Cell, Vol. 19, 845–854, April 1980, Copyright © 1980 by MIT

Phase Variation: Genetic Analysis of Switching Mutants

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Summary

Site-specific inversion of a controlling element is responsible for flagellar phase transition in Salmonella. When a 900 bp DNA sequence is in one configuration, it allows the expression of the *H2* gene, a structural gene which codes for the flagellar antigen. When it is in the opposite configuration, the *H2* gene is not expressed. A hybrid λ phage containing the invertible control region and the adjacent *H2* gene was constructed, and expression of the *H2* gene was shown to be regulated by the orientation of the inversion region. Transposon Tn5 insertion derivatives of this hybrid phage were isolated and λ*H2::Tn5* mutants defective for inversion (*H2* switching) were selected and characterized. Two classes of switching phenotypes were observed among the mutants—those which had slightly reduced frequencies of transition from expression of the *H2* gene (*H2* on) to nonexpression (*H2* off) (intermediate class) and those in which the frequency of transition was reduced at least three orders of magnitude (null class). Physical mapping of the Tn5 insertion sites revealed that in all mutants the insertion was located inside the inversion region. Tn5 insertion sites in the null class of mutants defined a region of DNA including approximately 500 bp which was necessary for inversion. Genetic complementation tests showed that these λ*H2::Tn5* mutants could invert the *H2* gene control element if the 500 bp region was introduced in the trans configuration. It is concluded that a gene is located inside the inversion segment and codes for a protein which is required for the inversion event. Furthermore, the two sites at which the crossover event occurred functioned in a cis configuration and were required for inversion. The presence of a gene which is involved in controlling site-specific recombination events may be a general feature of transposon-like elements.

Introduction

Regulation of gene expression by mechanisms involving site-specific recombination has been suggested by recent work with a variety of experimental systems. In eucaryotes, genetic experiments with the yeast mating-type system (Hicks, Strathern and Herskowitz, 1977; Kushner, Blair and Herskowitz, 1979) and molecular cloning experiments involving the immunoglobulin genes in mice (Sakano et al., 1979) support the idea that specific genetic rearrangements determine the nature of gene expression. In procaryotes, the inversion of the G loop region of bacteriophage Mu DNA has been correlated with the infectivity of the phage particle (Bukhari and Ambrosio, 1978; Kamp, et al., 1978). We have shown that the mechanism of phase variation in Salmonella involves a specific rearrangement of DNA structure (Zieg et al., 1977; Zieg, Hilmen and Simon, 1978; Silverman et al., 1979b).

In Salmonella, two genes code for the major flagellar structural protein, flagellin. These two genes, the *H1* and *H2* genes, map in different regions of the Salmonella genome (Lederberg and Edwards, 1953). The phenomenon of phase variation refers to the ability of the cell to alternate or switch between expression of the two flagellin structural genes. This variation of antigenicity presumably allows Salmonella to evade the host immune response. The frequency with which cells undergo phase transition varies with different Salmonella strains from 10^{-3} to 10^{-5} per bacterium per generation (Stocker, 1949). The alternative expression of the *H1* and *H2* genes is controlled by the state of a genetic element linked to the *H2* gene (Lederberg and Iino, 1956). Another gene, *rhl*, linked to and coordinately expressed with *H2*, codes for a repressor substance that prevents expression of the *H1* gene (Fujita, Yamaguchi and Iino, 1973; Silverman, Zieg and Simon, 1979a). Thus when a cell is expressing the *H2* gene it also expresses *rhl*. This results in the repression of the *H1* gene, and only *H2*-type flagella are formed. When a cell is in phase 1, neither the *H2* nor *rhl* gene products are synthesized, and the *H1* gene can be expressed, leading to the formation of *H1*-type flagella.

To understand the mechanism of phase variation at the molecular level, recombinant molecules which carried the *H1* and *H2* gene regions were constructed and cloned in E. coli, where the phase variation effect could be reproduced. Genetic and physical analysis of the recombinant DNA molecules showed that inversion of a 900 bp region adjacent to the *H2* gene controlled the expression of this gene: in one orientation the *H2* operon was "on" and in the opposite orientation the *H2* operon was "off" (Zieg et al., 1977). The inversion of this control region which contains the *H2* operon promoter was found to be site-specific and independent of the RecA recombination system of E. coli (Zieg et al., 1978; Silverman et al., 1979b). These observations are summarized in Figure 1.

Inversion of a controlling element explains the oscillatory nature of *H2* operon expression, but to describe the mechanism which controls the frequency of the phase variation phenomenon precisely we have attempted to define by genetic techniques functions which are necessary for the inversion process. Two genetic alterations which affect the frequency of tran-

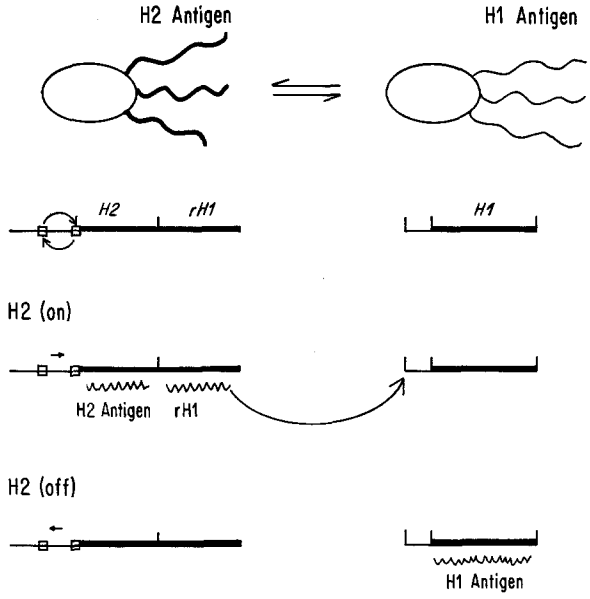

Figure 1. Model for the Alternation of Expression of the *H1* and *H2* Genes in Salmonella

Expression of the flagellar serotypes is regulated by the orientation of an invertible DNA sequence adjacent to the *H2* operon. When the *H2* operon is transcribed, the *H2* and *rhl* gene products are synthesized and the *rhl* (repressor of *H1*) gene produce prevents expression of the *H1* gene (H2 phase). When the *H2* operon is not transcribed, the *H1* gene product is formed (*H1* phase).

sition are known. One is a variant termed vH2$^-$ which was found in natural populations of Salmonella (Iino, 1961). It restricts phase transitions and was shown to map adjacent to the *H2* gene. The other is a deletion which removes about 50% of the DNA sequences on one side of the invertible region and fixes the *H2* gene in the *H2* (on) state (Silverman et al., 1979b). To further define the functions involved, a variety of mutants are required. To this end, a hybrid λ phage was constructed which contained the *H2* gene with its invertible control region. Convenient techniques were devised to measure switching of the state of expression of the *H2* gene on this hybrid λ, and transposon Tn5 was used to introduce insertion mutations into the hybrid. This report describes the isolation of mutants defective in the phase transition process. These mutants define a region of DNA inside the inversion region which codes for a gene whose product is necessary for the inversion process.

