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Epitope Mapping and Characterization of the Infectious Hematopoietic Necrosis Virus Glycoprotein, Using Fusion Proteins Synthesized in *Escherichia coli*†

L. XU,[1] D. V. MOURICH,[1] H. M. ENGELKING,[1] S. RISTOW,[2] J. ARNZEN,[2] AND J. C. LEONG[1]*

Department of Microbiology, Oregon State University, Corvallis, Oregon 97331-3804,[1] and Department of Animal Sciences, Washington State University, Pullman, Washington 99164-6332[2]

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A characterization of the antigenic determinants (epitopes) of the glycoprotein (G) of infectious hematopoietic necrosis virus was made by expressing different regions of the G gene in *Escherichia coli*. A cDNA copy of the G gene was divided into four fragments by *Taq*I digestion, and the fragments were subcloned into pATH vectors, placing the expression of each G gene fragment under control of the *trpE* promoter. The resulting plasmids, pXL2, pXL3, and pXL7, encoded *trpE*-G fusion proteins subsequently detected with anti-infectious hematopoietic necrosis virus sera by Western immunoblots. A comparison of reactivities of the fusion proteins encoded by these plasmids was made by Western immunoblot and radioimmunoassay with a number of anti-G specific monoclonal antibodies (MAbs). The nonneutralizing MAb 136J reacted with the *trpE*-G fusion protein encoded by pXL3 and fusion proteins encoded by plasmids p52G and p618G, which were described in previous studies (R. D. Gilmore, Jr., H. M. Engelking, D. S. Manning, and J. C. Leong. Bio/Technology 6:295–300, 1988). Another nonneutralizing MAb, 2F, bound to the pXL3 fusion protein, and the neutralizing MAb RB/B5 recognized the pXL7 fusion protein. All fusion proteins were tested as vaccines in rainbow trout fry. Although significant protection was induced by all fusion proteins, the pXL3 fusion protein was most effective as a vaccine.

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus associated with severe epizootic disease in juvenile salmonid fish (17, 23). IHNV infections have resulted in large economic losses to salmon and trout industries. Presently, there are no chemotherapeutic agents or licensed vaccines available for prevention or control of the disease; however, upon immunization with killed IHNV, fish produce protective serum antibody which neutralizes IHNV in vitro (1). Because attenuated and killed vaccines are extremely expensive and have not proven totally efficacious (2, 13), the aquaculture industry is looking for advances in recombinant DNA technology to provide economical and effective vaccines.

The IHNV virion contains an unsegmented negative-sense single-stranded RNA genome of approximately 11,000 nucleotides encoding five structural proteins and one nonvirion protein (11, 15, 16). The glycoprotein, found on the surface of IHNV, has been identified as the single viral protein which induces the production of neutralizing antibodies in both rabbits and fish. With the glycoprotein, cross-protective immunity in fish against challenge with different electrophoretic types of IHNV is obtained (5). It therefore may be possible to develop an economical subunit vaccine for controlling the disease patterned after the glycoprotein of a single type of IHNV. Polyvalent rabbit antiserum raised against enzymatically deglycosylated virus retains virus-neutralizing activity and reacts with glycosylated G protein in Western immunoblots (5), indicating that different types of IHNV share a neutralizing epitope(s) not involving carbohydrate side chains.

Previous work with rabies virus glycoprotein showed that expression of the full-length glycoprotein gene in bacteria resulted in a denatured protein unable to produce a protective immune response, presumably because inappropriate folding mechanisms for disulfide bond formation existed in *Escherichia coli* (14). Thus, we sought smaller portions of the IHNV glycoprotein gene coding for one or a few epitopes, instead of the intact gene, for construction of recombinant plasmids for bacterial expression (6).

