A CI-Independent Form of Replicative Inhibition: Turn Off of Early Replication of Bacteriophage Lambda

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Abstract

Several earlier studies have described an unusual exclusion phenotype exhibited by cells with plasmids carrying a portion of the replication region of phage lambda. Cells exhibiting this inhibition phenotype (IP) prevent the plating of homo-immune and hybrid hetero-immune lambdoid phages. We have attempted to define aspects of IP, and show that IP is directed to repl. phages. IP was observed in cells with plasmids containing a λ DNA fragment including oop, encoding a short OOP micro RNA, and part of the lambda origin of replication, oriL, defined by iteron sequences ITN1-4 and an adjacent high AT-rich sequence. Transcription of the intact oop sequence from its promoter, pD, is required for IP, as are iterons ITN3-4, but not the high AT-rich portion of oriL. The results suggest that IP silencing is directed to theta mode replication initiation from an infecting replλ genome, or an induced replλ prophage. Phage mutations suppressing IP, i.e., Sip, map within, or adjacent to cro or in O, or both. Our results for plasmid based IP suggest the hypothesis that there is a natural mechanism for silencing early theta-mode replication initiation, i.e. the buildup of λ genomes with oopα oriLα-p sequence.

Introduction

Normal cellular immunity to λ infection arises upon the lysogenic conversion of E. coli cells by a λ prophage. The CI repressor protein encoded by the prophage binds to the o2 and oR operator sites, each with three repressor binding sites, e.g., o2α, o2β, o2γ, within the immλ gene cluster pE:oriL-rexB-rexA-cI-pM-cro. CI protein within a λ lysogenic cell blocks transcription of the phage genes situated downstream from the major leftward and rightward phage promoters pR and pE [1], both from the resident prophage, or when a homo-immune immλ phage infects the cells. The variant λvir efficiently forms plaques on cells lysogenized by λ, because it carries point mutations v2 in o2α, v1 in o2β, and v3 in oRγ [2]. Transcription from pR (Fig. 1A) is required for expression of genes cro-ηλ-O-P, respectively encoding a second repressor (Cro) that binds to oRγ an unstable stimulator (CII) of the establishment mode of θ transcription from promoter pE [3]; and the repλ replication initiation cassette including genes O, P, and the origin (oriL) within O site, which participate in oR-dependent bidirectional (theta mode) replication initiation. The gene sof, is transcribed from promoter pD [4] opposite orientation from pR, partially overlaps the terminal end of θL, and encodes a short self-terminating antisense RNA (OOP), opposing CII expression [5]. Part of sof and pD share a 33 bp region of high sequence homology within lambda phages (Fig S1). The organizational similarity within the region encoding the η λ-like-sofα-“oriL”-O-like-P-like genes for lambda phages is shown in Fig S2.

The dual infection of a λ lysogen with two phages, a homo-immune immλ phage and a hybrid hetero-immune λimm434 phage, each of which share an identical replλ replication initiation cassette, revealed that the immλ434 phage predominated by 20-fold over the immλ phage in the cell burst [6]. The impaired replication of the homo-immune immλ phage, described as replicative inhibition, which we consider herein “CI-dependent” was explained by the assumption that CI repressor molecules made by the λ prophage in the co-infected lysogenic cells prevented replication of the homo-immune phage, when the λ replication initiation proteins (gpO and gpP) were provided in trans by the hetero-immune phage. The observations that CI-dependent replicative inhibition was suppressed by mutations in oR causing pD to become insensitive to repression, or by base changes creating new promoter sites downstream from pR, as exemplified by cl7 and four r’ (replication inhibition constitutive) mutations [7], provided support for an argument that transcription from pR (transcriptional activation) was required in ci for theta-mode replication initiation, and that replicative inhibition was explained by CI repressor in the lysogen preventing transcriptional activation of replication initiation from the co-infecting immλ, replλ phage.

Plasmids termed λdv were derived from phage λvir [8,9]. They encode the immλ, and replλ regions and are capable of autonomous replication. Early studies with cells transformed with λdv suggested that the cells acquired an unusual immunity or exclusion phenotype [8,10] and inhibited plating by homo-immune phages, including λvir, and hetero-immune hybrid phages as λimm434. Some other hetero-immune phages (e.g., λimm21 and λimm80) that were presumably replλ were able to escape the inhibition, i.e., could plate efficiently on cells transformed with λdv [8,10]. The ability of cells with λdv plasmids to inhibit λvir development was rationalized by
the suggestion that cells with this plasmid make more CI repressor than would a cell with a single prophage, and the higher levels of repressor would eventually bind the altered vir operators [8]. However, CI levels were not actually measured. No explanation was provided for the inhibition of imm434 development. When RNA transcription levels from cells with dv1 plasmid were measured, it was found that little or no cI transcription was detected, showing that the inhibition of homo-immune infecting phage development by dv plasmid was not due to CI repressor activity. It was proposed [10] that the dv-mediated inhibition of infecting rep prophage development represents a competition for bacterial protein(s) between the plasmid and an infecting phage, and that the site for the competition was different in the imm21 and imm80 phages that escaped the inhibition.

Independently, Rao and Rogers [12] demonstrated that cells containing a pBR322/dv hybrid plasmid that included the imml and repl regions exhibited an inhibition phenotype (referred to herein as “IP”), that prevented the plating of vir and imm434 infecting phage, but allowed imm21 to plate at high EOP. They reported isolating mutants of vir and imm434 which formed plaques at high EOP on cells with the plasmid, but the causative mutations were not further identified. Another inhibition phenotype, termed nonimmune exclusion (NIE) [13], was specific for imml and imm434 phages that were repl. NIE was exhibited by a variety of engineered cells with thermally induced (CI-inactivated) cryptic rep prophage deleted.

Figure 1. Replication-targeted inhibition of rep prophage plating. A. Plasmid cloned λ DNA fragments used to map the sequence requirement(s) for an inhibition phenotype (IP). B. Genomic region spanning five contiguous and partially homologous genes of phages λ and P22 (see Fig. 52). Phage λ is naturally missing the orf48 gene between oop and O that is present between oop and 18 in P22 [37,51]. C. Assay for EOP, defined as phage titer on strain 594 (with one of the indicated plasmids) / titer on 594 cells, where plating on 594 = EOP of 1.0. All of the plasmids shown were derived from pBR322. The oop+ oriλ+ plasmid used was p27. The DNA substitution of the “ice” [16] sequence of λ to make plasmid Δice oop+ oriλ+ (= p50) is shown in Fig. S3A. Numbers in brackets represent standard error values.

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for attL through kil, all genes rightward of P, and had acquired mutations inactivating P [14]. Seven independent λ se (suppress exclusion) mutations of λ wt (wild type) were isolated from NIE phenotype cells having a cro27 mutation in the cryptic prophage. The se defects were point mutations within oop2 (se100a, identical to mutation v1; and se101b) and within phenotypes, IP, by constructing plasmids with portions of Plasmid-mediated Inhibition Phenotype (IP)

Table 1. E. coli K12 and Bacteriophage λ Strains.

| Bacteria and phages | Relevant Genotype | Hayes lab # and source |
|---------------------|-------------------|------------------------|
| S94 [70] (presumably nR594[71]) | Sup+ cells; F− lac–335 galK2 galT22 rplL179 iN(rmD–rmE)1 | B10 [70]; Bachmann [71] |
| W3350 [72] (W3350A ) | Sup+ cells; F− lac–335 galK2 galT22 iN(rmD–rmE)1 | B12, Campbell & Balbinder, 1958, cited in [72]; Bachmann [71] |
| W3350 dnaAI-GrpD55 | dnaAI-GrpD55 mal/F089:Tn10 Tet^{8} | nB15; Bull & Hayes [36] |
| TC600 | thrL leuU6 fhuA21 lacY1 glnV44 el4 gfpR200 thi1supE | B8; Bachmann [71] |
| Y836 | Strain with cryptic λ (Ts)857 prophage ^ | Y836; Hayes and Hayes [13], derived from strain SA431 [73] |
| S94(λcR857)^b | imm1, c(Ts)857 rep1 prophage | nF1016; this work |
| S94(λcR857(18,12)P22)^b | imm1, c(Ts)857 repP22 prophage | nY1111; this work |
| λcR857 | c(Ts)857 rep1 | 1002; Hayes [49] |
| λcii72 | cI^ rep1 | 999; Hayes [49] |
| λcR857(18,12)P22 | imm1, c(Ts)857 repP22 = λ hy106 | 998; Hayes & Hayes [13] |
| λcII act II | 326-bp deletion of cII in λ c^- | 992; L. Thomason [50] |
| λcII papa | (= wild type cI^) | 241; Hayes & Hayes [13] |
| λc9017 | cI^, 9-bp duplication at 38341. [2] | 1006; Hayes & Hayes [13] |
| λse100a | orf 37796, GC->TA, CI^- phenotype | 1003; Hayes & Hayes [13] |
| λse101b | orf 37985, CG->AT, CI^- phenotype | 1004; Hayes & Hayes [13] |
| λse109b | orf 38009, CG->AT, CI^- phenotype | 1005; Hayes & Hayes [13] |
| λimm34 cl#5 | imm34 cl | 957, Hayes et al.[14] |
| λimm34 Anii5 | deletion NiniR recombination functions ^ | 969; Hayes et al. [18] |
| λbio275 imm343 | deletion of NiliN recombination functions ^ | 958; Hayes et al. [18] |
| λbio275 imm34 Anii5 | deletion of NiliN and NiniR functions | 952; Hayes et al. [18] |

The λ prophage genes int-xis-exo-bet-gam-ki1 in strain Y836 were substituted with bio275 [13]. The strain carries the chromosomal deletion Δ31[33] that removes genes rightward from ninB in prophage through mooA in host, including prophage genes orf146 (orf) – J22 i.e., all the late genes required for cell lysis and phage morphogenesis. A map of the cryptic lambda prophage in strain Y836 is drawn in Fig. 4A.

