Retrovirus silencer blocking by the cHS4 insulator is CTCF independent

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ABSTRACT

Silencing of retrovirus vectors poses a significant obstacle to genetic manipulation of stem cells and their use in gene therapy. We describe a mammalian silencer blocking assay using insulator elements positioned between retrovirus silencer elements and an LCRβ-globin reporter transgene. In transgenic mice, we show that retrovirus silencers are blocked by the cHS4 insulator. Silencer blocking is independent of the CTCF binding site and is most effective when flanking the internal reporter transgene. These data distinguish silencer blocking activity by cHS4 from its enhancer blocking activity. Retrovirus vectors can be created at high titer with one but not two internal dimer cHS4 cores. cHS4 in the LTRs has no effect on expression in transduced F9 cells, suggesting that position effect blocking is not sufficient to escape silencing. The Drosophila insulators gypsy and Scs fail to block silencing in transgenic mice, but gypsy stimulates vector expression 2-fold when located in the LTRs of an infectious retrovirus. The silencer blocking assay complements existing insulator assays in mammalian cells, provides new insight into mechanisms of insulation and is a valuable tool to identify additional silencer blocking insulators that cooperate with cHS4 to improve stem cell retrovirus vector design.

INTRODUCTION

The cHS4 insulator is located at the 5’ boundary between highly compacted chromatin and the transcriptionally active β-globin locus in chicken erythroid cells (1). Like other insulators, cHS4 blocks enhancer function on promoters and protects transgenes from position effects (2,3). The enhancer blocking activity of cHS4 has been attributed to CTCF binding at the FII binding site of the 250 bp insulator core (Fig. 1A) (3). CTCF also mediates enhancer blocking activity in the H19/ Igf2 locus (4). In contrast, transacting factors involved in cHS4 position effecting blocking activity are unknown (5) but appear to prevent the spread of histone deacetylation rather than the spread of DNA methylation (6,7). Otte and colleagues developed an assay to test silencer blocking activities of insulators against LexA silencing factor fusion proteins recruited to episomal reporter genes in human cells (8). Multimerized cHS4 cores and the Drosophila Scs and gypsy elements block all or most silencing in this assay (8). Silencer blocking by the gypsy insulator has also been tested in transgenic Drosophila (9,10). However, there is currently no silencer blocking insulator assay that uses a natural mammalian silencer in a genomic chromatin environment.

The Moloney murine leukemia virus (MoMLV) long terminal repeat (LTR) represents a powerful silencer in embryonic mammalian cells (11,12). Multiple mechanisms are involved in retrovirus silencing (13). Retrovirus vectors in transduced murine erythroleukemia (MEL) cells express a reporter gene prior to a two-step extinction process that correlates with an increase in methylation density on the provirus (14) and MeCP2 recruitment coupled with histone H3 deacetylation (15). However, in de novo methylase null embryonic stem (ES) cells silencing is rapidly established by 3 days post-infection (16). Chromatin modifications are involved in this silencing process as silent retrovirus transgenes in mice are marked by a repressive histone code composed of deacetylated H3, acetylated H4 and bound histone H1 (16). Histone acetylation levels are also altered during silencing of adenov-associated virus vectors (17).
In order to assay mammalian silencer elements, it is important to have a robust reporter gene. The human β-globin locus control region (LCR) directs high level β-globin expression in transgenic mice (18). We have shown that the BGT14 LCRβ-globin transgene composed of 5′ HS2, HS3 and HS4 is expressed at all integration sites (19). When this or other LCRβ-globin transgenes are linked to MoMLV or murine stem cell virus (MSCV) sequences, β-globin expression is silenced in transgenic mice (16,20). Mutation of all four known silencer elements in the self-inactivating (SIN) HSC1 retrovirus vector permits LCRβ-globin transgene expression at 6/10 integration sites, indicating that some unknown silencers remain (21). These data demonstrate that retrovirus silencing is dominant to LCR activity in mice and can be ameliorated but not eliminated by SIN vectors.

The monomer 1.2 kb cHS4 insulator in retrovirus vector LTRs limits position effects in transduced MEL cells (22,23) but not in ES cells (23). To develop a retrovirus vector that expresses to high levels in embryonic cells, we reasoned it was important to identify insulator elements that can block retrovirus silencers and to protect the reporter gene by flanking it internally with insulators (11,24). To this end, we devised a mammalian silencer blocking assay in transgenic mice and tested insulated retrovirus constructs.

