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Nucleotide sequence of the operators of λ ultravirulent mutants

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ABSTRACT

The nucleotide sequence of the operators of ultravirulent mutants of λ, able to grow on host cells with elevated repressor levels, was determined. It appears that ultravirulence in λ requires multiple mutational events at the operator sequences. O_L1, O_L2, and O_L3 operator sites are the target of mutational changes in ultravirulent phages indicating that these sites participate *in vivo* in repression of the P_L promoter. No changes were found in the O_R3 sequence, in contrast there is a mutation in O_R2 and two mutations in O_R1, in both λ668 and λ2668 phages. This mutated operator structure accounts for the constitutive expression of their P_R promoter either in cells overproducing the λ repressor or in cells overproducing the *cro* gene product. A model of the structure of the λ operator site is proposed. The nucleotide sequence in each site can be divided into two functionally different subsets, one of which is recognized by the repressor while the other stabilizes the repressor-operator interaction.

INTRODUCTION

The *cI* gene of bacteriophage λ codes for a protein termed the λ repressor, which binds strongly to multiple sites in phage DNA (1, 2). The repressor binding sites are organized into two separate operators (O_L and O_R) located several thousand base pairs apart. Each operator contains three 17 base pair sequences (O_L1, 2, 3 ; O_R1, 2, 3) which have been proposed as repressor contact sites (3-5). The binding sites are similar but not identical in their nucleotide sequence and differ in their affinity for repressor (6).

In a lysogenic bacterium, the repressor acts to prevent the expression of early genes required for viral DNA replication and for lytic genes activation (for reviews see reference 7). In this way the repressor keeps silent virtually all phage genes in the prophage state. The repressor also prevents development of a superinfecting phage - this property is designated lysogen immunity (8). In doing this, the repressor binds to the O_L and

the O_R operators and blocks the initiation of RNA transcription at the corresponding P_L and P_R promoters (9-13). Binding of RNA-polymerase to these promoters is sterically prevented by the repressor bound to the operators because the promoter sequences partially overlap the operator sequences (3, 14). Beside P_R, a second promoter, P_{RM}, that controls repressor synthesis in λ lysogens, is located in the O_R control region. The repressor must be bound to the O_R1 and O_R2 operator sites to prevent transcription from P_R, and bound to the O_R3 operator site to prevent transcription from P_{RM} (see reference 5 for reviews).

Constitutive expression of functions controlled by the P_R and the P_L promoters leads to virulence, that is to say the ability of a phage to overcome immunity. In the classical λ virulent phage (λvir), isolated by Jacob and Wollman (8), this constitutive expression is caused by mutations in both operators that decrease their affinity for λ repressor (9, 10, 15).

Phage λ vir is no longer able to grow on host bacteria when the repressor level increases several fold above the one normally displayed by a lysogenic cell (16, 17). Bacteria with such elevated repressor levels have been constructed by inserting the phage cI gene into plasmids such as pKB252 (16).

Discrete elevated cellular levels of the λ repressor were correlated with increasing degree of immunity by isolating phage λ mutants with stepwise increased virulence (17). These phages, defined as ultravirulent, were divided into three groups on the basis of their ability to grow in cells containing more and more λ repressor (17). Their ultravirulence was thought (17) to result from multiple mutations in the operator regions leading to a decrease in the affinity of the λ repressor for the operator sites.

In this publication we determined the primary structure of the operators of ultravirulent phage mutants. We have shown that their ultravirulent phenotype actually results in an accumulation of sequence changes at the operator sites.

MATERIALS AND METHODS

Phage strains : The isolation of λ 146, λ 169, λ 668 and λ 2668 ultravirulent phages was described elsewhere (17). The ultravirulence degree of these phages is : 2668 = 668 > 169 > 146 (17). Phages λ 146, λ169 and λ668 have as common ancestor phage λ 11 (8) ; therefore they are

expected to carry the v2 and the v3 mutations respectively located in O_L1 and in O_R1 ; phage λ 2668, derived from λ vir (8), must also carry the v1 mutation located in O_R2 (3). All the ultravirulent phages have the b2 deletion (18) that removes about 13 % of λ DNA (19). The "wild" type reference phage was λ cIts2Sam7 (obtained from M. Ptashne). The cIts2 mutation generates an additional HindIII cleavage site in the cI gene.

λ repressor, enzymes and chemicals : Purified repressor was a gift from B. Sauer and from M. Fanica and P. Moreau. The repressor was titrated using the DNA binding assay as described elsewhere (13, 37). One mole of λrepressor is defined as the amount that retains one mole of λ DNA on a nitrocellulose filter. Restriction endonucleases were purchased from Biolabs. Restriction enzymes were used as recommended by the manufacturer. Bacterial alkaline phosphatase and polynucleotide kinase were from P.L. Biochemicals. Chemicals utilized were : dimethyl sulfate (Aldrich), hydrazine (Eastman Kodak), acrylamide and bis-acrylamide (2 fold crystallized grade, Serva). Piperidine from Merck was redistilled under vacuum.

Growth of phages and DNA preparations : Ultravirulent mutant phages were propagated by infection of E. coli C600 bacteria grown in L Broth at low m.o.i (0.01 phage/bacterium). Phage purification and DNA extraction were done as described elsewhere (20). DNA of λ cIts2Sam7 phage was a gift from A. Levine.

