The Nucleic Acid Melting Activity of *Escherichia coli* CspE Is Critical for Transcription Antitermination and Cold Acclimation of Cells*

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Members of bacterial Csp (cold-shock protein) family promote cellular adaptation to low temperature and participate in many other aspects of gene expression regulation through mechanisms that are not yet fully elucidated. Csp proteins interact with single-stranded nucleic acids and destabilize nucleic acid secondary structures. Some Csp proteins also act as transcription antiterminators *in vivo* and in *vitro*. Here, we selected a mutation in the cloned cspE gene that abolished CspE-induced transcription antitermination. *In vitro*, mutant CspE showed RNA binding activity similar to that of the wild-type CspE but was unable to destabilize nucleic acid secondary structures. Thus, nucleic acid melting ability of CspE and its transcription antitermination activity are correlated. *In vivo*, mutant cspE was functional with respect to up-regulation of expression of *rpoS*, but, unlike the wild-type cspE, it did not complement the cold-sensitive phenotype of the quadruple Δ*cspaΔcspbΔcspgΔcspE* deletion strain. Thus, the nucleic acid-melting activity of Csp is critical for its proto-typical function of supporting low temperature survival of the cell.

When an exponentially growing culture of *Escherichia coli* is shifted from 30 to 15 °C, the cells exhibit a cold-shock response (1). This response is characterized by a transient arrest of cell growth during which a number of genes are induced, in contrast to a severe inhibition of general protein synthesis. Among the cold-shock proteins, the most prominent is CspA. Other cold-inducible proteins include transcription factor NusA (2), polynucleotide phosphorylase (3), initiation factor IF2 (4), RecA (5), histone-like protein H-NS (6), DNA gyrase (7), ribosome-associated factors RbfA (8), and CsdA (9).

In *E. coli*, there are nine Csp proteins, from CspA through CspI, of which CspA, CspB, CspG, and CspI are cold-shock-inducible. CspC and CspE are constitutively produced at 37 °C, whereas CspD is induced upon nutritional deprivation. The induction patterns of CspE and CspH are not known (for review see Refs. 10 and 11). CspA homologues are widely distributed in prokaryotes, and CspA is homologous to the cold-shock domain in human Y-box protein YB-1 and the Y-box proteins from other eukaryotes (for review see Refs. 12 and 13). None of the CspA homologues appear to be singularly responsible for cold-shock adaptation, because deletions in any one of the *csp* genes do not result in cold sensitivity. The double and triple deletion mutations in *E. coli cspa* (Δ*cspaΔcspb*), *ΔcspaΔcspg*, *ΔcspAΔcspg*, *ΔcspaΔcspI*, or *ΔcspaΔcspbΔcspg* did not result in cold sensitivity (14). In a triple deletion strain of *ΔcspaΔcspbΔcspg*, CspE accumulated at low temperatures, suggesting that members of the CspA family may functionally substitute for each other during cold acclimation of cells (14).

In addition to their apparent, but poorly understood role during cold-shock response, many cellular processes appear to respond to changes in Csp protein concentrations even at higher temperature. In *E. coli*, the presence of camphor leads to chromosome decondensation. Overproduction of CspE led to camphor resistance and chromosome condensation (15). CspC and CspE were also shown to be involved in the regulation of the expression of *rpoS*, a gene encoding a global stress response regulator, and *uspA*, a gene encoding a protein that is induced by numerous stresses (16).

In *vitro*, CspE binds to RNA with a low sequence specificity and a low binding affinity (17). CspB, CspC, and CspE are able to more selectively bind RNA and single-strand DNA (18). Binding of CspA to RNA destabilizes RNA secondary structures and, hence, it presumably facilitates translation at low temperatures (17). Hanna and Liu (19) demonstrated the interaction between CspE with the nascent RNA in transcription elongation complexes, suggesting that this protein is involved in transcription regulation. They also found that purified CspE interfered with Q-mediated transcription antitermination. It has also been shown that CspA, CspE, and CspC decreased transcription termination at several intrinsic terminators and also affected transcription pausing (20). Recently, it was found that purified CspE impedes poly(A)-mediated 3′- to 5′-exonucleolytic decay by polynucleotide phosphorylase by interfering with its digestion through the poly(A) tail and inhibits both internal cleavage and poly(A) tail removal by RNAse E (21).

