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Mutations in the gene 5 DNA polymerase of bacteriophage T7 suppress the dominant lethal phenotype of gene 2.5 ssDNA binding protein lacking the C-terminal phenylalanine

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Summary

Gene 2.5 of bacteriophage T7 encodes a ssDNA binding protein (gp2.5) essential for DNA replication. The C-terminal phenylalanine of gp2.5 is critical for function and mutations in that position are dominant lethal. In order to identify gp2.5 interactions we designed a screen for suppressors of gp2.5 lacking the C-terminal phenylalanine. Screening for suppressors of dominant lethal mutations of essential genes is challenging as the phenotype prevents propagation. We select for phage encoding a dominant lethal version of gene 2.5, whose viability is recovered via second-site suppressor mutation(s). Functional gp2.5 is expressed *in trans* for propagation of the unviable phage and allows suppression to occur via natural selection. The isolated intragenic suppressors support the critical role of the C-terminal phenylalanine. Extragenic suppressor mutations occur in several genes encoding enzymes of DNA metabolism. We have focused on the suppressor mutations in gene 5 encoding the T7 DNA polymerase (gp5) as the gp5/gp2.5 interaction is well documented. The suppressor mutations in gene 5 are necessary and sufficient to suppress the lethal phenotype of gp2.5 lacking the C-terminal phenylalanine. The affected residues map in proximity to aromatic residues and to residues in contact with DNA in the crystal structure of T7 DNA polymerase-thioredoxin.

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Introduction

Single-stranded DNA (ssDNA)-binding proteins are abundant cellular proteins that are essential for DNA replication, recombination and repair in all systems studied to date (Lohman and Ferrari, 1994; Shereda *et al.*, 2008). Initially, these proteins were assigned the role of removing secondary structures in DNA that impede the progress of replication enzymes or of protecting regions of ssDNA from cleavage by nucleases. However, in recent years ssDNA-binding proteins have emerged as key components in recruiting and organizing proteins of the replication machinery via multiple protein–protein interactions (Lohman and Ferrari, 1994; Shereda *et al.*, 2008). Dissecting the multiple roles of ssDNA-binding proteins is a challenging task as they bind DNA in a non-specific manner and they are involved in processes that are critical for the survival of the cell. Consequently, conventional experimental approaches are limited in their usefulness. For example, the importance of the acidic C-terminal tails of prokaryotic ssDNA-binding proteins has been recognized for more than two decades (Lohman and Ferrari, 1994), yet the mechanistic aspects of their function remain unresolved.

The ssDNA-binding protein of bacteriophage T7 is encoded by gene 2.5, an essential gene for phage growth (Kim and Richardson, 1993). The gene 2.5 protein (gp2.5) is a dimer in solution and binds ssDNA with micromolar affinity (Kim *et al.*, 1992a). Similar to all prokaryotic ssDNA-binding proteins, gp2.5 has an acidic C-terminal tail (Fig. 1). Removal of the acidic C-terminal tail is lethal *in vivo* (Kim and Richardson, 1994). Gp2.5 lacking its C-terminal tail has a higher affinity for ssDNA and can no longer physically interact with the T7 DNA polymerase or helicase (Kim and Richardson, 1994). Gp2.5 also facilitates homologous base pairing, a function that does not require the C-terminal tail (Rezende *et al.*, 2003).

Gp2.5 is one of the four proteins necessary and sufficient to reconstitute the T7 replication fork *in vitro*. The other three proteins are the T7 gene 5 DNA polymerase, its processivity factor, *Escherichia coli* thioredoxin (trx) and the multifunctional gene 4 helicase/primase. The acidic C-terminal tail of gp2.5 is critical for the interactions

Fig. 1. Gp2.5 dominant lethal mutations used for suppressor screening.
A. Sequences of the C-terminal tails of mutants used for suppressor screens. The generation and the properties of all mutants were previously described in Marintcheva *et al.* (2006).
B. Sequences of the C-terminal tails of selected intragenic suppressors. Both suppressors were selected in the screen using gp2.5Δ1 lethal mutant.

of the protein with T7 DNA polymerase and helicase/primase as evidenced by their abolishment when the tail is deleted (Kim *et al.*, 1992b; Kim and Richardson, 1994). The C-terminal tail of gp2.5 binds to two positively charged loops located in the thumb subdomain of the DNA polymerase (Hamdan *et al.*, 2005). These two loops are located within the unique 72-amino-acid insert in the thumb subdomain to which the processivity factor, trx, binds. Thus, the C-terminal tail of gp2.5 could potentially interfere with all interaction pairs at the T7 replication fork, either by interacting directly or by competing with another partner for the same contact surface (Kim *et al.*, 1992b; Kim and Richardson, 1994; Notarnicola *et al.*, 1997; He *et al.*, 2003). The interactions of the C-terminus of gp2.5 could thus function to co-ordinate the multiple reactions occurring at the replication fork. In fact, gp2.5 is essential for establishing co-ordinated leading and lagging strand DNA synthesis in an *in vitro* reconstituted system (Nakai and Richardson, 1988; Lee *et al.*, 1998; 2002).

The acidic C-terminus of gp2.5 can be viewed as a mimic of the negatively charged phosphate backbone of ssDNA. As such it could bind in the positively charged DNA-binding cleft and compete for DNA binding. Accordingly, we have shown that a peptide corresponding to the acidic C-terminal tail of gp2.5 and ssDNA bind to the same surface of gp2.5 in a mutually exclusive manner (Marintcheva *et al.*, 2008). Upon binding ssDNA the C-terminal tail is displaced and readily available for protein–protein interactions.

The structures of several prokaryotic ssDNA-binding proteins have been solved (Shamoo *et al.*, 1995; Raghunathan *et al.*, 1997; Raghunathan *et al.*, 2000; Hollis *et al.*, 2001; Bernstein *et al.*, 2004; Savvides *et al.*, 2004; Saikrishnan *et al.*, 2005). Despite the absence of sequence homology, they share a signature oligosaccharide/oligonucleotide binding-fold, comprised of antiparallel β-sheets forming a barrel with a well-defined cleft. Structural and mutagenesis data have shown that ssDNA binds within their cleft via stacking and electrostatic interactions. Unfortunately, no structural information is available for the C-terminal tail of any free prokaryotic ssDNA-binding protein and it is generally thought that most likely the tail is unstructured (Savvides *et al.*, 2004). Structural analysis of *E. coli* SSB C-terminal peptide/RecQ helicase complex and *E. coli* SSB C-terminal peptide/Exonuclease I complex reveal similar mode of interactions (Lu and Keck, 2008; Shereda *et al.*, 2009).

