Phase variation in *Salmonella*: analysis of Hin recombinase and *hix* recombination site interaction in vivo

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The bacteriophage P22-based challenge phase selection was used to characterize the binding of *Salmonella* Hin recombinase to the wild-type *hixL* and *hixR* recombination sites, as well as to mutant and synthetic *hix* sequences in vivo. Hin recombinase binds to the *hixL* or *hixR* recombination sites and represses transcription from an upstream promoter in the challenge phage system. Hin-mediated repression results from Hin associating into multimers either prior to binding or during the binding process at the *hix* operator sites (cooperativity). The ability of Hin multimers to repress transcription is eliminated when the *hix* 13-bp half-sites are rotated to opposite sides of the DNA helix by inserting 4 bp between them. Insertion of 1 bp between half-sites reduces overall repression. Hin also binds one of the *hixL* half-sites to repress transcription, but only when high levels of Hin protein are present in the cell. Mutations have been identified in the *hix* sites that impair Hin binding. Five of the 26 bp in the *hix* sites are critical; sites with base-pair substitutions at these five positions show greatly reduced binding. Three additional base pairs make minor contributions to binding. These results are consistent with the results of binding studies between Hin and the *hix* sites in vitro.

[*Key Words*: Hin recombinase; DNA binding; phage P22]

Received January 28, 1988; revised version accepted June 2, 1988.

Salmonella can express each of two different flagellar antigens, H1 and H2, alternatively (Stocker 1949; Iino 1969). The mechanism that mediates flagella phase variation in *Salmonella* is the reversible inversion of a 996-bp segment of the chromosome (Fig. 1) (Zieg et al. 1977, 1978; Zieg and Simon 1980). In one orientation, this inversion segment places a promoter upstream of the *H2* flagellin and the *rH1* (repressor of H1) genes, resulting in the production of H2 flagella and the rH1 repressor protein. The rH1 repressor acts in *trans* to repress transcription of the *H1* flagellin gene, which maps at a different location on the *Salmonella* chromosome (Iino 1977; Silverman et al. 1979). Inversion of the DNA segment to the other orientation positions the promoter so that neither H2 nor rH1 are transcribed, and only H1 flagellin is produced. Thus, the inversion mechanism is an on/off switch for the two antigenically different *Salmonella* flagellin proteins.

The mechanism of phase variation has been characterized extensively at the molecular and biochemical level. Inversion of the 996-bp segment is the result of a site-specific recombination event between two partially homologous 26-bp recombination sites, *hixL* and *hixR* (Fig. 2), that flank the left and right ends of the invertible segment, respectively (Johnson and Simon 1985). The recombination reaction is mediated by the Hin recombinase, encoded by a gene (*hin*) within the invertible segment (Silverman and Simon 1980; Johnson et al. 1986). In addition to Hin protein and the *hixL* and *hixR* sites, the inversion reaction requires a DNA recombinational enhancer site and two other cell proteins, Fis and HU (Johnson and Simon 1985; Johnson et al. 1986). The Fis protein interacts directly with the enhancer, whereas the HU protein is thought to contribute to the efficiency of the recombination reaction by enhanced bending of the DNA (Bruist et al. 1987a; Johnson et al. 1987). All of these factors orchestrate the formation of a specific protein–DNA complex necessary for the breakage and rejoining of DNA at the *hixL* and *hixR* sites resulting in inversion (Bruist et al. 1987b).

Three other site-specific inversion mechanisms similar to the Hin system have been found in phages Mu and P1, and in the *Escherichia coli* defective prophage e14 (Plasterk et al. 1983). The Mu, P1, e14, and Hin systems share recombination site and recombinase protein sequence homology (Kutsukake and Iino 1980; Szekeley and Simon 1983). The Mu and P1 systems also require Fis and a recombinational enhancer and are thought to use a mechanism for site-specific inversion similar to the Hin system (Huber et al. 1985; Kahman et al. 1985, 1987).

In this paper, we describe the initial genetic characterization of the specific protein–DNA interactions be-

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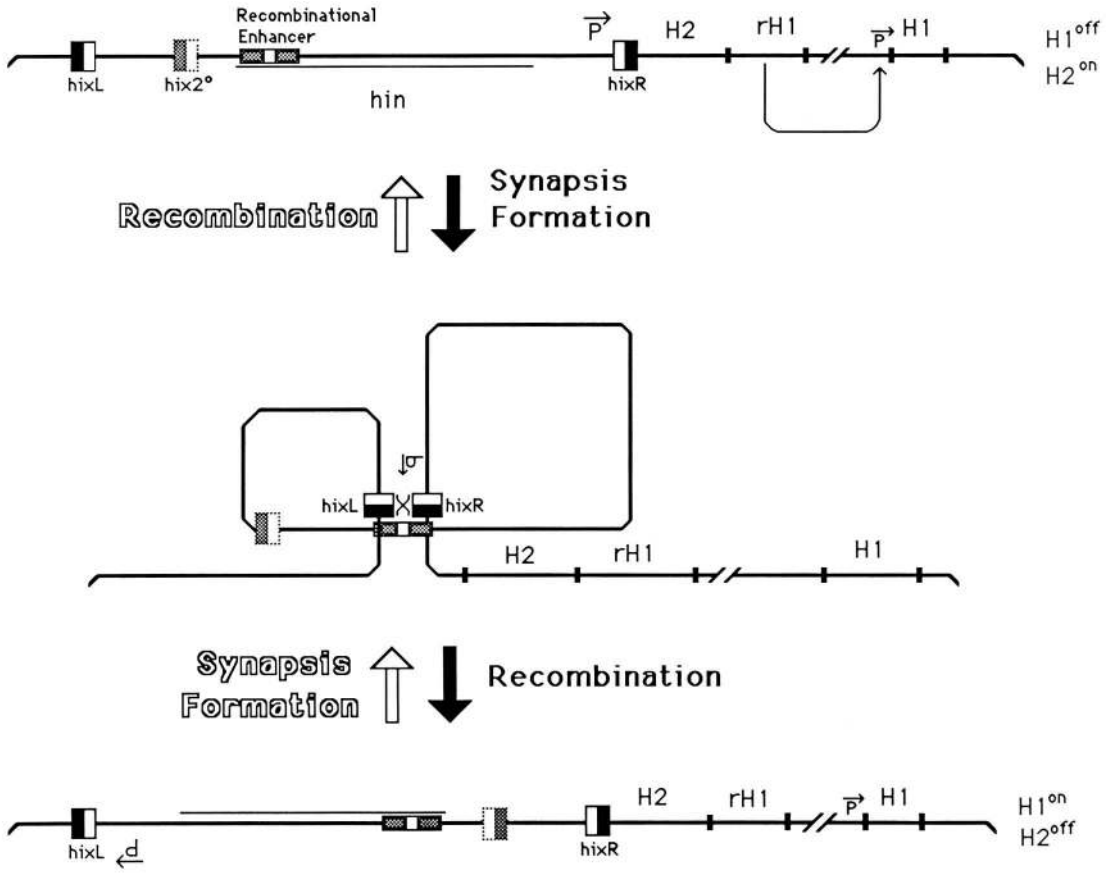