The IHNV glycoprotein gene of 1,609 nucleotides encoding a protein of 508 amino acids has been cloned and sequenced (10). Two recombinant plasmids, p52G and p618G, containing approximately 350 bases of the *Sau*3AI-digested IHNV G gene, have been constructed and expressed in bacteria as *trpE*-G fusion proteins. The two plasmids contain identical G gene fragments of 329 bp encoding amino acids (aa) 336 to 444, which were ligated in frame to the *trpE* protein, but they differ by additional G gene sequences which were ligated out of frame to the 3′ ends of the 329-bp fragments (6). The molecular masses of the fusion proteins expressed by p52G and p618G were 49 and 48 kDa, respectively. Bacterial lysates containing *trpE*-G fusion proteins made from p52G or p618G induced protective immunity against IHNV in laboratory trials (6) and led to the development of a prototype IHNV subunit vaccine (6). Despite the efficacy of the vaccine in laboratory trials, there still remained questions concerning the number of immunoprotective regions on the G protein and the identification of those regions recognized in fish. These questions led to the development of a number of expression clones containing different regions of the G protein. The construction of clones and the characterization of the epitopes of their expressed fusion proteins are described

* Corresponding author.

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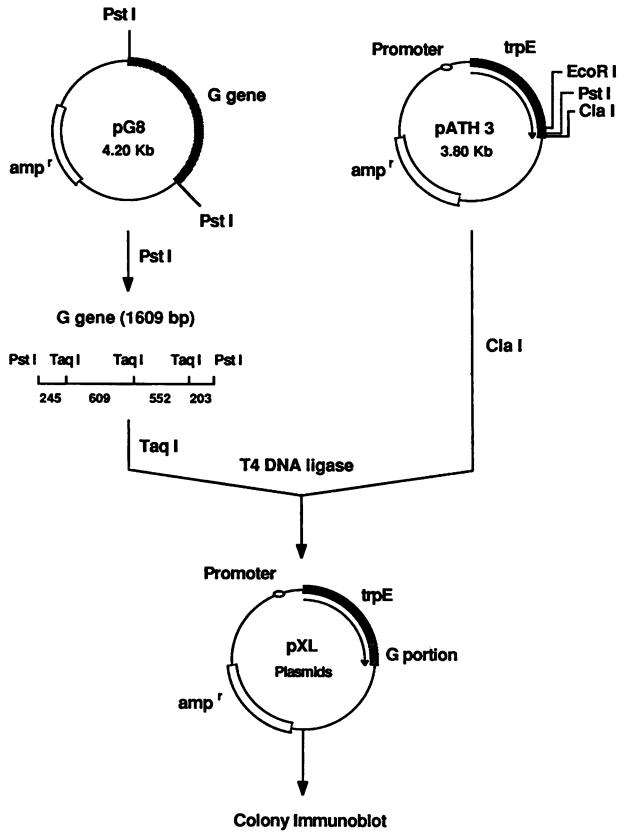

FIG. 1. Construction of *trpE*-G fusion protein expression plasmids. The 1.6-kb cDNA of the IHNV G gene isolated from the pG8 plasmid was digested with *Taq*I. The G gene fragments were purified and ligated to *Cla*I-digested pATH3.

here in an analysis of the antigenic nature of the IHNV G protein.

Portions of the G gene were subcloned into *trpE* expression vectors to create *trpE*-G fusion proteins (Fig. 1). *Taq*I digestion of a 1.6-kb *Pst*I fragment from pG8, which contained the entire G gene, resulted in four fragments: A (245 bp), B (609 bp), C (552 bp), and D (203 bp). Recombinant plasmids were constructed by the ligation of purified preparations of each fragment or of a mixture of all four fragments with the pATH3 vector which had been previously digested with *Cla*I. The ampicillin-resistant transformant colonies were screened for IHNV G protein expression by colony immunoblot (7) with rabbit polyclonal anti-IHNV sera. Three plasmids (pXL2, pXL3, and pXL7) isolated from three positive colonies were chosen for subsequent analyses. Direct plasmid DNA sequence analyses (8, 21) verified that pXL2 contained the *Taq*I B fragment and pXL3 contained the *Taq*I C fragment (Fig. 2). The pXL7 plasmid, which was derived from the ligation mixture of all four fragments, contained the 3′ external D fragment ligated in frame to the *trpE* gene. In addition, the 5′ external A fragment was ligated to the 3′ external fragment at the *Pst*I site in the same orientation as the G gene (Fig. 2).