In a separate communication we described a systematic study using site-directed mutagenesis and biochemical analyses to identify critical determinants for the function of the acidic C-terminus of gp2.5 (Marintcheva *et al.*, 2006). The overall negative charge of the C-terminal tail is important for its function *in vivo*; replacement of the charged residues with neutral amino acids abolishes its ability to support T7 phage lacking gene 2.5. We also found that the presence of a C-terminal aromatic amino acid is critical for the function of gp2.5. *In vitro*, the lethal phenotypes correlate with the ability of gp2.5 to physically interact with T7 DNA polymerase (Marintcheva *et al.*, 2006).

The dissection of the protein–protein interactions of gp2.5 at the replication fork has been difficult. The interactions are relatively weak, charge-mediated, and their specificity is not understood, thus making classical biochemical approaches not very informative. The availability of a collection of lethal mutations in the acidic C-terminus of gp2.5 (Rezende *et al.*, 2002; Marintcheva *et al.*, 2006) initially led us to look for suppressor mutations in other T7 replication genes. The identification of the proteins involved and the location and nature of the suppressor mutations should hopefully provide information on the interaction of gp2.5 with these proteins. Our initial attempts to select suppressor mutations proved unsuccessful and led to the realization that the mutants of interest are dominant lethal and significantly inhibit the growth of bacteriophage T7 (B. Marintcheva and C. C. Richardson, unpubl. data).

Here we report the design of a screen for second-site suppressors of dominant lethal mutations located in the C-terminal tail of gp2.5. Our experimental protocol selects for phage encoding a dominant lethal version of gene 2.5, whose viability is recovered via second-site suppressor mutation(s). Throughout the screen functional gp2.5 is expressed *in trans* to ensure propagation of the unviable phages and allow suppression to occur via natural selection. Our approach has identified both intragenic and

extragenic suppressors. The intragenic mutations reinforce the essential nature of the C-terminal phenylalanine discovered previously from *in vitro* mutagenesis studies (Marintcheva *et al*., 2006). Extragenic suppressor mutations were found in several genes, including gene 5, encoding enzymes of T7 DNA metabolism. We have focused our efforts on the suppressor mutations in T7 DNA polymerase as means to characterize further the significance of the gp2.5–gp5 interaction for the biology of bacteriophage T7.

Results

Suppressor screen design

Suppressor screens are a useful genetic tool for gaining insights into protein–protein networks. We have taken advantage of the availability of a collection of non-complementing mutants with amino-acid changes in the C-terminus of gp2.5 generated in previous studies (Rezende *et al*., 2002; Marintcheva *et al*., 2006). Two major challenges must be overcome to select suppressors of lethal mutations in gene 2.5. First, gp2.5 is an essential protein for T7 growth, and thus a wild-type gp2.5 must be available in the cell in order to propagate the suppressor phage carrying a copy of gene 2.5 harbouring a lethal mutation. Second, most of the lethal mutations in the C-terminal tail of gp2.5 are dominant lethal. For example, overexpression of gp2.5-Ins F231ΔF232, a dominant lethal mutant in which the C-terminal phenylalanine is moved to penultimate position (Fig. 1), used in this study reduces the efficiency of plating of wild-type T7 phage 100-fold, whereas overexpression of wild-type gp2.5 reduces the efficiency of plating of T7 phage by only 60%. These challenges were overcome by designing a screening procedure that uses two plasmids encoding gene 2.5. The first plasmid, pET-17b-gp2.5, provides wild-type gp2.5, whose function is indispensable for phage replication and recombination. The second plasmid, pACYC-gp2.5-mut, encodes the gene for the altered gp2.5 of interest, for which suppressors are desired (a total of four different screens are described in this work). This mutant gene 2.5 (designated as gp2.5-mut throughout this manuscript) is flanked by upstream and downstream sequences to ensure efficient recombination (Fig. 2). The latter plasmid is a low-copy-number plasmid and expresses gp2.5-mut from a relatively weak promoter, thus minimizing the dominant lethal effect of the altered protein.

