Cytotechnology **17**: 65–70, 1995.

Isolation of p10 gene from *Bombyx mori* nuclear polyhedrosis virus and study of its promoter activity in recombinant baculovirus vector system

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Received 18 April 1994; accepted in revised form 20 January 1995

Key words: baculovirus, *Bombyx mori*, expression vector, insect cell line, promoter activity, p10

Abstract

A homologue of *Autographa californica* NPV (AcNPV) p10 gene was identified and cloned from *Bombyx mori* NPV (BmNPV). BmNPV p10 gene encodes truncated protein of 70 amino acid residues that lacks carboxyl terminus comparing with the p10 protein encoded by AcNPV. The putative TATA box sequence and the ATAAG motif which is the consensus sequence of baculovirus very late promoter were conserved. A transfer vector, pBNT1, which includes the p10 promoter region of BmNPV for foreign gene expression was constructed. By using pBNT1, a recombinant BmNPV, Bmp10-Luc, in which the p10 gene was replaced by the firefly luciferase gene, was obtained. We also obtained another recombinant virus, BmPH-Luc, in which the polyhedrin gene was replaced by the luciferase gene. The luciferase activity detected in BoMo-15AIIc insect cells infected with Bmp10-Luc was approximately 50% of that infected with BmPH-Luc, suggesting that although both the p10 and polyhedrin promoters of BrnNPV are effective in high-level expression of foreign genes, the p10 promoter is not so strong as the polyhedrin promoter.

Introduction

The insect baculoviruses, *Autographa californica* nuclear polyhedrosis virus (AcNPV) and *Bombyx mori* NPV (BmNPV), are now widely used for high level expression of foreign genes (Luckow & Summers 1988; Maeda 1989a; Miller 1988; Reilly *et al.* 1992). In very late phase of AcNPV-infected, *Spodoptera frugiperda* cells, two viral proteins are abundantly synthesized. These are polyhedin, the major constituent of polyhedra, and p10. The p10 gene product is dispensable for the production of virus particles and polyhedron formation (Vlak *et al.* 1988). In the p10 protein of AcNPV, three functional domains are postulated. The amino-terminal domain is suggested to be involved in an aggregation or polymerization function (Vlak *et al.* 1988; Van Oers *et al.* 1993). The carboxyl terminus is involved in the formation of fibrillar structures (Van Oers *et al.*. 1993). The nuclear disintegration function may located in a central domain (Van Oers *et al.* 1993).

In both AcNPV and BmNPV expression vectors, the polyhedrin promoter has been utilized for the expression of many foreign genes and recently, in AcNPV vector system, several foreign genes were expressed using the p10 promoter (Vlak *et al.* 1990; Weyer *et al.* 1990; Vialard *et al.* 1990). Although the promoter activity of p10 is relatively lower than that of polyhedrin (Min & Bishop 1991; Roelvink *et al.* 1992), utilization of the p10 promoter for foreign gene expression enables recombinant viruses to be occluded in polyhedral inclusion bodies and, therefore, readily to be infectious for insects when administered *per os* (Vlak *et al.* 1990). Since the BmNPV vector system has a unique advantage of having an advanced system, the silkworm, for the mass production of foreign gene products (Maeda, 1989b Reis *et al.* 1992), construction of occluded recombinant viruses using the p10 promot-

er is expected to increase the utility of the silkworm production system. Recently, the sequence analysis of p10 gene of three BmNPVs was reported and showed that all possessed a deletion at +210 position, so that the encoded protein is truncated (Hu *et al.* 1994).

In this paper, we report the molecular cloning of BmNPV p10 gene from P6E clone. We constructed a transfer vector pBNT1, which includes the p10 promoter for foreign gene expression. We obtained recombinant BmNPVs and compared the activity of the p10 promoter with that of the polyhedrin promoter using the firefly luciferase gene (de Wet *et al.* 1987) as a reporter gene.

Materials and methods

Cells, virus and plasmids

The *Bombyx mori* cell line, NISES-BoMo-15AIIc (Inoue *et al.* 1990), derived from silkworm embryo, was maintained at 25 °C in MGM-448 medium (Mitsuhashi 1984) supplemented with 10% fetal bovine serum. The BmNPV P6E strain was used as a wild-type virus. The transfer vector pBm4, containing the polyhedrin promoter of BmNPV for foreign gene expression (Kobayashi *et al.* 1992), was used in this study for comparison of promoter activity.

