

Deletions of lambda phage locating a *prm* mutation within the rightward operator

(maintenance promoter for repressor synthesis/*in vivo* mRNA purification/nucleotide sequencing/deletion mutations of λ operator/repressor protection of operator)

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ABSTRACT Two deletion-substitution mutations of phage λ , *spi-113* and *spi-274*, are shown to remove about half of the rightward operator O_R . Physiological studies show that *spi-113* is still repressible. Thus, the 50 or so nucleotides of O_R deleted in this mutant are not absolutely essential for repression. *prm-116*, which inactivates the promoter essential for the maintenance of λ repressor synthesis, is located within O_R between the endpoints of *spi-113* and *spi-274*.

The rightward operator-promoter region of bacteriophage λ is a rather complex DNA region controlling transcription in divergent directions. As shown in Fig. 1, the major rightward promoter, P_R , controls transcription of the *cro*, *cII*, *O*, *P*, and *Q* genes. Very close to P_R is another promoter, *prm*, which controls leftward transcription of genes *cI* and *rex*. The product of gene *cI*, the λ repressor, binds to the rightward operator, O_R , thereby blocking transcription from P_R (repression) and stimulating transcription from *prm* (activation). (See refs. 1 and 2 for reviews.) We are interested in the molecular mechanism of these controls and have studied the nucleotide sequence relation of P_R , O_R , and *prm*. In this paper, we show that a *prm* mutation is located within O_R , and that about half of O_R is not essential for repression of the rightward operon.

To determine the nucleotide sequence of this control region, which we shall refer to here as O_R , we have taken advantage of the observation by Spiegelman *et al.* (3) that transcription of the *cI* gene can be initiated at the promoter, *pre*, located to the right of gene *cro*. This transcript was postulated to contain the sequence of O_R . By hybridization to DNA from appropriate deletion mutants of λ shown in Fig. 1, and subsequent RNase treatment, we have purified a fragment of this *pre* transcript containing O_R sequences. These sequences have been determined and compared with the complete nucleotide sequence of O_R independently determined by Walz and Pirrotta (4) and by Maniatis *et al.* (5). In addition we have investigated the amount of O_R DNA remaining in the deletions.

MATERIALS AND METHODS

Phage and Bacteria. Most of these have been described (6). All *spi* phages used here are deletion-substitution mutants extending from *att* into and beyond *cI* and contain the *nin-5* mutation to allow plaque formation (7). *spi-325* and *spi-330*, ending between *cI60* and *prm-116*, and *spi-329*, *spi-331*, *spi-332*, and *spi-335*, ending between *prm-116* and

V_s387, were isolated from M72 (*λxis-1 nin-5*) as described (6).

Phage λ *cI857 prm-116 susO29*, from Yen and Gussin (8), was crossed with *spi-B nin-5* to give λ *cI857 prm-116*, selected as a clear-plaque phage at 32° on a *recA* nonsuppressor (Su^-) strain (6). This was crossed with *λb2*, from Kellenberger *et al.* (9) with enrichment by citrate treatment (10) to give *λb2 cI857 prm-116*. $\phi 80 imm^\lambda cI857 prm-116$ was obtained by crossing $\phi 80 imm^\lambda$ (11) and λ *cI857 prm-116 susO29*, selecting a clear-plaque phage on a λ -resistant Su^- strain at 32°. The authenticity of the *prm-116*-containing phage was confirmed by their ability to complement *cY42* and by the presence of a clear-plaque mutation located between the endpoints of *spi-113* and *spi-274* (8, 6).