Results

E. coli is monophasic and has only one flagellin-specifying gene (*hag*), which is analogous to the *H1* gene of Salmonella. A hybrid λ phage (λ*fla 157*) was constructed by inserting a 3.75 bp Eco RI endonuclease restriction fragment that carried the *H2* gene derived from Salmonella onto a λ cloning vehicle. A Hag$^-$ E. coli strain lysogenized with the hybrid λ phage alternates between the nonflagellate and the flagellate (H2 serotype) phenotype. Cells with these two phenotypes could be conveniently distinguished by their susceptibility to the flagellotropic phage χ (Silverman et al., 1979b). Cells lysogenized with *H2* in the "on" configuration were sensitive to this phage, while lysogens with *H2* in the "off" configuration were resistant. The proportion of cells in a population in either state could be measured as a function of the number of generations of growth, and thus the frequency of phase transition could be determined (see Experimental Procedures).

To isolate λ*H2* derivatives which were mutagenized by transposon Tn5 insertion (λ*H2::Tn5*), the hybrid phage was grown in an E. coli strain with the Tn5 transposon inserted in the *ilv* gene (see Berg, 1977; Kleckner, Roth and Botstein, 1977). The resulting population of phage was then used to lysogenize a Hag$^-$ E. coli strain, and selection was applied for phage-mediated transduction of the kanamycin determinant residing on the Tn5 transposon (see Figure 2). In this way, a large number of lysogens which contained λ*H2::Tn5* insertions were collected. As a qualitative method to screen λ*H2::Tn5* phage with switching defects, clones of these lysogens were inoculated onto motility agar plates containing the flagellotropic phage χ. A lysogen clone in the *H2* (off) configuration or a lysogen in the *H2* (on) configuration which was capable of transition to the *H2* (off) phase would have been resistant to the flagellotropic phage. Only those lysogens harboring λ*H2::Tn5* which contained the *H2* gene in the "on" configuration and had a marked decrease in the frequency of transition to the "off" phase would be sensitive to the flagellotropic phage (see Figure 2). Using this screening method, putative switching mutants were chosen for further analysis from among 3000 λ*H2::Tn5* lysogens. The frequency of phase transition of these candidates was then measured. The *H2* (on) to *H2* (off) frequency for λ*H2::Tn5* lysogens with normal phase transition was approximately 10^{-2} per cell per generation. Among the mutant candidates, thirteen independently isolated clones showed reduced *H2* switching frequencies (see Table 1).

Two classes of mutant phenotypes were apparent: one (intermediate class) showed approximately one fifth the frequency of *H2* switching, while a second (null class) showed an approximately 1000 fold reduction in the frequency of phase transition. These phenotypes were characteristic of the hybrid λ, since the isolated phage could be used to prepare new lysogens which had the same altered frequency of phase transition. Furthermore, the effect of the insertion was symmetrical; that is, *H2* (off) derivatives of mutants which switched from *H2* (on) to *H2* (off) at reduced frequencies showed similar reductions in transition in the opposite direction from *H2* (off) to *H2* (on).

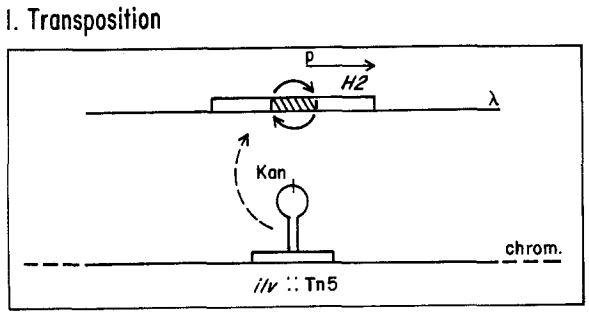

Figure 2. Isolation of Tn5-Induced *H2* Switching Mutants

Tn5 transposition to a hybrid λ phase containing the *H2* gene region resulted when this phage was grown in an E. coli strain containing Tn5 inserted in the chromosomal *ilv* gene (step 1). λ*H2::Tn5* insertion derivatives were isolated from the phage population by selection for lysogen cells which carried λ hybrids with the Tn5 kanamycin resistance determinant (step 2). Lysogens of λ*H2::Tn5* switching mutants were recognized by sensitivity to the flagellotropic phage χ (step 3).

Table 1. Switching Phenotypes of λ*H2* Mutants

λ Lysogen[a]	Mutant	Switching Frequency (per Generation)[b]
λ*fla157*	λ*H2*(wt)	1×10^{-2}
λ*fla250*	λ*H2*::Tn5	2×10^{-3}
λ*fla252*		
λ*fla255*		
λ*fla257*		
λ*fla242*	λ*H2*::Tn5	1×10^{-5}
λ*fla243*		
λ*fla244*		
λ*fla245*		
λ*fla247*		
λ*fla248*		
λ*fla251*		
λ*fla253*		
λ*fla254*		
λ*fla380*	λ*H2*(Δ)	1×10^{-2}
λ*fla385*		
λ*fla378*	λ*H2*(Δ)	2×10^{-5}
λ*fla381*		
λ*fla364*	λ*H2*(Δ)	$<1 \times 10^{-6}$
Mutant Class		wt / Intermediate / Null

[a] λ*H2* mutants were used to lysogenize E. coli Hag⁻ strain MS6302. See Figures 3 and 4 for description of hybrid λ.

[b] *H2* switching of lysogens was measured in the *H2* (on) to *H2* (off) direction as described in Experimental Procedures. Actual values varied ±50% of those shown above.

