Inducible and irreversible control of gene expression using a single transgene

Edya Fuhrmann-Benzakein, Irène García-Gabay[1], Michael S. Pepper, Jean-Dominique Vassalli and Pedro Luis Herrera*

Department of Morphology and [1]Department of Pathology, University of Geneva Medical School, 1 Rue Michel-Servet, CH-1211 Geneva 4, Switzerland

Received July 11, 2000; Revised and Accepted October 6, 2000

ABSTRACT

Experimental or therapeutic designs involving the conditional expression of genes often require the use of two different transgenes; this can represent a major undertaking. One of these systems takes advantage of inducible recombinases. Here we show a novel use of such enzymes, in that an inducible recombinase-encoding sequence can function to both block the transcription of a gene placed downstream and, subsequently, irreversibly activate transcription of this very same gene. This double function, which circumvents the need for two transgenes, can be achieved by flanking the inducible recombinase gene by two of its target sequences. In our design we used as the inducible recombinase gene the Cre-ER^T gene flanked by two loxP sites. This cassette was placed between a mouse phosphoglycerate kinase promoter and the enhanced green fluorescent protein (EGFP) coding sequence. Massive EGFP gene expression in BHK cells bearing this transgene was observed upon administration of 4-hydroxytamoxifen (4-OHT), the inducer of the recombinant activity of Cre-ER^T. In the absence of 4-OHT EGFP production was prevented. Because of its simplicity (only a single transgene needs to be used) this strategy is of obvious interest in certain protocols of gene or cell therapy and in a variety of experimental designs in which conditional expression of genes is required.

INTRODUCTION

The emergence of new molecular tools has allowed the design and generation of transgenic mice carrying subtle mutations whose expression can be targeted both spatially and temporally using cell type-specific or inducible promoters, (1,2). The Cre–loxP system was initially devised to inactivate genes in selected tissues, rather than in the whole organism (3,4). A subsequent development of this system allowed exquisite temporal control of the recombinant activity of Cre in selected cells *in vivo*. One of these approaches consists of a fusion enzyme, Cre-ER^T, in which the ligand-binding domain of a mutated estrogen receptor (ER^T) that recognizes the anti-estrogen tamoxifen or its derivative 4-hydroxytamoxifen (4-OHT) has been added to Cre (5–7). Accordingly, Cre-mediated recombination of genomic DNA is 4-OHT-dependent in mice bearing a Cre-ER^T transgene (5,6). This approach has been used to control the expression of transgenes by generating double transgenic mice bearing both a Cre-ER^T-encoding transgene and a second transgene (i.e. the gene of interest) carrying target sequences (called loxP) for Cre (5,6).

Here we show that inducible recombinases can be used to achieve the temporal control of expression of a given transgene-encoded sequence without the necessity of developing a binary system (i.e. without a requirement for two different transgenes). The originality underlying this approach is that the recombinase coding region serves both to block the transcription of a downstream gene and as the source of the inducible recombining activity that can permanently remove this block to transcription. In our design the inducible recombinase-encoding sequence (we used the Cre-ER^T gene) flanked by two of its target sequences (the loxP sites) was placed upstream of the gene of interest (enhanced green fluorescent protein, EGFP) within the same DNA molecule, so as to prevent production of the latter. Expression of the downstream gene occurred after excision of the loxP-flanked sequences, i.e. upon administration of the inducing agent (4-OHT).

Because of its simplicity (only a single transgene needs to be introduced), in addition to its *in vitro* and *in vivo* experimental potential, this strategy is of interest for the control of therapeutic transgenes.

MATERIALS AND METHODS

Transgene design: construction of plasmid pPI-PGK-loxP-Cre-ER^T-loxP-EGFP-neo^r

A 2 kb *Eco*RI fragment containing the Cre-ER^T gene from plasmid pCMVCre-ER^T (5) was cloned into a pBS-KS II vector (Stratagene) containing a rabbit β-globin polyadenylation (pA) signal (8). Subsequently, an *Eco*RI blunted–*Kpn*I fragment bearing the Cre-ER^T/β-globin pA gene was inserted into pBS-246 (Gibco BRL, catalog no. 10348-019) (3) at *Kpn*I–*Eco*RV, between two loxP sites. A *Not*I blunted–*Spe*I fragment containing the loxP-Cre-ER^T-loxP cassette was inserted between the *Xho*I blunted and *Nhe*I sites of pPI-hVEGF165-neo^r (a kind gift of C.

