A genetic selection for temperature-sensitive variants of the gene *V* protein of bacteriophage *f1*

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ABSTRACT

Complementary negative and positive genetic selections based on the activity of a plasmid-encoded bacteriophage *f1* gene *V* are developed. The negative selection is based on an activity of the gene *V* protein in *E. coli* cells which markedly reduces the infection of those cells by *f1*-related viruses. In order to select against cells expressing active gene *V* protein, the cells are infected with the phage R386, a derivative of *f1* which confers resistance to chloramphenicol, and are plated in the presence of the antibiotic. Those cells which contain gene *V* protein are infrequently infected with the virus and are unable to grow in the presence of chloramphenicol; those which do not contain the gene *V* protein are readily infected and can grow in the presence of the antibiotic. The positive genetic selection consists of excising the gene *V* sequences from the plasmids and using them to replace the gene *V* of a bacteriophage *f1* derivative containing an amber mutation in gene *V*. Only those genes which encode an active gene *V* protein can support phage growth and yield plaques. The two genetic selections can be combined in order to yield a substantial enrichment for genes encoding temperature-sensitive gene *V* proteins.

INTRODUCTION

The gene *V* protein of bacteriophage *f1* is a small dimeric protein which binds to single-stranded DNA and RNA (for reviews, see references 1 and 2). After the initial stages of infection of *E. coli* by this phage, the gene *V* protein inhibits replication of viral strand DNA and instead allows the DNA to be packaged into virions (3). The gene *V* protein also inhibits translation of viral gene II mRNA (4-6). With these two functions, the gene *V* protein effectively switches viral replication from *rf* -> *rf* synthesis to *rf*-> *ss* synthesis, and simultaneously regulates the level of the gene II protein which is required in both stages of replication. The gene *V* protein binds to essentially any single-stranded DNA or RNA, but its repression of gene II translation suggests that it has a specific interaction with gene II mRNA (6).

Because the gene *V* protein is readily obtainable in large quantity, it has been frequently used as a model for the study of single-strand nucleic acid-protein interactions. The three-dimensional structure of the protein has been determined by x-ray crystallography (7) and the contacts between the gene *V* protein and nucleic acids have been investigated by a variety of techniques, including nuclear magnetic resonance, fluorescence, and chemical modification studies (8-10).

Recently, we have developed a plasmid-based system for expression and mutagenesis of a synthetic gene $\bar{V}$ which may prove useful in studying the relationship between the sequence of gene $\bar{V}$ protein and its folding, stability, and nucleic-acid binding properties (11). In this work we describe complementary positive and negative genetic selections based on activities of the gene $\bar{V}$ protein. We then show that these selections can be used to considerably enrich a mixed culture of *E. coli* expressing mutant and wild-type genes $\bar{V}$ for a temperature-sensitive gene $\bar{V}$ phenotype.

MATERIALS AND METHODS

Bacteria, phage, plasmids, and media

The wild-type male strain *E. coli* K38 (HfrC *phoA tonA22 garB10 ompF relA1 pit10 spoT1 spoT2* λ , 12, 13) and its derivatives K561 (K38 *lacI^Q*, 14) and K870 (K38 *supP*, 6) as well as the phage f1 temperature-sensitive derivative R111 (5) and the chloramphenicol-resistant derivative R386 (M. Russel and P. Model, personal communication) were obtained from P. Model. The phage R386 consists of the *Cm^r* gene from pSK114 (15) on a *Pst* I fragment cloned into the unique *Pst* I site of f1 derivative CGF3 (16). The plaques are somewhat turbid. The phage renders its host *Cm^r*, but not at high efficiency, presumably because of killing. *E. coli* JM110 (17) was from J. Messing. The helper phage M13K07 (18) was obtained from Pharmacia. Plasmids pMC-9 (19) and pSC101 (20) were obtained from M. Casadaban. Plasmid pMC-9 contains, within a fragment bounded by *Eco* RI restriction sites, the *lacI* promoter and gene with the *lacI^Q* mutation. Plasmid pTT18 (Fig. 1) is derived from plasmid pEMBL-8 (21) and contains the *tac* promoter followed by a synthetic phage f1 gene $\bar{V}$ (11). Plasmid pTT19 is derived from plasmid pTT18 and contains amber codons in place of leucine 37 and leucine 44 of gene $\bar{V}$. Plasmid pTT20 is derived from a related plasmid (pTT12) and contains a single amber codon in place of leucine 37 of the synthetic gene $\bar{V}$.

