

DNA sampling: a method for probing protein binding at specific loci on bacterial chromosomes

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ABSTRACT

We describe a protocol, DNA sampling, for the rapid isolation of specific segments of DNA, together with bound proteins, from *Escherichia coli* K-12. The DNA to be sampled is generated as a discrete fragment within cells by the yeast I-SceI meganuclease, and is purified using FLAG-tagged LacI repressor and beads carrying anti-FLAG antibody. We illustrate the method by investigating the proteins bound to the colicin K gene regulatory region, either before or after induction of the colicin K gene promoter.

INTRODUCTION

Transcription in bacteria is controlled by a complex network of transcription factors that bind to gene regulatory regions and regulate promoter activity (1). The latest estimate suggests that the genome of *Escherichia coli* K-12 contains over 1800 promoters that are regulated by nearly 300 different proteins (2). Over 800 of these promoters have now been studied by the well-established combination of genetics, bioinformatics and biochemistry, using purified proteins, and it is clear that most regulatory regions are complex with many different proteins interacting (3).

Until recently, rapid identification of all the protein factors binding at a particular regulatory region has been problematic. However, methods that use target DNA molecules to trap specific proteins from crude cell extracts have now been developed (4–8). Most of these exploit advances in mass spectrometry that enable identification of subfemtomole amounts of proteins (8,9). Our aim in this work was to develop an alternative protocol that would enable rapid isolation, direct from *E. coli* K-12 cells, of specific DNA fragments together with attached proteins. We reasoned that this would reduce potential artefacts that might arise when crude cell extracts are

incubated with DNA fragments, and would also provide a simple way of detecting changes in protein binding at a particular locus as cell growth conditions change. Hence we describe ‘DNA sampling’, in which the target DNA segment is cloned into a low copy number plasmid at a site that is adjacent to multiple operator binding sites for the LacI repressor and between two target sites for the yeast I-SceI meganuclease. Induction of I-SceI expression leads to the liberation of a DNA fragment carrying the region to be sampled, together with LacI repressor-binding sites, and co-induction of bacteriophage lambda Gam protein ensures its stability. We explain how a host-encoded tagged LacI facilitates purification of the fragment together with accompanying proteins that can be identified by gel electrophoresis and mass spectrometry. We describe an experiment in which proteins binding to the promoter that regulates expression of the *E. coli* colicin K gene (*cka*) were sampled either before or after induction with nalidixic acid.

MATERIALS AND METHODS

Bacterial strains and growth conditions

A derivative of *E. coli* K-12 strain MG1655 (CGS7740) (10) that had been engineered, by the gene gorging method of Herring *et al.* (11) to express a 3xFLAG-tagged *lacI* gene product was used in this work (D.J.L., unpublished results). DNA sampling experiments were performed on cells grown in minimal salts medium (MSM) (12) supplemented with 0.2% glucose, chloramphenicol (25 µg/ml) and tetracycline (30 µg/ml). For induction of the bacterial SOS response, 8.5 µg/ml, of nalidixic acid was added to cultures. This is a sub-inhibitory concentration as determined by the broth dilution method (13).

Construction of pRW902

Plasmid pRW902 carries an EcoRI-HindIII fragment with the *E. coli cka* promoter region cloned immediately downstream of five LacI operator sites with two flanking 18-bp

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target sites for the yeast meganuclease I-SceI (Figure 1). pRW902 was constructed in three steps, using synthetic oligos listed in Table 1, starting from plasmid pRW50, a low copy number broad host range RK2 derivative encoding resistance to tetracycline, that carries unique EcoRI and HindIII sites (14). First, the *cka* promoter region from plasmid pKCT1 (15) was amplified by PCR using primers SceI_up and Cka_down, the product was cut with MfeI and HindIII and cloned between the EcoRI and HindIII sites of pRW50, to give an intermediate plasmid carrying an I-SceI site upstream of the *cka* promoter region on an EcoRI-HindIII fragment. Second, PCR, with primers Lac_up and Lac_down and a template given by Peter McGlynn (University of Aberdeen), was used to generate an MfeI-EcoRI fragment carrying five wild-type LacI operators. This fragment was cloned into the EcoRI site of the intermediate plasmid, resulting in a derivative carrying an I-SceI site and five LacI operators upstream of the *cka* promoter region on an EcoRI-HindIII fragment. Third, an I-SceI site was inserted downstream of the HindIII site in this derivative by cloning