**MATERIALS AND METHODS**

**Transgene construction**

The transgene constructs are derived from BGT32 (16). BGT60 was generated by inserting the 2.4 kb EcoRI–BamHI cHS4 insulator dimer from JC13-1 (2), using NotI linkers, into the NotI site between the MSCV 5′-LTR and the LCRβ-globin transgene. BGT108 was made by placing the 500 bp KpnI cHS4 insulator core dimer fragment from pNi-CD (3) into the NdeI site between the MSCV 5′-LTR and the LCRβ-globin transgene and into the NotI site between the LCRβ-globin transgene and the 3′-LTR. BGT91 was produced by inserting a 360 bp EcoRI–BamHI fragment from pII/III-InsQ, which contains four copies of footprints II and III of cHS4 (25), into both the NdeI site near the 5′-LTR and the NotI site near the 3′-LTR of the LCRβ-globin transgene in BGT58, which contains the HSC1 3′-LTR (21). BGT139 was made by inserting the SpeI fragment containing the dimer ΔII cHS4 core from pGI(Δ2D)2S/P (from G. Felsenfeld) by blunt end ligation sequentially into the two NotI sites in BGT32. BGT140 was made by replacing the BGT108 3.2 kb NdeI fragment with a 2.7 kb BGT32 NdeI fragment to remove the dimer cHS4 cores located between the MSCV 5′-LTR and β-globin LCR. BGT141 and BGT142 were generated by inserting the 480 bp gypsy insulator as a NotI fragment from EK642 (from P. Geyer) and the 1.0 kb NotI Scs insulator fragment from EK854 (from P. Geyer) into the BGT140 NotI site between the MSCV 5′-LTR and LCRβ-globin reporter, respectively. BGT143 was made by inserting the SpeI dimer ΔII cHS4 core fragment into the blunt NotI site of BGT140.

**Transgenic mice**

Transgenic mice were produced in FVB fertilized eggs as previously described (19). Day 15.5 post-injection fetal mice were dissected and DNA extracted from head tissue while the fetal liver was saved frozen in two halves for future analyses. Transgenic fetuses were identified by slot blot hybridization of fetal head DNA with the 0.9 kb EcoRI±BamHI β-globin intron 2 (βivs2) probe using standard techniques.

**DNA analysis**

Transgene copy number was determined by digesting transgenic fetal head DNA with either EcoRI or BamHI, which both cut once within the transgene. Southern blots were probed with the βivs2 fragment by standard procedures and copy number determination was performed using a Molecular Dynamics PhosphorImager. Transgene mosaicism was determined by digesting fetal liver DNA with Accl or SstI, which both cut twice within the human β-globin gene, and probing with the βivs2 fragment to compare transgene band intensities to the single copy B26 transgenic bred line (19). Loading of each lane was controlled by co-hybridization with a probe for the endogenous mouse Thy-1 gene. Percent transgenic cells in...
each founder was determined using a Molecular Dynamics PhosphorImager and the formula:

\[(Tg \beta/\beta g \text{mThy}-1)/(B26 \beta/\beta \times \text{copy number/B26 mThy-1})\]

where Tg indicates transgenic, H\(\beta\) indicates human \(\beta\)-globin, m-Thy-1 is mouse Thy-1 and B26 indicates single copy bred line B26.

**RNA analysis**

Fetal liver RNA was extracted and S1 nuclease analysis performed as described (19). Percent expression levels were calculated according to the formula (Hu\(\beta\)/Mo\(\beta\)) \times 100% and normalized by setting the \(\mu\)DL4 RNA at 50% to account for probe-specific activity differences between experiments. Percent expression per transgene copy was calculated as (2 Mo\(\beta\) genes/transgene copy number) \times (% expression/transgenic) \times 100.

