The double mechanism of incompatibility between λ plasmids and *Escherichia coli* *dnaA(ts)* host cells

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For plasmids derived from bacteriophage λ , the initiation of bidirectional DNA replication from *ori λ* depends on the stimulation of transcription from the *p_R* promoter by the host replication initiator protein DnaA. Certain *Escherichia coli* *dnaA(ts)* mutants cannot be transformed by wild-type λ plasmids even at the temperature permissive to cell growth. This plasmid–host incompatibility appeared to be due to inefficient stimulation of transcription from the *p_R* promoter by the mutant DnaA protein. This paper shows that there is a second mechanism for the incompatibility between λ plasmids and *dnaA(ts)* hosts, exemplified in this study by the *dnaA46* mutant. This is based on the competition between the λ P protein and the host DnaA and DnaC proteins for DnaB helicase. Both mechanisms must be operative for the incompatibility.

Keywords: λ plasmid replication, *dnaA* mutants, DnaA protein functions, transcriptional activation of origin, DnaB helicase

INTRODUCTION

Plasmids derived from bacteriophage λ (called λ plasmids) bear the replication region of the phage genome and can be maintained in the host (*Escherichia coli*) cells at a certain copy number (for a review see Taylor & Węgrzyn, 1995). There are two λ -encoded replication proteins, O and P. The O protein binds to the *ori λ* region (located within the O gene) and initiates the assembly of the replication complex. The host-encoded helicase, DnaB, is delivered to *ori λ* due to its interaction with the P protein, which also has an affinity to the O protein. Since the P–DnaB interaction is very tight, the action of molecular chaperones, DnaK, DnaJ and GrpE, is necessary for remodelling the nucleoprotein complex in such a way that DnaB is liberated as active helicase from P-mediated inhibition. Additionally, a process called transcriptional activation of the origin, i.e. transcription entering this region, is necessary for efficient initiation of DNA replication from *ori λ* . This transcription starts from the *p_R* promoter located several hundred base pairs upstream of *ori λ* (Taylor & Węgrzyn, 1995).

Recent studies have revealed that although the assembly of the λ replication complex is necessary for initiation of λ plasmid DNA replication, this process plays no regulatory role (for a review see Węgrzyn & Węgrzyn, 2001). In fact, after one replication round the λ replication complex is inherited by one of two daughter plasmid copies (Węgrzyn & Taylor, 1992). The complex may function in subsequent replication rounds (Węgrzyn *et al.*, 1996a), but requires a trigger signal which seems to be provided by transcriptional activation of *ori λ* (Szalewska-Pałasz *et al.*, 1994; Szalewska-Pałasz & Węgrzyn, 1994; Węgrzyn *et al.*, 1996a). Thus, this process is crucial for regulating the frequency of replication initiation at *ori λ* (Taylor & Węgrzyn, 1995, 1998; Węgrzyn & Węgrzyn, 2001). It was demonstrated recently that the *p_R* promoter is stimulated by DnaA protein (Szalewska-Pałasz *et al.*, 1998a), the host replication initiator. Therefore, DnaA controls initiation of λ DNA replication by stimulating the transcriptional activation of *ori λ* .

Wild-type λ plasmids cannot transform certain *E. coli* temperature-sensitive (*ts*) mutants in the *dnaA* gene, *dnaA46*, *dnaA204* and *dnaA508*, even at a temperature permissive for bacterial growth (Węgrzyn *et al.*, 1996b). This incompatibility between λ plasmids and *dnaA(ts)*

Abbreviation: aCT, autoclaved chlortetracycline.

mutants presumably acts at the level of replication initiation, and therefore is comparable to incompatibility between two plasmid replicons that cannot co-exist in a host cell. It can be abolished by specific mutations in the λ *P* gene, called π (Kur *et al.*, 1987; Węgrzyn *et al.*, 1996b). However, neither wild-type λ plasmids nor π mutants can replicate in all tested *dnaA*(ts) mutants at elevated (above 42 °C) temperatures (Kur *et al.*, 1987; Węgrzyn *et al.*, 1996b).

The aim of this work was to investigate the mechanism(s) of the incompatibility between λ plasmids and certain *dnaA*(ts) mutants, exemplified in this study by the *dnaA46* mutant. We suspected that such studies would provide important information about the role of transcription in the control of DNA replication and about the functions of the DnaA protein.