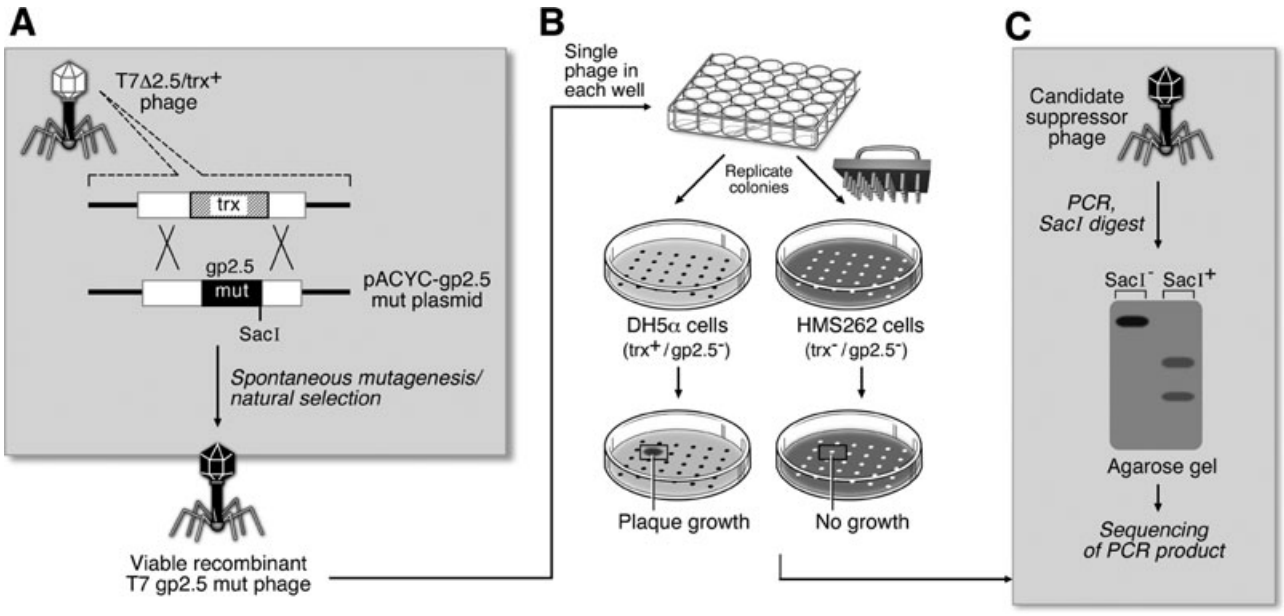

Fig. 2. Suppressor screen design and screening.
A. Selection of viable phages. *E. coli* DH5α cells were transformed with pET17b-gp2.5-wt plasmid encoding gp2.5 wild-type and with pACYC-gp2.5-mut plasmid encoding a gp2.5 mutant of interest flanked by 400 bp upstream and 200 bp downstream sequences. The cells were infected with T7Δ2.5/trx+ phage at multiplicity of infection of 1 and the culture grown until complete lysis. The lysate was plated on DH5α cells and individual plaques were picked for further analysis.
B. Candidate suppressors selection. Candidate suppressors were transferred to microtitre plate and plated on DH5α and HMS262 cells to identify phages product of homologous recombination. Plaques that grew on DH5α cells and did not grow on *E. coli* HMS 262 cells were selected and subjected on further characterization.
C. Screening for the presence of SacI marker. Gene 2.5 and flanking sequences were amplified using PCR, the PCR products purified and digested with SacI. The presence of the SacI marker was verified by agarose gel electrophoresis. $SacI^+$ plaques were sequenced to identify the presence of the corresponding gp2.5 lethal mutation and potential suppressor mutations.

The general design of the suppressor screen is outlined in Fig. 2. *E. coli* DH5α cells transformed with pET-17b-gp2.5 and pACYC-gp2.5-mut plasmids were grown to an OD_{600} of 0.2 and infected with T7Δ2.5/trx bacteriophage at a multiplicity of infection of 1. T7Δ2.5/trxA has gene 2.5 replaced with the *E. coli* trxA gene encoding thioredoxin that further serves as a selection marker in the screen. In order to select for recombinant phage that acquired mutations that render gp2.5-mut functional, the lysate was plated on *E. coli* DH5α cells. Only phages that are self-sufficient for growth will form plaques on these cells. Parental phages (T7Δ2.5/trx) are selected against as they lack gp2.5 that is essential for replication. Recombinant phages are products of recombination events within the 400 bp upstream and 200 bp downstream gene 2.5 sequences that are included as sequences flanking gene 2.5 in the pACYC construct (Fig. 2). Recombinants carrying a copy of the gp2.5-mut are also selected against unless a compensatory mutation(s) has occurred that render gp2.5 functional. The suppressor phages that are able to grow on *E. coli* DH5α are recombinant phages that have acquired compensatory mutations, resulting in suppression or a reversion of the original lethal mutation (i.e. the compensatory mutations recover the wild-type gp2.5 sequence as opposed to arising outside the C-terminal tail harbouring dominant lethal mutation). In this screen we sought selection of both intragenic and extragenic suppressors. Intragenic supressors should provide valuable information about the contact(s) of the acidic C-terminus of gp2.5 protein on the surface of the protein itself. In contrast, extragenic suppressors should identify interacting partners and the location of the interaction surface.

Controls for the integrity of the screen

We have carried out suppressor screens with four different lethal mutants in the acidic C-terminal tail of gp2.5. We have used the gp2.5Δ1 and gp2.5InsF231ΔF232 mutants (Fig. 1), both of which have a lower affinity for T7 DNA polymerase as measured by surface plasmon resonance (Marintcheva *et al*., 2006). In the first altered protein the C-terminal phenylalanine has been deleted, while in the second the C-terminal phenylalanine has been moved in penultimate position. As a control we have used gp2.5 F232L and gp2.5F232R with amino acid substitutions for the C-terminal phenylalanine, and which also bind to T7 DNA polymerase somewhat weaker than does wild-type gp2.5. However, both of the genes encoding these altered proteins can easily revert back to wild-type by undergoing one or two single mutations respectively. All mutants fail to complement the growth of the T7 Δ2.5/trx phage lacking gene 2.5 and do not support DNA replication *in vivo* as evidenced by pulse labeling of DNA with [^{3}H] thymidine (Marintcheva *et al*., 2006). These properties provided an opportunity to use a strong 'growth versus no growth' selection for our screens, as well as for providing a means for monitoring the integrity of the screen: viable phages are expected to arise more frequently when only one point mutation is required to revert the lethal phenotype (gp2.5F232L mutant), and less frequently when two point mutations are required (gp2.5F232R).

Selection and suppressor identification

Lysates from each of the four screens described above were plated on *E. coli* DH5α in order to select for viable plaques, i.e. candidate suppressor phages. Suppressors were expected to arise as a result of two molecular events: (i) recombination between the T7Δ2.5/trx phage genome and the corresponding gene 2.5-mut flanked by upstream and downstream T7 sequences (Fig. 2); and (ii) spontaneous mutagenesis/natural selection resulting in mutations that compensate the lethal phenotype of gp2.5-mut. Recombinant phages should lack the *trx* gene, which is replaced by the mutant gene 2.5. In addition, a silent SacI restriction site was engineered immediately after the gp2.5-mut stop codon in pYCAC-gp2.5-mut plasmid and was used as a molecular marker to follow up recombinant phages. The genome of T7 phage does not contain any SacI restriction sites itself, thus allowing us fast and efficient way to discriminate parental phages ($SacI^-$) and recombinant phages ($SacI^+$). As gene 2.5 is an essential gene recombinant phages will not be viable unless spontaneous compensatory mutation(s) arise. Compensatory mutations could arise in: (i) genes different from gp 2.5, resulting in extragenic suppressors or (ii) within gp2.5 itself resulting in intragenic suppressors. If the intragenic suppression takes place within the region, where the original gp2.5 dominant lethal mutation is located, it could produce pseudorevertants, i.e. recombinant phages, in which gp2.5 mut is reversed to gp2.5 wild-type. These phages, however, will be *trx*$^-$/*SacI*$^+$ but will not harbour a lethal mutation in gene 2.5.