Figure 1. Hin-mediated site-specific recombination. The reversible, Hin-mediated recombination mechanism that switches *Salmonella* flagellin production between the H1 and H2 flagellin antigens is shown. (*Top*) The *H2* promoter provides a transcription initiation site for transcription and subsequent translation of the *H2* and *rH1* genes. The *rH1* gene encodes a protein that acts to repress transcription from the *H1* promoter. Thus, only the H2 flagellin is produced in the *top* orientation. Hin mediates a site-specific recombination reaction between the *hixL* and *hixR* recombination sites. The reaction also requires a host protein, Fis, which interacts directly with the recombinational enhancer sequence and probably the Hin protein as well to form a synaptonemal-like complex necessary for recombination to occur. The recombinational event inverts the segment of the chromosome between the *hix* sites. (*Bottom*) There is no promoter to express the *H2* and *rH1* genes, and only the H1 flagellin is produced.

tween Hin recombinase and the *hix* recombination sites in vivo. We have used the bacteriophage P22 challenge phage selection, which is designed to assay the relative strengths of protein–DNA interactions in vivo (Benson et al. 1986). In this system, Hin binding to a *hix* site is assayed as a repressor binding to its operator site. We have replaced the normal operator for the P22 *ant* gene (Mnt binding site) with various *hix* sites that may act as alternate operator sequences for controlling *ant* gene expression (Fig. 3). When Hin is absent or cannot bind a given *hix* operator site, the *ant* gene is derepressed, and its product, antirepressor, is produced constitutively.

Figure 2. The sequence of the *hixL*, *hixR*, and *hixS* sites as they are read counterclockwise on the *Salmonella* linkage map.

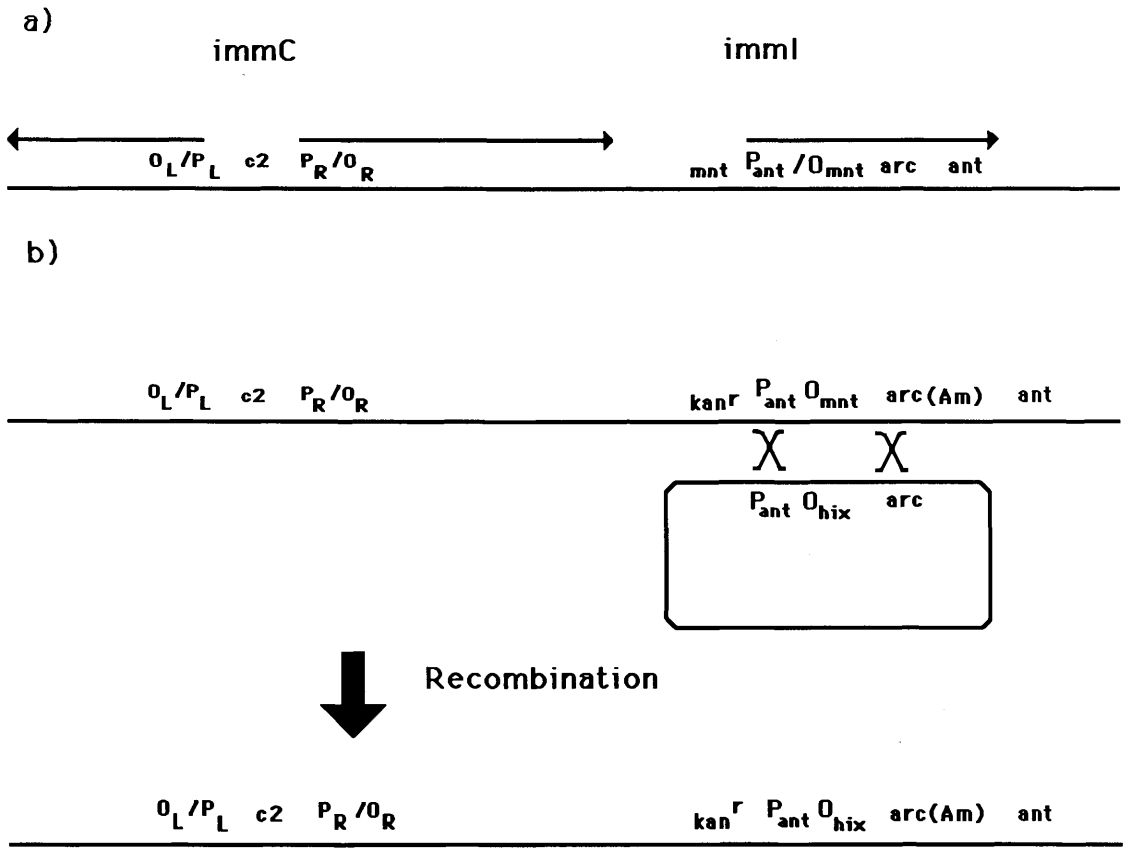

Figure 3. Construction of the P22 *hix* challenge phages. (*a*) A diagram of the wild-type phage P22 showing the immunity regions. (*b*) A recombination event between P22 phage [*Kn9 arcH*(am)] and the pPY190 plasmid with a *hix* sequence cloned into the O_{mnt} site. This results in a challenge phage in which transcription from P_{ant} is regulated by Hin, which can bind to the O_{hix} site and repress transcription.

Antirepressor inhibits the activity of P22 *c2* repressor (analogous in function with phage λ*c*I repressor), prevents the establishment of lysogeny, and channels the superinfecting phage into lytic development (Susskind and Youderian 1983). When Hin is present and binds a given *hix* site, transcription of the *ant* gene is repressed, and the challenge phage carrying the *hix* site can lysogenize the *Salmonella* host.

The recovery of lysogens is by direct selection because the challenge phage carries a kanamycin resistance (Kan^r) determinant (Youderian et al. 1983). The relative binding efficiency of Hin protein to a particular site is measured as the frequency of lysogeny of a challenge phage with this site, a value that ranges over eight orders of magnitude. In this assay, the level of Hin can be varied, because Hin is expressed from the *tac* promoter, which is under the control of Lac repressor. The level of Lac repressor and, therefore, *hin* transcription can be increased or decreased by increasing or decreasing the level of the inducer of Lac repressor, isopropyl β-D-thiogalactopyranoside (IPTG). The relative binding efficiencies of Hin to different *hix* sites can thus be inferred by assaying the frequency of lysogeny of challenge phages with different *hix* sites at varying IPTG concentrations.

Results

Use of the P22 challenge phage selection to study Hin binding to hix *recombination sites in vivo*

To study binding of Hin recombinase to various *hix* sites in vivo, the bacteriophage P22 challenge phage selection was used (Benson et al. 1986). This selection is based on the genetic properties of the P22 *immI* region (Fig. 3) (Susskind and Youderian 1983). The *ant* gene product, antirepressor, inhibits the activity of the primary P22 repressor, *c2*. The *ant* gene, in turn, is regulated by the Mnt repressor, which binds a single, symmetric site (the *ant* operator region) located at the startpoint of transcription of the *ant* gene, preventing transcription of the *ant* gene. If *ant* is not repressed, P22 cannot lysogenize its *Salmonella* host.

By constructing a P22 phage that has a Kan^r gene in place of the *mnt* structural gene, lysogens can be selected and the frequency of lysogenization readily measured. The *ant* operator region has been replaced by a multiple cloning site for inserting synthetic operators at the *ant* gene transcriptional startpoint. Any DNA segment whose in vivo binding to a particular protein is to be characterized is inserted at the *ant* operator site. When a protein can bind the artificial operator at the *ant*

operator region, *ant* is repressed and lysogenization frequencies are determined by measuring the frequencies of Kan^r survivors. This system has been applied successfully to study in vivo binding of the *E. coli* Lac, Gal, and Trp repressors, phage λcI and Cro repressors, and the site-specific recombinase FLP protein of yeast *Saccharomyces cerevisiae* to their respective DNA-binding sites (Benson et al. 1986; Bass et al. 1987; Lebreton et al. 1988).