The relevance, if any, of all of these biochemical functions of CspA homologues to their physiological function is not known. In the present paper, we explored the physiological relevance of transcription antitermination activity of CspE by mutational analysis of a cloned cspE gene. We show that a mutation of an
evolutionarily conserved histidine residue, His32, which resides within RNP2, a putative RNA-binding site of CspE, abolishes transcription antitermination in vivo and in vitro. The mutation leaves the RNA-binding activity of CspE unaffected but abolishes the nucleic acid-melting activity of CspE. The mutant CspE overexpression does not complement the cold sensitivity of the ΔcspΔcspβcspαcspγcspE mutant. Thus, the methods of Lerner et al. were used to design a screening assay for antitermination-deficient mutants (3). As a splint for ligation the oligonucleotide was used: 5′-GAGTGGTCACGTCGCTAAC- TTTTATCTGTGCTTCCCTATGCACCGCCGACGAGCAGCTGAAACAT CTCCTCGTAAGAACCCT-DABCYL. It was made by ligating the two oligos: 5′-half-tetramethyl rhodamine-AGGGTTCTTTGTGGTGTTTTTATCTGTGCTTCCCTATGCACCGCCGACGAGCAGCTGAAACAT CCTCTCGTAAGAACCCT-DABCYL. The 88-nucleotide RNA fragment to which CspE binds preferentially as shown previously by SELEX (systematic evolution of ligands by exponential enrichment) was used, and the filter binding assays were carried out as described before (18). T7 RNA polymerase reaction was carried out in the presence of [α-32P]UTP to prepare the RNA probe. The RNA was then purified by phenol-chloroform extraction and ethanol precipitation and was quantitated by urea-acrylamide gel electrophoresis. RNA concentration was estimated by quantitating %P incorporation.

Binding assay was carried out in a 15-µl reaction mixture containing binding buffer (10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, 10 mM KCl, 7.4% glycerol) and 50 fmol of RNA. After incubation on ice for 20 min, samples were analyzed by filter binding assays. Variable amounts of proteins were incubated with constant amounts (50 fmol) of RNA. The reaction mixtures were passed through nitrocellulose filters, which were washed thoroughly to remove unbound RNA. Radioactivity retained on filter was measured by liquid scintillation counter. About 1% of the input radioactivity was detected as background in the absence of any protein in the reaction mixture. This background was subtracted from the measured amounts and used for the specific binding.

Western Blot—The Western blot analysis was carried out as described previously (16). Wild-type cells containing pBINII, or the pBINII plasmids containing open reading frames of cspE or its mutants were grown at 37 °C in M9 medium. The exponentially growing cells at an A600 of 0.5 were treated with 1 mM IPTG for 30 min and were then concentrated by centrifugation at 13,000 x g. The resulting cell pellets were suspended in SDS-loading buffer, and the proteins were resolved by SDS-PAGE. The Western blots were prepared according to the antibody manufacturer's protocol (Neolone).

**Molecular Beacons**—The molecular beacons were a gift from Dr. Sanjay Tyagi (New York University). The beacon used in this study was a 82-nucleotide-long hairpin-shaped molecule labeled with a fluorescein and a quencher: tetracyclolymethyl rhodamine-AGGGTTCTTTGTGGTGT GAGTGGTCACGTCGCTAAC-TTTTATCTGTGCTTCCCTATGCACCGCCGACGAGCAGCTGAAACAT CTCCTCGTAAGAACCCT-DABCYL. It was made by ligating the two oligos: 5′-half-tetramethyl rhodamine-AGGGTTCTTTGTGGTGTTTTTATCTGTGCTTCCCTATGCACCGCCGACGAGCAGCTGAAACAT CCTCTCGTAAGAACCCT-DABCYL. As a splint for ligation the following oligonucleotide was used: 5′-TACCCGATCTGCTGCTGGC GCTATGGGAAGCCAGATATAGAAA-3′. The ligated full-length product was purified on a 10% acrylamide gel containing 7 M urea. The product was eluted in GE buffer and precipitated with ethanol.

