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NinR- and Red-Mediated Phage-Prophage Marker Rescue Recombination in *Escherichia coli*: Recovery of a Nonhomologous *imm*λ DNA Segment by Infecting λ*imm*434 Phages

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ABSTRACT

We examined the requirement of λ recombination functions for marker rescue of cryptic prophage genes within the *Escherichia coli* chromosome. We infected lysogenic host cells with λ*imm*434 phages and selected for recombinant *imm*λ phages that had exchanged the *imm*434 region of the infecting phage for the heterologous 2.6-kb *imm*λ region from the prophage. Phage-encoded activity, provided by either Red or NinR functions, was required for the substitution. Red^- phages with ΔNinR, internal NinR deletions of *rap-ninH*, or *orf-ninC* were 117-, 12-, and 5-fold reduced for *imm*λ rescue in a Rec^+ host, suggesting the participation of several NinR activities. RecA was essential for NinR-dependent *imm*λ rescue, but had slight influence on Red-dependent rescue. The host recombination activities RecBCD, RecJ, and RecQ participated in NinR-dependent recombination while they served to inhibit Red-mediated *imm*λ rescue. The opposite effects of several host functions toward NinR- and Red-dependent *imm*λ rescue explains why the independent pathways were not additive in a Rec^+ host and why the NinR-dependent pathway appeared dominant. We measured the influence of the host recombination functions and DnaB on the appearance of *ori*λ-dependent replication initiation and whether *ori*λ replication initiation was required for *imm*λ marker rescue.

MARKER rescue recombination to produce gene substitutions involves exchanges within regions of homology straddling a marker of interest. Strong modern evidence for the shuffling of phage gene modules in nature is provided by the stx phages and prophages of *Escherichia coli*, which share the genome organization of bacteriophage λ (Brüssow *et al.* 2004). Early λ workers identified phage-prophage marker rescue, where an infecting λ was capable of rescuing a gene present on a homologous cryptic prophage in a lysogenic cell. Signer and Weil (1968) used a spot test involving the rescue of an *h* (unspecified host range) marker from rec^+ cells with a cryptic λ prophage (deleted for a large portion of prophage, including the *imm* region) that was infected by λh^{λ}, and λ*h* recombinants were selected on host cells that were resistant to infection by λh^{λ} but sensitive to λ*h*. Using this assay, Signer and Weil (1968) were able to screen hydroxylamine-treated infecting phage for deficiency in marker rescue. Several mutants with reduced ability to rescue prophage markers were subsequently mapped as recombination-defective *red* mutants. Echols and Gingery (1968) recovered λsus^+ recombinants that were formed by marker rescue between an infecting λ*sus* phage and a defective prophage in a lysogen. Both studies concluded that Red functions of λ were required for phage-prophage marker rescue in *E. coli* hosts defective for the host *recA* function. The λ Red-dependent recombination activity (reviewed by Stahl 1998; Kuzminov 1999; Court *et al.* 2002) depends upon the expression of λ genes *exo* and *bet* (or Redα, Redβ; combined, Red) along with *gam*. Red-dependent recombination is initiated by double-strand breaks, and when marked Red^+ λ phages infect cells blocked for DNA replication, the λ × λ exchanges are focused near the *cos* ends, the only site of an initiating double-strand break (Tarkowski *et al.* 2002). Murphy and coworkers (Murphy 1998; Murphy *et al.* 2000) constructed an *E. coli* strain in which the cellular *recBCD* genes (Smith 2001) were replaced with *exo-bet* and placed under *lac* promoter control. They found that the λ activities supported recombination between the cellular chromosome and linear DNA fragments at an elevated level. Recombination in these Δ*recBCD* cells, lacking Gam, depended upon Exo and Beta, was greatly reduced in *recA* mutants, and required host recombination genes *recQ*, *recO*, *recR*, *recF*, and *ruvC*, but not *recJ* or *recG* (Murphy 1998; Poteete *et al.* 1999; Murphy *et al.* 2000; Poteete and Fenton 2000). The λ Red functions can facilitate chromosomal engineering, *i.e.*, substituting or disrupting genes in an *E. coli* chromosome or plasmid

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(Yu et al. 2000; Court et al. 2002). In the absence of *E. coli* methyl-directed mismatch repair activity, the inheritance of markers from single-strand DNA oligonucleotides required only the Bet function (Constantino and Court 2003).

The co-integration of a plasmid into a phage, each sharing limited DNA homology, is shown as a single reciprocal crossover addition reaction (Figure 1A). King and Richardson (1986) and Shen and Huang (1986) found that packaged plasmid-phage co-integrates were formed by homologous recombination proceeding predominantly via the *E. coli* RecBCD pathway (reviewed by Kowalczykowski *et al.* 1994; Stahl 1998; Kuzminov 1999) when cells were infected with λ int^- red^- or Δ*int-gam* phages sharing limited DNA sequence homology with a small plasmid carried within the cell. Hollifield *et al.* (1987) found that the formation of phage-plasmid co-integrates by Δ*int-gam* phage Charon 4A (Blattner *et al.* 1977) depended on an encoded phage function mapping between λ genes *P* and *Q* within the NinR (Kröger and Hobom 1982) interval, defined by deletion Δ*nin5* and including the genes *ren* (Toothman and Herskowitz 1980) and nine open reading frames designated *ninA–ninI* (Daniels *et al.* 1983). They identified the *rap* function (recombination adept with plasmid) that mapped to orf204 (*ninG* gene) as being required for RecBCD pathway-mediated formation of phage-plasmid co-integrates. Even though Charon 4A was Δ*nin5*, it encoded a similar determinant to *rap* that mapped within phage Φ80-derived sequences included in the cloning vector. The *rap* product (Hollifield *et al.* 1987; Stahl *et al.* 1995; Tarkowski *et al.* 2002) represents a branch-specific endonuclease that targets recombination intermediates generated *in vivo* and *in vitro* (Sharples *et al.* 1998, 1999). It can function as a genuine Holliday junction resolvase when presented with DNA substrates containing sufficient homology at the crossover (Sharples *et al.* 2004) and can substitute for the *E. coli* RuvC Holliday junction resolvase in λ Red recombination (Poteete *et al.* 2002). Yet another λ recombination function removed by Δ*nin5* was localized by Sawitzke and Stahl (1992) to *orf146* (*ninB* gene) and termed *orf* because it could complement single mutations in the host genes *recO*, *recR*, and *recF* in *recBC sbcB sbcC* cells and thus function in RecF-dependent pathway crossovers between two co-infecting phages. Orf, with a monomer molecular mass of 16.6 kDa, was found to have pleiotropic effects on recombination, replication, and repair in *E. coli.* Orf suppresses the mutant phenotype not only of *recF*, *recO*, and *recR*, but also of *ruvAB* and *ruvC* (Poteete and Fenton 2000; Poteete *et al.* 2002; Poteete 2004).

The intercrossing between two phage genomes is routinely drawn as a single splice reaction. Motamedi *et al.* (1999) demonstrated efficient homologous recombination between co-infecting *int-gam*-defective ΔNinR phages in rec^+ hosts, showing that the host could provide needed recombination functions for phage-phage recombination and that the Red or NinR phage activities were not required. This observation supported a prior report by Stahl *et al.* (1995) that Rap function had no effect on the frequency of recombination between co-infecting phages sharing DNA homology, *i.e.*, λ × λ exchange in an infected cell. In Red-mediated λ × λ crosses occurring when λDNA replication was blocked, Tarkowski *et al.* (2002) showed that the NinR products Orf and Rap influenced end focusing at double-strand breaks, but that Δ*nin5* had little or no effect on the outcome of such crosses in a $recA^-$ host.

This study was undertaken on the basis of observations from a rapid marker rescue test (functional immunity assay: Hayes and Hayes 1986; Hayes 1991) in which cells with a cryptic *imm*λ prophage were stabbed to a fresh overlay plate containing cells lysogenized with λ*imm*434 plus added free λ*imm*434*cI* phage. Marker exchange between the cryptic prophage and the infecting λ*imm*434*cI* phage (Figure 1B) yielded λ*imm*λ recombinant phage that were revealed by a lysis spot (up to a 0.5-cm radius) surrounding the stabbed colony formed in the cell lawn of the λ*imm*434 lysogen. The formation of *imm*λ recombinant phages arose even when the stabbed cells (with cryptic *imm*λ prophage) carried a null mutation in the key host recombination functions *recA* (trace lysis), *recB*, *recD*, *recG*, *recJ*, and *ruvC* or were double null mutants *recA recD*, *recD recF*, and *recF recJ*. In these crosses, the ~1.4-kb *imm*434 DNA interval on the infecting phage (Figure 1C) was substituted by rescue of the heterologous ~2.6-kb *imm*λ DNA interval (which included the additional genes *rexA-rexB*) present in the cryptic λ prophage (K. Asai and S. Hayes, unpublished results). We examined whether rescue of the *imm*λ region depended solely upon gene products encoded by the infecting phage. We found that λ's Red or its NinR each function in the absence of the other to support *imm*λ exchange. The host requirements for the Red- or Nin-mediated *imm*λ rescue differed significantly. The two systems were partially competitive, rather than additive, when both were expressed. We monitored the influence of DnaB and host recombination functions on DNA replication from *ori*λ to assess the participation of prophage replication initiation in *imm*λ rescue.