METHODS

Bacterial strains and plasmids. *Escherichia coli* wild-type strain MG1655 (Jensen, 1993) and a series of its *dnaA* and/or $\Delta seqA::Tn10$ derivatives (described by Węgrzyn *et al.*, 1999) were used. The following plasmids were employed: pBR322 (Bolivar *et al.*, 1977), pKB2 (wild-type λ plasmid harbouring a kanamycin-resistance gene) (Kur *et al.*, 1987), pCB104 (wild-type λ plasmid harbouring a chloramphenicol-resistance gene) (Boyd & Sheratt, 1995), pAW6 (a derivative of pCB104 but bearing the $\pi A66$ mutation) (Węgrzyn *et al.*, 1995), pTC λ 1 (an artificial λ plasmid containing the p_{tet} promoter instead of p_R) (Herman-Antosiewicz *et al.*, 1998b), pTC λ 1 π [a derivative of pTC λ 1 but bearing the $\pi A66$ mutation; this plasmid was constructed by insertion of the *PvuII*–*PvuII* fragment of pAW6, bearing the replication region, into the *SmaI* site of plasmid pCattTrE18 (Herman-Antosiewicz *et al.*, 1998b) to produce pAW10, and then by deletion of the *NotI*–*NotI* fragment of pAW10 encompassing *oriPBR322*], and a series of pINGK-derived plasmids (pINGK is a cloning vector with the *ara* promoter) bearing the *dnaB* (pINB), *dnaC* (pINC) or *dnaB* and *dnaC* genes (pINBC) under control of the *ara* promoter (Allen & Kornberg, 1991).

Culture medium and growth conditions. All experiments with bacterial cultures were performed using LB medium (Sambrook *et al.*, 1989). Cultivations were performed in shake flasks at 30 °C.

Estimation of the efficiency of transformation and plasmid copy number. The procedures described by Węgrzyn *et al.* (1996b) were used.

Electron microscopic analysis of protein–DNA interactions. The interactions between DnaA protein and linear DNA fragments were investigated using electron microscopy as described previously (Szalewska-Pałasz *et al.*, 1998c; Konopa *et al.*, 1999). The DNA fragment, biotinylated at one end and encompassing the region of wild-type λ plasmid from p_R to *ori λ* , was prepared by PCR using pKB2 plasmid DNA as a template as described previously (Szalewska-Pałasz *et al.*, 1998c). The analogous DNA fragment encompassing the region of pTC λ 1 from p_{tet} to *ori λ* was also prepared by PCR as described above but using pTC λ 1 plasmid DNA as a template and the following primers: 5'-CTG CTC TAC ACC TAG CTT CT-3' and 5'-TTC TCT GAC GAA TAA TCT TT-3' (biotinylated at the 5' end).

RESULTS

Impaired stimulation of the p_R promoter is responsible for the incompatibility between λ plasmids and *dnaA* mutants

To test whether a few-fold decrease in the activity of the p_R promoter, as observed in the *dnaA46* mutant (Szalewska-Pałasz *et al.*, 1998b), is responsible for the incompatibility between this mutant and λ plasmids, we used an artificial λ plasmid (pTC λ 1) which contains the p_{tet} promoter instead of p_R (Herman-Antosiewicz *et al.*, 1998b). It was found previously that activity of a p_{tet} –*lacZ* fusion is independent of the *dnaA* gene function (Herman-Antosiewicz *et al.*, 1998a). Here we show, using electron microscopic techniques, that in contrast to the p_R promoter region, the DnaA protein does not interact specifically with DNA regions located at or near the p_{tet} promoter. When a fragment of wild-type λ plasmid containing the p_R promoter was investigated, a large peak representing a significant number of DnaA molecules bound to the promoter region was observed in a statistical analysis of electron microscopic pictures of several hundred DnaA–DNA complexes (Fig. 1). There was no such a peak at the promoter region when an analogous fragment of plasmid pTC λ 1, which bears p_{tet} instead of p_R , was investigated (Fig. 1).

