Targeted transgenesis at the HPRT locus: an efficient strategy to achieve tightly controlled in vivo conditional expression with the tet system

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Palais G, Nguyen Dinh Cat A, Friedman H, Panek-Hluet N, Millet A, Tronche F, Gellen B, Mercadier J, Peterson A, Jaisser F. Targeted transgenesis at the HPRT locus: an efficient strategy to achieve tightly controlled in vivo conditional expression with the tet system. Physiol Genomics 37: 140–146, 2009. First published January 13, 2009; doi:10.1152/physiolgenomics.90328.2008.—The tet-inducible system has been widely used to achieve conditional gene expression in genetically modified mice. To alleviate the frequent difficulties associated with recovery of relevant transgenic founders, we tested whether a controlled strategy of transgenesis would support reliable cell-specific, doxycycline (Dox)-controlled transgene expression in vivo. Taking advantage of the potent hypoxanthine-aminopterin-thymidine selection strategy and an embryonic stem (ES) cell line supporting efficient germ-line transmission, we used hypoxanthine phosphoribosyltransferase (HPRT) targeting to insert a single copy tet-inducible construct designed to allow both glucocorticoid receptor (GR) and β-galactosidase (β-Gal) expression. Conditional, Dox-dependent GR and β-Gal expression was evidenced in targeted ES cells. Breeding ES-derived single copy transgenic mice with mice bearing appropriate tet transactivators resulted in β-Gal expression both qualitatively and quantitatively similar to that observed in mice with random integration of the same construct. Interestingly, GR expression in mice was dependent on transgene orientation in the HPRT locus while embryonic stem cell expression was not. Thus, a conditional construct inserted in single copy and in predetermined orientation at the HPRT locus demonstrated a Dox-dependent gene expression phenotype in adult mice suggesting that controlled insertion of tet-inducible constructs at the HPRT locus can provide an efficient alternative strategy to reproducibly generate animal models with tetracycline-induced transgene expression.

hypoaxanthine phosphoribosyltransferase; tetracycline; mouse

TETRACYCLINE-DEPENDENT REGULATORY systems (tet systems), developed in the laboratory of Herman Bujard, allow stringent control over gene expression in a wide range of cultured cells as well as in transgenic animals (4, 32). Hallmarks of the system include tight control, the ability to regulate gene activity in a tissue-specific manner, a doxycycline dose-dependent response, as well as the possibility of reverting to the control situation, when desired, by stopping transgene expression. However, due to the sensitivity of the tetO minimal promoter to positional effects, expression of the operator construct is sometimes leaky, requiring that several operator lines are generated prior to recovering one that expresses the transgene in a conditional manner.

Randomly integrated constructs are often subjected to position effects that impair tissue specificity and/or expression level, sometimes leading to complete silencing of expression (6). Moreover, potential disruption of an endogenous gene at the insertion site requires that multiple lines derived from independent founders be analyzed. To avoid such position effects, targeted transgenesis into a permissive locus, neutral for the activity of exogenous enhancers, would be an efficient alternative (20). Reproducibility of both expression levels and cell-type specificity would also allow meaningful comparisons to be made amongst a series of constructs. Also, docking constructs in such permissive loci by gene targeting in embryonic stem (ES) cells would allow precise control of integration site, copy number, and construct orientation before the time-consuming and costly generation of transgenic lines. Rosa26 and hypoxanthine phosphoribosyltransferase (HPRT) are among the permissive loci that have been identified so far (20, 43). HPRT has the distinct advantage of also supporting efficient selection for the anticipated homologous recombination event using hypoxanthine-aminopterin-thymidine (HAT) media, wherein cell survival is dependent upon HPRT activity. When ES cells bearing a partially deleted and null HPRT locus are transfected with a targeting construct containing the deleted HPRT sequences and the experimental transgene, homologous recombination at the HPRT locus simultaneously restores functional HPRT expression and inserts a single construct copy in known orientation at a predetermined site 5′ of the HPRT locus (5). HPRT expression renders the cell resistant to the toxic effects of HAT media (9). This strategy avoids the necessity of introducing one or more selectable markers in the targeting construct that often have deleterious effects on subsequent levels and patterns of transgene expression (5). Moreover, the HPRT locus has proven to be exquisitely permissive for several promoter/enhancer combinations that drive transgene expression, including inducible Cre recombinase, in a wide variety of cell types including heart (17), muscle (38), kidney (7, 23), prostate (24), myelinating glia (8, 12) endothelial cells (11, 16, 30), and smooth muscle (35).