MATERIALS AND METHODS

Bacteria: *E. coli* strains are shown in Table 1. Strain Y836 (infected host drawn in Figure 1B) carries a cryptic prophage, is SA500 F^- *his87 relA1 strA181* (λ*bio*275 *cI*[Ts]*857*Δ*431*), and was made from Y832 (Hayes and Hayes 1986) derived from SA431 (Adhya *et al.* 1968; Stevens *et al.* 1971), which is a derivative of SA302 [AM3100 str^r *his87* (λ*cI*[Ts]*857*)], itself a λ-lysogen of SA500 F^- *his87 relA1 strA181* (Hayes 1991). The *cI* repressor (CI) in these strains, in addition to being temperature sensitive (Ts), carries an Ind mutation (Hayes and Hayes 1986). Y836 was made by replacing the genes *int-cIII* (Daniels *et al.* 1983) in Y832/SA431 with *bio*275 DNA (Figure 1B) from

TABLE 1

***E. coli* strains**

Bacterial strain	Relevant genotype	Reference/source
W3350A (= W3350)	F^- *lac3350 galK2* IN(*rrnD-rrnE*)	Hayes and Hayes (1986)
W3350(λ*imm*434-T)	Strong *imm*434 CI activity	Hayes and Hayes (1986)
TC600	*thr1 leuB6 fhuA21 lacY1 glnV44 e14*$^-$ *glpR200 thi1 supE*	Bachmann (1987) (from C600)
N100	*recA*	S. Hayes collection
SA500	F^- *his87 relA1 strA181 tsx83*(?)	Hayes (1991) (see text)
SA431	SA500(λ*cI*[Ts]*857* Δ*orf146-chlA*)	Adhya *et al.* (1968); Stevens *et al.* (1971)
SA439	SA500(λ*cI*[Ts]*857* Δ*P-chlA*)	Adhya *et al.* (1968); Stevens *et al.* (1971)
Y832	SA500(λ*cI*[Ts]*857*Δ431=*orf146-chlA*)	Hayes (1991); Hayes *et al.* (1990)
Y836	SA500(λ*bio*275 *cI*[Ts]*857* Δ431	Hayes and Hayes (1986); Hayes *et al.* (1990)
B832	C600 *arg*::Tn*5 nusAhy302 recD1009 suII*	F. W. Stahl
FC40 (= SMR624)	Δ(*srlR-recA*)::Tn*10*	Harris *et al.* (1994); S. M. Rosenberg via H. Bull
SMR692	*recD*6001::miniTn*10*-kan	Harris *et al.* (1994); S. M. Rosenberg via H. Bull
FWS-A354 (= SMR1)	*recB*21 *argA*::Tn*10 sbcA*	Stahl *et al.* (1980); S. M. Rosenberg via H. Bull
SZ635/Szigety	594 r^-m^+ *ruvC*53 *eda51*::Tn*10*	Sharples *et al.* (1990); S. M. Rosenberg via H. Bull
DPB271	MG1655 *recD*1903::miniTn*10*	Biek and Cohen (1986); S. M. Rosenberg via H. Bull
MVM401	*recF*400::Tn*5 sulA*::Mud1 *cam*	Madiraju and Clark (1991)
N2731/SMR600	*recG*258::miniTn*10*-kan	Lloyd and Buckman (1991); S. M. Rosenberg via H. Bull
JC12-123/FWS-A548	AB1157 *recJ*284::Tn*10*	Lovett and Clark (1985); S. M. Rosenberg via H. Bull
W3350 *grpD55*	*grpD55* (allele of *dnaB*) *malF*3089::Tn*10*	Bull and Hayes (1996); M. Horbay and S. Hayes, unpublished results)
SMR572	C600 Δ(*srlR-recA*)::Tn*10* [pRP42]	S. M. Rosenberg via H. Bull
Y836[pCI$^+$]	Amp^R *imm*λ CI$^+$ high copy	M. Horbay, this laboratory
TC600[pRP42]	Amp^R *imm*434 CI$^+$	K. Asai, this laboratory
W3350[pRP42]	Amp^R *imm*434 CI$^+$	K. Asai, this laboratory
N100[pRP42]	Amp^R *imm*434 CI$^+$	A. Chu, this laboratory
Y836 *grpD55*	*grpD55* (allele of *dnaB*) *malF*::Tn*10*	K. Asai, this laboratory
Y836 Δ*recA*	*recA* (Δ*srlR-recA*306::Tn*10*)	K. Asai, this laboratory
Y836 *recB*	*recB*21 *argA*::Tn*10*	K. Asai, this laboratory
Y836 *ruvC*	*ruvC*53 *eda*51::Tn*10*	K. Asai, this laboratory
Y836 *recD*	*recD*1903:: miniTn*10*	K. Asai, this laboratory
Y836 Δ*recA recD*	*recA* (Δ*srlR-recA*306::Tn*10*) *recD*6001::miniTn*10*-kan	K. Asai, this laboratory
Y836 *recD recF*	*recD*1903::miniTn*10 recF*400::kan	K. Asai/N. Pastershank, this laboratory
Y836 *recF*	*recF*400::Tn*5*	N. Pastershank, this laboratory
Y836 *recF recJ*	*recF*400::Tn*5 recJ*284::Tn*10*	N. Pastershank, this laboratory
Y836 *recJ*	*recJ*284::Tn*10*	N. Pastershank, this laboratory
Y836 *recG*	*recG*258::miniTn*10*-kan	K. Asai, this laboratory

the transducing phage λ*bio*275 (Hayes and Hayes 1986; Hayes *et al.* 1990). Both strains Y836 and Y832/SA431 are deleted for the NinR functions (Kröger and Hobom 1982; Daniels *et al.* 1983; Cheng *et al.* 1995) by Δ431, where the left endpoint of Δ431 is between 40,764 and 40,810 bp λDNA (Hayes 1991) within *orf146* (*ninB*). The variants of Y836 were made by P1vir transduction of appropriate markers. The *recA* character of the donor alleles and the *recA* transductants was demonstrated by showing that Fec$^-$ λ-phage, *e.g.*, those defective for *exo-bet-gam*, as λΔ*int-red-gam imm*434 (λ*bio*275*imm*434), did not form plaques on these *recA* hosts. In contrast, the *recD recA* variant permitted efficient plating by Fec$^-$ λ phage (Amundsen *et al.* 1986). The *grpD55* marker (Saito and Uchida 1978) that was moved into Y836 from W3350 *grpD55 malF*::Tn*10* was genetically mapped to *dnaB* (Bull and Hayes 1996) and shown by sequence analysis (M. Horbay and S. Hayes, unpublished results) to be an allele of *dnaB*.

Plasmids: pCH1 includes λ bases 34,449–41,732 (Hayes *et al.* 1997). pHB30$^{nl42\text{-}9}$ (abbreviated pCI$^+$) was derived from pHB30 (Bull 1995), which includes pBR322 (bases 375–4286) and λ*cI*[Ts]*857* (bases 34,499–34,696, 36,965–38,103, and 38,814–40,806; see Figure 2B). pCI$^+$ was a *cI*[Ts]*857* to *cI*$^+$ (λ 37,742 T to C) revertant (isolated and sequenced by M. Horbay) of pHB30. It expresses sufficiently high levels of CI repressor to prevent the plating of λ*cI* mutants and λ*vir* (data not shown). pRP42 is Amp^R ColE1 and was from M. Ptashne via SMR/HB (Table 1) and expresses the *imm*434 CI$^+$ repressor. Cells with pRP42 infected at 39° with λ*cI*72 yielded no λ*imm*434 recombinants (frequency $<1 \times 10^{-8}$) when scored on TC600(λ) lysogens. Plasmids pTP914 and pTP915 were from A. R. Poteete. Plasmid pTP914 (Poteete *et al.* 2002) is *Aat*II-*galK* (N-terminal end) p_{mac} (TTTACA:-35; TATAAT:-10; RBS) *rap Sac*I Kan^R from Tn*903-galK* (N-terminal end) *Bam*HI pBR322:*ori-bla*, where the *Apa*I-p_{mac}-*rap-Sac*I interval was cloned into pTP838 (Murphy *et al.* 2000), and in pTP915 *gfp* replaces *rap*. Poteete *et al.* (2002) reported that the Rap$^+$ phenotype

in pTP914 was unaffected by the presence or absence of the *lac* inducer IPTG, suggesting a basal level of expression of *rap* from p_{mac}.