Localization of the Tn5 Insertions

Restriction fragment analysis was used to determine the location of the Tn5 insertions in the DNA of the thirteen λ*H2::Tn5* hybrid phage with switching defects, one hybrid phage with a H2⁻ phenotype and 19 hybrid phage with nondefective *H2* switching phenotypes. Initially, the phage DNA was restricted with Eco RI, which cleaves the λ*H2::Tn5* genome into five discrete restriction fragments. Insertion of transposon Tn5, which contains no Eco RI site in 5200 bp of length, into a particular Eco RI fragment markedly altered the size of that restriction fragment. Transposon Tn5 was localized in the 3.75 bp *H2* gene Eco RI insert in the case of all λ*H2::Tn5* mutants and the H2⁻ λ*H2::Tn5* hybrid, but with all hybrid phage with a nondefective switching phenotype Tn5 insertion was located in other regions of the hybrid λ genome. Further restriction analysis gave the approximate position of insertion within the Eco RI fragment and the orientation of insertion of the Tn5 transposon (data not shown). To more precisely locate the points of insertions, the mutant λ*H2::Tn5* hybrids were restricted with Hpa II, which cleaved the *H2* gene insert into several well characterized fragments. The fragments were identified using the Southern blotting technique. Fragments with homology to the *H2* gene insert were detected by hybridization with a probe containing DNA from the central region of the *H2* gene insert (see Figure 3). From an analysis of the nature of the Hpa II restriction fragments from the region of Tn5 insertions, the location of each Tn5 insertion was unambiguously determined. For example, with the hybrid phage λ*fla243*, Tn5 insertion was in the 120 bp Hpa II fragment. Thus the 120 bp fragment disappears and two new fragments, resulting from the fusion of the 120 bp sequences to the arms of the Tn5, appear. Furthermore, the restriction pattern of the wild-type phage λ*fla157* contains fragments that are

characteristic of the *H2* (on) orientation (700 and 400 bp) and of the *H2* (off) orientation (560 and 540 bp). In the restriction pattern of λ*fla243* the two restriction fragments characteristic of the *H2* (off) orientation (540 and 560 bp) were missing. This demonstrates that there is little or no switching at the molecular level. The phenotype of a lysogen containing this phage—that is, the null level of transition—is consistent with this observation. Transposon Tn5 insertion in hybrid phage λ*fla250* was in the 700 bp fragment, but fragments characteristic of the "off" orientation were also present. Thus, again in agreement with the phenotype of this phage, inversion occurred at intermediate frequencies. From these results and other restriction analysis, the location of Tn5 insertions in the mutant λ*H2::Tn5* hybrids shown in Figure 3 was determined. With all the switching mutants (either intermediate or null class) the location of Tn5 insertion was clearly within the invertible control region. With hybrid λ*fla249*, which has an H2$^-$ phenotype, Tn5 insertion had occurred in a region known to contain the *H2* structural gene (Silverman et al., 1979b).

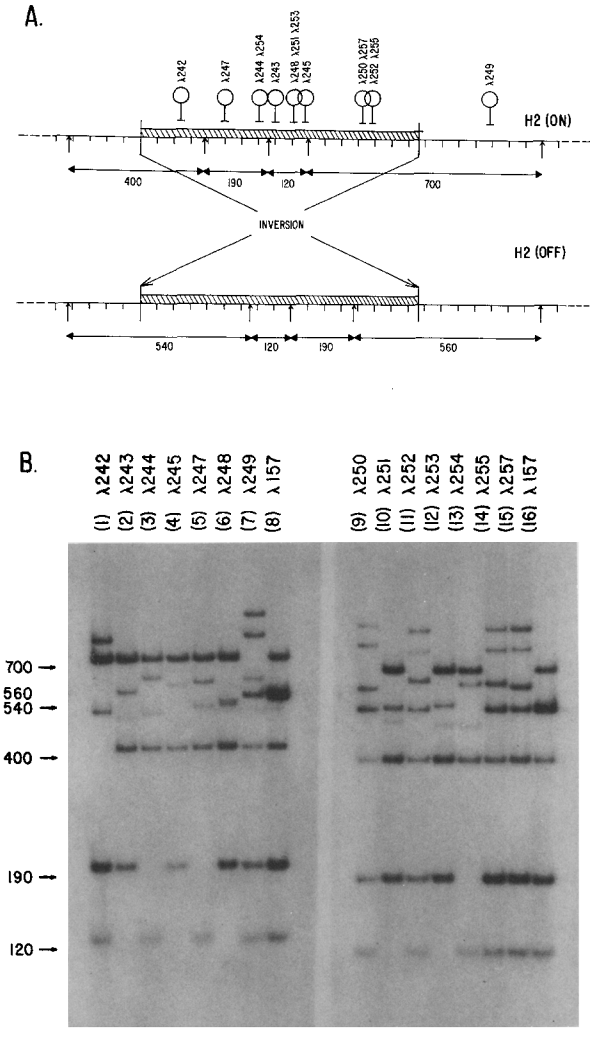

Figure 3. Location of Tn5 Insertions in *H2* Switching Mutants

The location of Tn5 insertions in mutant phage described in Table 1 is shown in (A). The inversion region (cross-hatched) and adjacent DNA sequences (*H2* gene on right) are shown in both *H2* (on) and *H2* (off) configurations. Restriction of DNA at Hpa II sites (vertical arrows) resulted in six DNA fragments. The 700 and 400 bp fragments are characteristic of the *H2* (on) phase and the 560 and 540 bp fragments are characteristic of the *H2* (off) phase. Length of DNA is marked in 50 bp intervals (vertical lines) and positions of Tn5 insertion are accurate to approximately 25 bp. Tn5 positions were determined in part from analysis of Hpa II restriction fragments of λ*H2::Tn5* DNA shown in (B). Hpa II fragments from mutant phage were transferred to nitrocellulose paper from acrylamide gels and hybridized to a ^{32}P-labeled probe (PJZ121) containing *H2* region sequences. Fragments characteristic of wild-type *H2* phage DNA (λ*fla157*) are shown at left. Interpretation of pattern of Hpa II fragments from Tn5 insertion mutants is described in the text.

