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Phase Variation and the Hin Protein: In Vivo Activity Measurements, Protein Overproduction, and Purification

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The alternate expression of the *Salmonella* flagellin genes *H1* and *H2* is controlled by the orientation of a 995-base-pair invertible segment of DNA located at the 5′ end of the *H2* gene. The *hin* gene, which is encoded within the invertible region, is essential for the inversion of this DNA segment. We cloned the *hin* gene into *Escherichia coli* and placed it under the control of the p_L promoter of bacteriophage λ. These cells overproduced the Hin protein. In vivo inversion activity was measured by using a recombinant λ phage which contains the *H2* and *lacZ* genes under the control of the invertible region. Using this phage, we showed that the amount of inversion activity is proportional to the amount of Hin protein in the cell. An inactive form of the protein was purified by using the unusual solubility properties of the overproduced protein. The amino acid composition of the protein agreed with the DNA sequence of the *hin* gene. Antibodies were made to the isolated protein. These antibodies cross-reacted with two other unidentified *E. coli* proteins.

Phase variation in *Salmonella typhimurium* refers to the alternate expression of the flagellin genes *H1* and *H2*. The change in expression from one flagellin gene to the other is the result of a switch in the orientation of an invertible 995-base-pair segment of DNA at the 5′ end of the *H2* gene (24). The orientation of this DNA segment controls which of the two flagellin genes is expressed. In one orientation (*H2* on), a promoter within this DNA segment directs the transcription of the *H2* gene and the *rh1* gene. *rh1* encodes a 16,000-molecular-weight protein which represses expression of the *H1* gene. In the *H2* off orientation, the promoter is directed away from the *H2* gene and neither *H2* nor *rhl* is expressed. In this state, no repressor is made, so the *H1* gene is expressed (19).

Switching of the 995-base-pair segment of DNA requires three genetic elements: two *cis*-acting sites, located at the ends of the segment, and the *hin* gene, located completely within the invertible segment. The two *cis*-acting sites contain identical 14-base-pair sequences which are in an inverted repeat orientation (25). These sequences are referred to as IRL and IRR for inverted repeat left and right. IRL is the repeated sequence most distant from the *H2* gene; IRR is the closest to the *H2* gene. Inversion results from homologous site-specific recombination within these two sequences. When one of the repeated sequences is inverted such that the two sequences are in a direct repeat configuration, recombination results in the deletion of the DNA between the repeated sequences (15).

The *hin* gene product, Hin, is a 20,000-molecular-weight protein, which acts in *trans* to give switching (17). It is presumed to be the recombinase which aligns the repeated sequences and performs the DNA strand exchange to give inverson. It is not known whether Hin can function alone or whether it requires additional factors provided by the cell.

To further characterize the recombination reaction, the *S. typhimurium H2* operon, which includes the invertible region with the *hin* gene and the *H2* and *rhl* gene, was cloned into *Escherichia coli* on a plasmid. Inversion occurs in *E. coli* and is dependent on the *hin* gene (24). This plasmid has been used for the development of strains and assays for the purification of the *hin* gene product. In this work, we describe plasmids used for the overproduction of the Hin protein and substrates which are used to measure the switching reaction in vivo. We show that increases in the level of Hin protein increased the inversion rate. The overproduced protein was purified in an inactive form. This protein was used to produce antibodies to Hin which could be used in assays to quantitate amounts of Hin.

MATERIALS AND METHODS

Bacterial strains and media. The *E. coli* K-12 strains used in this work were CSH4rh (F^- *trp lacZ rpsL thi recA hag*) and MA800 (F^-, prototrophic; a gift from A. Hoyt), a gal^+ transductant of a cryptic λ prophage from N4830 (5) into W3102 (F^- *galK2*). MA800 expresses the λ repressor *c*I857 and the antiterminator *N*. The prophage is deleted from *cro* to beyond *attR* and from *ral* through *int*. LB, NZ, and mot media were made as prevously described (2, 9, 11). Top agar was composed of 10 g of NZ amine, 8 g of NaCl, 2 g of $MgCl_2 \cdot 6H_2O$, and 7 g of agar in 1 liter of water.

Plasmid purification and constructions. Plasmids were purified by alkaline extraction (1) and when necessary purified further on a CsCl density gradient (11). Restriction enzymes, exonuclease BAL31, and DNA ligase were used according to the recommendations of the distributors.

Phage construction. Switching activity was measured in vivo by using a recombinant phage, λfla406, which contains the *lacZ* gene under the control of the invertible region. The phage can direct the synthesis of β-galactosidase only when the invertible region is in the *H2* on orientation. Thus, in a *lacZ* mutant host, switching the inversion region from off to on results in the ability to ferment lactose. λfla406 was a gift from M. Silverman; a schematic of it is shown in Fig. 1. The phage is imm^{434} and encodes all of the functions necessary for both lytic growth and lysogeny. Phage stocks were grown on CSH4rh in NZCYM medium by the method of Maniatis et al. (11).

Phage growth for switching assay. Strains were assayed in vivo for switching activity by using the phage λfla406. Cells to be tested were grown to a density of ca. 2×10^8 cells per

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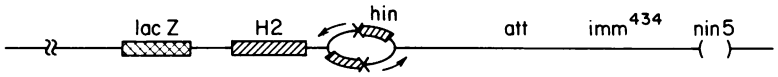

FIG. 1. A schematic of the phage λfla406 showing the relative orientation of *hin* and the inversion region, *H2*, and *lacZ*. The X indicates the deletion in *hin*, and the arrow represents the *H2* promoter.

ml in LB medium with 0.1% maltose. The 2×10^8 cells were spun down and resuspended in 25 μl of 50 μM NaCl–10 mM Tris-hydrochloride (pH 7.8)–10 mM $MgCl_2$–0.01% gelatin containing 10^8 phage. After adsorption of the phage, the cells were washed twice, resuspended in NZ medium, and incubated at 30°C for one cycle of phage growth (2 h). Chloroform was added to kill lysogens, and then the progeny phage were plated out with CSH4rh on NZ plates with the dye 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal).