Phages: λΔ*nin5* (Hayes and Hayes 1986) was used for introducing the *nin5* deletion into the *imm*434 phages. λ*imm*434 is λ*imm*434 *cI* as described in Hayes *et al.* (1998) (lysate 668a). λ*imm*434 Δ*nin5* was made by crossing λΔ*nin5* × λ*bio*275 *imm*434 Δ*nin5*. λ*bio*275 *imm*434 Δ*nin5* was prepared by crossing λΔ*nin5* × λ*bio*275 *imm*434 *cI*BG[Ts]. λ*bio*275 *imm*434 *cI*BG[Ts] was prepared by crossing λ*imm*434 *cI*BG[Ts] (Hayes *et al.* 1998) × λ*bio*275. (Thus, all of the *imm*434 phages, except λ*imm*434 *cI*, were *cI*BG[Ts] and formed clear plaques at 37°.) λ*cI*[Ts]*857* Δ*d2* (stock MMS1892) and λ*cI*[Ts]*857* Δ*d5* (stock MMS1891) were from F. W. Stahl. λ*imm*434 Δ*d2* was prepared by crossing λ*bio*275 *imm*434 Δ*nin5* × λ*cI*[Ts]*857* Δ*d2*. λ*imm*434 Δ*d5* was prepared by crossing λ*bio*275 *imm*434 Δ*nin5* × λ*cI*[Ts]*857* Δ*d5*. λ*bio*275 *imm*434 Δ*d2* was prepared by crossing λ*bio*275 *imm*434 Δ*nin5* × λ*imm*434 Δ*d2*. λ*bio*275 *imm*434 Δ*d5* was prepared by crossing λ*bio*275 *imm*434 Δ*nin5* × λ*imm*434 Δ*d5*. The phages employed for marker rescue assays are diagrammed in Figure 1C. The *nusA* strain distinguishes phages with Δ*nin5*, which are able to form plaques on it, from phages that cannot, *i.e.*, λ wild type or phages with Δ*d2* or Δ*d5* (F. W. Stahl, personal communication). In the experiments reported here, the *int-xis-hin-exo-bet-gam-kil* gene interval (representing λ bases 27,731–∼33,303; Daniels *et al.* 1983) on some λ*imm*434 infecting phages, or within the cryptic *imm*λ prophage in strain Y836, was replaced ("Δ*int-red-gam*") with *bio*275 *E. coli* chromosomal DNA present on specialized transducing phage λ*bio*275. Fec$^-$ phages with *bio*275 gene replacements do not form plaques on *recA* hosts. Phage λ*imm*434 (Kaiser and Jacob 1957) carries a substitution of nonhomologous phage 434 DNA for a 2.66-kb *imm*λ DNA region (35,584–38,245 bp λ) present in phage λ and in the cryptic λ prophage (Daniels *et al.* 1983). Each *imm* region encodes the respective immunity-specific genes *cI* and *cro* and the promoters p_L and p_R, except the *imm*λ also includes genes *rexA-rexB* (1.28 kb not present in *imm*434). Phages designated ΔNinR carry the *nin5* deletion of DNA bases 40,503–43,307, including *ren-ninA–ninI* (Daniels *et al.* 1983; Hollifield *et al.* 1987); Δ*d2* removes λDNA bases 40,943–41,810, including the C-terminal half of *orf* (*ninB*) and most of *ninC* from the N-terminal end, and Δ*d5* removes λDNA bases 42,925–43,183, including the C-terminal end of *rap* (*ninG*) and most of *ninH* from the N-terminal end (Hollifield *et al.* 1987).

Assay for replication initiation from induced cryptic λ prophage: Single-colony isolates of strain Y836 and 13 variants were inoculated into tryptone broth (TB; 10 g Bacto tryptone, 5 g NaCl/liter) and grown overnight to saturation. Duplicate 20-ml subcultutures made from 1/100 culture dilutions were prepared and grown in a shaking water bath at 30° to midlog (A_{575} ∼ 0.35) in fresh TB. Cell aliquots were diluted in buffer (0.01 m NaCl and 0.01 m Tris HCL, pH 7.8) and spread on TB agar plates incubated at 30° and 42° for 48 hr to determine cell titer and cell viability (frequency of survivor clones) upon induction of the cryptic prophage. One of the duplicate subcultures at midlog was induced for expression of the genes on the cryptic λ prophage by shaking the culture for 15 sec at 60° and then placing it in a shaking 42° water bath for an additional 60 min. The parallel 30° culture was grown for an additional 60 min at 30°. At the end of the 60-min growth period, DNA was prepared from the 30° and 42° culture cells using the QIAGEN (Chatsworth, CA) DNAeasy kit that can process 2×10^9 cells. A culture volume equivalent to 2×10^9 cells was processed per DNA sample per filter. Duplicate DNA preparations were prepared for each culture sample at 30° and 42°. The duplicate DNA samples were combined, an aliquot was diluted 1/10 in TE* buffer (0.01 m Tris HCl, pH 7.6 or 8.0, 0.001 m Na_2EDTA), and the DNA concentration was determined by spectrophotometer. Aliquots of the DNA estimated at 6.0 μg were ethanol precipitated and the pellets resuspended in 16 μl TE* for use in Southern blot analysis. A digoxigenin-dUTP (Dig)-labeled λDNA probe was prepared by amplifying pCH1 plasmid DNA (300 ng) using PCR primers L22 (λ bases 5′-38517–38534) and R24 (λ bases 5′-40298–40281) and the Dig Hy Prime DNA labeling detection kit (Roche Applied Science). The DNA probe band (Figure 2B) from the PCR was purified by electrophoresis on a 0.7% agarose gel, extracted using the QIAGEN gel extraction procedure, eluted, and concentrated to 2.0 μg/32 μl. The extracted band was converted to a hybridization probe using the Dig-labeling procedure. The DNA probe concentration was estimated from spot coloration on a filter to be ∼25 ng/μl. Each DNA preparation (2 μg), from cultures of Y836 and variants, was digested with *Bst*EII at 60° and run on a 0.7% agarose gel, with 1 μg of λ*cI*72 DNA digested with *Bst*EII per gel as a control for band size. The gels were processed for blotting to GeneScreen Plus (DuPont, Wilmington, DE) filter paper. The Southern blots were hybridized using the Dig-labeled DNA probe at a concentration of 25 ng/ml of hybridization solution and bands were visualized using antidigoxigenin-AP according to the Dig Hy Prime labeling/detection protocol.

Marker rescue assays for *imm*λ recombinants: The recombination event involved the rescue of a 2.6-kb nonhomologous region of prophage DNA that is flanked by homologous regions of ∼2 kb shared by both the prophage and the infecting phage. The infections were slightly varied from the procedure in Hayes *et al.* (1998). Phage of 5×10^8 were mixed with $\sim 1 \times 10^8$ cells, placed in an air incubator at 39° for 15 min, diluted 0.01 into TB, and shaken 90 min at the indicated infection temperature. The lysate was clarified and plated onto sensitive cells to assay total phage and onto *imm*434 cells to assay for *imm*λ recombinants. All cultures were made from a fresh colony on a TB agar plate (TB + 11 g Bacto agar per liter) or plate containing the antibiotic(s) corresponding to the strain's resistance marker(s). The averaged results and standard errors are for multiple independent assays. The practical cutoff for marker rescue of *imm*λ in the crosses was set to $\leq 1 \times 10^{-5}$, considered a background value even when no *imm*λ recombinant PFU were obtained. Recombinant *imm*λ phages were detected by plaque formation on W3350 or TC600 host cells lysogenized by λ*imm*434-T or transformed by plasmid pRP42. The *imm*λ rescue frequency was determined by dividing the titer of the recombinants by the total phage titer for the clarified 90-min lysate obtained on sensitive TC600 cells.

RESULTS

Requirement of phage genes for *imm*λ marker rescue: We examined the phage requirement for rescuing *imm*λ from the cryptic prophage in strain Y836 (Figure 1B) using λ*imm*434 infecting phages (Figure 1C). Phage Δ*int-red-gam* ΔNinR (Table 2, line Y836, column 5) essentially failed to support *imm*λ marker rescue at 30° or 39°. Thus, phage-encoded activity is needed for gene swapping, and the *E. coli* recombination functions provided in the absence of the phage activities do not suffice. We term the phage-dependent acquisition of heterologous genes the kleptomania (KM) phenotype, since functions are acquired or replaced without selective necessity. The Int-Red-Gam$^+$ functions, provided by λ*imm*434 Δ*nin5* phage, or the NinR$^+$ functions, provided by λ*bio*275

FIGURE 1.—(A) Homologous recombination between common sequence (rectangle) in phage and plasmid forming a phage-plasmid co-integrate structure (see text). SHEN and HUANG (1986) and HOLLIFIELD *et al.* (1987) used infecting phages that carried amber mutations in both λ genes *A* and *B* and were deleted for recombination functions *int-xis-exo-bet-gam* and the genes within the $NinR^{\lambda}$ region, which were substituted by another set of genes from $Nin^{\Phi 80}$. (B) Phage-prophage marker rescue of the *imm*λ genes from a cryptic prophage (lacking a *cos* site) within the bacterial chromosome by an infecting *imm*434 phage. The open rectangles are DNA strands of a circular *E. coli* chromosome; solid lines are λDNA, as infecting phage or as cryptic prophage within *E. coli* chromosome; the solid rectangle is *imm*434; the stretched diamond structure is *imm*λ; the stippled rectangle is *bio*275 substitution of *E.* coli DNA for the λ prophage genes *int-cIII* in the Y836 chromosome (and in infecting phages in C); the solid triangle on map of Y836 represents the Δ431 deletion removing rightward of λ prophage genes and adjacent chromosomal genes. The actual λ bases that were deleted or substituted are described in MATERIALS AND METHODS. The drawing is to scale, except that the sizes of the *imm*434 and *imm*λ intervals were made equal when *imm*434 was smaller by ~1.2 kb. (C) Maps of infecting phages used in Tables 2–5. The deletion *nin5* on infecting phage encroached on the interval of λ base homology to the right of the *imm*434 marker (B) between the cryptic prophage and the infecting phage, reducing it from ~2519 (or 2565) bp (with Δ431 endpoint) to ~2257 bp. The *bio*275 substitution on the infecting phage increased base homology to the left of the *imm*434 marker: the 2281-bp λ homology was unchanged, but homology was increased by the size of the *bio*275 substitution (see HAYES *et al.* 1990) carried on both prophage and infecting Δ*int-red-gam* phage.