Isolation of D3', a Transducing Phage Carrying the $\Delta H1$ Deletion Endpoint within the *cro* Gene. Strain M72 (*λbio256 cI857 ΔH1*), constructed by Dr. Helen Greer, has the gene order *gal attλ bio N^λ cI^λ O_R^λ ΔH1*. $\Delta H1$ deletes the λ prophage from within the *cro* gene to the right prophage end, as well as the host *bio*, *uvrB*, and *chlA* genes (12). This strain was lysogenized with $\lambda imm^{21} cI^{TS} susS7$ (13), briefly exposed to ultraviolet light, and thermally induced. From the resulting lysate, Bio^+ (biotin-independent) transductants of strain M72 *bioD19* were obtained at about 10^{-6} per plaque-forming unit. About half of the transductants were also λ -immune. Lysates of 9 out of 98 such transductants contained *bio* transducing phages. Four of these formed plaques on a λ lysogen, but not on a λimm^{21} lysogen. In crosses with $\lambda susN7 V_1 V_3$, all produced 3–4% turbid-plaque recombinants ($V_1^+ V_3^+$ or V_1^+) selected on an $Su^- \lambda imm^{21}$ lysogen, showing they contain O_R of λ . The one used here is isolate D3' and has the presumed structure:

A att bio256 N^λ cI^λ O_R^λ bact imm^{21} O R where "bact" represents bacterial genes from the right of the $\Delta H1$ deletion endpoint.

Purification of *pre* mRNA. Labeling and purification of ^{32}P -labeled *pre* mRNA from λ -infected cells has been described (14). For isolation of band 5 (see *text*), the RNA was subjected to a second hybridization to D3' *l*-strand DNA and the treatment following the first hybridization was repeated. Routinely, from 20 ml of culture containing 50 mCi of $^{32}P_i$, 0.1 to 1×10^6 cpm were recovered in individual bands with a purity of 50–90%, as judged by fingerprint analysis.

RESULTS

***spi*-Phage Operator DNA Fragments Protected from DNase by λ Repressor.** λ repressor binds to its operator DNA such that DNA fragments of increasing size are protected from DNase digestion as the repressor to operator ratio is increased (20). Beginning at about 30 base pairs (bp), the sizes of the fragments increase by discrete increments of

Abbreviations: Su^- , suppressor-negative phenotype; bp, base pairs.

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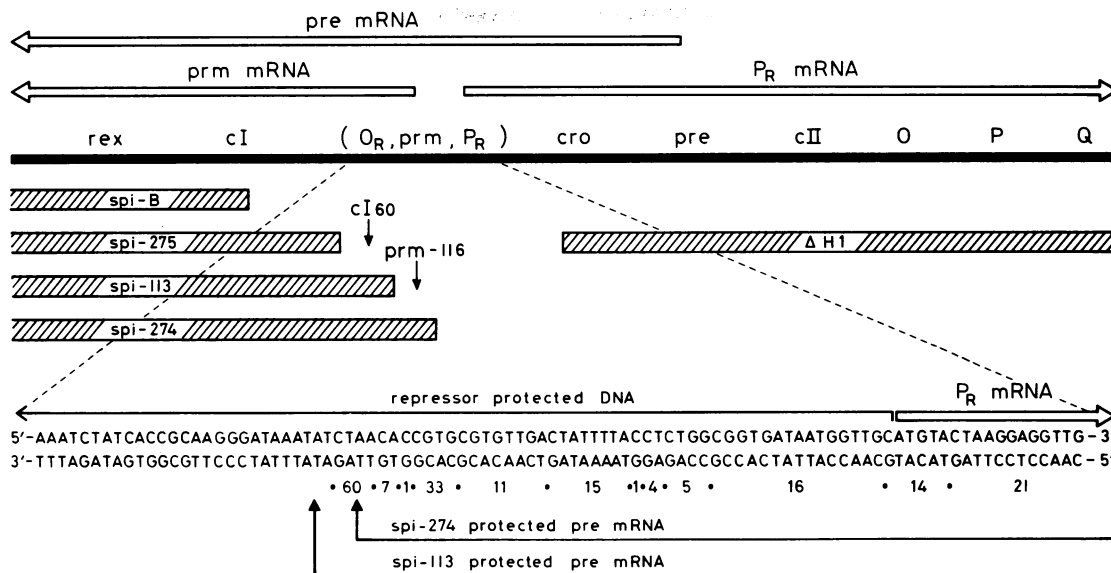