In the present study, we inserted a tet-inducible construct at the mouse HPRT locus to determine if the HPRT environment is sufficiently neutral and permissive to support tightly regu-
lateral conditional expression of such inducible transgenes in adult mice. Targeted transgenesis of an inducible construct was achieved and the efficiency of the tet system was assessed both ex vivo, in targeted ES cells, and in vivo, in mice bred to also contain an appropriate transactivator. We show that insertion of such constructs in single copy at the HPRT locus provides an environment that is ideally suited for both robust and tightly regulated inducible transgene expression in vivo.

**METHODS**

**Generation of knock-in mice.** The AseI-SalI fragment of pb13 (1), including the teto and lacZ sequences, was inserted between the XhoI and EcoRV sites of a pBS vector (Stratagene, La Jolla, CA) lacking the original PstI site, resulting in pBS-tetoLacZ. The KpnI-DraII fragment of pcDNAhGR (kindly provided by M.-E. Rafestin-Oblin), including the full-length human glucocorticoid receptor (GR) coding sequence and the BGH polyadenylation signal, was blunt-ended and inserted into the PstI site of pBS-tetoLacZ, resulting in the pBS-teto-LacZ construct. The NotI-NotI insert was subcloned into the blunt EcoRI sites of the pENTR1A vector (Invitrogen, Carlsbad, CA). The GR-teto-LacZ insert was transferred by site-specific recombination using the Gateway cloning enzymes (Invitrogen) into the HPRT Gateway destination vector (13) upstream from the human HPRT promoter and exons I and II that are missing in the HPRT Gateway targeting vector with the Gateway system, as described elsewhere (31). The resulting targeting construct was electroporated into the BPES11 cell line that contains the HPRT deletion originally described by Hooper et al. (19). To generate the BPES11 cell line, mice were derived from BK4 cells (kindly provided by S. Bronson). Following a series of crosses, the mice were derived from BK4 cells (kindly provided by S. Bronson) and used to generate the LacZ-teto-GR mouse line.

**Stable transgenesis of an inducible construct was achieved by electroporation of the BPES11 cell line, which contains the HPRT deletion, onto a highly mixed C57BL/6 and 129 background from which the HPRT promoter and exons I and II that are missing in the HPRT locus.** The GR-teto-LacZ insert was transferred by site-specific recombination using the Gateway cloning enzymes (Invitrogen) into the HPRT Gateway destination vector (13) upstream from the human HPRT promoter and exons I and II that are missing in the HPRT Gateway targeting vector with the Gateway system, as described elsewhere (31). The resulting targeting construct was electroporated into the BPES11 cell line that contains the HPRT deletion originally described by Hooper et al. (19). To generate the BPES11 cell line, mice were derived from BK4 cells (kindly provided by S. Bronson). Following a series of crosses, the mice were derived from BK4 cells (kindly provided by S. Bronson) and used to generate the LacZ-teto-GR mouse line.

All mice were fed with standard chow (A03; Scientific Animal Food Engineering, Epinay sur Orge, France) and provided with tap water ad libitum. When required, drinking water was substituted with a solution containing 2 mg/ml doxycycline (Dox) (D-9891; Sigma). Mice were kept under an artificial light-dark cycle of 12 h and all experiments were performed during the light phase. Mice were housed in microisolator cages with standard commercial diet and water ad libitum. All animal care and experimental procedures were conducted in accordance with the guidelines established by the French government and were approved by the local ethics committee (MC-00013.01) of the University of Lyon 1.

**Innovative Methodology**

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(clone 8) were plated at a density of 3 × 10⁵ cells/well in six-well plates 24 h before transfection. For each well, 1 µg of the linearized construct and 4 µl of Reagent Plus (Invitrogen) were incubated at room temperature (RT) in 100 µl of OPTIMEM (Invitrogen) for 15 min. Then, 100 µl of a 3% Lipofectamine/OPTIMEM solution was added and incubated again for 15 min at RT. Thereafter, an additional 800 µl of OPTIMEM was added to each well after 4 h, and medium was replaced by fresh ES medium. Forty-eight hours after transfection, cells were transferred to ES medium supplemented with 300 µg/ml Geneticin G418 (Invitrogen). Two G418-resistant clones, hereafter called CMV-tetO2/GR-tetO-LacZ BPES, were recovered and amplified.