Purification of fragments bearing λ operators on sucrose gradients : About ten picomoles of each mutant phage DNA were cleaved with BglII and centrifuged in a sucrose gradient to purify two DNA fragments, called I and II, bearing respectively the O_L and the O_R operator (Fig. 1). Fragment I was cleaved with EcoRI. This hydrolysis generated three fragments : the large fragment Ic, fragment Ib and fragment Ia still containing the entire O_L operator (Fig. 1). After centrifugation in a sucrose gradient, fragment Ia was well separated from fragment Ic but not from fragment Ib. Sucrose gradients, performed as described elsewhere (20), were run 17 h at 26,000 rev/min.

Preparation of 5' ^{32}P labeled DNA fragments and DNA nucleotide sequence : DNA fragments recovered from sucrose gradients were hydrolyzed with appropriate restriction endonucleases cleaving in, or near to, the operator sequences. Restriction fragments were dephosphorylated by alkaline phosphatase which was then inactivated by alkaline treatment (21). DNA fragments were 5' labelled with γ ^{32}P ATP (NEN ; specific activity 2,500

Ci/mM) and polynucleotide kinase (22). Labelled fragments were fractionated by gel electrophoresis and operator containing DNA fragments were eluted from the gel (20). The two labelled ends were separated by polyacrylamide gel electrophoresis after restriction with another enzyme or denaturation of the DNA fragment (22). DNA fragments labelled at one 5' end were eluted from the preparative gel and dissolved in water. Chemical reactions, specific for G, A+G, C+T, T and A/C, giving a stronger band for A than C, were performed (22, 23, 24). Sequence reaction products dissolved in the loading solution were heat denatured and fractionated on acrylamide sequencing gels one millimeter thick.

RESULTS

I - Strategy used for sequence determination

In order to determine the primary structure of the operators from ultravirulent mutant phages, using the chemical degradation method of Maxam and Gilbert (22), restriction DNA fragments had to be prepared labelled at one end close to the operator sequence. Such fragments could be purified from wild type λDNA by exploiting the fact that λ repressor binds them with high specificity. Since repressor affinity for operator DNA from ultravirulent mutants was strongly reduced (data not shown) the above purification procedure could not be used. Therefore suitable operator containing DNA fragments had to be recognized in a total DNA hydrolysate, either on the basis of their electrophoretic mobility or using the corresponding wild type fragments trapped by λ repressor in the DNA hydrolysate as markers on gels. We found that the identification of the wanted DNA fragments and their specific end labelling were improved when partially purified phage operator DNA was used instead of the entire λ DNA molecule. Consequently we used for sequence determination a strategy the steps of which can be summarized as follows :

1) The mutated DNA was cleaved with restriction enzymes allowing large scale purification of phage operators on two separate DNA fragments called II and Ia (Fig. 1).

2) These fragments were hydrolyzed with appropriate restriction endonucleases so that λDNA is cleaved in, or close to, the operator sequences. These hydrolyses generate a relatively small number of DNA fragments (Fig. 2) since most of non-operator phage DNA (about 95% of non-O_R and 80% of non-O_L) was previously eliminated. Consequently DNA fragments were efficien-

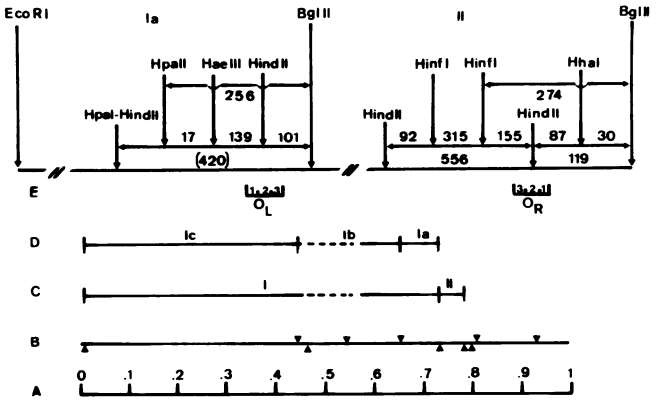


FIGURE 1 : Cleavage sites of restriction endonucleases used for sequence determination.

A) Physical map co-ordinates for λ DNA.

B) Cleavage sites in λDNA for EcoRI (▼) and for BglII (▲). Map locations are from Thomas and Davis (36) and from Pirrotta (25).

C) Operator containing DNA fragments generated by cleavage of λ b2 DNA with BglII. Fragments are designated so as to avoid confusion with the nomenclature employed by Pirrotta (25) for BglII wild type DNA fragments.

D) Fragments generated by cleavage of the BglII fragment I with EcoRI. Broken lines in c) and in d) indicate the portion of λ DNA removed by the b2 deletion.

E) Expanded map of fragments, Ia and II, showing restriction endonuclease sites in and around the operators. O_R and O_L indicate the right and the left operator ; 1, 2, 3 are repressor binding sites. Vertical arrows indicate restriction endonuclease cleavage sites ; except for the HindII/-HpaI site (26), their exact position is known from DNA or RNA sequence data. Distances between sites expressed in base pairs are not drawn to scale. Approximate distances, not calculated from sequence data, are in brackets.