FIG. 1. Genetic and physical structure of the region surrounding the rightward operator of phage λ . *Open arrows* at the top indicate the directions and approximate initiation and termination points of the mRNAs of the genes (*rex* through *Q*) shown just below. The extents of the deletions used here (*spi*'s and $\Delta H1$) are shown by the *hatched bars*. The mutations *cI60* and *prm-116* are located between the deletion endpoints as shown. These genetic and transcriptional relations are not drawn to scale. The DNA nucleotide sequence of the region containing O_R , *prm*, and P_R is from refs. 4 and 5, as is the region of DNA protected by repressor from DNase and the point of initiation of P_R mRNA. Hyphens between the nucleotides are omitted for clarity. The locations of some T₁-oligonucleotides of *pre* mRNA, shown in Fig. 4, are indicated by their numbers. The extents of protection of *pre* mRNA by *l*-strand DNA from *spi-274* and *spi-113* are indicated by the arrows at the bottom.

about 15 bp to about 105 bp. For O_R the first fragment protected occurs farthest to the right in the region indicated in Fig. 1 (21).

In order to determine the amount of O_R DNA remaining in *spi* deletion-substitution mutants, λ repressor was bound to operator-containing DNA fragments isolated from these mutants, whose deletions extend increasingly into and beyond gene *cI* (see Fig. 1). In each reaction the amount of repressor was calculated to be at least 40 times the amount necessary to bind all operator-containing DNA. The repressor-bound fragments were treated with pancreatic DNase, the protected fragments were purified, and their sizes were compared as shown in Fig. 2. Under these conditions, two major fragments of DNA were obtained from *spi-B* and *spi-275*. Only the shorter was obtained from *spi-113*. The shorter fragment was estimated to be 40–60 bp and the longer 70–100 bp, by comparison with λ DNA fragments generated by endonuclease *HpaII*. If no repressor was added, no protected fragments were seen. Considering the genetic structure of the *spi* mutants, we assume that the larger fragment contains the DNA sequences of the smaller, plus about 30–45 bp to the left. Since *spi-113* yielded only the shorter fragment, we conclude that at least some of the operator DNA sequences are missing in this mutant and that the endpoint of the *spi-113* deletion lies within the rightward operator. Nucleotide sequencing results presented below indicate the extent of this deletion.

Isolation and Identification of *pre* mRNA Covering O_R . As suggested in Fig. 1, *pre* mRNA should contain a transcript of O_R . We have identified this mRNA and isolated a short fragment covering part of O_R , using deletion mutations entering the *pre* mRNA region from the right and from the left. RNA was hybridized to deletion DNA and the RNA chain was cut very near the deletion endpoint with RNase. ³²P-labeled *pre* mRNA was purified from cells infected with a derivative of λ (14). After hybridization to *l*-strand DNA (complementary to leftward-directed tran-

scripts) from deletion mutants shown in Fig. 1 and cleavage with pancreatic RNase, the protected RNA molecules were separated by gel electrophoresis (Fig. 3). Hybridization to *spi-274 l*-strand DNA yielded three species of RNA: *oop* and band 3, both of which originate from the right of *pre* (ref. 22 and unpublished observations), and band 2. Band 2 derives from *pre* mRNA, since, unlike *oop* and band 3, its size is changed upon subsequent hybridization to $\Delta H1 l$ -strand DNA and RNase cleavage. As a result of such hybridization, band 2 is replaced by a shorter RNA, band 5, about 115 nucleotides long.

Hybridizations to *spi-113* and to *spi-275* followed by hybridization to $\Delta H1$ produce band 5 RNAs of increasing length (Fig. 3), as predicted from the genetic mapping of these deletions (6). Since O_R mutations can be rescued from all of these deletions, band 5 must be a transcript of at least part of O_R . Further, we conclude that the ends of the band 5 prepared with *spi-274* and *spi-113* are generated by RNase cleavage at or near the *spi* deletion endpoint. (The length of the *pre* mRNA fragment is not affected by RNase treatment when it is hybridized to *spi-275 l*-strand DNA, showing that this RNA fragment does not extend beyond the *spi-275* endpoint. This indicates that the end of this fragment is generated by some event, presumably within the cell, prior to hybridization.)