Transposition-Generated Deletions

Tn5 stimulates the formation of deletions adjacent to its point of insertion (Berg, 1977; Kleckner, 1977). The deletions usually have one endpoint within the transposable element and extend in either direction from that point into adjacent sequences. Deletion mutants of hybrid λ*H2::Tn5* phage generated by Tn5 transposition were obtained by chelating agent (Na pyrophosphate) selection (Parkinson and Huskey, 1971). Deletions originating at Tn5 insertion points inside the inversion region in λ*fla250*, λ*fla252*, λ*fla255* and λ*fla257* are particularly interesting, since they retain most of the phage transition function, and the loss of remaining function can be correlated with deletions. 72 deletions were selected. All of these deletions had lost the kanamycin determinant. Some of these were the result of apparently precise excision events, and phase transition and the integrity of the restriction fragments was completely restored. Others resulted in a variety of deletions. These were analyzed genetically and by Southern transfer hybridization (Southern, 1975). The orientation of the deletion could be easily determined, since one fragment resulting from the insertion remained unchanged while the other fragment was either shortened or eliminated. The size

The location of Tn5 insertion could be correlated with the mutant phenotype. The four λ*fla* phages that showed an intermediate switching phenotype all carried the Tn5 insertion within a 100 bp sequence inside the inversion region (see Figure 3). All mutant phage with the null phenotype contained Tn5 insertions within the 120, 190 and 400 bp fragments, including a target of about 500 bp. It is of interest to note that elongation of the 900 bp inversion region by insertion of the 5200 bp Tn5 transposon apparently had little effect (approximately 5 fold) on the frequency of phase transition (for example, λ*fla250*, λ*fla252*, λ*fla255*, λ*fla257*). On the other hand, Tn5 insertion into the 500 bp region defined by hybrids such as λ*fla245* drastically reduced the ability of the region to invert.

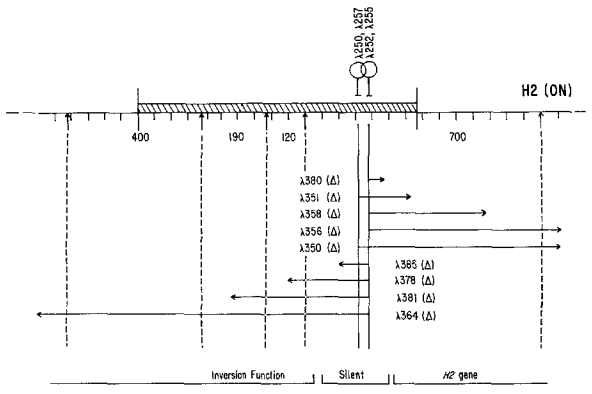

Figure 4. Deletion Mutants Derived from λ*H2::Tn5* Phage

Deletion mutants which arose by Tn5-mediated transposition were derived from four λ*H2::Tn5* mutants (intermediate switching class) shown above the Hpa II restriction map of the *H2* region in the *H2* (on) phase. One terminus of the deletion is within an arm of the Tn5 element (solid vertical lines), and deletion extends through Tn5 DNA (kanamycin determinant is deleted) to another terminus in *H2* DNA (horizontal arrows). Deletion endpoints shown in *H2* DNA are imprecise, but were accurate relative to Hpa II sites (vertical dotted lines), to crossover points for inversion (vertical line at ends of inversion sequence), and to other deletion endpoints. Mapping of deletions was by restriction analysis such as that shown in Figure 3B. Functions defined by the phenotype of the mutant phage are summarized at the bottom of the figure. Other details of the map are the same as Figure 3A.

of deletions shown Figure 4 is approximate, since it is not clear exactly how much of the Tn5 transposon remains fused to the *H2* sequence. An estimate of the minimum size of the *H2* region that was removed could be made, however, since the deletion endpoints were accurate relative to Hpa II restriction sites, crossover points for inversion and other deletion endpoints. The ability of the remaining material to invert could be determined from the presence or absence of the restriction fragments characteristic of inversion. Figure 4 summarizes the results of the deletion mapping experiments.

On the basis of our examination of the physical and genetic properties of these phages, the following conclusions were drawn. First, deletion past the crossover points for the inversion event always abolished inversion. Thus inversion did not occur with hybrids λ*fla358*, λ*fla350*, λ*fla356* and λ*fla364*. Second, inversion did not take place if the deletion extended into the region previously defined as necessary for inversion. Thus deletion into the 120 or 190 bp fragments, for example, λ*fla378* and λ*fla381*, markedly reduced inversion, and extensive deletion, as in λ*fla364*, completely eliminated inversion. On the other hand, λ*fla385* showed inversion. In fact, the *H2* switching phenotype of hybrid λ*fla385* was similar to that of the wild-type *H2* phage (λ*fla157*) rather than that of the parent λ*H2::Tn5* hybrid (λ*fla255*). The reduction in switching frequency observed with the intermediate class switching mutants (that is, λ*fla255*) could therefore be attributed to elongation of the inversion region by insertion of the Tn5 element and not to the interruption of a particular switching function located at the point of Tn5 insertion. Third, λ*fla380* and λ*fla351* showed wild-type switching frequencies as measured by restriction analysis; however, hybrid λ*fla351* had lost the ability to express the *H2* gene. As expected, hybrids λ*fla350*, λ*fla356* and λ*fla358* also had the H2$^-$ phenotype. These data suggest that the location of the *H2* gene promotor is probably within the first 100 bp of the invertible control region. We have not determined whether any of the coding sequences for the *H2* gene product are also located within this segment. In addition to the sites where inversion takes place, these regions, shown in Figure 4, have been defined: a region containing the *H2* gene and its promotor, a region inside the invertible segment which has little apparent effect on switching frequency (described as "silent" in Figure 4), and a region of approximately 500 bp inside the invertible segment which is necessary for *H2* gene switching.