Identification of the p10 gene homologue in BmNPV

Total genomic DNA of BmNPV was digested with several restriction enzymes and separated on agarose gel. The separated fragments were transferred onto a positive charged nylon membrane (Sambrook *et al.* 1989). To identify the p10 gene homologue in BmNPV genome, a DNA fragment containing AcNPV p10 coding region (Kuzio *et al.* 1984) was amplified by the polymerase chain reaction (PCR). In the PCR, the genomic DNA of AcNPV was submitted to 25 PCR cycles (1 min at 92°C, 2 min at 50°C and 3 min at 72°C) followed by a 7 min final extension at 72°C, using a Model HB-TR1 thermal reactor (HYBAID Co.). The reaction mixture (100 µl) was 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % gelatin, 100 pg of the genomic DNA of AcNPV, 2.5 units of Taq polymerase (Promega) and 40 pmol each of the synthesized primers, 5′GAGCAAGAAAATAAAACGCC 3′ and 5′ATGGAGTTTATGCAGTACAC 3′. The amplified

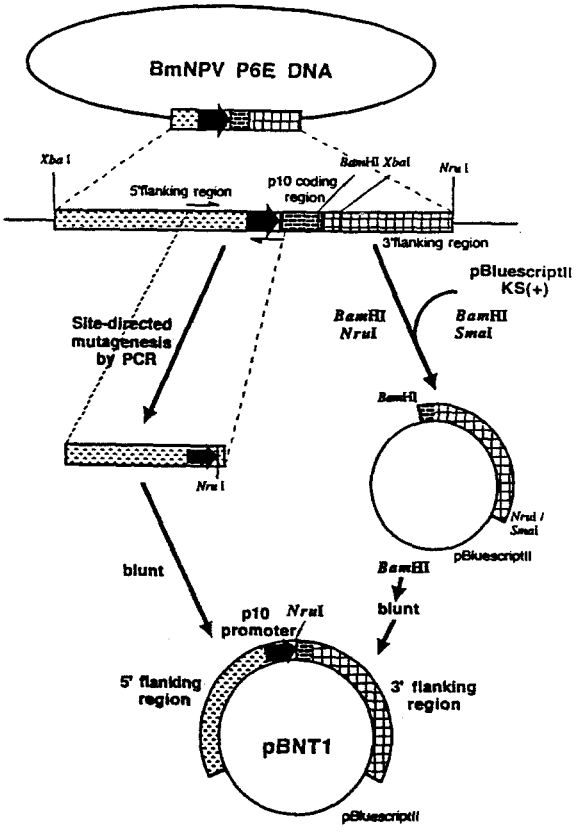

Fig. 1. Construction of a p10-based transfer vector pBNT1. The arrows show the direction of transcription.

DNA fragment was labeled with [^{32}P]dCTP and used for hybridization as the AcNPV p10 gene probe.

The DNA fragments with molecular size of 2 kb, 6 kb, and 9 kb were hybridized with the probe when the genomic DNA was digested with *Xho*I, *Xba*I, and *Nru*I, respectively. The automated DNA sequencer 373A(Applied Biosystems Inc.) was used for sequencing the 2 kb *Xho*I fragment. An open reading frame with 213-nucleotide-long was found in this fragment, and identified as the p10 gene of BmNPV based on a comparison with the published sequence of the p10 gene of AcNPV (Kuzio *et al.* 1984).

