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BRIEF REPORT

Mutagenesis of a cAMP Response Element within the Latency-Associated Transcript Promoter of HSV-1 Reduces Adrenergic Reactivation

David C. Bloom,*[,1] Jack G. Stevens,† James M. Hill,‡ and Robert K. Tran*

*Department of Microbiology, Arizona State University, Box 872701, Tempe, Arizona 85287; †Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, California 90095; and ‡Department of Ophthalmology, LSU Medical Center, New Orleans, Louisiana 70112

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Mutagenesis of a cyclic AMP response element (CRE) within the LAT promoter of HSV-1 reduces the ability of LAT expression to be induced in transient assays, but has only a minimal impact on reactivation of the virus in *in vitro* systems. Here we show that a CRE mutation results in a significant reduction of adrenergically induced reactivation *in vivo* in the rabbit eye model. Spontaneous reactivation frequencies were also reduced. In addition, we demonstrate that this mutation has no effect on the amount of LAT expressed during latency when compared with the parent, 17*syn*+, and the rescuant. These results indicate a greater effect of CRE on induced reactivation *in vivo* than in *in vitro* systems, but also suggest that the CRE in the LAT promoter is not autonomous in conducting the reactivation signal.

INTRODUCTION

Herpes simplex viruses establish life-long latent infections within sensory ganglia neurons and periodically reactivate in response to environmental stimuli such as stress (for a review see Stevens, 1989). Control of the ability to reactivate has been shown to reside within the latency associated transcription unit (LAT) of HSV-1 (Block *et al.,* 1993; Bloom *et al.,* 1994; Hill *et al.,* 1990; Sawtell and Thompson, 1992) and HSV-2 (Krause *et al.,* 1995), and viruses with deletions of the LAT promoter show reduced reactivation in both *in vitro* and *in vivo* models (Block *et al.,* 1993; Bloom *et al.,* 1994; Devi-Rao *et al.,* 1994; Hill *et al.,* 1990; Krause *et al.,* 1995; Leib *et al.,* 1989; Perng *et al.,* 1994). Although genetic evidence for the involvement of LAT in reactivation has been obtained, the actual mechanism by which LAT orchestrates this process is unknown. Central to understanding this mechanism is identifying the trigger for initiation of the reactivation event. Because viruses with LAT promoter deletions are restricted in their ability to reactivate, a great deal of attention has been directed toward identifying elements within the LAT promoter that could act as switches for this process. Notably, two cAMP-response elements (CRE) have been identified, one at −43 to −36 (Leib *et al.,* 1991) and the other at −85 to −78 (Kenny *et al.,* 1994) and these elements have been proposed to trigger reactivation via a second messenger pathway involving cAMP. This type of mechanism would be capable of translating stress signals from the cell to the latent viral genome.

Initially, the CRE at −43 was shown to be critical for induction of LAT expression in transient assays by dibutyryl cAMP, forskolin, nerve growth factor, or phorbol 12-myristate 13-acetate (Leib *et al.,* 1991). In addition, reactivation of wild-type virus in explant cocultivation experiments was found to be inhibited by cyclic AMP antagonists. These data supported the hypothesis that cAMP plays an important role in HSV reactivation, and that the CRE at −43 in the LAT promoter is central to this process. Subsequent experiments with site-directed CRE mutants showed that a modest, but statistically significant, reduction in reactivation following *in vitro* cocultivation of mouse trigeminal ganglia was attributable to the CRE mutation, suggesting that CRE plays some role in this process (Rader *et al.,* 1993).

One question raised by these studies is what impact the CRE mutation would have *in vivo* in an induced reactivation model. Previous studies have shown a number of differences between *in vivo* reactivation models (in mouse, rabbit, or guinea pig) and *in vitro* mouse models (Block *et al.,* 1993; Bloom *et al.,* 1994; Devi-Rao *et al.,* 1994; Hill *et al.,* 1996). One possibility is that induced reactivation of a CRE mutant *in vivo* would be more extensively impaired than *in vitro* reactivation. Because the previous CRE mutants were constructed in a KOS strain background, and the impact of these mutations impact on epinephrine-induced reactivation could not be readily assessed (Hill *et al.,* 1987), we constructed recombinant

[1] To whom correspondence and reprint requests should be addressed. Fax: (602) 965-2272. E-mail: dcbloom@asuvm.inre.asu.edu.