Heat induction of the Hin protein. Cells containing plasmids that encoded a temperature-inducible *hin* gene were grown in 5-ml cultures at 30°C to a cell density of 2×10^8, induced by incubation at 42°C, harvested, and dissolved in 0.1 ml of sodium dodecyl sulfate (SDS) sample buffer. Samples (10 μl) were run on 18% polyacrylamide gels.

Plasmids for Hin protein overproduction. The plasmids used for this work are illustrated in Fig. 2. The complete invertible region is contained on the plasmid pJZ143 (23). The *hin* gene and IRL are located on a *Bst*N1 restriction fragment of pJZ143. This fragment was inserted into the *Cla*I restriction site of pBR322 to make the plasmid pES201 (E. Szekely, Ph.D. thesis, University of California, San Diego, 1982; Fig. 2a). Synthesis of the Hin protein was enhanced by inserting the strong promoter p_L of bacteriophage λ in front of the *hin* gene. A 2.7-kilobase *Eco*RI restriction fragment from λ (*bio*256 *c*I857 *cro*27 *S*am7; see Hedgpeth et al. [7]), containing the genes *c*I857 and *N* and the promoters p_L and p_R, was inserted into pES201 to make pGM5 (G. Mandel and M. Simon, unpublished data). The fragment is oriented such that the *hin* gene is under the control of p_L (Fig. 2b).

It was thought that the overproduction of the *c*I857 repressor might partially inhibit Hin overproduction; therefore, the *c*I857 gene was deleted from the plasmid, and a host which encoded the *c*I857 gene in its chromosome was used. For the construction of the *c*I857 deletion plasmid, a derivative of pBR322, pMFB8, was made in which the *Eco*RI and *Cla*I restriction sites were destroyed by restricting, filling-in with DNA polymerase large fragment, and then religating. A *Hin*dIII restriction fragment from pGM5 which carries only p_L, *N*, IRL, and *hin* was inserted into the *Hin*dIII restriction site of pMFB8, to give pMFB9. This plasmid has a unique *Eco*RI restriction site between p_L and *hin* and has a unique *Cla*I restriction site within the *hin* gene (Fig. 2c). As a control, another plasmid (pMFB10) was made by creating a frame-shift mutation within the *hin* gene by restricting and filling-in the *Cla*I restriction site and then religating (Fig. 2d).

The efficiency of the transcription of the *hin* gene was further increased by reducing the distance between p_L and *hin*. This was done by removing an *Eco*RI-*Hpa*I restriction fragment between p_L and *hin* on pMFB9 and then treating the linearized plasmid with the exonuclease BAL 31. The plasmids produced by this treatment were religated and transformed into MA800 (Fig. 2e).

Hin protein purification. Six liters of the Hin-overproducing strain MA800 with pMFB14 were grown to 2×10^8 cells per ml at 30°C, heated to 42°C, and grown for 8 h more. The cells were harvested and resuspended in 100 ml of 50 mM Tris-hydrochloride (pH 8.0)–200 mM NaCl–5 mM dithiothreitol–2 mM EDTA–10% sucrose and frozen and stored at −70°C. The thawed cells were then sonicated and centrifuged at 23,000 × *g* for 15 min. The pellet, which contained the Hin protein, was washed with 30 ml of 10 M urea–1.6% (vol/vol) Triton X-100 in P-E buffer (10 mM sodium phosphate [pH 7.8], 5 mM EDTA) and centrifuged for 30 min at 140,000 × *g* at 15°C. The pellet was then washed in 25 ml of 2.5 M guanidine hydrochloride in P-E buffer and centrifuged for 30 min at 140,000 × *g* at 5°C. The Hin protein was extracted from the pellet with 10 ml of 5 M guanidine hydrochloride in P-E buffer and clarified by centrifugation for 45 min at 200,000 × *g* at 5°C. The Hin protein in the supernatant was precipitated by dialysis overnight against P-E buffer. The precipitate could be solubilized in either 2% SDS at 80°C or 5 M guanidine hydrochloride. The protein gave a single band on SDS-polyacrylamide gel electrophoresis.

Electrophoresis and protein transfer experiments. SDS-polyacrylamide slab gel electrophoresis was done by a modification of the method of Laemmli (10) which increases the Tris-hydrochloride concentration to 0.5 M in the running gel. Proteins in SDS-gels were transferred to nitrocellulose electrophoretically, and specific proteins were visualized with rabbit antibodies and goat anti-rabbit antibodies coupled to horseradish peroxidase by the method of Towbin et al. (22). The antibody-stained proteins were quantitated by scanning positive transparencies of the gel at 485 nm and integrating the peaks. The concentrations were determined by using a standard curve generated from purified Hin samples run on the same gel.

Antibody production and affinity purification. The Hin protein was futher purified by preparative electrophoresis on an SDS–15% polyacrylamide gel; the Hin-containing band was cut out, and the protein was electroeluted (8). New Zealand white rabbits were each injected with 1.3 mg of Hin with complete Freund adjuvant. After 4 weeks, booster injections of 0.5 mg of Hin each were given every 2 weeks either subcutaneously with incomplete Freund adjuvant or intravenously. Bleedings were done 7 days after booster injections. The titer and specificity of the antisera were tested by using dot blots of purified Hin on nitrocellulose (6) and by protein gel transfer experiments described above using whole *E. coli* extracts.

