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]

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*Salmonella* can express each of two different flagellar antigens, H1 and H2, alternatively [Stocker 1949; lino 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 [lino 1977; Silverman and Simon 1979]. Inversion of the DNA segment to the other orientation places 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 lino 1980; Sekely 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-
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.

Figure 2. The sequence of the hixL, hixR, and hixS sites as they are read counterclockwise on the Salmonella linkage map.
Antirepressor inhibits the activity of P22 c2 repressor (analogous in function with phage λcl 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') 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 imml 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' 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 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' survivors. This system has been applied successfully to study in vivo binding of the E. coli Lac, Gal, and Trp repressors, phage λcl 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 (≈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 pK66 plasmid that harbors both the lacF 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' survivors at each different IPTG concentration were measured. As shown in Figure 4, when MS1868 [pK66] 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 auto-regulation. 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' lysogens as a function of increasing amounts of IPTG. The recipient in all assays is MS1868 [pK66]. In these assays, Hin is expressed from the tac promoter in plasmid pK66 whose transcription is regulated by the LacO repressor also encoded in pK66. 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 [pK66] 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
21,000 molecules per cell at an IPTG concentration of $6 \times 10^{-4}$ 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 $hixL$
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.

\[
\begin{align*}
\text{hixL(1)} & \quad 5'-\text{P}^{\ast}T_{\text{P}}TCTTGAACCCAAAGTGTGGAT\text{A}A-\text{arc-ant} \\
\text{hixL(II)} & \quad 5'-\text{P}^{\ast}TCTTGAACCCAAAGTGTGGAT\text{A}A-\text{arc-ant} \\
\text{hixR(1)} & \quad 5'-\text{P}^{\ast}TTTCTTTGGAAGGTGGTTGGAT\text{A}A-\text{arc-ant} \\
\text{hixR(II)} & \quad 5'-\text{P}^{\ast}TTTCTTTGGAAGGTGGTTGGAT\text{A}A-\text{arc-ant} \\
\frac{1}{2} \text{hixC(1)} & \quad 5'-\text{P}^{\ast}TTTCTTTGGAAGGTGGTTGGAT\text{A}A-\text{arc-ant} \\
\frac{1}{2} \text{hixC(II)} & \quad 5'-\text{P}^{\ast}TTTCTTTGGAAGGTGGTTGGAT\text{A}A-\text{arc-ant} \\
\frac{1}{2} \text{hixL(1)} & \quad 5'-\text{P}^{\ast}TTTCTTTGGAAGGTGGTTGGAT\text{A}A-\text{arc-ant} \\
\frac{1}{2} \text{hixL(II)} & \quad 5'-\text{P}^{\ast}TTTCTTTGGAAGGTGGTTGGAT\text{A}A-\text{arc-ant} \\
\frac{1}{2} \text{hixR(1)} & \quad 5'-\text{P}^{\ast}TTTCTTTGGAAGGTGGTTGGAT\text{A}A-\text{arc-ant} \\
\frac{1}{2} \text{hixR(II)} & \quad 5'-\text{P}^{\ast}TTTCTTTGGAAGGTGGTTGGAT\text{A}A-\text{arc-ant} \\
\text{hixL(I)+1} & \quad 5'-\text{P}^{\ast}TTTCTTTGGAAGGTGGTTGGAT\text{A}A-\text{arc-ant} \\
\text{hixC} & \quad 5'-\text{P}^{\ast}TTTCTTTGGAAGGTGGTTGGAT\text{A}A-\text{arc-ant}
\end{align*}
\]

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 Ncol restriction site. Plasmid DNA with the hixL-Ncol site was cut with Ncol, filled in, and religated to construct a plasmid with the hixL + 4 sequence.

The results of infections with challenge phages carrying the hixL-Ncol, hixL + 1, and hixL + 4 sites are shown in Figure 7. The frequency of lysogeny of Hin-producing cells infected with the hixL-Ncol 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 c2 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 c2 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.
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 7](image_url)

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

![Figure 8](image_url)

**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 aI 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.
fusion

<table>
<thead>
<tr>
<th>Fusion</th>
<th>β-Galactosidase activity</th>
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<tr>
<td>hixL-lac</td>
<td>3000</td>
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<tr>
<td>hixL (+1)-lac</td>
<td>3300</td>
</tr>
<tr>
<td>hixL(Ncol)-lac</td>
<td>2500</td>
</tr>
<tr>
<td>hixL(+4)-lac</td>
<td>4600</td>
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<tr>
<td>hixC-lac</td>
<td>3500</td>
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*β-Galactosidase activity is expressed as nanomoles per minute per optical density unit [650 nm].

