Bacteriophage P4 sut1: a mutation suppressing transcription termination

Susanna Terzano,† Ilaria Oliva,‡ Francesca Forti, Claudia Sala, Francesca Magnoni, Gianni Dehò and Daniela Ghisotti

Dipartimento di Scienze Biomolecolari e Biotecnologie, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy

In the *Escherichia coli* satellite phage P4, transcription starting from PLE is prevalently controlled via premature termination at several termination sites. We identified a spontaneous mutation, P4 sut1 (suppression of termination), in the natural stop codon of P4 orf151 that, by elongating translation, suppresses transcription termination at the downstream t151 site. Both the translational and the transcriptional profile of P4 sut1 differed from those of P4 wild-type. First of all, P4 sut1 did not express Orf151, but a higher molecular mass protein, compatible with the 303 codon open reading frame generated by the fusion of orf151, cnr and the intervening 138 nt. Moreover, after infection of *E. coli*, the mutant expressed a very low amount of the 1.3 and 1.7 kb transcripts originating at PLE and PLL promoters, respectively, and terminating at the intracistronic t151 site, whereas correspondingly higher amounts of the 4.1 and 4.5 kb RNAs arising from the same promoters and covering the entire operon were detected. Thus the sut1 mutation converts a natural stop codon into a sense codon, suppresses a natural intracistronic termination site and leads to overexpression of the downstream cnr and z genes. This correlates with the inability of P4 sut1 to propagate in the plasmid state. By cloning different P4 DNA fragments, we mapped the t151 transcription termination site within the 7633–7361 region between orf151 and gene cnr. A potential stem–loop structure, resembling the structure of a Rho-independent termination site, was predicted by mfold sequence analysis at 7414–7385.

INTRODUCTION

Bacteriophage P4 is an *Escherichia coli* satellite phage that depends on a helper phage, such as P2, for its lytic growth (reviewed by Briani et al., 2001; Dehò & Ghisotti, 2006). The helper phage provides all the structural proteins that are assembled by P4 to build ‘small’ heads, suitable for its reduced-size genome. In the absence of P2, P4 can propagate in the plasmid state, replicating autonomously. Alternatively, P4 can lysogenize the bacterial host, integrating its genome into the bacterial chromosome.

The choice between the lysogenic and the plasmid/lytic mode of propagation depends on the regulated expression of two genes, cnr and z, required for P4 DNA replication. Both genes are located in the distal part of the P4 left polycistronic operon, whose transcription is controlled by two promoters, the constitutive promoter PLE and the upstream inducible promoter PLL (Fig. 1). Early after infection, PLE activity results in the expression of three main transcripts: a 4.1 kb RNA covering the entire operon (genes cl, kil, o, orf151, cnr and z) terminating at t5, a 1.3 kb RNA that terminates at t151 between orf151 and cnr and short transcripts about 0.3 kb long (Dehò et al., 1988, 1992).

About 20 min after infection, transcription starting at PLL is prematurely terminated at several termination sites (t1, t9, timm) within the first 300 nt downstream of the transcription starting site (Briani et al., 2000). The P4 factor that controls premature transcription termination is a small RNA, the CI RNA, produced by processing of longer transcripts. The CI RNA induces transcription termination at t1 and timm by pairing with the nascent transcript (Briani et al., 2000; Forti et al., 2002). In this way, it prevents transcription of the distal part of the polycistronic operon, promotes entry into the lysogenic state and confers immunity to superinfection of the host.

The plasmid/lytic mode of propagation of P4 is associated with the late activation of the PLL promoter (Dehò et al., 1988, 1992). Two main transcripts 4.5 and 1.7 kb long, terminating at t5 and t151, respectively, are produced. When transcription starts at PLL, two additional genes, vis and eta, encoded in the 5′ end of the transcript, are expressed. Eta covers the transcription termination sites t9, t4 and timm. As a consequence, when transcription starts from PLL,
transcription termination is prevented by eta translation (Forti et al., 1999).