*imm*434, independently supported rescue of *imm*λ from the cryptic prophage (Table 2). This shows that λ encodes two distinct genetic mechanisms for the KM phenotype. Marker rescue of *imm*λ was favored by the $NinR^+$ functions acting alone and was 117-fold higher at 39° than the basal level observed with Δ*int-red-gam* ΔNinR phage infection.

NinR activities in Rec^+ hosts: The possibility that multiple NinR functions participate in $NinR^+$-dependent *imm*λ rescue was tested by infections with Δ*int-red-gam* phages deleted within NinR for *orf-ninC* or for *rap-ninH*. Deletion of the NinR region by Δ*nin5* reduced *imm*λ rescue by 117-fold (Table 2), whereas deletions of *orf-ninC* or of *rap-ninH* yielded 5-fold and 12-fold reductions (Table 3), suggesting the participation of the deleted functions, as well as that of unaccounted NinR activities, in *imm*λ rescue. The very inhibitory effect of Δ*nin5* on marker rescue is probably not explained by its encroachment on the rightward recombination interval, *i.e.*, shortening it ~10% (Figure 1, B and C).

Y836 cells containing a pBR322-derived multicopy plasmid expressing *rap* (pTP914), or a *gfp* control (pTP915), were infected to determine if Rap made by the plasmid could complement for the λ functions removed by Δ*nin5*, Δ*d5*, Δ*d2*, and even Δ*int-red-gam* (Table 4). Adding the Gfp plasmid pTP915 to Y836 cells increased the background level for *imm*λ rescue by phage Δ*int-red-gam* ΔNinR by 4-fold and the Rap plasmid pTP914 increased the background >10-fold [*i.e.*, background 3 in Y836, 12 in Y836(pTP915), and 32 in Y836(pTP914) cells]. The presence of Rap provided by pTP914 did not substitute for all of the functions removed by ΔNinR [*i.e.*, comparing Y836(pTR914) infections by Δ*int-red-gam* $NinR^+$ and Δ*int-red-gam* ΔNinR phages]; however, *imm*λ rescue was complemented ~8-fold [*i.e.*, comparing Δ*int-red-gam* Δ*rap-ninH* infections of Y836(pTP914) and Y836 (pTP915)]. Clearly, more than one NinR activity must be removed to incapacitate NinR-dependent *imm*λ rescue, and Rap expression alone is insufficient to complement fully Δ*nin5* phages for the loss of $NinR^+$-dependent *imm*λ marker rescue. These findings further support a proposal that the NinR region encodes several functions that aid *imm*λ rescue in an otherwise Rec^+ host.

Host and phage functions influencing phage-prophage marker rescue at 39°: The influence of host recombination functions on the KM phenotype was examined. Do the mechanisms for *imm*λ rescue involve the same key host recombination activities? We show that, in the absence of Red activity, the NinR-dependent *imm*λ rescue activity: (i) was reduced by >300-fold and 88-fold, respectively, by mutations in the host genes *recA* and *recJ*; (ii) was reduced 7- to 10-fold by inactivation of the host genes *recB* or *recD* and by the modification (mutation *grpD55*) of *dnaB*; (iii) was reduced 2- to 3-fold by inactivation of *recF* and *recQ*. We also show that (iv) a *recF* defect can partially suppress the loss of *recJ*; (v) a *recF* defect suppresses and stimulates NinR-dependent recombina-

TABLE 2

Marker rescue of *imm*λ from cryptic prophage

Infected host strains	Infecting *imm*434 phages[a]			
	Red$^+$ NinR$^+$	Red$^+$ ΔNinR	Δ*int-red-gam* NinR$^+$	Δ*int-red-gam* ΔNinR
Y836[b]	202 (37)	139 (28)	352 (92)	3 (<1)
Y836 *grpD55*	22 (9)	59 (1)	44 (13)	2 (1)
Y836 *recA*	43 (6)	102 (47)	≤1 (<1)	≤1 (<1)
Y836 *recB*	24 (1)	527 (332)	35 (5)	2 (<1)
Y836 *recD*	128 (23)	549 (245)	50 (14)	≤1 (<1)
Y836 *recD recA*	197 (38)	189 (50)	≤1 (<1)	≤1 (<1)
Y836 *recD recF*	1283 (71)	988 (154)	683 (255)	26 (4)
Y836 *recF*	259 (32)	135 (25)	132 (23)	≤1 (<1)
Y836 *recF recJ*	582 (48)	587 (82)	38 (12)	8 (6)
Y836 *recJ*	763 (55)	508 (117)	4 (4)	≤1 (<1)
Y836 *recQ*	347 (37)	928 (99)	130 (23)	12 (7)

Frequency of *imm*λ marker rescue from cryptic prophage in host ($\times 10^{-5}$) for infections carried out at 39°. Standard error is shown in parentheses.

[a] Designations used: Red$^+$ NinR$^+$, λ*imm*434; Δ*int-red-gam* ΔNinR, λ*bio*275 *imm*434 Δ*nin5*; Δ*int-red-gam* NinR$^+$, λ*bio*275 *imm*434; and Red$^+$ ΔNinR, λ*imm*434 Δ*nin5*.

[b] Frequency of *imm*λ marker rescue from cryptic prophage in Y836 ($\times 10^{-5}$) for infections carried out at 30° for phages: Red$^+$ NinR$^+$, 7(3); Red$^+$ ΔNinR, 19(2); Δ*int-red-gam* NinR$^+$, 39(20), and Δ*int-red-gam* ΔNinR, 2(1).

tion in the absence of *recD*, whereas each separate mutation was inhibitory; and (vi) as noted above, *imm*λ rescue moderately, or partially, required NinR activities encoded within *rap-ninH* and *orf-ninC*. In contrast, in absence of NinR activity, the Red$^+$ system activity: (i) appeared independent of defects in the host genes *recA* and *recF*; (ii) was stimulated 4-fold by defects in the host genes *recB*, *recD*, *recJ*, and by *recF recJ* double mutations; (iii) was stimulated 7-fold by defects in *recQ* and by *recD recF* double mutations; and (iv) was reduced (∼2-fold) by the *grpD55* allele of *dnaB*.

TABLE 3

Influence of NinR region mutations Δ*d2* and Δ*d5* on *imm*λ marker rescue

Infected host strains	Infecting Δ*int-red-gam imm*434 phages	
	Δ*orf-ninC* [Δ*d2*][a]	Δ*rap-ninH* [Δ*d5*][b]
Y836	73 (10)	28 (4)
Y836 *recA*	≤1 (<1)	≤1 (<1)
Y836 *recB*	80 (16)	21 (2)
Y836 *recD*	83 (6)	62 (10)
Y836 *recD recA*	≤1 (<1)	≤1 (<1)
Y836 *recD recF*	2 (1)	14 (2)
Y836 *recF*	49 (3)	51 (9)
Y836 *recF recJ*	60 (6)	15 (1)
Y836 *recJ*	110 (16)	35 (12)
Y836 *recQ*	95 (7)	37 (4)

Frequency of *imm*λ marker rescue from cryptic prophage in host ($\times 10^{-5}$) for infections carried out at 39°. Standard error is shown in parentheses.

[a] Phage λ*bio*275 *imm*434 Δ*d2*.

[b] Phage λ*bio*275 *imm*434 Δ*d5*.

The distinct requirements for the host functions strongly suggest that individual Red-dependent or NinR-dependent mechanisms support *imm*λ rescue. The RecBCD complex activity was inhibitory to the Red-dependent activity, since *imm*λ rescue was significantly stimulated in Red$^+$ΔNinR phage infections of hosts defective for *recB* or *recD* (Table 2). In contrast, it was stimulatory to NinR recombination (*recB*$^-$ and *recD*$^-$ mutations were inhibitory). Similarly, RecQ activity stimulates NinR-dependent rescue (*recQ*$^-$ is inhibitory) and inhibits Red-dependent rescue (*recQ*$^-$ is stimulatory). RecJ powerfully stimulated NinR-dependent *imm*λ rescue (*recJ*$^-$ was strongly inhibitory) but inhibited Red-dependent rescue (*recJ*$^-$ is stimulatory). RecF appears to have a slight stimulatory effect on NinR-dependent *imm*λ rescue, but no effect on Red-dependent *imm*λ rescue. The inhibitory effects of RecBCD and RecJ on Red-dependent *imm*λ rescue appear to be independent since the increased level of *imm*λ rescue was the same in *recJ*, *recJ recF*, *recD*, and *recB* mutants, also suggesting that the inhibitory effect of RecJ was independent of Recf. The inhibitory effect of RecBCD on Red-mediated *imm*λ rescue increased in the absence of Recf.

