). Mutants that were not affected at the level of protein stability were first identified by Western blot with anti-Hin antibody on a subset of 154 R^- mutants. Within this subset, 34 independent mutants had qualitatively wild-type or near wild-type, steady-state levels of Hin protein in extracts. DNA sequence analysis of these 34 mutants revealed 25 different single amino acid substitutions that resulted in the R^- phenotype without affecting protein stability. Three of these substitutions, G139E, G139R, and A166V, are located in the DNA-binding domain (amino acids 138–190) of the 190-amino-acid Hin protein (Glasgow *et al.* 1989; Sluka *et al.* 1990; Hughes *et al.* 1992; Feng *et al.* 1994b), while the remaining 22 substitutions reside in what is referred to as the catalytic domain (amino acids 1–137; Adams *et al.* 1997).

The *in vivo* DNA-binding "challenge phage" assay was used to screen for Hin's DNA-binding activity independent of the recombination activity (see materials and methods). Using the *hixC* challenge phage, the subset of 154 *hin* R^- mutants were assayed for their ability to bind the *hixC* recombination site *in vivo.* 17/22 R^- mutants that did not affect protein levels and had changes in amino acids of the catalytic domain were proficient in binding the *hixC* recombination site (B^+). The remaining five mutants were not binding proficient. Thus, the challenge phage screen was able to identify binding-proficient, recombination-deficient (B^+ R^-) mutants directly using a simple *in vivo* plate assay. The R^- mutants affecting amino acid positions 139 and 166 of the DNA-binding domain, which did not affect protein levels, were deficient in binding the *hixC* recombination site (B^-). The ability of *hin* mutants to bind the *hixC* site *in vivo* is sufficient to identify *hin* mutants that do not affect protein stability and are specific to steps in the recombination reaction that occur after *hix* DNA recognition (steps 3, 4, 5, or 6 defined above). Of the remaining 296 R^- *hin* mutants, 37 were found to be of the B^+ R^- phenotype in the challenge phage assay using the *hixC* and *hixL* phages. DNA sequence analysis of these B^+ R^- mutants identified an additional 10 single amino acids substitutions in the catalytic domain resulting in the B^+ R^- phenotype. This screen eliminated *hin* mutants defective in protein stability and DNA binding (78% of the original 154 screened) and allowed us to focus on the 27 *hin* mutants specific to the DNA recombination reaction (B^+ R^-). These 27 mutants were also proficient in binding the *hixL* and *hixC* sites in the challenge phage assay.

The challenge phage system detects Hin-mediated invertasome formation *in vivo* and segregates *hin* mutants into distinct genetic classes: The 27 distinct B^+ R^- *hin* amino acid substitution mutants isolated above were tested for their ability to bind and repress the *ant* gene using the 10G, Tet-10G, and Inv-10G challenge phages. The results presented in Figure 4 show that the *hin* mutants of the B^+ R^- class fell into five genetic classes. A sixth class includes the G139R, G139E, and A166V B^- R^- mutants that are defective in the initial binding step and, therefore, also defective in recombination (B^- R^-).

One class (E) of mutant B^+ R^- Hin proteins binds the 10G site more efficiently than the wild-type Hin protein (Figure 4, E *vs.* F). Therefore, they are designated as having an enhanced binding affinity in that they can bind the 10G mutant site as efficiently as a wild-type *hix* site, whereas WT Hin does not. These mutants may also have an additional downstream effect in recombination. The R43H and R69C substitutions had also been previously isolated by their ability to bind mutant *hix* sites, and a more extensive characterization of these mutants has been presented elsewhere (Adams *et al.* 1997).

A second class (A and B) of B^+ R^- mutants showed increased suppression for the Inv-10G phage assays compared to 10G, similar to wild-type Hin (Figure 4, A and B). They are predicted to be capable of invertasome formation and have been designated the I^+ class among the B^+ R^- *hin* mutants. The recombination defect for this class is predicted to be specific to the DNA cleavage, strand rotation, religation, and release steps in the reaction.

Within this I^+ class are two subclasses. The first class (A) suppressed the defective 10G site equal to or better than WT Hin, when comparing the Inv-10G phage FOL to the 10G phage FOL (Figure 4A). It is important to note that some of the mutants in the larger I^+ class exhibit a lower FOL by the 10G phage compared to WT