Fluorescence measurements were performed on an LS-5B spectrophotometer (PerkinElmer Life Sciences), using 1-cm path length quartz cuvettes (Hellma). The temperature of the cuvette was controlled by a circulating bath. The excitation and emission emissions were 555 and 557 nm, respectively. The fluorescence of a 100-nM solution of 32 nt mRNA molecule dissolved in 20 mM Tris-HCl, pH 7.5, containing 1 mM MgCl2 was monitored as CspE or its mutant proteins (1.5 µg) were added. The reactions were carried out at room temperature. To check the effect of degradation of CspE by trypsin (50 times excess), the reactions were carried out at 37 °C.

**RESULTS**

Isolation of CspE Mutant Proteins Deficient in Transcription Antitermination—Previously we had reported the use of E. coli strain RL211 designed by Landick et al. (25) to demonstrate in vivo transcription antitermination activity of CspE proteins (20). This strain contains the cat gene preceded by a strong ρ-independent trpL terminator and is therefore sensitive to chloramphenicol. When transcription termination at trpL is reduced, the cat gene is expressed and the cells become resistant to chloramphenicol. Cells overproducing CspE or its IPTG-inducible pBINII plasmid former colonies on chloramphenicol-containing medium, indicating that CspE overproduction caused transcription antitermination at the trpL terminator in vivo (20). In the present study, we used this property of CspE to design a screening assay for antitermination-deficient mutants. The cspE gene was subjected to random PCR mutagen-
The band corresponding to overexpressed CspE is indicated.


Site-directed mutagenesis was next used to separate the two point mutations to create corresponding single mutants, CspE-N7K and CspE-H32R. We then tested if the overproduction of CspE proteins carrying these mutations can cause transcription antitermination in vivo using the RL211 cells as described above. Aliquots of the RL711 cells transformed with plasmids overexpressing the wild-type CspE, the double mutant, or the single mutants were spotted on LB plates containing ampicillin and IPTG, in the presence or in the absence of chloramphenicol. Cells carrying the pINIII vector alone were used as control. As expected, all cells exhibited growth in the absence of chloramphenicol, however, only those overproducing wild-type CspE or the N7K mutant were able to grow in the presence of chloramphenicol. The results of overnight cell growth are presented. B - SDS-PAGE analysis of induction pattern of CspE and its mutants. Cells with pINIII vector alone, lane 1; pINIIcspE, lane 2; pINIIcspE-N7K, lane 3; pINIIcspE-N7K-H32R, lane 4; and pINIIcspE-H32R, lane 5. The band corresponding to overexpressed CspE is indicated.

The N7K mutant was more stable ($T_m = 62 \, ^\circC$) than the wild-type CspE ($T_m = 51 \, ^\circC$) with a minimum around 217 nm, a characteristic of an unstacked $\beta$-strand structure. The double mutant spectra varied a little, however, this difference in the CD spectra is marginal. The tertiary structure profile shown in Fig. 2B indicates that the double mutant and the H32R mutant are not significantly different in their tertiary packing than the wild-type CspE; however, mutant N7K showed more differences compared with the wild-type CspE. Finally, analysis of the melting patterns (Fig. 2C) showed that the H32R mutant was less stable ($T_m = 51 \, ^\circC$) than wild-type CspE ($T_m = 62 \, ^\circC$), whereas mutant N7K was more stable ($T_m = 68 \, ^\circC$) than the latter. Interestingly, the double mutant showed stability ($T_m = 62 \, ^\circC$) similar to that of the wild-type CspE. The effect of the mutations on the transcription antitermination seen is not a manifestation of the differences in the stability of these proteins, because (i) in the present communication, the effect of CspE mutations on antitermination of transcription was checked at 25–37 $^\circC$, wherein the stability of mutant proteins was not significantly different from that of the wild-type CspE, and (ii) the double mutant with stability similar to that of the wild-type CspE did not show transcription antitermination activity.