NinR activities in Rec$^-$ hosts: The influence of host mutations on *imm*λ marker rescue by Δ*int-red-gam* phages deleted for part of the NinR region was examined (Table 3). We observed two unanticipated results: (1) The deletion of *orf-ninC* or of *rap-ninH* suppressed the requirement of *recJ* for NinR$^+$-dependent *imm*λ rescue by 27- and 9-fold, respectively (*i.e.*, compared to Δ*int-red-gam* NinR$^+$ infection of Y836 *recJ*, Table 2), and (2) there was a dramatic loss in *imm*λ rescue in a *recD recF* host for infections by Δ*int-red-gam* Δ*d2* or Δ*d5* compared to

TABLE 4

Rap participation and *imm*λ marker rescue

	Infected host strains		
Infecting *imm*434 phages	Y836[a]	Y836 [pTP914 Rap$^+$][b]	Y836 [pTP915 Gfp][b]
Red$^+$ ΔNinR	139 (28)	145 (15)	155 (15)
Δ*int-red-gam* NinR$^+$	352 (92)[c]	394 (43)	267 (38)
Δ*int-red-gam* ΔNinR	3 (<1)	32 (3)	12 (2)
Δ*int-red-gam* Δ*rap-ninH* [Δ*d5*]	28 (4)	86 (3)	11 (2)
Δ*int-red-gam* Δ*orf-ninC* [Δ*d2*]	73 (10)	132 (1)	33 (9)

Frequency of *imm*λ marker rescue from cryptic prophage in host ($\times 10^{-5}$).
[a] The results in this column are copied from Tables 2 and 3 for comparison.
[b] The results shown each represent an average for two independent infection reactions conducted in parallel.
[c] The result shown was for an average of seven independent determinations at different times.

a Δ*int-red-gam* NinR$^+$ infection (Table 2), and the levels were even lower than those for the Δ*int-red-gam* ΔNinR infection. We noted earlier that RecF was not required for Red activity, but in the absence of RecD, proved somewhat inhibitory to both Red- and NinR-dependent *imm*λ rescue. Was the level of RecF inhibition magnified by removal of the *orf-ninC* or *rap-ninH* genes within NinR? (Δ*d2* and Δ*d5* do not reduce the size of the recombining interval as both lie to the right of the interval between *imm*λ and Δ431; see Figure 1.)

Prophage replication initiation from *ori*λ stimulates *imm*λ rescue by infecting *imm*434 phage: λ prophage replication initiation and gene expression are repressed by the prophage CI857 repressor in strain Y836 and its variants for cells grown at 30°. However, upon shifting the cells to ≥38°, the repressor is thermally denatured and transcription initiates from the λ promoters p_L and p_R, the early genes *N* and *cro-cII-O-P* are expressed, and bidirectional replication is initiated from *ori*λ within gene *O* (Figure 2A). Hayes (1979) observed an increase of 5–6 λDNA copies by 20 min after shifting cells with a nonexcising cryptic prophage λ*cI*857Δ*Q-Jb* from 30° to 42°. Subsequently, C. Hayes and S. Hayes (unpublished results) found that *ori*λ-dependent replication forks induced in Y836 escaped bidirectionally into the contiguous *E. coli* chromosome, *e.g.*, replicating the adjacent *gal* operon 15–20 kb left of *attL*, but stalled <200 kb from *ori*λ.

The initiation of *ori*λ replication in strain Y836 from the Δ*int-red-gam-kil* prophage is shown by comparing DNA amplification of the 3675-bp *Bst*EII fragment (Figure 2C), including *ori*λ, from thermally induced and noninduced cultures. Replication from *ori*λ escapes rightward as seen by amplification of the ~4250-bp *Bst*EII fragment (Hayes *et al.* 1990), which is mainly *E. coli* DNA. The addition of a multicopy pCI$^+$ plasmid (7298-bp band, Figure 2C), expressing wild-type CI$^+$ repressor, prevented *ori*λ replication initiation, *i.e.*, amplification of the 3675-bp prophage fragment.

The initiation of divergent λ replication forks from the derepressed cryptic prophage in Y836 was accompanied by cell killing (Hayes *et al.* 1990), with the frequency of mutant survivor cells appearing at $<10^{-5}$. Figure 2, C and D (separate assays and gels), shows survivor frequencies of Y836 CFU at 1 or 2×10^{-6}, respectively. The addition of the pCI$^+$ plasmid to Y836 cells prevented the loss in cell viability seen when the cells were shifted to 39° (Table 5) or 42° (Figure 2C) due to CI$^+$ inhibition of λ prophage induction.

The *imm*λ rescue from Y836 cells infected at 39° by *imm*434 Red$^+$ or NinR$^+$ phages was seven- to ninefold higher than that for the parallel infections carried out at 30°, where the prophage genes are repressed (Table 2, 30° infection results in footnote "c"). We sought to determine if the increased frequency for *imm*λ rescue was temperature specific or if it was influenced by transcription and *ori*λ replication initiation from the induced cryptic prophage. In Table 5 we show that (i) the plasmid loss from Y836[pCI$^+$] cells grown at 39° was <0.5% (*i.e.*, <1/220 colonies) per experiment, greatly limiting the possibility for *imm*λ rescue from cells with induced prophages that had lost pCI$^+$; (ii) the frequency of *imm*λ recombinants formed at 39°, where both the Red$^+$ and NinR$^+$ recombination functions are expressed from λ*imm*434 (which is insensitive to the CI^λ repressor), was 212-fold below that for the infection of Y836 cells (without plasmid) by λ*imm*434 at 39°; and (iii) Y836[pCI$^+$] cells were 7-fold more inhibitory for *imm*λ rescue at 39° than wereY836 cells infected at 30° (Table 5 and footnote c in Table 2). We conclude that *ori*λ-dependent replication initiation, or simply the derepression of λ gene expression, stimulates *imm*λ rescue. Relevant to these observations, (i) the formation of λ*imm*434 progeny phage particles released from the infected Y836[pCI$^+$] cells was not reduced by pCI$^+$, and (ii) viable *imm*λ recombinants were not formed upon infecting W3350[pCI$^+$] cells (data not shown). (Note that while pCI$^+$ includes a small portion of λDNA within *sieB* left of *imm*λ and the stretch *O*[C-terminal half]-*P-ren* right of *imm*λ, it is deleted for the gene sequences

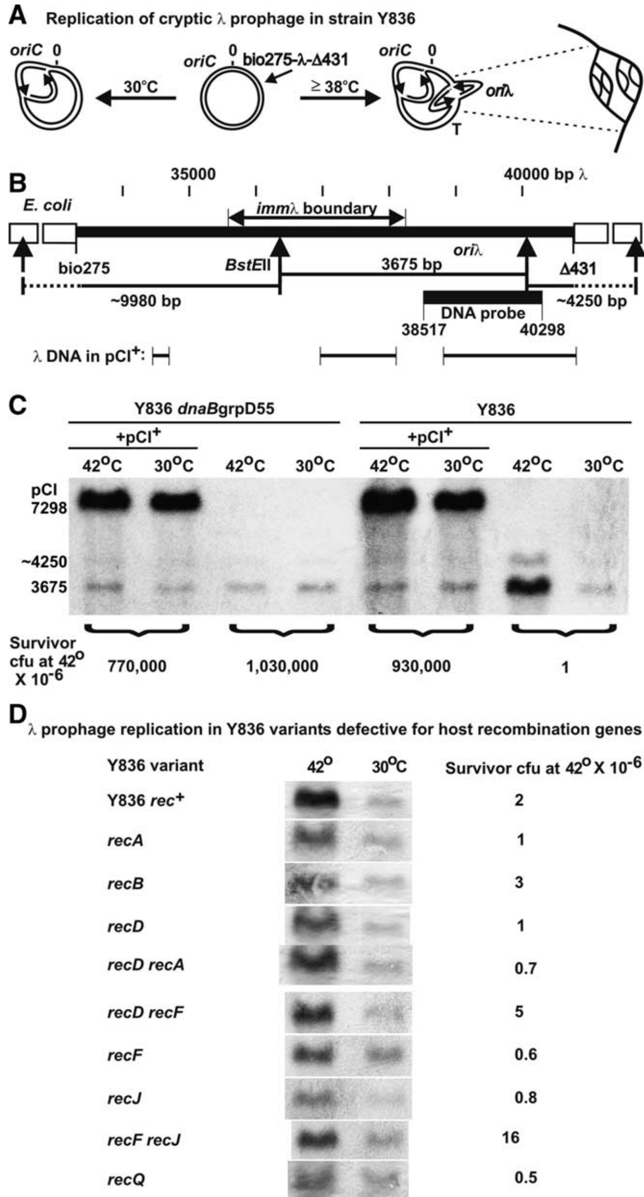

Figure 2.—Assay for prophage replication initiation from *ori*λ. (A) The nonexcisable cryptic λ fragment inserted (short arrow) within the *E. coli* chromosome remains repressed at 30° where the prophage repressor is active. Shifting cells to ≥38° inactivates the CI857 repressor for λ prophage transcription and replication initiation from *ori*λ. Multiple λ bidirectional replication initiation events from *ori*λ generate the onion-skin replication structure drawn at right. (B) λDNA (thick solid line) fragment within the *E. coli* chromosome (open boxes), *Bst*EII restriction sites within λ and the *E. coli* chromosome showing bands generated by cleavage (Hayes *et al.* 1990), and the region amplified to prepare a DNA probe. (C) Assay for replication initiation from *ori*λ upon shifting culture cells to 42° to induce the cryptic prophage. Cells with plasmid pCI^+ are not derepressed for λ transcription or *ori*λ replication at 42°, permitting a comparison between any chromosomal increase for cells shifted for 1 hr at 42° with *ori*λ replication initiation arising from the derepressed cryptic prophage in Y836 cells without the plasmid that were shifted for 1 hr to 42°. (See text for discussion of survivor CFU at 42°.) (D) The influence of host recombination defects on λDNA synthesis initiated from *ori*λ.

sieB-N-p_L-rexB-rexA and *cro-cII-O* (Figure 2B) so that any potential *imm*λ recombinants rescued by λ*imm*434 infection would be defective for growth.)