Complementation Analysis

Tn5 insertions in a region of approximately 500 bp inside the inversion sequence (that is, λ*fla251* and so on) resulted in loss of a function necessary for normal *H2* gene switching. This region of DNA is well separated from the sites where crossover takes place during inversion—indeed, some Tn5 insertions which drastically reduce inversion are located approximately 500 bp from the crossover points. It is conceivable that this region functions as a site which acts in a cis manner to activate inversion; for example, this region might act as a recognition site for a protein which catalyzes the inversion process. Alternatively, this region might be a gene and code for a protein factor which functions in trans to catalyze switching. To test the latter explanation, a genetic arrangement was devised to determine whether the defect in the λ*H2::Tn5* switching mutants could be complemented in trans. Figure 5 summarizes the strategy for a test by hybrid phage or plasmids containing the putative gene necessary for switching. E. coli cells were co-infected with a λ*H2::Tn5* mutant (*H2* fixed on) and a λ*H2* deletion mutant which contained the region of DNA necessary for inversion, the mixture of phage was isolated, Kan^r lysogens of a Hag$^-$ strain were prepared and the H2 phenotype of the lysogen was measured. The trans complementing phage was either deletion mutant λ*fla350* or λ*fla356* (see Figure 5), both of which were H2$^-$ and incapable of inversion but contained the distal portion of the inversion region. To eliminate the production of recombinant phage which might mimic the phenotype of a mutant λ*H2::Tn5*, infection was done with Red$^-$ λ derivatives in a RecBC$^-$ strain. The results of this complementation analysis are shown in Table 2. It is apparent that when the region containing the putative inversion controlling gene was provided in trans, a significant proportion of

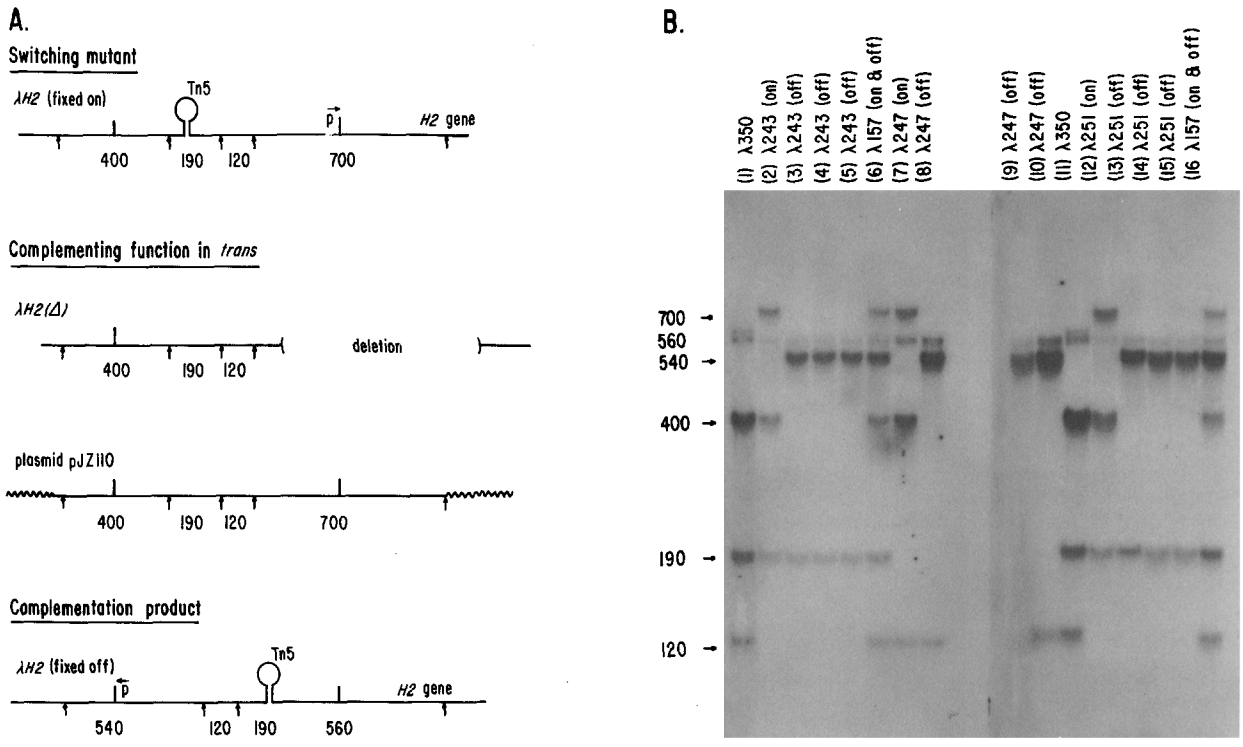

Figure 5. Complementation Analysis of Switching Mutants

The genetic test to determine whether λ*H2::Tn5* mutants could be complemented to switch, *H2* (on) to *H2* (off), by providing a function in trans is shown in (A). Null class λ*H2::Tn5* switching mutants in the *H2* (on) phase were used to infect cells which were either co-infected with a λ*H2* deletion mutant (trans function donor) or which already carried a hybrid *H2* plasmid (trans function donor). The resulting phage lysate was used to form λ*H2::Tn5* lysogens, and the H2 phenotype of the lysogen was then measured by the χ phage resistance test. Table 2 summarizes the results of this test. DNA purified from λ*H2::Tn5* mutants in the *H2* (on) configuration, a λ*H2* deletion derivative used for trans complementation, and λ*H2::Tn5* mutants which had been complemented to switch to the $H2^-$ phenotype (χ phage-resistant lysogen) were examined by hybridization analysis of Hpa II restriction fragments (see Figure 3B and Experimental Procedures). Figure 5B shows the result of this analysis. The source of the DNA was: wild-type phage λ*fla241* (lanes 6, 16); trans function donor λ*fla350* (lanes 1, 11); and λ*H2::Tn5* mutants λ*fla243* (lanes 2, 3, 4, 5), λ*fla247* (lanes 7, 8, 9, 10) and λ*fla251* (lanes 12, 13, 14, 15). DNA from λ*H2::Tn5* phage in the *H2* (on) phase was used in lanes 2, 7, 12 and DNA from λ*H2::Tn5* phage which had been complemented to switch to *H2* (off) was used in lanes 3, 8, 13 (trans donor was λ*fla350*), lanes 4, 9, 14 (trans donor was pJZ110) and lanes 5, 10, 15 (trans donor was pJZ143). The Hpa II fusion fragments generated by Tn5 insertion (see Figure 3B) were apparent only upon long exposure of the DNA blot. Phage λ*fla241* is identical to λ*fla157* (Figure 3B and Table 1) except that it contains a Tn5 insertion in a dispensable part of the λ genome.