Transcription from the p_{tet} promoter in pTC λ 1 is induced by autoclaved chlortetracycline (aCT). After autoclaving, chlortetracycline loses its antibiotic activity but retains the ability to induce the p_{tet} promoter (see Herman-Antosiewicz *et al.*, 1998b and references therein). Without induction pTC λ 1 cannot replicate in *E. coli* cells (Herman-Antosiewicz *et al.*, 1998b). We measured the efficiency of transformation of *dnaA*⁺ and *dnaA46* hosts with pTC λ 1 at different concentrations of aCT, resulting in different activities of the p_{tet} promoter. We found that both wild-type and *dnaA46* hosts were efficiently transformed by plasmid pTC λ 1, which bears the wild-type *P* gene, in the presence of aCT (Table 1). Above 0.005 $\mu\text{g ml}^{-1}$, the concentration of aCT has a relatively small effect on the efficiency of transformation (Table 1). These results indicate that when the activity of the promoter responsible for transcription of the λ replication region is independent of *dnaA* gene function, the incompatibility between the *dnaA46* mutant and λ plasmids bearing the wild-type *P* gene is abolished.

It was demonstrated previously that the copy number of pTC λ 1 in wild-type cells increases with an increase in the concentration of aCT in the medium (Herman-Antosiewicz *et al.*, 1998a, b). We found that this is also the case for the *dnaA46* host, but at a somewhat lower level (Table 1). Therefore, there is no significant influence of the *dnaA46* allele on the replication initiated at *ori λ* at 30 °C when transcription of the replication region is DnaA-independent.

We investigated the efficiency of transformation of *dnaA*⁺ and *dnaA46* hosts with a derivative of plasmid pTC λ 1, bearing the π mutation (pTC λ 1 π). For efficient transformation of the wild-type host by this plasmid, a

Fig. 1. Electron microscopic analysis of the interaction of DnaA protein with replication regions of plasmids pKB2 and pTC λ 1. At the top, a genetic map of the investigated fragment of the wild-type λ plasmid (like pKB2) is shown, including the promoters, terminators and transcripts (long arrows). The positions and orientations of DnaA boxes are marked by short arrows; these boxes are numbered according to Szalewska-Pałasz *et al.* (1998c). Below, the results of the DNA-DnaA interactions are presented for the DNA fragment described above, and further below, for the analogous fragment of the pTC λ 1 plasmid, whose map is also shown. Examples of protein-DNA complexes are presented at the bottom of the figure.

considerably higher concentration of aCT (and thus considerably more efficient transcription of the replication region) was required relative to the pTC λ 1

plasmid (Table 1). This requirement was abolished by the *dnaA46* mutation (Table 1). The copy number of pTC λ 1 π was significantly lower than that of pTC λ 1 in both host strains, and this difference was especially pronounced at low aCT concentrations (Table 1). The specific defect of the π mutation in pTC λ 1 π is a weaker interaction of the mutated P protein with DnaB helicase (Konieczny & Marszałek, 1995). The observation of more efficient establishment of this plasmid in the *dnaA46* host as compared to the *dnaA*⁺ host suggests that a competition for DnaB helicase might play a role in the incompatibility of the wild-type λ plasmid and *dnaA*(ts) hosts, in addition to impaired stimulation of transcriptional activation of *ori* λ .

λ plasmid-*dnaA46* incompatibility at different levels of *dnaB* and *dnaC* expression

The λ P protein interacts with DnaB to form a replication complex at *ori* λ (for a review see Taylor & Węgrzyn, 1995). DnaA also interacts with DnaB, and this interaction is required for the delivery of the helicase to *oriC* (Marszałek & Kaguni, 1994; Sutton *et al.*, 1998; Seitz *et al.*, 2000). Therefore, we suspected that the P protein might compete with the host replication initiator DnaA for DnaB, in addition to the previously reported competition between P and DnaC (Taylor & Węgrzyn, 1995 and references therein).

To test this, we measured the efficiency of transformation of *dnaA*⁺ and *dnaA46* hosts with λ plasmids bearing either wild-type or π alleles of the P gene under conditions of normal expression of *dnaB* and *dnaC*, and in cells overexpressing one or both of these genes. As expected, we found that wild-type λ plasmid (pCB104) could not transform the *dnaA46* mutant, in contrast to the wild-type host (Table 2). Corroborating previous reports, both hosts were transformed efficiently with the $\lambda\pi$ mutant plasmid (Table 2). We found that overexpression of *dnaC* or *dnaB* and *dnaC* together (but not *dnaB* alone) resulted in effective transformation of the *dnaA46* host by a wild-type λ plasmid, although the efficiency of this transformation was lower than that measured for *dnaA*⁺ bacteria (Table 2). We checked that these relatively rare *dnaA46* transformants contained wild-type λ plasmids rather than π mutants because plasmid DNA isolated from these clones could transform wild-type cells but not the *dnaA46* mutant (data not shown).