A P4 virulent mutant, vir1, is insensitive to P4 immunity, expresses persistently the replication genes cnr and a and, upon infection of an E. coli cell in the absence of the P2 helper, establishes the plasmid state in about 100% of the infected cells (Dehò et al., 1992). vir1 is a promoter up-mutation in the −10 region of Pll that makes transcription from this promoter constitutive (Lin, 1984; Dehò et al., 1988).

A coordinated expression of the cnr and a replicative genes is not only required for the maintenance of the lysogenic state but also seems to be critical for stable plasmid propagation; indeed, overexpression of Cnr and a proteins by a plasmid causes P4 over-replication and cell lethality ensues (Terzano et al., 1994).

In this work we report the identification of a new spontaneous mutation that eliminates the natural stop codon of orf151, preventing transcription termination at the downstream t151 site and thus causing overexpression of the replicative genes. The effect of the mutation is described and the t151 transcription termination site is mapped.

**METHODS**

**Bacterial strains, bacteriophages and plasmids.** The E. coli strains, bacteriophages and plasmids used are listed in Table 1. The coordinates are from the P4 complete genome as reported in GenBank accession no. X51522, corrected +1 because of a G:C insertion at 2687 (Piazzolla et al., 2006). The P4 DNA fragments indicated were obtained by digestion with restriction enzymes of P4 DNA and cloned in the vectors indicated. The P4 regions carried by pGM840, pGM841 and pGM842 were amplified by PCR, cloned and sequenced.

**Table 1.** Bacterial strains, bacteriophages and plasmids

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-1a</td>
<td>E. coli C, prototrophic</td>
<td>Sasaki &amp; Bertani (1965)</td>
</tr>
<tr>
<td>C-1843</td>
<td>Isogenic with C-1844</td>
<td>Sunshine et al. (1971)</td>
</tr>
<tr>
<td>C-1844</td>
<td>suA, polarity suppressor</td>
<td>Sunshine et al. (1971)</td>
</tr>
<tr>
<td>C-5204</td>
<td>str-1 supD</td>
<td>Dehò (1983)</td>
</tr>
<tr>
<td>C-5205</td>
<td>str-1 supD (P2)</td>
<td>Dehò (1983)</td>
</tr>
<tr>
<td>C-5218</td>
<td>Double P2 and P4 lysogen</td>
<td>Dehò (1983)</td>
</tr>
<tr>
<td>C-5586</td>
<td>C-5204 hbl-1</td>
<td>Piazza et al. (1996)</td>
</tr>
<tr>
<td>DS410</td>
<td>min</td>
<td>Reeve (1977)</td>
</tr>
<tr>
<td><strong>Bacteriophages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P4 sut1</td>
<td>TA to GC transversion at 7445</td>
<td>This work</td>
</tr>
<tr>
<td>P4 vir1</td>
<td>Promoter up mutation in Pll (GC to AT transition at 9100)</td>
<td>Lindqvist &amp; Six (1971)</td>
</tr>
<tr>
<td>P4 Vir3</td>
<td>Spontaneous strong virulent mutant, harbours vir1 and sut1 mutations</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGM256</td>
<td>P4 sut1 7630–7336 in pUC19</td>
<td>This work</td>
</tr>
<tr>
<td>pGM257</td>
<td>P4 7630–7336 in pUC19</td>
<td>This work</td>
</tr>
<tr>
<td>pGM274</td>
<td>P4 7946–4261 in pUC19</td>
<td>This work</td>
</tr>
<tr>
<td>pGM275</td>
<td>P4 sut1 7946–4261 in pUC19</td>
<td>This work</td>
</tr>
<tr>
<td>pGM331</td>
<td>tRNA&lt;sup&gt;α&lt;/sup&gt; reporter expressed from ptac</td>
<td>Briani et al. (1996)</td>
</tr>
<tr>
<td>pGM840</td>
<td>P4 7633–7311 in pGM331</td>
<td>This work</td>
</tr>
<tr>
<td>pGM841</td>
<td>P4 7633–7361 in pGM331</td>
<td>This work</td>
</tr>
<tr>
<td>pGM842</td>
<td>P4 7633–7411 in pGM331</td>
<td>This work</td>
</tr>
<tr>
<td>pUC19</td>
<td>Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
</tbody>
</table>