Viable phages could also arise via illegitimate recombination. In this scenario, wild-type gene 2.5 originating from pET-17b-gp2.5 could be inserted anywhere in the genome of T7Δgp2.5/trx^+ at a very low frequency due to the lack of flanking sequences promoting homologous recombination. Such phages will be able to propagate independently and expected to carry a copy of the *trx* gene, i.e. to be trx+/gp2.5-wt+/$SacI^-$.

Regardless of the nature and the location of the second-site mutation(s), all candidate suppressors should meet three criteria: (i) they should not contain a copy of the *trx* gene, (ii) they should contain the SacI restriction site and (iii) they should contain gene 2.5-mut as a part of

Table 1. Screening and characterization of candidate suppressors.

	Candidate suppressors[a]				Revertants[e]		Suppressors[f]		Trx$^+$ phages[g]	
Screen	No.	Freq.[b]	Trx$^-$ phages[c]	SacI$^+$ phages[d]	No.	Freq.[b]	No.	Freq.[b]	No.	Freq.[b]
gp2.5F232L	21	0.280	19	15	15	0.2	0	0	2	0.026
gp2.5F232R	19	0.038	3	3	3	0.006	0	0	16	0.032
gp2.5Δ1	18	0.014	2	2	0	0	2	0.0016	16	0.013
gp2.5-InsF231ΔF232	11	0.009	5	5	0	0	5	0.004	6	0.048

a. Candidate suppressors are defined as phages, originating from suppressor screen lysate and independently growing on plain DH5α cells.
b. All frequencies are calculated as number of plaques observed per microlitre of screened lysate. On average, the produced lysates contained 7×10^9 phages ml^{-1}.
c. Trx$^-$ phages were selected by parallel plating of selected plaques on HMS262 (trx$^-$) and DH5α (trx$^+$) cell strains. Phages growing only on DH5α cell were classified as trx$^-$.
d. Recombinant phages that possess artificially engineered SacI silent restriction site.
e. SacI$^+$ phages that do not carry gp2.5 lethal mutation.
f. SacI$^+$ phages that carry gp2.5 lethal mutation and extragenic or intragenic second-site mutation(s).
g. Trx$^+$ phages were selected by parallel plating of the candidate suppressors plaques on HMS262 (trx$^-$) and DH5α. Plaques that grow on HMS262 cell strain were classified as trx$^+$.

the phage genome. To confirm the absence of the *trx* gene all plaques that grew on DH5α cells were picked and transferred to a microtitre plate (Fig. 2). Using a replica plater (Sigma) the plaques were plated on Petri dishes overlaid with soft agar containing DH5α or HMS262 cells. The latter lack the gene for thioredoxin that is nonessential for the *E. coli* growth but essential for T7 growth. Thus, phages that are able to grow on *E. coli* HMS262 must contain the *trx* gene in their genomes, and therefore they are not a product of homologous recombination within the gp2.5 coding region. Throughout all four screens they arise with an average frequency of 0.03 plaques per microlitre of cell lysate (Table 1). The same frequency was observed in the control for illegitimate recombination in which *E. coli* HMS262 (trx$^-$) were transformed with pET17bgp2.5 plasmid and infected with T7Δgp2.5/trx phage. Therefore, phage growth on DH5α cells versus phage growth on HMS262 cells provided means to eliminate candidate suppressors that contain a copy of the wild-type 2.5 gene inserted via illegitimate recombination from the screen. Such phages could not provide useful information on the nature of the interactions of the C-terminal tail of gp2.5 during the T7 life cycle.

Candidate suppressors, which grew on DH5α cells, but did not grow on *E. coli* HMS262 cells, were further analysed. The region around the stop codon of gene 2.5 that is expected to carry the SacI site in the recombinant phages was amplified by PCR and the PCR products purified and digested with SacI (Fig. 1C). If the SacI site is present then digestion with SacI will produce DNA fragments with lengths of 550 and 300 bp. If the SacI site is not present the PCR product will remain uncut (850 bp) (Fig. 1C). All candidate suppressors were, in fact, cleaved by SacI (Table 1), demonstrating that the candidate suppressors derive from products of recombination between the T7Δ2.5/trx phage and pACYCgp2.5-mut plasmid. The PCR products were sequenced to determine the presence of the dominant lethal mutation in the gp2.5 C-terminus (gp2.5-mut). All candidate suppressors in the screens with gp2.5-F232L and gp2.5-F232R mutants were found to be pseudorevertants (Table 1). This finding is not surprising as only a single point mutation is needed to convert the codon for leucine (CTC) to a codon for phenylalanine (TTC), i.e. the reversion is a result of C to T transition. Two single point mutations are needed to convert the codon for arginine (CGC) to phenylalanine (TTC), i.e. the reversion is a result of C to T transition and G to T transversion. Correspondingly, the frequency of the leucine (L) to phenylalanine (F) reversion was 0.2 plaques μl^{-1} lysate (~1 in 5×10^6 phage particles, see Table 1), and the frequency of the arginine (R) to phenylalanine (F) reversion was 0.006 plaques μl^{-1} (~1 in 1.7×10^8 phage particles, see Table 1). For comparison, a control for evaluation of the recombination frequency was performed. HMS262 cells transformed with pACYCgp2.5-wt were infected with T7Δ2.5/trx$^+$ phage, and the resulting cell lysates were tittered on *E. coli* DH5α and HMS 262 cells. The titre on *E. coli* HMS262 estimates the total number of phage particles in the lysate, whereas the titre on *E. coli* DH5α estimates the number of recombinant phages (T7gp2.5/trx$^-$). The results show that one recombinant phage arises per 10^6 phage particles.