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in strain 17syn+ and evaluated its ability to reactivate in the rabbit eye model.

MATERIALS AND METHODS

Viruses, cell lines, and culture conditions

HSV-1 strain 17*syn*+ was propagated on rabbit skin (RS) cells. The RS cells were also employed to prepare and titrate virus stocks, and for transfection experiments, and were maintained in MEM (Life Technologies, Gaithersburg, MD) supplemented with 5% calf serum, 250 U penicillin, 250 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 292 μg/ml L-glutamine/ml.

Construction of the recombinant 17*CRE*

The plasmid pJGS 1 was created by site-directed mutagenesis of the plasmid pDB23 with the oligonucleotide 5′ GTT TTT GCA GAT CTT TCT CAG CCT T 3′ (corresponding to the region from 118,752–118,776 nt on the HSV genome) using the *dut-/ung- selection method of Kunkel (1985; Zwaagstra et al.,* 1991). pDB23 contains a 1.2-kb region encompassing the LAT promoter in pBluescript. The oligonucleotide used for mutagenesis introduced a unique *Bgl*II site into the resulting plasmid, pJGS1, which facilitated screening. The plasmid was sequenced to confirm that the CRE mutation had been properly introduced and along with 17*syn*+ DNA was used to cotransfect RS cells. Recombinants were screened by standard techniques using Tetramethyl ammonium chloride (TMAC) hybridization (Ausubel *et al.,* 1993) with the oligonucleotide used for the mutagenesis as the probe. The recombinant 17*CRE* was further characterized by Southern blot hybridization, as well as DNA sequence analysis of the LAT promoter (both copies), to verify the sequence of the CRE mutation. The rescue of 17*CRE* was created by cotransfecting 17*CRE* along with the plasmid pDB23 to restore the wild-type CRE sequences. This transfection was also screened using TMAC hybridization with the oligonucleotide GTT TTT GCT GCG TCA TCT CAG CCT T (118,752–118,776 nt), which corresponds to the wild-type sequence.

Analysis of reactivation in the rabbit model

Rabbits were latently infected with HSV and were reactivated using methods previously described (Bloom *et al.,* 1994; Hill *et al.,* 1986, 1987). Briefly, each eye of 2-kg New Zealand White rabbits was inoculated with $1-2 \times 10^5$ PFU of virus. The eyes were examined for corneal epithelial lesions as evidence of acute infection and rabbits were monitored for 40 days prior to induction. Spontaneous shedding was assessed by daily swabbing of the eyes from days 20 through 38 postinoculation.

The rabbits were induced to reactivate by transcorneal iontophoresis of 0.01% epinephrine (0.8 mA for 8 min) once a day for 3 consecutive days. Eye swabs were performed daily for up to 7 days postinduction and assayed on primary rabbit kidney cell monolayers for the detection of infectious virus.