**Retrovirus vector construction**

The MSCV-based vector construct A was generated by linking the MSCV 5'-LTR/gag sequences as a 2.1 kb NdeI–XhoI fragment to a 1.3 kb XhoI–ClaI PKG-EGFP reporter gene and a 3.6 kb ClaI–NdeI fragment from BGT140 containing the dimer chHS4 core insulator and the MSCV 3'-LTR. Construct B was made by blunt end ligation of the 500 bp EcoRI–KpnI chHS4 insulator dimer core fragment from pNI-CD into the HpaI site between the MSCV 5'-LTR and PKG-EGFP of construct A. Constructs C and D were made by blunt end ligation of the SpeI fragment containing the dimer \(\Delta\)II chHS4 core from pGI(\(\Delta\)II)\(\Delta\)S/P into the HpaI site in construct A. The HSC1-based vector construct E was made by PCR amplification of the gypsy insulator from EK642 with primers LP5 (5'-CGAGCTGAGATCGGATTCGAC-3') and LP3 (5'-CGAGCTGAGATCGGATTCGAC-3') that add multiple restriction sites to both ends. The PCR product was digested with SacI and inserted into the SacI site in the HSC1 3'-LTR. Construct F was made by subcloning the chHS4 insulator dimer core fragment into pE7K10 (from P. Geyer) and PCR amplification using primers LP5 and LP3 prior to introduction into the SacI site in the HSC1 3'-LTR. Construct G was made by PCR amplification using primers LP5 and LP3 of the 1.0 kb Sce fragment from pEK854 and insertion into the SacI site in the HSC1 3'-LTR. The DNA sequence of all PCR products was confirmed.

**Virus production, infection and flow cytometry**

Ecootropic Phoenix packaging cells were transfected with 20 \(\mu\)g virus vector construct DNA. Virus supernatant was infected into F9 and NIH 3T3 cells in triplicate with polybrene at 8 \(\mu\)g/ml. GFP expression was analyzed 3 days after infection by flow cytometry on a FacScan (Becton Dickinson) after propidium iodide staining to exclude dead cells.

**RESULTS**

**Retrovirus silencer blocking assay**

The MSCV retrovirus vector completely silences human LCR\(\beta\)-globin reporter genes resulting in expression in only 1/8 transgenic mice (16). To investigate whether the chHS4 insulator blocks retrovirus silencing, we placed a dimerized copy of the 1.2 kb chHS4 fragment (Fig. 1A) between the MSCV 5'-LTR/gag sequences and a human LCR\(\beta\)-globin reporter gene to create the BGT60 construct (Fig. 1B). This LCR\(\beta\)-globin reporter (BGT14) contains the human \(\beta\)-globin gene regulated by an 815 bp \(\beta\)-globin promoter and 5'-HS2, HS3 and HS4 of the \(\beta\)-globin LCR (19). Alone, the BGT14 LCR\(\beta\)-globin reporter directs RNA expression in 14/14 transgenic mice, ranging from 16 to 71% per copy of the endogenous mouse \(\beta\)-globin gene (19). BGT60 founder transgenic mice were generated as previously described (19). DNA from embryonic day 15.5 founder mice was analyzed by Southern blot for transgene copy number and intactness and fetal liver DNA was analyzed for transgene mosaicism.

Expression from BGT60 transgenes was analyzed by S1 nuclease protection assays on day 15.5 fetal liver RNA (Fig. 2A). The \(\mu\)D14 transgenic mouse line was used as a positive control that expresses to 50% levels the two mouse \(\beta\)-major genes or to 100% a single mouse \(\beta\)-major gene (19). BGT60 transgenic animals expressed human \(\beta\)-globin RNA in 8/8 animals, demonstrating silencer blocking activity by the dimer chHS4 insulator. However, only the three copy BGT60 animal expresses at a level of 48% per transgene copy which is within the normal range of 16–71%. The remaining seven mice express within a very low range (0.3–6% per copy). These data show that the dimer chHS4 insulator blocks retrovirus silencing but does not restore normal per copy transgene expression levels.