λ plasmid-*dnaA*(ts) incompatibility is abolished in Δ *seqA dnaA*(ts) double mutant strains

E. coli Δ *seqA* cells lack a protein which binds preferentially to hemimethylated DNA containing GATC sites (Lu *et al.*, 1994). In wild-type cells, *oriC* remains hemimethylated for one-third of the cell cycle and is sequestered from initiation for this time, termed the eclipse period. In *seqA* mutants the temperature-sensitive growth phenotype of the *dnaA46* mutant is suppressed (Lu *et al.*, 1994).

Table 1. Efficiency of transformation and copy number of plasmids pTC λ 1 and pTC λ 1 π in *E. coli dnaA*⁺ and *dnaA46* hosts at different concentrations of aCT in the medium

Efficiency of transformation (e.o.t.) is expressed as number of transformants per μ g plasmid DNA. Plasmid copy number (c.n.) is expressed as number of plasmids per cell. The values presented are means from three experiments. ND, Not determined.

aCT (μ g ml ⁻¹)	Efficiency of transformation and plasmid copy number							
	<i>dnaA</i> ⁺				<i>dnaA46</i>			
	pTC λ 1		pTC λ 1 π		pTC λ 1		pTC λ 1 π	
	e.o.t.	c.n.	e.o.t.	c.n.	e.o.t.	c.n.	e.o.t.	c.n.
0	< 10 ¹	ND	< 10 ¹	ND	< 10 ¹	ND	< 10 ¹	ND
0.005	4.5 $\times$ 10 ³	10	< 10 ¹	ND	1.6 $\times$ 10 ⁴	5	7.3 $\times$ 10 ³	2
0.01	4.6 $\times$ 10 ³	25	< 10 ¹	ND	1.6 $\times$ 10 ⁴	10	8.8 $\times$ 10 ³	3
0.05	2.1 $\times$ 10 ⁴	28	< 10 ¹	ND	2.2 $\times$ 10 ⁴	13	1.0 $\times$ 10 ⁴	2
0.1	1.8 $\times$ 10 ⁴	33	8.8 $\times$ 10 ²	2	2.8 $\times$ 10 ⁴	17	1.3 $\times$ 10 ⁴	5
0.5	2.1 $\times$ 10 ⁴	60	7.9 $\times$ 10 ³	3	2.8 $\times$ 10 ⁴	32	5.4 $\times$ 10 ⁴	10
1	2.0 $\times$ 10 ⁴	70	8.8 $\times$ 10 ³	19	1.8 $\times$ 10 ⁴	44	4.5 $\times$ 10 ⁴	50
5	1.7 $\times$ 10 ⁴	75	6.9 $\times$ 10 ³	20	7.3 $\times$ 10 ³	61	6.2 $\times$ 10 ⁴	38

Table 2. Efficiency of transformation of *E. coli dnaA*⁺ and *dnaA46* strains by λ P⁺ and λ π plasmids under conditions of *dnaB* and/or *dnaC* overexpression