We have constructed a set of *hix* challenge phages, including phages with wild-type *hix* sequences and phages with mutant sequences. The wild-type sequences are *hixL*, *hixR*, and a secondary Hin-binding site (*hixS*) located upstream of the *hin* translation initiation region in *Salmonella* and are shown in Figure 2. The *hixS* site binds Hin weakly in vitro and may be part of an autogenous regulatory mechanism (Bruist et al. 1987b). The *hixS* site is unrelated to the enhancer site that binds the Fis protein and is necessary for the inversion reaction. Challenge phages were constructed that placed the *hixL*, *hixR*, and *hixS* sites into the operator locus for the P22 *ant* gene. We found that none of these phages is able to lysogenize *Salmonella typhimurium* strains ($\lesssim 10^{-8}$), unless they carry Hin-producing plasmids. This suggests that the level of Hin produced from the chromosome is not sufficient to repress these challenge phages.

The challenge phage assays were all performed on strain MS1868, carrying the pKH66 plasmid that harbors both the *lacI*^{Q} gene and the *hin* gene expressed from the *tac* promoter. Hin expression in cells to be infected with challenge phage was induced by the addition of IPTG to the growth medium prior to infection. The levels of Hin were varied by varying the amounts of IPTG, and the frequencies of Kan^r survivors at each different IPTG concentration were measured. As shown in Figure 4, when MS1868 (pKH66) is grown at low IPTG concentrations, only a small fraction of cells survive infection by challenge with either the *hixL* or *hixR* sites. As the IPTG concentration is increased in small increments between 6×10^{-5} and 6×10^{-4} M, the frequency of lysogeny of these challenge phages increases dramatically, from 10^{-4} to >10%. The increase in the frequency of lysogeny responds sigmoidally to an increase in IPTG concentration and, therefore, the level of Hin. It was found (see below) that the concentration of Hin in the cell also increases sigmoidally over this range of IPTG concentrations, but the total increase is only threefold.

The precise response to challenge phage depends on the nature of the *hix* sites and also on the disposition of the site with respect to the *ant* promoter. Similar lysogenization curves are obtained for challenge phages with the *hixL* sequence in either orientation. In contrast, the *hixR* challenge phages show different dose–response curves for each of the two orientations of *hixR*. One of the *hixR* challenge phages shows a biphasic curve (Fig. 4) demonstrating orientation dependence of the challenge phage selection. When the challenge phage selection was performed with the *hixS* site, no lysogeny was detected in either orientation. This result suggests (but does not rule out) that Hin does not bind appreciably to the *hixS* site, and this site probably plays little role in Hin autoregulation. These results demonstrate that the challenge phage assay can be used to characterize binding of Hin to the *hixL* and *hixR* recombination sites in vivo.

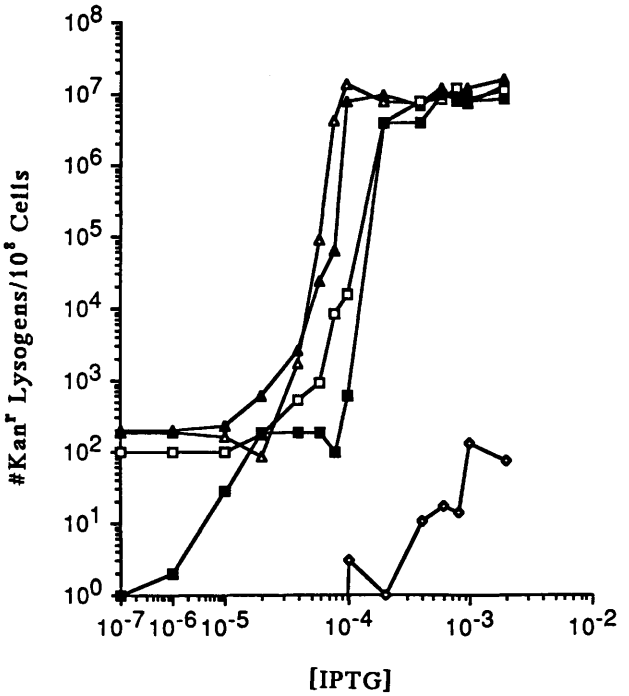

Figure 4. Challenge phage assays with the *hixL* [orientations I (△) and II (▲)], *hixR* [I (■) and II (□)], and half-site [I (◇) and II] challenge phages. These curves represent the frequency of Kan^r lysogens as a function of increasing amounts of IPTG. The recipient in all assays is MS1868(pKH66). In these assays, Hin is expressed from the *tac* promoter in plasmid pKH66 whose transcription is regulated by the LacI^Q repressor also encoded in pKH66. The levels of Hin are increased by increasing levels of Lac inducer, IPTG. The curves show relative binding efficiencies of Hin to the *hix* sites in the different challenge phages tested. Of the six half-site challenge phages tested, only the 1/2 *hixC* (orientation I, see Fig. 6) was able to lysogenize the recipient cells.

Determination of in vivo Hin concentration as a function of increasing IPTG levels

To confirm that small increases in IPTG concentrations correlate directly to small increases in Hin levels, the levels of Hin protein produced in vivo in strain MS1868 (pKH66) induced with the IPTG concentrations used in the challenge phage selections were determined by Western blot analysis. As shown in Figure 5A, small increases in IPTG concentrations result in gradual increases in Hin levels. By scanning the Hin bands in Figure 5A with a densitometer (LKB 2022 Ultrascan Laser Densitometer) and comparing the densities to those for purified Hin standards, the number of Hin molecules per cell was found to increase from ~7000 molecules per cell at an IPTG concentration of 2×10^{-5} M to