Construction of p10-based transfer vector pBNT1 from BmNPV

To construct a p10-based transfer vector, a 3.0 kb *Bam*HI-*Nru*I fragment and a 6.0 kb *Xba*I fragment were isolated separately from BmNPV genome and cloned into pBluescriptIIKS(+) (Fig. 1). The synthesized primers 5′GCGCGACAGAATAAAATCAAA

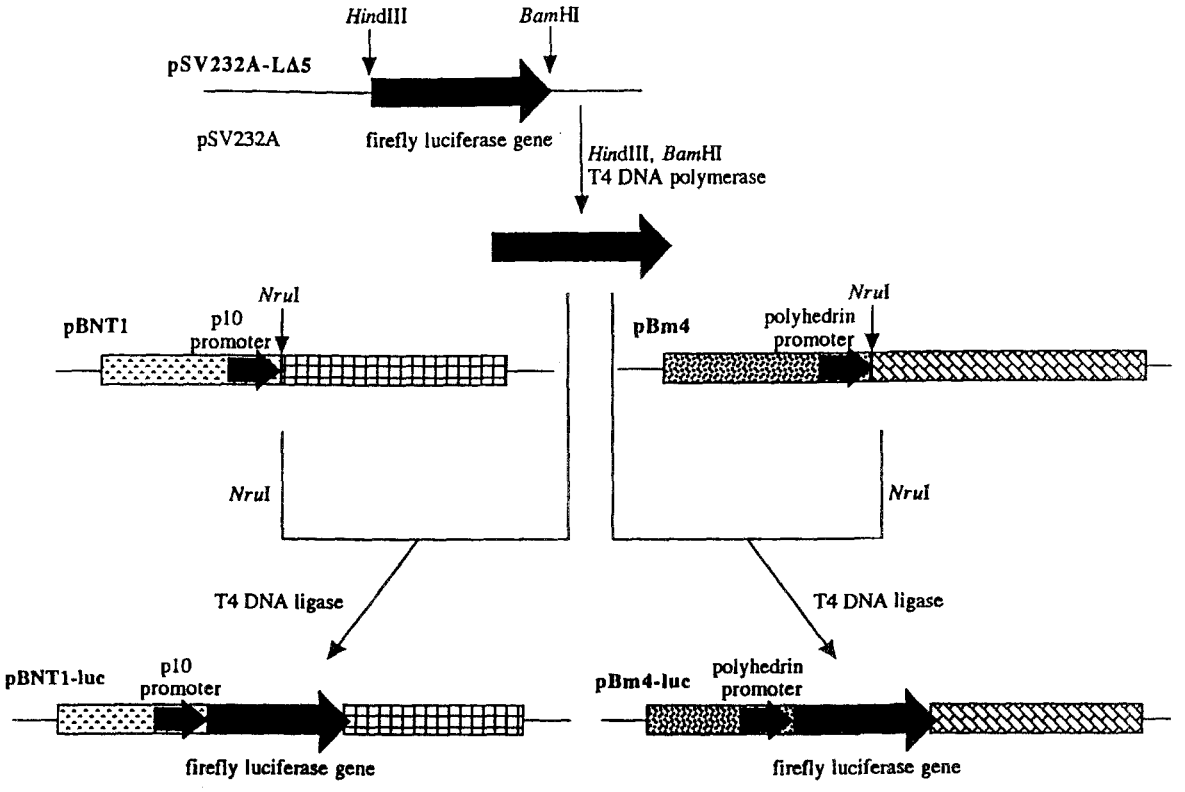

Fig. 2. Construction of transfer plasmids pBNT1-luc and pBm4-luc. The 2.4-kb *Hind*III-*Bam*HI fragment containing the coding region of the firefly luciferase gene was excised from the plasmid pSV232A-LΔ5′, filled with T4 DNA poly-merase and ligated into the *Nru*I sites of the transfer vectors, pBNT1 and pBm4.The arrows show the direction of transcription.

3′ and 5′TAAT*CG*C*GATAGTAAATAAAATG 3′ were used in PCR for amplification of a 2.4 kb fragment which consists of just upstream of the p10 initiation codon and for site-directed mutagenesis (the bases T, G, and C indicated by * were introduced in place of A, A, and T, respectively, in order to abolish the ATG initiation codon and to create a *Nru*I site indicated by an underline). The PCR conditions were the same as that stated above. This 2.4 kb fragment was ligated into just upstream of the 3.0 kb *Bam*HI-*Nru*I fragment to make the transfer vector pBNT1. As a result, this vector has a unique *Nru*I site for inserting foreign genes immediately downstream of the BmNPV p10 gene promoter.