The NiniR region deleted by Δnin1 removes λ bases 40,503–43,307, i.e., ren-nin1 – nin1 (including orf–ninC and rap-ninH); the NiliN region substituted by bio275 replaces genes int-xis-hin-exo-bet-gam-ki1, representing λ bases 27,731–33,303 [18].

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Results

Plasmid-mediated Inhibition Phenotype (IP)

The bacterial strains, phage, plasmids and primers for modifying plasmids are described in Tables 1, 2, 3. Plasmid pCH1, theoretically identical to the IP plasmid described by Rao and Rogers [12], and deletion derivatives as p25 and others (Table 2, Figs 1A) were made to determine which λ sequences were responsible for IP. Plasmids pCH1 and p25 inhibited the phage burst following thermal induction of a prophage was compared to replicative inhibition than phage was eliminated. We have shown that CI-independent, vir point mutations in λ vir was eliminated. We have shown that CI-independent, rep[857] prophage a Y836; Hayes and Hayes [13], derived from strain SA431 [73].

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plasmid (Fig. 2B); but, when the plasmid was altered by changing the -10 region for rep, or removing the ω-oo-p-ω, or ω-λ, regions, no inhibition of repl prophage development was observed, in agreement with the plating results in Fig. 1C.

We examined if a cloned intact 1450 bp Bgl II DNA fragment with KanR (derived from Tn903) within pUC4K was inserted within the remaining Bgl II sites.

Initially 45 random bases were chosen, but then some bases were modified to remove the possibility for secondary structure (hairpin) formation.

Table 2. Plasmids.

| Plasmid | λ bases | Bases from pBR322 |
|---------|---------|-------------------|
| pCH1b   | 34500–41731 | 1–375, 376–4361 |
| p25b    | 34500–39354 | 1–187, 376–4361 |
| p26b    | 38215–39354 | 1–187, 376–4361 |
| p27b    | 38215–39168b | 1–187, 376–4361 |
| p27Rb   | 38359–39168b | 1–187, 376–4361 |
| p28b    | 38815–39354 | 1–187, 376–4361 |
| p29b    | 38215–38835 | 1–187, 376–4361 |
| p50b    | 38568–39168b | 188–4359 |
| p51b    | 38568–38759, 38820–39168c | 188–4359 |
| p51kan  | 38568–38759, 38814–39168d | 188–4359 |
| p52b    | 38568–38759, 38814–39168d | 188–4359 |
| p27Rpo1– | 38359–38683, 38689–39168, bases 38684–38688 (ATTAT) replaced with GCGCG | 2297–4359 |
| p27R-R450OOP | 38359–38629, 38675–39168, bases 38630–38674 substituted | 2297–4359 |
| p27RAAT | 38359–39127 | 2297–4359 |
| p27RAITN1–4 | 38359–39043, 39120–39168b | 2297–4359 |
| p27RAITN3–4 | 38359–39077, 39120–39168b | 2297–4359 |
| pCIP-O-timm | modified 35799–35824, 37203–38036, 38686–39582 | 1–3, 651–4361 |
| P434pRP-O-timm | modified 35799–35824, 37203–37464, 38686–39582 | 1–3, 651–4361 |

*All plasmids were prepared in this laboratory. Described in [11], some illustrated in Fig. 1A. Described in [37,69], illustrated in Figures 1, S3.

Table 3. Primers used for plasmid modification.

| Plasmid | Unique Primersa | Sequence |
|---------|-----------------|----------|
| p27RAAT | Lpo1 | 5’-CACACGGCATATGGTTCGACAAAC |
| p27RAAT | RAAT1 | 5’-AAGAATTCCTTGGTCTCCTTCTT |
| p27Rpo1– | Rpo2 | 5’-TCTGTGATTTGGTTACGCCGAGCATTG |
| p27Rpo1– | Rpo3 | 5’-TCACGAGATCTTGGCGCAATATACAGCA |
| p27Rpo1– | Rpo4 | 5’-AAGAATTCCTTGGACAAATATCT |
| p27R-R450OOP | LROOP3 | 5’-GGTCTGACAAAGCTCTTGGTTCGACAAAC |
| p27R-R450OOP | RROOP2 | 5’-CTTCTGACTATATTCTGCTCTTTT |
| p27RAITN1–4 | LAITN1–4 | 5’-AAAGACGCAATATGTCGACAAATATACAGCA |
| p27RAITN1–4 | RAITN1–4 | 5’-TCTGTGATTTGGTTACGCCGAGCATTG |
| p27RAITN3–4 | LAITN3–4 | 5’-CGTCTGACAAAGCTCTTGGTTCGACAAAC |
| p27RAITN3–4 | RAITN3–4 | 5’-TCTGTGACAAAGCTCTTGGTTCGACAAAC |

*a and R primer sequences are from the lambda l-strand (coding strand for cl) and r-strand (coding strand for cl and oop) sequences, respectively. doi:10.1371/journal.pone.0036498.t003
To distinguish whether the transcription of the downstream oop sequence, or just transcription initiation from the p90 promoter was required for IP, the coding sequence of oop was modified in plasmid p27R-R45OOP (Fig. 3C). Nucleotides 2–46 of oop were replaced with a randomly chosen sequence, edited to remove internal secondary structure formation. For maintaining the self-terminating stem-loop structure of oop, the distal 31 nucleotides of oop were retained, as was the first base pair of the oop sequence, corresponding to 5' pppG of OOP RNA. p27R-R45OOP was unable to serve as an antisense RNA to inactivate OII and it was defective for IP (Fig. 3D, columns 1–3). The results with plasmids p27R<sub>pR<sup>-</sup></sub> and p27R-R45OOP suggest that transcription of the intact oop sequence is required for IP, rather than just transcription initiation from p90.

The aor<sub>l</sub> sequence comprises bases 39034–39160 within gene O (Fig. 3B), with four 18 bp iteron (ITN1–4) sequences joined to a 38 bp high AT-rich sequence. For maintaining the self-terminating stem-loop structure of oop, the distal 31 nucleotides of oop were retained, as was the first base pair of the oop sequence, corresponding to 5' pppG of OOP RNA. p27R-R45OOP was unable to serve as an antisense RNA to inactivate OII and it was defective for IP (Fig. 3D, columns 1–3). The results with plasmids p27R<sub>pR<sup>-</sup></sub> and p27R-R45OOP suggest that transcription of the intact oop sequence is required for IP, rather than just transcription initiation from p90.

### Table 4. Averaged EOP on host cells +/- plasmids with cloned O gene<sup>a</sup>

| Phage    | 594 | 594(pclpR-O-timm)<sup>b</sup> | 594[p434(pR-O-timm)]<sup>b</sup> |
|----------|-----|-----------------------------|----------------------------------|
|          | 30<sup>d</sup> | 39<sup>d</sup> | 42<sup>d</sup> | 30<sup>e</sup> | 42<sup>e</sup> |
| 3978     | 0.75 | 0.74 | 1.0   | 1.0   | 1.0   |
| 3979     | 0.75 | 0.74 | 1.0   | 1.0   | 1.0   |
| 3947     | 0.75 | 0.74 | 1.0   | 1.0   | 1.0   |

<sup>a</sup>The average EOP per indicated phage was relative to that phage plating on strain 594 cells. The standard errors were all < 0.05.

<sup>b</sup>The precise O sequence (ATG = 38686–39582 plus TAA stop codon that replaces normal TGA stop at end of O) was cloned to make plasmids pclpR-O-timm and p434(pR-O-timm). In each plasmid, gene O occupies the position corresponding to λ gene cro (in phage) and the consensus Shine Dalgarno sequence for cro was maintained ahead of O in pclpR-O-timm (75,76). In p434(pR-O-timm), the SD differed by one bp compared to the SD in pclpR-O-timm because of the slightly different sequence ahead of O in imm434 DNA (77). The O gene within pclpR-O-timm is transcribed from p90 and regulated by cII(T5)857 repressor: at 30° O is repressed, at 39° and 42° O is expressed, or fully expressed. Gene O is constitutively expressed from p90 in p434(pR-O-timm).

<sup>c</sup>The column for plating at 30°, 39° and 42° yielded equivalent phase titers on 594 and the EOP was set to 1. Plaques ranged between 0.5–2 mm in diameter.

<sup>d</sup>Plaques formed were tiny.

<sup>e</sup>Plaques ranged from 0.3–1 mm diameter.

<sup>f</sup>nd is not done, since equivalent results were expected as seen for λcII72.