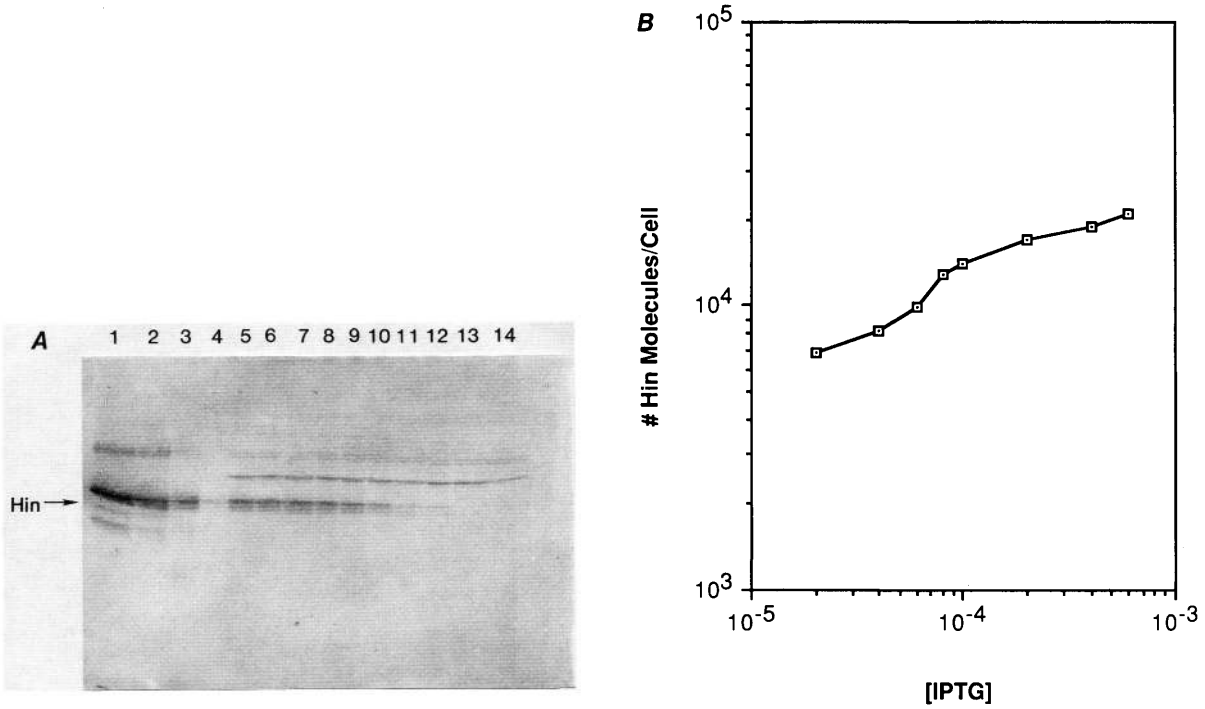

Figure 5. (*A*) Variable Hin induction by IPTG. Strain MS1868 carrying plasmid pKH66 was grown in the presence of varying IPTG levels (see Materials and methods), and the levels of Hin produced were assayed by antibody staining. Lanes *1–4* contain 500, 250, 100, and 50 ng of purified Hin protein, respectively. Lanes *5–12* contain extracts of MS1868 carrying pKH66 induced with 6×10^{-4}, 4×10^{-4}, 2×10^{-4}, 8×10^{-4}, 8×10^{-5}, 6×10^{-5}, 4×10^{-5}, and 2×10^{-5} M IPTG, respectively. Lane *13* contains uninduced cells and lane *14* contains MS1868 without plasmid. There is an additional band picked up by the antibody, which migrates slower than Hin. This band is present in both *E. coli* and *S. typhimurium* and its presence is independent of the presence or absence of Hin protein in the cell (Bruist and Simon 1984). (*B*) The number of Hin molecules per cell under different IPTG-inducing concentrations. The Hin bands in the Western blots in *A* and other similar blots (data not shown) were scanned with a densitometer, and these densities were compared to those of purified Hin standards to estimate the number of Hin molecules per cell in the presence of different concentrations of IPTG inducer.

21,000 molecules per cell at an IPTG concentration of 6×10^{-4} M. Although Hin levels increase only threefold over this range of IPTG concentrations, the increase is sigmoidal (Fig. 5B).

Challenge phage assays on hix *half-sites*

The above result that repression at the *hixR* operator shows a difference depending on orientation suggests that there may be differential binding to each of the half-sites in vivo, as has been shown in vitro (Bruist et al. 1987b). Challenge phages were constructed with each of the three different *hix* half-sites from *hixL* and *hixR* (Figs. 2 and 6). The half-sites in *hixL* and *hixR* that are inverted during the recombination reaction are identical. The two half-sites that are not inverted differ from the common, inverted half-site and from one another. Nine out of 13 bp in the *hixL* half-sites, and 8 out of 13 bp in the *hixR* half-sites are symmetrically related.

When the challenge phage assay was performed on each of the three different half-sites in both possible orientations (Fig. 6), we observed that only one of the sites in one orientation showed significant binding to Hin (Fig. 4). The lysogeny resulting from Hin binding to the common half-site, as shown in Figure 4, is at a low frequency compared to Hin binding to the *hix* full sites, and the increase in the lysogenization frequency with increases in IPTG levels is small compared to the that with full sites. Furthermore, the half-site does not show the sigmoidal increases in lysogenization with small increases in IPTG levels, as was observed using full sites. Thus, Hin is unlike most characterized repressors because it is able to recognize and bind to a half-site.

The binding of Hin to mutant hix *operator sequences*

All of the above results are consistent with the hypothesis that at least two molecules of Hin bind the natural *hix* sites in a cooperative manner. We tested the possible cooperativity of Hin binding by assaying the ability of Hin to repress transcription in mutant challenge phages in which the *hixL* half-sites have been separated and rotated with respect to one another by small insertions of 1 or 4 bp between the half-sites. A mutant *hixL* site with one extra base at the dyad (*hixL* + 1) was isolated as a spontaneous mutant of a *hixL* challenge phage defective in binding Hin (see below). The *hixL*

hixL(I) 5'-P_{ant}-TTATCAAAAACCTTGGTTTTCAAGAA-arc-ant

hixL(II) 5'-P_{ant}-TTCTTGAAAACCAAGGTTTTTGATAA-arc-ant

hixR(I) 5'-P_{ant}-TTTTCCTTTTGGAAGGTTTTTGATAA-arc-ant

hixR(II) 5'-P_{ant}-TTATCAAAAACCTTCCAAAAGGAAAA-arc-ant

½ hixC(I) 5'-P_{ant}-TTATCAAAAACCA-arc-ant

½ hixC(II) 5'-P_{ant}-TGGTTTTTGATAA-arc-ant

½ hixL(I) 5'-P_{ant}-TTCTTGAAAACCA-arc-ant

½ hixL(II) 5'-P_{ant}-TGGTTTTCAAGAA-arc-ant

½ hixR(I) 5'-P_{ant}-TTTTCCTTTTGGA-arc-ant

½ hixR(II) 5'-P_{ant}-TCCAAAAGGAAAA-arc-ant

hixL(I)+1 5'-P_{ant}-TTATCAAAAACCTTTGGTTTTCAAGAA-arc-ant

hixC 5'-P_{ant}-TTATCAAAAACCATGGTTTTTGATAA-arc-ant

Figure 6. Orientation of the *hix* sequences in the different challenge phages with respect to the *ant* gene promoter. For *hixL* and *hixR*, orientation I is the orientation read 5′–3′ clockwise on the *Salmonella* chromosome, as presented in the standard linkage map (Sanderson and Roth 1983); orientation II is the orientation read 5′–3′ counterclockwise on the *Salmonella* chromosome. The 1/2 *hixC* (I and II) sequences represent the half-site common to both *hixL* and *hixR* in each orientation. The 1/2 *hixL* and 1/2 *hixR* sequences represent the half-sites unique to *hixL* and *hixR*, respectively.

mutant with four extra bases between the half-sites (*hixL* + 4) was constructed from a plasmid with a *hixL* site in which one of the central A : T base pairs is changed to a T : A base pair, creating an *Nco*I restriction site. Plasmid DNA with the *hixL–Nco*I site was cut with *Nco*I, filled in, and religated to construct a plasmid with the *hixL* + 4 sequence.

The results of infections with challenge phages carrying the *hixL–Nco*I, *hixL* + 1, and *hixL* + 4 sites are shown in Figure 7. The frequency of lysogeny of Hin-producing cells infected with the *hixL–Nco*I challenge phage responds to increasing IPTG concentrations in a way indistinguishable from cells infected with a challenge phage carrying the wild-type *hixL* site (Figs. 4 and 7). Cells infected with the *hixL* + 1 phage show up to 20-fold reduced frequencies of lysogeny, depending on the IPTG concentration. The *hixL* + 1 phage still shows a sigmoidal binding curve, and the half-maximal frequency of lysogenization occurs at the same IPTG concentration as the half-maximal frequency for *hixL*. Only a very low level of lysogeny was detected with the *hixL* + 4 site at the highest IPTG concentrations.