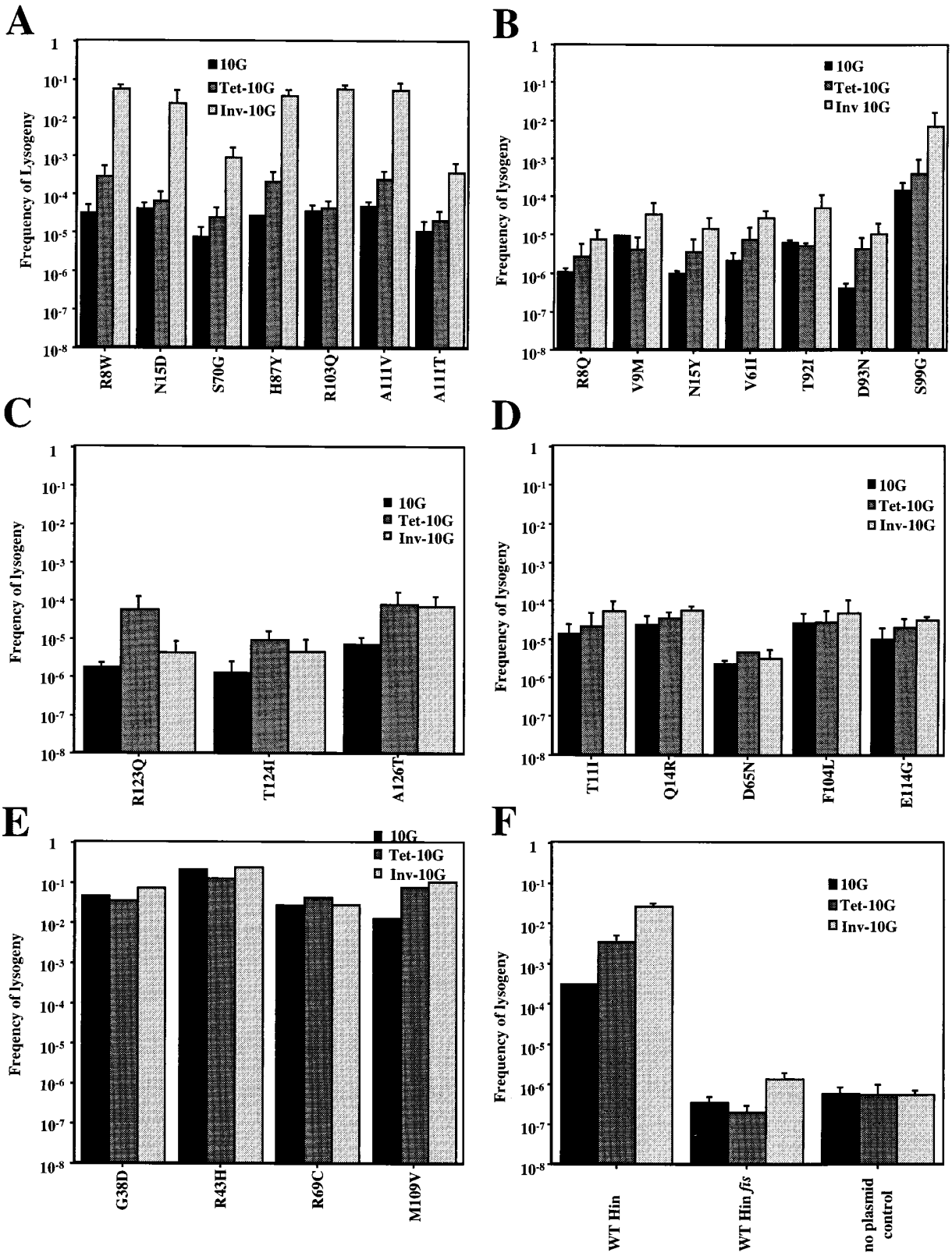

Figure 4.—Genetic classification of Hin mutants based on results from at least two independent assays with challenge phages 10G, Tet-10G, and Inv-10G. Frequencies of lysogeny are the average of at least three independent assays with the estimated standard deviation based on a sample ($n - 1$) shown in the error bars: (A) I^+ class, (B) $I^{\pm}$ class, (C) T^+ I^- class, (D) T^- I^- class, (E) enhanced binding affinity class, and (F) wild-type Hin and wild-type Hin in a *fis* strain, as well as the no plasmid controls. Similar results were obtained in a chromosomal *hin* background (data not shown).

Hin, which prompted the use of the ratio of FOL on the Inv-10G phage to the FOL on the 10G phage compared to this ratio for WT Hin as the main criterion for distinguishing the I^+ class. The second I^+ subclass (B) maintained a reduced capability (~20%) to form the invertasome *in vivo* by these same criteria, compared to WT Hin, and was designated $I^{\pm}$ (Figure 4B).

A third class of B^+ R^- mutants (C) showed some suppression only in the Tet-10G phage and not in the Inv-10G phage, and has been designated the T^+ I^- class (Figure 4C). The main criteria used to distinguish this class were the ratio of the Tet-10G FOL *vs.* the 10G FOL and the absence of better suppression on the Inv-10G phage *vs.* the Tet-10G phage. It is important to note that some of the mutants in the T^+ I^- class exhibit a lower FOL by the 10G phage compared to WT Hin, which prompted the use of the ratio of FOL on the Tet-10G phage to the FOL by the 10G phage, compared to this ratio for WT Hin, as one criterion for distinguishing the T^+ I^- class. Because they show suppression with the Tet-10G phage and not the Inv-10G phage, they are predicted to be proficient in tetramer formation through the pairing of Hin dimers, but unable to generate the invertasome. A negative effect by the addition of the enhancer on FOL for the R123Q mutant was observed with the Inv-10G construct compared to the Tet-10G construct (Figure 4C). This may indicate a disruption of a Hin/Fis interaction required for efficient formation of an invertasome-like structure on the Inv-10G phage that is not required for tetramer formation on the Tet-10G construct. This T^+ I^- class also falls into two subclasses. Because the *hixR* site was the only wild-type *hix* site where WT Hin exhibited a Fis^- dependence for binding, we hypothesized that some interaction is required between Hin and Fis to bind *hixR* efficiently. It was expected that mutants in the T^+ I^- class may be disrupted for this type of putative interaction between Hin and Fis. The *in vivo* binding reflected by the FOL in the T124I mutant on the *hixR* challenge phage in fis^+ strains resembled that seen for the wild-type Hin protein in a fis^- background (Figure 5). The *hixL* data are included as a positive control to rule out a general binding defect for the T124I and A131V mutants. The R123Q and A126T mutants of the T^+ I^- class bound the *hixR* challenge phage about as well as WT Hin (Figure 5).