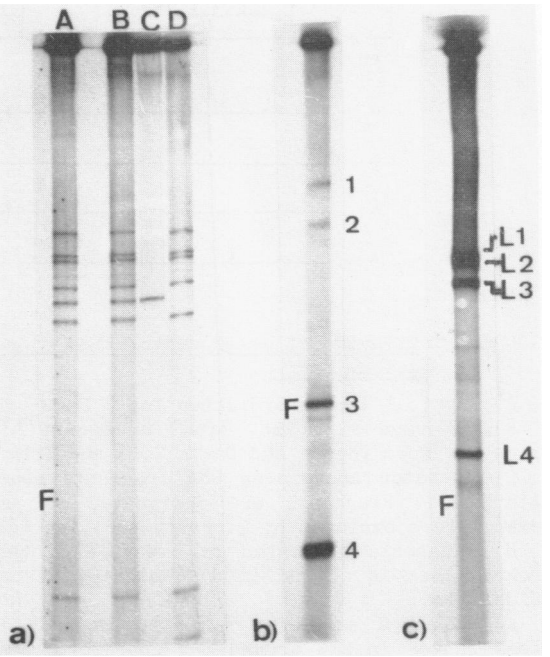
tly end labelled and those containing operator sequences were easily identified (Fig. 2).

3) Once purified fragments labelled at both 5' ends were prepared, we used either the DNA denaturation technique or the cleavage with appropriate restriction endonucleases to obtain DNA fragments labelled at one 5' end for sequence determination (see Results section II and III).

II - Mutational changes in O_L

The 420bp HpaI-BglII fragment (Fig. 1) labelled at both ends was purified from ultravirulent phage mutant DNAs and from wild type phage DNA as described in the legend of figure 2c. This fragment was hydrolyzed with

FIGURE 2 : Characterization of restriction fragments containing operators.



a) HinfI-BglII bearing O_R: The BglII fragment II from wild type or λ 668 phage DNAs was hydrolyzed with HinfI and end labelled. Excess of γ^{32}P ATP was eliminated by loading the DNA preparations on 1 ml sephadex G100 columns. An aliquot of the wild type DNA digest was treated with λ repressor. The reaction mixture was as described elsewhere (37). Repressor was in about ten-fold molar excess over operators. The mixture was filtered on a Schleicher and Schuell B-6 membrane filter. DNA fragments in the filtrate were recovered as well as operator containing DNA fragments retained on the filter which were eluted by denaturing the repressor protein in Tris 30mM pH 7.6, 0.2% SDS. All DNA samples were electrophoresed on a 6% polyacrylamide slab gel. A and B : HinfI digest of BglII fragment II from λ 668 (A) or λ wt (B) DNAs. C and D : fragments from wt DNA digest retained on (C) or passed through (D) the filter after repressor treatment.

b) HindII-BglII bearing O_R1 : The BglII fragment II from λ 2668 DNA was hydrolyzed with HindII and end labelled. The hydrolysate was fractionated on a 5% polyacrylamide slab gel. Fragment 1 and fragment 4 were recovered from the gel and hydrolyzed with HinfI or HhaI. The cleavage patterns obtained (data not shown) indicate that fragment 1 is the HindII-HindII 556 bp fragment bearing O_R2 and O_R3 and fragment 4 is the HindII-BglII 119 bp fragment bearing O_R1 (see fig. 1).

c) HpaI-BglII bearing O_L : A mixture of fragments Ia and Ib (generated from BglII fragment I cleaved with EcoRI) from λ 2668 phage DNA was hydrolyzed with HpaI and end labelled. The hydrolysate was fractionated on a 5% polyacrylamide slab gel. Fragment L1 was recovered and cleaved with HpaII or HaeIII. The cleavage patterns obtained (data not shown) indicate that this fragment is the HpaI-BglII 420 bp fragment carrying O_L (see fig. 1).

F indicates the position of the xylene cyanol dye.

HpaII to generate a fragment labelled at the BglII end that contains the entire left operator. A sequence of about 130 nucleotides from the labelled end was determined. Figure 3 shows sequencing gels used to establish the O_L nucleotide sequences.

There is perfect agreement between the wild type sequence (Table 1) we determined and that published (4, 27) by others. Several base pair changes, transitions as well as transversions, were found in the DNA sequence from