Intragenic suppressors

Two intragenic supressors (Table 1) were identified in the screen using the gp2.5Δ1 mutant (Fig. 1). Interestingly, the suppression restored a phenylalanine residue in the C-terminal position via several single point mutations, consistent with the low frequency of candidate suppressors (Table 2). In both suppressor phages, the overall length of the acidic C-terminal tail was one residue shorter

Table 2. Suppressor mutations identification.

gp2.5 mutation used for screening	Affected gene	Nucleotide(s) change	Amino-acid change	Type of event
gp2.5F232L	gene 2.5	TTC → CTC	L232F	Pseudoreversion
gp2.5F232R	gene 2.5	TTC → CGC	R232F	
gp2.5ΔF	gene 2.5	GAC → TTC	D231F	Intragenic suppression
		GGC → GAA	D230D	
gp2.5-InsF231ΔF232	gene 3	AAC → ACG	N65T	Extragenic suppression
	gene 7	GAA → CAA	E42Q	
		GGA → GAA	G51E	
	gene 5	GGG → AAG	G371K	
		GCA → ACA	A411T	
		ACG → ATG	T258M	
	gene 5.3	ACA → TCA	T32S	
	gene 6	TGG → TGT	W42C	
		GAG → GAT	E120D	

The class II genes of all candidate suppressors were sequenced. The highlighted letters reflect the nucleotide changes found during the sequence analysis.

than the acidic C-terminal tail of the wild-type gp2.5. In the first suppressor the C-terminal phenylalanine was restored by two transversions converting the codon for D231 (GAC) into a phenylalanine codon (TTC) (Table 2). When compared with wild-type gp2,5 the isolated intragenic suppressor would be designated as gp2.5 ΔD231 (Fig. 1). In the second suppressor the C-terminal phenylalanine was restored by the same two transversions observed in the first intragenic suppressor. In addition, the codon for glycine-230 was mutated to that for aspartate via a single transition (GGA→GAA) (Table 2). When compared with wild-type gp2.5, the isolated intragenic suppressor could be designated as gp2.5ΔG230 (Fig. 1). Sequence analysis revealed no additional mutations in any of the replication associated (Class II) genes, confirming that the identified suppressors are true intragenic suppressors.

Extragenic suppressors

Several extragenic suppressors were identified from the screen using the gp2.5-InsF231ΔF232 mutant (Fig. 1). The sequencing of gene 2.5 revealed that the original mutation is present in the genome of the suppressor phage and that no additional mutations have arisen within gene 2.5. Sequencing of the Class II genes of T7 revealed mutations (Table 2) in gene 5 (DNA polymerase), gene 3 (endonuclease), gene 6 (exonuclease) and gene 5.3 (unknown function). Original plaques were plaque purified three times and amplified to produce suppressor phages master stocks. Small aliquots from the master stocks were used to prepare genomic DNA and confirm the presence of the suppressor mutations as well as the original mutation in gene 2.5.

Gp2.5 is known to physically interact with T7 gene 5 DNA polymerase-thioredoxin and with gene 4 helicase-primase. In the current study, however, we have chosen to focus on the suppressor mutations identified within the DNA polymerase gene. The interaction of gp2.5 with T7 DNA polymerase has been more extensively characterized biochemically than have the interactions with the other gene products.

Mutations in gene 5 DNA polymerase are true suppressors

One of the suppressors identified has one amino-acid change in gene 5 of T7 (gp5-G371K) whereas the other has two (gp5-A411T/T258M). As our DNA sequence analysis involved only Class II genes and not the entire phage genome, it is possible that other mutations exist and contribute to the observed phenotype. To exclude this possibility we carried out a two-gene complementation assay. For the purpose of this analysis we constructed a T7 lacking gene 2.5 and gene 5. In addition, the thioredoxin (*trxA*) and cytidine monokinase kinase (*cmk*) were inserted in the respective positions. Both *trxA* and *cmk* are essential for the growth of T7 phage in *E. coli*, but *E. coli* itself requires neither gene (Qimron *et al.*, 2006). A plasmid carrying gene 2.5-InsF231ΔF232 (the gp2.5-mut, against which a second-site suppressor in gp5 was selected) and a plasmid carrying the corresponding suppressor gene 5 mutations were transformed into an *E. coli* K12/*trxA*$^{-}$/*cmk*$^{-}$ strain. Then, an infection with T7Δ2.5/*trxA*$^{+}$/Δ5/*cmk*$^{+}$ phage was carried out and the ability of each pair of mutations (lethal gp2.5 mutation/gp5 suppressor mutation) to complement the growth of the phage lacking gp2.5 and gp5 genes was determined (Table 3). The suppressor mutations were able to support the growth of the T7Δ2.5Δ5 deletion phage in a pair with the original gp2.5-InsF231ΔF232 mutation they were selected against. In contrast, the combination of wild-type gp5 and gp2.5-InsF231ΔF232 was not able to support the growth of the double deletion phage. We conclude that the iden-

Table 3. Gp5-G371K and gp5-A411T/T258M are true suppressors of gp2.5-Ins F231ΔF232 mutation.

gp2.5	gp5	Viral titre
Wild-type	wt	7×10^8
	wt	0
gp2.5-InsF231ΔF232	G371K	3×10^6
	A411T/T258M	5×10^6
	A411T	0
	T258M	0

Two-gene complementation assay was performed to demonstrate that the selected gp5 mutants are true suppressors of gp2.5-Ins F231ΔF232 mutation. *E. coli* K12 cells lacking the trx and cmk genes were transformed with pGP5-based plasmid encoding for wild-type or mutant gp5 and pACYC-based plasmid encoding for wild-type or mutant gp2.5. Infection with a T7Δgp2.5/trx$^+$/Δgp5/cmk$^+$ was carried out in liquid culture and the resulting lysates titrated on agar plates overlaid with soft agar containing DH5α cells. The phage titres are an average of three repetitions.
wt, Wild-type.

tified secondary mutations are true suppressor mutations. The observed suppression is specific as gp5-G371K and gp5-A411T/T258M mutants support productive infection only in the presence of gp2.5-InsF231ΔF232 and not gp2.5F232L variant (data not shown).

The suppressor mutations in T7 gene 5 (DNA polymerase) support the growth of T7Δ5 bacteriophage

In order to determine if the gene 5 suppressor mutants encode functional DNA polymerase, we examined their ability to support the growth of T7Δ5 bacteriophage (Fig. 3A). Remarkably, one of the suppressor mutations complemented almost as well as the wild-type gene 5 (efficiency of plating 0.9 versus 1), whereas the other exhibited approximately a twofold reduction (efficiency of plating 0.6 versus 1). Preliminary biochemical analysis suggests that the polymerase activity of the gene 5 proteins harbouring the suppressor mutations are comparable to that of wild-type T7 DNA polymerase using a primed M13 DNA primer-template (S. Ghosh, B. Marintcheva and C. Richardson, unpubl. data). This result is not surprising as the suppressor mutations are located outside the active site of the polymerase (see below). When overexpressed the gp5 variants carrying suppressor mutations are somewhat inhibitory for the growth of wild-type T7 phage (Fig. 3B).