Hin-specific antibodies were isolated on a Hin-agarose column, which was made by coupling 7 mg of electroeluted Hin in 5 ml of 0.2 M $NaHCO_3$–0.2% SDS to 2.5 ml of Sepharose CL-4B (Pharmacia Fine Chemicals) by the method of March et al. (12). About 60% of the Hin was covalently coupled to the agarose. Immunoglobulins from the rabbit sera were precipitated by adding ammonium sulfate to 33% saturation. The pellet was washed with 33%-saturated ammonium sulfate, resuspended in 100 mM NaCl–20 mM sodium phosphate (pH 7.3), and dialyzed against the same buffer. Protein derived from 10 ml of antiserum was passed over the Hin-agarose column and the column was washed with 15 ml of NaCl-phosphate buffer and eluted with 100 mM NaCl–200 mM acetic acid. The eluted antibody was immediately brought to pH 7.5 with Tris base.

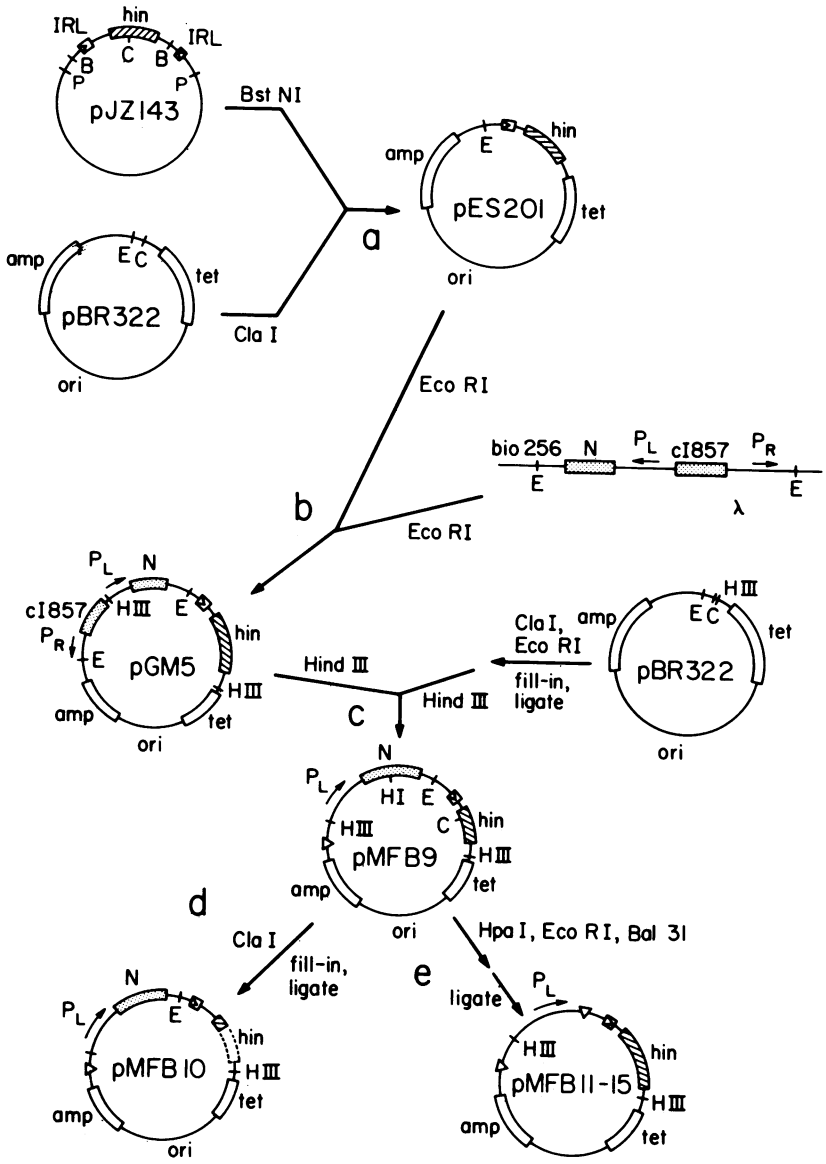

FIG. 2. The construction scheme for plasmids pGM5, pMFB9, pMFB10, and pMFB14 which are used in making Hin-overproducing strains. Restriction sites: B, *Bst*NI; C, *Cla*I; E, *Eco*RI; HI, *Hpa*I; HIII, *Hin*dIII; P, *Pst*I.

RESULTS

Overproduction of the Hin protein. The DNA sequence of the *hin* gene predicts that it will direct the synthesis of a 20,000-molecular-weight protein. The plasmid pMFB9 carries the *hin* gene under the control of the λ promoter p_L. When this plasmid is carried by a strain which provides the temperature-sensitive repressor *c*I857 (MA800), synthesis of a 20,000-molecular-weight protein is induced by heating the culture to 42°C for 5 min. This was demonstrated by SDS-polyacrylamide gel electrophoresis of whole-cell extracts from induced and uninduced cultures (Fig. 3a). In a control experiment, pMFB9 was replaced by pMFB10 which contains a two-base insertion in the *hin* gene. The control strain showed no heat induction of a 20,000-molecular-weight protein, indicating that the *hin* gene was required for its expression (Fig. 3b). Other minor proteins of different molecular weights were heat induced; however, these were seen with both pMFB9 and pMFB10. The same level of induction of the 20,000-molecular-weight protein was seen in a strain carrying a plasmid which also encodes the *c*I857 repressor (pGM5), indicating that there was little effect of the *c*I857 gene copy number on the level of expression of Hin from p_L.