Sequence Studies of O_R Transcript. T₁ fingerprints of band 5 RNAs isolated after hybridization to *spi-274* and *spi-113 l*-strand DNA are shown in Fig. 4. The compositions and, in most cases, the complete sequences of the oligonucleotides were determined (23). Over half of the oligonucleotides can be placed within the complete sequence of O_R determined by others (4, 5). With two exceptions noted below, the remaining oligonucleotides come from the region between O_R and $\Delta H1$.

The oligonucleotides from the 3'-ends of the fragments are readily distinguished as the only ones not containing G. Band 5 isolated after protection by *spi-274 l*-strand DNA

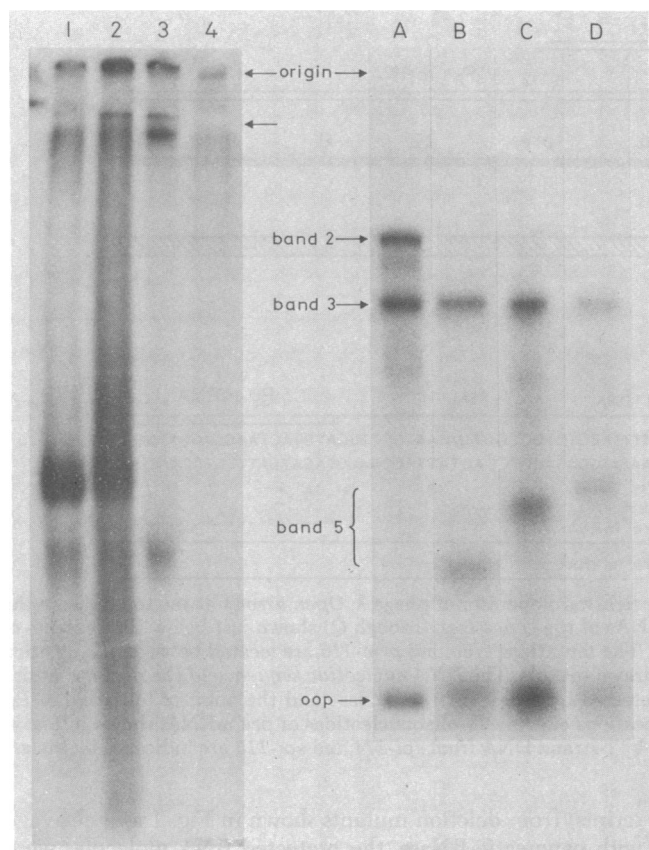


FIG. 2 (left). Repressor-protected DNA fragments from *spi* deletion phages. [^{32}P]DNA was prepared from double lysogens of genotype M72chl-6 (λ xis-1 cI857 susS7, λ spi-nin-5 susS7), which were grown at 30° to $\text{OD}_{600} = 1$ in Tris medium (14) containing 1 mM K_2HPO_4 . After heating at 42° for 10 min the cultures were centrifuged and resuspended in Tris medium containing 1 mCi/ml of $^{32}\text{P}_i$ (Amersham) at 37°. The cultures were incubated for 1 hr, phage was purified, and DNA was extracted as described (15). The [^{32}P]DNA was sheared by sonic oscillation and diluted into repressor binding solution (16–18). Repressor (16–18) sufficient to bind all operator-containing fragments was added and after 10 min incubation on ice the mixtures were filtered through Millipore filters, and operator-containing DNA was recovered (19). The solutions of recovered operator-containing DNA were adjusted to contain 1 ml of repressor binding solution. Each mixture contained sonic operator fragments from about 5 μg of λ DNA. Repressor solution (3 μl), sufficient to bind 180 μg of intact λ DNA, was added and the mixtures were incubated 10 min on ice. Then, 50 μl DNase (Worthington, 1 mg/ml) was added and incubation was continued for 3 min. The mixtures were filtered as above, and after elution from the filters the protected fragments were precipitated in ethanol with 50 μg of *Escherichia coli* tRNA. The fragments were electrophoresed 14 hr at 150 V in a 2 mm \times 10 cm \times 15 cm, 12% polyacrylamide gel with a 1 cm 7% polyacrylamide “stacking gel” in Tris-EDTA-borate buffer (14) plus 10 mM MgCl_2 . The gel was dried on Whatman no. 1 paper and exposed to Kodak “no-screen” x-ray film. 1, *spi-B* DNA; 2, *spi-275* DNA; 3, *spi-113* DNA; 4, *spi-275* DNA digested with DNase in the absence of repressor. The lower arrow indicates the interface between the 7% and 12% acrylamide.