The H32R Substitution Results in Loss of Transcription Antitermination in Vitro—Next we attempted to further characterize the effect of H32R mutation on transcription antitermination in vitro by using purified proteins. Stalled E. coli RNA polymerase elongation complexes were prepared on DNA template containing the T7 A1 promoter followed by a r-independent terminator tR2 (26). Transcription was resumed by the addition of NTPs in the presence or in the absence of CspE proteins. As reported previously and as seen from Fig. 3, the wild-type CspE decreased transcription termination at tR2. The N7K mutant protein also decreased transcription termination, but the double mutant and the H32R mutant had no effect. The mean readability efficiency values (RE), defined as the fraction of the run-off transcripts of the total transcripts produced, were calculated for three independent experiments and are presented in Fig. 3. As can be seen, RE was less than 30% in the control reaction in the absence of CspE, in the presence of the double mutant and the H32R mutant. Addition of the wild-type CspE or the N7K mutant protein resulted in increase in the RE values to 55 and 48%, respectively. These results are consistent with the in vivo data shown above and suggest that single amino acid substitution of Asn at CspE position 7 does not affect the ability of CspE to cause transcription antitermination. In contrast, the substitution of His at CspE position 32 with Arg abolishes this activity.

Transcription Antitermination Defect of CspE Mutants Is Due to the Reduced RNA Melting Activity—In E. coli, an intrinsic transcription terminator signal is encoded in RNA and consists of two essential elements: a stem loop structure followed by a stretch of U residues. To act as a transcription antiterminator, CspE presumably must bind the nascent RNA. Thus, the defect in CspE-induced antitermination can be the trivial consequence of the absence of RNA binding. CspE can act as an “RNA chaperone” destabilizing RNA secondary structures and increasing RNA susceptibility to RNase attack (17). Therefore, the defect in CspE-induced antitermination can also be due to defects in RNA melting by mutant CspE. To select between these two possibilities, we tested whether CspE mutants were impaired in their (i) RNA-binding activity or (ii) RNA-melting activity.

We used an 88-nucleotide-long radioactively labeled RNA to check the RNA binding of CspE and its mutants by filter...
binding. The RNA fragment was previously selected as a preferred substrate of CspE binding by SELEX (18). The apparent $K_d$ value of the binding reaction was defined as the concentration of protein at which half of maximum binding was obtained as described by Kajita et al. (27). As seen from Fig. 4, the $K_d$ value for CspE was 0.3 $\mu$M. These values are consistent with the previous report (18). The calculated $K_d$ values for the double mutant, and N7K and H32R mutants were 0.35, 0.06, and 0.09 $\times$ 10$^{-6}$ M, respectively. Thus, the double mutant bound the RNA substrate with approximately the same efficiency as the wild-type CspE. On the other hand, both the N7K and the H32R mutants showed significantly higher RNA binding than the wild-type CspE. It is not clear at this point how a combination of the two single mutations that increase CspE binding to RNA results in lower, “wild-type” binding efficiency. Be as it may, the results suggest that the inability of CspE carrying the H32R substitution to elicit transcription antitermination is not due to decreased RNA binding activity.

To determine the effects of CspE mutations on nucleic acid melting we used molecular beacon system designed by Tyagi and Kramer (28). The molecular beacon is a single-stranded nucleic acid molecule that possesses a stem and a loop structure. A fluorescent moiety is attached to the end of one arm, and a nonfluorescent quenching moiety is attached to the end of the other arm. The stem keeps these two moieties in close proximity to each other, resulting in efficient quenching of the fluorophore fluorescence. When a protein “opens up” the hairpin loop structure, the arms of the beacon move apart causing the fluorophore and the quencher to move away from each other as well. Because the fluorophore is no longer in close proximity to the quencher, its fluorescence will increase. Purified CspE was added to the beacon, and the fluorescence was monitored as described under “Experimental Procedures.” As seen from Fig. 5A, the addition of the wild-type CspE resulted in the stably increased beacon fluorescence. Next, the experiment was repeated, but trypsin was added to the reaction mixture after the maximum fluorescence was achieved. This resulted in a slow decrease in the fluorescence as the tryptic degradation of CspE occurred, because the two arms of the beacon came back together bringing back the quencher in close proximity of the fluorophore again. A control experiment showed that CspE alone did not have any fluorescence. We conclude that (i) increased fluorescence of the beacon is due to nucleic acid melting caused by CspE, and (ii) molecular beacon

![Fig. 2.](#) Circular dichroism (CD) and $T_m$ of E. coli CspE and its mutants. A, secondary structure CD was measured from 140 to 260 nm. B, tertiary structure CD was measured from 260 to 320 nm. C, thermal folding of CspE and its mutants was monitored using changes in negative ellipticity at 222 nm. Wild-type CspE, open circles; CspE-N7K-H32R, closed circles; CspE-N7K, open triangles; CspE-H32R, closed triangles.