In pMFB9, the distance from p_L to the start of the *hin* gene is about 1.8 kilobases as determined by restriction mapping (data not shown). This distance was reduced by using the exonuclease BAL 31. Plasmids resulting from this treatment were transformed into MA800, and individual colonies were screened for temperature-induced Hin overproduction by using SDS-polyacrylamide gel electrophoresis. Several colonies displayed a significant enhancement of Hin protein production; plasmids from these were designated pMFB11 through pMFB15. The best overproducing strain contained the plasmid pMFB14. In making these plasmids, the plasmid copy of the *N* gene was destroyed; however, *N* is still

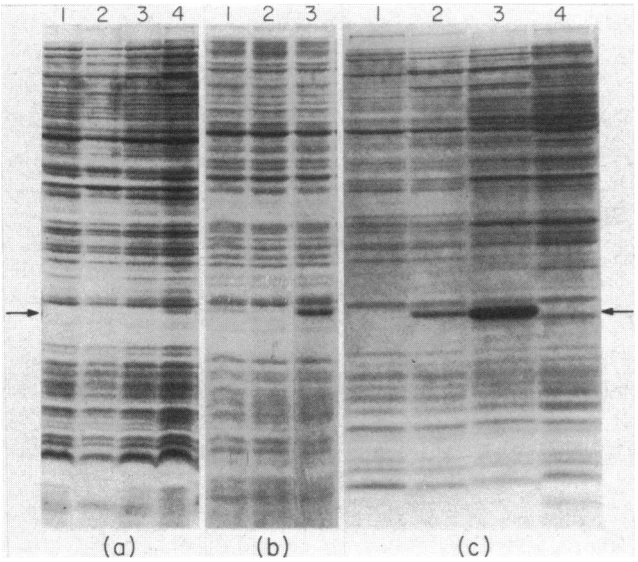

FIG. 3. Demonstration of the induction of Hin by gel electrophoresis. (a) Strains of *E. coli* MA800 containing different plasmids were grown at 30°C and then induced for 15 min at 42°C. The arrow points to the 20,000-molecular-weight Hin protein. Lanes 1 and 2 show whole-cell extracts of uninduced and induced MA800; lanes 3 and 4 show uninduced and induced MA800 carrying a plasmid with *hin* under p_L control (pMFB9). (b) Lanes 1, 2, and 3 show extracts of cells induced for 15 min with pMFB9, the *hin*-frame-shifted control plasmid (pMFB10), and the strong overproduction-directing plasmid (pMFB14), respectively. (c) Lanes 1 and 2 show extracts of exponential cultures of uninduced and 1-h-induced MA800 carrying pMFB14. Lanes 3 and 4 show extracts of the same cultures grown to the stationary phase. The culture for lane 3 was grown at 42°C.

expressed from the chromosome of MA800. In pMFB14, the distance from p_L to *hin* was determined to be about 400 base pairs. Moving p_L closer to *hin* enhanced the amount of Hin protein synthesized by about fivefold (Fig. 3b). When MA800 with pMFB14 was grown to stationary phase at 42°C, an even greater amount of Hin protein was made (Fig. 3c).

Solubilization and purification of the Hin protein. The unusual solubility properties of the overproduced Hin protein were used for its purification. In all of the inducible strains, after induction, most of the Hin protein was found in the pellet of lysed extracts after a 10-min centrifugation at 25,000 × *g*. Small amounts of Hin did remain in solution. The amount of soluble Hin was maximized when MA800 carrying pMFB9 was induced for only 3 min and lysed in low-salt buffer (25 mM NaCl) at low cell concentrations (1 g of cells per 100 ml of lysis buffer). Most of the Hin protein in the pellet of MA800 carrying pMFB9 which had been induced for 5 min could be solubilized with 3 M urea. When the induction time was increased to 15 min, 6 M urea was required to solubilize all of the Hin. In the most extreme case, when the stronger overproducing strain which carries pMFB14 was grown to stationary phase at 42°C, Hin was not solubilized by either 10 M urea with 1.6% Triton X-100 or 2.5 M guanidine hydrochloride; however, the Hin protein in this pellet was solubilized by 5 M guanidine hydrochloride or 80°C 2% SDS. The solubilized Hin protein precipitated when the guanidine or SDS was dialyzed away. At concentrations of less than 1 mg/ml, solubilized Hin did remain in solution when dialyzed against 1 M urea or 0.1% SDS. Few other *E. coli* proteins have these solubility properties; therefore, these properties provided a rapid method for purifying large quantities of Hin protein. From 6 liters of culture, 20 mg of Hin protein was isolated. An amino acid composition analysis of the purified protein was in agreement with the composition predicted from the DNA sequence of the *hin* gene. When 5 μg of the isolated protein was run on an SDS-polyacrylamide gel, some minor contaminants were seen. Therefore, the Hin protein was further purified by preparative gel electrophoresis before injecting it into rabbits for antibody induction or coupling to agarose for antibody isolation.

Antibodies to Hin and other proteins. The electrophoretically purified Hin protein was used to make rabbit antibodies to Hin. Figure 4 (lanes 1 to 4) shows an SDS-polyacrylamide gel transferred onto nitrocellulose and stained with the purified antibody to Hin. In strains in which Hin was expressed from the wild-type promoter, those carrying