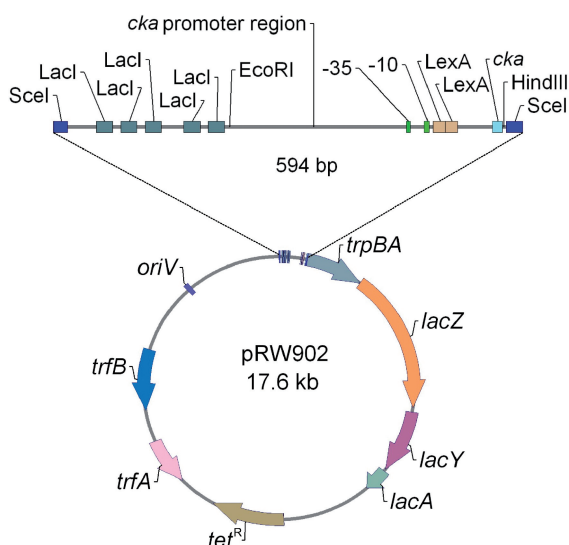


Figure 1. Schematic representation of pRW902 plasmid. The pRW902 construct carries five operator sites for LacI just upstream of the promoter region of the *cka* gene and flanking them are the two target sites for the yeast meganuclease I-SceI. Different DNA segments to be sampled for DNA-binding proteins can be cloned into the EcoRI and HindIII restriction sites. The plasmid map was drawn to scale using Redasoft Visual Cloning 3.2.

a HindIII-SacI fragment that had been generated following a PCR reaction with pRW50 as a template and primers SacI_up and SacI_down. The base sequence of all new constructs was checked using the University of Birmingham Functional Genomics Unit. The complete sequence of pRW902, together with annotation, is shown in the Supplementary Data.

Construction of pACBSR-DL1

Plasmid pACBSR, which encodes I-SceI and the bacteriophage lambda Red system both under the control of an arabinose-dependent promoter, was obtained from Scarab genomics (11). Plasmid pACBSR-DL1 is a derivative lacking the bacteriophage lambda *exo* and *bet* genes. To construct this derivative, the internal SphI-SphI DNA fragment encoding the bacteriophage lambda Red genes was replaced with a smaller SphI-SphI fragment encoding just the Gam protein, that was generated after PCR using primers Gam_up and Gam_down and pACBSR as a template. Plasmids pACBSR and pACBSR-DL1 both carry a chloramphenicol resistance determinant.

Sample preparation

Escherichia coli strain MG1655 encoding 3× FLAG-tagged LacI was co-transformed with pRW902 and pACBSR-DL1. Overnight cultures of transformants were diluted 1:200 into media and grown with aeration at 37°C. When cells reached an OD₆₀₀ of 0.6, 0.4% L-arabinose was added to induce expression of the I-SceI meganuclease and the bacteriophage lambda Gam protein. After 20 min, bacteria were harvested by centrifugation (15 min, 8000 r.p.m., room temperature). Cell pellets were re-suspended in 20 ml of extraction buffer [50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10% sucrose, 2 mM MgCl₂, 0.1% (v/v) Triton X-100, 200 µg/ml phenylmethylsulfonyl fluoride, 4 µg/ml pepstatin] and a Roche Diagnostics protease inhibitor cocktail tablet was added. RNase A (300 µg/ml) and lysozyme (400 µg/ml) were then added and, after incubation at room temperature for 10 min, each mixture was cooled on ice and sonicated for three periods of 30 s. Samples were then centrifuged at 18 000 r.p.m. for 20 min at 4°C to give a clear lysate. In experiments to induce the *cka* promoter, parallel cultures were grown and 8.5 µg/ml of nalidixic acid was added to one culture when the OD₆₀₀ reached 0.3. The DNA content of lysates was checked by electrophoresis on 1.2%

Table 1. Primers used in the present work (underlines denote restriction sites)