the mutant population switched to the H2 (off) phenotype. In fact, mutant λ*H2::Tn5* could be complemented to switch to *H2* (off) at almost the same frequency as λ*H2::Tn5* mutants with the intermediate switching phenotype grown without a complementing phage. Phage DNA was purified from the *H2* (off) (χ phage-resistant) lysogens carrying the λ*H2::Tn5* mutants and subjected to restriction analysis. It was clear that the DNA from these lysogens was indeed in the *H2* (off) configuration (see Figure 5B). For example, DNA from λ*fla251* grown without complementing phage showed only the 700 and 400 bp fragments characteristics of the *H2* (on) configuration, but DNA from an $H2^-$ lysogen (χ phage-resistant) containing λ*fla251* previously grown with a complementing phage showed only the 540 and 560 bp fragments characteristic of the *H2* (off) configuration. Another complementation scheme was used in which the trans complementing region was provided by hybrid plasmids that had in common only the 900 bp *H2* inversion region (Zieg et al., 1978). In this case also (see Figure 5 and Table 2) the λ*H2::Tn5* switching mutants (null class) could be complemented to switch from *H2* (on) to *H2* (off).

These results suggest that there is a gene of approximately 500 bp in length in the inversion region which encodes a product that functions to cause inversions of the *H2* control region. Measurement of complementation of switching was also carried out with λ*H2::Tn5* mutants set initially in *H2* (off) configuration, and these mutants could be complemented to switch to *H2* (on) by providing the same DNA region in trans used for *H2* (on) to *H2* (off) complementation. Thus this factor acts to induce inversion in both directions. Even though *H2* switching was reduced by three orders of magnitude in the mutants, the *H2* phenotype was not absolutely fixed. The low residual level of H2 switching [both *H2* (on) to *H2* (off) and *H2* (off) to *H2* (on)] was observed with the λ*H2::Tn5* mutants only when a Rec^+Hag^- E. coli lysogen was examined.

Table 2. Complementation of λ*H2::Tn5* Switching Mutants

λ*H2::Tn5* Mutant[a]	λ or Plasmid in Trans[b]	Switching Frequency (per Generation)[c]	*hin* Function
λ*fla241*		8×10^{-3}	+
λ*fla243*, λ*fla244* λ*fla247*, λ*fla251*		5×10^{-6}	–
λ*fla243*, λ*fla244* λ*fla247*, λ*fla251*	λ*fla350*, λ*fla356*	1×10^{-3}	+
λfla243, λfla244 λfla247, λfla251	λ*fla364*, λ*fla381*	5×10^{-6}	–
λ*fla243*, λ*fla244* λ*fla247*, λ*fla251*	pBR322	5×10^{-6}	–
λ*fla243*, λ*fla244* λ*fla247*, λ*fla251*	pJZ110, pJZ121, pJZ143	2×10^{-3}	+

[a] All λ*H2::Tn5* are Red⁻ derivatives. Hybrid λ*fla241* has wild-type switching function and is used for comparison with mutant phage.
[b] See Figure 3 for description of hybrid phage used for trans complementation and Zieg et al. (1978) for description of plasmids.
[c] See Experimental Procedures for detailed explanation of complementation test. Switching frequencies are averages, with actual values varying ±50%.

When RecA⁻Hag⁻ lysogens were examined, no "spontaneous" switching was observed. Apparently the host recombination system (RecA) can mediate a low but measurable level of inversion of the *H2* control region. This "spontaneous" switching was observed with deletion derivatives of the λ*H2::Tn5*, but only if the inversion crossover regions were intact, and deletion derivatives of λ*H2::Tn5* phage could also be complemented to switch, but only if the inversion crossover regions were present (data not shown). This indicates again that cis-acting sites located at the cross-over points are absolutely essential for *H2* control region inversion.

Discussion

The 900 bp invertible region that controls phase variation includes a gene which encodes a product required for its own inversion. This *hin* gene is defined by deletion and insertion mutations that are localized within a DNA sequence of approximately 500 bp. An intact *hin* gene is required for phase transition, and the *hin* gene product promotes site-specific inversion in both directions. Genetic complementation tests show that the *hin* gene product acts in the trans configuration. Its presence increases the frequency of inversion by at least three orders of magnitude. Furthermore, the *hin* function is not sensitive to the size of the region that is inverted. Thus insertion of Tn5 into the silent region of the invertible segment, which increases its size by 5200 bp, and deletions which decrease its size by approximately 100 bp have only small effects on the frequency of phase transition. On the other hand, deletions that remove the sequences which contain the crossover points completely eliminate inversion. Genetic complementation cannot overcome the effects of these deletions. The crossover sites therefore behave as cis-acting elements whose participation is required for site-specific inversion.

The simplest interpretation of our data is that the *hin* gene product is a protein that catalyzes site-specific inversion. We have identified a 19,000 dalton polypeptide that is encoded by sequences within the inversion region (M. Silverman and M. Simon, manuscript in preparation) and may therefore be the product of the *hin* gene. It would function as an enzyme which is able to recognize sequences at the crossover points and catalyze the inversion event. Alternatively, it may participate in the inversion process by conferring site specificity to some more general recombination system which is endogenous to E. coli and Salmonella.

The *hin*-mediated inversion occurs independently of the RecA or RecBC systems (Zieg et al., 1978). Even in the absence of the *hin* gene, however, there is still a low residual level of phase variation. This is seen clearly when the frequency of transition is compared in deletions that are missing the *hin* function and those that have lost the crossover sequences. The loss of the crossover sequence completely eliminates all of the residual phase transition events. Furthermore, the low level residual switching in the Hin⁻ mutants disappears if the genes are put in a RecA host. These results, taken together, suggest that phase transition can occur through a "legitimate" recombinational mechanism, albeit at very low frequencies (approximately 10^{-6} per cell per generation). The crossover points may contain homologous sequences that can be recognized by the RecA system and lead to inversion (Anderson and Roth, 1977). The *hin*-mediated system provides a site-specific, RecA-independent pathway which allows phase transition to occur at much higher frequencies (10^{-2} per cell per generation).

In addition to the *hin* and the crossover functions, deletions define a region inside the invertible segment which is necessary for *H2* gene expression. We suggest that this deletion defines a promotor sequence which is located close to the crossover point. In previous work, we have shown that transcription of the *H2* gene is controlled by a sequence inside the inversion region, and phase transition operates by connecting or disconnecting this promotor from the adjacent *H2* structural gene sequence (Silverman et al., 1979b). The insertions and deletions also define a short sequence (100–200 bp) between the promotor and the *hin* gene sequences which does not appear to be required for phase variation. These sequences could possibly code for function necessary for the regulation of *H2* promotor activity. The scheme pre-

sented in Figure 6 summarizes our conclusions about the functions encoded by the DNA involved in phase transition.