Both A411T and T258M mutations in gp5-A411T/T258M are required for suppressor function

One of the gp5 suppressor mutants acquired two single mutation: A411T and T258T. In order to separate the contribution of each mutation to the observed suppressor phenotype, we cloned each point mutation on separate plasmids and examined each for its ability to suppress the gp2.5-InsF231ΔF232 mutation. Neither single mutant was able to do so (Table 3), demonstrating that both mutations are required for suppression.

Discussion

ssDNA binding proteins are universal components of the genome maintenance machinery. As such they bind DNA, protect its integrity, as well as recruit and organize proteins with various enzymatic activities depending on the nature of the DNA substrate. ssDNA-binding proteins of prokaryotes and their viruses mediate protein–protein interactions via their acidic C-terminal tails. The tail is highly flexible and unstructured, most likely reflecting the 'need' to accommodate interactions with various partners. In addition, the tail binds, at least transiently in the ssDNA-binding cleft of the molecule, protecting the cleft from random binding to negatively charged surfaces. Most important, this intramolecular binding bridges the DNA-binding and the protein–protein interactions of the molecule.

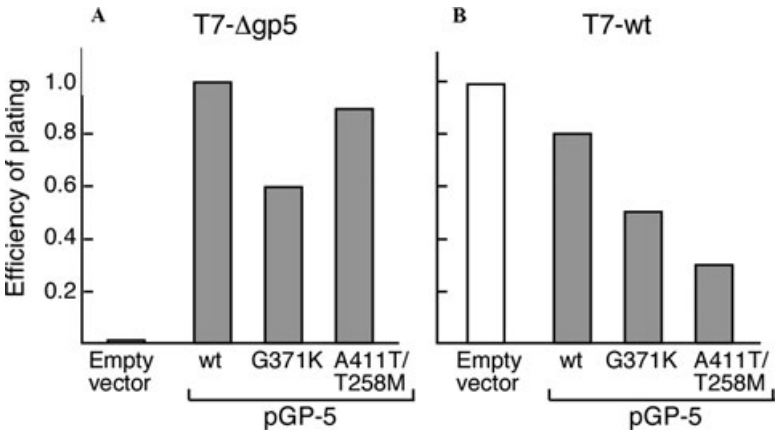

Fig. 3. Efficiency of plating T7Δgp5 and T7 wild-type phages on cells overexpressing gp5 variants.
A. Complementation assay. Wild-type and mutant gp5 plasmid were transformed in *E. coli* DH5α cells and the cells were infected with T7Δgp5 phage. The plating efficiencies were calculated as a ratio of the number of plaques observed for each gp5 variant and the number of plaques observed for wild-type gp5.
B. Gp5 overexpression is inhibitory for wild-type T7 phage growth. DH5α cells transformed with wild-type and mutant gp5 variants were infected with wild-type T7 phage and efficiency of plating calculated as a ratio of the number of plaques observed for each gp5 variant and the number of plaques observed for the cell strain transformed with empty vector.

In the current study we sought to identify interaction partners *in vivo* of the acidic C-terminal tail of the ssDNA binding protein, gp2.5, encoded by bacteriophage T7. Using the newly designed approach we obtained both intragenic and extragenic suppressors. The identified intragenic suppressors confirmed the critical nature of the C-terminal aromatic residues, previously identified from *in vitro* mutagenesis studies. Extragenic suppressor mutations were found in known and novel interaction partners. Among these extragenic suppressors are mutations in gene 5, the gene encoding T7 DNA polymerase, a known interaction partner of gp2.5 (Kim *et al*., 1992b). We demonstrate that the mutations in gene 5 are true suppressors and propose a mechanism for suppression based on the amino-acid changes in T7 DNA polymerase.

Selection of suppressors of dominant lethal mutants

Studies on dominant-lethal mutations of essential genes are challenging as the lethal phenotype limits the approaches that can be used. Dominant lethality is not a surprising phenomenon for ssDNA-binding proteins as they are multifunctional, oligomeric and abundant, as well as being involved in multiple DNA processes. Theoretically, any mutation that impairs the ability of a ssDNA protein to bind to DNA could result in the sequestering of interaction partners in the cell as opposed to 'bringing' them to the ssDNA. On the other hand, mutations that abolish protein–protein interactions could also result in ineffective recruitment of enzymes to the relevant DNA substrates. In some cases, the overexpression of wild-type protein, itself, is sufficient to interfere with the integrity of the biochemical processes. For example, over production of wild-type gp2.5 reduces T7 phage growth by 60% (data no shown).

The suppressor selection scheme presented here solves the problem of dominant lethality by using two plasmids: a low-copy plasmid expressing the dominant lethal allele of gene 2.5 from a weak promoter and a high-copy plasmid expressing wild-type gene 2.5 from strong promoter. The successful selection of suppressors relies on the ability of the lethal mutant to recombine and the availability of a functional gp2.5 to propagate the recombinant genome(s) so that natural selection occurs. Recombination is enhanced by cloning the lethal version of gene 2.5 with long-flanking sequences. It is critical the reaction to be performed in liquid media allowing recombinant phages unlimited access to host cells to be propagated several times. Our screen was able to identify previously described as well as new interaction partners of gp2.5. Surprisingly, the screen did not identify previously known interaction with T7 gene 4 helicase/primase. One possibility is that the scale of the screening was insufficient or that the C-terminal phenylalanine of gp2.5 is irrelevant in the interaction. In summary, we have developed a screening scheme that allows for selection of second-site suppressors of dominant lethal alleles of one of the viral essential genes of bacteriophage T7. The scheme can be used to select suppressors to any dominant lethal allele of viral essential gene providing appropriate genetic markers are available.