Primer	Sequence
SceI_up	5'- <u>CCCCAATTG</u> TAGGGATAACAGGGTAAT <u>GAATTC</u> TTTGTCATGCTCATATCC-3'
Cka_down	5'-CCCAAGCTTGTCTTTAGCCATAAAATTCCTC-3'
Lac_up	5'- <u>CCCCAATTG</u> AGCCAGCACGCTAGCTAGC-3'
Lac_down	5'- <u>CCC</u> GAATTC <u>CCC</u> AGAAATTGTTATCCGCTCACAATTCGTGC-3'
SacI_up	5'- <u>CCC</u> AAGCTT <u>TAGGGATAACAGGGTAAT</u> TTGTCAGTGC <u>GCAAAA</u> GATCC-3'
SacI_down	5'-GCCACCATCCAGTGCAGGAGCTC-3'
Gam_up	5'-GTGGCATGCTAAGGAGGTTATAAAAAATGGATATTAATACTG-3'
Gam_down	5'-GACGCATGCTTATACCTCTGAATCAATATC-3'

agarose gels after clean up with a Qiagen DNA isolation kit (Hilden, Germany). In a control experiment, equal volumes of cultures containing the starting *E. coli* strain MG1655, and engineered MG1655 encoding 3× FLAG-tagged LacI that had been co-transformed with pRW902 and pACBSR-DL1, were mixed together immediately prior to arabinose induction, harvesting and lysis.

Isolation and analysis of protein–DNA complexes

Clear lysates were mixed with 25 mg of Dynabeads (M270-epoxy; Invitrogen) cross-linked to mouse anti-FLAG antibody (F3165, Sigma) and incubated on ice for 10 min with gentle agitation. Dynabeads were collected with a magnet and washed five times with 1 ml of wash buffer [20 mM HEPES pH 7.4, 100 mM NaCl, 0.1% (v/v) Tween 20] in 2 ml unsiliconized tubes (Eppendorf). The sampled protein–DNA complexes were eluted from the anti-FLAG antibody with 500 µl of elution buffer (0.3 M NH₄OH, 0.5 M EDTA pH 8.0), mixing on a rotating wheel for 5 min, and supernatant was removed from the beads and transferred to a fresh unsiliconized tube. DNA in the supernatant was purified by phenol–chloroform extraction and ethanol precipitated and analyzed by electrophoresis in 1.2% agarose gels. Proteins in the supernatant were vacuum dried and dissolved in SDS–PAGE loading buffer containing 10 mM *tris*(2-carboxymethyl)phosphine–HCl (Sigma) at 95°C for 5 min. Samples were then alkylated by addition of 50 mM iodoacetamide (Sigma) for 30 min at room temperature, resolved by SDS–PAGE in 4–12% gradient gels (Invitrogen), and visualized by Coomassie blue staining. Quantities and relative stoichiometries of DNA and proteins in the complexes were determined after scanning agarose gels and SDS–PAGE gels, that had been calibrated with the NEB 100-bp DNA ladder and Invitrogen SeeBlue Plus 2 protein markers respectively. Bio-Rad Quantity One software was used for quantification of different bands and the presence of LacI repressor in each sample was exploited as an internal reference. To identify different proteins in SDS–PAGE gels, 1 mm gel slices were excised and destained and digested with trypsin (Promega) for 4 h at 37°C as previously described (16,17). Peptides from each slice were analyzed on a Thermo-Finnigan FT-ICR mass spectrometer using a NanoMate chip-based electrospray system operated by the University of Birmingham Functional Genomics and Proteomics Unit.

RESULTS

We constructed a derivative of the broad host range low copy number RK2 plasmid with two DNA sites for the yeast I-SceI meganuclease that flank a DNA segment carrying five LacI repressor-binding targets adjacent to unique EcoRI and HindIII sites. Plasmid pRW902, illustrated in Figure 1, carries a 330-bp fragment with the promoter region of the *E. coli* colicin K (*cka*) gene between the EcoRI and HindIII sites. Our goal was to identify the different proteins that bind to the *cka* regulatory region. To do this, pRW902 was transformed into

a derivative of *E. coli* K-12 strain MG1655 in which the *lacI* gene had been modified to encode a 3× FLAG-tagged LacI repressor. We also introduced plasmid pACBSR-DL1 that encodes I-SceI and the bacteriophage lambda Gam protein, under the control of an arabinose inducible promoter. We reasoned that, after induction by arabinose, a discrete DNA fragment carrying the *cka* promoter adjacent to five LacI repressor targets would be generated, and that it would be stable due to inhibition of RecBCD by the Gam protein (18). Our strategy, outlined in Figure 2, was then to use immunoaffinity methods to isolate the DNA fragment together with accompanying proteins, which we would identify using gel electrophoresis and mass spectroscopy.