X-Gal staining assay. CMV-tetO2/GR-tetO-LacZ BPES cells were incubated with or without 4 µg/ml Dox for 24 h. Cells were then rinsed with PBS buffer, fixed for 5 min at 4°C with 2% paraformaldehyde in PBS buffer, rinsed again three times with PBS buffer, and incubated for 3 h at RT with 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma-Aldrich) in X-Gal buffer [2 mM MgCl₂, 5 mM K₂Fe(CN)₆, 3H₂O, 5 mM K₃Fe(CN)₆, 150 mM NaCl, in 1X PBS buffer].

β-Galactosidase activity assay. β-Galactosidase (β-Gal) activity was evaluated in CMV-tetO2/GR-tetO-LacZ BPES cells incubated in media with or without 4 µg/ml Dox for 48 h. Culture medium was discarded, and cells were lysed in 250 µl of lysis buffer for β-Gal assay (29). Absorption at λ₆ 420 nm was recorded for triplicate experiments and values expressed as means ± SE.

Western blot analysis. Cells and tissues were lysed in buffer containing 50 mM Tris·HCl, 1 mM EDTA, 1 mM EGTA, 200 mM NaCl, 1% SDS, 1 mM DTT, 2 mM PMSF. We separated 20 µg of protein by SDS-PAGE and transferred it to nitrocellulose membrane (Amersham, Biosciences Europe, Orsay, France). The blot was blocked overnight at 4°C with 5% nonfat dry milk in TBS-T (20 mmol/l Tris·HCl, 137 mmol/l NaCl, 0.1% Tween 20). The membrane was then incubated for 1 h at RT with polyclonal antibodies against β-Gal (Capell, ICN Biomedicals) 1/1,000, or actin (Sigma) 1/10,000. After washing the membrane (3 × 5 min in TBS-T), we incubated it with the secondary anti-rabbit antibody at a dilution of 1/5,000 for 1 h at RT. The secondary antibody was detected by a chemiluminescent technology, Heidelberg, Germany) 1/1,000, or actin (Sigma) 1/10,000. Absorption at λ₆ 420 nm was recorded for triplicate experiments and values expressed as means ± SE.

RESULTS

The strategy used to knock-in constructs into the HPRT locus is depicted in Fig. 1. When the targeting vector, bearing the construct of interest (Fig. 1A), undergoes homologous recombination at the HPRT locus, it restores expression of HPRT allowing selection of recombinant ES cells in HAT media (Fig. 1A). For the experiments reported here, homologous recombination at the partially deleted HPRT locus in BPES cells simultaneously restores function of the HPRT locus and inserts a single copy of the bidirectional GR-tetO-LacZ construct (Fig. 1B), shown previously to be functional in mice (31). In cells that also carry an appropriate transactivator transgene, this bifunctional construct should support inducible expression of both β-Gal and GR. Transgene expression was first analyzed in ES cells. Ten independently targeted BPES clones (hereafter called GR-tetO-LacZ BPES) were obtained and characterized. As expected, all contained the transgenic sequences and none exhibited β-Gal expression in the absence of the transactivator (not shown). To assess whether the bidirectional construct was functional as a single copy construct docked in the HPRT locus, one of the GR-tetO-LacZ BPES clones was transfected with a tetON2 expression cassette in which expression of the rtTA2-M2 transactivator (37) is controlled by the CMV promoter. A neomycin-resistant double-transgenic subclone (CMV-tetON2/GR-tetO-LacZ BPES) was recovered, and expression of both β-Gal and GR was examined following incubation with or without Dox. X-Gal staining (Fig. 2A), β-Gal activity (Fig. 2B), and accumulation of β-Gal protein (Fig. 2C) all demonstrated that the tet system was functional; i.e., β-Gal expression only occurred when cells were stimulated with Dox. GR protein expression was also inducible in the presence of Dox (Fig. 2C). Notably, no expression was observed in monotransgenic (MT) GR-tetO-LacZ BPES cells (not shown) or in CMV-tetON2/GR-tetO-LacZ BPES cells in the absence of Dox (Fig. 2, A–C), demonstrating that the tetO promoter is not active in the absence of the transactivator. Thus, despite the proximity of the GR-tetO-LacZ construct to the ubiquitously expressed HPRT housekeeping gene, the tetO promoter remains tightly controlled and highly inducible.
Transgenic mouse lines derived from the same recombinant GR-tetO-LacZ BPES clone examined above were evaluated to determine first, if detectable expression in vivo could be realized from a single copy construct and second, if HPRT enhancers adjacent to the insertion site altered conditional expression. This analysis was complemented with a comparison to a conditional mouse model obtained by classical transgenesis (Dox-dependent expression, tissue specificity, expression level).