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**Figure 2. Thermal Induction of rep<sub>L</sub> or the rep<sub>P22</sub> -hybrid λ.cl857 prophages.** Lysogenic cultures of strain 594 were grown at 30° and each prophage was thermally induced by shifting the culture from 30° to 42° at time 0. A. Thermally induced rep<sub>L</sub> prophage. B. Thermally induced rep<sub>L</sub> prophage. The results represent the averages for 2 independent assays. Plasmids within lysogenic cells: square, Po<sup>+</sup> oop<sup>+</sup> ori<sup>L</sup>; results shown for p27R, but identical results were observed for p27T; triangle, Po<sup>+</sup> oop<sup>−</sup> ori<sup>L</sup>; inverted triangle, Δ(to-oop-Po)α<sup>L</sup> (ITN-AT); diamond, cI-oop-Po<sup>−</sup> Δori<sup>L</sup>; circle, none (no plasmid). The standard deviation is shown for all of the data points, but is too small for visualization in some data intervals.

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**Dissecting IP sequence requirement(s)**

The spacing interval between the to-op-p90 sequence and ori<sub>L</sub> in p50 was modified by deletion or insertion (Fig. S3D) to learn if the spatial orientation between these two regions was important for IP. All the modified versions of p50, i.e., p51, p51kan, and p52, retained IP (Fig. S3C–D). We asked if transcription of oop from p90 participated in IP by inactivating the -10 region of p90, replacing the sequence ATGAT by GGGCGG in p27R to stringently assess a requirement for oop expression from a high copy ori<sub>L</sub> plasmid. The resulting plasmid, p27R<sub>pR<sup>-</sup></sub> (Fig. 3C), no longer expressed oop, as determined by the OOP antisense phenotype/cII inactivation assay (see Materials and Methods) and was defective for IP (Fig. 3D), suggesting that transcription from p90 is essential for IP.
Figure 3. Replication silencing of repλ phages requires oop, and iterons (ITN) from oriλ. A. The non-excisable cryptic λ fragment (short arrow) inserted within the E. coli chromosome in strain Y836 [13,35] remains repressed at 30°C where the prophage repressor is active. Shifting cells to about 39°C inactivates the CI857 repressor that prevents prophage transcription and replication initiation from oriλ. Multiple bidirectional replication initiation events from oriλ generate the onion-skin replication structure drawn at right. B. Map showing oop-oriλ region. The DNA sequence for oriλ, shown as a rectangle around ITN-AT within gene O has four repeated 18 bp iteron sequences (ITN1 to ITN4), each separated by a 38 bp high AT-rich sequence. The genes cII and O are each shown truncated and are transcribed rightward from pR. The
preferred template for packaging λ DNA into phage heads. The sigma mode arises about 15 min after phage infection of cells [29–32]. Skalka et al. [31] stated that replication via the “early mode occurs only once or twice, after which rolling circle [late] replication predominates.” They suggested that a direct, internal origin of RK occurs only once or twice, after which rolling circle (late) replication predominates. The upstream control gene for the turn-off of early replication either “does not exist”, or “must not be expressed in the absence of replication” because early replication products accumulate (after infection or induction) when concatemer formation is destabilized in λ gam mutants, or under fer+ conditions (including both λ red and host recA mutations). The chromosome in strain Y836 (Table 1; Fig. 4A) has an engineered cryptic λ prophage deleted for recombination genes int-uvs-exo-bet-gam-kil involved in general and site specific recombination [13] and for genes oriH+ (≡ off) – Jh2, including genes required for cell lysis and phage morphogenesis [33], but it encodes the HimM and repl2 regions. Transcription of O–P from pR is prevented at 30°C by the ε[Jts]657 encoded temperature sensitive repressor. Inactivating the CI repressor, by shifting cells grown at 30°C to 42°C, triggers oril-dependent bi-directional replication initiation from the trapped λ fragment. Initiated replication forks escape leftward and rightward beyond the λ fragment and into the E. coli chromosome. This event is lethal to the cell and was termed Replicative-Killing [7], i.e., RK phenotype [18,34]. Survivor cells that escape Replicative-Killing (RK+ mutants) arise within the RK+ starting cells and were found to possess mutations that prevented replication initiation from oril [13,14,33–35]. Transducing a dnaB mutation (GrpD55) that prevents λ replication initiation (but not E. coli DNA synthesis) into the RK+ Y836 cells can fully suppress Replicative-Killing without interfering with gene expression from the induced λ fragment [19]. We examined whether plasmids exhibiting the IP phenotype could suppress Replicative-Killing (Fig. 3D, rightward columns e–g). The viability of RK+ Y836 cells shifted from 30°C to 42°C was <0.00001. Similar results were seen when Y836 was transformed with p27R+R45OOP, p27RAITN1–4, or to a lesser extent with p27R/Po+, indicating that these three plasmids do not suppress the RK+ phenotype. Cells transformed with plasmids p27R and p27R4AT suppressed Replicative-Killing at 42°C, suggesting that they interfered with (silenced) theta-mode replication initiation from the chromosomal λ fragment.

We examined if the IP-plasmids could block replication initiation from a thermally induced ε[Jts]657 λ fragment within the Y836 chromosome. Replication initiation arising from the oril region of the induced cryptic prophage was assessed by probing for a 1774 bp NdeI fragment (Fig. 4A–C) following NdeI digestion of the Y836 cell chromosome. The probe to the NdeI fragment overlapped with each of the λ fragments in the plasmids introduced into Y836, permitting an internal measure of plasmid copy increase. Theta-mode replication initiation increased by about 3-fold from oril when Y836 cells without a plasmid were shifted from 30°C to 42°C (Fig. 4C). The plasmid p27R+plasmid p27R solely inhibited theta mode replication initiation, in full agreement with the data showing that this plasmid blocked Replicative-killing (Fig. 3C). Cells with p27RAITN1–4, with a deletion of the four iterons (but not the AT-rich region) was not inhibitory; whereas, the converse plasmid p27RAAT, modified to remove the high AT-rich sequence but containing ITN1–4, was fully inhibitory to theta-mode replication initiation from the prophage oril site. The intensity of the replication increase was not as robust as previously seen [Fig. 2 in [18]] where the probe was larger and could detect two λ prophage restriction fragments (i.e., 3675 bp oril band, and a 4250 bp band showing escape replication), possibly because of the high level of competition for the probe by the λ DNA within the plasmids. Two of the 1774 bp bands at 42°C for cells where oril replication initiation was inhibited decreased slightly compared to their 30°C counterparts. This may represent some level of DNA extraction variation, or it could be real and represent fragment destruction resulting from abortive oril replication initiation from the induced prophage in these strains.

Escape from IP

We previously showed [18] that marker rescue for HimM recombinants was below the level of detection for Y836 dnaB- GrpD55 host cells infected with himM434 plage deleted for λ genome regions NinL (int-red-gam recombination functions) and NinR [ren-ninA-ninK, including Orf and Rapi] (Table 1 in [18]). The same result was seen for Y836 recA host cells infected with himM434 versions of NinR ΔNinL and ΔNinR ΔNinL-phages (Table 2, lines 2–3 in [18]). The GrpD55 locus was suggested linked to dnaB [36], and Horbay [37] subsequently determined by sequence analysis that it represented two missense mutations within dnaB.
The dnaB-GrpD55 mutation confers a temperature sensitive phenotype for λ, plating but does not prevent E. coli replication, cell growth [36]. The EOP of λ on strain W3350 dnaB-GrpD55 was significantly reduced compared to W3350 (EOP set = 1.0). The respective EOP’s at 30°, 40° or 42° on the dnaB-GrpD55 host were 0.08, 0.01, <0.0001 (for λE:B57); 0.2, 0.002, <0.0001 (for λimm4344;ΔI) and 0.04, 0.04, <0.0001 (for λimm4344ΔNinR), showing increasing temperature sensitivity for λ, replication, while the E. coli dnaB-GrpD55 host was able to form effective cell lawns at the elevated temperatures. We define “free-loader” coefficient, as a measure of phage progeny for infections at multiplicity of infection (MOI) 5, per the phage progeny from infections at MOI 0.01 (see Discussion). The availability of λ, recombination functions within an infected cell can influence the free-loader coefficient. W3350 dnaB-GrpD55 recA4 cells were infected at MOI of 5 or 0.01 with λ, deleted for the NinL, NinR, or both recombination regions, then incubated for 90 minutes at 42° and plated for phage burst. Infections with phages λimm4344NinL+λNinR, λimm4344NinRΔ-NinL, λimm4344ΔNinR NinL+, and λimm434 ΔNinLANinR yielded respective coefficients of 1065 (+/− 18 std. error), 502 (+/− 31), 156 (+/− 10), and 111 (+/− 27), suggesting that the λ, NinR and NinL recombination functions can influence phage burst from multiply infected cells where the infecting phages are blocked for theta-mode oriλ-dependent replication initiation by the dnaB-GrpD55 mutation. This result supports our prior suggestion [18] that ori-specific theta-mode replication initiation, dependent upon P-DnaB interaction, can be bypassed in multiply infected cells, i.e., phage replication can likely be driven by intermediates derived via homologous recombination between co-infecting phage genomes.