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 to 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
Hin recombinase/hix site interactions

HixL
(35 spont, 34 UV-induced)

HixR
(15 Spont)

HixC
(124 UV-induced)

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 lysoenzogene. 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 Ks9 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 Ks9 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 Smal site of plasmid [Bass et al. 1987]. Reaction mixtures contained 1 µl Smal-digested pPY190 DNA (1 mg/ml), 6.5 µl hybridized unphosphorylated oligonucleotides [1–10 mg/ml], 1.5 µl 10 x DNA ligase/kinase buffer [0.66 M Tris-HCl (pH 7.5), 100 mM MgCl2, 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). Ligation reactions 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 (AmpR) transformants were screened for insertions of the synthetic hix sites by digestion of plasmid DNA with EcoRI, followed by 5% polyacrylamide gel electrophoresis. The pPY190 plasmid has 341-bp sequence that includes the Smal cloning site. Plasmids that had a larger EcoRI 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 lacP promoter. The original Pms571-hin construct was on a high-copy-number plasmid pMS571 [Johnson and Simon 1985]. In strains carrying pMS571, levels of IPTG >10^{-4} 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 LacP promoter 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_{lac}-hin fragment of plasmid PMS571 into the BamHI site of plasmid pMS421. The pMS571 plasmid was partially digested with BamHI and electrophoresed on a 1% agarose gel. The 1.1-kb fragment was purified and ligated into BamHI-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 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 Mud lysogen selection [Hughes and Roth 1984]], chloramphenicol [12.5 µg/ml], kanamycin sulfate [40 µg/ml], spectinomycin [Spc; 100 µg/ml], streptomycin [Str; 50 µg/ml], and tetracycline [10 µg/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 x 10^{8} to 2 x 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 x 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 KanR phenotype.
Hin recombinase/hix site interactions

**Table 2. List of strains and plasmids**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Source*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>D. Hanahan</td>
</tr>
<tr>
<td></td>
<td>M. Casadaban</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td></td>
</tr>
<tr>
<td>DS162:leuA414 [Am] supD Fels+</td>
<td></td>
</tr>
<tr>
<td>DS163:leuA414 supE40 Fels+</td>
<td></td>
</tr>
<tr>
<td>DS167:leuA414 supE40 Fels+ ataA::[P22 sieA44 Ap2 DEL7283 [im] [Ap]]</td>
<td></td>
</tr>
<tr>
<td>DS168:leuA414 hsdSB Fels+</td>
<td></td>
</tr>
<tr>
<td>DS168:leuA414 hsdSB endA Fels+</td>
<td></td>
</tr>
<tr>
<td>DS158:leuA414 supE40 Fels+</td>
<td></td>
</tr>
<tr>
<td>SM2544:leuA414 supE40 hsdSB recA1 Fels+</td>
<td></td>
</tr>
<tr>
<td>SL4213:hsdL6 hsdSA29 galE946 meta22 metE55 ilv-452 rpsL120 y1-404 H1-b H2-c,n,x nml- Fels+</td>
<td></td>
</tr>
<tr>
<td>TH564:leuA414 supE40 Fels+ ataA::[P22 sieA44 Ap7 tifpr184 Δ[mnt-al] [Ap]]</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMS421 [pSC101 origin, lacO2, Str, Spe]</td>
<td>M. Susskind</td>
</tr>
<tr>
<td>pKH66 [pMS421 P~Hin+]</td>
<td>this paper</td>
</tr>
<tr>
<td>pPY190 [pBR322, Pant-arc-ant']</td>
<td>P. Youderian</td>
</tr>
</tbody>
</table>

* 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 SM1868 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 SM1868 with plasmid pKH66 was grown overnight at 37°C in LB medium containing Str and Spe to maintain plasmid selection. The culture was diluted 100-fold in LB plus Str and Spe and grown to O.D. 0.8 and then diluted 4-fold into LB plus Str and Spe 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**

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**References**


Hughes et al.


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

K T Hughes, P Youderian and M I Simon

*Genes Dev.* 1988, 2:
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References

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