*The first coordinate of the P4 regions is cloned proximal to plac or ptac.
**Media and chemicals.** *E. coli* was grown in LD broth (Ghisotti et al., 1992), with the addition of $5 \times 10^{-3}$ M CaCl$_2$ for P4 adsorption. M9 minimal medium was described by Lindahl et al. (1970). Streptomycin (100 μg ml$^{-1}$), ampicillin (100 μg ml$^{-1}$) and IPTG (40 μg ml$^{-1}$) were added when required.

**One step growth.** The bacterial strain C-5204 was grown in LD broth, supplemented with 5 mM CaCl$_2$, to an optical density equivalent to $1 \times 10^6$ cells ml$^{-1}$ and infected with either P4$^+$ or P4 sut1 at an m.o.i. of 10 and incubated at 37°C. After 10 min, phage adsorption was measured and P2 antiserum (K = 1) was added to eliminate the unadsorbed phages; at 15 min from infection, the cultures were assayed to determine the titre of cells surviving the infection and of the cells yielding phage; a sample was diluted $10^3$-fold and incubated with aeration; at 90 min from infection, the P4 titre was measured.

**P4 plasmid state propagation.** Bacterial strain C-1a growth, infection with P4 and measurement of the survivors were performed as in the one-step growth experiment. The morphology of the surviving clones was determined as 'rosette'-type colonies (pP4 carriers) and normal colonies (Deho et al., 1984). The presence of P4 immunity was assessed by the cox test (Alano et al., 1986).

**Analysis of plasmid-encoded proteins.** Minicells were prepared from strain DS410 carrying the plasmids indicated as described by Reeve (1979). The purified minicells were suspended in M9 minimal medium containing 2 μg methionine ml$^{-1}$ and 100 μg D-cycloserine ml$^{-1}$, incubated at 37°C for 60 min, pelleted and suspended at a concentration of $2 \times 10^{10}$ cells ml$^{-1}$ in the same medium in the presence of $^{[35]S}$methionine [800 Ci mmol$^{-1}$ (29.6 TBq mmol$^{-1}$)]. The radioactively labelled proteins were separated by SDS-PAGE. P4 proteins of known size were run in the same gel and used as molecular mass markers (not shown).

**Northern blot hybridization.** RNA was extracted from *E. coli* C-1a and from P4-infected cells and fractionated by 1.5 % formamide–formic acid (1:5) at 40°C through a BioRad gel. RNA was transferred onto Hybond-N filter membranes by vacuum blotting. Hybridization to specific riboprobes was performed as described by Deho et al. (1988). The riboprobe covers the P4 8419–8775 region, 151 riboprobe covers the P4 7332–7631 region and 861 probe is the oligonucleotide 5'-TGTTTCTGTGTTAAATGTATCCGGC-3', complementary to the initial part of the *ptac* transcript.

**In vitro recombination.** In vitro recombination was performed by cutting circular DNA from P4 and P4 Vir3 with a set of restriction enzymes. The DNA fragments were separated by gel electrophoresis and different P4$^+$ and P4 Vir3 fragments were ligated to construct the reciprocal recombinants. We found serendipitously that P4 Vir3 was unable to plate on strain C-5586, which harbours the bfl-1 mutation in *pup* (Piazza et al., 1996; Regonesi et al., 2004). Thus, upon transformation of C-2505 with the ligated DNAs, the lysate was plated on C-5205 and single plaques were tested on C-2505, on the double P2–P4 lysogenic strain C-5218 and on C-5586. Four different phenotypes could be observed: the parental wild-type phage, which formed turbid plaques on C-5205; the other parental P4 Vir3 mutant, which was able to plate on C-5218 and unable to plate on C-5586; P4 virulent recombinants, which formed clear plaques on C-5205 and could plate both on C-5218 and on C-5586; and a second class of recombinants, which formed semiturbid plaques on C-5205 and were unable to plate on C-5586. Sequencing of the recombinant mutant fragments showed that P4 Vir3 carries two mutations: the first one was mapped with the already known P4 *vir1* (Lindqvist & Six, 1971); the second one, named *sut1*, was mapped within the 6728–7044 P4 fragment and was a T7443G transversion.