The critical nature of the C-terminal phenylalanine of gp2.5

Previous studies of the determinants critical for the function of the C-terminal tail of gp2.5 identified an aromatic residue in the C-terminal position as well as the importance of acidic residues (Marintcheva *et al*., 2006). The critical nature of the C-terminal aromatic residue is most likely dictated by its interaction with a hydrophobic patch/pocket with tight geometrical constrains (Marintcheva *et al*., 2006). In this study we selected for intragenic suppressors of mutants lacking the C-terminal phenylalanine or of one in which the phenylalanine was changed from the C-terminal position to the penultimate. To our surprise, no such mutations were identified. Instead, we selected only for mutations that restored the C-terminal phenylalanine, i.e. effectively 'repeating' *in vivo* our rationally designed *in vitro* experiments. One of the selected suppressors is in fact gp2.5Δ1F (Marintcheva *et al*., 2006) lacking D231 (Fig. 1). Genetically, it complements the growth of T7Δ2.5 phage and exhibits properties similar to that of the wild-type with regard to its ability to bind to ssDNA and T7 DNA polymerase (Marintcheva *et al*., 2006). The second suppressor lacks G230 (Fig. 1), a residue that is not critical for gp2.5 function as evidenced by the viability of the G230C mutant (B. Marintcheva and C.C. Richardson, unpubl. data), as well as the viability of the gp2.5Δ6F mutant lacking six amino acids from the C-terminus of gp2.5 but preserving the C-terminal phenylalanine (Marintcheva *et al*., 2006). Although the nature of the selected intragenic suppressors does not allow us to identify what are the contacts of the C-terminal phenylalane on the surface of gp2.5, it provides excellent example of 'natural selection unfolding on a scale of single experiment', as well as confirms independently the critical role of the presence of aromatic residue in C-terminal position of gp2.5.

Location of the suppressor mutations in the crystal structure of T7 DNA polymerase

The crystal structure of the T7 gene 5 DNA polymerase in complex with thioredoxin, a primer-template, and a deoxynucleoside triphosphate has been determined at 2.8 A resolution (Doublie *et al*., 1998). Therefore, in order to gain insight on the mechanism of suppression and the

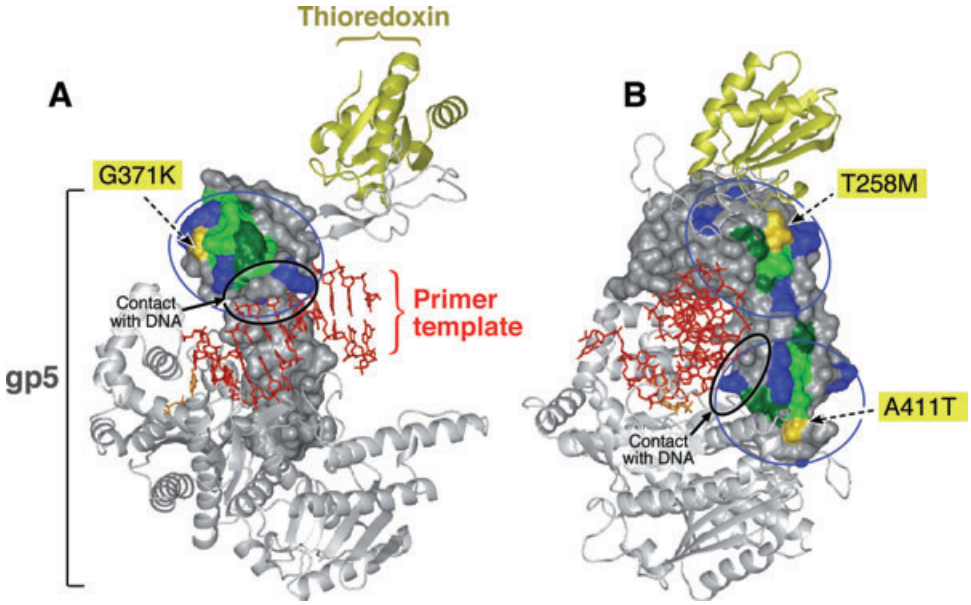

Fig. 4. Mapping the suppressor mutations on the structure of gp5/thioredoxin/primer/template complex. The positions of the identified suppressor mutations were mapped on the structure of the gp5/trx/primer-template complex (Doublie *et al*., 1998). The gp5 is coloured in grey, the thioredoxin is coloured in light yellow, the primer-template DNA is coloured in red and the nucleotide in the polymerase active site is coloured in orange.
A. Position of G371K mutation. The G371K mutation is coloured in yellow. Hydrophobic residues in close proximity to G371K are coloured in green. Dark green depicts aromatic residues (W351, Y356). Positively charged residues in close proximity to G371K mutation are coloured in blue (E535, E546). Residues in direct contact with DNA (V364, D366) and in close proximity to G371 K mutation are circled with a black oval.
B. Position of the A411T and T258M mutations. The A411T and T258M mutations are coloured in yellow. Adjacent aromatic residues (Y409, F234 and F261, F334 respectively) are coloured in dark green. Hydrophobic and charged residues are coloured as described in (A). Residues in direct contact with DNA (K405, D403) and in close proximity to A411T mutation are circled with a black oval.

interaction between the C-terminus of gp2.5 and T7 DNA polymerase, we mapped the positions of the suppressor mutations on the 3D structure of the complex (Fig. 4). The lysine resulting from the suppressor mutation G371K lies in close proximity to residues V364 and D366 reported to be in contact with the DNA (Doublie *et al*., 1998). Similarly, in gp5 A411T/T258M the A411 residue is in close proximity to residues K405 and D403 involved in direct contacts with DNA. Interestingly, residues with aromatic side-chains (dark green) are also found in proximity to the suppressor mutations. It is tempting to speculate that the essential phenylalanine at the tip of the C-terminal tail interacts with at least some of these aromatic side-chains. If so, the suppressor mutations could introduce small changes in the hydrophobic pocket allowing the phenylalanine in the penultimate position to be accommodated.

Such a mechanism is in concert with a recently proposed mechanism for the physical and functional interaction of the C-terminal tail of *E. coli* SSB protein with Exonuclease I (Lu and Keck, 2008) and RecQ helicase (Lu and Keck, 2008; Shereda *et al*., 2009). Despite the absence of homology both proteins interact with the C-terminal tail of SSB in a strikingly similar manner. Both the hydrophobic moiety and the α-carboxyl of the residue are involved in the interaction. The α-carboxyl group forms ionic bond with conserved residue from the 'basic lip' of the hydrophobic pocket that accommodates the aromatic ring. Negatively charged residues from the tail interact electrostatically with residues from the 'basic ridge' of the hydrophobic pocket (Lu and Keck, 2008; Shereda *et al*., 2009).