Figure 3A–C illustrates experiments in which the DNA content of lysates was analyzed after growing cells either in minimal media or in nutrient rich broth. Since the *cka* promoter is known to be repressed by the LexA repressor, and, thus, induced by the SOS response, the experiment was performed either before or after induction by nalidixic acid (19–21). Arabinose was added to cells to induce expression of I-SceI and Gam and cells were harvested after different times. The results show that, in each case, after 20 min induction, a linear DNA fragment is generated. The time courses shown in Figure 3 suggest that 20 min may be the optimal time for fragment generation by this protocol. In a parallel experiment, the efficiency of DNA cleavage by I-SceI was assayed by comparing cell counts after plating on solid media either with or without tetracycline (recall that pRW902 encodes resistance to tetracycline). These measurements showed that, after addition of arabinose, less than 1% of colonies carry functional pRW902.

DNA–protein complexes were purified using anti-FLAG antibody as described in Materials and Methods section, and DNA in the complexes was analyzed by electrophoresis. The result, illustrated in Figure 3D, confirms that the procedure resulted in purification of the 600-bp DNA fragment with five LacI operators and the *cka* promoter region. We estimate that 200 ml of bacterial culture yields ~0.2 µg of DNA fragment. SDS–PAGE combined with mass spectrometry was used to analyze the proteins in the complexes and Figure 4 shows stained gels of the proteins from samples isolated both before and after the addition of nalidixic acid. As expected, the complexes contain a major band corresponding to the FLAG-tagged LacI and a band corresponding to the LexA repressor. Quantification of the relative levels of protein and DNA in the complex isolated from cells before nalidixic acid treatment suggests stoichiometries of 10–11 LacI molecules and four to six LexA molecules per DNA fragment. The analysis also reveals the presence of RNA polymerase in the complex. Addition of nalidixic acid results in a decrease in the relative level of LexA repressor and an increase in the level of RNA polymerase. The complexes contain other proteins at lower levels, including the small nucleoid-associated proteins, H-NS, FIS and HU (22,23), Lon protease (87 kDa) and the different subunits of pyruvate dehydrogenase (99 kDa, 66 kDa and 56 kDa).

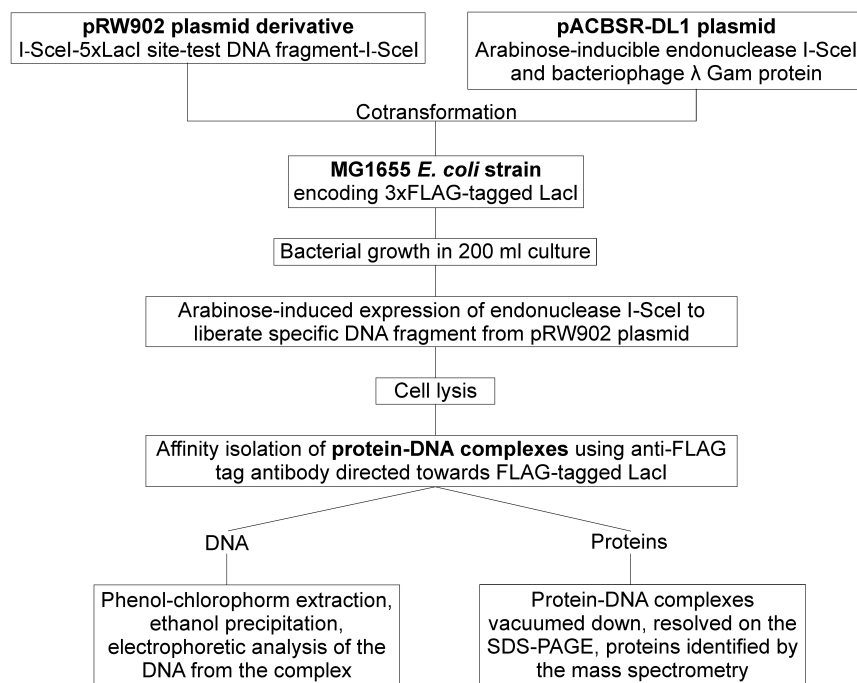


Figure 2. Outline of DNA sampling.