The results from Fig’s 1, 2, 3 and 4 suggest that IP serves to block / silence replication initiation from oriλ. We examined whether IP could be bypassed, comparing the bursts from singly infected (low MOI, 0.01), or multiply infected (high MOI, 5) cells (Table 5). Infections of wild type host strains W3350 and 594 at either MOI’s of 5 or 0.01 with recI, or recP22 phages produced essentially equivalent bursts. A similar result was seen for recP22 phage infections of W3350 dnaB-GrpD55 cells at either MOI 5 or
0.1. There was essentially no burst (background level) when the repEl phage infected W3350 dnaB-GrpD55 cells at an MOI of 0.01; however, the phage burst was equivalent to that on the W3350 cells when the W3350 dnaB-GrpD55 cells were multiply infected at an MOI of 5. Thus, while the altered DnaB protein [GrpD55 allele] interferes with the P-DnaB interaction required for theta-mode λ replication initiation, it can still apparently drive λ or E. coli DNA synthesis that is independent of P. Placing multiple copies of a recombination proficient λ genome within a cell appears to bypass the P-DnaB interaction at oriL, required for the theta-mode of λ replication initiation. Similarly, 594 cells with plasmid p27R (oop* opl*) prevented phage burst from cells infected at MOI 0.01. But when these same cells were infected at MOI 5, IP was suppressed (suppressed). 594 cells with p27R p27, which is defective for IP, yielded an essentially similar repEl phage burst at MOI 0.01 as when 594 cells without the plasmid were infected. These results suggest that IP serves to silence / inhibit theta-mode oriL replication initiation and that multiple copies of recombination-proficient λ genomes can, at some level, bypass this essential requirement for replication initiation from a single prophage or from one infecting λ genome.

Suppression of Inhibition Phenotype (Sip) by λ mutants and hybrids

We looked for a target of IP by i) characterizing 10 independent (Sip) mutants of λcI857B (Fig. 5) and ii) by screening for IP-escape, testing λ mutants and hybrid phages (Fig's S4, S5). We first asked if insertion by homologous recombination (of the AmpR oop* opl* plasmid into the infecting phage) was responsible for Sip (Fig. S6 and Supplemental Methods S1), and eliminated this possibility. The cl - P regions were sequenced for 10 independent Sip phage isolates, and for λcI857B cro27 with a null mutation in cro, Fig. S5 [11,28,38,40]. Three sip mutations, Sip 1, 2, 7 arose at two sites in O to the left of the ITN sequences, of which mutations Sip 2 and 7 introduced different changes in the same codon by altering position 38922. Five other Sip mutations (3, 6, 7, 8, 9, and 10) introduced missense changes within cro. Another Sip mutation (Sip 4) altered the ribosomal binding (SD) site for cro and another (Sip 5) changed the base preceding the AUG for cro. One of the sip phage (Sip2) was mutated in both cro and O. By conventional logic, the Sip mutations in cro might function by reducing Cro down regulation of pL and thus increase O expression, or the Sip mutations in O increase O expression or activity.

Alternatively, several of the Sip mutants conferred missense mutations in an 81 codon open reading frame, PreX; these included five sip mutations (of which Sip6 eliminated the PreX start codon); plus the “se” mutations (described above) introduced missense changes into PreX (Fig. S7). PreX can only be expressed via high level establishment mode pL[pRE-cI-rexA-rexB mRNA synthesis (i.e., 20–100X level of pM-cI transcription [28,39,40]), requiring CI activation at pL [3]. The pL-cI transcript is antisense to cro, and the possible PreX reading frame from it would overlap 15 codons at the N-terminal end of cl, all of ORF/pR region, and 35 codons of cro, and would be expressed from the same reading frame as cro, but the opposite coding strand (Fig. S7).

Since six of the λcI857B-derived Sip mutants produced five missense changes in cro (two independent Sip mutations, 8 and 10, each changed base pair 31813 in cro), we examined if any Sip mutants exhibited the λcI857B cro27 plating phenotype. Phage λcI857B cro27 has the interesting property of forming plaques at 37–39°, but not at 30° or 42° [38,40–42], and of exhibiting a phenotype within infected cells termed Cro lethality [See [40] for a discussion of Cro lethality concept relative to rexA-rexB expression, translational frameshift sites within [43], and possible effect upon [14] high levels of pL[pRE-cI-rexA-rexB expression (Fig. S7A,B) from an induced or-defective λ lysisogen or infecting phage.) Our isolate of λcI857B cro27 carried a single G-A transition (Arg to Gln) at base 30153 in cro (Fig. 5), nullifying cro activity. Only the Sip7 phage shared a nearly similar plating phenotype with λcI857B cro27 by forming faint plaques at an EOP of <10-3 at 30°, tiny-faint plaques at EOP 0.5 at 42°, and 1 mm clear plaques at 37 and 39°. Sip phages 1–6 and 8–10 formed 0.5–1.0 mm turbid plaques on 594 host cells at 30°, and about 1 mm clear plaques at 37°. Only the Sip 4 and 8 phages plated with slightly reduced EOP, i.e., by 3 or 13-fold, at 30° compared to 37°. Alternatively, we asked if λcI857B cro27 can escape IP, i.e., if it shares properties with the λcI857B Sip phages, and found that the cro27 allele did not confer a Sip phenotype (Table S1). Thus, simply inactivating Cro does not directly confer a Sip phenotype, and so the Sip mutations must have another effect.

The inability of the repEl phage λcI857 to escape IP was not modulated by the CI repressor, reflected by equally IP-sensitive repEl phages λwt (cl†), and phenotypically CI† (lysogenization-defective) phages: λcI72 (cl‡), and by phages with CI-defective phenotype that escape replicative inhibition, i.e., λeR/pR point mutations (λse mutants: 100a, 101b, and 109b [Table 1, Fig. S7C]), and λcI90c17 (Table 1), where pR-independent transcription [44,45] arises via the c17 insertion downstream from pR. The repEl phages λeR, λin121cI and λinm434cI partially escaped IP, plating with EOP’s of 0.1 or higher (Fig. S4A), but their plaque sizes were reduced. The sequence of λinm434cI was identical to λ throughout the cl-O interval (Fig. S5). cI is mutated in both oop and opl at bases 37979 and 38007 [2,34], respectively, although, it is unclear what other mutations it possesses. The λinm21 hybrid had base alterations within the cl-oop overlap (Fig. S5) and a silent TGC to TGT codon change at 39,035 (not shown), one base left of the ITN1 sequence in O.

Plaque size is a qualitative measure of phage development or burst, and we previously found that impeding λ replication significantly reduced normal plaque size [18]. Thorough examination revealed that the plaques formed by λinm434cI on 594[oop* opl*] cells were barely visible, i.e., 5% of their normal diameter on 594 host cells (Fig.S4C) and λinm21cI plaques were 35% their normal diameter. Plaques formed on 594[arp*] cells by the repEl phages (Fig. S4C) were reduced in plaque diameter by

**Table 5. opl*-dependent DNA replication inhibition is bypassed in multiply infected cells.**

| Host Strain | Burst of infecting phage per cell at indicated MOI a | λcI857B [repEl] | λcI857B/(6,13)P22 [repF22] |
|-------------|-----------------------------------------------------|-----------------|-----------------------------|
| λcI857B [repEl] | W3350 MOI 0.01 | 35.5±/–0.3 | 31.0±/–0.7 | 13.8+/–1.4 | 11.3+/–1.1 | 3.4+/–1.4 |
| λcI857B [repEl] | W3350 MOI 0.01 | 31.6+/–13.1 | 1.3+/–0.7 | 19.2+/–7.3 | 17.1+/–3.2 |
| λcI857B [repEl] | dnaBGrpD55 | 25.8+/–0.3 | 2.3+/–0.8 | 16.4+/–3.8 | 9.4+/–0.8 |
| λcI857B [repEl] | 594[pBR322] | 22.4+/–1.4 | 26.0+/–1.23 | 9.0+/–1.0 | 5.7+/–0.1 |
| λcI857B [repEl] | 594[arp*] | 21.1+/–8.2 | 1.1+/–0.7 | 6.8+/–0.6 | 6.7+/–0.7 |

aBurst at 110 min after infecting cells. The results are expressed as phage burst (phage particles released per infective center) +/- standard error. Each value represents the average of ≥2 separate trials.

bSee [78] for host requirements for growth of λ-P22 hybrid.
about half, in agreement with the observations that oop \( ^{+} \) plasmids partially interfere with phage maturation.

To help ascertain why the \( \nu_p \) phages \( \lambda \text{imm}34\text{eI} \), and to a greater extent \( \lambda \text{imm}21\text{I} \), partially escaped IP, their oop- orp regions were sequenced (Fig. S5). While phage 434 has three base changes within the oop sequence, the \( \lambda \text{imm}34\text{eI} \) hybrid sequence was equivalent to \( \lambda \). The \( \lambda \text{imm}21\text{I} \) hybrid shared the same sequence as phage 21, with an expected altered sequence within \( \text{cll} \) left of oop, and differences within the oop / cll overlap region (Fig. S1, S3). The \( \lambda / \text{P22} \) hybrid, i.e., \( \lambda \text{cI}\text{Ts}[57]/\lambda / \text{P22} \) that was insensitive to IP, carried the \( \lambda \) version of cll, yet differed by one base (37673) within oop, by one base (36689) just right of the common -10 sequence (ATTAGG) for the oop promoter, \( \rho_o \), and completely diverged rightward from the \( \lambda \) sequence at base -19 (38694) within \( \rho_o \), so that the -35 region’s for \( \rho_o \) promoters for \( \lambda \) and for \( \lambda / \text{P22} \) hybrid were distinct (Fig.S5) as were downstream \( \lambda \) genes O-P [2] and P22 genes oopA-18–12 [46] (Fig. S2).