The distribution of the electron density of the C-terminal peptide of *E. coli* SSB protein with exonuclease I (Lu and Keck, 2008) suggests that the interaction relies mainly on the very C-terminal part of the tail. The authors report well-defined electron density only for the last three amino acids from a 10-mer peptide. This finding parallels our results from site-directed mutagenesis, showing that the C-terminal tail of gp2.5 can be shortened to some extent without function disruption as long as C-terminal aromatic residue is present.

Most likely, the length of the C-terminal tail is not a strict constrain for function as means to accommodate multiple interaction partners. The G371K mutation introduces significant change in the size and the charge of the amino acid at position 371 in the protein. The replacement creates new options for long distance hydrogen bonding or salt bridges, which were not possible when the position is occupied by glycine. The introduced lysine could favour electrostatic interactions (Fig. 4A) between the 'fingers'

domain (depicted in spacefill mode) and the polymerase domain (depicted in ribbon and holding nucleotide and DNA primer/template). Such an interaction might 'pull' the pocket where the end of the C-terminal tail binds, so that now it can accommodate one extra amino acid.

In contrast, the point mutations in gp5A411T/T258M do not introduce drastic alterations in terms or charge or size of the amino acid in each respective position. Interestingly, the A411T and T258M mutations occupy symmetrical positions on both sides of the DNA template binding surface. The symmetrical position of A411T and T258M and the nature of the individual residue might be the reason why both mutations are necessary for the suppressor properties of the gp5-A411T/T258M mutant.

The identification and genetic characterization of mutations suppressing the lethal phenotype of gp2.5ΔF232InsF231 mutants reported here create an opportunity for further explorations of the role of the C-terminus of gp2.5 at the T7 replisome. Biochemical characterization of the mutant/suppressor mutant pairs has the potential to provide valuable insights in the dynamics of protein–protein interactions at the replisome. The selection procedure for suppressors of dominant-lethal mutations in essential genes can also be used in any model system, providing appropriate genetic markers are available, thus offering an excellent tool to explore the significance of dominat lethal phehotypes.

Experimental procedures

Plasmids, bacterial strains and phages

pET-17b-gp2.5-wt and pGP5 plasmids containing wild-type copy of gp2.5 and gp5, respectively, were previously described (Rezende *et al.*, 2002). pACYC-gp2.5 containing a copy of wild-type gp2.5 gene, flanked by 400 bp upstream and 200 bp downstream sequences, was generated by PCR. The region of interest was amplified using wild-type T7 phage DNA and cloned into pACYC 184 vector. The pACYC-gp2.5-F232L, pACYC-gp2.5-F232R, pACYC-gp2.5ΔF232 and pACYC-gp2.5-InsF231ΔF232 plasmids were generated by *in vitro* mutagenesis, using pACYC-gp2.5-wt as a template and mega-primer two-step PCR approach (Colosimo *et al.*, 1999) to introduce the corresponding mutations. A diagnostic SacI silent restriction site was introduced immediately after the stop codon for gp2.5, thus creating a convenient marker. pGP5-G371K, pGP5-A411T/T258M, pGP5-A411T and pGP5-T258M were generated by *in vitro* mutagenesis using pGP5 plasmid as a template and Quick change mutagenesis kit (Stratagene). The sequence of all plasmids was confirmed by sequencing.

The following bacterial strains were used throughout this study: DH5α (Novagen), HMS262 (*F*$^+$*hsdR pro leu*$^-$*lac*$^-$*thi*$^-$*supE tonA*$^-$*trxA*$^-$ genotype) (laboratory collection), K12 *trx*$^-$ and K12 *cmk*$^-$ were obtained from Keio collection (Baba *et al.*, 2006), K12trx-*cmk*$^-$ strain was constructed as described below.

The following previously described phages were used in this study: T7 wild-type and T7 Δ2.5/trx$^+$ (Kim and Richardson, 1993). The double deletion phage lacking gene 2.5 and gene 5 was constructed as described below.

Constructing trxA::kan *and* cmk::tetA *double gene replacements*

A mutant *E. coli* K-12 BW25113-*trxA*::*kan* from the Keio collection was used as a parent strain for introducing an additional *cmk*::*tetA* gene replacement essentially as described for introducing the first replacement, with some modifications. A PCR reaction was used to amplify the *tetA* resistance marker from pACYC184 plasmid (New England Biolabs, Ipswich, MA). The following primers were used to produce a *tetA* cassette flanked by 50 bp homology to the sequences upstream and downstream the *cmk* gene: '*cmk tetA* for' – ATGCGCGCGGTTATGTTAACGGTACGCCTGTTTTAAGGA GATAAAGATGA ATGAAATCTAACAATGCGCT and '*cmk tetA* rev' – ACATACCCGCTGTCATTCCATTGCAACGGGGGTAC TGCAAATTCGGTCGC TCCGTTAGCGAGGTGCCGCC.

PCR products were digested with DpnI in order to eliminate the resistance marker of the pACYC184 template. The products were then purified using PCR product purification kit (Qiagen, Valencia, CA).

Escherichia coli K-12 BW25113-*trxA*::*kan* carrying the Red helper plasmid pKD46 were grown in 500 ml Luria–Bertani medium with kanamycin, ampicillin and 1 mM L-arabinose at 30°C to an OD_{600} of 0.3. Electroporation-competent cells were prepared by washing the culture three times with ice-cold-10% glycerol and finally re-suspending the pellet with 500 μl ice-cold-10% glycerol. Approximately 50 μl of competent cells was mixed with 400 ng of the PCR fragment in an ice-cold 0.1 cm cuvette (Invitrogen, Carlsbad, CA). Cells were electroporated at 1.8 kV, immediately followed by the addition of 1 ml of SOC medium. After incubation for 2 h at 37°C, one-tenth portion was spread onto agar plate to select for *tet*^{R} colonies. Sequencing of a PCR product, which amplified the region of the *cmk* gene replacement, verified the desired cassette insertion.

Constructing T7Δgp2.5/trx$^+$*Δgp5/cmk*$^+$ *phage*

T7Δgp2.5/trx$^+$Δgp5/cmk$^+$ phage *was* generated by recombination between T7 Δgp2.5/trx$^+$ phage and a pGp5-*cmk* plasmid encoding the *cmk* gene flanked by sequences upstream (400 bp) and downstream (200 bp) of gene *5* following a previously described protocol (Qimron *et al.*, 2006). The gene replacement was confirmed by direct sequencing of the genomic DNA from the recombinant phage and master phage stock was prepared after three steps of plaque purification.

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