Bacterial strains and phage were propagated as described (16). Single isolates of plasmids were obtained by transformation with purified plasmid and isolation of single colonies or by transduction of plasmid DNA into a male host strain. In the latter case, plasmids were packaged by helper phage M13K07, transduced into strain K561, and single colonies were isolated.

The media used was 2YT (22) except for storage of colonies on plates, in which case minimal A media was used (22). Ampicillin, when required, was used at a concentration of 150 μ g/ml in liquid media and 50 μ g/ml in plates. Tetracycline, where required, was used at 12.5 μ g/ml, and chloramphenicol, where required, was used at 30 μ g/ml. All growth was at 37° except as noted; growth in liquid media was with vigorous shaking except as noted.

Competent cells were prepared by the calcium chloride procedure (23) or by a high-efficiency method (24).

DNA sequencing

Sequencing was carried out by the chain termination method (25) using α -³⁵S-dATP as described (26). Single-stranded plasmid DNA ('+' strand) was obtained by infection of *E. coli* K561 containing the appropriate plasmid with the helper phage M13K07 (18).

Construction of *lacI*^Q plasmid pTT15

Plasmid pMC-9 contains the *lacI*^Q mutation in the *lacI* promoter and gene between two *Eco* RI restriction sites, and plasmid pSC101 contains a single *Eco* RI site. The 1.9 kb *Eco* RI-*Eco* RI fragment of pMC-9 containing the *lacI* gene was inserted into the corresponding site of pSC101 by standard methods (27). The resulting 11.2 kb plasmid, conferring tetracycline resistance and overexpression of the *lac* repressor on its host, was named pTT15. Overnight cultures, cultures from -80° permanents, and plates with strains containing plasmid pTT15 were grown in the presence of tetracycline (12.5 µg/ml), but the antibiotic was otherwise generally omitted.

Construction of temperature-sensitive gene V plasmid pTT21

The temperature-sensitive fl mutant R111 contains a single C->T base change leading to the substitution of cysteine for arginine-82 in the gene V protein. The corresponding substitution was made in the synthetic gene V in plasmid pTT18, yielding plasmid pTT21. This plasmid was constructed by direct ligation of a mutagenic oligodeoxynucleotide onto a gapped heteroduplex as follows (28). A heteroduplex consisting of the uracil-containing '+' strand of plasmid pTT18, and the '-' strand of pTT18 lacking the fragment between the *Xba* I and *Sna* BI sites on this plasmid was constructed and purified. The phosphorylated oligodeoxynucleotide 5'-dCAAGCATAACGATCAATCATAAGAGAACCGAAGTGG-3' (TCT25) was a gift of P. Gardner and D. Steiner and was purified by polyacrylamide gel electrophoresis (29). This oligodeoxynucleotide is complementary to the gap in the heteroduplex at all points except the base in bold type, and was ligated directly to the heteroduplex. The ligated DNA was used to transform competent K561 cells. Sixteen of the resulting transformants were checked for the production of active gene V protein at 42° by comparing their growth rates in the presence and absence of the inducer 1 mM isopropylthiogalactoside (IPTG). Cells expressing the wild-type gene V protein at a high level grow very poorly. Fourteen of the sixteen isolates tested showed equal growth in the presence and absence of IPTG at 42°, indicating that they did not produce active gene V protein at this temperature. One of these was sequenced between the *Sna* BI and *Xba* I sites used to construct it and was found to have the expected single base change (CGC -> TGC; Arg82 -> Cys). This plasmid was named pTT21.

Construction of the gene V amber mutant phage f1T2

Plasmid pTT20 contains the synthetic gene V from plasmid pTT12, but the fragment of this plasmid between the restriction sites for *Mlu* I and *Kpn* I contains an amber codon at leucine 37. This fragment was ligated by standard methods (23) between the corresponding sites of phage f1T1. Phage f1T1 contains the entire synthetic gene V from plasmid pTT12 (11). The ligated DNA was used to transform competent *E. coli* K870 (*supP*). Five plaques were tested for growth on K38 (*sup^Q*) and K870. Three grew only on K870, and one of these was named f1T2. Phage f1T2 grows well on K870 (*supP*, leucine-inserting) but not on JM110 (*supE*, glutamine-inserting).

