Isolation and characterization of ϕ X174 mutants carrying lethal missense mutations in gene G

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Received 30 June 1982

ABSTRACT

A previously constructed *Escherichia coli* transformant carrying a functional copy of bacteriophage ϕ X174 gene G on a plasmid, p ϕ XG, was used to isolate gene G mutants carrying temperature sensitive and lethal missense mutations. Two of the mutations have been characterized by sequencing: one carries a G→A transition at residue 2821 producing a Gly→Ser change in codon 143 of the G spike protein; the other carries an A→G transition at residue 2678 producing Glu→Gly change in codon 95. Sequencing DNA from 2 other mutants carrying lethal mutations that are rescued with p ϕ XG did not reveal any changes in the coding sequence. The lesion is believed to be in the intercistronic region between genes F and G. The adsorption kinetics for these mutants appear to be normal. Their burst size is about 25% that of wild type ϕ X174 on the host carrying p ϕ XG. These results along with previous results from the senior author's laboratory demonstrate that p ϕ XG can be used to rescue any gene G mutant of ϕ X174 regardless of the nature of the mutation involved.

INTRODUCTION

Gene G of ϕ X174 was the first essential gene from a virulent phage to be cloned (1). So far it is the only ϕ X gene to be cloned successfully (2). The plasmid bearing the gene, p ϕ XG, is a critical part of the system we have been developing to study the molecular mechanisms of mutations by carcinogens (3,4). We have shown that p ϕ XG will rescue gene G nonsense mutants (1,3) as well as a site-specific deletion-frameshift mutant (5). Recently p ϕ XG has been used to isolate a spontaneous mutant carrying a deletion-insertion frameshift in gene G (Bhanot and Chambers, unpublished results). To complete the calibration of the gene G mutant rescue system, we have used p ϕ XG to screen for lethal missense mutants. While one expects mutants of this type to be 'rescued' by p ϕ XG, it is important to demonstrate this experimentally, because phenotypic mixing of wild type gene G spike protein produced by p ϕ XG and non-functional G spike protein from the mutant phage might produce non-infectious virus particles. This paper describes the use of p ϕ XG to selectively screen

for and isolate gene G mutants carrying lethal missense mutations. The base changes responsible for the loss of gene G spike protein activity have been characterized in two of the nine new mutants described here.

MATERIALS AND METHODS

Bacterial strains, phage and various media have been described previously (3).

Mutagenesis

Freshly prepared NaNO_2 (1 mL of 0.1 M) was mixed with 1 mL of sodium acetate buffer (0.1 M, pH 4.5) containing 10^{12} "wild type" ϕX174 . After ten min at 37° (~ 0.1% survival), 0.1 mL aliquots of suitably diluted phage were mixed with HF4738/ ϕXG in R soft agar and plated on R plates to give approximately one hundred plaques per plate. Individual plaques were picked with toothpicks and gridded sequentially on lawns of HF4738/ ϕXG and HF4738. Mutants were identified and isolated from the HF4738/ ϕXG grid.

Preparation of RC mutant stocks

Phage extracted from a single plaque were diluted and replated on HF4738/ ϕXG grown to midlog phase in R medium and concentrated by centrifugation to about 5×10^9 cells/mL. Individual plaques (20-40) of each mutant were extracted with 1.1 mL of BE buffer (0.005 M $\text{Na}_2\text{B}_4\text{O}_7 + 10^{-4}$ M EDTA) overnight at 4° . Aliquots (0.1 mL) of each suspension were plated without dilution on midlog phase E. coli. Those extracts with less than 200 wild type phage/mL were pooled and concentrated ten-fold in vacuo at 25° . These stock solutions were filtered through sterile Millipore filters. These stocks had 10^9 - 10^{10} phage/mL and $<10^{-6}$ wild type phage. Except for RC4, the titers of these isolates remained constant during storage at 4° for several months. Two independent preparations of RC4 lost 50% of their original titer during three months' storage. RC2 and RC3 consistently gave individual plaques with higher wild type content than the others.