Construction of recombinant viruses

A 2.4 kb *Hind*III-*Bam*HI fragment containing cDNA of the firefly luciferase gene was isolated from the plasmid p5V232A-LΔ5′ (de Wet *et al.* 1987), blunted with T4 DNA polymerase and ligated into the *Nru*I site of the transfer vector pBNT1 (Fig. 2). The same fragment was also ligated into the *Nru*I site of the polyhedrin-based transfer vector pBm4. The resulting plasmids, pBNT1-luc and pBm4-luc, were independently cotransfected with the genomic DNA of wild-type BmNPV into BoMo-15AIIc cells using the calcium phosphate precipitation technique (Maeda 1989). Six days after cotransfection, the culture supernatant was collected and two recombinant viruses, Bmp10-Luc and BmPH-Luc, in which the p10 gene and the polyhedrin gene were replaced with the luciferase gene were isolated by the endpoint dilution method on 96-well plates (Maeda 1989). Three times of repeat of further endpoint dilution was performed to clone the viruses genetically. The purity of viruses was confirmed by extracting the viral genomic DNAs and analysis by southern blotting after restriction enzyme digestion (data not shown). The recombinant viruses were propagated and stored at −20°C subsequent to use.

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AcNPV            ... ... ... ... ... ... ... ... ... ... ... .A. ... ... ... ...
BmNPV            TAC AAA GAT TCA GAA ATA CGC ATC ACT TAC AAC AGG GGG GAC TAT GAA  -133
BmNPV            Tyr Lys Asp Ser Glu Ile Arg Ile Thr Tyr Asn Arg Gly Asp Tyr Glu
AcNPV             -   -   -   -   -   -   -   -   -   -   -   -  Lys  -   -   -   -

... ... ... ... ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ..T ..T ...
ATT ATG CAT TTG AGG ATG TCG GGA CCT TTA ATT CAA CCC AAC ACA ATA TAT TAC AGC TAA   -73
Ile Met His Leu Arg Met Ser Gly Pro Leu Ile Gln Pro Asn Thr Ile Tyr Tyr Ser ***
 -   -   -   -   -   - Pro  -   -   -   -   -   -   -   -   -   -   -   -   -

.............C....C.....................AC.........................A...
ATAAGAATTATTATTAAATTATTTGTATATTAATTAAAATCTTATACTGTAAATTACATTTTATTTACTATC         -1

... ... ... ... ... ... ... ..G .A. ... ... ... ... G.. A.. ... ... ... ... ...
ATG TCA AAG CCT AAC GTT TTG ACA CGA ATT TTA GAC GCC ATT GCG GAA ACT AAC ACA AAG    60
Met Ser Lys Pro Asn Val Leu Thr Arg Ile Leu Asp Ala Ile Ala Glu Thr Asn Thr Lys
 -   -   -   -   -   -   -   - Gln  -   -   -   - Val Thr  -   -   -   -   -

... ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ... ... .T. ... ... ...
GTT GAC AGT GTT CAA ACT CAG TTA AAC GGG CTG GAA GAA TCG TTC CAG CCT TTG GAC GGT   120
Val Asp Ser Val Gln Thr Gln Leu Asn Gly Leu Glu Glu Ser Phe Gln Pro Leu Asp Gly
 -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   - Leu  -   -   -

... ... ... ... ... ..C ... C.. ... ... ..G ... ... ... ... ... ... ... ... ... ...
TTG CCC GCT CAA TTG ACT GAT TTT AAC ACT AAA ATC TCA GAA ATT CAA TCC ATA TTG ACC   180
Leu Pro Ala Gln Leu Thr Asp Phe Asn Thr Lys Ile Ser Glu Ile Gln Ser Ile Leu Thr
 -   -   -   -   -   -   - Leu  -   -   -   -   -   -   -   -   -   -   -   -
                             ↓
..C ... .T. .T. ... ... ... ... ... ..A ... ... ... .A. ... ... .G. ... ... ...   240
GGT GAC ACT GCT CCG GAT CCT CCA GAC TC- CTA AAG CCT ACG CTG AAA ATC CAA GCT TTT   239
Gly Asp Thr Ala Pro Asp Pro Pro Asp Ser  ** *
 -   - Ile Val  -   -   -   -   -   - Leu Lys Pro Lys Leu Lys Ser Gln Ala Phe