The sequences that code for phase transition are similar to those involved in the G loop inversion in bacteriophage Mu. The *hin* gene function appears to be analogous to the *gin* gene function, which has been shown to be required for G loop inversion in bacteriophage Mu (Chow, Kahmann and Kamp, 1977). In fact, it appears that trans-acting functions required for site-specific events are generally found to be associated with the region of DNA in which the recombination event occurs. For example, the *int* gene is λ maps adjacent to the *att* site, where it mediates integrative recombination (Gottesman and Weisberg, 1971; Nash, 1977). Furthermore, the Tn3 transposon appears to code for polypeptides that function to catalyze and regulate transposition (Chou et al., 1979; Heffron et al., 1979). The possibility has been raised that even short sequences such as the IS-1 sequence may code for polypeptides that could be involved in IS transposition (MacHattie and Shapiro, 1978; Ohtsubo, Ohmori and Ohtsubo, 1978). The ability to code for factors required for site-specific recombination may be a general feature of transposon-like sequences and controlling elements.

In the case of Tn3 there are factors which control the frequency of transposition that are also part of the transposable sequence. One could imagine that the frequency of phase transition could be regulated by changes in the *hin* gene or in the promotor region that controls its expression. Furthermore, the specific sequences at the crossover points may also be important in regulating the frequency of inversion. The differences in the frequency of phase transition in naturally occurring Salmonella strains may be the result of such variations. One possible scenario for the origin of the phase system is that it may have arisen from the association of a primordial *H1* gene with a transposable element that also carried functions that could mediate site-specific inversion. Subsequent mutation could have provided the optimum frequency of phase transition for different Salmonella strains.

The DNA sequence of the *H2* region is currently being determined. It will allow a more precise definition of *H2* switching function and closer comparison with other systems that show site-specific recombination. The invertible sequence represents a new class of regulatory elements that effect gene expression via site-specific recombination. It is possible that such mechanisms operate in both eucaryotic and procaryotic cells to allow the cell to express alternate genes for a specific function. This type of system could provide diversity, particularly for functions that involve surface properties and are required for the physical interaction of cells with each other or with the environment. It could also be the prototype for regulatory events involved in cell differentiation and development.

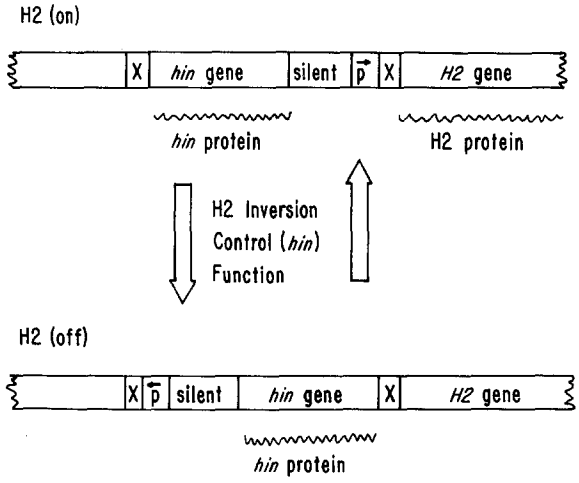

Figure 6. Model for Inversion of *H2* Control Region

Switching of *H2* gene expression is controlled by the orientation of an invertible region of DNA which contains a promotor for the H2 operon. Inversion of the control region requires specific sites (X) at the crossover points and is catalyzed by the product of a gene (*hin*) residing within the invertible region.

Experimental Procedures

Isolation of *H2* Switching Mutants

The construction of hybrid λ*fla157* (λ*H2*) which contains the 3.75 kb *H2* gene insert is described in Silverman et al. (1979b). Hybrid λ*fla157* has λ functions necessary for lysogenization, and its genome is small enough to accommodate Tn5 insertions and remain an acceptable size for DNA packaging. To obtain Tn5 insertion, λ*fla157* is grown lytically in E. coli strain DB1358 obtained from D. Berg (Washington University, St. Louis, Missouri). The resultant phage population should contain λ*H2::Tn5* phage at a frequency of approximately 10^{-4} (Berg, 1977; Kleckner, 1977; Kleckner, Roth and Botstein, 1977). Tn5 insertion phage were recovered by infection (multiplicity ≃1) of Hag^- kanamycin-sensitive strain MS6302 (Silverman et al., 1979b) with selection for Tn5-linked kanamycin resistance. Hybrid λ*H2::Tn5* lysogenic colonies on L-agar plates containing 40 μg/ml kanamycin (Sigma Chemical, St. Louis, Missouri) were recloned and screened for *H2* gene switching defects. Hybrid λ*H2::Tn5* switching mutants isolated in the *H2* fixed on orientation as lysogens of Hag^- E. coli strain MS6302 had a stable $H2^+$ phenotype and could be distinguished from lysogens of hybrid phage with functional *H2* switching which expressed both $H2^+$ and $H2^-$ phenotypes. Since the flagellotropic phage χ infects only $H2^+$ lysogens, lysogens with mutant λ*H2::Tn5* phage fixed in the *H2* (on) phase would be sensitive to χ, while lysogens with λ*H2::Tn5* phage which can switch to *H2* (off) would be resistant. Approximately 3000 kanamycin-resistant λ*H2::Tn5* lysogens were tested for χ sensitivity by inoculation onto motility agar plates with soft agar overlays containing 2×10^9 phage. (See Komeda, Silverman and Simon, 1978, for composition of motility agar plates and other media used for growth of bacteria and phage.) 100 candidate λ*H2::Tn5* phage with mutant *H2* switching phenotypes were saved as lysogens and analyzed quantitatively for the frequency of phase transition. Deletion mutants derived from λ*H2::Tn5* phage by Tn5-mediated transposition were selected by virtue of their resistance to chelating agents (Parkinson and Huskey, 1971; Ross, Swan and Kleckner, 1979). Selection of deletion mutants of various λ*fla* phage has been described (Silverman and Simon, 1977). Only those λ deletion mutants which lost the kanamycin determinant were saved for further analysis.