Isolation of DNA for sequencing

Approximately 10^8 phage from each of the stocks prepared as described above were mixed with HF4738/ ϕXG in R soft agar and plated on R plates. Ten plates for each mutant were grown to confluent lysis. The soft agar layers were scraped from the plates, pooled and homogenized in 100 mL of the TE buffer described above. After centrifugation to remove the agar, the supernatant solutions were concentrated in vacuo to approximately 10 mL. Titers of about 10^{10} phage/mL with a wild type frequency of about 5×10^{-4} were obtained. These preparations were used to infect two 2.5 L cultures of

HF4738/p ϕ XG grown to 2×10^8 cells/mL in 3XD medium; the multiplicity of infection in each case was 0.1. The phage were isolated using the polyethylene glycol procedure (6) and banded in CsCl (0.625 g/mL). The phage band was collected and dialyzed against BE buffer (10 mM Tris-HCl + 1 mM EDTA, pH 8) and then concentrated five-fold in vacuo. DNA was extracted as described by Sinsheimer (7). The ethanol precipitate was dissolved in 0.1 mL of the BE buffer described above. The absorbance of these solutions at 260 nm was 15-20. The solutions were stored at 4°.

Preparation of restriction fragments

ϕ X RF DNA from the mutant ϕ XGam3 OB1 (3,8) was prepared as described previously (5). The restriction fragment Hha I 614 was prepared from approximately 20 A_{260} units of this DNA. The Hha I 614 fragment was isolated by polyacrylamide gel electrophoresis and used to prepare the following restriction fragments: Hpa II 179, Hinf I 102, Hinf I 200, and Hinf I 312. The fragments Hind II 85 and Hind II 79 were prepared from the Hinf I 312 fragment. The fragments Mbo II 89 and Mbo II 121 were prepared directly from six A_{260} units of the RF DNA.

DNA sequencing

The chain terminator method (9) was used.

Adsorption of phage mutants on E. Coli C

Adsorption was measured in the following mixtures: 2×10^7 cells/mL grown in KC broth and treated with 3 mM KCN for 10 min and stored in ice until used, phage, 2 mM Ca^{2+} and 3 mM KCN in KC broth. A series of experiments using multiplicities of infection ranging from 1 to 5 were carried out. Adsorption was allowed to take place at 37° without shaking. Aliquots (0.1 mL) were removed at 2, 5, 10, and 15 min, pipeted into 0.9 mL of chilled KC broth and centrifuged immediately at high speed for 4 min. Unadsorbed phage were titrated immediately on HF4738/p ϕ G grown to mid-log. Apparent rate constants were determined from semilog plots.

Burst size

The one step growth procedure described by Adams (10) was followed using HF4738/p ϕ XG as the host for the adsorption and plating. Rabbit antiserum directed against ϕ X174 was diluted 100 fold and used to inactivate unadsorbed phages.

RESULTS

HF4738/p ϕ XG is a ϕ X174 sensitive, Su^+ , recA transformant of E. coli that carries a functional copy of ϕ X gene G on a plasmid (1). The parent HF4738

gives a suppression pattern that indicates it inserts glutamine at amber codons (3). When a mutagen producing primarily base substitutions is used, HF4738 and HF4738/p ϕ XG can be used to screen mutagenized phage for gene G mutants of ϕ X174 carrying either temperature sensitive or lethal mutations.

ϕ X174 was mutagenized to 0.1% survivors with HNO₂ and plated on HF4738/p ϕ XG. Individual plaques were gridded sequentially from a single toothpick stab onto prepouring lawns of HF4738 and HF4738/p ϕ XG. By comparing plaques on the two plates, nine mutants were identified out of the first 500 plaques examined. Each of these was isolated and purified to less than 5×10^{-6} wild type phage as described in the methods section. These were named RC1-RC9. RC1 proved to be a gene G temperature sensitive mutant. RC2-RC8 were neither temperature sensitive nor suppressible with the ϕ X^S suppressors we have on hand (amber Su1⁺, Su2⁺ and Su3⁺; ochre Su⁺; opal Su⁺).