Replication initiation at *ori*λ requires an interaction between the λ *P* gene product and the host DnaB helicase (Mallory *et al.* 1990). Bull and Hayes (1996) transduced the mutation *grpD*55 from the original isolate (Saito and Uchida 1978) into W3350 and mapped it to *dnaB*. The *grpD*55 allele in the original isolate and in the allele moved into W3350 were sequenced and found to have identical dual missense mutations within *dnaB* (M. Horbay and S. Hayes, unpublished results). λ forms plaques (at high efficiency) on *E. coli* W3350 *dnaB*grpD55 cells at 30° but not at the restrictive temperature of ≥39° (Bull and Hayes 1996), suggesting that the P:DnaB interaction required for *ori*λ replication breaks down at ≥39°. However, the *dnaB*grpD55 allele does not significantly interfere with *E. coli* replication, since the cells can form a lawn on plates incubated at 39°–42°. The influence of *dnaB*grpD55 on *ori*λ replication initiation in Y836 was examined. The *dnaB* allele strongly prevented *ori*λ replication initiation from the 3675-bp prophage fragment and suppressed cell killing by the induced λ prophage (Figure 2C).

The formation of *imm*λ recombinants from infections of Y836 *dnaB*grpD55 cells at 39° was reduced by nine-, eight-, and twofold in Red^+ $NinR^+$, Δ*int-red-gam* $NinR^+$, and Red^+ ΔNinR phage infections (Table 2). We conclude that NinR-dependent *imm*λ marker rescue is notably stimulated by replication initiation from the cryptic λ prophage fragment, whereas Red-dependent *imm*λ rescue is much less dependent upon this event, suggesting again that λ encodes two mechanistically distinct recombination activities.

Studies being reported elsewhere (M. Horbay, C. Hayes and S. Hayes, unpublished results) are relevant to these observations. We asked if the absence of plaque formation on W3350 *dnaB*grpD55 cells at 42° reflected the absence of a phage burst. We quantified plaque formation, free phage, and phage bursts from cells infected at MOI's of 0.01 and 5 at 30° and 42° with different *ori*λ phages and with the *ori*P22 hybrid phage λ*cI*857 (*18,12*)P22, which is insensitive to the influence of the *dnaB*grpD55 allele. Recombination-driven phage replication was able to bypass the formal *ori*λ replication initiation step required for generating phage progeny if two homologous phage genomes entered a cell (M. Horbay, C. Hayes and S. Hayes, unpublished results), whereas the low MOI infections yielded no burst at the restrictive temperature, in agreement with the plaque assay results, but strong burst at 30°.

Formation of λ*imm*λ recombinants: We observed a >1000-fold difference in the frequencies of *imm*λ recombinant phages formed upon varying the availability of phage and host functions (Table 2). At the low end, λΔ*int-red-gam* $NinR^+$ and ΔNinR phages produced no *imm*λ recombinants upon infection of the *recA* host. Was

TABLE 5

CI+ limits *imm*λ rescue in Y836 at 39° following λ*imm*434 infection

Host Y836[pCI+] [a]	Cell viability TB + Amp/TB [a] 30°	39°	Colonies with plasmid loss at 39° [b]	Recombination frequency ($\times 10^{-5}$) [c] 30°	39°
Experiment 1	0.91	1.05	0/220	<0.3	0.5
Experiment 2	1.04	1.02	0/220	<0.1	1.4

Plasmid pCI+ is multicopy and expresses the *cI*+ gene encoding CI repressor of λ (see MATERIALS AND METHODS).

[a] Single colonies of Y836[pCI+] were prepared on TB + Ampicillin (100 μg/ml) agar plates, and a single colony was used to inoculate a culture of TB + Amp (100 μg/ml). Cell aliquots were infected and inoculated into TB (Experiment 1) or TB + Amp (100 μg/ml) (Experiment 2) for 90 min and the recombination frequency was determined. The same cell culture was subsequently plated on TB and on TB + Amp plates and incubated at 30° or 39°; the cell titer on TB + Amp plates was divided by the cell titer on TB plates. The growth of culture cells on TB plates does not require that cells maintain plasmid.

[b] Single colonies on TB plates incubated at 39° were stabbed to TB + Amp and TB plates, respectively, for measuring the proportion of Y836 cells that had lost pCI+. In Experiment 1, all colonies from two dilution plates were picked.

[c] The frequency of *imm*λ recombinants from the crosses were determined by dividing the PFU titer on TC600[pRP42] cells by the PFU titer on TC600 cells.

this because *imm*λ exchange occurred but the formation of a mature *imm*λ particle required phage replication, because exchange between the infecting phage and prophage could not occur, or because there was an interdependence of replication and recombination? (We will discuss phage maturation without replication, *i.e.*, the "free-loader" concept, below.) HAYES (1979; see FURTH and WICKNER 1983) surveyed the requirement of host replication proteins for *ori*λ replication initiation. We are unaware of a similar assay for the influence of recombination proteins on replication initiation from *ori*λ. Since *imm*λ marker rescue was diminished in the absence of *ori*λ replication initiation from prophage (*i.e.*, by CI blocking prophage induction or the conditional defect of the *dnaB*grpD55 allele), it was important to learn if host recombination functions influenced the appearance of replication initiation from *ori*λ.

We assayed *ori*λ replication from variants of Y836 with null mutations in host recombination genes (Figure 2D). In comparison to results seen for the Rec+ parent (Figure 2, C and D), the appearance of the onion-skin complex, representing multiple bidirectional replication initiation events (Figure 2A) arising from *ori*λ, was reduced in all of the variants, especially those with null alleles in *recA, recB, recD*, *recQ*, and *recJ*. Addressing the above question, *ori*λ replication initiation, albeit reduced, proceeded in the *recA* host. Thus, λΔ*int-red-gam* phages should undergo *ori*λ replication initiation in this host, and thus the absence of *imm*λ recombinants likely involves a requirement for RecA in phage-prophage exchange or maturation.

The most significant defect in DNA amplification from *ori*λ was seen in the Y836 *recJ* variant. (The strongest of three DNA amplification assays is shown in Figure 2D.) The *recJ* effect was suppressed partially by an additional null mutation in *recF*. Another experiment with Y836 *recJ*, which was identical to that for Y836 *dnaB*GrpD55 (Figure 2C, *i.e.*, one culture set included pCI+ to visualize the *ori*λ prophage bands from 30° and 42° cultures with blocked prophage induction), revealed little if any amplification of the *ori*λ band (data not shown) from the induced Y836 *recJ* culture cells. These results suggest that *recJ* participates in the appearance of the onion-skin DNA complex initiated from *ori*λ (Figure 2, A and C). However, as described below, λ will form a plaque on a *recJ* host.

NinR and *recJ* influence plaque size: The plaque size of λ*imm*434 variants on *E. coli* hosts was measured in parallel at 30°. Plaques (>15/phage/host; ≤10% standard error) averaged 0.35 mm for Δ*int-red-gam* NinR+ and Δ*int-red-gam* ΔNinR phages on TC600 Rec+, and, respectively, 0.35 and 0.18 mm on Y836 and 0.22 and 0.16 mm on Y836 *recJ*. Clearly, the *ori*λ phages somehow replicate and mature on a host defective for *recJ*, but the extent of phage progeny released was compromised if we roughly assume that plaque size has some relationship to phage burst. The NinR+ activity doubled the plaque size of Δ*int-red-gam* phage on Y836 and somewhat suppressed for the loss of *recJ*. Even in the presence of Red activity, the NinR region strongly contributed to plaque size, since in the same assay λ*imm434cI* formed 0.90 and 0.53 mm plaques on TC600 and Y836 cells, whereas λ*imm434*ΔNinR plaques were 0.44 and 0.18 mm, respectively.

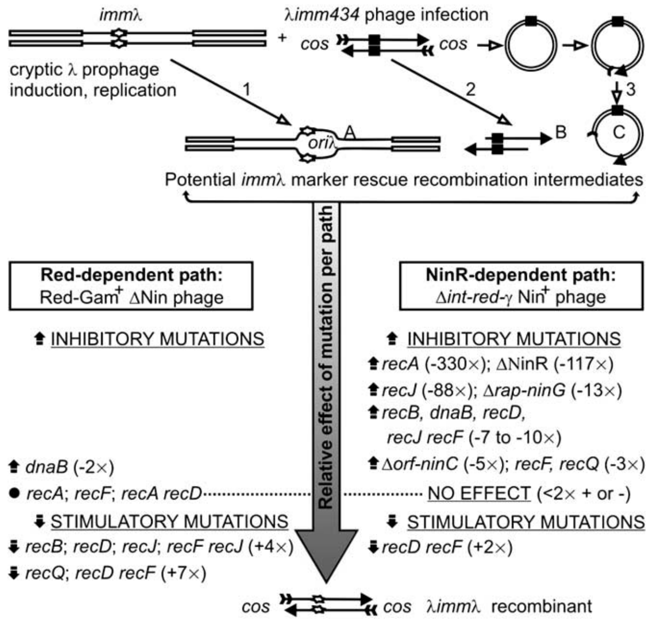

Figure 3.—Summary of recombination factors influencing NinR-dependent and Red-dependent phage-prophage marker rescue. The open rectangles are DNA strands of circular *E. coli* chromosome; thin lines are λDNA, as infecting phage or as cryptic prophage within the *E. coli* chromosome; the solid rectangle is *imm*434; the stretched diamond is *imm*λ; the feathered tails of arrows and the solid arrowheads respectively represent the 5′- and 3′-ends of phage DNA strands. The potential *imm*λ recombination intermediates include: 1, replication D-loop formed within cryptic prophage after replication initiation from *ori*λ when the cells are placed at or above 39°, generating intermediate A; 2, exonucleolytic activity at 5′-ends of linear infecting λ*imm*434 phage, resulting in 3′-single-stranded DNA overhangs generating intermediate B; 3, a nick is introduced into the circularized monomeric phage genome [or cos end(s) remains unligated], which is converted to a gap by the joint action of a DNA helicase and a single-stranded 5′–3′ exonuclease producing intermediate C.

