Recombinational Switch for Gene Expression

Abstract. *Flagellar antigens are specified by two genes, H1 and H2. The expression of these genes is regulated such that only one gene activity, or phase, is expressed at a given time. Molecular cloning techniques were used to isolate the segments of* Salmonella *DNA which contain these genetic loci. Heteroduplex analyses revealed an anomaly in the cloned fragment, that is, an apparent inversion, which was shown to be adjacent to the H2 gene. A correlation was demonstrated between the phase state of the H2 gene and the sequence of the adjacent segment. We propose that an inversion of this region is the phase-determining event in flagellar gene expression in* Salmonella.

Phase variation in *Salmonella* is an example of a regulatory mechanism that involves a switch in gene expression mediated by a metastable change at the level of the DNA. This was initially referred to by Lederberg and Iino (*1*) as a change in the "local state" of the gene, and it was suggested that such changes could be involved in cell differentiation and development. The molecular mechanism of this change is not known. However, it has become possible to obtain relatively large amounts of the specific DNA that carries the phase switch so that the DNA can be examined directly. We describe experiments that suggest that phase variation results from a directed inversion adjacent to one of the genes that controls the structure of the *Salmonella* flagellar antigen.

Phase variation was discovered by Andrewes 55 years ago (*2*). He found that mass cultures derived from a single clone of *Salmonella typhimurium* would agglutinate with two different antiserums directed against their flagellar filaments. Twenty-five years later, Stocker (*3*) showed that the variation was the result of the ability of *Salmonella* strains to switch from one flagellar antigen (phase) to another with a small probability which was two to three orders of magnitude higher than the frequency of mutation. Lederberg and Iino (*1*) investigated the nature of the switch by genetic techniques. They concluded that (i) the structure of the flagellar filament was specified by two genes, H1 and H2; (ii) the ability to switch from the expression of one gene to another was controlled by a genetic element linked to the H2 gene; (iii) the state of expression of the H2 gene could be transduced, and, therefore, the switch involved a change at the DNA level. The kinds of mechanisms that have been proposed to account for phase variation fall

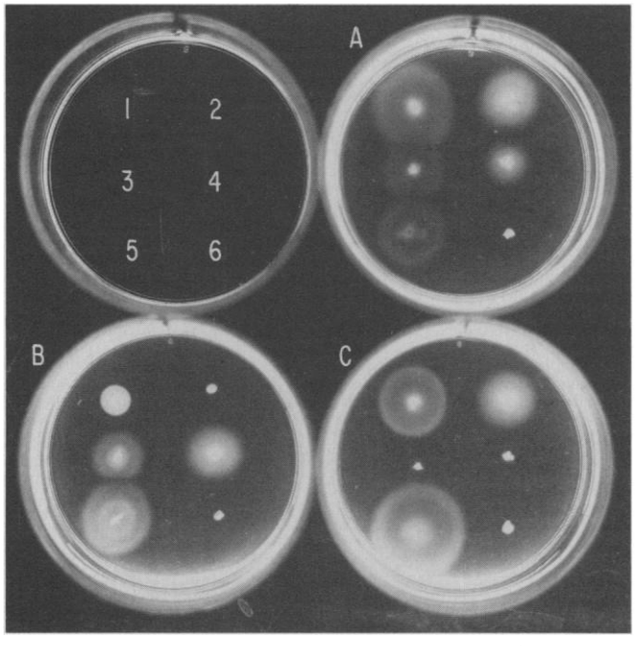

Fig. 1 (above). Serological tests illustrating flagellar antigen expression. No antiserum was added to the motility agar in plate A; antiserums to $H1^b$ and to $H2^{enx}$ were added to plates B and C, respectively. Anti-$H1^b$ was obtained from Burroughs-Wellcome Co., Greenville, North Carolina, and anti-$H2^{enx}$ was obtained from Lee Laboratories, Grayson, Georgia. Identification of the clones is as follows: (position 1) *Salmonella* phase 1–on (SL4213), (position 2) $H1^b$ clone (C600 Hag^-), (position 3) *Salmonella* phase 2–on (SL4213), (position 4) $H2^{enx}$ clone (C600 Hag^-), (position 5) C600 Hag^+, (position 6) C600 Hag^-. $H2^{enx}$ clones were immobilized only in anti-$H2^{enx}$ while $H1^b$ clones were immobilized only in anti-$H1^b$. Motility of C600 Hag^- was not affected by either antiserum.