FIG. 3 (right). ^{32}P -Labeled λ RNAs purified by hybridization to *l*-strand DNA from *spi* deletion phages. RNA was extracted from $\phi 80imm^+c^+$ -infected M72 chl-6, hybridized to the indicated *l*-strand DNA, treated with pancreatic RNase, and electrophoresed on polyacrylamide gels as described in *Materials and Methods* and ref. 14. In B, C, and D, RNA was hybridized sequentially to *spi l*-strand DNA and to D3' *l*-strand DNA containing the $\Delta H1$ deletion. (A) *spi-274*; (B) *spi-274 - \Delta H1*; (C) *spi-113 - \Delta H1*; (D) *spi-275 - \Delta H1*. Lane A is from one experiment, and lanes B, C, and D from another.

contains U-Up and U-U>p (oligonucleotides 34 and 8 in Fig. 4A), demonstrating that this molecule ends with the sequence G-U-Up. Because (5'-3')d(G-T-T) occurs only once on the lower DNA strand shown in Fig. 1, this result places the limit of *spi-274* protection at the position shown.

When *spi-113 l*-strand DNA is used to protect the RNA, the oligonucleotides U-Up and U-U>p are replaced by U-U-A-Gp, oligonucleotide 60 in Fig. 4B. In addition, oligonucleotide 61, A-Up, is present as the new 3'-end. These results place the limit of *spi-113* protection four nucleotides to the left of that of *spi-274* as shown in Fig. 1. [Although two other (5'-3')d(G-A-T) sequences are present 19 and 23 nucleotides to the left, these are ruled out by the absence of the very long oligonucleotide which would occur in the intervening sequence.]

Location of the *prm-116* Mutation. The mutation *prm-116*, which abolishes the activity of *prm*, has been mapped between the ends of deletions *spi-113* and *spi-274* (8, 6). We have located the nucleotide change occurring in *pre* mRNA purified from cells infected with a derivative of λ *prm-116*. A T_1 fingerprint of this RNA, isolated with protection by *spi-113*, is shown in Fig. 4C. All of the oligonucleotides in wild-type RNA (Fig. 4B) occur in the mutant RNA except oligonucleotide 60, U-U-A-Gp, which is missing (less than 3% of wild type). This result is consistent with the location of this oligonucleotide between the limits of protection by *spi-113* and *spi-274*, and the genetic location of *prm-116* between these deletions. Since oligonucleotide 61, A-Up, is still present in the mutant, (1.1 moles compared to 1.2 moles from wild-type RNA), the Gp of U-U-A-Gp must still be located within the nucleotide sequence (5'-3')d(G-T-T-A) shown in Fig. 1.

A number of additional oligonucleotides are seen in Fig. 4C. Most of these are identical to oligonucleotides from RNA in band 3 seen in Fig. 3. They probably derive from fragments of band 3 running on the gel at the same position as band 5. The presence of these contaminants precludes the unambiguous identification of the nucleotide change in *prm-116*, although many single nucleotide changes can be ruled out due to the absence of the predicted T_1 oligonucleotides.