Several HPRT knock-in male chimeras stably transmitted the transgene to the next generation. DT mice were obtained by breeding F1 GR-tetO-LacZ mice with the previously characterized MHC-tetOFF strain, CamKII-tetOFF strain, or the LAP-tetON2 strain. As expected, X-Gal staining of tissues from an MHC-tetOFF/GR-tetO-LacZ DT male mouse revealed high-level, homogeneous β-Gal expression in cardiomyocytes (Fig. 3A) but no expression in liver, kidney, muscle, or brain (Fig. 3B). Such cardiomyocyte-specific expression was tightly controlled since β-Gal expression was not observed in the heart of an MT GR-tetO-LacZ mouse (Fig. 3A, left and right panels). Furthermore, β-Gal expression could be prevented if Dox was administered to DT mice (Fig. 3C), demonstrating that the tet system is fully functional allowing detectable, inducible, and tissue-specific expression of a tet operator-controlled construct inserted at HPRT.

Since targeted transgenesis in the HPRT locus normally results in insertion of a single construct copy, we compared the level of β-Gal expression to that observed in DT mice obtained after mating the same cardiac-specific transactivator strain with a GR-tetO-LacZ line obtained by random integration (thereafter called GR-tetO-LacZ random) and previously used to assess the role of the glucocorticoid receptor on cardiac function (31). This line expressed β-Gal in a conditional manner when crossed with an appropriate transactivator strain, while expression was absent in the MT GR-tetO-LacZ random mice indicating functional but tightly controlled transgene expression. Western blot analyses indicated that β-Gal expression in MHC-tetOFF/GR-tetO-LacZ DT mice was roughly similar to that observed in DT mice obtained with a randomly integrated GR-tetO-LacZ construct (Fig. 3, D and E). To assess whether the ability to conditionally express the GR-tetO-LacZ HPRT transgene was restricted to cardiomyocytes or also efficient in other tissues, GR-tetO-LacZ mice were crossed with CamKII-tetOFF (Fig. 4A and B) or LAP-tetON2 (Fig. 4, C and D) mice, leading to the generation of CamKII-tetOFF/GR-tetO-LacZ and LAP-tetON2/GR-tetO-LacZ male DT mice, respectively. The CamKII-tetOFF mice express the rtTA transactivator in the forebrain (27), while the LAP-tetON2 mice express the rtTA2S-S2 variant in hepatocytes and renal proximal tubular cells (15). Conditional β-Gal expression was observed in the forebrain of CamKII-tetOFF/GR-tetO-LacZ DT mice (Fig. 4A and B) or in the hepatocytes and renal proximal tubules in the LAP-tetON2/GR-tetO-LacZ DT mice after 3 wk Dox induction (Fig. 3C and D). Thus, the GR-tetO-LacZ construct

![Fig. 3](image-url)
targeted to the HPRT locus can be activated in various cell types depending on the transactivator expression pattern.

As mentioned above, when the GR-tetO-LacZ construct was docked at HPRT, both β-Gal and GR were expressed at readily detectable levels in the presence of CMVtetON2 in the resulting ES clones (Fig. 2C). Surprisingly, while β-Gal expression was robust in the heart, liver, kidney, or brain of the various BPES-derived DT mice, no GR expression could be detected (Fig. 5A). As robust GR expression was observed when the operator construct was randomly integrated (Fig. 5A), the HPRT locus may support expression of some constructs in an orientation-dependent manner. We therefore generated the LacZ-tetO-GR mouse line in which the GR-tetO-LacZ construct was inserted in the reverse orientation. As predicted by an orientation-dependent model, conditional expression of both GR and β-Gal was observed in the heart of MHC-tetOFF/LacZ-tetO-GR male DT mice with GR accumulating to levels similar to male DT mice bearing a randomly inserted GR-tetO-LacZ construct (Fig. 5B).