DISCUSSION

We summarize in Figure 3 the influence of phage and host recombination functions on the rescue of a cryptic prophage *imm*λ region by an infecting heteroimmune λ*imm*434 phage. Major differences in the *imm*λ marker rescue requirements were seen for infecting phages deleted for Δ*int-red-gam* or for NinR. This suggests the occurrence of two distinct phage-encoded recombination pathways, the Red-dependent path and the NinR-dependent path. The deletion of both paths eliminated *imm*λ marker rescue, even in the full presence of wild-type host recombination functions.

To make sense of the many observations, we consider the following: Some mutants have unexpected phenotypes, for which we cannot rule out strain or allele-specific effects. However, in summary, the host recombination activities RecBCD, RecF, RecJ, and RecQ stimulated (participated in) NinR-dependent recombination while they served to reduce (inhibit) Red-mediated *imm*λ marker rescue (Figure 3). RecA was essential for NinR, but had slight influence on Red-dependent recombination. RecF was not required for Red activity, but in absence of RecD, proved somewhat inhibitory to both Red- and NinR-dependent *imm*λ rescue. The Red-dependent *imm*λ rescue was mainly unaffected by host mutations in *recA, recF*, or a double mutant in *recA recD*, but was stimulated by host mutations in *recB, recD, recJ*, and *recQ* and double mutations in *recF recJ* and *recD recF*, whose products may compete for recombination intermediates generated by Red pathway functions. Thus, several of the host functions had opposite effects toward NinR- and Red-dependent *imm*λ rescue, *e.g.*, as noted, RecBCD stimulated NinR but inhibited the Red-dependent recombination. Because of these opposite effects, the combination of both Red$^+$ and NinR$^+$ recombination activities expressed by a λ*imm*434 infecting phage (*i.e.*, the sum of independent Red$^+$- and NinR$^+$-dependent frequencies) was not additive for *imm*λ rescue in the Rec$^+$ Y836 host. Due to the inhibitory effect of several of the host functions on Red and their stimulatory effect on NinR-dependent recombination, we suggest that most of the *imm*λ rescue seen with the Red$^+$ NinR$^+$ infections of Rec$^+$ Y836 cells is NinR$^+$ dependent; *i.e.*, NinR$^+$ is the dominant pathway in a Rec$^+$ host.

It was suggested that the λ Red functions Beta and Exo (double-stranded 5′-exonuclease) share functionality with RecA and RecJ (Corrette-Bennett and Lovett 1995). While RecA mediates recombination via single-strand invasion, Exo and Beta generate recombinants by a process called single-strand annealing (see Court *et al.* 2002), possibly analogous to double-strand break repair in yeast. Our results showing a fourfold stimulation of Red-dependent marker rescue (by ΔNinR phages) in the absence of *recJ*, which encodes a single-stranded 5′ exonuclease (Lovett and Kolodner 1989), agree with observations by Murphy (1998) and with the finding that RecJ participates in Red-mediated λ × λ recombination in the absence of Rap activity (Tarkowski *et al.* 2002).

The requirements for phage-prophage marker rescue have gone mainly unexplored since the studies by Echols and Gingery (1968) and Signer and Weil (1968), who suggested that λ's Int or Red functions support marker rescue recombination in a RecA$^-$ host. The results reported here agree and show that the Red-dependent pathway is capable of eliciting substantial *imm*λ rescue in the absence of RecA activity. However, the investigators in the 1960s suggested that the host Rec$^+$ functions could provide for marker rescue recombination in the absence of Red functions. This was unsupported by our results showing virtually no *imm*λ phage-prophage marker

rescue in the absence of the Red or the NinR functions encoded by phage λ. We explain the latter discrepancy by assuming that the marker rescue infection assays carried out in the absence of Red functions actually provided unrecognized NinR$^+$ functionality within the infecting phages employed, which was attributed to the host Rec$^+$ functions.

Hollifield *et al.* (1987) identified the NinR function *rap* as an essential requirement for phage-plasmid co-integration at a site of shared DNA homology (Figure 1A) using infecting phages deleted for *int-gam.* The Rap protein was identified as a Ser/Thr phosphatase (Voegtli *et al.* 2000) and as a Holliday junction resolvase (Sharples *et al.* 2004). We show that the *nin5* deletion of NinR functions on a Δ*int-red-gam* infecting phage was complemented partially by expressing *rap* from a plasmid within the infected cells. The deletion of *rap-ninH* on a Δ*int-red-gam* infecting phage inhibited *imm*λ recombination 9-fold, while similarly providing Rap from a plasmid within the infected cells increased *imm*λ rescue. The partial complementation of both Δ*nin5* and Δ*rap-ninH* deletions by Rap expressed from a plasmid indirectly implies that Rap is important for *imm*λ rescue, but it is unlikely to be the only participating NinR function. An analogous infection with a Δ*orf-ninC* phage similarly decreased *imm*λ rescue (∼5-fold). Thus, the *orf* and/or *ninC* product contributes, along with Rap (and possibly NinH), to NinR-pathway-dependent recombination. Of relevance to this study, Stahl *et al.* (1995) reported a very modest (2.5-fold) effect of Rap on the frequency of apparent patch (substitution) recombinant formation between a plasmid and a phage whereas the production of co-integrate (apparent single splice or addition) products was stimulated 17-fold.

The formation of NinR-dependent *imm*λ recombinants was far lower at 30° than at 39°. Two procedures that inhibited replication initiation from the cryptic prophage, *i.e.*, the addition to cells of a CI$^+$ plasmid or of the *grpD55* allele of *dnaB* (both shown to prevent *ori*λ replication initiation), both reduced *imm*λ marker rescue by 212- and 9-fold, respectively, at 39°. Thus, *imm*λ marker rescue is enhanced by *ori*λ replication initiation from the cryptic prophage. The products of λ genes *O-P* participate in loading the host *dnaB* product at *ori*λ (Learn *et al.* 1997), contributing eventually to the open DNA complex represented by intermediate A in Figure 3, which likely is subjected to replicative inhibition (see Hayes and Hayes 1986) by CI due to a requirement for transcriptional activation of *ori*λ (Dove *et al.* 1969; Furth and Wickner 1983). An enhancement of *imm*λ recombination due to the formation of intermediate A could involve DnaB driving branch migration (Kaplan and O'Donnell 2002). The greater inhibition of *imm*λ rescue in Y836[pCI$^+$] than in Y836 *dnaB*grpD55 cells at 39° or in Y836 cells (with CI857 repressor) at 30° may result from excess CI$^+$ severely inhibiting λ transcriptional derepression.

The amplification level of onion-skin replication was not a critical requirement for NinR-dependent *imm*λ marker rescue, although its formation clearly proved of equal importance to the requirements for *recB* and *recD.* The accumulation of the onion-skin product of *ori*λ replication initiation was greatly reduced in the *recJ* host and was eliminated in the *dnaB*grpD55 strain. Yet the *recJ* mutation was significantly more inhibitory to NinR-dependent *imm*λ marker rescue than was the altered *dnaB* allele. Moderate *ori*λ replication accumulated as an onion-skin intermediate in strains defective for *recA*, *recB*, and *recD*, yet there was an absolute requirement of RecA and an intermediate requirement of RecB and RecD for NinR-dependent *imm*λ rescue. *recQ* encodes a helicase that translocates in the 3′ → 5′ direction when bound to single-stranded DNA (Umezu *et al.* 1990). Amplification of the onion-skin product of *ori*λ replication initiation was minimal in *recQ* mutants, but there was less than a threefold reduction (observed in ∼30 separate assays) in the level of NinR-dependent *imm*λ rescue.