Host*	Efficiency of transformation†			
	pCB104 (λ P ⁺)		pAW6 (λ π)	
	– Ara	+ Ara	– Ara	+ Ara
<i>dnaA</i> ⁺	9.9 $\times$ 10 ³	1.0 $\times$ 10 ⁴	4.9 $\times$ 10 ³	4.6 $\times$ 10 ³
<i>dnaA</i> ⁺ /pINGK	1.4 $\times$ 10 ⁴	1.2 $\times$ 10 ⁴	4.6 $\times$ 10 ³	4.8 $\times$ 10 ³
<i>dnaA</i> ⁺ /pINB	1.6 $\times$ 10 ⁴	1.5 $\times$ 10 ⁴	5.6 $\times$ 10 ³	5.8 $\times$ 10 ³
<i>dnaA</i> ⁺ /pINC	1.1 $\times$ 10 ⁴	6.3 $\times$ 10 ³	6.8 $\times$ 10 ³	5.3 $\times$ 10 ³
<i>dnaA</i> ⁺ /pINBC	9.1 $\times$ 10 ³	9.1 $\times$ 10 ³	5.0 $\times$ 10 ³	5.2 $\times$ 10 ³
<i>dnaA46</i>	< 10 ¹	< 10 ¹	6.4 $\times$ 10 ³	7.1 $\times$ 10 ³
<i>dnaA46</i> /pINGK	< 10 ¹	< 10 ¹	5.4 $\times$ 10 ³	5.6 $\times$ 10 ³
<i>dnaA46</i> /pINB	< 10 ¹	< 10 ¹	5.7 $\times$ 10 ³	5.3 $\times$ 10 ³
<i>dnaA46</i> /pINC	< 10 ¹	1.0 $\times$ 10 ²	5.6 $\times$ 10 ³	5.9 $\times$ 10 ³
<i>dnaA46</i> /pINBC	< 10 ¹	4.2 $\times$ 10 ²	2.0 $\times$ 10 ³	5.4 $\times$ 10 ³

* Plasmid pINGK is a vector. Plasmids pINB, pINC and pINBC are derivatives of pINGK bearing *dnaB*, *dnaC*, and both *dnaB* and *dnaC* genes, respectively, under control of the *ara* promoter.

† Efficiency of transformation is expressed as number of transformants per μ g plasmid DNA. The values presented are means from three experiments. Plasmids pCB104 (a λ plasmid bearing the wild-type *P* gene) and pAW6 (a pCB104 homologue but bearing the π A66 mutation) were used. Following incubation of competent cells with plasmid DNA and a temperature shift (see Sambrook *et al.*, 1989, for details), further incubation of bacteria was performed in LB medium and then (after spreading) on LB plates (– Ara) or on LB plates supplemented with 0.05% arabinose (+ Ara).

We measured the efficiency of transformation of *dnaA*⁺ *seqA*⁺, *dnaA*(ts) *seqA*⁺, *dnaA*⁺ Δ *seqA*, and *dnaA*(ts) Δ *seqA* hosts with λ plasmids bearing either wild-type or π alleles of the *P* gene (Table 3). Efficient transformation was found in all combinations tested, with the exceptions of single-mutant *dnaA46*, *dnaA204* and *dnaA508* hosts and the wild-type λ plasmid. Despite a high number of transformants, the colonies obtained in the *dnaA46* Δ *seqA* host were rather small, and those of the *dnaA508* Δ *seqA* host were tiny (Table 3). These results show that the incompatibility between λ plasmids and *dnaA*(ts) hosts is abolished in the Δ *seqA* background.

DISCUSSION

The aim of this study was to investigate the mechanism of the incompatibility between λ plasmids and certain host temperature-sensitive mutants in the *dnaA* gene at a temperature permissive for cell growth. The experiments presented here not only provided the answer to this question, but also shed some light on the functions of DnaA and the role of the transcriptional activation of the *origin* in the regulation of λ DNA replication. Our results indicate that there are two mechanisms leading to the incompatibility between λ plasmids and *dnaA*(ts) mutants, and a combination of both these processes is lethal for the host cells (under antibiotic selection pressure) due to inhibition of replication of either plasmid (bearing an antibiotic-resistance gene) or host chromosome or both.

The first mechanism is based on the finding that moderately decreased transcription of the λ replication

Table 3. Transformation of *E. coli* *dnaA*⁺ *seqA*⁺, *dnaA*⁺ Δ *seqA*, *dnaA*(ts) *seqA*⁺, and *dnaA*(ts) Δ *seqA* strains by λ plasmids

Size of colonies is indicated by pluses, from + + + + for normal-sized colonies to + for very small colonies. – indicates efficiency of transformation < 10¹ transformants per μ g plasmid DNA. In all ‘plus’ cases the efficiency of transformation was > 10⁴ transformants per μ g plasmid DNA.