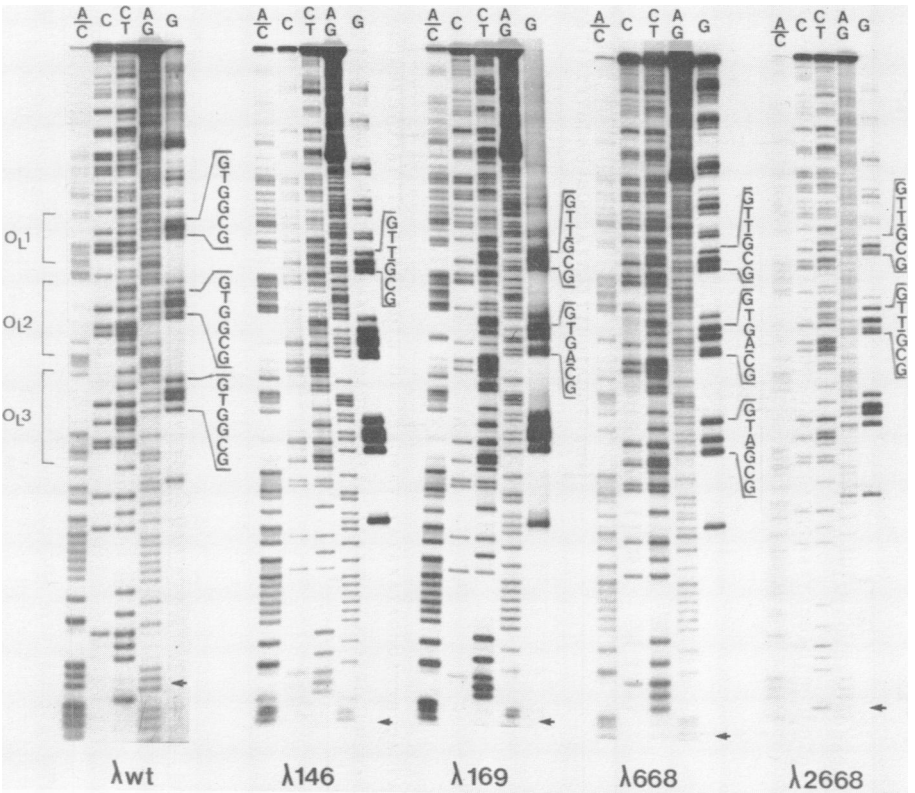


FIGURE 3 : O_L sequencing gels.

The *BglII-HpaI* fragment (asterisks denote labelled ends) purified as indicated in fig. 2c was cleaved with HpaII to remove the labelled HpaI end. Products of such hydrolysis were fractionated on a 6% polyacrylamide slab gel. The *BglII-HpaII DNA fragment bearing O_L was eluted from the gel and subjected to the Maxam-Gilbert chemical degradation. Products were fractionated on a 16% sequencing gel. Arrows give the position of the xylene cyanol marker dye. Bands corresponding to O_L1, O_L2 and O_L3 sequences are indicated. Representative DNA sequences are written. Bases differing from the wild type sequence are underlined.

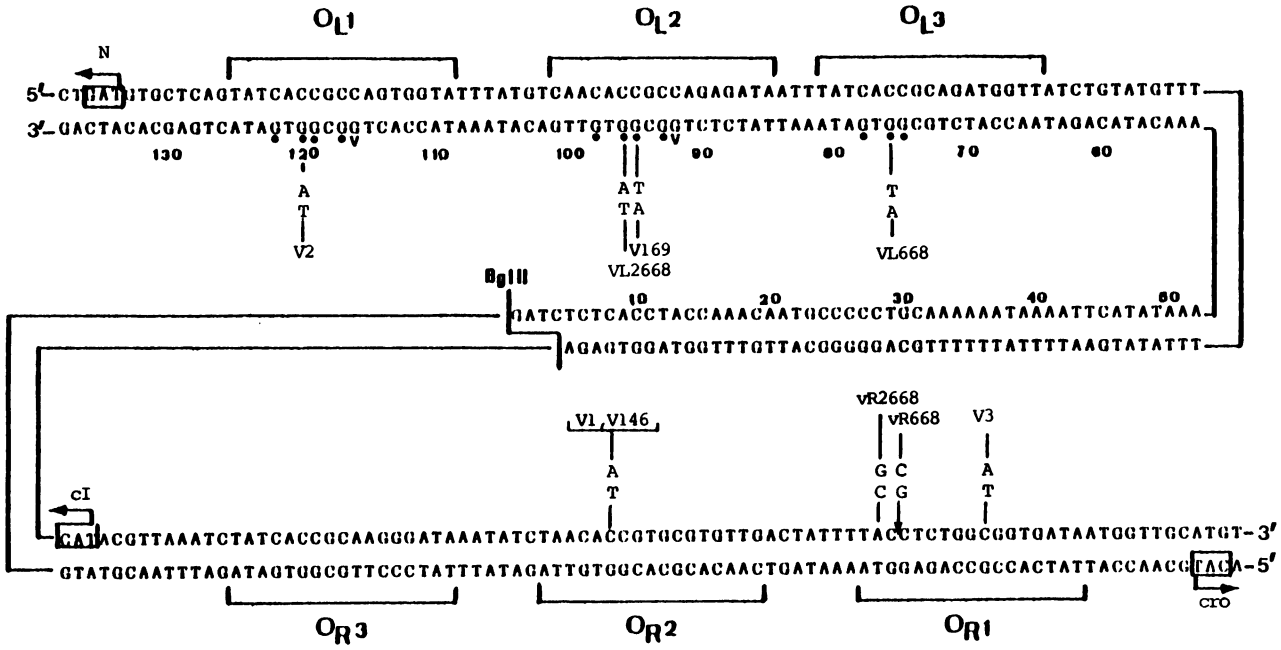


TABLE 1 : Sequence changes in the operators from ultravirulent phage mutants.