A fourth class (D) of B^+ R^- mutants showed no suppression by either *hixL* in the Tet-10G phage or *hixL* plus the RE in the Inv-10G phage compared to their respective FOL on the 10G phage (Figure 4D). If the Tet-10G phage does indeed detect tetramer formation, then these mutations are predicted to impair the ability of the protein to synapse the 10G and *hixL* sites, yielding a tetramer. Consequently, they are also predicted to be defective in invertasome formation. These are designated T^- I^-.

A final mutant, A131V, could not be specifically classified. Phenotypically, using the *hixL*, 10G, Tet-10G, and Inv-10G, it behaved like a T^- I^- mutant in the TH2128 background (data not shown). However, unlike all other mutants, A131V gave a B^+ R^- phenotype for one of the two orientations of *hixL* at the *ant* operator site of the phage, *hixL(II)*. The *(I)* and *(II)* designations refer to the two possible orientations of the *hixL* site relative to the *ant* gene promoter in the challenge phage construct (see materials and methods). Using the *hixL(I)* challenge phage, we obtained a FOL of 10^{-6}. All other B^+ R^- mutants showed no preference for the orientation of the *hixL* site in the challenge phage (data not shown).

Biochemical characterization of B^+ R^- Hin mutant proteins: Biochemical analysis was performed on the various B^+ R^- *hin* mutants to assess the correlation between the step in recombination that is blocked, as inferred by the *in vivo* challenge phage data, and the step in recombination that is blocked, as defined by standard biochemical assays (Haykinson *et al.* 1996). The ability of mutant proteins to assemble various nucleoprotein intermediates can be detected using the *in vitro* Hin-mediated DNA cleavage and inversion assays. Purified Hin and Fis proteins catalyze DNA inversion on a supercoiled plasmid DNA substrate that carries *hixL* recombination sites and the RE. If the assay is done in the absence of Mg^{2+} and in the presence of EDTA and ethylene glycol, Hin-mediated DNA cleavage occurs, but not recombination (Johnson and Bruist 1989). This provides a biochemical assay for Hin-mediated DNA cleavage.

Cell extracts from *E. coli recA1 endA1* strains overexpressing wild-type or B^+ R^- Hin mutant proteins from the low-copy-number (pKH66 parent) plasmid vector were screened for DNA inversion (Mg^{2+} present) and cleavage (Mg^{2+} absent, EDTA and ethylene glycol present) activities using a supercoiled plasmid containing

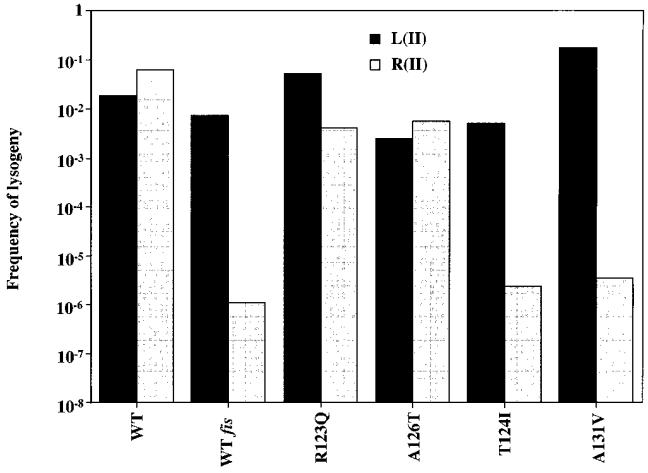

Figure 5.—Representative results from challenge phage assays with the *hixL(II)* and *hixR(II)* phages on mutants from the T^+ I^- class, A131V, and wild-type controls.