These results are a direct consequence of the ability of Hin to bind the mutant and wild-type *hix* operator sites and are not due to the effects of cloned operator sequences on the intrinsic activity of the *ant* promoter. To demonstrate this, we constructed fusions of the *lac* operon to the *ant* promoter with each of the different *hix* wild-type and mutant sites at the operator locus on single-copy P22 prophages (Fig. 8), and measured the amount of β-galactosidase made from each hybrid operon. As shown in Table 1, all fusions direct the synthesis of similar, high levels of β-galactosidase.

Construction and properties of a perfectly symmetric hix *site*

We have shown that one of the three different half-sites in *hixL* and *hixR* apparently binds Hin more tightly than the other two do. The ability of one of the *hix* half-sites to bind Hin more tightly than the others suggested that a perfectly symmetric *hix* site with a pair of the tightest binding half-site, *hixC*, might act as a better operator site than either *hixL* or *hixR*. Therefore, we synthesized *hixC* and inserted this site into the challenge phage *ant* operator locus. As shown in Figure 7, we found that the *hixC* sequence is a slightly better operator site for Hin repressor than *hixL* is. Also, we found that by using the *hixC* challenge phage, we could detect an apparent low level of repression even by Hin made from the chromosome.

Isolation and DNA sequence analysis of hix *operator mutants defective in binding Hin*

A strain carrying a P22 prophage that expresses *c*2 repressor and a plasmid that expresses Hin (MS1582/pKH66) is immune to superinfection by challenge phages with *hixL*, *hixR*, or *hixC* sites. In the absence of the Hin-producing plasmid, superinfecting challenge phages express antirepressor, inhibit *c*2 repression, and form plaques efficiently. When challenge phages with *hix* sites are plated on a P22 lysogen that produces Hin, plaques that grow on the immune host arise at a low frequency (10^{-5}). Phage from such plaques include mutants that carry *hix* operator mutations, which prevent repression by Hin protein and thereby permit the constitutive synthesis of antirepressor.

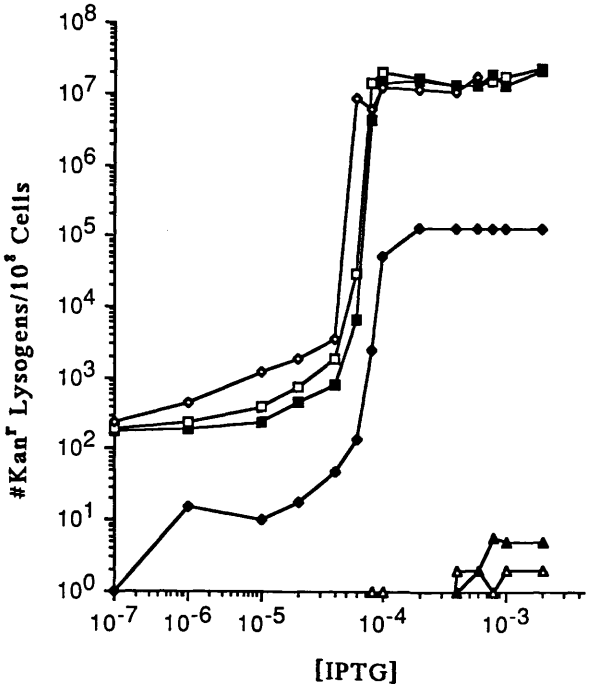

Figure 7. Challenge phage assays with the *hixL*(*Nco*I) [orientations I (□) and II (■)], *hixC* (◇), *hixL* + 1(I) (◆), and *hixL* + 4(*Nco*I) [I (▲) and II (△)] challenge phages. The curves show frequency of lysogeny as a function of increasing IPTG levels (increasing Hin levels).

Independent plaque-forming mutants derived from *hixL* challenge phages were isolated on such a host, and the DNA sequence of the *hix* region of each mutant phage was determined. Initially, we sequenced 33 independent spontaneous mutations in *hixL*. A majority of these (25) were deletions, 3 were single base pair insertions, and 1 was a substitution of a three G : C base pair run for three A : T base pairs.

Although the deletion and insertion mutations we characterized were somewhat informative, we wanted to isolate single base pair substitution mutations to identify the particular DNA base pairs in the *hix* sequences that interact directly with the Hin protein in vivo. To enrich for single base pair substitution mutations, we irradiated *hixL*, *hixR*, or *hixC* phage with ultraviolet (UV) light prior to infection of an immune host that carried a plasmid (pGW1700) expressing the *mucAB* genes. The *mucAB* genes are derived from the conjugative *Salmonella* plasmid pKM101, and their expression increases the frequency of base pair substitutions in UV-irradiated targets (Perry and Walker 1982).

The results of the analysis of mutant *hix* binding sites are shown in Figure 9. Only single base pair frameshift or substitution mutations are represented. Among mutant *hixR* phages, all of the single base pair substitutions were found in the tighter binding half-site. We found three single base pair substitutions among 15 mutations sequenced in *hixR*. These include an A : T to G : C substitution at position 4; a change in a base shown to be important in minor groove contacts between Hin and *hixR* in vitro (Sluka et al. 1987; A. Glasgow and M. Simon, unpubl.). Among 34 UV-induced *hixL* mutations, we found four different single base pair substitutions that change base pairs at positions 5 and 6 in both half-sites. Again, these four base pairs have been shown to be involved in minor groove contacts between Hin and *hixL*, based on the results of methylation protection and interference studies in vitro (A. Glasgow and M. Simon, unpubl.). Of 124 UV-induced mutations in the *hixC* challenge phage, we recovered single base pair sub-

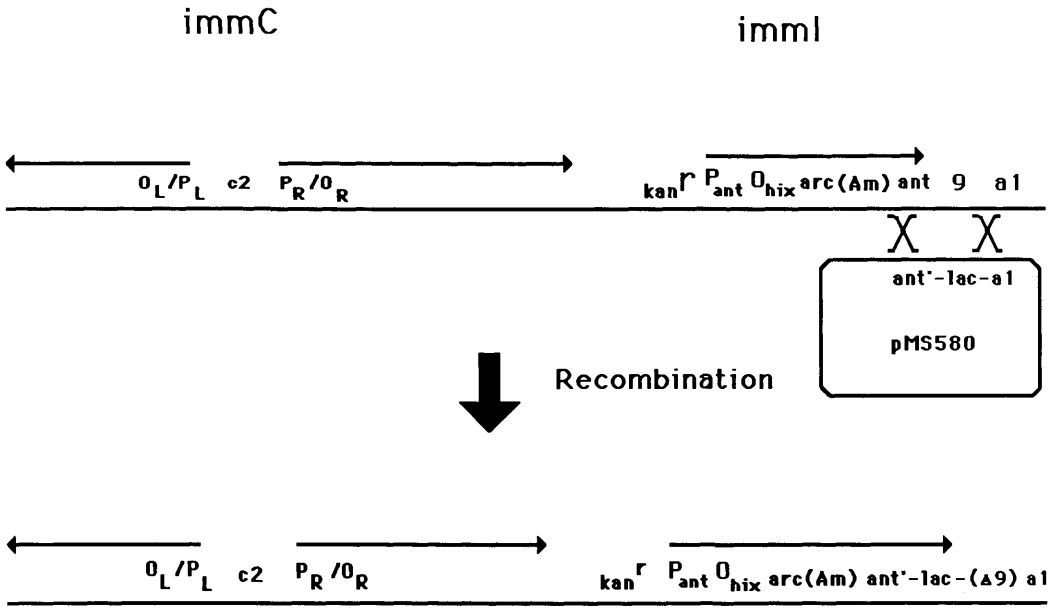

Figure 8. Construction of challenge phages with the *lac* operon under the control of the different *hix* operator sequences. Recombination between pMS580 and P22 phages at the *ant* and *a1* loci results in a phage carrying a fusion of the *lac* operon to the *ant* promoter. Recombinants are easily screened as blue plaques on X-gal-containing media.