Generation of site-directed random mutants

A set of point mutations located between the restriction sites for the enzymes *Xba* I and *Sna* BI was generated by ligation of a mixture of oligonucleotides into a gapped heteroduplex DNA (28). The oligonucleotides in the mixture span the gap between the sites for these restriction enzymes and were synthesized such that at each position, the base corresponding to the pTT18 sequence was incorporated at a frequency of 95.5%, and the

remaining three nucleotides each at 1.5%. The oligonucleotides contained, on average, two base substitutions from pTT18. The mutagenesis procedure yielded approximately 2500 independent isolates.

Negative genetic selection based on gene V protein-dependent inhibition of infection by R386

An overnight culture of *E. coli* K561 pTT15 containing the plasmid to be tested (e.g., pTT18, encoding the synthetic gene *V*) is diluted 1:100 in 1 ml of 2YT media containing ampicillin (150 µg/ml), and is grown to a cell density of 3×10^8 cells/ml (O.D.₆₅₀ ~ 0.5). IPTG is then added to a concentration of 1 mM, and the culture is shaken at 37° for 30 minutes to induce the expression of gene *V*. (For isolation of temperature-sensitive mutants, this IPTG induction step is carried out at 42°.) Then, in a 37° (or 42°) 1.5 ml plastic tube, 20 µl of this culture is added to 2 µl of a stock of bacteriophage R386 containing 3×10^{10} phage/ml. The tube is capped and left at 37° (or 42°), without shaking, for 30 minutes to allow the phage to infect those cells which are susceptible to infection. Then 1 ml of 2YT media containing ampicillin (150 µg/ml) and chloramphenicol (30 µg/ml) is added, the contents of the tube are mixed, and the tube is placed at room temperature. Within 20 minutes, 10 µl of the infected culture, or 10 µl of a 1:100 dilution of this culture are plated on 2YT media containing ampicillin (50 µg/ml) and chloramphenicol (30 µg/ml). The plates are left at 37° for about 20 hours to allow the infected cells to grow.

Positive selection based on gene V protein requirement for propagation of phage f1T1

A pool of *E. coli* K561 containing plasmid pTT15 (*lacI*^Q), replicative-form phage R386 DNA, and the plasmids to be selected (e.g., pTT18) was obtained from the negative selection procedure described above. Plasmid DNA was isolated from the pool of colonies by the alkaline lysis method, and 25 to 300 ng of the 4.3 kB plasmid pTT18 (or its equivalent) were separated from the plasmid pTT15 (11.2 kB) and the R386 rf DNA (8.3 kB) by electrophoresis in 1% low melting temperature agarose, followed by removal of that part of the gel containing the pTT18 DNA.

The plasmid DNA in agarose was heated to 70°, the mixture was diluted so as to lower the agarose concentration to 0.2%, and the DNA was cleaved at 37° by the restriction enzymes *Mlu* I and *Kpn* I. There are sites for these enzymes near the 5' and 3' ends of gene *V*, respectively (Fig. 1). The mixture was extracted with phenol, then with chloroform. The purified DNA was precipitated with ethanol and resuspended at a concentration of about 5 ng/µl in water.

Alternatively, plasmid DNA was purified from agarose gels using Gene Clean (Bio101). This DNA was used to transform K561 cells in order to increase the amount of DNA with which to work, and plasmid DNA was obtained from ampicillin-selected transformants using the alkaline lysis method. This DNA was cut with the restriction enzymes *Mlu* I and *Kpn* I, purified with Gene Clean and resuspended at a concentration of about 5 ng/µl in water.

The restriction enzyme-cleaved plasmid DNA was then ligated to phage f1T2 rf DNA, cleaved with the same enzymes. Phage f1T2 contains a synthetic gene *V* with an amber codon instead of leucine 37, and has restriction sites for *Mlu* I and *Kpn* I. The plasmid DNA (5 ng) was added to the phage DNA (4 ng) in a volume of 5 µl of 30 mM tris (hydroxymethyl) aminomethane pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol and 1 mM ATP,

containing 15 units/ml of T4 DNA ligase (Boehringer Mannheim). The ligation mixture was left at 16° overnight, then 2.5 µl were used to transform competent K38 cells. The transformants were plated along with 200 µl of a fresh culture of K38 in 2YT top agar and were left at 37° (or 34° for selection of temperature-sensitive mutants) overnight. Plaques were transferred to 1 ml of 2YT media which was then placed at 65° for 10 minutes to kill bacteria.