Meyer *et al.* (24) have determined by different methods that the sequence (5'-3')d(G-T-T-A-G) is (5'-3')d(G-T-T-A-A) in their derivative of *prm-116*. This is inconsistent with our results because of the presence of A-Up and the absence of U-U-A-A-A-Up in Fig. 4C. At present we do not know whether this discrepancy is due to differences in our strains or in our methods.

Repressibility of Deletions Ending within O_R . The complexity of O_R led us to question the role of the various segments of this region. We have thus examined, by several indirect tests, the repressibility of *spi-274*, *spi-113*, and six other deletions with similar genetic endpoints.

spi-113 and *spi* deletions ending to its left form stable double lysogens at 30° with λ cI857. These phages do not grow in lysogens and are unable to help $\lambda imm^{21} susO$ upon co-infection of a lysogen. These results, shown in Table 1 and ref. 6, show that the region of O_R remaining in *spi-113*, *spi-325*, and *spi-330* is sufficient for repression under these conditions. However, these phages can help $\lambda imm^{434} susO$, presumably due to a slight escape from repression, augmented by *N* gene product from λimm^{434} but not from λimm^{21} (25). We suppose either that the left part of O_R is required for complete repression, or that there is transcription starting

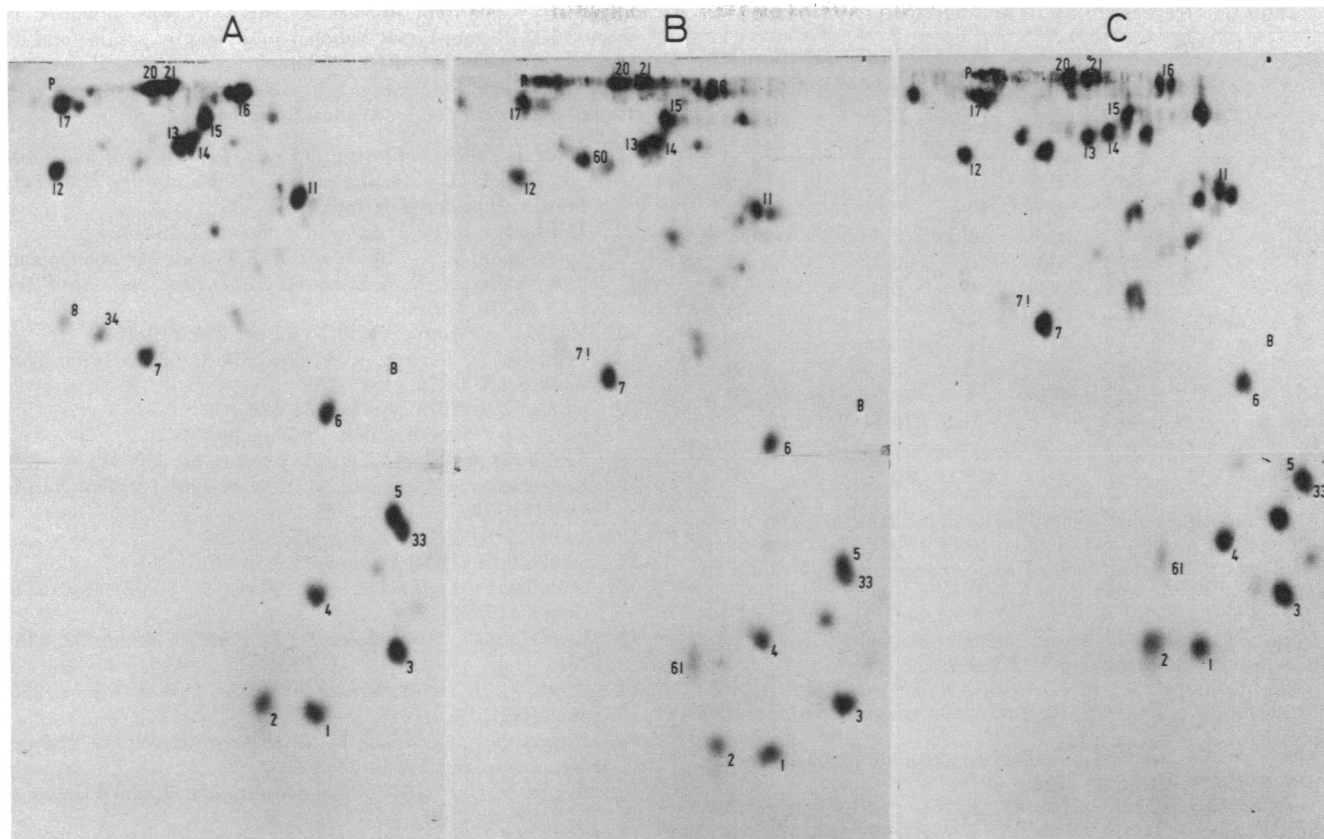


FIG. 4. T_1 RNase fingerprints of $[^{32}P]$ mRNA fragment band 5. RNA was prepared and purified as described in *Materials and Methods* and Fig. 3. (A) Wild-type *pre* mRNA from $\phi 80imm^{\lambda c^+}$ sequentially hybridized to *spi-274* and to $\Delta H1$. (B) Wild-type *pre* mRNA from $\phi 80imm^{\lambda c^+}$ hybridized to *spi-113* and $\Delta H1$. (C) *prm-116 pre* mRNA from $\lambda b2cI857 prm-116$ hybridized to *spi-113* and $\Delta H1$. In other experiments, $\lambda b2$ gave results similar to B, and $\phi 80imm^{\lambda cI857 prm-116}$ gave results similar to C. Numbered oligonucleotides derive from *pre* mRNA, as deduced from relative molar yields in fingerprints A and B ranging between 0.75 and 1.2, except for oligonucleotides 1, 2, 3, 4, 7, and 12, which occur in multiple molar yield, and 11, which occurred in 0.60 moles relative to the others. Unnumbered oligonucleotides derive from band 3 RNA (see Fig. 3) as deduced from relative molar