Table 1. *β-Galactosidase activities of* hix–lac *fusion strains*

Fusion	β-Galactosidase activity[a]
hixL–lac	3000
hixL (+1)*–lac*	3300
hixL (*NcoI*)*–lac*	2500
hixL (+4)*–lac*	4600
hixC–lac	3500

[a] β-Galactosidase activity is expressed as nanomoles per minute per optical density unit (650 nm).

stitutions in the same four base pairs involved in minor groove contacts between Hin and the *hixL* sequence. Only changes of A : T to G : C or A : T to C : G were obtained in these four base pairs.

Conclusions

To understand the Hin-mediated recombination reaction, we are attempting to study each of the individual steps involved in this ordered pathway of events. Here, we report the genetic characterization of the first step of the inversion reaction, the recognition and binding of Hin to the *hix* recombination sites in vivo. Hin recognizes and binds specific DNA segments. In addition to protein–DNA interactions, Hin is presumably involved in protein–protein interactions during the inversion reaction: (1) Hin forms multimers in the DNA-binding step of the reaction; (2) Hin multimers bound at the *hixL* and *hixR* sites may have a second interaction site required to form a larger multimer and bring the two different regions together; (3) Hin may also bind to the Fis protein as well, although Fis is not required to bring the two *hix* sites together (R.C. Johnson and M.I. Simon, unpubl.). Finally, Hin catalyzes a site-specific recombination reaction and releases itself from the inversion product.

To characterize the binding of Hin to the *hix* recombination sites in vivo, we have cloned *hix* sites at the startpoint of transcription of the phage P22 *ant* promoter and have examined the ability of Hin to bind the *hix* sites and repress the *ant* promoter. At first, it was unclear whether a recombinase would act as a repressor, i.e., whether or not Hin would bind tightly enough to a *hix* operator to repress transcription from the *ant* promoter. Nonetheless, we have found that Hin binds sufficiently to challenge phage DNA that includes either *hixL* or *hixR* sites so that the challenge phage selection could be used to study Hin binding in vivo. Hin apparently represses better at the *hixL* operator than at the *hixR* operator. This phenotype enables us to study the binding of Hin to *hix* recombination sites in vivo and to separate binding from subsequent steps in the recombination reaction. These results are consistent with results found in the characterization of the in vitro properties of Hin binding to the *hix* sites (Sluka et al. 1987; A. Glasgow and M. Simon, unpubl.).

One of the unexpected results of this study is the finding that Hin will bind tighter to one of the three natural *hix* half-sites, the one common to both *hixL* and *hixR*. Hin binds less well to the unique *hixL* and *hixR* half-sites (Fig. 4). This suggests that the Hin protein has a very high affinity for binding to the individual half-sites. The sequence of the common half-site that binds tightest to Hin was used to construct a perfectly symmetric *hix* site, *hixC*. The use of the *hixC* site will allow us to study the effects of symmetric changes in the *hix* sequence on binding to Hin.

The nature of the possible cooperative interaction of Hin with the *hix* sites was characterized by assaying the binding of Hin to *hixL* sites having either 1 or 4 bp inserted between the *hixL* half-sites. The result of the *hixL* + 4 challenge phage is consistent with multimeric binding of Hin to each half-site. We anticipated that the 4-bp insertion would rotate the half-sites to different faces of the DNA helix and thereby greatly impair multimeric binding. As expected, insertion of 4 bp between the *hixL* half-sites eliminates multimeric binding, as measured by the challenge selection. Hin-mediated repression of the *hixL* + 4 phage is not as great as that with the *hixC* half-site alone. This is probably due to competition between the two rotated half-sites in the *hixL* + 4 phage in binding to Hin, thereby reducing overall binding to the promoter-proximal half-site necessary for repression to occur.

The *hixL* + 1 site, with a single base-pair insertion, appears defective in binding. The single base-pair insertion does not interfere with the sigmoidal lysogenization curve, but the complex formed between the mutant *hixL* + 1 site and Hin appears not to be as stable as with the *hixL* site, because repression and therefore apparent binding of the *hixL* + 1 phage are down at all IPTG concentrations. If cooperative binding was reflected by the sigmoidal lysogenization curve, the low lysogenization frequencies at low Hin concentrations would represent monomer binding. If this were due to cooperative binding of monomers, the *hixL* + 1 challenge phage should not exhibit reduced lysogenization frequencies at low IPTG levels as compared with *hixL*. The fact that it does suggests that Hin binds to the *hix* sites as a multimer at all IPTG levels and the sigmoidal lysogenization curve is an intrinsic property of repression of the *ant* promoter and does not reflect cooperative binding of monomers.

The repression of *ant* by the binding of Hin to *hixR* was found to be orientation dependent (Fig. 4). Hin represses when *hixR* is in the orientation that places the tighter binding half-site proximal to the startpoint of *ant* transcription better than when the poorer half-site is promoter proximal. One explanation consistent with this result is that it reflects a difference in the competition between RNA polymerase and Hin for binding to the promoter–operator region in these two situations. When the *hixR* site is oriented with respect to the *ant* promoter so that the poorer *hixR* half-site is promoter proximal, a higher concentration of Hin is required to repress transcription. It may be that when the weaker binding half-site of *hixR* is promoter proximal, RNA polymerase can outcompete (or essentially 'peel off') the

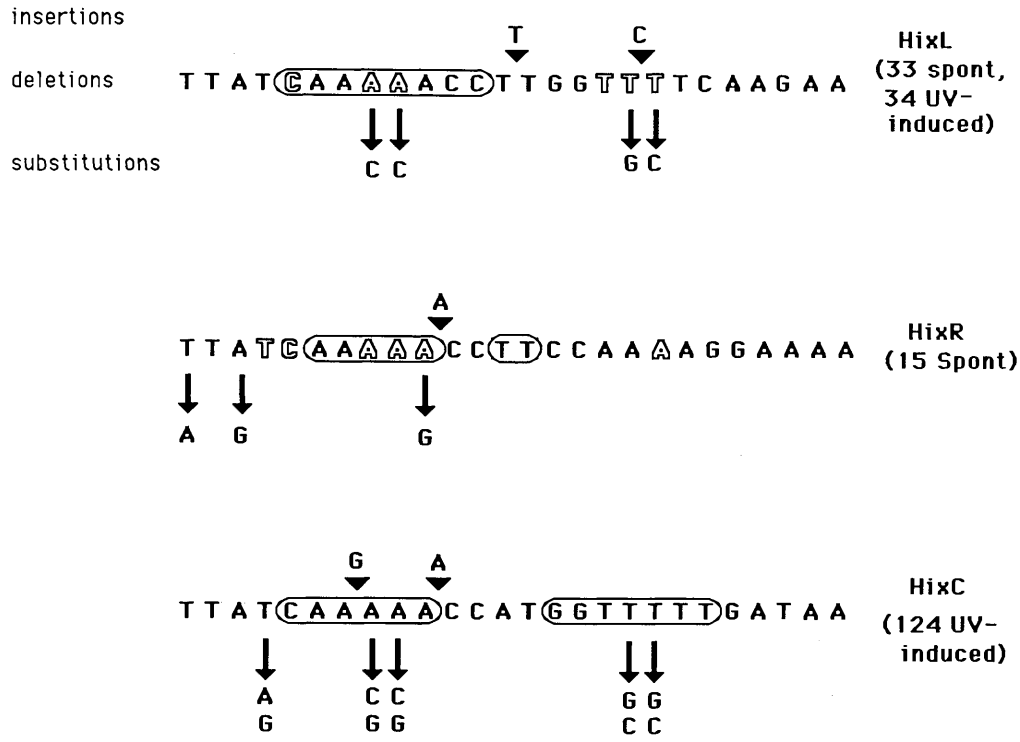

Figure 9. Single base changes in *hixL*, *hixR*, and *hixC* which reduce binding to Hin. The circled bases are those that are reduced for binding Hin when singly deleted. The outlined bases in *hixL* and *hixR* are those base pairs that prevent Hin binding in vitro when methylated (A. Glasgow and M. Simon, unpubl.).

bound Hin multimer at the lower Hin concentrations.