All of the \( \nu_p \) phages formed plaques with \( \approx 120\% \) larger diameters on 594[oop\( ^{+} \)] vs. 594 cells (Fig. S4C), suggesting that OOP RNA can stimulate \( \nu_p \) lytic growth. The C-terminal 53 nt including the stop codon for gene cll overlap the 3′-end of oop (Fig. S1). The last 17 amino acids of cll are not required for CII activity, but this region is necessary for CII regulation by OOP [5].

The infection of \( \nu_p \)-\( \lambda \) phages into cells with plasmids expressing OOP micro RNA, which is antisense to cll [47] (Fig. 2), creates a cll-defective phenotype [40] resulting in clear plaques at 30° even for the hybrid \( \lambda / \text{P22} \) gene cll21 gave turbid plaques on 594, but clear on 594[oop\( ^{+} \)] host cells, suggesting that the five base changes within the oop / cll overlap region do not prevent OOP RNA (made from oop\( ^{+} \) plasmid) from serving as an antisense RNA to cll expression from imm21. Clearly, infecting cll\( ^{+} \) phages into cells expressing OOP RNA creates a phenotypic cll-defective condition, characterized by no \( \rho_p \text{recX-cll-rxsA-recI} \) transcription, no cro antisense RNA, and lytic phage growth. Thus, we did not consider it relevant to evaluate independent missense cll\( ^{+} \) phages, all of which map left of the cll/opp overlap [3]. In hundreds of cro\( ^{+} \)cll\( ^{+} \) prophage induction experiments, for example [4,28,39,40,49], no l-strand transcription attributable to \( \rho_p \) was ever detected (Hayes lab results). This result, coupled with our current understanding of the role of OOP as an antisense regulator of cll expression, suggests that the synthesis of OOP RNA under the conditions described herein will prevent \( \rho_p \) transcription from infecting phage or induced prophage. But, an OOP block to \( \rho_p \) transcription is insufficient on its own to explain CI-independent IP, i.e., oop\( ^{+} \) \( \Delta \nu_p \) plasmids were defective in IP.

We examined the IP-sensitivity of a phage deleted for cll-oop. The interval between AUG for cll and second codon for \( O \) in phage \( \lambda / \text{imm}34\text{eI} \) (\( \equiv \text{opp} \)) [50] was deleted (i.e., \( \lambda \) bp 38363–38688; we confirmed by sequencing two isolates). The deletion fused the retained -35 region of the oop promoter, \( \rho_o \) (leftward from bp –14 at 38609), with the sequence left of the second codon for cll (bp 38362), changing the -10 region for \( \rho_{o} \) from ATTAGG to CATATG, which might still support \( \rho_{o} \)-dependent leftward transcription. The \( \lambda / \text{imm}34\text{eI} \) phage partially escaped IP, forming pinprick-ghost plaques (impractical to quantitate/measure) on 594[oop\( ^{+} \text{-imm}^{+} \)] cell lawns; yet it was capable of forming large clear plaques at EOP of 1 on 594 and 594[oop\( ^{+} \)] cells. Further analysis is needed to explain the paradox that \( \nu_p \) phages retaining the cll-oop region are sensitive to IP (requiring OOP and \( \nu_p \)) yet their development is not curtailed by the presence of competing \( \nu_p \) plasmids; whereas, deleting cll-oop has the opposite effect.

### Discussion

**Replicative inhibition**

We previously showed that the hybrid phage \( \lambda / \text{P22} \) [18,12]P22, with the \( \nu_p \) region swapped by \( \nu_p \)P22, was extremely sensitive to CI-dependent replicative inhibition, and by comparison, \( \lambda / \text{P22} \), the \( \lambda \) se mutants, and \( \lambda / \text{b90c17} \) were respectively 4.6, 27–76, and
173 fold less sensitive [13]. This result illustrates that CI-dependent replicative inhibition does not directly target the \( \sigma\) region, but rather, transcriptional activation of \( \sigma\). In contrast, the \( \text{repP22} \) phage escaped CI-independent replicative inhibition; whereas, the \( \text{repH} \) phages as \( \lambda CI \), the \( \sigma \) se mutants, and \( \lambda P90 \) c17 were fully sensitive. Therefore, we would assert that the CI-dependent (blocking transcriptional activation of the \( \sigma\) region) and the CI-independent (IP directed theta mode replication silencing) forms of replicative inhibition are completely distinct, and that their mechanisms are likely different, even if they share the same end result.

### Requirement for IP

We have provided additional understanding of the observation, termed here IP (Inhibition Phenotype), whereby host cells with plasmids containing the \( oop\)-ori\( \lambda \) region of the lambda genome inhibited plating. This region includes several cis-acting target sites, for example, the iteron sequences, ITN1-4, bounded by \( O\) protein and sites for promoter, \( pO\), and terminator, \( tO\), for the 77nt OOP micro RNA (Fig. 5S) [51]. In summary: i) Plasmids containing the \( \lambda, \text{Oop}-\text{pO}\) through \( \text{ori}\( \lambda \) DNA sequence inhibited the development of \( \text{rep}\)\( \lambda \) infecting, or an induced \( \lambda CB57 \) prophage, and neither the \( oop\) nor \( \text{ori}\( \lambda \) regions, separately, could account for IP. ii) IP was independent of the activity of \( \lambda\) repressors CI and Cro. iii) A \( \lambda CB57\) hybrid with \( \text{rep}\)\( \lambda \) was insensitive to plasmids containing the \( \lambda, \text{Oop}-\text{pO}\)\( \lambda \), and \( \text{ori}\( \lambda \) DNA sequences, suggesting that IP is directed to a \( \text{rep}\)\( \lambda \) function. iv) Sequence analysis revealed that the \( \text{rep}\)\( \lambda \) hybrid contained \( \text{imu}\( \lambda \)\), an essentially intact (one base change) \( oop\) sequence, a hybrid \( pO\) promoter with a \( \lambda\) -10 region and P22 -35 region, and the substitution of \( \lambda\) genes \( O-P\) with P22 genes \( \text{orf}48-18-12\) [37,51]. v) OOP RNA synthesis from the \( oop\) plasmids channeled both the \( \lambda\)/P22 and \( \text{imm}\)\( \lambda\) plages into a lytic mode to form clear plaques, suggesting the level of OOP RNA made was sufficient to serve as an antisense regulator of \( \text{I}\) expression of \( \text{I}\) function(s). [5,47]. vi) A dissection of the contributions to IP revealed that an \( oop\) plasmid deleted for the AT rich region of \( \text{ori}\( \lambda \)\) was fully functional for IP, \( oop\) plasmids deleted for ITN1-4 or ITN3-4 were defective for IP, and \( \text{ori}\( \lambda \)\)-containing plasmids substituted for 45bp within \( oop\), or inactivating the \( pO\) promoter for \( \text{ori}\( \lambda \)\) transcription, were defective for IP.

### Phage escape from IP

In summary: i) Two types of full escape from \( oop\) \( \text{ori}\( \lambda \)\) plasmid-dependent IP were observed: i substitution of \( O-P\) in \( \lambda\) by \( \text{orf}18-10-12\) in the \( \text{rep}\)\( \lambda \) hybrid (Fig. 5S) enabled the hybrid to escape IP, even though its \( \text{I}\) expression was inactivated by OOP RNA; and ii) Sip mutations within or near cro or in \( O\) suppressed IP. 2) Some \( \text{rep}\)\( \lambda \) phage partially escaped \( oop\) \( \text{ori}\( \lambda \)\) plasmid-dependent IP, but phage development was retarded (as evidenced by reduced EOP and plaque size). 3) Phages that could escape CI-dependent replicative inhibition were unable to suppress IP. This result refutes a hypothesis that natural or mutational events that increase transcription from \( pR\), e.g., by limiting Cro or CI binding to \( oq\), or introducing downstream promoters, will augment transcriptional activation of \( oq\), and in turn promote theta-mode-\( \text{ori}\( \lambda \)\)-dependent replication initiation, and suppress IP. Another explanation is needed. Anderl and Klein [52] suggested that if the ratio of DNA:O protein is increased, theta-mode replication initiation will be inhibited due to titration of O protein, which suggests that plasmid-borne \( \text{ori}\( \lambda \)\) iteron sites could act as competitor origins, sequestering the O protein made by infecting \( \text{rep}\)\( \lambda \) phages. The “handcuffing” analogy for dimer formation [25] between O proteins binding to the iteron sequences in several \( \text{ori}\( \lambda \)\) sites could serve as a model for blocking the formation / completion / processing of a preprimosomal complex. The minimum molar ratio [53] of O protein:ori\( \lambda \) (termed O-some [54] complex) that was required for strand unwinding was 20:1. When additional \( \text{ori}\( \lambda \)\) regions are present, or if multiple interacting O-ori\( \lambda \) complexes are formed, it is unlikely that this molar ratio will be achieved. Our results suggest that handcuffing cannot account for IP, even if multiple ori\( \lambda \) targets bind excess O protein. Cells with multiple copies of two plasmids lacking oop sequence, but encoding an intact gene O/\( \text{ori}\( \lambda \)\), did not reduce EOP, i.e., exhibit IP, whether or not O was expressed.