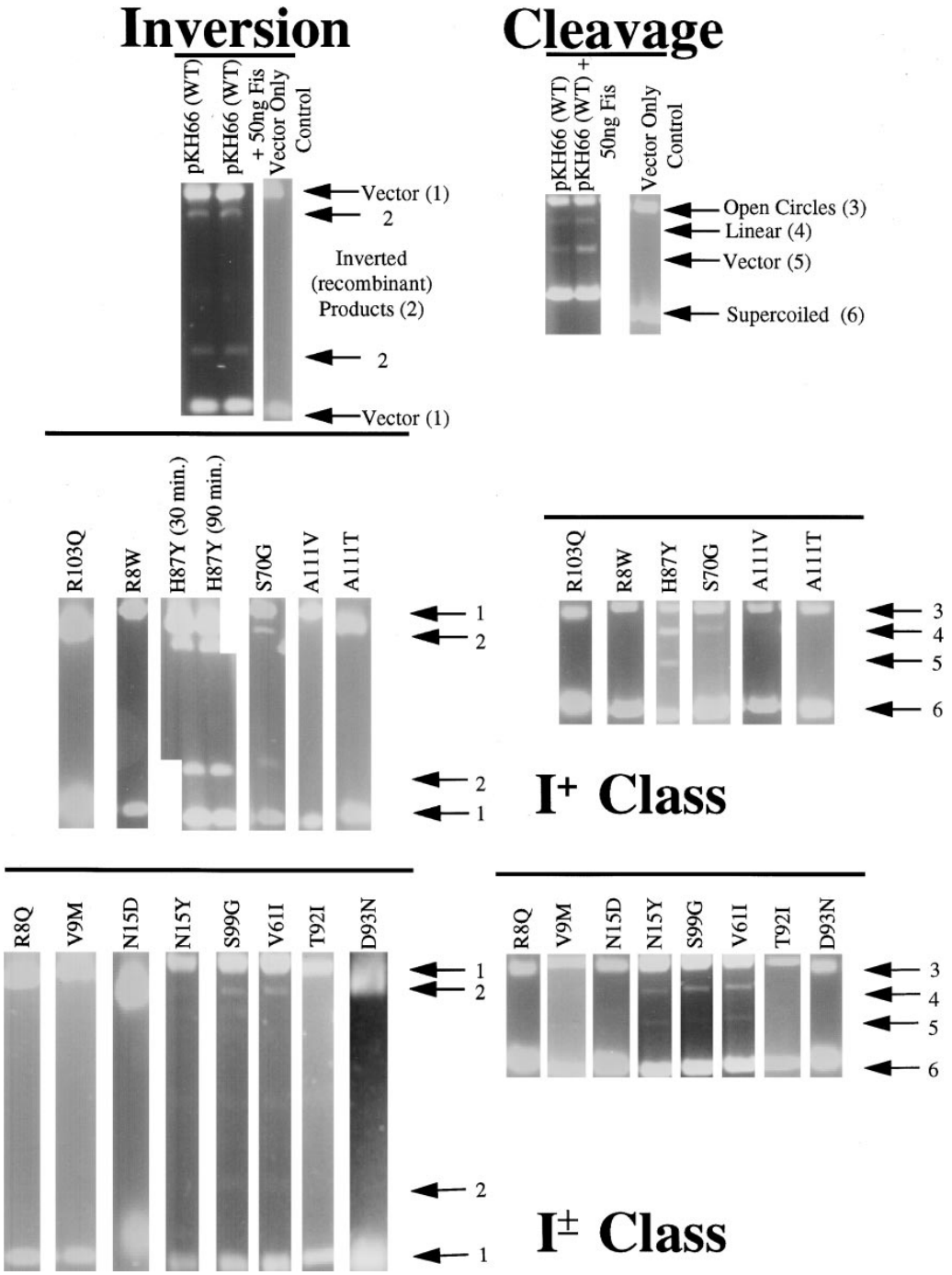

Figure 6.—Hin-mediated DNA inversion and DNA cleavage assays with crude lysates prepared from *E. coli* cells overexpressing wild-type and various *hin* mutants, labeled in the diagram directly above the gel lane. Inversion moves two restriction endonuclease cleavage sites with respect to each other. Restriction endonuclease cleavage after inversion yields two different-sized bands labeled "Inverted (recombinant) Products." At least two independent crude lysates were prepared on each strain, for which data are shown, and both showed similar results. A single-cleavage event by Hin yields a linear product; a double-cleavage event excises the DNA flanked by the *hix* sites yielding the vector and excised insert (not shown).

two symmetrically oriented *hixL* sites and the enhancer as a substrate (see materials and methods). All of the mutants tested with these cell extracts were qualitatively able to bind (as well as WT) either the *hixC*, *hixL*, or *hixR* sites *in vitro* by gel mobility shift assays when using 20–40 μg of total protein per reaction (data not shown). No binding activity for either of these sites was discernible using 40 μg of an extract prepared with the vector only as a negative control (data not shown). This is in agreement with the *in vivo* B^+ phenotype of the challenge phage assay. All but five B^+ R^- *hin* mutants failed to catalyze the DNA inversion reaction *in vitro* (Figure 6 and Table 1). These mutants were H87Y, S70G, S99G, V61I, and A131V. The H87Y and S70G mutants are from the I^+ class of B^+ R^- alleles, the S99G and V61I mutants are from the $I^\pm$ class of B^+ R^- alleles, while the A131V mutant did not fit into a specific class. It was expected that only leaky mutants would be corrected for DNA inversion by changing the reaction conditions from *in vivo* to *in vitro*. The S99G and V61I mutants exhibited qualitatively lower inversion activities than the wild-type protein or the other *in vitro* inversion-proficient mutants. The H87Y, S70G, S99G, V61I, and A131V mutants were also proficient in the *in vitro* DNA cleavage reaction. Because DNA cleavage is required for inversion, this was the expected result for these mutants.