... C.. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...              285
GAA TTC GAT TCA GAC GCT CGT CGT GGT AAA CGC AGT TCC AAG TAA                        284

Glu Leu Asp Ser Asp Ala Arg Arg Gly Lys Arg Ser Ser Lys ***
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Fig. 3. Nucleotide sequence of the BmNPV p10 gene and surrounding region, with the corresponding sequence of the AcNPV genome (Kuzio *et al.* 1984) shown above it. The first nucleotide of the ATG initiation codon has been designated number +1. Dots indicate identity of the nucleotides. The predicted amino acid sequence of BmNPV p10 as well as of p26 homologue are shown beneath the nucleotide sequence of the BmNPV genome. The predicted amino acid sequence of AcNPV p10 as well as p26 are also shown beneath that of BmNPV. Dashes indicate identical amino acids. The putative TATA box sequence is boxed and the ATAAG motif, the consensus sequence of baculovirus very late promoter, is double-underlined. The one nucleotide deletion is found at the position of +210 (arrow) and the termination codon (TAA) is appeared at +211.

Luciferase assay

BoMo-15AIIc cells (2.0×10^5) were subcultured in 35 mm plastic dishes and allowed to attach for 24 h. Cells were inoculated at an M.O.I. of 10 $TCID_{50}$ unit per cell with either the Bmp10-Luc or BmPH-Luc virus in 1 ml of medium. After 2 h, the medium was replaced with 2 ml of fresh medium. Luciferase Assay System™ (Promega) was used to lyse the cells and quantify the expressed luciferase. The cells were lysed with 100 µl of lysis buffer and 10 µl of lysate was used for the assay. The luminometer Lumat LB9501 (Berthold Inc.) was used for the luciferase assay.

Results and discussion

The complete nucleotide sequence of the p10 gene with some of its 5′ and 3′ flanking sequences is shown in Fig. 3. Comparing with the AcNPV, the nucleotide

sequence homology of this region is 94.4%. Both the putative TATA box sequence at the position of −86 and the ATAAG motif, the consensus sequence of the baculovirus very late gene promoters (Vlak *et al.* 1990) at −72 were conserved in the 5′ flanking region of BmNPV p10 gene. The open reading frame of AcNPV p26 gene (Liu *et al.* 1986) terminating with TAA codon at −75 was also found in BmNPV. In the coding region of BmNPV p10 gene, there is a deletion of an adenine residue at +210, and the resulting frame shift creates TAA termination codon at +211, while the TAA termination codon used in AcNPV p10 gene (Kuzio *et al.* 1984) locates at +282. This one nucleotide deletion and resulting frame shift were confirmed by sequencing three independent clones and it is also observed in other strains of BmNPV (Hu *et al.* 1994). Thus the p10 homologue of BmNPV is capable of encoding truncated protein of 70 amino acids with a molecular weight of approximately 7.7 kDa. The carboxyl terminus of p10 protein is involved with the formation of fibrillar structures (Van Oers *et al.* 1993). This is consistent with no one have reported the observation of conspicuous fibrillar structures in BmNPV infected cells by electron microscopy.

The p10-based transfer vector pBNT1 (Fig. 1) was constructed to permit insertion of foreign genes into the virus genome under the control of the p10 promoter, instead of p10 coding sequence. To compare the promoter activity of p10 with that of polyhedrin, the firefly luciferase gene was introduced into pBNT1 and the polyhedrin-based transfer vector pBm4 (Fig. 2). By means of homologous recombination between these recombinant vector plasmids and wild-type BmNPV DNA, recombinant BmNPVs, Bmp10-Luc and BmPH-Luc, that contain the luciferase gene instead of p10 and polyhedrin, respectively, were obtained. In contrast with BmPH-Luc, which was unable to form polyhedra, normal polyhedra, in which virions were occluded, were formed in Bmp10-Luc infected BoMo-15AIIc cells. Bmp10-Luc could replicate and form the occlusion body in cultured cells with no obvious difference from the wild type BmNPV. Thus it was confirmed that, as with AcNPV, the p10 gene is dispensable for BmNPV replication cycle in cells.