![Fig. 3.](#) CspE mutations affect transcription antitermination in vitro. The in vitro transcription assays were carried out as described under “Experimental Procedures.” DNA template containing the T7A1 promoter fused to the tr2 terminator was used, and CspE or its mutants were included in the reaction mixtures. The products were analyzed by urea-PAGE (7 M urea/10% polyacrylamide). RO and tr2 indicate the runoff and the tR2-terminated transcripts, respectively. Lower panel, results of quantification of the gel shown at the top panel. The readthrough efficiency was calculated as described under “Results” and “Discussion.”

![Fig. 4.](#) RNA binding of CspE and its mutants. Filter binding assays (see “Experimental Procedures”) were performed using variable amounts of each protein and constant amount (50 fmol) of the 32P-labeled RNA probe. Wild-type CspE, open squares; CspE-N7K-H32R, open triangles; CspE-N7K, closed squares; CspE-H32R, open circles.

The values shown are the mean values of three independent binding experiments.
RNA-melting activity. We used CspE mutants to check whether nucleic acid-melting function is relevant for two of the in vivo functions of CspE.

It has been shown that CspC and CspE are involved in the regulation of expression of E. coli rpoS, a gene coding for the stationary phase sigma factor, $\sigma^S$. This regulation is mainly at post-transcriptional level, and mRNA stabilization is one of the important aspects of this regulation. It is believed that mRNA stabilization is due to Csp binding to the rpoS mRNA (16). Western blot analysis using monoclonal anti-$\sigma^S$ antibodies showed that overexpression of the wild-type CspE resulted in a $\sim$4-fold up-regulation of rpoS, as expected (Fig. 6, see also Ref. 16). Overexpression of any of the three CspE mutants resulted in a similar up-regulation of rpoS (Fig. 6). Therefore, we conclude that nucleic acid melting and/or transcription antitermination functions of CspE are not involved in the rpoS up-regulation.

Next we examined if nucleic acid melting and/or transcription antitermination functions of CspE have any relevance to its ability to complement the cold-sensitive phenotype of the quadruple deletion strain of E. coli. E. coli CspA family consists of nine homologous members. The functions of family members appear to be partially redundant. A quadruple deletion strain $\Delta$espA$\Delta$espB$\Delta$espG$\Delta$espE exhibits cold sensitivity at 15 °C, which is complemented by overproduction of any one of CspA homologues except CspD. We introduced pISSI-based wild-type CspE, mutant CspE-overproducing plasmids, or the pISSI vector alone in the quadruple deletion strain and growth at 15 and 37 °C was examined on LB plates with and without 0.1 mM IPTG. The low levels of IPTG were used, because higher concentrations of the inducer were found to be toxic to CspE plasmid-bearing cells.

As seen from Fig. 7, all clones grew at 37 °C, except that the growth of cells carrying the pISSI espE-N7K plasmid was inhibited in the presence of IPTG, suggesting that overproduc-
tion of CspE-N7K is toxic for this particular deletion strain. As reported previously, the pINIII vector allows easy expression of a cloned gene even in the absence of inducer and similar inhibition of colony formation in the presence of IPTG was seen with CspB-, CspC-, and CspH-overproducing plasmids (14). Toxicity associated with the overproduction of CspE-N7K was not seen in the E. coli strains such as the wild-type JM83 strain or the RL211 strain. Furthermore, overproduction of the double-mutant CspE had no adverse effect on cell growth at 37 °C (Fig. 7), as if the H32R substitution modulated the toxic effect of the N7K substitution. It is possible that both the toxicity of induced cspE-N7K and the ability to complement the C phenotype when uninduced are simply due to higher levels of cspE-N7K expression. Indeed, quantitative analysis of the wild-type and mutant CspE overproduction showed that CspE-N7K was consistently overproduced to higher levels than other CspE proteins (~1.5-fold higher, data not shown).