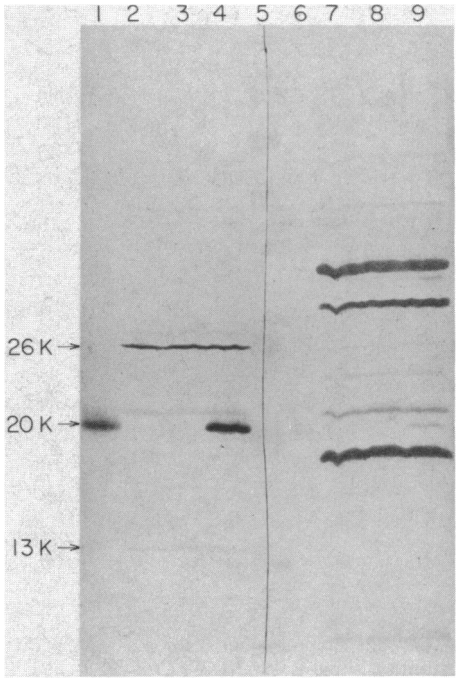

FIG. 4. Antibody staining of Hin and other proteins. Whole-cell extracts from 10^8 cells were run on an SDS-polyacrylamide gel, and the proteins in the gel were transferred to nitrocellulose and specifically stained with antibodies. Lane 1 contains purified Hin; lane 2 contains a whole-cell extract of CSH4rh carrying pES201, a plasmid with *hin* and its wild-type promoter; lanes 3 and 4 contain whole-cell extracts of the Hin-inducible strain carrying pMFB9 uninduced and induced for 15 min. Lane 5 contains prestained molecular weight standards (myosin, 200,000; phosphorylase B, 92,500; bovine serum albumin, 68,000; ovalbumin, 43,000; α-chymotrypsinogen, 25,700; β-lactoglobin, 18,400; and cytochrome *c*, 12,300). Lanes 6 to 9 duplicate lanes 1 to 4, respectively. Lanes 1 to 4 were stained with affinity-purified antibody to Hin. The Hin protein is at the limit of detection when it was uninduced (lanes 2 and 3) but is clearly visible after induction (lane 4). Two proteins, molecular weights 26,000 and 13,000, stained clearly in all of the whole-cell extracts, indicating they cross-reacted with the Hin antibody. Lanes 6 through 9 were stained with rabbit immune sera which was depleted of antibodies to Hin by passage over a Hin-agarose column. Although many proteins are stained, the three proteins which were stained well by antibodies to Hin are stained poorly.

pES201 and uninduced pMFB9, the amount of Hin protein produced was at the limit of detection. However, when Hin was expressed from p_L (induced strains carrying pMFB9), Hin was the major protein band stained.

E. coli contains at least two other proteins which cross-reacted with antibodies to Hin. These ran at molecular weights 26,000 and 13,000 and are shown in Fig. 4, lanes 1 to 4. As a control, the Hin antiserum was depleted of antibodies to Hin by passing it over the Hin-agarose column. In Fig. 4, lanes 6 to 9 contain the same samples as lanes 1 to 4 but they were stained with the depleted antiserum. Hin and the 26,000- and 13,000-molecular-weight cross-reacting proteins were stained very weakly. This demonstrates that these three proteins reacted specifically with the anti-Hin antibodies in the antiserum. Both cross-reacting proteins were seen in similar amounts in several different *E. coli* strains tested, including strains which had no detectable background switching activity (see below).

In vivo switching assay. An in vivo switching assay was designed by using the recombinant phage λfla406 (Fig. 1). λfla406 contains the invertible region and the *H2* gene in an operon fusion with the *lacZ* gene such that the expression of *H2* and *lacZ* depends on the orientation of the invertible region. The *hin* gene in the invertible region contains a deletion; therefore, switching depends on host sources of the Hin protein. If initially all of the phage have the invertible region in the off orientation, that is, neither *H2* nor *lacZ* are expressed, the phage can be used to measure the amounts of Hin activity in a particular cell. The strain to be tested is infected and the phage is allowed to undergo one cycle of growth. If the host expresses any switching functions, some of the phage will have the invertible region switched to the on orientation while they are replicated. These switched phage will transduce both the Lac^+ and Hag^+ phenotypes. The progeny phage are plated on a strain which is both an *lacZ* and an *hag* mutant. In the presence of X-gal, any plaques of $lacZ^+$ phage are blue (e.g., Fig. 5). The assay has an internal control in that any blue plaque should contain hag^+ lysogens and forms a swarm when picked onto mot agar. The amount of switching activity in the strain tested will be reflected by the percentage of progeny phage that give blue plaques.

During the course of a single cycle of infection, some of the phage which have been switched on will be switched back off, especially at high levels of switching activity. Scott and Simon (15) have shown that plasmids containing the invertible region in the presence of Hin have equal amounts of invertible segment in each orientation at equilibrium. By using this equilibrium value of 50% on and by assuming a single, exponential approach to the equilibrium, a correction for back-switching can be calculated from the percent of the phage in the on orientation with the equation $A = 1/2\ln(1 - S/50\%)$, where A is the corrected switching activity and is given as the number of switching reactions per phage, and S is the observed percent of the phage giving blue plaques. Below 8% switching, the corrected switching activity and the percent of blue plaques are directly proportional within 10%. However, at higher activities this direct proportionality fails; for example, 18 and 30% blue correspond to corrected switching activities of 0.23 and 0.46.

As a part of the assay, the cells were washed twice after adsorption of the phage to remove any unadsorbed phage. By monitoring the washes and observing a time course of the infection, it was shown that greater than 90% of the phage were adsorbed and that less than 0.5% of the phage present after the first cycle of growth derived from unadsorbed phage.

Some *E. coli* strains will give a low frequency of switching without a copy of the *hin* gene (3, 13). No background switching was detected in CSH4rh, and MA800 gave about one switch per 10^4 phage after an overnight infection with λfla406. Because of its absence of background switching, CSH4rh was used to grow stocks of λfla406 which had less than one phage per 10^5 in the on orientation.