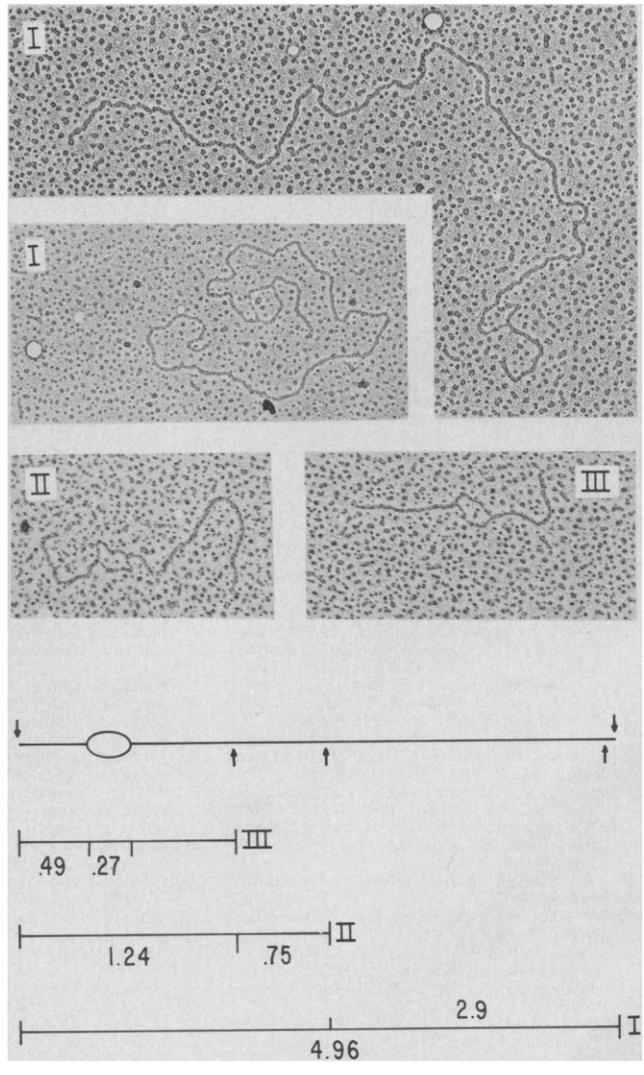

Fig. 2 (right). Heteroduplex analysis of cloned DNA. Electron micrographs: I and insert show reannealed fragment I derived from Eco RI endonuclease treatment of hybrid plasmid containing H2 gene; II and III show fragments derived by Eco RI and partial Sal I digestion. Restriction enzyme Eco RI was obtained from D. Helinski; Sal I was purchased from New England Bio-Labs, Beverly, Massachusetts. Below is a map of the $H2^{enx}$ DNA indicating positions of Eco RI sites (arrows above line) and Sal I sites (arrows below line). Covalently closed Col E1 was used as an internal standard in the measurements. Dimensions are shown in microns.