Fusion proteins were expressed in *E. coli* DH5α cells after indoleacrylic acid induction as previously described (6). The induced bacterial cells were harvested by centrifugation and lysed by high-speed agitation in a minibeadbeater (Biospec Products) with Tris-EDTA buffer (10 mM Tris–1 mM EDTA, pH 8.0). The *trpE*-G fusion proteins encoded by pXL2, pXL3, and pXL7 were detected by Western immunoblot (12, 20) with anti-IHNV serum, and the estimated molecular masses of each protein were 59.3, 57.3, and 43 kDa, respectively (Fig. 3). The *trpE* component of the fusion protein contributed 340 aa (37,400 kDa) to each fusion protein. These estimates were consistent with the molecular masses calculated from the derived amino acid sequence obtained from the plasmid DNA sequence analyses for pXL2 (aa 66 to 269), pXL3 (aa 270 to 453), and pXL7 (aa 454 to 508).

The location of linear antigenic determinants of the IHNV G protein were then determined by reaction with monoclonal antibodies (MAbs) on Western immunoblots (20). These results were compared with those for the proteins produced by p52G (aa 336 to 444) and p618G (aa 336 to 444). The study included 10 MAbs which had been shown to be reactive with the IHNV glycoprotein (2, 3). Only those MAbs which reacted with the IHNV G protein in Western immunoblots are described here. The nonneutralizing MAb 136J bound to the pXL3 (aa 270 to 453), p52G (aa 336 to 444), and p618G (aa 336 to 444) fusion proteins (Fig. 4A). The nonneutralizing MAb 2F reacted only with the pXL3 (aa 270 to 453) fusion protein (Fig. 4B). The pXL7 (aa 454 to 508) fusion protein was recognized by neutralizing MAb RB/B5 (provided by J. L. Fryer, Oregon State University [22]) (Fig. 4C). Table 1 summarizes the binding activity of each MAb to the *trpE*-G fusion proteins.

The pXL2 (aa 66 to 269) plasmid encoding a *trpE*-G fusion protein was recognized by anti-IHNV serum (Fig. 3) but not by any of the anti-G MAbs. Although there are two external *Taq*I-digested G gene fragments (totaling 510 nucleotides) inserted into pXL7 (aa 454 to 508), the *Taq*I D fragment was the only sequence expressed as part of the *trpE*-G fusion protein. Examination of the sequence indicated that there was a TAA termination codon at the end of the coding sequence in the *Taq*I D fragment (position 1547 in the original glycoprotein gene) and no consensus recognition site for translational initiation (19) of the *Taq*I A fragment sequence. Therefore, a peptide of only 55 aa from the G gene was encoded by pXL7 (aa 454 to 508). This peptide reacted with both anti-IHNV serum (Fig. 3) and the neutralizing MAb RB/B5 (Fig. 4C). The results indicated that there was a neutralizing epitope recognized by RB/B5 within this C-terminal region of 55 aa. A hydropathy plot of the amino acid sequence of the IHNV G protein indicated that a very large hydrophobic domain, presumably the transmembrane region, was encoded in the G fragment cloned into the pXL7 (aa 454 to 508) plasmid. This hydrophobic domain consists of 27 aa from 461 to 487. This would suggest that the

TABLE 1. Summary of the immunoblot characterization of G subunits by MAbs

IHNV isolate[a]	MAb	Ig type[b]	Neutralizing ability[c]	Reactivity of plasmids tested by Western blot assay[d]				
				pXL2	pXL3	pXL7	p52G	p618G
039-82-SR	136J	IgG	–	–	+	–	+	+
Cedar River	2F	IgM	–	–	+	–	–	–
Round Butte	RB/B5	IgM	+	–	–	+	–	–

[a] IHNV isolates that were used to generate the MAbs.
[b] Ig, Immunoglobulin.
[c] –, Unable to neutralize; +, able to neutralize.
[d] +, Reactivity; –, no reactivity.