**Dimer chHS4 core blocks retrovirus silencing**

To ascertain if the core insulator possesses silencer blocking activity, we surrounded the LCR\(\beta\)-globin transgene with dimer chHS4 cores and linked it to MSCV sequences to create BGT108 (Fig. 1B). This configuration tests the ability to block upstream retrovirus silencers in the construct and block downstream retrovirus silencers present in adjacent 3' transgene copies in multicopy concatameric arrays. The presence of two insulators internally placed with respect to the retrovirus sequences makes it a ‘double internal’ insulator configuration. S1 nuclease protection analysis of BGT108 expression levels are significantly increased in comparison to BGT60 transgenic animals (Fig. 2B). In contrast to BGT60 transgenic animals, BGT108 animals demonstrate transgene expression ranging between 7 and 64% per copy, consistent with normal per copy expression levels (16–71% per copy) of the LCR\(\beta\)-globin reporter alone (19). The BGT108 expression levels are significantly increased in comparison to BGT60 transgenic animals (\(P < 0.01\) by the Wilcoxin rank sum test). This indicates that the dimer chHS4 core blocks retrovirus silencing and that a double internal configuration restores normal per copy transgene expression levels.

**Silencer blocking by chHS4 core is CTCF independent**

The enhancer blocking activity of chHS4 resides in a 90 bp fragment encompassing DNA footprints II and III of the hypersensitive site (Fig. 1A), and ultimately was refined to the 42 bp footprint II fragment that binds CTCF (3). To test whether the CTCF binding site co-localizes with silencer
blocking activity, we placed four copies of the 90 bp fragment as a ‘quad’ cHS4 (3) on both sides of the LCRb-globin reporter. This reporter was linked to 5'-LTR/gag sequences of HSC1 (21), a SIN derivative of MSCV, to generate BGT91 (Fig. 1B). HSC1 sequences linked directly to the LCRb-globin reporter permit expression in 6/10 transgenic animals (21). Silencer blocking activity by the quad insulator should increase the number of expressing transgenic animals. In contrast, a reduction in this number would indicate that the quad insulator accentuates silencing. S1 nuclease protection analysis of BGT91 fetal liver RNA revealed LCRb-globin reporter expression in 0/15 animals (Fig. 2C). As the number of expressing transgenic animals is 0, this indicates that the quad cHS4 containing four CTCF binding sites not only fails to block silencing, but actually accentuates the phenomenon and functions as a silencer itself.

To confirm that the CTCF site is not required for silencer blocking, we surrounded the LCRb-globin reporter with double internal dimer DII cHS4 cores (Fig. 1A) (5) and linked it to MSCV sequences to create the BGT139 construct (Fig. 1B). S1 nuclease protection analysis (Fig. 2D) demonstrated transgene expression in 5/5 animals ranging from 5 to 67% per copy, equivalent to the 7–64% per copy levels obtained with the wild-type dimer cores in BGT108. Together these data demonstrate that silencer blocking by the dimer cHS4 core is independent of CTCF and therefore is distinct from enhancer blocking activity.

**Dimer cHS4 core transmits through a retrovirus vector**

Given that the dimer cHS4 core is active in the transgenic mouse silencer blocking assay, we attempted to make infectious MSCV retrovirus vector stocks. To determine whether the dimer cHS4 core can be transmitted through a retrovirus vector we inserted it internally between the PGK-EGFP reporter gene and the 3'-LTR in construct A (Fig. 3). In addition, we surrounded the reporter gene with double internal dimer cHS4 cores in construct B to test whether silencing can be blocked by this insulator configuration. Ecotropic virus stocks were generated and infected into F9 EC cells, a common indicator cell line in which retrovirus silencing is well documented (21,26), and into NIH 3T3 cells, which do not exhibit silencing. Flow cytometry of control MSCV-transduced cells demonstrates that EGFP mean fluorescence is ~200 in F9 cells, in comparison to 1140 in NIH 3T3 cells (Fig. 3). Not only is the mean fluorescence reduced in F9 cells, but the GFP+ percent also drops in F9 cells relative to NIH 3T3 cells (34 versus 50%), suggesting that some proviruses are completely silenced in F9 cells.

The titer of construct A was virtually unchanged (34% in F9 and 44% in NIH 3T3) from the control MSCV vector, demonstrating that a dimer cHS4 core can be transmitted efficiently by retrovirus vectors (Fig. 3). Mean fluorescence of construct A was slightly increased in F9 EC cells to 266 and reduced in NIH 3T3 cells to 739. To determine whether double internal dimer cHS4 cores can be transmitted through the retrovirus vector, we generated virus stocks with construct B. We were unable to detect significant numbers of GFP+ cells, demonstrating that this construct design is deleterious to retrovirus transmission. We were also unable to transmit double internal dimer cHS4 insulators through a series of neo containing vectors (data not shown). In addition, reducing the length of sequence homology by combining a dimer DII cHS4 core with the wild-type dimer cHS4 core failed to rescue high titer production in construct C (Fig. 3). The dimer DII cHS4
core in reverse orientation in construct D was also unable to rescue high titer production (Fig. 3), as was the use of a smaller dimer DII cHS4 core (Fig. 1A) in either orientation (data not shown). These results suggest that sequence homology interferes with virus titer and that two different silencer blocking insulators with no homology must be used.