In order to prove that these mutants carried lethal missense mutations four of them (RC2, 5, 7 and 8) were selected for sequencing. Our strategy was based on the restriction map shown in Fig. 1 (11). All the fragments shown were isolated in the usual manner (see Methods) and used as primers for sequencing. The results are summarized in Table 1. The following remarks are pertinent.

1. We read about 100, occasionally 150, residues from individual gels.
2. RC2, 5, 7 and 8 all have a normal start codon (ATG) at 2395 and a normal stop codon (TGA) at 2920. Particular attention was paid to position 2421 (T), 2616 (T), 2802 (G) and 2814 (T) because sequence changes

Fig. 1. Strategy for sequencing the relevant DNA from some ϕ X174 gene G mutants. The restriction endonuclease sites are shown by the vertical arrows; the restriction fragments isolated and their size in base pairs is shown by the double headed arrows. The span of DNA sequenced with these primers is indicated by the single headed horizontal arrows.

Table 1. Sequence changes in some ϕ X174 gene G mutants carrying lethal missense mutations

Mutant	Nucleotide	Change	Codons	Effects
RC2	2538	G→T	(ACG)Thr→(ACT)Thr	Silent
	2731	A→G	(AAT)Asn→(GAT)Asp	Permissive
	2811	C→T	(AAC)Asn→(AAT)Asn	Silent
	2821	G→A	(GGT)Gly→(AGT)Ser	Lethal
RC5	2731	A→G	(AAT)Asn→(GAT)Asp	Permissive
	2811	C→T	(AAC)Asn→(AAT)Asn	Silent
RC7	2731	A→G	(AAT)Asn→(GAT)Asp	Permissive
	2811	C→T	(AAC)Asn→(AAT)Asn	Silent
RC8	2678	A→G	(GAG)Glu→(GGG)Gly	Lethal
	2731	A→G	(AAT)Asn→(GAT)Asp	Permissive
	2811	C→T	(AAC)Asn→(AAT)Asn	Silent

have been reported at these positions in the revised ϕ X sequence (11). In each case the nucleotide found corresponded to the revised sequence.

3. RC2, 5, 7 and 8 differed at residues 2731 and 2811 from the most recently published sequence (11). These represent changes in our wild-type stock. The nucleotide we found at 2731 corresponds to the G reported in the first ϕ X sequence (12). The change at 2731 gives an Asn→Asp insertion which is permissive; the change at 2811 occurs at the "wobble" position of an Asn codon and so is silent.

4. RC2 shows microheterogeneity at position 2538; equal amounts of G and T were found. This represents a silent transversion (G→T; Thr→Thr). Because of the way our virus stocks were prepared (see Methods), this change cannot be present in our original RC2 stock; nor can it arise from the mutagenesis step. It appears to have arisen as a spontaneous mutation in the particular phage plaque used for preparation of viral DNA for sequencing.

5. Lethal missense changes have been found in RC2 and RC8. In RC2, a G→A transition at 2821 leads to a lethal Gly→Ser substitution. Thus RC2 is now designated ϕ XGms143 RC2. In RC8, an A→G transition at 2678 produces a lethal Glu→Gly substitution. Thus, RC8 is now designated ϕ XGms95 RC8. The 2678 change in RC8 occurs in one of the Hinf I sites; the absence of this restriction site in RC8 RF DNA confirms this

sequence change.

6. No unique changes were found in the translated region of gene G in RC5 or RC7. We suspect (from sequence and recombination data) that these mutants have lethal changes in the intercistronic region between genes F and G.

We have examined the adsorption of RC2, 4 and 5 on *E. coli* C at 37° in 0.8 mM Ca²⁺ with 3.33x10⁷ *E. coli* cells/mL and multiplicities of infection of 1.0. Each of these mutants as well as wild type phage gave an apparent rate constant for adsorption of 0.166 min⁻¹. This corresponds to 1.4x10⁻¹⁰ mL sec⁻¹. Fujimura and Kaesberg report 1.0x10⁻¹⁰ mL sec⁻¹ for wild-type ϕ X174 under quite different experimental conditions (13). Thus the adsorption of the RC mutants seems to be normal even though one (RC2) has a change in the gene G and another (RC5) seems to have a change in the intercistronic region between genes F and G.