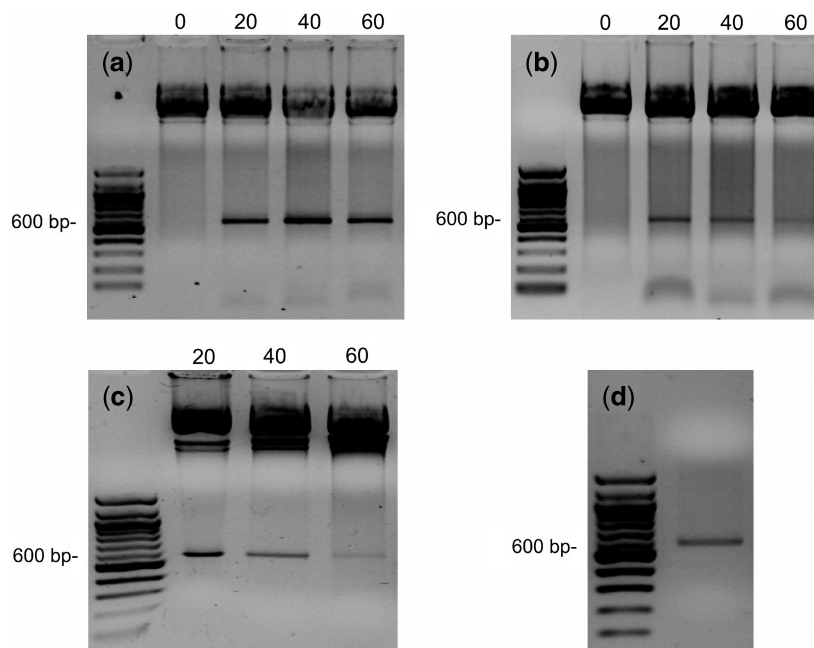


Figure 3. Analysis of DNA fragments generated by I-SceI. Panels A–C show a gel analysis of the DNA content of crude extracts harvested at different times (min) after induction of the expression of I-SceI and the bacteriophage lambda Gam protein. The extracts were made as described in Materials and methods section from cells grown in (A) MSM with 0.2% glucose, (B) MSM with 0.2% glucose and subjected to DNA damaging stress or (C) LB media. (D) shows a gel analysis of the DNA in the complex purified from the extract in panel A. The gel is calibrated with a 100-bp ladder.

DISCUSSION

Factors that interact at *E. coli* gene regulatory regions have, traditionally, been identified by a combination of genetics, bioinformatics and biochemistry (3). Recently, chromatin immunoprecipitation has been exploited to

identify all the different DNA targets that are bound by any particular protein (24). Here we have developed a complementary approach to attempt to identify all the different proteins that bind at any particular DNA target. The method, DNA sampling, develops a previous