Assay for temperature sensitivity

Temperature-sensitive phage were initially identified by touching a phage stock (about 10⁸ pfu/ml) with a sterile toothpick, transferring phage to lawns of K38 cells, and noting which phage formed plaques at 34° but not at 42°. To characterize the growth of the phage in more detail, dilutions of the phage stocks were mixed with K38 cells, plated in top agar, and grown at 34° and 42°.

RESULTS

Development of a negative selection based on activity of the gene V protein

A principal role of gene V protein in bacteriophage f1 propagation is to block viral DNA complementary strand synthesis (3, 30). It therefore seemed likely that a selection procedure could be based on this property. It was shown earlier that low-level production of gene V protein in *E. coli* cells markedly reduces the propagation of f1 phage introduced either by infection or transfection (30). A selection procedure involving infection by the phage R386, a derivative of phage f1 which confers resistance to chloramphenicol, was developed based on this property. Cells infected by R386 can grow in the presence of chloramphenicol. *E. coli* which express gene V at a high level, however, are very poorly infected by this phage, so that very few can grow in the presence of the antibiotic.

This selection procedure was tested using the *E. coli* strain K561/pTT15, a male strain expressing the *lac* repressor at a very high level, and the plasmids pTT18, encoding a synthetic gene V, or pTT19, identical to pTT18 except that it contains two amber codons in gene V. The strains were grown, treated with IPTG to induce gene V expression, infected with phage R386, and plated as described in METHODS, with the modifications listed.

Table I shows the number of colonies obtained from plating approximately 10⁵ cells, subjected to variations of this procedure, in the presence of chloramphenicol. Rows 1-6 of Table I show that in the absence of IPTG, about 4000 colonies are obtained from this procedure, indicating that about 4% of the cells are infected by R386 phage. As increasing concentrations of IPTG are added, fewer cells are infected, so that when 1 mM IPTG is added only about 20 colonies are obtained, corresponding to infection of only 0.02% of the cells.

The low fraction of cells infected even in the absence of IPTG may be due to several factors. First, not all cells infected by R386 are viable (Model, P., personal communication). Second, the plasmid pTT18 contains the phage f1 replication origin. Plasmids containing these sequences are known to interfere with phage propagation (31). Finally, some gene V protein may be produced by these cells even in the absence of IPTG (see below).

In the preceding experiments, IPTG, where added, was present throughout the infection process. Rows 7-10 of Table I indicate that addition

Table I. Infection of strains K561/pTT15 containing plasmids pTT18 (encoding wild-type gene γ protein,) or pTT19 (encoding an amber mutant gene γ) with phage R386 under various conditions, followed by plating in the presence of chloramphenicol.

	Plasmid	IPTG		R386 infection		Number of colonies obtained on chloramphenicol plates	
		concentration (mM)	time before infection (min)	phage/ml during infection	time between infection and chloramphenicol addition (min)	-IPTG	+IPTG
1.	pTT18	0.00	30	3×10^{10}	30	4000	-
2.	pTT18	0.01	30	3×10^{10}	30		7200
3.	pTT18	0.02	30	3×10^{10}	30		3200
4.	pTT18	0.05	30	3×10^{10}	30		1400
5.	pTT18	0.10	30	3×10^{10}	30		104
6.	pTT18	1.00	30	3×10^{10}	30		19
7.	pTT18	1.00	0	3×10^{10}	30	3200	4400
8.	pTT18	1.00	15	3×10^{10}	30		58
9.	pTT18	1.00	30	3×10^{10}	30	4000	20
10.	pTT18	1.00	45	3×10^{10}	30		4
11.	pTT18	1.00	30	3×10^{10}	2	250	2
12.	pTT18	1.00	30	3×10^{10}	30	4800	22
13.	pTT18	1.00	30	3×10^{10}	60	4800	9
14.	pTT18	1.00	30	0	30	0	0
15.	pTT18	1.00	30	0.3×10^{10}	30	2000	0
16.	pTT18	1.00	30	3×10^{10}	30	4200	100
17.	pTT18	1.00	30	10×10^{10}	30	6400	64
18.	pTT19	1.00	30	3×10^{10}	30	8000	8000

of IPTG before initiation of infection is required for substantial inhibition of infection. In this set of experiments, aliquots from a single culture were removed and treated with IPTG at various times, then all cultures were simultaneously infected with the phage R386.