The sequence written is from the wild type λ DNA (4, 30, 27, 38, this paper). The repressor binding sites are as proposed (3, 4). The sequence changes observed in mutant phage DNAs are indicated. Bases in O_L are numbered from the BglII cleavage site. Guanines that are protected by repressor from methylation are indicated by a dot and those whose reaction rate is increased are indicated by a caret. Only guanines in the lower DNA strand are shown. Guanines protected in O_L1 are from Humayun, Kleid and Ptashne (29). The transcription initiation sites for genes N, cro and cI are indicated.

ultravirulent mutant phages (Table 1). All the sequence changes lie in the proposed (3, 4) repressor binding sites. The transversion C:G ⟶ A:T was found in O_L1 from all ultravirulent phages. This base pair change corresponds to the v2 mutation (27) carried by the parental phage λ 11. The transition C:G ⟶ T:A, v169, was found in O_L2 from λ169 and λ668 phages. The transition C:G ⟶ T:A, vL668, was observed in O_L3 from phage λ 668. The order in which mutations arose in the genealogy of the ultravirulent mutant phages is summarized in Table 2. The highest degree of constitutivity in this phage filiation results in three sequence changes in O_L. Another ultravirulent phage, λ 2668, coming from a different filiation (17) shows, beside the v2 base pair change, the transversion C:G ⟶ A:T, vL2668, located in O_L2.

Gilbert, Maxam and Mirzabekov (28) have shown that a protein bound to a DNA molecule can suppress or enhance the rate of chemical methylation by dimethylsulfate of certain bases within its recognition sequence. Since all the changes observed in the mutated operators lie in the GCGGTG sequence, this sequence must be recognized by the repressor.

In order to test in the GCGGTG hexanucleotide G residues shielded by bound repressor, we performed a dimethylsulfate experiment on O_L2 and O_L3 DNA sequences similar to that undertaken by Humayun, Kleid and Ptashne (29) on O_L1. We used DNA fragments bearing O_L from λ146 and λ169 phages. The

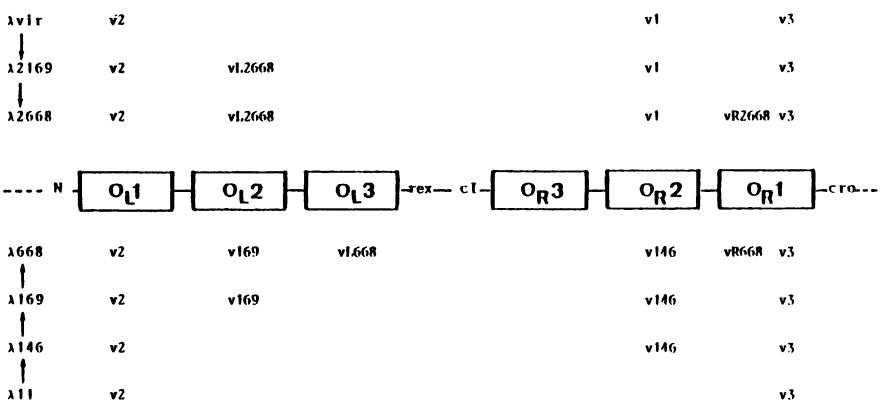


TABLE 2 : Genetic structure of operators from ultravirulent mutants of λ.

The genetic structure of operators from each mutant phage is given. The phage's derivation is indicated by arrows. The genetic structure of λ 2169 phage operators is only putative.

λ146 fragment carries wild type O_L2 and O_L3 sequences and the λ169 fragment carries only the wild type O_L3 sequence. DNA was methylated by dimethylsulfate in the presence or absence of λ repressor and then subjected to G-specific cleavage of methylated DNA (Fig. 4).

The pattern of G residues affected by bound protein in O_L2 is similar to that observed in O_L1. The repressor protected all G residues in the GCGGTG sequence and enhanced the methylation of a flanking G (Table 1). In contrast only three on four G residues in this hexanucleotide were protected by repressor bound to O_L3, the first being unaffected.

The fact that the repressor protected one fewer guanine in O_L3 is consistent with the different order of repressor affinities for O_L3 as compared with O_L2 and O_L1 operator sites.

The v2 mutation in O_L1 and the v169 mutation in O_L2 change the wild type sequence that becomes GCGTTG and GCAGTG respectively. We found that repressor did not protect any G residue in these mutated sequences from methylation. This result indicates that the repressor must actually be bound to the operator sequences to affect the pattern of methylated guanines.

III - Mutational changes in O_R

The O_R sequence was established on both complementary DNA strands. The

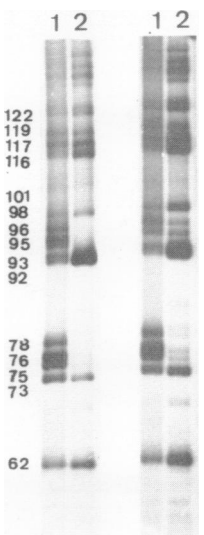


FIGURE 4 : Gel fractionation of G-specific cleavage products in O_L methylated in the absence or in the presence of repressor.

The HpaII-BglII* fragment (asterisk denotes the labelled end), bearing O_L, from λ169 and λ146 phage DNAs was dissolved in 180 µl DMS buffer (50mM sodium cacodylate pH 8.0, 10mM $MgCl_2$, 0.1mM EDTA) and 20 µl of λrepressor (more than 25-fold molar excess over operators, see Materials and Methods) (column 2) or 20 µl of λ repressor buffer (10mM Tris pH 8.0, 0.1mM EDTA, 0.1mM DTT, 200mM KCl, 5% glycerol) (column 1) were added. The mixtures were incubated at 20° for 10 min and methylation was then performed for 15 min at the same temparature with 100mM dimethyl sulfate. Methylated DNA was depurinated with piperidine (22) and G-specific cleavage products were fractionated on a 16% sequencing gel. Bands corresponding to guanines in and around O_L are numbered according to Table 1. Guanine 95 and guanine 120 are respectively missing in the v169 O_L2 sequence and in the v2 O_L1 sequence.