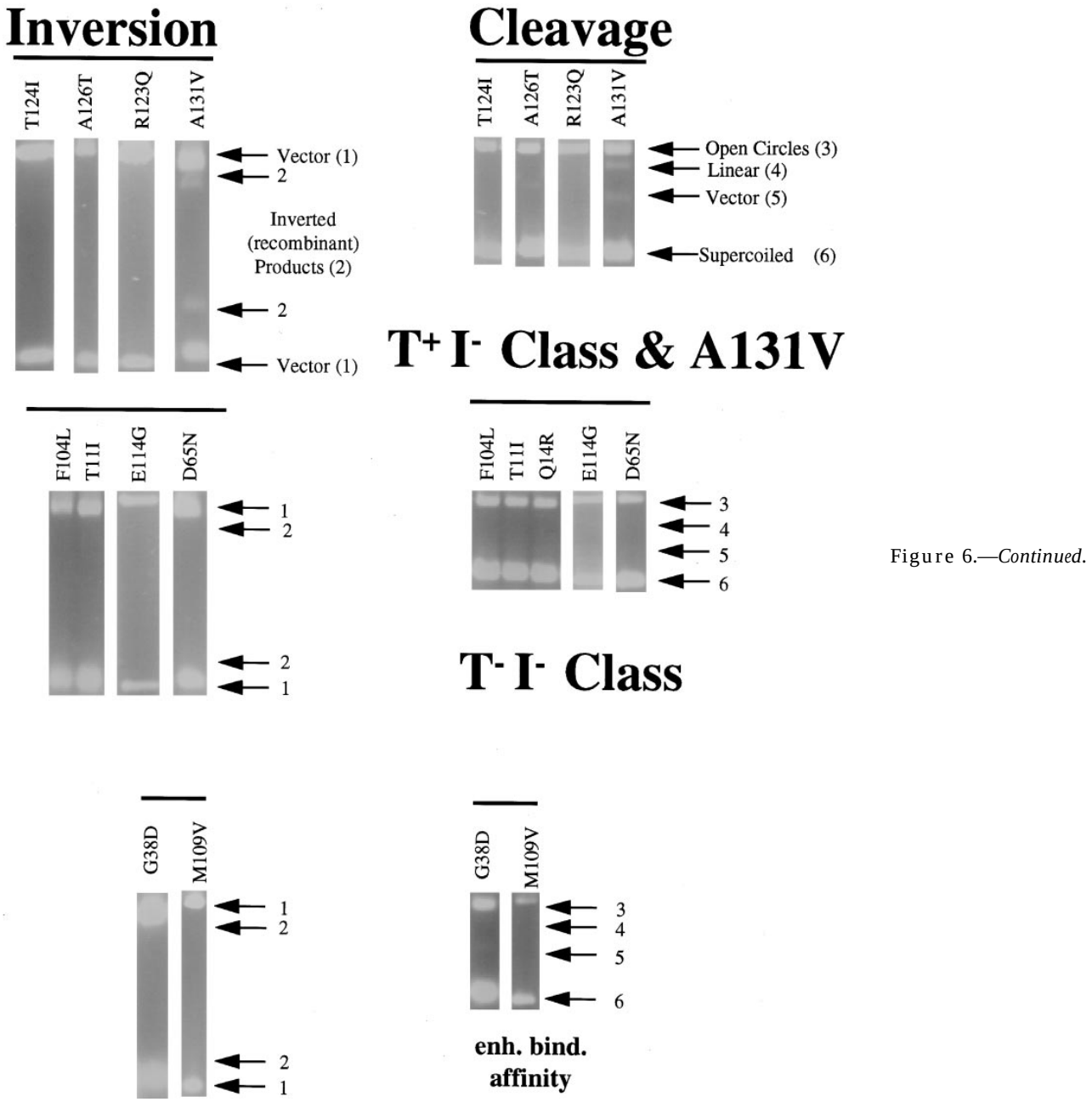

Figure 6.—*Continued.*

The N15Y mutant within the $I^{\pm}$ class exhibited qualitatively lower cleavage activity than the wild-type protein but no inversion activity. The A126T mutant within T^{+} class yielded only the linear product band in cleavage assays, presumably because double-strand cleavage, but not inversion, takes place at one *hix* site under the conditions tested (Figure 6 and Table 1).

DISCUSSION

Genetic classification of recombination-specific Hin mutants: We had been successful in using the P22-based challenge phage system in combination with molecular and biochemical studies to characterize the binding of Hin to the *hix* DNA sites (Hughes *et al.* 1992). Subsequent publication of the three-dimensional structure of the Hin-binding domain cocrystallized with the *hix* DNA half-site showed complete agreement between the results obtained by both methods (Feng *et al.* 1994b). In addition, the genetic data revealed the quantitative importance of minor groove interactions to overall DNA binding. Having used the P22 challenge phage system to successfully separate the DNA-binding step from the remaining steps of the recombination reaction, we decided to modify the system in ways that might allow the genetic characterization of the holy grail of the site-specific recombination reaction, the molecular process of DNA strand exchange. How could we design the challenge phage to obtain amino acid substitutions that were defective in specific recombination steps?