The time allowed between addition of the phage R386 and the addition of chloramphenicol seems not to be critical (Table I, rows 11-13), as the same results were obtained after either 30 or 60 minutes of growth after infection. The titer of the infecting phage affects the fraction of cells which become infected, as indicated by Table I, rows 14-17. This titer also appears to affect the ratio of infected cells in the presence and absence of inducer, with the greatest difference found with a low titer of infecting phage.

Finally, row 18 shows that when a strain containing an inactive gene γ (K561/pTT15/pTT19) is infected by phage R386, the number of cells infected is not dependent on whether IPTG is added. The slightly higher yield of infected cells, compared to infection of K561/pTT15/pTT18 in the absence of IPTG, suggests that there is some gene γ protein synthesis by the latter strain even without induction.

Test of the negative genetic selection with a temperature-sensitive variant of the gene V protein

The bacteriophage f1 derivative R111 is temperature sensitive for growth (5), due to the substitution of cysteine for arginine-82 in its gene V protein (unpublished data). The phage grows at 34° - 37°, but not at 42°. A single base change was incorporated into the synthetic gene V of plasmid pTT18 so that it encoded the temperature-sensitive gene V protein (see METHODS), yielding plasmid pTT21.

The inhibition of phage R386 infection by the wild-type gene V protein from plasmid pTT18, and by the temperature-sensitive gene V protein from plasmid pTT21 were tested. This was done as described above, except that the induction of gene V expression and the infection with phage R386 were each for only 15 minutes, without shaking, and both induction and infection were carried out at 34°, 37°, or 42°. Table II shows the results of plating approximately 7.5×10^4 cells in the presence of chloramphenicol at the end of this procedure.

Somewhat surprisingly, neither the wild-type nor the temperature-sensitive gene V proteins showed a marked temperature dependence of their inhibition of infection by the phage R386. Instead, the temperature-sensitive gene V protein appears relatively inactive in this test at all temperatures, only inhibiting infection by about 60%. That the gene V in plasmid pTT21 does in fact lead to a temperature-sensitive phenotype is demonstrated below (see Table III). The relative inactivity of the temperature-sensitive gene V protein at even the permissive temperature is consistent with earlier work on the repression of gene II translation by gene V protein (5). It was found in that work that repression was more pronounced at the permissive temperature than at the restrictive temperature, but the repression in both cases was much less than by the wild-type protein. It therefore seems likely that the gene V protein encoded by phage R111 is marginally active and temperature-sensitive, and that in our tests the activity is so low that little temperature dependence is observed.

Support of phage growth as a positive selection for gene V protein

The negative selection described above can be used to enrich a population of plasmids for those which do not encode active gene V protein. The resulting plasmids might encode generally inactive gene V proteins, partially inactive proteins, or conditionally inactive proteins. If a strong positive selection procedure were available, the partially or conditionally inactive proteins could be directly selected from this pool.

A strong positive selection can be accomplished by transfer of a gene V to be selected from a plasmid directly into a derivative of phage f1. For this purpose, phage f1T2, containing the synthetic gene V, but containing one amber codon, is used. In this case, only those phage which receive an active gene V can grow and form plaques on a non-suppressing host.

Enrichment for a temperature-sensitive mutant by application of negative and positive selections

The usefulness of the complementary genetic selections described here were tested by using them to enrich for a temperature-sensitive mutant. This test was carried out using three plasmids: pTT18, containing the synthetic gene V; pTT19, derived from pTT18 but containing two amber codons in gene V; and pTT21, encoding the temperature-sensitive gene V protein. Cultures of K561/pTT15 containing each of these three plasmids were grown

Table II. Infection of K561/pTT15 and plasmids encoding wild-type gene V protein (pTT18) and temperature-sensitive gene V protein (pTT21) with phage R386 at various temperatures, followed by plating in the presence of chloramphenicol.

Plasmid	Temperature	Number of colonies obtained in presence of chloramphenicol	
		-IPTG	+IPTG
pTT18 (wild-type)	34°	7200	72
	37°	7200	82
	42°	2600	15
pTT21 (ts mutant)	34°	12000	4000
	37°	10000	4200
	42°	10000	5300

to 3×10^8 cells/ml and were then mixed in varying proportions. The mixed cultures were subjected first to the negative selection based on inhibition of phage R386 infection at 42°, enriching for plasmids which do not encode gene V protein which is active at high temperature. Segments containing gene V were then excised from the plasmids obtained in this step, and were used to replace the gene V in phage f1T2, which contains an amber codon in gene V. The resulting plaques were grown at 34°, selecting for those phage which express active gene V protein at low temperature. Taken as a whole, it was expected that this protocol would enrich for the temperature-sensitive gene V of plasmid pTT21 relative to both the wild type gene V of plasmid pTT18 and the null mutant of plasmid pTT19.