The induction of *ori*λ-dependent replication from a cryptic prophage kills the host cell, probably by several mechanisms (Datta *et al.* 2005). Rare survivor cells include mutants with large deletions removing part of or the entire λ fragment (Hayes *et al.* 1990; Hayes 1991). The endpoints in 435 such deletions arising from cells with an induced λ*cI*857Δ431 prophage were mapped and none of the deletion endpoints was at or near *attL* (Hayes 1991). Thus, the attractive possibility that the *int* product *efficiently* promotes unequal crossing over between *att* and secondary sites within the prophage or bacterial chromosome to generate deletions was unsupported. The *int-cIII* prophage segment in the λ*cI*857Δ431 prophage was substituted in strain Y836 to reduce further the possibility for concerted illegitimate deletion of the prophage fragment. The appearance of *imm*λ recombinants from λ*imm*434 Δ*nin5* infections was ascribed to Red-dependent λ recombination activities, but also includes the potential for *int*-dependent *attB* × *attP* site-specific recombination driving marker rescue; however, we provide no further evidence for or against this possibility. We note that Enquist and Skalka (1973) found no evidence for Int-mediated recombination between circular monomers to produce packagable concatemers; *i.e.*, no more phages were produced on infection of *recA*$^-$ cells with *int*$^+$*red*$^-$ *gam*$^-$ point mutants than with λ*bio11 int*$^-$ *red*$^-$ *gam*$^-$ phage. We consider unlikely the possibility that a significant fraction of *imm*λ marker rescue is explained by illegitimate excision of the replicating cryptic prophage out of the chromosome, *i.e.*, enabling the infecting λ to recombine with a prophage circle rather than with the chromosome. The excision of a λ fragment (circular or not) from a replicating cryptic prophage imbedded within the chromosome was not observed here by gel blotting studies

that identified the single-copy 3675-bp *ori*λ fragment excised by *Bst*EII from a noninduced prophage.

Whether promoted by Red or by NinR, mechanistically, the detection of *imm*λ marker rescue from a chromosomal cryptic prophage, lacking *cos*, requires gene substitution and replacement of a functionally equivalent (although nonhomologous) DNA module in the *imm*434 infecting phage plus packaging of the recombinant genome to form a mature phage particle. Models for DNA substitution involve two independent splice events flanking *imm*λ or a single invasion event extended by branch migration, leaving a large patch of unpaired DNA that is ultimately resolved into distinct daughter copies by replication. The quite different requirements for Red- *vs.* NinR-promoted *imm*λ rescue suggest independent mechanisms.

The formation of mature *imm*λ recombinant particles "should" also depend upon sufficient replication of the recombinant molecule to produce a molecular intermediate able to serve as a substrate for DNA packaging, *i.e.*, one with two *cos* sites. Packaged phage genomes are occasionally formed from substrates with less than two *cos* sites (Little and Gottesman 1971; Yarmolinsky 1971; Enquist and Skalka 1973). Similarly, *imm*λ lysogens grown in heavy medium and then infected with *imm*434 in light medium yielded some viable phage particles containing DNA (fully heavy repressed prophage) predominantly made before infection (Ptashne 1965); the thermal induction of a λ prophage in a lysogenic cell unable to synthesize DNA at the inducing temperature also yielded some infective centers, requiring the formation of a mature virus particle (Fangman and Feiss 1969). Several of the experiments described here included an infection of cells on which plaque formation was virtually abolished: (1) Δ*int-red-gam* phage plated onto *recA*-defective hosts and (2) *ori*λ phage plated onto *dnaB* grpD55 host cells at restrictive temperature. In (1), the absence of plaque formation is explained by the "classical" Red-Rec bypass model (see Smith 1983; Amundsen *et al.* 1986). Linear multimers (concatemers) formed via late stage λ rolling-circle replication are destroyed by the *Exo*V activity of the RecBCD complex in the absence of Gam, and a dimer is not formed by recombination between two monomeric circles in the absence of RecA, resulting in the absence of a molecular substrate with two *cos* sites for λ genome packaging. *recD* null mutants (of $recA^+$ cells) have a hyperrecombination phenotype (Kuzminov 1999). The introduction of a $recD^-$ mutation into a $recA^-$ host restores the ability of Red^- phage to form a plaque on a *recA* host, somehow suppressing the destruction of linear concatemers by *Exo*V (Amundsen *et al.* 1986). Linear multimers are produced by plasmids in *recBC sbcBC* (*Exo*V- and *Exo*I-defective) cells and are formed by the rolling-circle type of plasmid DNA replication dependent upon the RecF pathway genes *recA* (Biek and Cohen 1986), *recF*, *recJ* (Cohen and Clark 1986; Silberstein and Cohen 1987), *recO*, and *recQ* (Kusano *et al.* 1989). Indeed, the link between accelerated concatemer formation and plasmid instability led to the identification of *recD* mutants (Biek and Cohen 1986).

Stahl *et al.* (1997) suggested that λDNA replicated in *recA* mutant cells is a good substrate for annealing, providing DNA ends, presumably as tips of rolling circles. We showed that defects in host recombination genes modulated the accumulation of the onion-skin replication product, suggesting that the recombination proteins are required for either *ori*λ replication or the stability of the accumulated DNA, a topic requiring further analysis. (For example, the product from the double *recD recA* or *recD recF* mutants is greater than that from the three single mutations.) We suggest that the onion-skin product is a target for host nucleases and that some of the DNA copies of this region are broken and degraded. Presumably, the broken ends arising from θ (*ori*λ) replication could stimulate recombination by serving as a substrate for strand invasion or by invading the replicon of the infecting phage. This may help explain why *imm*λ marker rescue increased when the cryptic prophage was able to initiate *ori*λ replication at 39° and reduced when *ori*λ replication was blocked. No *imm*λ recombinants were detected from λΔ*int-red-gam imm*434 $NinR^+$ infections of *recA* or *recA recD* hosts, even though *ori*λ replication should occur from prophage and infecting phage in both hosts, and in the *recA recD* host, linear concatemers should be stabilized. The absence of *imm*λ recombinants for the λΔ*int-red-gam imm*434 $NinR^+$ infection of a *recA recD* host provides strong support for the requirement of RecA for NinR-dependent marker rescue, since neither replication nor the potential to form stable linear concatemers for genome packaging was limiting.

In plating situation (2) above, the absence of plaque formation by an *ori*λ phage on *dnaB*grpD55 host cells at restrictive temperature is explained by an inability of the infecting phage to initiate replication, as observed. Nevertheless, when the *ori*λ infecting phages were $NinR^+$ or Red^+, mature *imm*λ *ori*λ recombinants arose in the lysates of the infected *dnaB*grpD55 cells. We offer two observations that demonstrate that these results are not without precedent:

1. Biophysical studies: McMilin and Russo (1972, p. 55) reported, "under conditions which block λ DNA duplication, unduplicated λ DNA can mature, including molecules which have recombined in the host." Stahl *et al.* (1972) extended this observation and coined the term "free-loader" phage to describe phage produced under replication-blocked conditions, whose synthesis depended upon bacterial and phage recombination systems.
2. Infections at MOI >1: Sclafani and Wechsler (1981) showed that at low MOI (0.1) no λ phage particles were produced in cells lacking a functional

dnaB product, yet at high MOI a significant proportion of the cells can produce phage. M. Horbay, C. Hayes and S. Hayes (unpublished results) found that *ori*λ replication initiation is needed for a phage burst when the MOI of the infecting phage is <1 (*e.g.*, the situation in a plaque assay with many-fold excess cells to phage). But infections introducing two phage genomes per cell yield bursts of up to 30 phage/infected cell following infections into cells where *ori*λ replication is blocked.

From each of these four separate observations, we assume that recombination mechanisms can bypass the need for *ori*λ replication to produce a mature phage. (Motamedi *et al.* 1999 and Kuzminov 1999 discuss postulates for recombination intermediates initiating replication.) The *imm*λ bursts, shown here to depend upon Red or NinR phage functions, suggest that phage-prophage recombination will permit the maturation of a phage particle in a host cell where both the prophage and the infecting phage are blocked for *ori*λ replication initiation. Similarly, plasmids of diverse origin sometimes generate open circular by-products with single-stranded tails, *i.e.*, linear multimers, in cells defective for *Exo*V and *Exo*I (Cohen and Clark 1986; Silberstein and Cohen 1987). The dependence of plasmid linear multimer formation on the functional *recA*, *recF*, and *recJ* genes was interpreted to reflect integrative recombination between tails and plasmid circles operating as an additional mechanism for tail elongation (see Kusano *et al.* 1989). Silberstein *et al.* (1990) examined λ-mediated synthesis of plasmid linear multimers and proposed that (a) concatemer formation involved RecE, RecF, or Red pathway-dependent recombination between DNA double-stranded ends and (b) recombination-dependent priming of DNA synthesis at a 3′ OH end of an external, single-stranded DNA that invades a homologous sequence on a circular template. Recombination-independent models for switching from the early θ-mode to the late σ-mode of λ linear multimer replication include DNA synthesis at a 3′ OH end of a nicked strand and displacement of the 5′-end or the conversion of *ori*λ-dependent bidirectional θ-replication to unidirectional replication accompanied by a break in the lagging strand downstream from the replication fork remaining active.

Clark *et al.* (2001) described seven short boundary sequence intervals, each highly conserved per interval, which divided the metabolic regions of many lambdoid phages into six modules (Campbell and Botstein 1982). They proposed that the boundary sequences were "foci for genetic recombination" in lambdoid phages and served to assort the modules and to increase genetic mosaicism. BLASTP search results (Altshul *et al.* 1997) for the λNinR region reading frames reveal widely shared homologies with other phages and bacterial genomes. We propose that these NinR clusters (KM modules) have a role in stimulating exchanges between the boundary sequences of infecting phages and cellular prophages and thus participate in driving phage evolutionary diversity (Hendrix *et al.* 1999; Kameyama *et al.* 1999; Weisberg *et al.* 1999; Juhala *et al.* 2000; Johansen *et al.* 2001; Brüssow *et al.* 2004).

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