Quantitation of HSV DNA and LAT transcripts

Rabbit trigeminal ganglia were snap frozen, and the tissue was extracted using Trizol (Life Technologies, Gaithersburg, MD) and separated into RNA and DNA fractions according to the manufacturer's instructions. PCRs were performed in an Ericomp Thermalcycler (San Diego, CA). For the detection of HSV DNA, 50-μl reactions containing 0.5 μg of ganglionic DNA; a 0.5 μM concentration of each primer (primer 1, 5′ CAT CAC CGA CCC GGA GAG GGA 3′ [nt 65,866–65,886]; primer 2, 5′ GGG CCA GGC GCT TGT TGG TGT 3′[nt 65,957–65,937]); 1.5 m*M* Tris (pH 8.8); 16.6 m*M* ammonium sulfate; 6.7 m*M* $MgCl_2$; 0.17 mg bovine serum albumin per milliliter; a 1.25 m*M* concentration each of dGTP, dCTP, dATP, and dTTP; 1 μCi of [^{32}P]dATP (~6,000 Ci/mmol); and 2.5 U of *Taq* polymerase (Perkin–Elmer) were amplified at 94, 68, and 72°, with the first cycle having a duration of 3 min at each temperature; an additional 30 cycles were for 1 min at each temperature. The 92-bp HSV-specific product was resolved on a 7.5% polyacrylamide gel and visualized by autoradiography. For the detection and quantitation of HSV LAT RNA, reverse transcription reactions were performed using 0.5 μg of the ganglionic RNA, 10 pmol of random hexamer primers (Pharmacia); 12.5 μM each of dGTP, dCTP, dTTP, and dATP (Pharmacia); 150 m*M* KCl; 100 m*M* Tris, pH 8.3; 6 m*M* $MgCl_2$; 20 m*M* dithiothreitol; 20 U RNasin (Promega); and 200 U of Mo-MuLV reverse transcriptase (Life Technologies) in a final reaction volume of 20 μl. Reactions were carried out for 60 min at 37° and terminated by incubation at 100° for 10 min. PCR was performed using $\frac{1}{5}$ of each reverse transcription reaction; a 0.5 μM concentration of each primer (primer 1, 5′ CGG CGA CAT CCT CCC CCT AAG C 3′ [nt 118,888–118,908]; primer 2, 5′ GAC AGA CGA ACG AAA CAT TCC G 3′ [nt 119,036–119,015]); 67 m*M* Tris (pH 8.8); 16.6 m*M* ammonium sulfate; 4.0 m*M* $MgCl_2$; 0.17 mg bovine serum albumin per milliliter; a 1.25 m*M* concentration each of dGTP, dCTP, dATP, and dTTP; 1 μCi of [^{32}P]dATP (~6,000 Ci/mmol); and 2.5 U of *Taq* polymerase (Perkin–Elmer) were amplified at 94, 68, and 72°, with the first cycle having a duration of 3 min at each temperature; an additional 30 cycles were for 1 min at each temperature. The 149-bp HSV-LAT-specific product was resolved on a 5% polyacrylamide gel and visualized by autoradiography. For all experiments, a primer pair specific for rabbit actin (primer 1, 5′ AAG ATC TGG CAC CAC ACC TT 3′; primer 2, 5′ CGA ACA TGA TCT GGG TCA TC 3′) was used as an internal standard for quantitating total DNA and RNA recovery. To determine levels of DNA in the Trizol RNA fractions, as well as to act as a control for the reverse transcription reactions, an

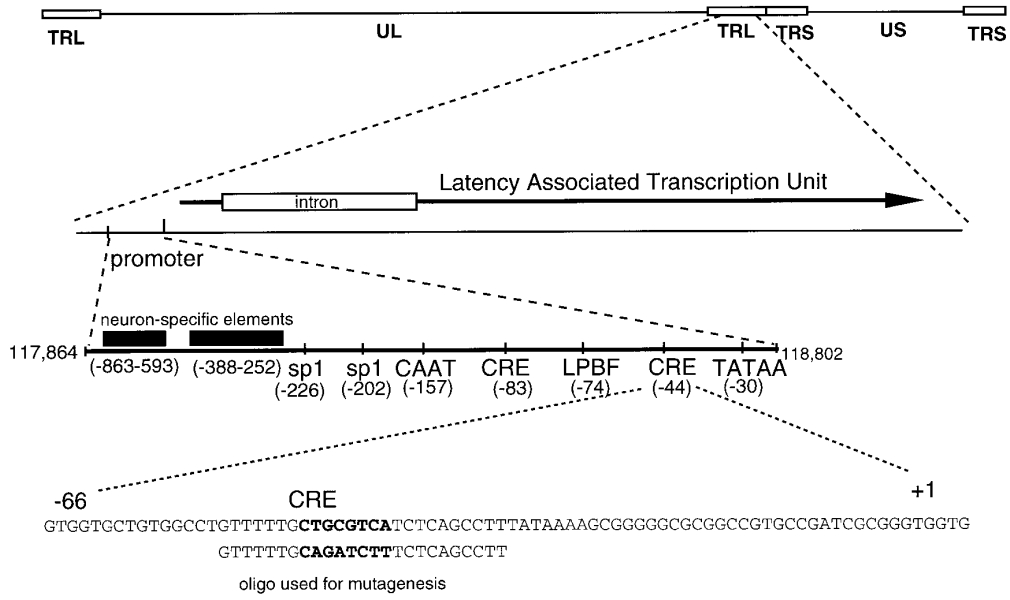

FIG. 1. Construction of the virus 17*CRE.* Shown at the top is the HSV genome and depicted below is the location of the LAT region, including an expanded view of the LAT promoter. The locations of a number of transcriptional elements located within the LAT promoter are indicated (Kunkel, 1985; Zwaagstra *et al.,* 1991).