Table III shows the results of this experiment. When plasmid pTT18, containing the wild-type gene V, was present in the host K561/pTT15, very

Table III. Combination of negative and positive genetic selections applied to K561/pTT15 and plasmids encoding wild-type gene V protein (pTT18), a null mutant (pTT19), and a temperature-sensitive gene V protein (pTT21).

	Fraction of cells in culture containing plasmid:			Number of colonies obtained from 10^5 cells in the presence of chloramphenicol	Number of plaques obtained per nano-gram of plasmid DNA	Number of plaques tested	Fraction of plaques containing temperature sensitive phage
	(w.t.) pTT18	(null) pTT19	(ts) pTT21				
1.	100	0	0	60	>600*	5	0%
2.	0	100	0	8000	0	n.a.**	n.a.
3.	0	0	100	4000	520	5	100%
4.	99	0	1	140	1100	10	40%
5.	90	0	10	800	320	10	100%
6.	50	50	0	2400	0	n.a.	n.a.
7.	50	49	1	8000	3	2	50%
8.	50	40	10	3000	18	10	100%

*Less than 0.5 ng of plasmid DNA was used in this transformation and 320 plaques were obtained.

**n.a. Not applicable.

Figure 1. Schematic diagram of portion of plasmid pTT18 containing the synthetic gene *V*. The numbering system corresponds to that of pEMBL-8 (21).

few cells were infected by phage R386 after IPTG induction. For those which did become infected, however, plasmid which contained an active gene *V* could be isolated. After replacing the amber-mutant gene *V* of phage f1T2, this gene *V* allowed growth of the phage on the non-suppressing host (K38).

Cells harboring plasmid pTT19, encoding the null mutant gene *V*, were readily infected by the phage R386 at 42°. The gene *V* sequences obtained from these plasmids, as expected, did not support growth of phage f1T2 on K38.

The cells carrying only the conditional mutant plasmid pTT21 were infected by phage R386 at 42° and the mutant gene *V* sequences did support phage growth at 34° on the non-suppressing host K38, also as expected. As the previous set of experiments demonstrated, the infection of the strain carrying plasmid pTT21 could have been carried out at 37° or 34° with similar results.

When various combinations of the three cultures were mixed and subjected to the two selection steps, the proportion of temperature-sensitive genes *V* derived from plasmid pTT21 was enriched relative to that of the wild-type gene by a factor of about 40. This is approximately as expected, as cells containing plasmid pTT21 yielded about 65 times as many colonies in the negative selection as those containing plasmid pTT18, and both were expected to support phage growth at 34°. The enrichment of the conditional mutant relative to the wild-type gene is therefore accomplished only in the first step of this procedure. None of the null mutant gene *V* was obtained at all at the end of this procedure, of course, as such a gene will not support growth of the phage f1T2. Consequently, the procedure enriches 40-fold for the conditional mutant relative to the wild-type, but it enriches much more strongly for the conditional mutant relative to the null mutant.

Application of the selection for temperature-sensitive mutants to a pool of gene *V* variants

This selection procedure was applied to a collection of variant plasmids differing from the parent pTT18 by an average of 2 nucleotides within the region bounded by sites for the restriction enzymes *Kpn* I and *Sna* BI (Fig. 1). This region encodes residues 35-50 of the gene *V* protein. At the conclusion of the selection procedure, a total of 162 plaques were obtained. Of these, 109 were found to contain temperature-sensitive phage. The sequences of

Table IV. Temperature-sensitive mutants obtained from application of genetic selection procedure to gene V variants with substitutions in residues 35-50.

Mutant	Number of nucleotide substitutions	Amino acid substitutions	Number obtained with this sequence
M21.6	1	Asp 36-> Asn	1
M21.35	2	Asp 36 -> Asn Thr 48 -> Asn	14
M21.88	1	Gly 38 -> Ala	1
M21.23	1	Asp 50 ->His	14

the gene V region of 32 of these temperature-sensitive phage were determined, and five different phage were found. One of these was a derivative of bacteriophage M13 and did not contain the synthetic gene V from plasmid pTT18. The origin of this phage is unknown and it was not considered further.