Figure 4 shows the time course of luciferase expression in the BoMo-15AIIc cells infected with Bmp10-Luc and BmPH-Luc. Endogenous luciferase activity was neither detected in uninfected cells nor cells infected with wild type virus. Luciferase activity was detected in the cells infected with both Bmp10-Luc and BmPH-Luc. As expected from the sequence homology between BmNPV and AcNPV in the p10 promoter, the strong luciferase activity was detected in the Bmp10-Luc-infected BoMo-15AIIc cells. At 60 hours post-infection, the amount of luciferase expressed by Bmp10-Luc reached maximum and was approximately 50% of that by BmPH-Luc, which expressed the luciferase gene under control of the polyhedrin promoter. Therefore, the promoter activity of p10 appears to be relatively lower than the polyhedrin promoter. The similar results have been obtained in comparative studies on the promoter functions between p10 and polyhedrin of AcNPV (Weyer *et al.* 1990; Min & Bisho 1991; Roelvink *et al.* 1992).

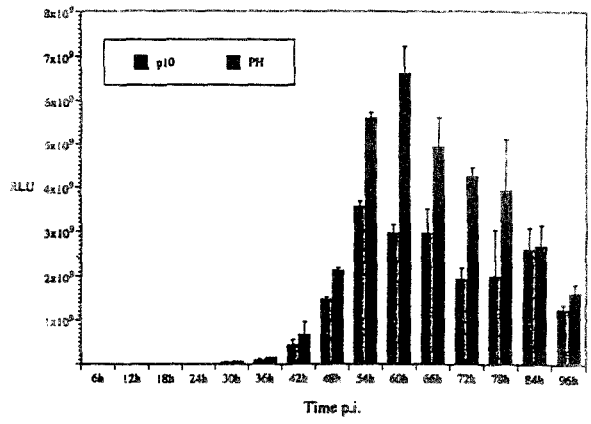

Fig. 4. Expression of luciferase in NISES-BoMo-15AIIc cells. A monolayer of BoMo-15AIIc cells (2.0×10^5) in 35 mm dish were infected with recombinant BmNPVs at an M.O.I. of 10 $TCID_{50}$ unit per cell. At 6 h intervals after infection, the cells were harvested and the cell lysate was used for luciferase assay. Data for each time point was derived from four independent sets of infected cells.

In the AcNPV expression vectors, some vectors were constructed based upon the p10 promoter for foreign gene expression (Vlak *et al.* 1990; Weyer *et al.* 1990). In other vectors, p10 promoter is used for the expression of selectable marker gene such as β-galactosidase (Vialard *et al.* 1990). These studies demonstrated that the p10 promoter, like the polyhedrin promoter, is a very effective promoter for high-level expression of foreign gene.

Previously the firefly luciferase gene was expressed under control of the polyhedrin promoter of AcNPV in Sf9 insect cells (Hasnain & Nakhai 1990). The expressed luciferase was retained intracellularly in Sf9 cells. In this study, luciferase expressed by the recombinant BmNPVs was also predominantly detected in the lysate of BoMo-15AIIc cells and luciferase activi-

ty of the cell culture supernatant was less than 1% of the cell lysate (data not shown).

The BmNPV vector system has an unique advantage of expressing the foreign genes in the silkworm larvae at a large scale. Previous studies have demonstrated that the expression of some proteins, such as interleukin-3 (Miyajima *et al.* 1987), α-amidating enzyme (AE-II) (Kobayashi *et al.* 1992), porcine growth hormone (PGH) (Kawai *et al.* 1992) and human α-interferon (Maeda *et al.* 1985), were significantly higher in concentration in the hemolymph of silkworm larvae than culture supernatant or lysate of insect cells. In these studies, silkworm larvae were injected with budded virus. In contrast, recombinant viruses constructed with the p10-based transfer vector pBNT1 can form occluded virus, and which can be used for infecting a large number of silkworm larvae easily by oral ingestion. Further studies on *in vivo* expression of the luciferase gene using the occluded Bmp10-Luc are in progress.

Acknowledgements

We thank Dr. Subrata Chowdhury for critical reading of the manuscript, and Dr. Li Rong Deng for the instruction of luciferase assay.

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