BglII-HinfI 274 bp fragment (Fig. 1) from ultravirulent phage DNAs was purified as described in the legend of figure 2a. The DNA fragment labelled at both ends was denatured and strands were separated by gel electrophoresis. DNA sequences covering O_R were derived from each labelled end. An O_R1 sequence, complementary to that read from the BglII end, was also established from the HindII labelled end of the HindII-HhaI fragment (Fig. 1). Representative gels used for sequence determination are shown in figure 5 and 6. The sequence changes observed are summarized in Table 1 and the order in which they arose in the phage's filiation is given in Table 2.

The O_R3 sequence from ultravirulent mutants did not differ from the wild type published sequence (4, 30). The transversion C:G→A:T, v146, was observed in O_R2 from phage λ146 and its derivatives. This sequence change is identical to that caused by the v1 mutation (3). Phages λ 146 and λ 169 showed no additional changes in the O_R1 sequence except for the C:G →A:T transversion, v3 (3) carried by the parental phage λ 11. In contrast phage λ668 acquired the insertion of the C:G base pair, vR668, in its O_R1 sequence. In a similar way the ultravirulent phage λ 2668, beside the v1 and the v3 sequence changes carried by its ancestor phage λ vir, acquired the transition A:T →G:C, vR2668, located in O_R1.

DISCUSSION

Ultravirulence in λ requires multiple mutational events

The primary structure of the operators from ultravirulent λ phages was determined. Changes were found in the DNA sequences proposed (3, 4, 5) as repressor binding sites. Six and five mutational changes were found respectively in λ668 and in λ2668 the most ultravirulent phages we examined. Even though we cannot exclude that these phages have acquired mutations elsewhere it seems that their ultravirulent phenotype results in an accumulation of sequence changes at the operator sites. Because operator and promoter sequences interpenetrate, it is not surprising that several mutational events are required for ultravirulence in λ. A single mutational event, such as a deletion removing the operator sequences would also delete the promoter sequences.

In vitro repressor binds to operator sites in O_L with the following affinity order $O_L1 > O_L2 > O_L3$. *In vivo* site occupation must be a function of the concentration of repressor in the cell. Consequently, it is expected that O_L1 first and then O_L2 and O_L3 will be the target of operator

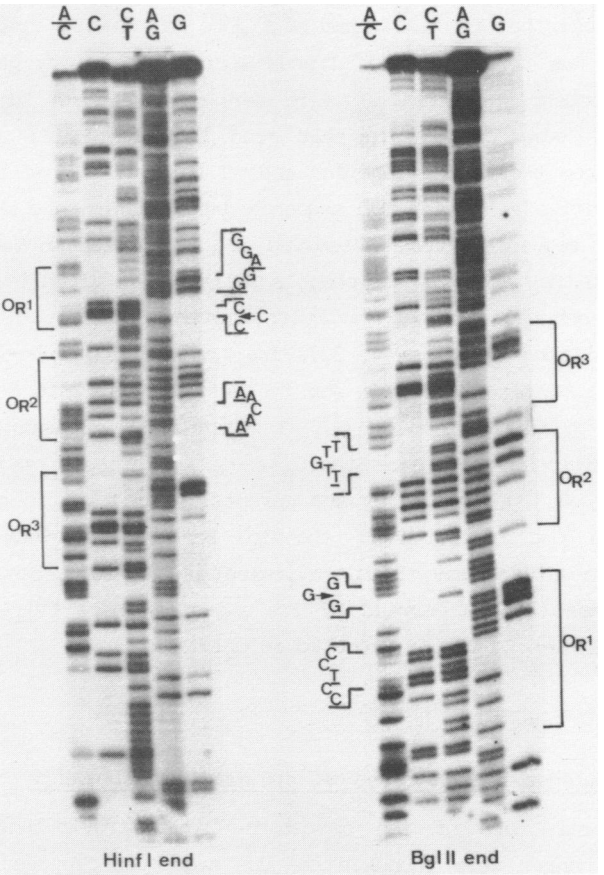


FIGURE 5 : O_R sequencing gels.

The *HinfI-BglII* fragment bearing O_R (asterisks denote labelled ends) from phage λ668 DNA purified as described in fig. 3a was heat denatured. Strands were separated by electrophoresis on a 5% polyacrylamide slab gel. The two single stranded DNA fragments labelled at one end were eluted from the gel and subjected to the Maxam-Gilbert chemical degradation. Products were fractionated on a 16% sequencing gel that was run until the xylene cyanol marker had migrated down to 76 cm from the top. The position of the O_R1, O_R2 and O_R3 repressor binding sites is indicated. Representative sequences from the mutant DNA are written. Bases differing from the wild type sequence are underlined. The insertions are indicated by arrows.