TABLE 1

Activities of mutant Hin proteins *in vitro*

Mutation	Cleavage	Inversion	Class
Wild-type	+	+	
Vector only	−	−	
R8W	−	−	I^+
S70G	+	+	I^+
H87Y	+	+	I^+
R103Q	−	−	I^+
A111V	−	−	I^+
A111T	−	−	I^+
R8Q	−	−	$I^\pm$
V9M	−	−	$I^\pm$
N15D	−	−	$I^\pm$
N15Y	±	−	$I^\pm$
V61I	±	±	$I^\pm$
T92I	−	−	$I^\pm$
D93N	−	−	$I^\pm$
S99G	±	±	$I^\pm$
R123Q	−	−	T^+I^-
T124I	−	−	T^+I^-
A126T	(s)	−	T^+I^-
T11I	−	−	T^-I^-
D65N	−	−	T^-I^-
F104L	−	−	T^-I^-
E114G	−	−	T^-I^-
A131V	+	+	None
G38D	−	−	Enh. bind.
M109V	−	−	affinity

Summary of *in vitro* cleavage and inversion data from Figure 6. In the cleavage column, (+) indicates the presence of double-*hix* cleavage events, whereas (s) denotes double-strand cleavage events, presumably at one *hix* site. For the inversion column, (+) indicates the presence of inverted (recombinant) product in the reaction. For either column, (±) indicates qualitatively less cleavage or inversion product(s) relative to the amount of product(s) visible in the wild-type control lanes. A minimum of two crude lysate preparations was assayed for each protein. In all cases, results were similar from independently prepared lysates.

Using a variety of mutagenic methods, hundreds of *hin* mutants defective in the recombination reaction were obtained (R^-). Those able to bind in the *hixC* challenge phage system were labeled as binding proficient (B^+). Because they are able to bind the *hixC* site, we presume that these mutants make normal levels of protein; this was confirmed by Western analysis (not shown).

These Hin mutants could be further classified *in vivo* using the Tet-10G and Inv-10G phage assays. At least six classes of R^- *hin* mutants could be defined *in vivo* using the following challenge phages: (A) I^+, presumably proficient in invertasome formation, (B) $I^\pm$, (C) $T^+ I^-$, (D) $T^- I^-$, presumably deficient in tetramer and invertasome formation, (E) enhanced binders, and one other class of mutants disrupted in DNA binding. It is possible that any one mutation could affect more than one function, or that it may have more complex allosteric effects. These factors complicated the genetic classifications of the mutants, and some differences within classes have been presented. However, the Tet-10G and Inv-10G challenge phages allowed the *in vivo* identification and classification of recombination intermediates in the Hin/Fis catalyzed DNA inversion reaction that results in flagellar phase variation in Salmonella sp.

Binding of Hin to defective *hix* sites *in vivo* is dependent on Fis: Unexpectedly, the suppression using the Tet-10G phage was found to be Fis dependent. Subsequent *in vivo* analysis of Hin binding in the *hixC*, *hixL*, *hixR*, and 10G challenge phages revealed that Hin binding in the *hixC* and *hixL* phage assays was Fis independent, while Hin binding in the *hixR* and 10G phages was Fis dependent. The relative binding affinity of purified Hin to the 10G site is only ~40% of the binding affinity to a *hixC* site, as measured *in vitro* using filter-binding assays (Hughes *et al.* 1992). In addition, it was shown previously that only one of the half-sites in the *hixR* sequence, the consensus half-site, is primarily responsible for binding Hin *in vivo* and *in vitro* (Hughes *et al.* 1988; Glasgow *et al.* 1989). *In vivo*, the *hixC* and *hixL* sequences bind Hin with a higher affinity than the 10G sequence. This suggests that Fis-dependent binding in the Tet-10G phage results in the 10G mutant site being below a minimal binding threshold, even with an upstream *hixL* wild-type site present in *fis* strains. Fis-dependent binding to the *hixR* site suggests that Fis may have a dual role in the DNA inversion reaction. One role is to bind the recombinational enhancer and interact with Hin/*hix* complexes to form the invertasome structure. The other role of Fis may be to bind Hin, either bound or about to bind, to *hix* sites (especially *hixR*), and this complex may then trap the enhancer. This second role may be required because Hin is limiting in the cell, and the *hixR* sequence is defective in binding Hin, when compared to the *hixL* site *in vivo*, in the absence of Fis.

Another interpretation of these results is that these amino acid substitutions affect a Fis-dependent conformational change in Hin that normally enhances the ability of Hin to bind the 10G and *hixR* sites. The R8W mutant is able to bind the *hixR(I)* challenge phage efficiently, even in a *fis*$^-$ strain (data not shown). This gain of function property of the R8W mutant over WT Hin for binding *hixR(I)* in *fis*$^-$ strains is consistent with this alternative interpretation of our results.

Taken together, these data are also consistent with a model recently proposed for the Hin recombination reaction that holds that the primary rate-limiting step for assembling the invertasome may be a Fis-dependent conformational adjustment in Hin that is required to initiate concerted DNA cleavage (Haykinson *et al.* 1996). By interfering with or altering such a potential conformational change in Hin, the mutations from the $T^+ I^-$ class may be causing the observed cleavage and/or inversion defects. These results suggest a role for Fis

before invertasome formation that is independent of the recombinational enhancer, although nonspecific DNA binding may still be required.