The binding of Hin to the *hixL* half-sites is also orientation dependent. The dependence of the frequency of lysogeny of a challenge phage on the orientation of a cloned half-site may not reflect a difference in Hin binding; rather, the correct position of the bound Hin repressor with respect to the *ant* promoter could be a critical determinant of the ability of Hin to repress transcription. The results of in vitro experiments suggest that Hin binds the minor groove in the center of the *hix* dyad and the major groove at the outside regions (A. Glasgow and M. Simon, unpubl.). Thus, a full site would bind the same faces of the DNA relative to the *ant* promoter regardless of orientation. In contrast, Hin would bind the half-site in the major groove in the promoter-proximal portion in one orientation and the minor groove in the promoter-proximal portion of the *hix* half-site in the other orientation. Thus, the orientation of the half-site would determine which side of the DNA helix bound relative to the *ant* promoter, which may greatly affect the ability of Hin to repress transcription.

The binding of Hin to *hix* sites in vivo is tight enough to construct a host immune to infection by the *hixL*, *hixR*, and *hixC* challenge phages. Such a host carries a P22 *c2+* prophage and a plasmid expressing Hin. The *hixL*, *hixR*, and *hixC* challenge phages cannot form plaques on this strain, because Hin is able to repress *ant* transcription. On the basis of this phenotype, we have selected mutants that result from DNA base changes in *hix* sites that impair Hin binding. Rare plaques that arise when *hix* challenge phages are plated on a 'Hin-immune' host carry mutations in their *hix* sites. More than 200 *hix* operator-constitutive mutations were sequenced to determine which base pairs in the *hix* sites are essential for Hin binding in vivo. Sequence analysis of *hixL* and *hixC* mutations shows that the A : T base pairs at positions 5, 6, and 10 in the *hix* site are critical for binding. These results are consistent with studies showing that A : T base pairs at positions 4, 5, and 6 prevent Hin binding in vitro when methylated in the minor groove, (A. Glasgow and M. Simon, unpubl.). The C : G base pair at position 9 also prevents Hin binding when methylated in the major groove.

We did not recover A : T to T : A transversion mutations at positions 5 and 6 of the *hix* site. Hin binds both *hixL*, which has A : T base pairs at positions 5 and 6 and T : A base pairs at positions −5 and −6, and *hixR* which has A : T base pairs in both half-sites. Hin may contact these runs of A : T base pairs in the minor groove at positions 5 and 6. All of the mutations in *hixR* that impair binding to Hin were found in the tighter binding half-site. In addition, three base substitution mutations were obtained that were not observed among the 150 *hixL* and *hixC* mutants sequenced. This may suggest that the *hixR* sequence is more sensitive to base substitution mutation, because one *hixR* half-site is defective in

binding when compared to the other half-site. We presume that weaker contact points between Hin and *hix* were identified by mutations in *hixR* that were not found in *hixL* or *hixC*.

The results presented here demonstrate the utility of the challenge phage selection in the characterization of the in vivo binding properties of Hin recombinase to its recombination sites. A second advantage of using Hin to repress *ant* transcription in bacteriophage P22 is the ability to construct an immune host allowing for the direct selection of *hix* site mutants defective in binding to Hin. We hope to use these mutants to select for Hin suppressor mutants that can regain the ability to bind to mutant *hix* sites and allow the mutant challenge phages to lysogenize. Such studies will enable us to determine the specific amino acids in Hin that make specific base contacts in the *hix* sites.

Materials and methods

Bacteria and bacteriophage strains

All strains used in this study and their sources are listed in Table 2. All *S. typhimurium* strains are derived from LT2 strain DB7000 (Susskind 1980). *E. coli* strains are derived from K-12. P22 *Kn9 arcH1605* (am), the parent of the *hix*-challenge phages, has been described (Youderian et al. 1983). The *hix*-challenge phages were constructed by crosses between P22 *Kn9 arcH1605* and plasmids in which various *hix* sequences had been cloned in place of the *ant* operator (Fig. 3).

Plasmids

Plasmids used in this study and their sources are listed in Table 1. The construction of plasmids carrying *hix* DNA sequences at the P22 *ant* operator locus (Fig. 3) was done essentially as described for other operator sequences (Benson et al. 1986). A linker tailing protocol was used to clone synthetic *hix* sites (double-stranded oligonucleotides) into the *Sma*I site of plasmid (Bass et al. 1987). Reaction mixtures contained 1 μl *Sma*I-digested pPY190 DNA (1 mg/ml), 6.5 μl hybridized unphosphorylated oligonucleotides (1–10 mg/ml), 1.5 μl 10× DNA ligase/kinase buffer [0.66 M Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 2 mg/ml bovine serum albumin], 3 μl 10 mM ATP, 3 μl 100 mM dithiothreitol, and 1.5 μl of T4 DNA ligase (New England Biolabs). Ligations were carried out overnight at room temperature. After ligation, 5 μl of 3 M sodium acetate (pH 5) and 80 μl of cold (−20°C) ethanol were added. The solutions were mixed and stored at −70°C for 10 min. The DNA/protein mixtures were pelleted in a microfuge (15 min at 4°C), rinsed with 70% ethanol, 30% 10 mM Tris-HCl (pH 7.5), and 0.1 mM EDTA and vacuum-dried. Dried pellets were resuspended in 20 μl of 10 mM Tris-HCl (pH 7.5) and incubated at 65°C for 5 min. A 66-μl aliquot of H buffer [100 mM NaCl, 10 mM Tris-HCl (pH 7.5), 5 mM EDTA] was added to each mixture, the mixtures were incubated at 65°C for 10 min, set at room temperature for 10 min, and then put on ice or frozen for later use. A 20-μl aliquot of each solution was used to transform 200 μl of frozen and thawed competent DH1 cells. Plasmids carried by ampicillin-resistant (Amp^r) transformants were screened for insertions of the synthetic *hix* sites by digestion of plasmid DNA with *Eco*RI, followed by 5% polyacrylamide gel electrophoresis. The pPY190 plasmid has 341-bp sequence that includes the *Sma*I cloning site. Plasmids that had a larger *Eco*RI fragment than the parent were kept as putative inserts.