into two general classes: (i) an alteration in the genetic arrangement at the H2 locus, for example, the insertion of a heterologous sequence or the inversion of an adjacent region (*4, 5*); and (ii) modification of a regulatory sequence (*6*), for example, specific methylation of the promotor region in the H2 gene. In order to test these hypotheses, the region carrying phase variation in *Salmonella* was cloned. A strain of *Escherichia coli* which did not synthesize the flagellar antigen (C600 mK^- rK^-, Hag^-) was used as recipient. This strain was shown to form abortive transductants with transducing phage grown on *Salmonella*. The transductants expressed either phase 1 or phase 2 of *Salmonella* in *E. coli*. The donor material was prepared by treating *Salmonella* DNA from strain SL 4213 (*7*) with Eco RI endonuclease and forming hybrid molecules with restricted colicinogenic factor E1 (Col E1). The mixture of transformed (*8*) cells was streaked onto motility agar containing colicin, and the swarms that emerged were picked and tested with antiserums specific for *Salmonella* phase 1 ($H1^b$) flagella (anti-$H1^b$) and *Salmonella* phase 2 ($H2^{enx}$) flagella (anti-$H2^{enx}$). Figure 1 shows that there were two kinds of clones: (i) those that were able to swim in the presence of anti-$H2^{enx}$ but not anti-$H1^b$ and, therefore, carried the structural gene for $H1^b$ and (ii) those that were able to swim in the presence of anti-$H1^b$ but not anti-$H2^{enx}$ and carried the gene for $H2^{enx}$. The ability to show phase variation is tightly linked to the $H2^{enx}$ gene. DNA was isolated from this strain and used to retransform the C600 Hag^- cells. One hundred single colonies were picked and stabbed onto motility agar. After 1 day, 98 of these showed some level of motility, and two were apparently nonmotile. However, when the nonmotile clones were again streaked on agar they showed motile swarms. The heterogeneity of response suggested that the strain was undergoing phase transition. Thus, the DNA prepared from this strain should be a mixture of molecules carrying phase 2 in the on-state and phase 2 in the off-state. If the switch were the result of an anomaly at the DNA level, we should be able to see it by denaturing and reannealing the $H2^{enx}$ DNA. If phase transition involved the insertion, deletion, or inversion of DNA, a fraction of the denatured DNA would consist of heteroduplexes containing a "deletion loop" or an "inversion bubble." On the other hand, if modification, that is, methylation, were involved, we would not expect to see anything distinctive about the reannealed DNA. Before denaturation and renaturation, the DNA had no anomalous features. However, after renaturation between 5 to 10 percent of the molecules had a structure similar to an "inversion bubble" (*9*) (Fig. 2).

The DNA was further characterized by using restriction endonucleases. Treatment with Eco RI endonuclease separated the *Salmonella* DNA (fragment I) from the Col factor DNA. The *Salmonella* DNA was 4.96 ± .04 μm in length, corresponding to a molecular weight of 9.9×10^6. The "inversion bubble" was 0.27 ± .03 μm in length corresponding to 750 bases. The short arm adjacent to the inversion was 0.49 ± .04 μm corresponding to 1450 base pairs. The DNA had three sites for Sal I endonuclease. Figure 2 shows the positions of the Sal I sites as determined from electron microscopy measurements and agarose gel electrophoresis.