Homology between Hin and γδ resolvase—models for invertasome structure: The Hin recombinase is a member of a large family of bacterial DNA invertases whose members include Gin from phage Mu (Kamp and Kahmann 1978), Cin from phage P1 (Kutsukake and Iino 1980), Pin from the e14 prophage of *E. coli* (Plasterk and Van de Putte 1985), and others (Glasgow *et al.* 1989b). In addition, Hin shares ~40% amino acid homology with DNA resolvases from the Tn*3* and γδ transposons (Feng *et al.* 1994a; Rice and Steitz 1994). Crystallographic studies revealed that the Hin DNA-binding domain and the γδ resolvase DNA binding domain are almost identical in their three-dimensional structure. Because of this homology, a hypothetical three-dimensional model of the Hin recombinase bound to the *hix* DNA sites has been constructed using the coordinates from the Hin-binding domain and *hix* half-site cocrystal structure for the Hin binding domain, as well as the γδ resolvase "catalytic domain" coordinates (Feng *et al.* 1994a). This model is used below in the interpretation of our genetic and biochemical analysis of the Hin B^+ R^- mutants isolated in this study.

Localization of mutants onto the predicted Hin structure provides clues about the structure of the invertasome *in vivo*: Mutants proficient in invertasome formation *in vivo* (I^+ class) are hypothesized to be blocked at a step subsequent to invertasome formation. Such defects may affect one or more of the following: (1) DNA cleavage, (2) strand exchange, (3) religation, and (4) dissociation of the invertasome complex from DNA. Some mutants in this class localize to or are near the presumed active site in Hin. Based on data from the γδ resolvase system, the Arg-8, Val-9, Asn-15, and Ser-70 positions identified here are candidates for being either part of the Hin active site or directly involved in catalysis