**RESULTS**

**P4 sut1 eliminates the stop codon of orf151 and suppresses transcription termination at *t*$_{151}$**

In an attempt to isolate new virulent mutants of phage P4, we plated several independent P4$^+$ phage lysates on C-5218, an *E. coli* strain lysogenic for P2 and P4, looking for clear plaques. A spontaneous strong virulent mutant, P4 Vir3, was found to harbour two different mutations: the first one was identical to the previously isolated *vir1* mutation in P$_{LL}$, a base substitution that makes P$_{LL}$ independent from positive activators (Lindqvist & Six, 1971). The second mutation, *sut1* (suppression of termination), was mapped by *in vitro* recombination within the P4 6728–7776 region, as described in Methods. Sequencing of this region revealed a T7443G transversion (Fig. 2) that eliminates the stop codon of orf151 and creates a fusion open reading frame of 303 codons (orf303), which includes orf151 and the downstream *car* gene joined by the intervening 138 nt.

Using a minicell system for analysis of plasmid-encoded proteins (Reeve, 1979), we detected Orf151, Cnr and *z* polypeptides in minicells harbouring pGM274, which carries the wild-type P4 7946–4261 region, whereas pGM275, which carries the corresponding P4 *sut1* DNA fragment, expressed Cnr and *z* but not Orf151 (Fig. 3a). Orf303 was partially covered by proteins of the plasmid resistance genes of similar size and was not clearly visible.

P4 *sut1* formed clearer plaques than P4 wild-type and gave a higher burst size upon infection of the P2 lysogenic strain C-5204 (Table 2). In the absence of the helper phage (infection of strain C-1a), P4 *sut1* could establish lysogeny upon infection at the same frequency as the wild-type, but we could not detect P4 plasmid carriers ('rosette'-type colonies; Deho et al. 1984) (Table 3). Moreover, infection of C-1a...
with the double mutant P4 vir1 sut1 (that is, P4 Vir3) caused extensive cell lethality (0.04 % survivors). The few ‘rosette’ colonies among the survivors were scarcely viable and could not be subcultured.

The data above suggest that the sut1 mutation might alter the transcription of the P4 left operon, causing higher expression of its promoter-distal part. Thus, we compared by Northern blotting the transcription profile of the left operon in P4 sut1 and P4 + after infection of strain C-1a (Fig. 3b). In P4 sut1, an about fivefold increase of the 4.1 kb transcript and the reduction or absence of the 1.3 kb RNA that ends at t151 were observed at 10 min after infection. At 20 min, the 4.1 kb transcript was reduced to a level comparable to P4 +. At 60 min, the 4.5 kb RNA was more intense, but the 1.7 kb transcript was not observed. Thus, the P4 sut1 mutation that abolishes the stop codon of orf151 causes suppression of transcription termination at t151. As a consequence, the cnr and α genes are presumably expressed at a higher level in the mutant phage. This correlates with the increased burst size in the P4 lytic cycle.

Characterization of the P4 t151 transcription termination site

The t151 transcription termination site is not dependent on the bacterial Rho factor, since the 1.3 and 1.7 kb RNAs are also present in rho2 hosts (both rho-102 and rho-15 strains; Briani et al., 1996).