additional set of samples were mock reverse-transcribed (no RTase added). Quantitation of bands was performed on a Phosphorimager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

RESULTS AND DISCUSSION

Based on published studies with the cellular CRE recognition sequences, we constructed a CRE mutant in which the natural CRE sequence (CTGCGTCA) was changed to CAGATCTT, a recognition sequence predicted to provide less than 10% binding of the CREB protein (Fig. 1). The parent strain was 17*syn*+ which, unlike KOS, can be induced to reactivate at high frequencies (Hill *et al.,* 1987). The recombinant (17*CRE*) showed no alterations in growth characteristics when compared to the rescuant (17*CRE*r) as assessed by multistep growth curves on rabbit skin cells (RS) *in vitro* (data not shown).

The recombinant, 17*CRE,* and the rescuant, 17*CRE*r, were analyzed for both spontaneous and induced reactivation phenotypes in rabbits by the ability to detect virus following eye swabs. 17*CRE* showed a marked reduction in reactivation frequency following epinephrine induction (Table 1); 9 of 24 (37%) eyes and 7 of 12 (58%) 17*CRE* infected rabbits were positive for reactivation compared to 7 of 10 (70%) eyes and 4 of 5 (80%) rabbits infected with the rescuant. Reactivation is not reduced as dramatically, however, as in the case of 17ΔPst (a LAT promoter deletion virus) and, in fact, is somewhat intermediate (58%) between the values observed for 17ΔPst (19%) and the wild type (78%). When the total positive swabs per total swabs are examined, however, 17*CRE* demonstrates a greater and more significant reduction, suggesting that the CRE mutation could impact the duration of the shedding episodes as well. Also of interest is the observation that the CRE mutation had an even greater effect on spontaneous reactivation than on induced reactivation; spontaneous reactivation frequencies for 17*CRE* were very similar to those of 17ΔPst.

DNA was extracted from 17*CRE* and 17*CRE*r latently infected rabbit trigeminal ganglia to determine whether the reduced reactivation observed with 17*CRE* was caused by an effect on the efficiency of the establishment of latency. However, there was no significant difference in the amounts of viral DNA detected in trigeminal ganglia from rabbits latently infected with either virus (Fig. 2A, and Table 2). Therefore, the decrease in reactivation is not the result of a replication/establishment defect.

To examine the impact of the CRE mutation on the expression of LAT during latent infection in the trigeminal ganglia, Trizol RNA fractions from the same ganglia used for the DNA determinations were subjected to quantitative RT-PCR analysis. As shown in Fig. 2B, the amount of LAT detected was similar in the 17*CRE* virus and its rescuant, indicating that the modification of the CRE site in the recombinant 17*CRE* does not cause a detectable difference in LAT expression during latency. Also, the amount of LAT expressed by this mutant in the rabbit trigeminal ganglia during latency was similar to that previously quantitated in the mouse (Ackland-Berglund *et al.,* 1995).

Although the total amounts of LAT and total amounts of viral DNA detected in the ganglia were the same for the mutant and the rescuant, we considered that there

TABLE 1

Influence of the LAT Promoter CRE Site on HSV Reactivation

Virus	Positive rabbits/ total rabbits	P^a	Positive eyes/ total eyes	P^a	Positive swabs/ total swabs	P^a
Spontaneous reactivation[b]						
17*syn*+	13/18 (72%)	—	19/33 (57%)	—	28/627 (4.4%)	—
17ΔPst	3/19 (16%)	0.0356	3/37 (8%)	0.0012	4/703 (0.7%)	0.0001
17*CRE*	4/21 (19%)	0.0448	5/42 (12%)	0.0042	8/798 (1.0%)	0.0001
17*CREr* (rescuant)	3/6 (50%)	0.7171	4/12 (33%)	0.5484	12/228 (5.2%)	0.7149
Epinephrine-induced reactivation[c]						
17*syn*+	11/14 (78%)	—	19/25 (76%)	—	45/175 (26%)	—
17ΔPst	3/16 (19%)	0.0580	3/32 (9%)	0.0008	6/224 (2.7%)	0.0001
17*CRE*	7/12 (58%)	0.780	9/24 (37%)	0.230	13/168 (7.7%)	0.0002
17*CREr* (rescuant)	4/5 (80%)	1.000	7/10 (70%)	1.000	22/70 (31%)	0.5459