### Theta-mode replication silencing by IP

The loading of DnaB onto ssDNA, formed by strand separation within the high-AT-rich region of \( \text{ori}\( \lambda \)\), was suggested to mark the end of the initiation phase of \( \lambda\) theta mode DNA replication [53]. Previously, we confirmed that theta-mode \( \text{ori}\( \lambda \)\)-dependent prophage replication initiation, which requires \( \text{P}\) interaction with, and loading of, DnaB, was inhibited if the host carried the dnaB-GrpD55 mutation, yet there was no obvious influence of this allele on \( E\) coli DNA propagation [18]. Herein, we observed that both theta-mode replication from \( \text{ori}\( \lambda \)\), and its manifestation, i.e., the Replicative Kiling of induced cells (dependent upon triggering theta-mode replication from a trapped, defective \( \lambda\) prophage) was prevented in cells with plasmids exhibiting IP. Both observations strongly suggest that theta-mode replication initiation is silenced, in \( \text{trans}\), by the \( oop\) \( \text{ori}\( \lambda \)\) plasmids. Blocks to theta-mode replication initiation from an infecting phage, by cellular \( oop\) \( \text{ori}\( \lambda \)\) plasmid copies or by the chromosomal dnaB-GrpD55 mutation, could be bypassed by multiply infecting such cells with \( \lambda\). This result is not without precedent. Freifelder et al. [56] infected nonpermissive cells at MOI’s between 0.01 and 40 with \( \lambda R57\)Pam3 phages that were variously inactivated for integration or Red recombination functions. For their Int” Red” variant, they showed an increase in phage burst of 240-fold between MOI’s of 0.01 (transmission coefficient 0.001) and 10 (transmission coefficient of 0.24), yet the \( \lambda R57\)Pam3 phage was unable to form plaques on nonpermissive cells; and in our hands the Pam3 mutation reverts at a frequency of \( <10^{-5}\). Freifelder et al. [56] concluded that if recombination is reduced, the ability to produce mature phage was markedly reduced. McMillin and Russo [57] reported that under conditions which block \( \lambda\) DNA duplication, unduplicated \( \lambda\) can mature, including molecules which have recombined in the host. Stahl et al. [58] extended this observation, coining the term “free-loader” phage to describe phage produced under replication-blocked conditions, whose synthesis depended upon bacterial and phage recombination systems. We borrowed this concept, using “free-loader coefficient” to describe the influence of phage recombination functions on \( \lambda\) progeny from infected dnaB-GrpD55 cells in which the infecting phage genome cannot initiate theta-mode replication. We showed that phage recombination functions from both NinL and NinR regions can influence by up to ten-fold the phage progeny released from multiply infected dnaB-GrpD55 host cells, supporting the Freifelder et al. [56] conclusion. Sclafani and Wechsler [59] showed that at low MOI, no \( \lambda\) particles were produced in cells lacking a functional dnaB product; yet at high MOI, a significant proportion of the cells can produce phage. Thus, the bypass of an ori\( \lambda\) replication block in multiply infected cells could depend upon a recombination-driven replication shunt, possibly analogous to the replisome inversion mechanism described by Poteete [60]. It is recognized that if a cell contains \( \geq 2\) circularized \( \lambda\) genomes, recombination between the monomers can produce an invading strand which could lead to rolling circle replication, independent of ori\( \lambda\) [61]. Presumably,
recombination / replication intermediates can be formed that produce packageable, concatemeric DNA by the introduction of a nick into one of the DNA strands of a λ monomer, enabling rolling circle replication initiating from the 3'-OH end of the nick, or by recombination between homologous λ DNA segments. It was proposed that double-strand break repair recombination intermediates in E. coli are capable of initiating and undergoing DNA replication [62,63]. It is possible that the circularized λ genomes produce linear multimers, formed by the rolling circle type of plasmid replication dependent on the RecF recombination pathway [64-67].

The potential to bypass theta-mode replication initiation via recombination suggests that there is no obligatory order / mechanism for triggering late mode λ replication from the early orλI-dependent replication products. Alternatively, the extensive evidence for a shift from early to a late replication mode supports the possibility that some natural mechanism can inhibit early theta-mode replication initiation. Two events come to mind where theta-mode replication initiation is undesired and would best be silenced. Theta-mode bidirectional replication forks arising from a λ DNA copy that is integrating, or has integrated, into the host chromosome will kill the potential lysogen via the escape replication (Replicative Killing). The initiation of theta replication from linear concatemeric DNA might inhibit genomic DNA packaging into the phage head. Our results for plasmid based IP suggest that there is a natural mechanism for silencing theta-mode replication initiation, i.e. the build up of λ genomes with opp^+ orλI^+ sequence.

**Toward a mechanism for IP**

There are a number of ways opp expression could influence transcriptional activation of orλI: i) OPP antisense RNA binding the βR transcript could promote degradation of the downstream CI-O-P transcript, in turn limiting transcriptional activation of orλI and O-P expression. ii) Cells expressing OPP antisense RNA can nullify CII formation, eliminating βR→pnX→CI→rexA→rexB transcription and the (little appreciated) potential of this mRNA to permit a) high CI repressor buildup, b) hypothetical oppX→rex expression, or c) high level βR-promoted antisense mRNA to cro expression, in turn, reducing Cro build up and interference with transcription from βR (Fig. S7). Since the repP22 phage λB57/18,12/P22 was insensitive to IP, yet almost fully shared the same CI→βR→cro→DI→opp sequence as repIP phages, it seems unlikely that the contribution of opp to CI-independent IP simply involves OPP serving as an antisense RNA to the βR→cII mRNA, or events that increase transcription from βR, but they might explain why cells with an opp^+ plasmid can stimulate phage maturation (i.e., support larger plaques).

Overall, the results suggest that OPP RNA expression from an opp-orλI DNA template increases the sensitivity of repI genomes to competing orλI sequences, with the outcome of silencing theta mode replication initiation from the orλI sites. This is a new idea in search of an explanation. Some form of molecular coupling between opp expression and orλI may serve to block the formation or completion of the preprimosomal complex. Several old observations remain a mystery regarding the regulation of opp expression. A low level of β0J transcription arises from a repressed prophage [4], which, if extrapolated would additively increase the level of OOP in cells with multiple opp^+ plasmids. This low level transcript was discovered because its expression increased about 40-fold between 5 to 12 minutes following the thermal induction of a cryptic λ prophage (as in Fig. 4) [4,28]. The increase was linked to phage replication, since a prophage deleted for P showed no OOP increase [4], nor was there an increase from intact λ prophages in cells with Ts host dnaB or dnaG genes, or prophage with O, P, or orλI mutations [49] which we have confirmed by sequence analysis. While one might explain this as a gene dosage effect, the level of induced opp expression was about the same from an induced defective prophage [49] as from an induced λB57/12p22 prophage defective for cell lysis (Table S2), where we typically see between 30 -200 fold increase in phage particles; or when λ was induced in cells with a Ts dnaE mutation blocking DNA fork progression [49]. This coupling between replication events at orλI, and opp expression, still requires an explanation.

**Materials and Methods**

**Reagents and media**

Growth experiments were carried out using tryptone broth (TB; 10 g Bacto-tryptone and 5 g NaCl per liter), TB plates (TB with 11 g Bacto-agar per liter) and TB top agar (TB with 6.5 g Bacto-agar per liter). Ampicillin was added to a final concentration of 50 μg/ml where required. B80 buffer (0.1 M NaCl, 0.01 M Tris-HCl, pH 7.6) was utilized for cell culture and phage dilutions, TE (0.01 M Na2 EDTA, 0.01 M Tris-HCl pH 7.6) and TE^+ (TE buffer with 0.001 M Na2 EDTA) buffers were used for DNA storage and manipulation of DNA, respectively, TM buffer (0.01 M MgSO4, 0.01 M Tris-HCl, pH 7.6) was used in phage burst assays. TBE buffer (0.089 M Boric acid, 0.002 M Na2EDTA, 0.089 M Tris-HCl, pH 8) was used to make agarose gels and as running buffer during electrophoresis. Restriction enzymes and T4 DNA ligase were from New England Biolabs. Tag DNA polymerase was from Invitrogen and New England Biolabs. Oligonucleotides were from Sigma Aldrich and Integrated DNA Technologies, Inc. Plasmid DNA was isolated using Promega Wizard Plus SV Mini and Midi prep, or Qiagen miniprep kits. DNA was isolated from gels using the Qiagen gel extraction kit, and reaction fragments were purified using the Qiagen QIA quick PCR purification kit.

**Bacteria, bacteriophage, and plasmids**

Table 1 shows the E. coli K-12 and bacteriophage strains and Table 2 and Fig.'s 1, 3, S3 show the plasmids employed. All of the plasmids were derived from plasmid pCH1 [11] prepared by ligating the λ34500-41731 BamHI fragment into the unique BamHI site of pBR322. The λ sequences are described by Daniels et al. [2]. The fragment orientation in pCH1: λ base pair 41731 was closest to the N-terminal end of the interrupted tet gene.