**gypsy does not block silencing**

To overcome this inability to transmit homologous insulators at two locations in a vector, we examined the possibility that an internal dimer cHS4 core might cooperate with a different internal insulator. The gypsy insulator is normally located just downstream of the retrotransposon 5′-LTR and therefore gypsy and Scs were inserted between the 5′-LTR/gag sequences and the LCRβ-globin reporter to make BGT141 and BGT142, respectively (Fig. 4A). The BGT141 construct containing the 480 bp gypsy insulator was expressed in 7/7 animals from 0.2±3.4% per copy, indicating that gypsy does not block silencing in this assay. Expression from the construct BGT142 containing a 1.0 kb Scs insulator was detected in 7/7 animals, but was more variable (<0.1±27% per copy), with the majority of integration sites expressing less than 4% per copy. Analogous gypsy and Scs constructs tested in a series of MSCV and HSC1 retrovirus vectors by infection into F9 cells also failed to cooperate reproducibly with the dimer cHS4 core (data not shown).

Although a dimer DII cHS4 core and a wild-type dimer cHS4 core cannot be transmitted together through the retrovirus vector constructs C and D shown in Figure 3, we tested whether these two elements can cooperate in the BGT143

**Figure 3.** One but not two internal dimer cHS4 cores can be transmitted through a retrovirus vector. (Left) Maps of the MSCV-based retrovirus vectors containing a PGK-EGFP reporter and internal dimer cHS4 cores (two black triangles) or dimer DII cHS4 cores (two gray triangles). (Right) The titer expressed as GFP+ cell percent and EGFP mean fluorescence detected by flow cytometry in F9 and NIH 3T3 cells infected in triplicate, with standard error shown.

**Figure 4.** Lack of cooperation by gypsy and Scs with the downstream dimer cHS4 core to block 5′-LTR silencing by MSCV in the transgenic mouse retrovirus silencer blocking assay. (A) Transgenic mouse constructs to test cooperative silencer blocking activity. Dimer cHS4 core (two black triangles), gypsy (diamond), Scs (heavy black cross) and dimer DII cHS4 cores (two gray triangles). (B) S1 nuclease analysis showing that the downstream dimer cHS4 core permits very low expression at all BGT140 integration sites. An internal gypsy or Scs fails to cooperate with the downstream dimer cHS4 core in BGT141 and BGT142, respectively. (C) S1 nuclease analysis showing strong cooperation between dimer DII cHS4 cores and wild-type cHS4 cores in BGT143 mice.
construct (Fig. 4A). Expression was detectable at 23–103% per copy level in 6/6 transgenic mice (Fig. 4C), showing that two non-identical cHS4 elements can cooperate to block retrovirus silencing.

**gypsy stimulates vector expression levels**

As two flanking gypsy elements can have greater insulator activity than one or two internal gypsy elements in Drosophila (27), we inserted gypsy into the 3′-LTR of HSC1 to create the retrovirus vector construct E. After retrovirus transduction, gypsy is positioned in both LTRs and hence flanks the silencer–reporter gene combination (Fig. 5). A 2-fold dilution of control HSC1 virus resulted in 20–35% GFP+ cells and mean fluorescence of 415 in F9 cells and 705 in NIH 3T3 cells. Undiluted virus stocks resulted in 16–28% GFP+ cells, suggesting a 2-fold reduction in titer. Construct E expressed mean fluorescent intensities of 907 in F9 cells and 1340 in NIH 3T3 cells, demonstrating that gypsy stimulates vector expression in both cell types. Notably, infectious retrovirus stock produced by construct E directed the highest level expression we have observed from the PGK promoter in transduced cells of embryonic origin.