[a] *P* values determined by two-tailed chi-square analysis.
[b] Eyes swabbed from days 20 through 38 postinoculation.
[c] Iontophoresis once a day for 3 consecutive days. Eyes swabs were performed for 7 consecutive days.

might be a difference in the total number of neurons expressing LAT during latent infections with the two viruses. *In situ* hybridization, using a LAT probe, of every fourth section of completely sectioned left trigeminal ganglia from the same rabbits analyzed for DNA and RNA by PCR showed no statistically significant differences in the number of LAT-positive neurons per section regardless of the virus (average LAT+ sites/section were 12.2 ± 3.6 for 17*CRE* and 7.4 ± 2.8 for 17*syn*+).

These results demonstrate that mutation of the seven-base pair CRE located at −43 relative to the transcriptional start site of the HSV LAT promoter reduces the frequency of both spontaneous and induced reactivation in the rabbit eye model. These results extend previous results conducted in *in vitro* cocultivation systems of mice (Rader *et al.,* 1993) to our *in vivo* induction model in rabbits. Of importance in the current study is the fact that the mutant, 17*CRE* seems to show a more dramatic reduction in reactivation potential *in vivo* in the rabbit (37% of total eyes, compared to 70% for the rescue) than *in vitro* in the mouse cocultivation system [73% of ganglia, compared to 93% for the rescue (Rader *et al.,* 1993)]. In