Measurement of *H2* Gene Expression

A quantitative method for measuring H2 gene switching, H2 (on) to H2 (off), has been described previously (Silverman et al., 1979b). As mentioned above, Hag$^-$ E. coli strains lysogenized with *H2* phage are either sensitive or resistant to the flagellotropic phage, depending upon the state of expression of the *H2* gene. To measure the *H2* switching frequencies, lysogen populations were initially enriched for the *H2* (on) phase by inoculating into motility agar where only *H2* (on) bacteria migrate and then growing cells harvested from the edge of a migrating swarm of bacteria. These *H2* (on) cells were grown for approximately 10 generations in L broth and diluted, and appropriate amounts were plated in overlay agar with and without 10^9 χ phage. The ratio of χ phage-resistant colonies to the total number of cells plated gave the fraction of cells which had switched to the *H2* (off) phase. This ratio divided by the number of generations the lysogen population had grown was used as the frequency of *H2* switching (see Stocker, 1949). Kanamycin was present in the bottom agar (motility plates) to exclude any cells not lysogenized with λ*H2::Tn5* phage. Measurement of *H2* (off) to *H2* (on) switching with the χ phage selection method was subject to large error. Because the switching products, *H2* (on) lysogens, were sensitive to χ phage, their presence was measured indirectly by calculating the difference between the total number of lysogens and the number of χ-resistant lysogens. Since the switching frequency in the mutants is low, the difference in the number of chi-resistant lysogens was small and therefore difficult to assess accurately. *H2* switching in this direction could be determined in a qualitative manner, however, by inoculating a particular lysogen in a zone on motility agar plates, and, after incubation at 37°C for 8 hr, estimating the number of motile swarms, *H2* (on), emanating from the region of inoculation. Only lysogens with very large differences in *H2* (off) to *H2* (on) switching frequencies could be differentiated by this method, as opposed to the former method, where differences in *H2* (on) to *H2* (off) frequency of ±25% were detectable. E. coli strain MS6302, which is Hag$^-$ RecA$^+$, was used for most switching measurements, but a RecA$^-$Hag$^-$ derivative of Cold Spring Harbor strain CSH4 was used to measure switching in a RecA$^-$ environment.

Complementation Analysis

Complementation of the *H2* switching defects in λ*H2::Tn5* mutants (null class) was measured by growing the λ*H2::Tn5* mutants in cells which were co-infected with another hybrid or which contained a hybrid plasmid, the latter containing DNA sequences being tested for trans-acting function. To eliminate the possibility of recombination between the λ*H2::Tn5* mutant and homologous regions on the λ or plasmid DNA in the trans configuration, Red$^-$ phage and a RecBC$^-$ host were used. RecBC$^-$ hosts supported growth of Red$^-$ phage, whereas RecA$^-$ hosts would not. Red$^-$ λ*H2::Tn5* derivatives were isolated by selection for their ability to grow on P2 lysogens (Spi$^-$ phenotype), and RecBC$^-$ strain JC5519 obtained from J. Clark (University of California, Berkeley) was used as host. Hybrid λ*H2::Tn5* synchronized in the *H2* (on) phase were obtained by ultraviolet induction of lysogens in the *H2* (on) phase. Host strain JC5519 was co-infected with a λ*H2::Tn5* mutant and a second λ (function donor) or host strain JC5519 harboring a hybrid plasma (function donor) was infected with the λ*H2::Tn5* mutant. Multiplicity of infection was approximately two, growth was at 37°C and one infection cycle was completed. Cells were infected with the resultant lysate until three cycles of phage growth were completed. This lysate was used to lysogenize Hag$^-$ strain MS6302. Infection of MS6302 was at a multiplicity of one at 30°C, and cells were allowed to grow for 16 hr, at which time they were diluted 1/100 into L broth containing 20 μg/ml kanamycin to select for λ*H2::Tn5* lysogens. After 8 hr of growth, the fraction of lysogens with λ*H2::Tn5* phage in the *H2* (off) phase was determined by the χ test (see above). The results in Table 2 are presented as the frequency of change of the *H2* phenotype [proportion *H2* (off) per generation], as are those in Table 1. However, since the λ*H2::Tn5* phage were grown not as lysogens, but vegetatively during the course of the complementation test, the term "generation" has a different meaning. Thus the values in Table 1 and Table 2 should not be compared directly. We have estimated the number doubling of the λ phage population during the course of the complementation test to be approximately 30, and use this value for "generation" in computing the switching frequencies shown for the complementation test.

The *H2* DNA sequences contained on the hybrid plasmids used in the complementation test are described in detail in Zieg et al. (1978). The *H2* DNA contained in plasmid pJZ110 is shown in Figure 5A. Plasmids pJZ121 and pJZ143 are recombinational variants derived from plasmid PJZ110. Plasmid pJZ121 contains the inversion sequence flanked on either side by part of the *H2* gene sequence (right arm in Figure 5A) and plasmid pJZ143 contains the inversion sequence flanked by the non-H2 gene sequence (left arm in Figure 5A). All three plasmids have in common the invertible region.

Restriction Analysis

Methods for restriction analysis of the *H2* gene region of hybrid λ phage have been described by Silverman et al. (1979b). Hpa II restriction fragments were separated electrophoretically on a 9% acrylamide gel. Where the method of Southern (1975) was used to make DNA transfer to nitocellulose paper, it was necessary to allow transfer to proceed for 24 hr to ensure complete transfer. ^{32}P-labeled probes were prepared from *H2* plasmids pJZ110 and pJZ121 (Silverman et al., 1979). When pJZ110 DNA was used as a probe for DNA bands transferred to nitrocellulose paper, one additional fragment between the 560 and 700 bp fragments was detected. This fragment is located outside the *H2* inversion region and is extraneous to this analysis. Isolation of hybrid λ DNA for restriction analysis was performed directly from phage lysates as described by Blattner et al. (1978).

Acknowledgments

We thank Dr. Janine Zieg for providing plasmid strains, Dr. Douglas Berg for providing Tn5 insertion strains, Dr. John Clark for Rec$^-$ strains, Dr. David Botstein for a restriction map of the Tn5 element, and Dr. Elizabeth Szekely for advice concerning the Southern transfer technique. This work was supported by grants from the NSF and the American Cancer Society.

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Received December 26, 1979

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