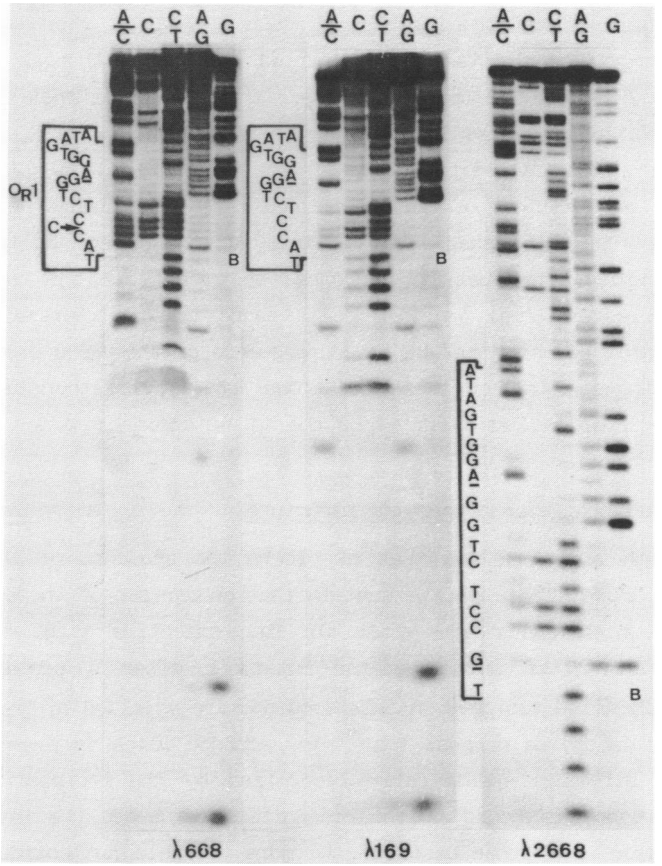


FIGURE 6 : O_R1 sequencing gels.

The *BglII-HindII* fragment (asterisks indicate labelled ends) purified as described in fig. 3b was cleaved with HhaI to remove the BglII labelled end. Cleavage products were separated by electrophoresis on a 6% polyacrylamide slab gel. The *HindII-HhaI fragment bearing O_R1 was eluted from the gel and subjected to the Maxam-Gilbert chemical degradation. Products from λ668 and λ169 phage DNAs were fractionated on a 25% sequencing gel and products from λ2668 phage DNA on a 16% sequencing gel. B gives the position of the bromophenol blue marker dye. Bands corresponding to the O_R1 sequence are indicated. Representative sequences from mutant DNAs are written. Bases differing from the wild type sequence are underlined. The insertion is indicated by an arrow.

constitutive mutations when the repressor level in the selective host cell increases. Our results strongly support such a model of repressor action. The most ultravirulent mutant λ 668 carried mutational changes in O_L1 as well as in O_L2 and in O_L3 operator sites ; this indicates that in vivo the three repressor binding sites participate in the repression of P_L.

Strong constitutive expression of P_R in λ 668 and in λ 2668 phages resulted in acquisition of an additional sequence change in O_R1. We postulate that in these phages the affinity for repressor of the primary repressor binding site must be decreased so greatly that a hundred-fold increased repressor level in host cells cannot ensure repression of P_R. Such a decreased affinity may be the consequence of the vR668 or the vR2668 sequence changes alone or these mutations may act together with the v3 mutation. Moreover phages must carry the v1 mutation in order to escape from repressor action at O_R2.

Mutational changes and operator structure

Through our work and that of others the sequence of 18 operator mutations is known (Table 3). We sustain that comparison of these sequences will reveal which bases are vital for the effective tight binding of λ repressor. Eleven of such operator mutations alter a pentanucleotide sequence $\frac{CGGTG}{GCCAC}$. All G residues in this sequence are shielded by the repressor (29, this paper).We suggest that this sequence which is present in all six operator sites is specifically recognized by the repressor protein. Once the repressor is bound to the recognition sequence, the interactions of the repressor with other bases of the more variable nucleotide sequence in each operator site will determine the stability of the repressor-operator complex. Bases in this sequence will be the target of operator constitutive mutations only when their nature and their relative position is vital for a stable repressor-operator interaction. This is the case of the A:T bp in position 2 and of the C:G bp in position 6 (Table 3). Note that in the wild type O_L3 a T:A bp is encountered in position 6, and that in the wild type O_R3 a C:G base pair is found in position 5. In both these operator sites the G residue in position 8 (upper strands in Table 3) is replaced by a T. The position of this G residue seems to be important for the effective repressor contact to the G residue in position 9. These particular features of the O_L3 and the O_R3 operator sites can tentatively account for their reduced affinity for repressor. We do not know the importance (if any) of bases at positions 1, 3, 15, 16, 17 in the proposed