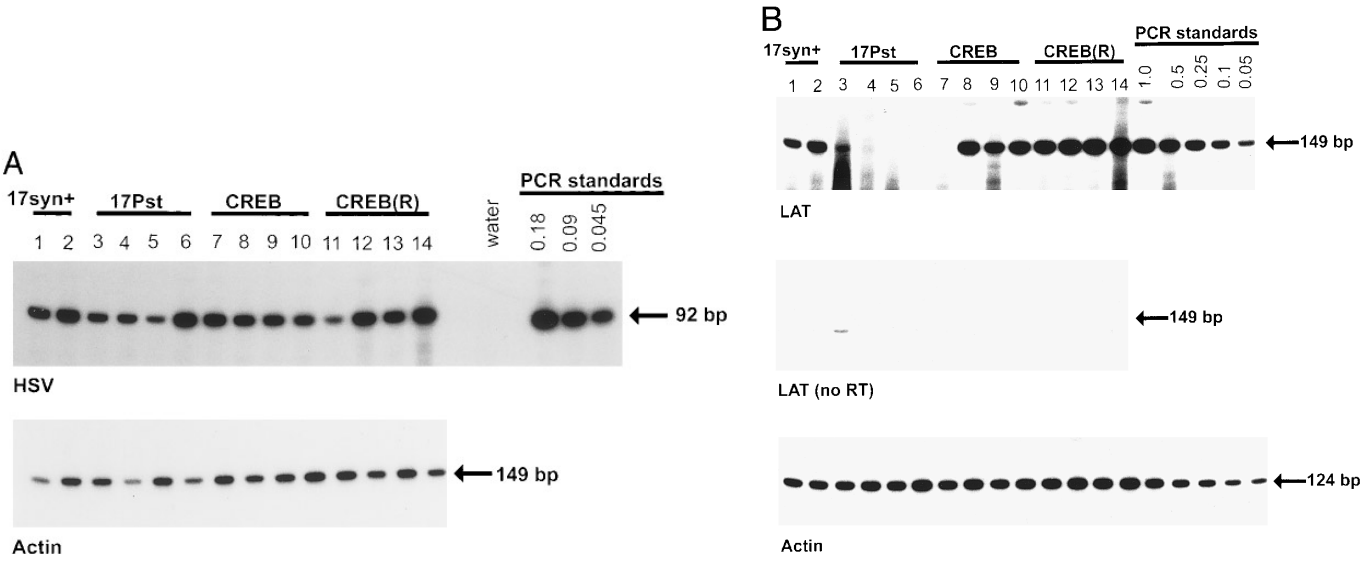

FIG. 2. PCR analysis of HSV DNA and LAT RNA in latently infected rabbit trigeminal ganglia. (A) Quantitation of viral DNA in latent rabbit trigeminal ganglia by PCR. The tissue was extracted using Trizol reagent (Life Technologies, Gaithersburg, MD) and separated into RNA and DNA fractions. The extracted DNA was subjected to PCR using a primer pair specific for the HSV polymerase gene that produces a 92-bp product. A primer pair specific for rabbit actin (124 bp product) was used as an internal standard. (B) Quantitation of LAT RNA in latently infected rabbit trigeminal ganglia. The extracted RNA fraction was reverse transcribed using random hexamer primers. The cDNA from the reverse transcription reactions (as well as a duplicate mock reverse transcription set) was subjected to PCR using a primer specific for the 5′ region of LAT (149-bp product) and rabbit actin. The PCR conditions and primers used are as previously described under Materials and Methods.

TABLE 2

Quantitation of HSV DNA Present in the Trigeminal Ganglia of Latently Infected Rabbits

Virus	Rabbit	HSV DNA[a]	Rabbit actin[a]	Ratio[b]	Average
17*syn*+	1	17.9	8.08	2.20	1.69 ± 0.51
	2	22.5	19.1	1.18	
17ΔPst	1	14.2	16.9	0.84	1.51 ± 1.04
	2	13.0	7.5	1.73	
	3	9.0	19.0	0.47	
	4	29.6	9.95	2.99	
17*CRE*	1	23.0	21.6	1.06	0.96 ± 0.17
	2	17.6	16.4	1.07	
	3	20.4	18.7	1.09	
	4	16.7	26.9	0.62	
17*CRE*r	1	9.8	23.8	0.41	1.13 ± 0.52
	2	23.2	17.3	1.34	
	3	18.5	23.0	0.80	
	4	27.8	14.2	1.96	

[a] HSV DNA and rabbit actin data corresponds with quantitation of the PCR fragments resulting from amplification with the samples with either a primer pair specific for HSV DNA polymerase primer pair or for rabbit actin (as described under Materials and Methods). The values presented are relative integrated intensities of the PCR analysis described in the legend to Fig. 2A.

[b] Ratio of relative intensities of HSV DNA/rabbit actin.

addition, this mutation could have an even greater effect on the rate of spontaneous reactivation than it does on the rate of induced reactivation. One explanation could be that the epinephrine induction causes a number of global responses that result in an unusually strong reactivation stimulus. Although the CRE located at −43 within the LAT promoter plays a role in reactivation, other components within the LAT promoter that are likely required for the full reactivation phenotype. Of interest is the existence of a second CRE recently identified within the LAT promoter (Kenny *et al.,* 1994). One possibility is that the second CRE is required separately or in concert with the −43 CRE in order to have full reactivation potential. Another possibility proposed by Lieb and coworkers is that structural elements within the LAT promoter contributes to reactivation (Ackland-Berglund *et al.,* 1995). In further support of this idea is the presence of a large number of CpGs within the LAT promoter region, which could be acting to change the structure of the DNA either singularly or as a result of methylation of the cytosines (Bloom *et al.,* 1996). This could increase or minimize the requirements for transcription factor binding within the region as previously described for other promoter systems (Parvin and Sharp, 1993). While these hypotheses address how LAT could be activated to induce reactivation, a question that remains to be answered concerns why mutation of the LAT CRE should affect reactivation if there is no obvious effect on the basal level of LAT transcription. Since it has been recently shown that the majority of latent neurons do not express LAT (Gressens and Martin, 1994; Mehta *et al.,* 1995), it is possible that induction of LAT within these (LAT-) neurons results in reactivation within these neurons. Therefore, future work may need to focus on levels of LAT expression at the level of individual neurons (perhaps neurons of a specific phenotype) rather than LAT expression at the gross tissue level.

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