**Plaque Assay**

repP22 = λB57 and repP22 = λI 857/18,12/P22 infected phages were plated on several plasmid-containing host cell strains to measure plasmid-mediated inhibition of phage plating. An aliquot (0.25 ml) of a fresh overnight cell culture was mixed with 5 ml of warm TB top agar and 0.1 ml of diluted repP22, or repP22 phage lysate, and poured over TB or TB+Amp plates. Plates were incubated at 30° overnight and plaques counted. The results were expressed as EOP, i.e. phage titer on 594[test plasmid] / phage titer on plasmid free host 594 cells.

**Prophage Induction Assay**

The repP22 and repP22 prophages were thermally induced in lysogenic cells transformed with plasmids containing various λ fragments. Lysogenic cells were grown at 30° in 20-ml TB (+/- Amp) in a shaking bath to A573 nm = 0.15. The dT31B57 prophage in the cells was synchronously induced by swirling the culture flask in a 55-60°C water bath for 15 seconds and then transferring to a 42° shaking water bath to denature the repressor. The culture absorbance was monitored at 30 minute intervals over

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**Bacteria, bacteriophage, and plasmids**

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five hours. Each culture assay was repeated, the several results were averaged and the standard error determined.

**Phage Burst**

Host cells transformed with plasmids containing various λ fragments were infected with a repλ or a repP22 phage at a high or low MOI. The phage particles released per infected cell (i.e. phage burst) were measured for each infection. Protocol: 16–18 hour culture cells grown at 30°C in TB (+/- Amp) were pelleted and resuspended in an equal volume of 1.0 buffer. A cell aliquot (0.1-ml) was mixed with 0.2-ml of ice cold 0.01 M MgCl₂/CaCl₂ plus an appropriate volume of sterile phage lysate needed for MOIs of 5 or 0.01. The cell-phage infection mix was held on ice for 15 min to permit phage attachment and then transferred (time zero for measuring infective centers) to a stationary 42°C air incubator for 10 min to permit phage infection. The cell-phage mixture was pelleted and resuspended (2X) in 1.0 buffer and the third cell pellet was resuspended in 0.4 ml pre-warmed 42°C TB. Half of the resuspended cells (0.2-ml) were inoculated to 20 ml TB (+/- Amp), incubated with shaking at 42°C, and aliquots were removed after 65 and 110 min from the time of inoculation to determine phage titer. The second half (0.2-ml) of the washed cell-phage mixture (first held 15 min on ice and then at 42°C for 10 min) was immediately pelleted. The supernatant was used to measure the unattached phage remaining after the attachment and infection steps, and the cell pellet was resuspended, diluted, and aliquots were mixed with sensitive cells, top agar, and overlayed on a TB agar plate. Each plaque that arose on the plate was from a potential infective center (an infected cell that has not yet lysed). The phage burst (number of phage released per number of potential infective center (an infected cell that has not yet lysed). One primer contained the λ sequence plus the stop codon for gene cII (extended from the left, is at position -33. Position 1 is set as -1). The lambda CII expression [48], resulting in an otherwise  λoop-ori, was utilized as an assay system to test the activity of the OOP gene. The last 17 codons of the λoop, plus the λoof-ori-p0, and λori-λoof-pO-*OOP* sequence. One primer contained the λoof-ori-p0 (5’-oof-ori-p0). The second primer was made to contain “random” bases (screened to eliminate secondary structures) replacing λ bases 38630–38674 of the wild type λoof-ori sequence. One primer contained the λ strand sequence (LROOP5) and the other contained the r-strand sequence (RROOP2) (Table 3). The p27R template was PCR amplified with the mutated primers and with primers LPo1 and RPo4. The second half (0.2-ml) of the washed cell-phage mixture was pelleted and resuspended (2X) in 1.0 buffer and the third cell pellet was resuspended in 0.4 ml pre-warmed 42°C TB. Half of the resuspended cells (0.2-ml) were inoculated to 20 ml TB (+/- Amp), incubated with shaking at 42°C, and aliquots were removed after 65 and 110 min from the time of inoculation to determine phage titer. The second half (0.2-ml) of the washed cell-phage mixture (first held 15 min on ice and then at 42°C for 10 min) was immediately pelleted. The supernatant was used to measure the unattached phage remaining after the attachment and infection steps, and the cell pellet was resuspended, diluted, and aliquots were mixed with sensitive cells, top agar, and overlayed on a TB agar plate. Each plaque that arose on the plate was from a potential infective center (an infected cell that has not yet lysed). The phage burst (number of phage released per number of potential infective centers) was determined for the 65 and 110 min infections, correcting for the phage particles that did not attach to cells.

**OOP Phenotype/CII Inactivation Assay**

The last 17 codons of cII are not required for CII activity, but are necessary for CII regulation by OOP [5]. The 终核 52 nucleotides plus the stop codon for gene cII overlap the 3’-end of oof. The expression of OOP antisense RNA from a plasmid prevents lambda CII expression [48], resulting in an otherwise CI-Independent Replicative Inhibition.

**Plasmid Sequence Modification**

We supplied primers and DNA template to the service at National Research Council/Plant Biotechnology Institute, Saskatoon to confirm the λ-region sequences for the plasmids employed and to verify the mutations introduced into plasmid p27R. PCR mutagenesis was used to modify the ωg-ωp-ωt and αλλ plasmid DNA sequences using the SOEing technique [68]. p27R/p0- (ωg-ωp-ωt-αλλ): For mutating the -10 region of the p0 promoter in p27R, two primers were made that contained the sequence 5’GGCCGG3 in place of the wt sequence 5’ATTAT3’ at λ bases 38664–38668. One primer contained the λ strand sequence λ bases 38671–38700 (LPo3) and the other contained the r-strand sequence λ bases 38700–38671 (RPo2) (Table 3). The p27R template was PCR amplified with the mutated primers and with primers LPo1 (5′ λori site and λ bases 38357–38372) and RPo4 (5′ EcoRI site and λ bases 39172–39153) in a two-step PCR technique. Both for this plasmid and for those described below, the final PCR product was digested with NdeI and EcoRI and ligated into the larger (~2000 bp Amp + CoEI origin) fragment resulting from p27R. NdeI and EcoRI digestion. p27R-AR50OP: Bases 2–46 of the ωp gene coding sequence in p27R were mutated. Two primers were made to contain “random” bases (screened to eliminate secondary structures) replacing λ bases 38630–38674 of the wild type ωp sequence. One primer contained the λ strand sequence (LROOP5) and the other contained the r-strand sequence (RROOP2) (Table 3). The p27R template was PCR amplified with the mutated primers and with primers LPo1 and RPo4 (Table 3), p27RAITN1–4: Two hybrid primers were made to delete iterons (ITN) 1–4, each with sequences flanking the iterons. LAITN1–4 contained the λ bases 39014–39043 fused to 39120–39144, while RAITN1–4 contained the same sequence on the r-strand (Table 3). These two primers, in conjunction with LPo1 and RPo4, were used for deleting λ bases 39044–39119 (i.e. 87 nt of ITNS 1–4), pHB27RAAT: Primers LPo1 (5′ λori site and λ bases 38357–38372) and RAAT1 (5′ EcoRI site and λ bases 39127–39119) were used to amplify the pH27R λ DNA fragment. The resulting PCR fragment was digested with NdeI and EcoRI and cloned into the 2000 bp pBR322 fragment from pH27R digested with NdeI and EcoRI. The plasmid pH27RAAT was shown to be deleted for λ bases 39,128–39172, removing the AT rich region of αλλ (Table 2).

**Isolation and sequencing Sip mutants**

λBl57 formed small plaques at a frequency of ≤10^{-6} on 594 cells. An individual plaque from ten separate isolations was transferred by a sterile toothpick to 10 ul buffer (10mM Tris-HCL, 10 mM MgCl₂, pH 7.6) and spread using sterile paper strips onto a fresh agar overlay of these cells. This procedure was repeated (as many as 13 times) yet always produced plaques that were heterogeneous in size on the 594_[λ-ori] cells. Each of the ten independent Sip phages were plated on 594 host cells (without plasmid) and a single plaque was used to prepare a phage lysate. Single plaques arising from these lysates were sequenced from gene cII into P (λ bases 37905–39191) using primers LHM29 (37905–37922: 5’-CGTCTGGAAGGCATGATTGG-3’), L22 (38517–38534: TGCTGCTTGCTGTTCTTG), RPG6 (38569–38552: CATTCGACAGCTGTCGTC), and R941 (39191-39175: TGGTCAGAGATCCGCC). The method is described in [18], only herein, chromosomal DNA was digested with NdeI, not EcoRI.