Burst sizes for wild type phage and mutants RC2-RC9 were measured from one step growth curves using HF4738/p ϕ XG as a host. The results are shown in Table 2. We suspect that the small burst sizes compared to wild type phage may reflect limiting amounts of normal gene G protein produced by p ϕ XG in the host or phenotypic mixing of wild type and mutant gene G spike proteins to produce non-infectious phage, or both.

DISCUSSION

The work described here adds two more well characterized ϕ X gene G mutants, ϕ XGms95 RC8 and ϕ XGms143 RC2, to the list of ϕ X174 mutants. A total

Table 2. Burst Size of gene G Mutants HF4738/p ϕ XG

Mutant	Burst size ^a
w.t.	170, 169
am9	40, 40
RC2	45, 55
RC3	32, 25
RC4	40, 45
RC5	58, 48
RC6	96, 42
RC7	42 -
RC8	48 -
^a Viable phage particles/cell. The results of duplicate experiments are shown.	

of five mutations in this gene have now been identified by sequencing. One of these is a nonsense mutant (3). The two described in this paper are lethal missense mutants. The other two have more extensive changes involving deletions, insertions, and frameshifts (5, Bhanot and Chambers, unpublished data). All of these mutants have been isolated with hosts containing a functional copy of gene G on a plasmid, ϕ XG. In this paper, we have described for the first time the use of ϕ XG to screen for a particular kind of gene G mutant. By judicious choice of mutagen and host cells, we were able to find 2 examples of the desired lethal missense mutants without difficulty. In addition, a temperature sensitive gene G mutant and six more nonsuppressible gene G mutants were isolated; the exact positions and nature of the changes in these mutants are still unknown. However, two of these (RC5 and RC7) have been studied by sequencing and no unique change has been found in the structural gene in either case. We suspect that the mutations in RC5 and RC7 are in the intercistronic region between genes F and G. This is a particularly interesting region of the viral DNA. It is known to have a ribosome binding site (14) and it has been suggested that it contains information important in the viral DNA or the mRNA structure (12,14). The specific recognition site for the protein n' is located in this region (residues 2301-2355) (15). It should be recalled that the changes in RC5 and 7, wherever they are, are lethal. Mutations in either the ribosome binding site or the n' recognition site might well be lethal. Thus, it will be interesting to identify the exact changes in these mutants that are responsible for the observed lethality.

This work completes the calibration of our ϕ XG rescue system. The loc-

Fig. 2. Mutations in gene G of ϕ X174 that have been characterized by sequencing. OB1= ϕ XGam3 OB1; ZH1= ϕ XGde40-66.2 ZH1; RC8= ϕ XGms95 RC8; RC2= ϕ XGms143 RC2; OB2= ϕ XGde143-147in50B OB2. The origin of the 50 base insertion in OB2 is unknown; it is not from ϕ X174 (Bhanot and Chambers, unpublished results). The intercistronic regions are shaded; deletions are cross hatched; point mutations are indicated as dots.

ations and nature of the gene G mutations we have now characterized are summarized in Fig. 2. We are now confident that any mutation in gene G can be rescued with ϕ XG. This is critical for the system we have developed for studying the molecular mechanisms of mutation by carcinogens since this system employs site specific covalent modifications of gene G, and in most cases one cannot predict the kind of mutation a particular adduct will produce. Since we are dealing with an essential gene most mutations will be either conditionally lethal or lethal, and we need a rescue system that is permissive for any kind of mutation. Taken together, the data summarized in Fig. 2 demonstrate that ϕ XG satisfies this requirement.

ACKNOWLEDGEMENTS

Part of this work was done at New York University School of Medicine and was supported by Grant 5 R01 CA6319-05 awarded by the National Cancer Institute, U.S. Department of Health, Education and Welfare. We are indebted to Dr. Zafri Humayun for providing mutagenized phage stock.

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