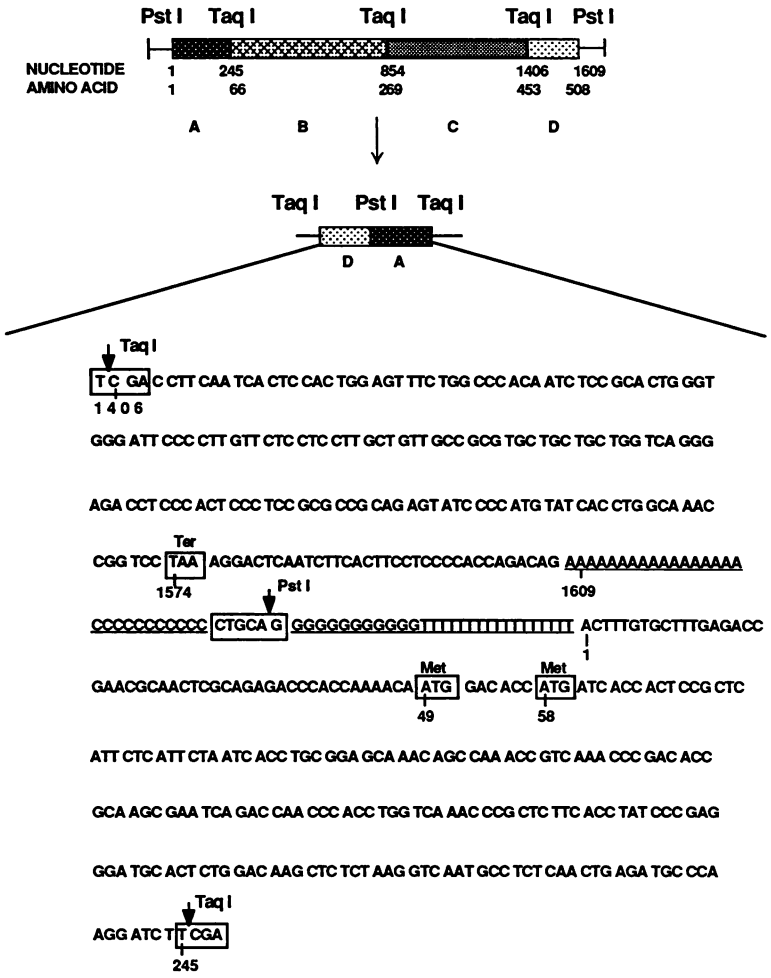

FIG. 2. DNA sequence of the cloned G gene fragments in the plasmid pXL7.

RB/B5 epitope is localized to the N-terminal region (aa 453 to 460) of the G fusion protein encoded by pXL7 (aa 454 to 508).

Although the plasmids pXL3 (aa 270 to 453), p52G (aa 336 to 444), and p618G (aa 336 to 444) had DNA inserts of different sizes, they all contained a common G gene sequence from nucleotides 1052 to 1379 (Fig. 5). The plasmid pXL3 (aa 270 to 453) contained an additional 198 nucleotides of the G gene sequence at the 5′ end. These plasmids expressed *trpE*-G fusion proteins of 57.3, 49, and 48 kDa, respectively. All of these proteins reacted with polyclonal anti-IHNV sera (Fig. 3) and the nonneutralizing MAb 136J (Fig. 4A) in Western immunoblots. This result indicated that the common 109-aa sequence (336 to 444) encoded by these three plasmids contained a linear epitope recognized by MAb 136J.

The MAb 2F recognized by Western immunoblot a unique epitope which was found only in the G subunit defined by pXL3 (aa 270 to 453) (Fig. 4B). This result indicated that there was a distinct linear epitope, 2F, located in the unique 66 aa at the N terminus of the pXL3 (aa 270 to 453)-encoded G protein sequence. MAb 2F was generated against the Cedar River strain of IHNV, while MAb 136J was raised against the 039-82-SR strain of IHNV. Since pXL3, a clone containing G gene sequences from the Round Butte strain of

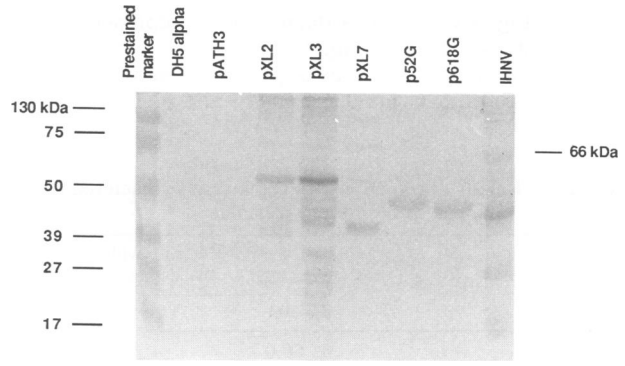

FIG. 3. Analysis of *trpE*-G fusion proteins. Bacterial lysates were resolved on sodium dodecyl sulfate–10% polyacrylamide gels. The proteins on the gels were transferred to nitrocellulose and analyzed with anti-IHNV serum. The *trpE*-G fusion proteins are shown. The major bands expressed by pXL2, pXL3, pXL7, p52G, and p618G migrated at positions of 59.3, 57.3, 43, 49, and 48 kDa, respectively.