yields of less than 0.30 in B, and their occurrence in fingerprints of band 3 (not shown). Electrophoresis in the first dimension (right to left) was on cellulose acetate, 5% (vol/vol) HOAc-pyridine (pH 3.5), 7 M urea; second dimension (top to bottom) was on Whatman DE81 DEAE-cellulose paper in 7% formic acid. B and P indicate the positions of the blue and pink marker dyes, respectively.

within the substituted bacterial DNA which is partially insensitive to repressor.

In contrast to these deletions, *spi-274* and four other deletions ending genetically in the same region are constitutive by all of the above tests, and have presumably lost some part of O_R essential for repression. Nevertheless, they still have some sensitivity to repressor, since they do not form plaques on a lysogen, unlike *lvir* multiple mutants and *spi* deletions entirely removing O_R , which are constitutive by all of the tests described here (6, 26, 27).

DISCUSSION

The data presented here show that two deletions, *spi-113* and *spi-274*, delete a considerable fraction of the rightward operator O_R of λ . This is shown directly for *spi-113*, because the DNA fragment protected from DNase digestion by repressor is smaller than that of other deletions ending further to the left, outside the operator. (See Fig. 1). In addition, the extent of protection of *pre* mRNA from RNase was determined for both *spi-113* and *spi-274*. These limits were placed within the complete O_R sequence shown in Fig. 1.

Repressor protects at least 100 nucleotides in O_R , and this provides an estimate of the length of O_R (20). Although *spi-113* contains only the right half of O_R , about 50 nucleotides, it is repressible by several tests. Therefore, the left 50 nu-

cleotides of O_R do not appear to be essential for efficient repression. Consistent with this interpretation, all operator-constitutive mutations which we have tested and all such mutations sequenced to date are located to the right of *spi-113* (refs. 6 and 28, and unpublished observations).

The mutation *prm-116* has been located genetically between the endpoints of *spi-113* and *spi-274*. Our biochemical studies of these deletions thus indicate that *prm-116* is located within O_R . Sequence analysis of the transcript of *prm-116* confirmed the location of this mutation within O_R , as shown in Fig. 1.