Four different temperature-sensitive phage contained a gene V derived from the mutagenized plasmid pTT18. As the remainder of these phage genomes were derived from phage f1T1, which is not temperature-sensitive, it was concluded that these phage are temperature-sensitive in gene V.

Table IV summarizes the amino acid changes in the four temperature-sensitive gene V proteins and the number of each mutant allele which were obtained. All four phage produce plaques similar to those produced by the parent phage f1T1 at 34°. The phage M21.35 (Asp 36->Asn/Thr 48 -> Asn) produces no visible plaques at 42°. The other three phage produce plaques much smaller than those made by the parent phage f1T1 at 42°. The four phage were not all obtained at the same frequency, indicating that there is some step at which the propagation of one sequence relative to another can vary significantly. One likely point for this to occur is during the step selecting for proteins which are not active at 42°. During this step the DNA corresponding to partially inactivated proteins might be expected to be obtained at a lower frequency than that corresponding to fully inactivated proteins. A second possibility is that not all nucleotide substitutions are equally represented in the starting pool of gene V mutants. As only single isolates of two of the phage were obtained, some additional amino acid substitutions in this region leading to a temperature-sensitive phenotype could probably be isolated.

The four temperature-sensitive mutants obtained might in principle be defective at high temperature for stability of the gene V protein dimer, for binding of the dimer to DNA, for formation of dimer-dimer contacts in the gene V protein-DNA macromolecular complex (1), or possibly for some other function. It seems likely, however, that at least some of the isolated dimers have a reduced thermal stability, as has been found for other temperature-sensitive lesions (32, 33). None of the residues altered in these mutants (Asp 36, Gly 38, Thr 48, Asp 50) are known to interact with the DNA bound to the gene V protein, while several have important interactions with other residues within the gene V protein dimer (7). Aspartate-50, for

example, is involved in a salt bridge with arginines 80 and 82. The substitution of histidine for aspartate at residue 50 might result in unfavorable electrostatic interactions, leading to a decreased stability of the protein. The side chain of threonine-48, while exposed to the solvent, is packed tightly next to the side chains of glutamate-30, leucine-32, and lysine 46, and it is possible that the substitution of the bulkier asparagine cannot be easily accommodated. Glycine-38 is the first residue in a turn, and the substitutions of alanine, a residue infrequently found in turns (34), may reduce the stability of the turn. On the other hand, for aspartate-36 no obvious electrostatic interactions are altered by the substitution of asparagine. Instead, the charge on this residue or its hydrogen-bonding might more indirectly affect the stability of the protein. Alternatively, it seems possible that aspartate-36 is involved in dimer-dimer contacts required for assembly of the gene V protein-DNA complex, or that it directly interacts with the DNA.

CONCLUSION

The DNA-binding activity of the bacteriophage f1 gene V protein can be used in an effective genetic selection against activity of this protein. The protein blocks replication of infecting viral DNA, so that only cells not expressing the gene V protein can be infected by a phage conferring chloramphenicol resistance. It is not certain at this point whether the prevention of infection is due to the direct binding of the gene V protein to the infecting single-stranded DNA or to repression of the phage gene II mRNA expression, or to both of these properties (30). Our recent observations (unpublished) that the gene V protein inhibits transduction of *E. coli* by single-stranded plasmid DNA not requiring f1 gene II protein for replication suggests, however, that the inhibition of infection and transduction is due to binding to the single-stranded DNA.

The gene V protein is required for viral propagation, so support of viral growth can be used as a strong positive selection for the activity of the gene V protein. This is carried out here by replacing an amber mutant of gene V in phage f1T2 with the segment of DNA encoding the gene to be tested, and growing the resulting phage on a non-suppressing host. In this way the only phage which can grow are those in which an active gene V has been introduced.

These selections can be combined to yield a considerable enrichment for a conditional mutant phenotype. In the test carried out here, the enrichment for a temperature-sensitive phenotype was 40-fold relative to the wild-type and very strong relative to a null mutant. This procedure has also been shown to be useful in the direct isolation of temperature-sensitive mutants of the bacteriophage f1 gene V, and four such mutants were obtained from a pool of gene V variants altered in the codons for amino acids 35-50.

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ABBREVIATIONS

Kb, kilobase pairs; IPTG isopropylthiogalactoside; pfu, plaque-forming units; rf, replicative form; ss, single-stranded.

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