To test whether the dimer cHS4 core and Scs could also function in the LTR, constructs F and G were generated and undiluted virus stocks transduced into the indicator cells (Fig. 5). F and G expression levels in NIH 3T3 and F9 cells were roughly equal to that directed by the control HSC1 vector alone, confirming that cHS4 located in a position effecting blocking position in the LTR fails to block retrovirus silencing in embryonic cells. These data demonstrate that gypsy and cHS4 have distinct activities in mammalian cells.

**DISCUSSION**

Silencing of retrovirus vectors is a concern for stem cell marking and genetic manipulation studies and may compromise expression of therapeutic genes during gene therapy applications. To overcome this obstacle, we designed a transgenic mouse silencer blocking assay to test the ability of insulator elements to block retrovirus silencing. Using this assay, we show that double internal dimer cHS4 core insulators block silencing by the MSCV retrovirus vector and that silencer blocking activity is distinct from enhancer blocking mediated by the CTCF site (3). The silencer blocking assay relies on the establishment of an epigenetic mark on retrovirus sequences and its spread to silence the linked LCRβ-globin transgene. Retrovirus-silenced LCRβ-globin transgenes in mice are marked by a repressive histone code (16). It has recently been proposed that cHS4 prevents condensed chromatin spread at the endogenous chicken β-globin locus (28). This model for cHS4 action predicts what we have observed, that cHS4 elements surrounding the reporter gene fully protect it from silencers. We show that four CTCF binding sites in the quad cHS4 are not capable of silencer blocking and in fact accentuate silencing, whereas the dimer ΔII cHS4 core has silencer blocking activity. Therefore, silencer blocking is CTCF independent. CTCF binding sites are also not required for cHS4 position effect blocking activity (5).

A monomer 1.2 kb cHS4 insulator in retrovirus LTRs reduces position effects from vectors transduced into MEL cells (22,23) but not into ES cells (23). As cHS4 insulator function is greatly enhanced when it is dimerized (29), silencer blocking activity could be more pronounced from dimer cHS4 core constructs, and the use of cores would also avoid the distance effects reported using the monomer 1.2 kb cHS4 element in bone marrow (30). Our results show that MSCV silencing can be only mildly attenuated in F9 cells by one dimer cHS4 core located internally, in agreement with the reported lack of insulation by a monomer 1.2 kb cHS4 located downstream of the 5′-LTR in a retrovirus vector (31). Dimer cHS4 cores located in the LTRs also fail to block silencing in F9 cells, indicating that silencing is established on the retrovirus sequences and is not solely a position effect of surrounding chromatin. These results suggest that dimer cHS4 cores must be placed directly between the virus silencer sequences and the reporter gene to escape silencing in stem cells.

As double internal insulator retrovirus vectors containing two dimer cHS4 cores could not be transmitted, we investigated the possibility that a downstream dimer cHS4 core might cooperate with a different insulator located upstream of the reporter gene. In the transgenic silencer blocking assay, a downstream dimer cHS4 core marginally increased expression. In this configuration, the downstream dimer cHS4 core would only block silencing that spread from 3′ adjacent transgene copies in a concatamer. Thus the level of LCRβ-globin reporter expression in constructs containing gypsy or Scs would depend entirely on their silencer blocking effects on the 5′-LTR. Neither gypsy nor Scs demonstrated silencer blocking activity in this assay.

The gypsy insulator located in the LTRs of the HSC1 retrovirus vector stimulated expression 2-fold to a mean fluorescence of 900 in F9 cells. In mammalian cells, gypsy is bound by the nuclear matrix in vitro and interacts with matrix proteins (32). Therefore, gypsy may increase the transcriptional potential of expressing proviruses by relocating them to transcriptionally more active locations in the nucleus or on the matrix (33). In fact, retrovirus vectors containing the interferon β MAR also increase expression in stem cells (34). Regardless of its mechanism, gypsy directed the highest GFP expression we have observed from the PGK promoter in retrovirus vector-transduced F9 cells.

To completely escape retrovirus silencing, it will be necessary to use two insulators with no sequence homology
that can be transmitted together at high titer in the double internal configuration. The ability of other insulator elements to cooperate with cHS4 can be tested with the transgenic mouse silencer blocking assay described here. Although cHS4 located in the LTRs failed to block retrovirus silencing, this vector design may improve retrovirus vector safety in stem cell gene therapy protocols by blocking insertional activation of surrounding genes.

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