**Supporting Information**

**Figure S1** Aligned conserved sequence regions for 23 lambdoid phages. Sequence regions were searched using a 33 nt region of sequence similarity between HK620 and λ (“sequence 5” in [79]). The bases in red show greater than 90% sequence homology. The sequence of OOP spans positions -90 (terminator end) through -10 (5’ end). The termination sequence for lambda gene cII, extending from the left, is at position -33. Position 1 is set as the ATG start for lambda gene 0, for P22_MJ50 homologue as hkaW, EC_CPI693_21, or a HK097 gp53 homologue of λ54 (see Fig. S2) [80]. An annotated version of this data was provided in the review.
The sequences were obtained and aligned using EBI's implementation of the ClustalW alignment algorithm (http://www.ebi.ac.uk/clustalw/) in full alignment mode as well as a hierarchical clustering method implemented in the Multalin program on the RNA servers (http://prodes.toulouse.inra.fr/multalin/multalin.html) using a DNA identity matrix and various penalties imposed on gap opening, none on extension. Sequences were obtained from the NCBI nucleotide database. Accession numbers and references are as follows. GI:215104; lambda; E. coli [81], GI:14988; 434; E. coli [77], GI:4539472; 21; E. coli [82]. GI:19911589; stx2I; E. coli O157:H7 Okaoyama O-27 [83]. GI:4583377; 933W; E. coli O157:H7 strain ELN593 [84]. GI:49523585; phi-4795; E. coli strain 4795/95 serotype 04:H4, unpublished. GI:7393813; H-19B; E. coli [85]. GI:9634119; HK229; E. coli [86]. GI:32128102; stx2I; E. coli O157:H7 Moriova V526 [87]. GI:32128102; stx1I; E. coli O157:H7 Moriova V526 [87]. GI:5881592; VT2-Sa; E. coli O157:H7 [88]. GI:6901504; HK097; E. coli [91]. GI:23343450; NlI2; E. coli O157:H7 strain NlI53, unpublished. P22-pbI; S. enterica serovar typhimurium [46]. GI:8439576; P22; S. enterica serovar typhimurium [89]. GI:1143407; ES18; S. typhimurium [90]. GI:13517599; HK620; E. coli H strain 2158 [79]. GI:51773702; CP-16; E. coli 1639/77 [91]. GI:24250761; ST4T; S. enterica serovar typhimurium [92]. GI:33334157; St6; Shigella flexneri [93]. GI:14800; Pho80-B; E. coli [94]. GI:46357884; ST104T; S. typhimurium DT104; phage 434 (GI:14988); phage 21 (GI:4539472); and phage P22 (AF527681). GI:21914413; AF212753.1, [95]. Sequence date from this laboratory are shown for: lambda = λB57 (DQ372056), λimm434I (DQ372053.1), λimm21I (DQ37204.1 being revised), and P22-Lambda hybrid = λB57(18,12)P22, representing Ahy106 from Dr. S. Hilliker (DQ372055.1); and are expanded and compared to sequences for 434, 21, and P22 in Fig. S5. (TIF) Figure S2 Comparative analysis of lambdoid phage maps. The regions cII-like, oop, orf, O-like and P-like are with reference to lambda gene map, e.g., gene cII of P22 is equivalent to cII of lambda. The numbers in boxes indicate RNA length in nucleotides (nt) for oop RNA, or amino acids per proteins cII, Orf, O or P, without specifying the level of gene homology. Color coding relates the similarity of protein length to lambda protein length. Locus identity was obtained using the conserved 33 bp high homology region sequence (Fig S1) ACTGATCT-CAATCCACAGAGCATTATCTGACAAA (from the promoter and 5’ end of oop RNA and BLASTed using an expectation value of 1000 and parameters to remove gapping penalty, each containing the conserved sequence with minimum 90% homology: lambda [J02459], 434 (V00633), 21 (AJ237660), Stx2 (AP004402), 933W (AF152520), phi 4795 (AJ556162), H-19B (AF034975), HK022 (NC_002166), Stx2 II (AP005154), Stx 1 (AP005153), VT2-Sa (AP000563), HK097 (AF069529), NlI2 (AJ143274), P22 (AF217253), ES18 (N37420), HK620 (AF335353), CP-1639 (AJ034858), ST4T (AY052786), St6 (AF547978), Phi-80 (X10365), and ST104T (AB102968). Examples of the open reading frame left of the O-like protein sequence are orfH in HK022 [80], and gene p43 in HK97, representing 162 nt (NC_002167). This figure was redrawn with modification from [51]. (TIF) Figure S3 Influence of spacing between oop and orf on repI-inhibition. Influence of spacing between oop and orf on repI inhibition. A. Plasmid p50 substitutes E. coli DNA from the specialized transducing phage λsp156 for the ‘‘ice’’ sequence of λ (Table 2) and was made by cloning the 684 bp EcoRV-EcoRI fragment from λsp156Δann5 [96] into the equivalent sites in pBR322 [89]. B. The stable predicted secondary structures of OOP RNA were obtained using the IxDi SciTools OligoAnalyzer 3.0 website. C. EOP of repI and repP22 phages on host cells with modified ΔcI Δorf Δl phasmids. The averaged data is shown. (Near identical results were seen for each of the phasmids transformed into E. coli strain W3350, where standard errors were negligible for the repI phage, and ranged between <0.1 to 0.28 for the repP22 phage on the different transformed cells.) D. Phasmid modifications to p50: λ DNA fragments in the DNA interval between oop and orf was varied by deletion or insertion (Table 2). (TIF) Figure S4 Plating-sensitivity to cells exhibiting inhibition phenotype (IP) and relative plaque size on cell lawns. A. Variation in susceptibility of repI phages to the IP. A 0.3 ml aliquot of fresh overnight stationary phase strain 594[p27R] cells (grown in TB+50 μg/ml Amp) were mixed with 0.1 ml of test phage and 3.0 ml of molten top agar and poured onto a TB plate. Plates were incubated overnight at 30°C and resulting plaques were counted. EOP was calculated as the titer on strain 594[p27R]/titer on 594. The results represent the average of at least two independent assays. Averaged EOP’s and standard errors values were: λWT (wild type), 5.17 ± 6.28 × 10^−5 ± 6.28 × 10^−5; λT2, 1.73 ± 10^−5 ± 6.99 ± 10^−5; λimm434I, 0.01 ± 0.04; λimm21, 0.70 ± 0.06; λttv, 0.41 ± 0.06; λE0 (c7, f1.0 × 10^−7 ± 2.0 × 10^−7; λttv mutants (λse100a, λse101B, λ109b) 1.51 × 10^−5 ± 2.21 × 10^−2. Notes: 1) The downstream promoter in λE0 [90] appeared not strong enough to suppress IP. 2) The phasmids employed in earlier studies [8,10,12] inhibited λttv, but each included ci repressor gene. We show (Table 4) that λttv was inhibited for plating at 30°C in cells with multiple copies of the O/repI plasmid version with ci from imm434, whereas, Fig S4A shows λttv is only partially inhibited by cells with oop orf phasmids without ci, thus, CI availability to bind or can increase repI phage sensitivity to IP. B. Portion of λ map showing region of DNA substitution for the imm11 and imm434 hybrid phages and the portion of λDNA present in phasmids transformed into strain 594. C. Strain 594 was grown overnight to stationary phase in TB [18]; alternatively, 594 transformed with one of the plasmids, shown in part B, was grown overnight in TB+AMP (50 μg/ml). The culture cells (0.25 ml) were mixed with 0.1 ml of phage lysate dilution plus 5 ml TB top agar [18], poured on TB agar plates, and incubated overnight at 30°C. Plaque size was determined using a tissue culture inverted microscope at 4× magnification with an eyepiece grid. Each grid interval was 0.045 mm at 4× magnification. Plaque diameters were measured as grid units, i.e., grids/plaque. Approximately 30 plaques were measured per assay phage on each of the host strains and the average plaque diameter and SE were determined. All assays for a given phage were performed in parallel on each of the host strains using same preparation of agar plates. (TIF) Figure S5 Sequence determination for distal cII-oop to O interval for l-hybrid imm434, imm21, and repP22 phages employed. Hybrid phage sequences compared to λ. The highlighted/underlined bases differ from λ; all data were from this laboratory except sequences for phages 434 and 21; sequence differences rightward from base 38698 are continued in Fig S1). Phage λimm21, which retains the repI, sequence, had a silent TGC to TGT codon change (not shown in Fig S5 or S1) at 39,033 (one base left of the ITN1 sequence in O). Lambda = λB57 (DQ372056) is as in [2]; λimm434I (DQ372053.1); λimm21I (DQ372054.1, being revised); and P22-Lambda hybrid = λB57(18,12)P22, representing Ahy106 from Dr. S. Hilliker
The partial comparative sequences for non-hybrid phages 434, 21 and P22 were: phage 434 (GI:14906; phage 21 (GI:459472), and phage P22 (AF327600.1; GI:21914413; AF217253.1).

**Table S1** EOP of cI857cro27 on host strains.

| Strain | EOP |
|--------|-----|
| E. coli | 100 |
| E. coli | 90 |
| E. coli | 80 |

**Supplemental Methods**

**Table S2** Relative OOP RNA transcription after prophase induction.

| Time (h) | Relative OOP RNA |
|---------|------------------|
| 0       | 100 |
| 1       | 90 |
| 2       | 80 |

**Acknowledgments**

This research followed up and extended earlier thesis [69] results for which we are grateful, and involved remaking and expanding upon initial constructs.

**Author Contributions**

Conceptualized and designed the experiments: SH MH. Performed the experiments: MH CH. Analyzed the data: SH MH CH. Wrote the paper: SH.

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