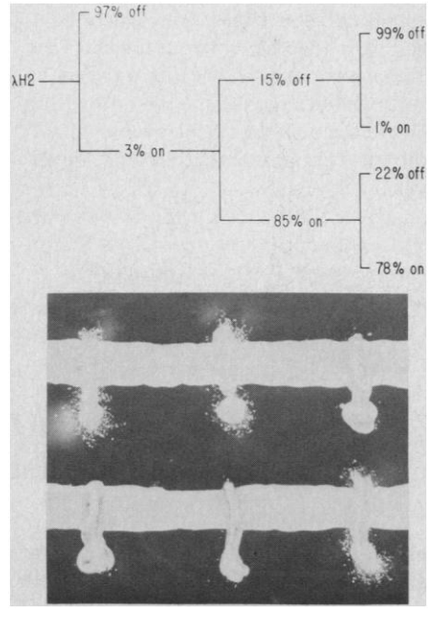

Fig. 3. Genetic properties of λ H2. Transductional crosses between λ H2 and an *E. coli* strain defective in flagellin synthesis is shown at the bottom. A tester strain, MS5014, was spread on motility agar and cross streaked with phage from individual plaques. Transduction of the H2 gene had two consequences: (i) swarms of motile cells occurred by recombination or by integration of the transducing phage; (ii) trails of immotile clones emanated from the region of infection. These resulted from abortive inheritance of the H2 gene and indicated complementation of the flagellin defect. λ H2 clones were of two types: (i) those that gave high levels of complementation, λ H2–on, that is, top left, top middle, and bottom right; (ii) those that gave very low levels of complementation, λ H2–off, that is, top right, bottom left, and bottom middle. The variation in the genetic properties of λ H2 clones is shown above. λ H2 was grown on overlay plates and harvested, and single plaques were selected. These were grown sufficiently to obtain enough phage for complementation tests. The forks in the diagram summarize the distribution of phenotypes obtained from single plaques.

If the "inversion bubble" were related to H2 gene expression, we might expect it to be adjacent to the H2 gene. The Col E1 factor does not have a site for Sal I endonuclease. Therefore, if we treat the plasmid DNA with Sal I, isolate the bands that are formed on agarose gel electrophoresis, and retransform with this DNA, we can delete parts of the *Salmonella* DNA and determine the effect of the deletion on the expression of the $H2^{enx}$ gene. When Col E1 factor carrying *Salmonella* DNA fragment II or fragment III was used for transformation they both gave motile enx-specific transformants. Single clones were picked from these transformants and the plasmid DNA was again isolated. It was found to carry only fragment II or fragment III. Therefore, both fragments must carry the $H2^{enx}$ structural gene. It is difficult to distinguish between the arms on either side of the "inversion bubble" in fragment III because they are of about the same length. However, flagellin, the product of the H2 gene, has an apparent molecular weight of about 50,000 (*10*), and either arm has DNA which is just sufficient to code for a polypeptide of that size. Therefore, the region carrying the inversion must either be part of the H2 structural gene or it must lie just outside the region that codes for H2. The inversion segment is therefore closely associated with the H2 gene.

The next question is whether the event that generates the "inversion bubble" correlates with phase transition. It is difficult to follow phase transition in cells carrying the hybrid-Col factor since each cell has a large number of copies (from 10 to 20) of the factor. We therefore transferred the DNA onto a λ phage vehicle. The λ phage Charon 1 designed by Blattner (*11*) could accept heterologous DNA as large as fragment I. The DNA was ligated with Eco RI–treated Charon 1 phage DNA and the mixture was used to transfect a recipient strain (C600 rK^-mK^-). Plaques were picked, and the phage were tested for their ability to transduce $H2^{enx}$ to *E. coli* that lacked the flagellin gene (strain MS5014) (*12*). Two kinds of plaques that transduced the H2 gene were found, those that gave high levels of transduction and those that gave low levels of transduction (Fig. 3). When single plaques from the λ population that gave low levels of transduction were retested, 1 percent were found to give high levels. When plaques from phage that gave high levels of complementation were retested, 80 percent of them gave high levels and 20

percent gave low levels. Figure 3 summarizes this analysis and shows the behavior of both classes of λ H2 phage in transductional tests. We assume that these two types represented the behavior of phage carrying phase 2–on, or phase 2–off. The process of phase transition was asymmetric, that is, the rate of transition from on to off appeared to be at least tenfold higher relative to the rate in the reverse direction (off to on). The picture is similar to that initially observed by Stocker (*3*). He found that the transition from phase 2–on to phase 2–off occurred at about ten times the frequency of the reverse transition. On the basis of these experiments, we can define two populations of λ phage, one that carries H2–off in almost all of the population and another which has mostly H2–on.