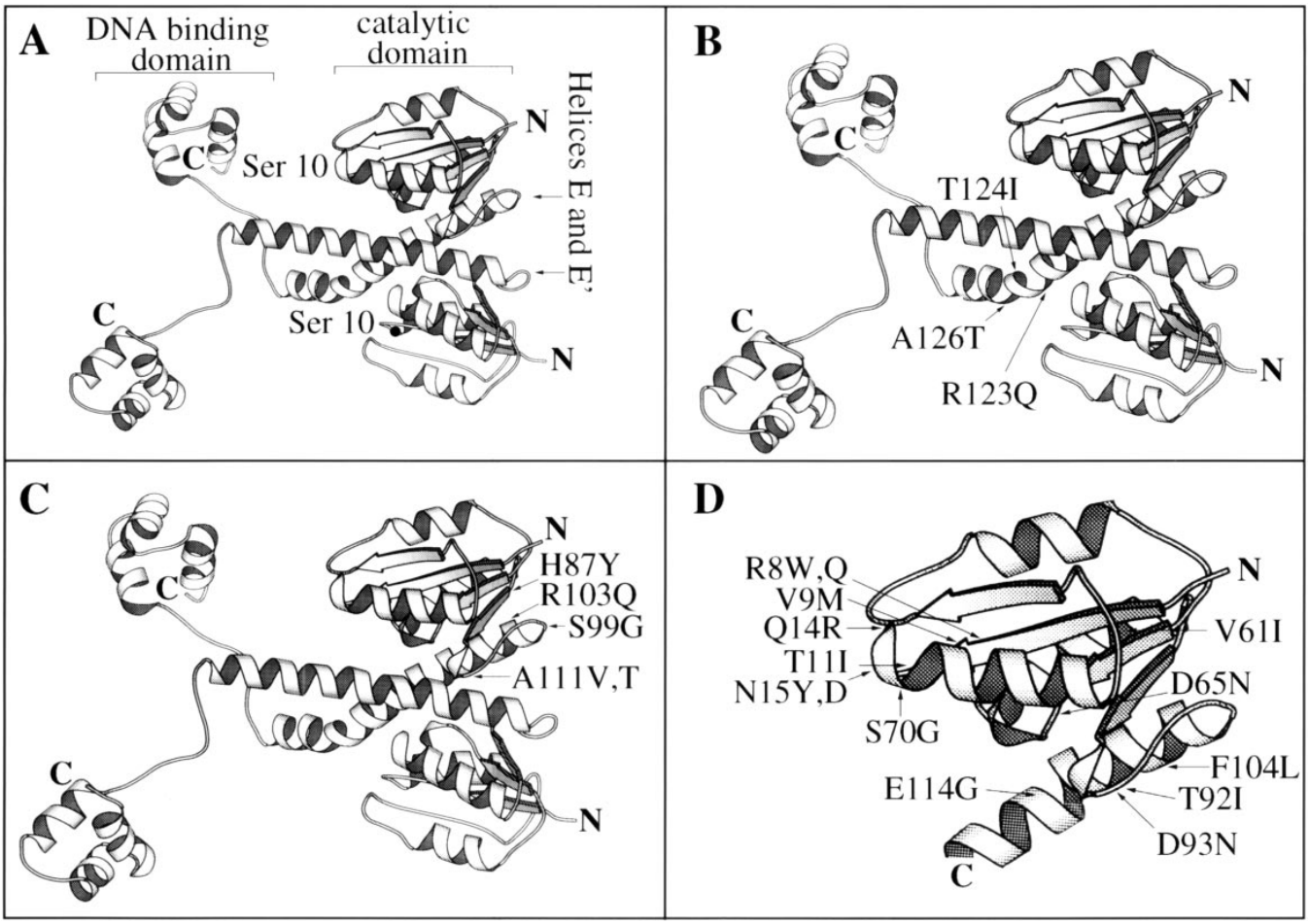

Figure 7.—Model of B^+ R^- *hin* mutants on the Hin dimer structure (Haykinson *et al.* 1996). (A) The DNA-binding domain is localized to the C terminus of Hin, while the N terminus makes up the catalytic domain. The active-site serine-10 is labeled with a ball in the one monomer where it is visible. The α helices E and E′ make up the dimer interface (Haykinson *et al.* 1996; Lim 1994). (B) The T^+ I^- class lies in the C-terminal half of the E helices. Only one of the Hin monomers is labeled. (C) Localization of the I^+ subclass amino acid substitutions H87Y, S99G, R103Q, A111V, and A111T. (D) One Hin monomer up to amino acid 120 is depicted with the T^+ I^- class and the $I^{\pm}$ subclass labeled. This figure was prepared using MOLSCRIPT (Kraulis 1991).

(Hatfull *et al.* 1987; Hughes *et al.* 1990; Boocock *et al.* 1995; Yang and Steitz 1995). In addition, the ability of the S70G mutant to carry out *in vitro* inversion also supports the assertion that these mutants are proficient in invertasome formation.

We modeled our B^+ R^- mutants to a hypothetical Hin structure that is based on a crystal structure of the Hin DNA-binding domain and that of the homologous γδ resolvase catalytic domain (Haykinson *et al.* 1996). The T^+ I^- class of mutants include R123Q, T124I, and A126T (Figure 4C). These lie in the C-terminal half of the E helices, as diagrammed in Figure 7B. By our genetic data, these mutants are predicted to be proficient in tetramer formation through the pairing of Hin dimers, but they are unable to generate the invertasome. Because the *in vivo* binding of the T124I and A131V mutants using the *hixR* challenge phage in *fis*$^+$ strains resembled that seen for the wild-type Hin protein in a *fis*$^-$ background (Figure 5), these may represent a region of Hin that interacts with Fis during the formation of the invertasome. The A126T and R123Q did not exhibit a *hixR*-binding defect, while T124I did. This may result from a more severe defect in Fis interactions with the T124I mutant. Mutants from the I^+ class did not exhibit *hixR*-binding defects, as may be predicted from the above model (O. Z. Nanassy and K. T. Hughes, unpublished results). Alternatively, this region in Hin may undergo a conformational change as a result of Hin/Fis interactions that lead to assembly of the invertasome, and these mutations interfere with this conformational change.

The amino acid substitutions H87Y, S99G, R103Q, A111V, and A111T of the I^+ subclass localize in areas of the protein distant from the presumed active site (Figure 7, A and C). The active site placement for Hin in this case was inferred from comparisons with the results from the γδ resolvase system (Yang and Steitz 1995). It is possible to imagine a surface extending across from the Hin His-87 through Ser-99, Arg-103, and Ala-111, as defining a potential dimer/dimer interface within the invertasome, based on the positions identified by our genetic I^+ subclass mutants. The residue affected by the Hin H87Y mutant (γδ Ser-89) is exposed at the amino end of β-strand 4 in the structure of the γδ resolvase catalytic domain (Sanderson *et al.* 1990). This region in γδ resolvase has been implicated in interactions between resolvase dimers bound to DNA within the resolvasome (Hughes *et al.* 1993). Therefore, our findings for this region in Hin are consistent with the results from the γδ resolvase system.

The T^- I^- class (T11I, Q14R, D65N, F104L, and E114G) is predicted to be defective in tetramer formation by our genetic data. Also, the $I^\pm$ subclass of the I^+ mutants (R8Q, V9M, N15Y, V61I, T92I, D93N, and S99G) had a reduced capability (~20%) to form the invertasome *in vivo*, compared to wild-type Hin. The combination of the $I^\pm$ subclass and T^- I^- class localize to three distinct regions: one region from amino acids 8–15, one region including amino acids V61, D65, and H87, and a third region in the N-terminal portion of helix E including amino acids 92–114 (Figure 7D). These two sets of mutants may have more complex allosteric effects on the recombination reaction after DNA binding. It is possible that the I^- mutants are just more severe than the $I^\pm$ subclass and actually affect the same steps in the reaction.

Until now, evidence for the invertasome was obtained only from studies using the wild-type protein *in vitro* (Heichman and Johnson 1990). Further biochemical characterization of purified mutants obtained from this study will allow us to substantiate the observed *in vivo* defects *in vitro*. The novel *in vivo* methodology outlined in this study may then prove to be an effective approach for visualizing the higher-order geometry of the Hin invertasome, as well as other nucleoprotein complexes, in their dynamic cellular states.

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