The results of the in vivo switching assay with MA800 carrying various plasmids are shown in Table 1 and Fig. 5. The multicopy plasmid pES201 carries *hin* with its wild-type promoter and no additional promoters. Strains carrying this plasmid gave less than 0.01 switch per progeny phage from a λfla406 infection at 30°C. The level of activity was unaffected by a 42°C induction pulse. The inducible strain (MA800) carrying pMFB9 gave about the same switching activity at 30°C as did strains with pES201. When cells carrying pMFB9 were given a 5-min induction at 42°C before infection, the

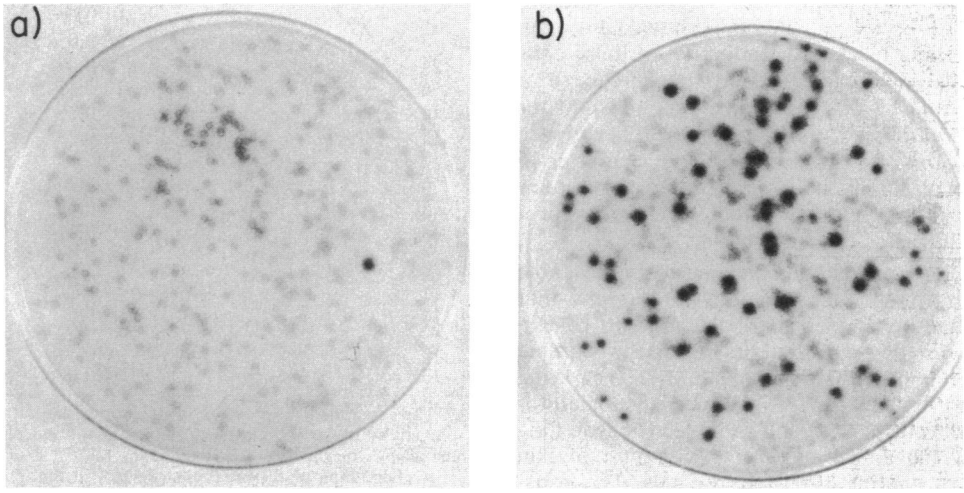

FIG. 5. Plaque assay for in vivo switching with λfla406. *E. coli* strain MA800 carrying pMFB9 was infected with λfla406 for 2 h and 15 min at 30°C. The progeny phage were plated with strain CSH4rh on NZ plates with the dye X-gal. (a) No temperature induction was given. (b) Cells were incubated 5 min at 42°C before phage infection.

TABLE 1. Switching activity of *E. coli* MA800 carrying various plasmids induced for different times at 42°C[a]

Plasmid	No. of blue plaques/total no. of plaques (switches per phage)				
	Grown at 30°C	Induced at 42°C for following times (min):			
		5	15	30	60	120
pES201	6/948 (0.006)	—	11/1477 (0.008)	—	—	—
pMFB9	5/992 (0.005)	148/1240 (0.14)	170/1109 (0.18)	111/710 (0.19)	59/575 (0.11)	138/1633 (0.09)
pMFB10	0/500 (<0.002)	—	0/2500 (<0.001)	—	—	—
pMFB14	16/680 (0.024)	62/408 (0.18)	32/548 (0.06)	—	—	—

[a] Cultures of MA800 carrying the indicated plasmid were grown at 30°C and induced at 42°C for the indicated time. The cultures were returned to 30°C and infected with λfla406 as described in the text. Switches per phage were calculated from the percent of blue progeny phage by the equation given in the text. —, No progeny phage were produced from an infection of MA800 carrying pMFB14 after a 2-h induction.

switching activity increased to 0.14 switch per phage, which is a greater than 20-fold enhancement of switching activity (Fig. 5). Longer periods of induction increased the amount of activity measured only slightly. The switching frequency for λfla406 from on to off (blue to colorless) was the same as the frequency from off to on (data not shown).

Since the time required for a single cycle of phage growth was about 2 h, it was important to show that the *hin* gene product was stable and present during a significant portion of the phage infection. When cells were incubated for 30 min at 30°C after a 10-min 42°C induction and then infected with λfla406, the level of activity was the same as when the cells were infected immediately after the induction. Thus, the Hin protein was stable in vivo for longer than 30 min (see also Fig. 6b).

The upper limit to the oberved percent switched was not an artifact of the phage itself. When a phage infection of MA800 carrying pMFB9 was allowed to go for 6 h at 30°C (ca. three cycles of phage growth) with an initial 10-min induction and a second induction after 3 h, 35% of the progeny gave blue plaques. This corresponded to 0.6 switches per phage, which was about three times the value after a single cycle of phage growth (16% blue, 0.19 switch per phage).

It was shown above that MA800 carrying pMFB14 induced much more Hin protein than MA800 carrying pMFB9. Strains with pMFB14 have a higher background at 30°C (Table 1), presumably due to some leakage from p_L. Short time induction (5 min) of this strain gave switching activities comparable to strains carrying pMFB9. However, after 15 min of induction, strains carrying pMFB14 showed a drop in the amount of switching measured. Induction of these cells slowed their growth greatly and caused the burst size of lambda to be small. The small burst size was constant for both λfla406 and λ *imm*^{434} and, thus, was not a consequence of the invertible region. The drop in switching seen after 15 min of induction may simply be an indication of the poor growth of the cells, or it may indicate that strongly overproducing cells can inactivate the Hin protein.

Switching activity proportional to Hin concentration. Table 2 and Fig. 6 illustrate two experiments which show that switching activity was proportional to the amount of Hin protein in the cell. The first experiment (Fig. 6a) was a time course of the induction of the Hin protein in MA800 carrying pMFB9. Samples were collected from cultures incubated for increasing lengths of time at 42°C and analyzed for switching activity by the λfla406 assay and for the amount of Hin protein by antiserum staining of transferred gels. The transfer to nitrocellulose and the staining were calibrated by running a Hin standard curve on the same gel as the samples. The 26,000-molecular-weight cross-reacting protein was used as an internal standard to normalize the total amount of protein in each lane. Levels of Hin less than 75 ng and greater than 500 ng were difficult to quantitate. The amount of Hin protein in 10^8 cells was plotted against the number of switches per phage as calculated by using the equation given above (Fig. 6c). From 2 to 6 min of induction, the increase in activity was proportional to the increase in the amount of protein. As mentioned above, after 10 min of induction, activity increased little, but Fig. 6a and Table 2 show that Hin protein synthesis continued. Other experiments shown in Table 1 indicate that there was no further increase in activity after 15 min of induction and that a drop of activity was seen after 1 or 2 h of induction.