The source of Hin used in this study is the moderate copy number plasmid PKH66, which expresses the *hin* gene from the *tac* promoter, under the control of the $lacI^Q$ gene. The original P_{tac}–*hin* construct was on a high-copy-number plasmid pMS571 (Johnson and Simon 1985). In strains carrying pMS571, levels of IPTG $>10^{-5}$ M were lethal to cell growth when plasmid selection was maintained. At high IPTG concentrations, the Hin protein aggregated into insoluble inclusion bodies. The accumulation of large amounts of these insoluble inclusion bodies may be the cause of lethality in cells where Hin is highly overproduced. In strains carrying pKH66, IPTG levels as high as 2 mM have no noticeable effects on cell growth. Thus, pKH66 provides a source of Hin that is well repressed by the $LacI^Q$ gene product but can be varied in response to the concentration of IPTG present in the growth medium. The pKH66 plasmid is present in low copy and presumably produces much less Hin when fully induced than from the pMS571 plasmid. The plasmid PKH66 was constructed by cloning the P_{tac}–*hin* fragment of plasmid PMS571 into the *Bam*HI site of plasmid pMS421. The pMS571 plasmid was partially digested with *Bam*HI and electrophoresed on a 1% agarose gel. The 1.1-kb fragment was purified and ligated into *Bam*HI-digested plasmid pMS421, resulting in plasmid pKH66.

Media

The E medium of Vogel and Bonner (Vogel and Bonner 1956), supplemented with 0.2% dextrose, was used as minimal medium. Alternative carbon sources were supplemented to 0.2% in E medium lacking citrate (Maloy and Roth 1983). Luria-Bertani (LB) medium (Difco Tryptone, 10 g/liter; Difco yeast extract, 5 g/liter; NaCl, 5 g/liter) was used as rich medium. Auxotrophic supplements were included in media at final concentrations described previously (Davis et al. 1980). Antibiotics (Sigma) were included in media as needed (final concentrations given): ampicillin [100 μg/ml for plasmid selection, 30 μg/ml for Mu*d* lysogen selection (Hughes and Roth 1984)], chloramphenicol (12.5 μl/ml), kanamycin sulfate (40 μl/ml), spectinomycin (Spc; 100 μl/ml), streptomycin [Str; 50 μl/ml for plasmid selection, 1 mg/ml for chromosomal *rpsL* (*strA*) mutant selection], and tetracycline (10 μl/ml). Bacterial cell growth was monitored at 540 nm in a Klett–Summerson colorimeter.

Transductional methods

For all transductional crosses, the high-frequency generalized transducing mutant of bacteriophage P22 (*HT105/1 int-201*) was used (Sanderson and Roth 1983). Selective plates were spread with 2×10^8 to 2×10^9 phage. Transductants were purified as described (Hughes and Roth 1985). For antibiotic selections other than ampicillin or tetracycline, phage and cells were mixed and left at room temperature for 1 hr prior to spreading on selective plates to permit expression of the antibiotic-resistance phenotype.

Challenge phage assay

An overnight culture of strain MS1868 carrying the Hin-producing plasmid pKH66 was diluted 100-fold into LB plus Str and Spc to maintain plasmid selection and was grown to a density of 100 Klett units (~6×10^8 cells/ml). Cells were diluted fourfold into the same medium plus varying amounts of IPTG and grown for 1 hr to permit the induction of Hin expression from plasmid pKH66. P22 *hix* challenge phage was added to a m.o.i. of 20, and infected cells were incubated for 1 hr at room temperature to allow the expression of the Kan^r phenotype.

Table 2. *List of strains and plasmids*

Strains	Source[a]
E. coli	
DH1:F$^-$ *thi-1 supE44 hsdR17 endA1 recA1 relA1 gyr-96* λ$^-$	D. Hanahan
MC1000:F$^-$ *thi araD139* Δ(*araBAOIC-leu*) *7679 galU galK lacY74 rpsL* λ$^-$	M. Casadaban
S. typhimurium	
MS1362:*leuA414* (Am) *supD* Fels$^-$	
MS1363:*leuA414 supE40* Fels$^-$	
MS1367:*leuA414 supE40* Fels$^-$ *ataA*::[P22 *sieA44 Ap2 DEL7283* (immI) (Ap^r)]	
MS1582:*leuA414 supE40* Fels$^-$ *ataA*::[P22 *sieA44 16*-am*H1455 tpfr-49*]	
MS1868:*leuA414 hsdSB* Fels$^-$	
MS1882:*leuA414 hsdSB endA* Fels$^-$	
MS1883:*leuA414 hsdSB supE40* Fels$^-$	
MS2544:*leuA414 supE40 hsdSB recA1* Fels$^-$	
SL4213:*hsdL6 hsdSA29 galE496 metA22 metE55 ilv-452 rpsL*120 xyl-404 H1-b H2-e,n,x *nml*$^-$ Fels$^-$	B. Stocker
TH564:*leuA414 supE40* Fels$^-$ *ataA*::[P22 *sieA44 Ap7 tpfr184* Δ(*mnt-a1*) (Ap^S)]	

Plasmids		Source
pMS571	(P_{tac}–Hin$^+$, Ap^r)	Johnson and Simon (1985)
pMS421	[pSC101 origin (low copy no.), *lacI*^{Q}, Str^r, Spc^r]	M. Susskind
pKH66	(pMS421 P_{tac}–Hin$^+$)	this paper
pPY190	(pBR322, P*ant-arc-ant'*)	P. Youderian
pMS580	(pBR322, P*ant-lacZYA'*-Δ9 *al*)	M. Susskind

[a] Unless indicated otherwise, all strains originated from M. Susskind's lab.

After 1 hr, dilutions of the adsorption mixture were plated on LB–Kan plates containing the same concentration of IPTG used for induction and incubated overnight at 37°C. As a negative control, strain MS1868 carrying plasmid pMS421, which is otherwise isogenic with pKH66 but lacks the Hin gene, was used. None of the *hix*-challenge phages were able to lysogenize this strain.

DNA sequence analysis

Plasmid DNA and double-stranded phage DNA were sequenced by the method of Sanger et al. (1977).

Western blot analysis of Hin levels

The ProtoBlot Immunoscreening System protocol (Promega) was used to assay Hin levels expressed from plasmid pKH66 under different induction conditions. Strain MS1883 with plasmid pKH66 was grown overnight at 37°C in LB medium containing Str and Spc to maintain plasmid selection. The culture was diluted 100-fold in LB plus Str and Spc and grown to O.D. 0.8 and then diluted 4-fold into LB plus Str and Spc and the following concentrations of IPTG (final molar concentration given): 2×10^{-5}, 4×10^{-5}, 6×10^{-5}, 8×10^{-5}, 8×10^{-4}, 2×10^{-4}, 4×10^{-4}, 6×10^{-4}. The cultures were grown for an additional hour at 37°C to allow for induction of Hin (final O.D. 5–6). Cells (10 ml) from each IPTG concentration used were pelleted by centrifugation and resuspended in 5 ml of LB. A unit sample of O.D. 1 of each culture was electrophoresed in a 15% SDS–polyacrylamide (4% stacking) gel and hybridized with Hin antibody prepared previously (Bruist and Simon 1984). Purified Hin (Johnson et al. 1986) was also run as a standard.

Acknowledgments

We thank Carol Lee for help with the DNA sequence and Western blot analysis; Steve Bass, Nicholas Benson, Brigitte Lebreton, Paul Sugiono, and Miriam Susskind for helpful discussions; and Anna Glasgow and Robert Bourret for critically reading this manuscript. This work was supported by grants from the National Science Foundation and from the Defense Advanced Research Projects Agency (DARPA) Institutional Research Initiative Program to M.I.S. and grant GM34982 from the National Institutes of Health to P.Y. K.T.H. was supported by fellowship GM11088 from the National Institutes of Health.

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Genes Dev. 1988, **2:**
Access the most recent version at doi:10.1101/gad.2.8.937