To better characterize t151, we cloned the P4 wild-type and P4 sut1 7630–7336 regions, which span the last codons of orf151, cnr and the intervening region, downstream of the plac promoter in pUC19. A translational fusion of the 5′ end of lacZ and the last codons of orf151 was created (Fig. 4a). In pGM257, translation terminates at the orf151 stop codon, whereas in pGM256, the sut1 mutation eliminates the stop codon and translation proceeds downstream. The E. coli C-1843 (Rho +) and C-1844 (Rho −) strains were transformed, RNA was extracted and analysed by Northern blotting using the 151 riboprobe (7332–7631 P4 region; Fig. 4b). A specific signal corresponding to an RNA that terminates at t151 was clearly present in pGM257 both in Rho + and in Rho − strains. The signal was much fainter in pGM256, which

Table 2. P4 sut1 lytic growth in E. coli C-5204

<table>
<thead>
<tr>
<th>Infecting phage</th>
<th>Survivors (%)</th>
<th>Yielders (%)</th>
<th>Phages produced/infected cell*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4</td>
<td>26</td>
<td>74</td>
<td>30</td>
</tr>
<tr>
<td>P4 sut1</td>
<td>23</td>
<td>74</td>
<td>82</td>
</tr>
</tbody>
</table>

*Phage was titrated 90 min after infection.

Table 3. P4 sut1 establishment of plasmid state in E. coli C-1a.

<table>
<thead>
<tr>
<th>Infecting phage</th>
<th>Survivors (%)</th>
<th>Lysogenic clones/ survivors (%)</th>
<th>pP4 carriers/ survivors (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4</td>
<td>77</td>
<td>99</td>
<td>0.4</td>
</tr>
<tr>
<td>P4 sut1</td>
<td>72</td>
<td>99</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>P4 vir1</td>
<td>74</td>
<td>&lt;1</td>
<td>100</td>
</tr>
<tr>
<td>P4 vir1 sut1</td>
<td>0.04</td>
<td>&lt;0.3</td>
<td>0.7†</td>
</tr>
</tbody>
</table>

*Colonies that express P4 immunity.
†Colonies of the ‘rosette’ type (Dehò et al., 1984), carrying plasmid P4.
‡Tiny colonies, low viability.
carries the P4 sut1 DNA fragment, confirming that the mutation in the translational stop exerts an effect on the downstream transcription termination at t151.

To map more precisely the t151 termination site, P4 DNA fragments spanning the 7633–7311 (pGM840), 7633–7361 (pGM841) and 7633–7411 (pGM842) regions were cloned in pGM331, between ptac and the tRNA Gly reporter gene (Fig. 5a). E. coli C-1a strain carrying pGM840, pGM841 or pGM842 was grown, transcription was induced from ptac by addition of IPTG, RNA was extracted and blotted as described in the legend of Fig. 5 and hybridized with the 861 oligonucleotide probe, complementary to 30 nt downstream of the ptac transcription start site. The same filter was hybridized with a probe complementary to the tRNA Gly reporter gene (data not shown). The full-length and t151-terminated RNAs are indicated to the left. (c) Secondary structure of the potential stem–loop downstream of 7411, according to the prediction made by the mfold RNA program (Zuker, 2003).

A potential stem–loop structure from 7414 to 7385 (Fig. 5c) is predicted by the mfold RNA program (Zuker, 2003). This structure is not present in pGM842 and may represent the transcription termination site.

**DISCUSSION**

**P4 sut1**

As in other better-characterized phages, such as λ, transcription termination and antitermination play an
important role in the control of satellite P4 gene expression, exemplified by P4 immunity and by the antitermination activity of P4 Psu protein at Rho-dependent terminators (polarity suppression; Sauer et al., 1981). In particular, expression of the left operon genes is essentially regulated by premature transcription termination. Both immunity establishment and maintenance are controlled by premature termination of transcription starting from Pl and t1 and t1imm (Briani et al., 1996, 2000; Forti et al., 2002); moreover, transcription termination at t11 controls differential expression of the promoter-distal genes of the left operon, both in the early (Pl) and in the late/plasmid (Pll) mode of transcription. In this paper we isolated and described a spontaneous mutation, sut1, in which a base substitution changes the stop codon of orf151 to sense.