Consistent with the previous report (14), plasmid overproducing wild-type cspE complemented the low temperature growth defect of the quadruple deletion strain, only in the presence of IPTG, whereas cells with pINIII vector alone did not form colonies at 15 °C either in the presence, or in the absence of IPTG. Similarly, plasmids overexpressing the double mutant and the H32R mutant did not complement the growth defect of the quadruple deletion strain. In contrast, cells carrying pINIIIcspE-N7K exhibited growth at 15 °C but only in the absence of IPTG, consistent with the result seen at 37 °C. This suggests that nucleic acid-melting/antitermination activities of CspE are critical for its function for cold acclimation of the cells.

**DISCUSSION**

Despite the extensive research on CspA and its homologues, it is not known exactly how these proteins help the cold acclimation of cells. At low temperatures, the secondary structures of RNA stabilize, which should slow down transcription elongation and ribosomal movement on RNA. Csps, acting like “RNA chaperones” could destabilize the secondary structures in RNA and thus facilitate transcription and translation (17).

In this paper we show that a CspE mutant selected for its inability to antiterminate transcription at high temperature is unable to complement cold sensitivity of quadruple csp deletion. In vitro, the mutant CspE protein binds RNA normally but is defective in destabilizing nucleic acid secondary structures. Thus, the RNA-chaperoning activity of CspE is essential for transcription antitermination function of the protein. Previously, we had shown that, in addition to reducing the efficiency of transcription termination on ρ-independent terminators, Csps reduce hairpin-induced transcription pausing. Taken together, the data demonstrate that CspE affects transcription termination by preventing the formation of the nascent RNA secondary structures, most likely the formation of the stem-loop structure at the terminator. Thus, the mechanism of Csp-induced antitermination appears to be similar to the recently deduced mechanism of transcription antitermination by λ N protein (29).

Previously we showed that (i) Csp proteins can act as transcription antiterminators in vivo and in vitro; (ii) at cold-shock, expression of promoter distal genes of the metY-rpsO operon, nusA, infB, rbfA, and pnp, is increased; and (iii) Csp overproduction causes similar increase in promoter distal metY-rpsO operon gene expression even at high temperature (20). This result provided the first evidence that transcription antitermination function of cold-shock-induced Csps is relevant, because the products of nusA, infB, rbfA, and pnp, which are NusA, IF2, RbfA, and PNP, respectively, are known to be induced at cold-shock and presumably enable the cells to adapt to low temperature. Our current results demonstrate that RNA-melting function of Csps is necessary for cellular adaptation to cold and are consistent with the idea that transcription antitermination function of Csps is linked to their cold-shock function. On the other hand, another function of CspE, its ability to up-regulate the rpoS gene expression, is unaffected by termination-altering mutations and is probably dependent only on its RNA-binding activity.

The three-dimensional structure of CspA from E. coli has been resolved by x-ray crystallography and NMR analysis (30–32). The structure consists of five antiparallel β-strands, β1 through β5, forming a β-barrel structure with two β-sheets. The two evolutionarily conserved RNA-binding motifs of CspA, RNP1 and RNP2, are located on the β2 and β3 strands. RNP1 Phe18 and Phe20, and RNP2 Phe31 and His33 form a compact surface-exposed site on the CspA three-dimensional structure. These residues are presumably involved in intercalation be-
between the bases of a nucleic acid target. Mutational analysis of E. coli CspA showed that single substitutions of Phe residues at positions 18, 20, and 31 by either Leu or Ser residues decreased DNA binding (33). Similarly, in the case of CspB from Bacillus subtilis, which is a homologue of E. coli CspA, substitution of Phe residues at positions 15, 17, and 27 by Ala and substitution of His at position 29 by Gln also abolished nucleic acid binding (34). Here, we show that substitution of E. coli CspE His32, which corresponds to E. coli CspA His33 and B. subtilis CspB His32, for Arg does not affect the RNA-binding activity, probably because Arg is capable of favorable electrostatic interaction with nucleic acids (34). The loss of the RNA-melting activity in H32R CspE mutant may be due to the difference in the side chains of His and Arg, because the imidazole ring of His is capable of intercalation between nucleic acid bases, whereas the guanidine group of Arg is not. According to this view then, in the wild-type CspE, His32 could participate in the initiation of nucleic acid secondary structure melting, which is further propagated by subsequent intercalation of Phe18, Phe20, and Phe31. Our ongoing site-specific mutational analysis of cspE and integrated biophysical, biochemical, and physiological analyses of CspE mutants should clarify this issue.

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