To determine whether the "inversion bubble" is correlated with expression of the different states of H2, DNA's from the different λ populations were prepared, denatured, and reannealed; and the frequency of inversions was measured. Three hundred molecules of λ H2–off DNA were scored, and 2.5 percent had the "inversion bubble." One hundred and fifty molecules of DNA carrying λ H2–on were scored, and 12 percent were found to carry the inversion. When equal amounts of these preparations were mixed, denatured, and reannealed, 34 percent of 150 molecules were found to have the inversion. The large increase in frequency obtained when the two populations were mixed indicated that it was the heteroduplex between phase 2–on and phase 2–off regions that formed the "inversion bubble." On the basis of these experiments we suggest that phase variation is determined by a specific region adjacent to the H2 gene which can undergo an inversion. In one configuration it allows the H2 gene and perhaps adjacent genes (*13*) to be transcribed. In the opposite configuration it does not allow H2 transcription. This hypothesis is shown schematically in Fig. 4. We cannot formally rule out the possibility that a specific transposition of heterologous DNA into the phase-controlling site is responsible for the "inversion bubble." However, in recent experiments DNA molecules associated by a duplex region which corresponded to the "inversion bubble" with nonhomologous single-stranded ends, have been observed. These results suggest that with respect to the "bubble" the notion that it is generated by an inversion is the simplest model that explains all of our observations. It raises questions about the specific mechanisms involved in regulating the transition. These can be approached experimentally with the use of the cloned DNA.

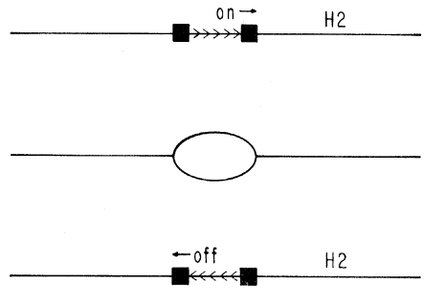

Fig. 4. A model for phase variation. An inversion of the sequence between the dark blocks could result in a change in the orientation of a promoter contained within the region or it could allow read through from a promoter outside the region.

Evidence in a variety of different systems has accumulated suggesting that a recombinational event, an inversion (*14*), or a transposition (*15*) can be involved in regulating gene expression. These findings taken together with our present view of phase variation suggest that it may be worthwhile to reexamine the notion that directed recombinational events play a role in cell differentiation and development.

JANINE ZIEG, MICHAEL SILVERMAN
MARCIA HILMEN, MELVIN SIMON
Department of Biology, University of California, San Diego, La Jolla 92093

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Gene Cloning and Containment Properties of Plasmid Col E1 and Its Derivatives

Abstract. *Colicinogenic plasmid E1 (Col E1) and Col E1 derivatives offer advantages as plasmid cloning vehicles with regard to both utility and biological containment. The Col E1 derivative pCR1 does not alter those essential characteristics of the enfeebled* Escherichia coli *strain χ1776 that make this strain particularly useful as a host-vehicle system for recombinant DNA research.*

In this report we discuss the properties of colicinogenic plasmid E1 (Col E1) and certain Col E1 derivatives obtained in our laboratory that make these plasmids particularly suited as cloning vehicles for recombinant DNA research.

Col E1 is a relatively small, covalently closed, circular DNA molecule (4.2 megadaltons in size) (*1*) that is present in multiple copies in *Escherichia coli*—about 20 to 30 molecules per cell (*2*). When grown in the presence of chloramphenicol, Col E1 is amplified; that is, bacterial chromosomal replication ceases, but Col E1 continues to replicate until approximately 45 percent of the cellular DNA is Col E1 DNA (*2, 3*). With the use of this amplification step, high yields of both Col E1 DNA and also any DNA sequence that is inserted into the plasmid are readily obtained (*4*).

Since Col E1 contains a nonessential region, a single site sensitive to the restriction endonuclease Eco RI, Eco RI–digested DNA can be inserted into the plasmid after cleavage with this restriction enzyme. Recombinant plasmids constructed by insertion at the Eco RI site are readily detected since they no longer produce the antibiotic protein colicin E1 (*4*). Col E1 is a nonconjugative plasmid; that is, it is unable to promote its own transfer from one bacterial cell to another (*5*). However, the conjugal transfer of Col E1 is promoted by self-transmissible (conjugative) plasmids (*6*). Conjugative plasmids vary greatly in their ability to promote the transfer of Col E1.

There are several unique derivatives of Col E1 that have been developed for use