At the molecular level, the mutation creates a fusion of orf151 and cnr which, in turn, suppresses Rho-independent transcription termination at the intergenic terminator t11. In the sut1 mutant, the 1.3 and 1.7 kb transcripts, originating from Pl and Pll, respectively, are produced in very low amounts, whereas the 4.1 and 4.5 kb RNAs are overproduced.

In the lytic cycle, the burst size of P4 sut1 is increased in comparison to P4+7. This may be caused by overproduction of the Cnr and x proteins encoded by the 4.1 and 4.5 kb RNAs and/or by a partial inactivity of the negative regulator Cnr, caused by its fusion in a 303 aa-long polypeptide. It is known that Cnr interacts with x, the P4 primase, and negatively controls P4 DNA replication initiation (Terzano et al., 1994; Ziegelin et al., 1997; Tocchetti et al., 2001). Although the amount of Cnr produced by P4 sut1 does not appear to decrease, the presence of the fusion protein might interfere with the interaction, being less efficient than normal. In both cases, P4 DNA over-replication may lead to an increased production and release of mature phages.

However, the t11 termination site appears more relevant for plasmid propagation than during the lytic cycle. In fact, plasmid propagation is completely prevented: no viable colonies that carried P4 sut1 in the plasmid state could be isolated either upon infection with P4 sut1 or upon infection with P4 sut1 vir1. Moreover, after P4 vir1 sut1 infection, extensive cell killing occurs in the infected cells. When the cnr and x proteins were overexpressed from a plasmid, the P4 lytic cycle was almost normal, but P4 vir1 was unable to propagate in the plasmid state, since the colonies were unstable and segregated cured clones (Terzano et al., 1994). This suggests that modulation of expression of the replication genes cnr and x has a primary role in P4 plasmid maintenance and that this regulation is normally achieved by transcription termination at t11. It is likely that x and Cnr overexpression prevents the correct control of plasmid replication, and cell lethality ensues.

**Characterization of t11**

The t11 transcription termination site was shown to be a weak Rho-independent terminator, located in the intergenic region between orf151 and cnr. Comparison of the P4 regions cloned in pGM841 and pGM842 (7633–7361 and 7633–7411, respectively) showed that the former contains a functional t11 site. Termination of transcription at t11 occurs in the absence of any P4-encoded protein, since it occurs also in pGM841 that did not express any P4 gene: the 65 terminal codons of orf151 are not likely to be translated, since no obvious ribosome-binding site or translation start codon exists.

Sequence analysis of the transcribed region with the mfold RNA program by Zuker (2003) showed a stem–loop structure between 7414 and 7385. The hairpin structure (ΔG = −16.5 kJ mol⁻¹) resembles a Rho-independent termination site and is preceded by an adenine-rich region (AAAAUUAA), conforming to the prediction of Lesnik et al. (2001). However, only two uracils are present immediately downstream. This may correlate with a relatively weak transcription termination.

The adenine-rich region is predicted to be unfolded, and the hairpin structure immediately downstream may induce transcription to pause and, eventually, to terminate prematurely at t11. The region in which t11 is located is intergenic between P4 orf151 and cnr and is normally not translated. The consequence of the sut1 mutation is connected to translation of the intergenic region: the presence of ribosomes will modify the structure of the mRNA, the formation of the hairpin will be prevented and transcription arrest inhibited.

Suppression of both Rho-dependent and Rho-independent transcription termination by translation of the nascent RNA is common in prokaryotes (Henkin & Yanofsky, 2002; Borukhov et al., 2005; Banerjee et al., 2006). However, while suppression of Rho-dependent termination appears readily explained by ribosome covering Rho-utilization sites, thus preventing Rho binding to mRNA, the mechanism of suppression at intrinsic terminators is less clear. It may be suggested that the oncoming ribosome may reduce the RNA pausing time at the newly formed stem–loop, thus decreasing the probability of transcription termination.

**ACKNOWLEDGEMENTS**

This work was supported by grant no. 2002053757-001 from the Ministero dell’Istruzione, dell’Università e della Ricerca, Rome, Italy, and by grant no. 01-0786 of INTAS.

**REFERENCES**