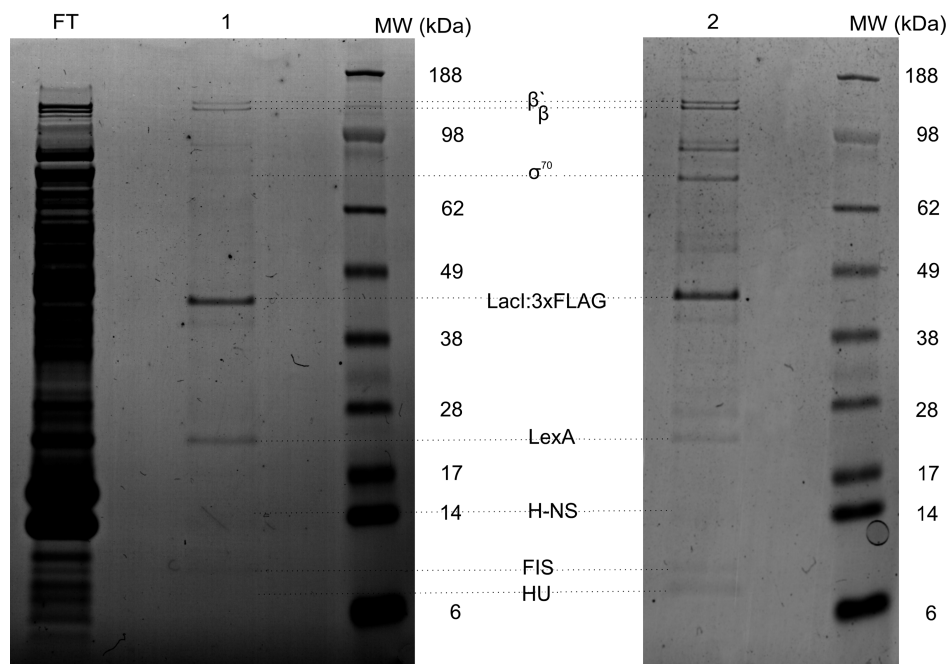


Figure 4. Analysis of proteins in purified complexes. SDS-PAGE analysis of proteins in *cka* regulatory region complexes, purified as described in Materials and Methods section. Lanes were loaded as follows: lane FT, flow-through; lane 1, affinity isolated proteins from bacteria grown in MSM with 0.2% glucose before induction; lane 2, affinity isolated proteins from bacteria strain grown in MSM with 0.2% glucose and treated with nalidixic acid to induce DNA damage. Protein identification was determined by mass spectrometry analysis and individual bands are labeled. The gel was calibrated with molecular weight markers (lanes MW). Other bands are the RNA polymerase α subunit (36 kDa), Lon protease (87 kDa), the different subunits of pyruvate dehydrogenase (99 kDa, 66 kDa and 56 kDa) and antibody that 'leaks' from the beads.

study in which a target DNA fragment was inserted into a plasmid, and proteins bound to this target were identified following the purification of plasmid-protein complexes (25). In DNA sampling, we exploit a yeast meganuclease to liberate specific DNA fragments, the bacteriophage lambda Gam protein to stabilize the fragments, an epitope-tagged LacI to facilitate the rapid purification of DNA-protein complexes, and state of the art mass spectroscopy to identify the proteins. In the experiment described here, we focused on the promoter of the colicin K gene (*cka*) that is known to be strongly repressed by global regulator LexA that sterically occludes RNA polymerase (19–21). The promoter can be induced by DNA damage and we show that DNA sampling can be used to follow this induction.

Most of the recently developed methods to identify specific DNA-binding proteins in bacteria use crude cell extracts and immobilized DNA fragments to reconstitute protein-DNA fragments, and attempt to mimic the conditions inside the cell (4–8). Here we used an alternative approach in which we attempt to purify DNA-protein complexes that had already formed inside the cell. As a control, we also purified complexes after mixing cells carrying epitope-tagged LacI and pRW902 with an equal quantity of MG1655 cells carrying unmodified LacI. We were unable to detect any unmodified LacI in the purified complexes, and this argues that most of the purified complex-bound protein must be bound to the DNA fragment before cell lysis.

In principle, the DNA sampling method outlined here could be used to investigate any DNA segment, and

should also be applicable to bacteria other than *E. coli*. The pRW902 plasmid derives from the broad host range RK2 plasmid and it is present in *E. coli* K-12 at six to eight copies per cell. Our experiments show that the 10–20 LacI repressor tetramers present per cell (26) ensure a sufficient yield of complexes. We have now run this protocol with a dozen different *E. coli* genome DNA fragments, including coding regions from within genes and have identified two shortcomings. First, samples are systematically 'contaminated' with Lon protease and pyruvate dehydrogenase which appear to bind directly to the affinity labeled beads. Second, gene regulatory proteins whose binding at targets is contingent on small ligands may escape detection. Future refinements exploiting cross-linking and competitor DNA may succeed in improving our protocol. Interestingly, a very recent paper (27) describes the purification of proteins associated with specific genomic loci in HeLa cell chromatin, exploiting hybridization rather than immunoaffinity, and including formaldehyde cross-linking and decross-linking steps. Clearly, a variety of approaches are possible.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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