In the second experiment (Fig. 6b), MA800 carrying pMFB9 was induced for 5 or 15 min at 42°C and then returned to 30°C. Samples were taken from the culture as it continued to grow at 30°C, diluting the cellular concentration of Hin. Activity and Hin concentrations were determined as before. The results are shown in Table 2 and given as crosses

TABLE 2. Time course of switching activity and Hin protein concentration during and after induction at 42°C[a]

Induction (min at 42°C)	Time (h) at 30°C after induction	Activity[b]	ng of Hin/10^8 cells[c]
0		0.002	<50
1		0.007	<50
2		0.007	60
3		0.034	120
4		0.095	200
6		0.17	390
10		0.20	~650
15		0.23	>750
0	0	0.004	<50
5	0	0.19	330
5	2	0.024	70
5	4	0.003	<50
5	6	0.006	<50
15	0	0.25	>750
15	2	0.065	190
15	4	0.010	70
15	6	0.006	<50

[a] The experimental procedure is given in the legend to Fig. 6.

[b] Activity is expressed as switches per phage, calculated from the percent blue plaques by the equation in the text.

[c] A photograph of the nitrocellulose blot in Fig. 5 and 6 was taken and printed on film and then scanned and integrated. A standard curve was determined by using the purified Hin samples and then used to determine the Hin concentration in the whole-cell extracts. The standard curve was only linear for 100 to 400 ng of Hin.

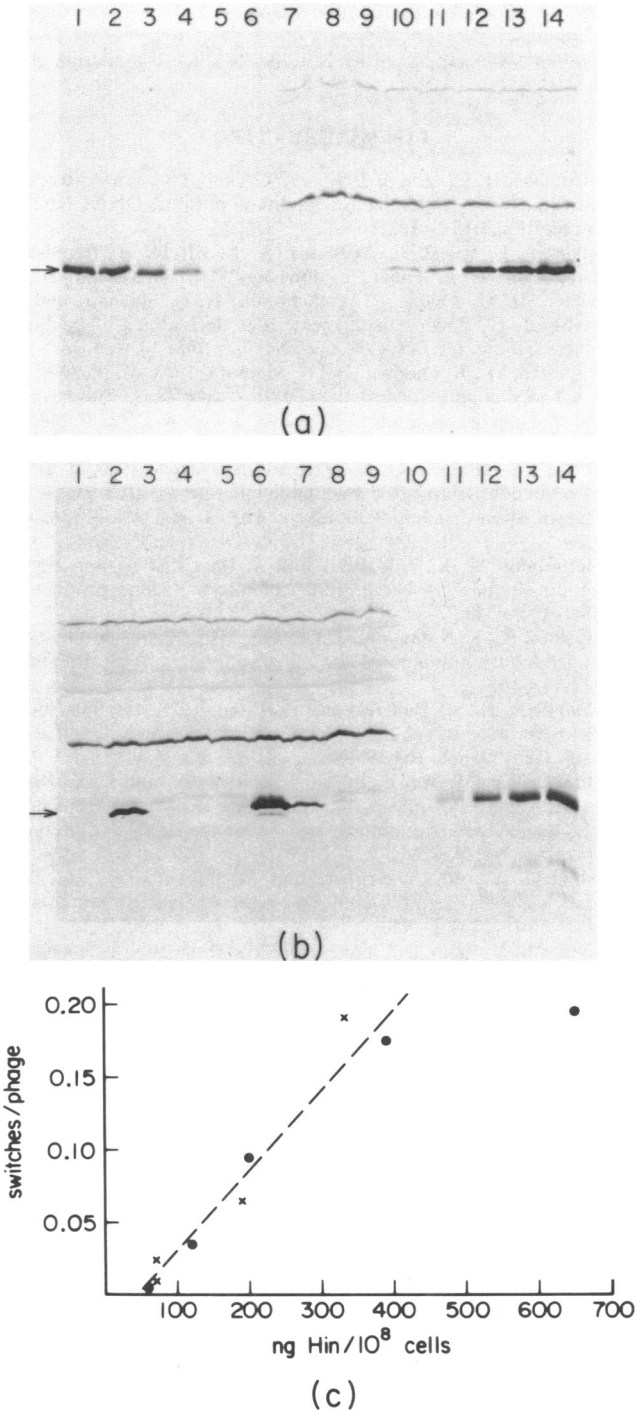

FIG. 6. Time course of Hin induction and dilution. (a) A culture of MA800 carrying pMFB9 was grown at 30°C and shifted to 42°C, and samples were removed after various amounts of time and put on ice. The optical density at 600 nm of each culture was determined and 10^8 cells were pelleted and mixed with SDS-gel sample buffer and run on a gel. Various amounts of purified Hin were run on the same gel. The proteins in the gel were transferred to nitrocellulose and stained with whole rabbit antiserum to Hin. Lanes 1 to 6 contain 500, 400, 300, 200, 100, and 50 ng of Hin. Lanes 7 to 14 show cultures induced for 0, 1, 2, 3, 4, 6, 10, and 15 min. (b) Separate cultures of MA800 carrying pMFB9 were induced for 5 and 15 min at 42°C and then shifted down to 30°C by diluting the cultures with cool medium and allowed to grow for several hours. Samples were taken at various times after induction and analyzed as in (a). Lane 1 contains an extract from uninduced cells. Lanes 2 to 5 show extracts from 5-min-induced cells immediately, 2, 4, and 6 h after induction. Lanes 6 to 9 show extracts made from 15-min-induced cells immediately, 2, 4, and 6 h after induction. Lanes 10 through 14 show 50, 100, 200, 300, and 400 ng of Hin protein. (c) The activities given in Table 2 were plotted against the protein concentrations determined from (a) and (b) above. Symbols: ●, cells grown at 30°C and then shifted to 42°C (a); ×, cells induced for 5 or 15 min at 42°C and then returned to 30°C (b).

on Fig. 6c. Hin activity dropped in proportion to the drop in the amount of Hin protein. The specific activity of Hin in the sample taken 2 h after the 15-min induction was comparable to that of freshly induced Hin.