**DISCUSSION**

Targeted insertion of constructs into a predetermined, neutral, and permissive environment in the genome provides a practical approach to generate transgenic mice with desirable expression phenotypes. The approach also supports the rapid generation of transgenic lines with reproducible features that allows direct qualitative and quantitative comparisons to be made between mutant constructs (20). In the present study we show that a controlled strategy of docking constructs at the HPRT locus supports tightly regulated in vivo inducible expression of tet-regulated constructs. Moreover, levels of expression are in a similar range to that realized from a multicopy randomly inserted construct. As with many X-linked loci, it has been shown that the HPRT gene is transcriptionally silent on the inactivated X chromosome in females. The targeted HPRT locus will be subject to random X-inactivation in heterozygous females. The expression of the transgene in all cells of the female requires the generation of females homozygous for the targeted transgene and heterozygous for the transactivator construct. An adequate breeding scheme should therefore be considered if females have to be analyzed.

![Fig. 4. Conditional expression in CamKII-tetOFF/GR-tetO-LacZ mice and LAP-tetON2/GR-tetO-LacZ mice. A: X-Gal staining revealed β-Gal expression in the forebrain, hippocampus, and olfactory bulb of CamKII-tetOFF/GR-tetO-LacZ male DT mice (DT) only, while no expression was present in wild-type (WT), transgenic GR-tetO-LacZ (GR-tetO-LacZ), or CamKII-tetOFF mice. B: as estimated by Western blot, β-Gal expression was observed in male DT mice with random or targeted integration, but not in MT GR-tetO-LacZ or GR-tetO-LacZ random mice. Actin was used as internal control for protein loading. C: X-Gal staining revealed homogeneous β-Gal expression in hepatocytes (Liver) and in the renal proximal tubular cells (kidney) of LAP-tetON2/GR-tetO-LacZ male DT mice upon Dox induction but not in GR-tetO-LacZ male/null transgenic mice (GR-tetO-LacZ), D: as estimated by Western blot, β-Gal expression was induced in male DT mice in liver and weakly in kidney upon Dox administration (DT +Dox), while expression was absent in male DT mice without Dox (DT −Dox) or in GR-tetO-LacZ male/null transgenic mice (GR-tetO-LacZ). Actin was used as internal control for protein loading.](http://physiolgenomics.physiology.org/)

![Fig. 5. Conditional GR expression in HPRT targeted BPES mice is orientation dependent. Western blot analysis indicated that GR was consistently expressed in the heart of MHC-tetOFF/LacZ-tetO-GR male DT mice (DT, random) but not in MHC-tetOFF/GR-tetO-LacZ male mice (DT, GR-tetO-LacZ), in contrast to β-Gal, which was expressed in both (A). This was not the case when the GR-tetO-LacZ was inserted in the reverse orientation leading to MHC-tetOFF/LacZ-tetO-GR male mice (DT, LacZ-tetO-GR) (B). Actin was used as internal control for protein loading.](http://physiolgenomics.physiology.org/)
Targeting of tet-dependent transgenes has been previously achieved in the HPRT locus allowing Dox-dependent expression of Xist (involved in X-chromosome inactivation) (40) HoxB4 (an homeotic selected gene) (22) and of shRNA-mir cassettes (39). In these examples, docking into the HPRT locus left a functional PGK-neo-polyA cassette that could interfere with gene expression in adult tissue. This type of interference has been shown for example when LoxP sites are inserted together with a neo selection cassette for conditional gene inactivation. In two of the cases, inducible transgene expression was only analyzed in ES cells or embryoid bodies but mice were not generated from the targeted ES cells (22, 40).

The present study is therefore, to our knowledge, the first to analyze the efficiency of HPRT-docked tet operator constructs in vivo in various organs from adult mice and in combination with several transactivator strains.