Relevant host genotype	Transformation of hosts by plasmids at 30 °C		
	pCB104 (λ P ⁺)	pAW6 (λ π)	pBR322
<i>dnaA</i> ⁺ <i>seqA</i> ⁺	+ + + +	+ + + +	+ + + +
<i>dnaA</i> ⁺ Δ <i>seqA</i>	+ + + +	+ + + +	+ + + +
<i>dnaA46</i> <i>seqA</i> ⁺	–	+ + + +	+ + + +
<i>dnaA46</i> Δ <i>seqA</i>	+ +	+ + + +	+ + + +
<i>dnaA204</i> <i>seqA</i> ⁺	–	+ + + +	+ + + +
<i>dnaA204</i> Δ <i>seqA</i>	+ + +	+ + + +	+ + + +
<i>dnaA508</i> <i>seqA</i> ⁺	–	+ + +	+ + +
<i>dnaA508</i> Δ <i>seqA</i>	+	+ + + +	+ + + +
<i>dnaA5</i> <i>seqA</i> ⁺	+ + + +	+ + + +	+ + + +
<i>dnaA5</i> Δ <i>seqA</i>	+ + +	+ + + +	+ + + +
<i>dnaA604</i> <i>seqA</i> ⁺	+ + + +	+ + + +	+ + + +
<i>dnaA604</i> Δ <i>seqA</i>	+ + + +	+ + + +	+ + + +

region, observed in the *dnaA46* mutant, as well as in other *dnaA* mutants which cannot be transformed by wild-type λ plasmids (Szalewska-Palasz *et al.*, 1998b), results in a dramatic impairment of the initiation of replication from *ori* λ . Since replacement of the DnaA-stimulated *p*_R promoter by a DnaA-independent *p*_{tet} promoter resulted in abolition of the incompatibility (Table 1), it seems that the main function of DnaA in the regulation of λ plasmid replication is the control of the efficiency of transcription of the replication region.

The results presented here (Table 2) suggest that a second mechanism leading to the incompatibility between λ plasmids and certain *dnaA* mutants exists. This mechanism is based on the competition between the λ P protein and the host DnaA and DnaC proteins for DnaB helicase. The P–DnaB and DnaC–DnaB interactions have been well known for a long time (for a review see Kornberg & Baker, 1992), and it was demonstrated that DnaA interacts with DnaB at the stage of the delivery of the helicase to *oriC* (Marszalek & Kaguni, 1994). DnaB is present at only about 20 molecules per cell (Kornberg & Baker, 1992), making it a likely candidate for competitive effects. It seems that P competes with DnaC for DnaB significantly more successfully in combination with DnaA46 than with wild-type DnaA.

The reason for this preference is at present not known. However, we believe that it is only indirectly due to DnaA–DnaB interaction. The interaction domains of the DnaA and DnaB proteins have recently been mapped (Seitz *et al.*, 2000). The physical contacts between these proteins involve residues 24–86 and 130–148 of DnaA. The product of the *dnaA46* allele contains two amino acid substitutions, A184V and H252Y (Hansen *et al.*,

1992), both located outside of the regions involved in interactions with DnaB. Therefore, a decreased efficiency of the DnaA46–DnaB interaction is unlikely. Moreover, the product of the *dnaA46* allele is not defective in the loading of DnaB at *oriC* (Seitz *et al.*, 2000; H. Seitz, C. Weigel & W. Messer, unpublished data). Therefore, we propose another explanation. The DnaA46 protein may have problems with *oriC* unwinding due to a reduced capacity for domain 3-mediated homo-oligomerization (Messer *et al.*, 2001). A partial sequestering of DnaB by the λ P protein in these mutants may cause severe problems for host chromosome replication as there are simply too few DnaB molecules available for interaction with DnaC and DnaA, preventing efficient formation of the replication complexes at *oriC*. This may lead to cell death irrespective of λ plasmid replication. This hypothesis is supported by the fact that wild-type λ plasmids can transform Δ *seqA* *dnaA* double mutants (Table 3). It seems that extension of the period for potential effective DnaA–DnaB interactions, observed in the *seqA* mutants, increases the probability of a successful competition of mutated DnaA with λ P for DnaB. In addition, the level of DnaA was found to be increased twofold in *seqA* mutants (von Freiesleben *et al.*, 1994; Torheim *et al.*, 2000). On the other hand, DnaA204 protein was reported to be stabilized in the Δ *seqA* host, whereas a lack of *seqA* gene function did not stabilize DnaA46 protein (Torheim *et al.*, 2000).

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