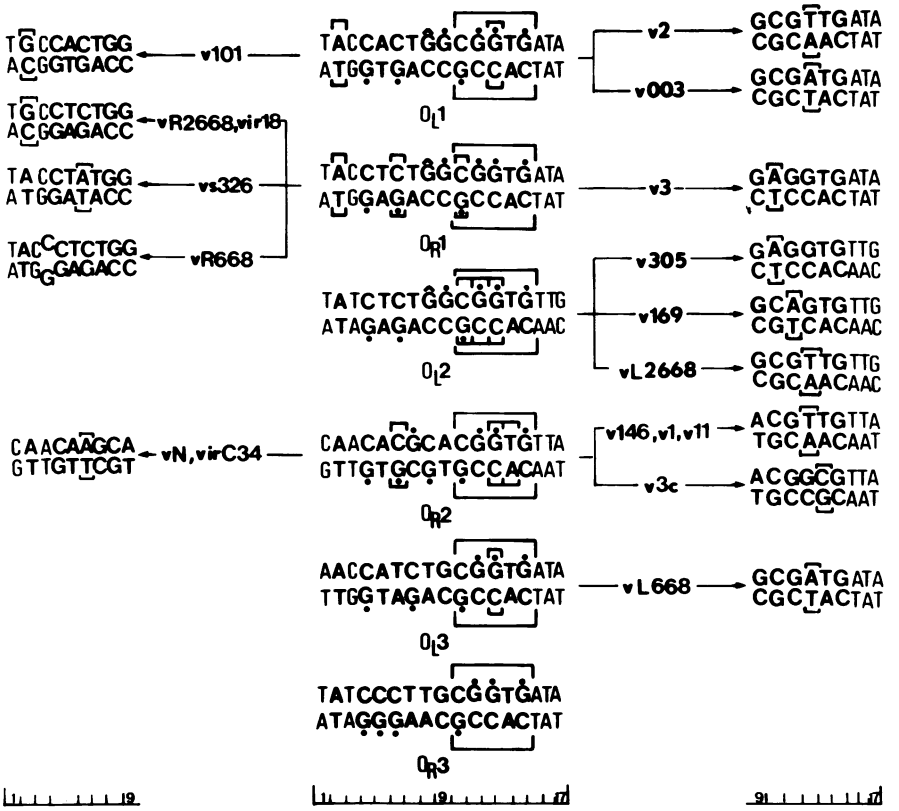


TABLE 3 : Comparison of sequences in operator sites.

The operator site sequences in Table I are written so as to bring out similarities. Guanines whose methylation is modified by repressor (29, this paper) are indicated as in Table I. Sites of known operator mutations are boxed (3, 27, 6, 39 and this paper) and mutated sequences are given. Bases in the proposed (3, 4) wild type sequences that we believe actually interact with repressor are indicated by bold face letters. The sequence thought to be recognized by the repressor is written in carets. Bases in mutant and wild type sequences are numbered as indicated.

Guanine in lower DNA strand in O_L1 have not yet been tested.

repressor binding sites. Note that the G residue at position 17 in O_L2 (see Results section II) and the G residue at position 1 in O_R2 (29) were not protected by bound repressor against methylation by dimethylsulfate. This was also the case of the G residues at position 3 in O_R1, in O_L1 and in O_L3 (29).

Structure of the mutated operators and escape of ultravirulent phages from cro repression

In the λ life cycle there is a second repressor protein, the product of gene cro, that functions midway to turn off the expression of the early genes (including that of cro itself) and of the cI gene (For review see reference 7). The cro protein recognize the same regions of DNA as does λrepressor (3, 32). Johnson, Meyer and Ptashne (33) have shown that this protein binds to the same three sites in O_R as does the λ repressor and protects from methylation a subset of those purines protected by λ repressor. Phages λ688 and λ2668 are insensitive to repression by the cro gene product (17) as the supervirulent mutant λ v2v3v_S326 isolated by Ordal (34) is. Changes conferred by vR668, vR2668 and v_S326 mutations are clustered in the O_R1 sequence (Table 3). These mutations may define a site essential for the cro protein-DNA interaction. Note that neither λ146 (v2v146v3) nor λ169 (v2v169v146v3) are supervirulent (17). On the other hand the v_S326 mutation alone, without v3, can allow constitutive expression from P_R under cro repression (6). It can be inferred that the v3 mutation does not affect the binding of the cro protein to DNA. This can also be predicted from the pattern of the G residues shielded by the cro protein in O_R1 (29) and from the sequence change caused by the v3 mutation.

Why do the most ultravirulent phages we examined escape from cro repression ? These phages carry the v3 mutation in O_R1 that alters the "recognition" sequence of λ repressor but, as we pointed out, does not affect the cro protein-DNA interaction. The acquisition by the phages of a second mutation, such as vR668 or vR2668, located in the "stabilizing sequence" in O_R1 will further decrease the affinity for repressor of the mutated O_R1 as it is required for ultravirulence. If bases in the "stabilizing sequence" (at most that affected by these mutations) are also vital for a stable cro protein-DNA complex, then it is expected that ultravirulent mutant phages derived from phage ancestors carrying the v3 mutation will also be insensitive to the cro repression.

Mutational pathways to ultravirulence

The comparison of the mutational changes in λ668 and in λ2668 phages indicates that the mutational pathway for producing ultravirulence in phage λ is not unique. Ultravirulence in λ 668 results in three mutations in the O_L operator, in contrast O_L from λ2668 shows only two mutational changes. The O_R operator of these two phages also differs - λ 668 O_R1 has an

insertion and λ2668 O_R1 a base pair change. One can speculate that an allele combination in O_L giving a relatively weak constitutive expression from P_L can confer an ultravirulent phenotype if the allele combination in O_R gives a strong constitutive expression from P_R. Sly, Rabideau and Kolber (35) have shown that in the classical λ vir the v2 mutation in O_R1 enhances virulence but is not required. It is possible that the combination of mutation v1vR2668v3 in λ2668 O_R gives a stronger constitutivity than the combination of v1vR668v3 mutations in the O_R operator of λ668 phage. It is also possible that the O_L3 mutation in λ668 increases its ultravirulence degree. R. Devoret informed us that λ668 phage shows higher constitutive expression of lytic functions than λ2668. A specific ultravirulent phenotype seems to depend on a given combination of alleles in the operator sites.

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