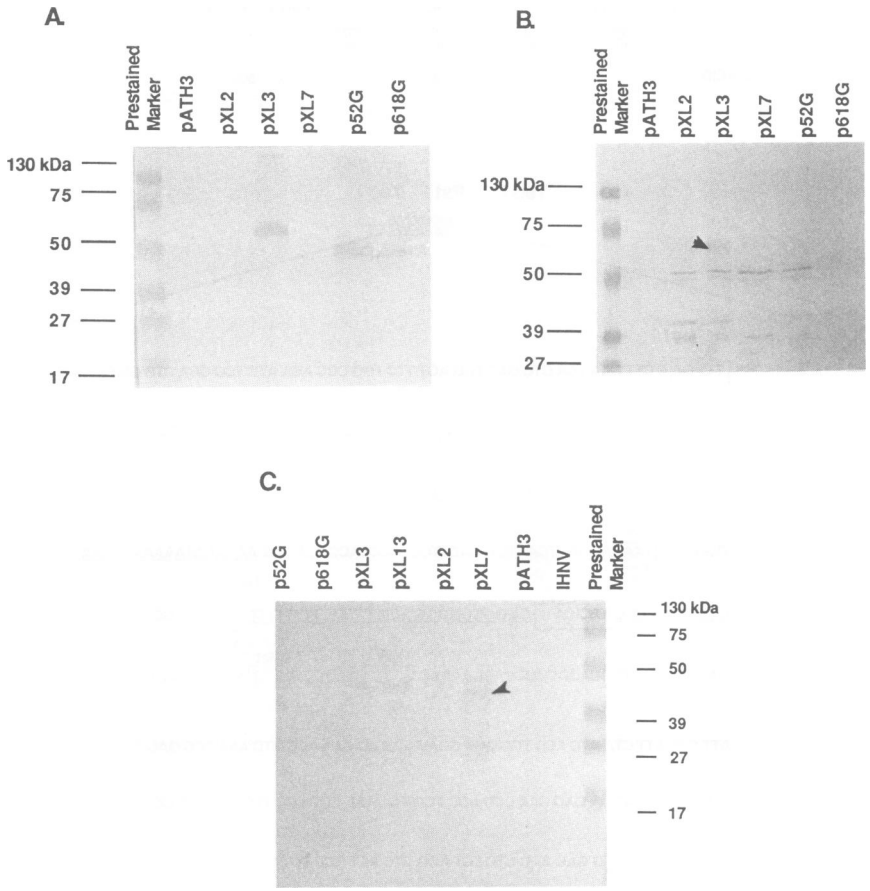

FIG. 4. Immunoblot characterization of the *trpE*-G fusion proteins with MAbs. Bacterial lysates were resolved on sodium dodecyl sulfate–10% polyacrylamide gels, and the proteins were transferred to nitrocellulose. Each blot was developed with the MAb indicated below. Each lane is marked at the top by the plasmid used to generate the protein samples that were added to that gel well. The location of the prestained molecular mass marker is shown on the side. (A) MAb 136J. The pXL3 (57.3-kDa), p52G (49-kDa), and p618G (48-kDa) fusion proteins were recognized by 136J. (B) MAb 2F. The pXL3 fusion protein (57.3 kDa) is indicated by the arrowhead. (C) MAb RB/B5. The pXL7 fusion protein (43 kDa) is indicated by the arrowhead. The band running just above the fusion protein band is a common cross-reactive polypeptide that is found in the bacterial lysates. The IHNV lane does not have any reactive bands because too little viral protein was used in this gel. However, RB/B5 does react with the glycoprotein of IHNV in immunoblots.

IHNV, reacted with the two MAbs, 136J and 2F, all three virus strains must contain common epitopes defined by 2F and 136J (Fig. 4A and B). A diagrammatic representation of these results is shown in Fig. 5.