The bidirectional operator construct of the tet system was designed to allow expression of two independent transcriptional cassettes using a bidirectional tetO promoter. It has been shown that balanced coexpression of two different transgenes could be achieved both ex vivo and in vivo (1, 18, 21). Surprisingly, we observed a clear dissociation between ex vivo and in vivo expression of the bidirectional construct when targeted to the HPRT locus. While the two transcriptional cassettes are functional in targeted ES cells, this is not the case in vivo. This is not related to a poor intrinsic efficiency of transcription/translation of the GR transgene since the same construct is efficiently processed in the GR-tetO-LacZ line obtained by classical transgenesis. However, in vivo, different β-Gal and GR protein stabilities may contribute to the present observations and the β-Gal protein is known for its long half life. Expression of both β-Gal and GR from the LacZ-tetO-GR transgene, integrated at the same position in the HPRT locus but in reverse orientation as the non-GR expressing GR-tetO-LacZ transgene, indicated that orientation is important. Indeed, an HPRT inserted LacZ reporter gene regulated by the proximal, unidirectional promoter of the myelin basic protein gene also was shown to be sensitive to orientation (12, 13). Recent evidence for regulatory partnerships between reporter constructs and the HPRT environment was observed by Tuason et al. (36). Further insight into such apparent regulatory partnerships may be possible through application of the recently introduced Chromosome Conformation Capture Carbon Copy technique such that the physical associations that develop between HPRT and construct regulatory sequences can be revealed (10). Of note, orientation-dependent expression has been reported recently also in the ROSA26 locus (34).

Besides HPRT, other loci have been proposed to be permissive and useful for targeted transgenesis. Few attempts have been made to develop an inducible system using targeted transgenesis strategies. Site-specific integration into the ColA1 locus (encoding for the collagen 1 isoform) of a tetO-EGFP cassette has been reported using two-step Frt-mediated targeting in ES cells together with a Rosa26 targeted tetON2 cassette (2). Expression was shown to be inducible in mice derived by tetraploid embryo complementation in many tissues, with the exception of brain and testis. Expression was patchy in lung, heart, and kidney (2). One caveat of this approach is that the plasmid backbone and the selection cassette that remained embedded in the ColA1 locus after targeting affected neighboring genes as well as the inducible transcription cassette. The Rosa26 locus has been shown to be ubiquitously expressed in embryos and has an extensive expression pattern in adults (14, 26). Targeted transgenesis of shRNA cassettes with the H1 or U6 promoters has been reported, allowing efficient in vivo knock-down in mouse embryos (33, 41) or adult tissues (33). Rosa26 has also been chosen for targeted transgenesis of tet-dependent inducible cassettes (25, 28). In these reports, the tet transactivator was placed under the control of the endogenous Rosa26 promoter, while the tetO cassette was co-integrated within the locus after gene targeting. In the paper of Mao et al. (25), a floxed selection cassette was inserted between the tetO promoter and the coding sequence of the gene of interest. The floxed selection cassette prevented the expression of the gene of interest until expression of a ligand-activated Cre recombinase allowed the Dox-dependent expression in chimeric embryos. Analyses in adult animals that would require germline transmission and breeding with an appropriate Cre recombinase strain remain to be done. A similar example was reported with targeted co-integration into the Rosa26 of both tTA and tetO-pdxIRES-eGFP transcription cassettes (28). Strathdee et al. (34) also used Rosa26 to support controlled transgenesis, targeting one allele with the tetON2 transactivator and the other with a tetO-eGFP cassette. Efficient Dox-dependent expression of eGFP was achieved in recombiant ES cells, was orientation dependent, and has yet to be assessed in ES-derived mice.

In the preceding examples, many of the desired features of controlled insertion events were realized. However, the targeting procedures used in each case were complex involving co-integrated cassettes, embedded selection cassettes, plasmid backbones, and Cre-mediated deletion of a stop cassette. In the present study we show that constructs docked at HPRT are conferred with a similar range of desirable expression features. In contrast to the above, due to the potent HPRT selection scheme, targeting is highly efficient and does not require an exogenous selection cassette. Moreover, the high germline transmission efficiency of the BPES cell line allows rapid generation of near 100% ES-derived mice, providing rapid production of the conditional model.

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