DISCUSSION

Silverman et al. (18) have demonstrated with a cell-free transcription-translation system that the *hin* gene product is a 20,000-molecular-weight protein. By engineering several plasmids which direct the overproduction of the Hin protein, we confirmed this result. The accumulated evidence for this is as follows: insertion of the bacteriophage λ promoter p_L in front of the *hin* gene and use of a temperature-sensitive repressor of the promoter caused a 20,000-molecular-weight protein to be induced when the temperature was raised; when the promoter was moved closer to the gene, presumably increasing the efficiency of the transcription of *hin*, the amount of 20,000-molecular-weight protein was increased; an identical plasmid which was altered by a two-base-pair frame-shift mutation within the *hin* gene failed to make any of the protein; and, finally, the amino acid composition of the purified 20,000-molecular-weight protein agreed with the DNA sequence of the *hin* gene.

Induction of Hin protein synthesis also increased switching activity. This was shown with an in vivo assay for switching activity that used the recombinant phage λfla406. The *lacZ* gene of λfla406 is turned on by switching activities. This assay showed that cells containing pES201, a multicopy-number plasmid which carries the *hin* gene with its wild-type promoter, switched about 1% of the phage during one lytic cycle of growth. Under conditions in which a single copy of *hin* is present in *S. typhimurium*, the highest frequency of phase variation observed is about one switch in 10^3 cells per generation (21). The assay showed that overproduction of the *hin* gene by using the promoter p_L could increase the switching activity up to 30-fold. By simultaneously measuring the in vivo activity and the amount of *hin* gene product, we showed that switching activity was proportional to the amount of Hin protein over at least a 10-fold range. This demonstrated that Hin is a limiting reagent needed for switching and that any other required cofactors or activities are present in a 10- to 20-fold excess.

It was not possible to get cells with enough Hin activity to give more than 20% switching during one lytic cycle of λfla406 growth. It may be that this upper limit of activity reflects the possible requirement for additional cellular proteins or factors needed for the recombination reaction. At high concentrations of Hin, the factors would become rate limiting. Since Hin activity could be increased 30-fold, these factors would have to be in excess over wild-type levels of Hin or be induced by Hin. Other explanations to the upper limit of activity should be considered. As the reaction approached the equilibrium value of 50%, increasingly more activity is needed to increase the percent switched an equal amount (e.g., a doubling of activity is needed to go from 18

to 30% switching). Furthermore, it was seen that cell growth and phage burst size decreased when large amounts of Hin were induced. The decrease in phage burst size was not caused by the presence of the invertible region on the chromosome. These observations may reflect stress put on the cell by overproduction of Hin, which somehow inhibited the switching reaction. It was also seen that the solubility properties of Hin changed as its level of induction was increased. It is possible that Hin failed to fold properly or was inactivated when it was synthesized in large amounts. Similar effects have been observed for the overproduction of other proteins in *E. coli*.

When Hin became several percent of the total cellular protein, it turned out to be very insoluble and hence easily purified. This purified protein was used to inject rabbits for the production of antibodies to Hin. The Hin antiserum was used to determine cellular concentrations of Hin.

The Hin antibodies were purified and used to demonstrate the existence of at least two cross-reacting antigens in *E. coli* with molecular weights of 26,000 and 13,000. It is unlikely that these cross-reactions were actually the consequence of contaminating antibodies in the isolated Hin antibodies, since the Hin protein was purified by preparative gel electrophoresis for both antibody induction and isolation. Some *E. coli* strains carry a homolog to the *hin* gene called *pin*. The *pin* gene has been sequenced (13), and its gene product has 60% amino acid sequence homology with Hin. However, it is six amino acids shorter than Hin, so its molecular weight is not consistent with the weights of the cross-reacting proteins. Furthermore, the *pin* gene product can switch the phase-variation invertible segment; however, CSH4rh has no detectable inversion activity, and yet it still has both cross-reacting proteins. It is possible that these two proteins belong to distinct site-specific recombination systems which contain immunologically cross-reacting domains with Hin-like functions; on the other hand, cross-reaction does not necessarily reflect functional homologies.

Phase variation is similar to the resolution of cointegrates of the transposon Tn*3*/γδ. In the Tn*3* system, resolvase, the product of the *tnpR* gene, catalyzes a site-specific recombination between two identical *res* sites which are in a direct-repeat configuration to separate a cointegrate into two replicons (4). Hin is involved in the same type of reaction when it causes the deletion of the region between direct repeats of IRL and IRR. Resolvase and Hin share a 35% amino acid sequence homology (20) and probably perform similar roles. Resolvase has been purified and demonstrated to resolve cointegrates in vitro in a simple system containing only substrate DNA, salt, buffer, magnesium, and no other cofactors (14).

Although the plasmids constructed in this study directed the strong overproduction of Hin, the protein produced was found to be insoluble and could not be solubilized in a native, functional form. Attempts to find in vitro switching activities in extracts made from cells carrying pMFB14 were unsuccessful, and attempts to renature the protein also failed. However, using cells carrying pMFB9 induced for short times, we have recently prepared extracts that display in vitro switching activity. The characterization of these extracts will be the subject of a subsequent report.

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