Lysates of induced cultures of pXL2 (aa 66 to 269), pXL3 (aa 270 to 453), p52G (aa 336 to 444), and pXL7 (aa 454 to 508) were used for the vaccination of 100 rainbow trout fry (0.4 g each) by immersion. Thirty days after the vaccination, the fry were challenged with 100 and 1,000 50% tissue culture infective doses of the Box Canyon IHNV per ml (a strain which has been classified as a type 2 isolate) (5). Moribund fish were collected on a daily basis for 30 days. The presence of virus in all these fish was confirmed by the production of characteristic cytopathic changes in tissue

TABLE 2. Relative protection induced in fish by bacterial lysates with different plasmids

Experimental group	% Mortality with virus challenge dose[a]	
	100	1,000
Control	56.0	75.0
pXL2	26.0	48.0
pXL3	2.5	14.0
p52G	19.0	56.0
pXL7	36.0	50.0

[a] Average percent mortality in three replicate samples of 30 fish each. The virus challenge dose is the number of 50% tissue culture infective doses per ml as described by Gilmore et al. (6).

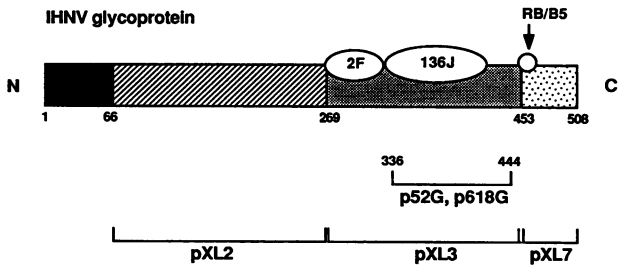

FIG. 5. Epitope map of the IHNV glycoprotein.

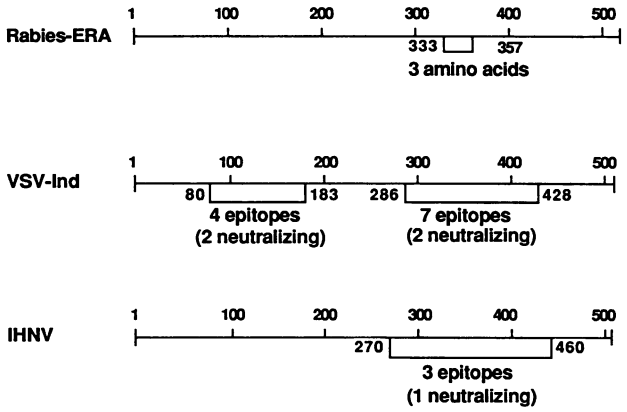

FIG. 6. Comparison of the locations of epitopes on the glycoproteins of IHNV, vesicular stomatitis virus (VSV-Ind), and rabies virus (rabies-ERA).

culture. Table 2 shows the average percent mortalities in each experimental group. The pXL3 (aa 270 to 453)-encoded region of the IHNV glycoprotein induced a strong immunoprotective response in fish. Although the regions encoded by pXL2 (aa 66 to 269), p52G (aa 336 to 444), and pXL7 (aa 454 to 508) also induced some protection, the pXL3 (aa 270 to 453) lysate was clearly much more protective as a vaccine.

Although there is a very low level of protein sequence homology between the IHNV glycoprotein and the glycoproteins of two other animal rhabdoviruses, vesicular stomatitis virus and rabies virus, the structural domains on these three glycoproteins (transmembrane region, signal peptide, positions of cysteines, and location of glycosylation sites) have also been conserved (10). It should also be possible to determine whether there is conservation among the antigenic structures of the rhabdovirus glycoproteins. The seven epitopes of the Indiana serotype of vesicular stomatitis virus, including two neutralizing epitopes, have been mapped to the amino acid sequence 286 to 428 (Fig. 6) (9). For the rabies virus (Evelyn-Rokitnicki-Abelseth strain) glycoprotein, amino acids Arg-333, Asn-336, and Gly-357 were found to be essential for neutralization (4, 18, 24). We have localized three epitopes of the IHNV glycoprotein in the region of aa 270 to 460 (Fig. 4A and B).

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