What is the nature of the *prm-116* mutation? As argued by Gussin *et al.* (29), it most likely blocks some event in the initiation of transcription from *prm*, since it does not block synthesis initiated at another promoter, *pre*, used for the establishment of repressor synthesis. Repressor itself is required for activation of transcription from *prm* (30, 31). Since the *prm-116* mutation is surrounded by repressor binding sites, this raises the possibility that *prm-116* eliminates the activation by repressor. A more direct effect of *prm-116* on the binding of RNA polymerase or on the initiation of RNA synthesis cannot be ruled out, however.

The existence of low affinity repressor-binding sequences between *prm* and gene *cI* raises the possibility that high concentrations of repressor may inhibit transcription from

Table 1. Repressibility of *spi* deletions ending in O_R

<i>spi</i> phage*	Burst size†		Helping‡ of $\lambda imm^{21} susO$		Helping‡ of $\lambda imm^{434} susO$	
	(-)	(λ^+)	(-)	(λ^+)	(-)	(λ^+)
<i>B</i>	62	0.5	9.7	0.1	11	0.3
<i>113</i>	97	0.9	11	0.5	10	12
<i>325</i>	82	0.3	12	0.4	9.5	12
<i>330</i>	99	0.3	12	0.4	10	12
<i>274</i>	47	57	9.4	5.4	7.1	11
<i>329</i>	35	57	9.5	11	8.0	7.2
<i>331</i>	32	120	12	12	9.7	7.1
<i>332</i>	38	130	12	13	11	9.1
<i>335</i>	43	97	11	13	7.2	9.1
None	—	—	0.1	0.1	0.1	0.1
None; $imm^{21} O^+$ or $imm^{434} O^+$			45	30	30	26

Cells were infected at a multiplicity of infection of about 5 with the indicated phages, and burst sizes, expressed as progeny phage per infected cell, were measured as described (6). Some values are the mean of two or more experiments, which gave results usually within 20% of each other. In co-infections, burst sizes of *spi* phages were between 10 and 30, except for superinfections of the λ^+ lysogen, where *spi-B* bursts were 0.1 and 0.2, and *spi-113*, *spi-325*, and *spi-330* bursts were 0.1 to 0.3 (with $\lambda imm^{21} susO$) or 4.1 to 7.4 (with $\lambda imm^{434} susO$). (-), infection of non-lysogen; (λ^+), infection of lysogen.

* All *spi* phages carry the *nin-5* mutation to allow plaque formation (7). *spi-B* ends within the *cl* gene (6).

† The lysogen was M72 SuIII⁺ (λ^+) and the non-lysogen was M72 SuIII⁻.

‡ $\lambda imm^{21} int red cl susO29$ and $\lambda imm^{434} int red cl susO8$ phages and *recA* Su⁻ (λ^+), and *recA* Su⁻ bacteria were those used previously (6). Burst sizes shown are for λimm^{21} and λimm^{434} .

prm. This would result in further autoregulation of the synthesis of this protein, as seen in many bacterial operons (32). Perhaps the primary role of the left half of O_R is to regulate leftward transcription from *prm*, while the right half regulates rightward transcription from P_R .

Recently Meyer *et al.* (24) have in fact observed that high concentrations of repressor repress *prm*-directed transcription in a cell-free system. Mutations affecting repressor binding in the right half of O_R do not drastically alter repression of *prm*. Reichardt (16–18) found that cells with 1, 2, or 3 prophages contained 1.0, 1.3, or 1.7 units of repressor, respectively, as if high concentrations of repressor partially prevented more from being made. A more indirect measure of repressor in intact cells earlier led Tomizawa and Ogawa to suggest this possibility (33).

Finally, we note that our isolation and analysis of *pre* mRNA, along with the sequence analysis of the rightward transcript from P_R (4, 22), unequivocally demonstrates the existence of bidirectional transcription of the λ genome, as deduced previously from hybridization studies (3).

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