*To whom correspondence should be addressed. Tel: +41 22 702 5225; Fax: +41 22 702 5260; Email: pedro.herrera@medecine.unige.ch

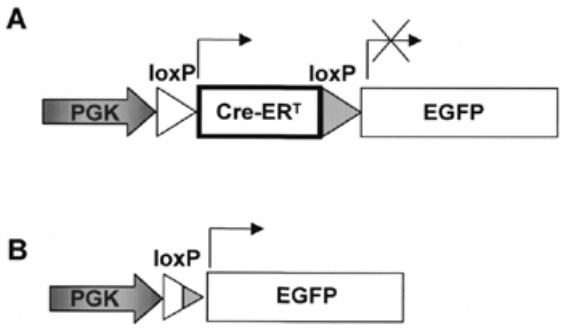

Figure 1. Transgenes present in the neomycin resistance gene-containing plasmids used to transfect BHK cells. (**A**) The loxP-Cre-ER^T-loxP cassette is placed between the mouse PGK gene promoter and the coding region of EGFP. (**B**) After excision of the loxP-flanked Cre-ER^T gene, the EGFP coding sequence can be transcribed.

Rinsch and P. Aebischer, Lausanne), immediately downstream of a mouse phosphoglycerate kinase (PGK) promoter and a small intron. We termed this plasmid pPI-PGK-loxP-Cre-ER^T-loxP-hVEGF-neo^r. The hVEGF coding region was then excised with a partial *Not*I/*Eco*RI double digestion. An EGFP gene-containing *Not*I–*Eco*RI fragment from plasmid pEGFP-1 (Clontech, catalog no. 6086-1) was used to replace the hVEGF gene, thus yielding plasmid pPI-PGK-loxP-Cre-ER^T-loxP-EGFP-neo^r (Fig. 1A).

Transfection and analysis of cells

Transformation of BHK cells. Given the spontaneous recombination of the Cre-ER^T gene in bacterial cells (see Results), the plasmid DNA preparations were purified in 0.7% agarose gels, after linearization with endonuclease *Eco*47III. This procedure resulted in very low yields of DNA, thus making normal transfection impossible. Therefore, using a phase contrast microscope (Zeiss Axiovert) and a microinjector (Inject+Matic), 0.1–0.2 pl of a solution containing ~50–100 DNA molecules was introduced into the nuclei of BHK cells grown to ~50% confluence (9). The plasmids injected were pPI-PGK-loxP-Cre-Er^T-loxP-EGFP and pPI-PGK-loxP-EGFP. About 100 cells were injected per culture dish; a minimum of three dishes was used per construct. G418 (Promega, LT no. 90421) (400 µg/ml) was used to select the cells for subsequent experiments.

Cells were maintained in standard culture conditions: DMEM containing 450 mM glucose (Gibco BRL, catalog no. 52100-039) supplemented with 10% FCS (Gibco BRL, catalog no. 10106-169) and 2 mM glutamine (Gibco BRL, catalog no. 25030-024).

4-OHT treatment. 4-OHT treatment began 10 days after the cells had been transformed. 4-OHT (Sigma, catalog no. H-7904) was dissolved in 100% ethanol (BDH, catalog no. 10107) at 1 mM; the final concentration was 1 µM (the final concentration of ethanol in the culture dishes was 0.01%). Cells were analyzed after 4 days exposure to 4-OHT.

EGFP expression analysis. Emission of fluorescence by either control or transfected BHK cells was monitored using an inverted Zeiss Axiovert microscope (equipped with a UV lamp). Cells were also analyzed using a fluorescence-activated cell sorter (model FACScan; Becton Dickinson) after trypsinization. Samples of 10 000 cells were counted in each experimental condition. The experiments were performed four times.

Statistical analysis. Experimental and control groups, before and after 4-OHT treatment, were compared using two-tailed *t*-tests.

PCR analysis. Two types of amplification by PCR were designed to assess for transgene recombination in the DNA extracted from the transformed BHK cells. The first two oligonucleotide primers used were located so that the 5′ (sense) oligonucleotide was within the loxP-flanked sequence (ER^T s2, 5′-GCT CTA CTT CAT CGC ATT CC-3′, in the ER^T coding region). The reverse primer (EGFP rev1, 5′-TCG CCC TCG AAC TTC ACC TC-3′) was in the EGFP gene. The size of the fragment amplified from the non-recombined transgene is 1266 bp, no amplification being possible after Cre-mediated excision. The second set of primers were placed flanking the floxed Cre-ER^T sequence: sense primer PGKpr1 (5′-GTA GCC TTG CAG AAG TTG GTC-3′, in the PGK promoter region) and EGFPrev1, described above. The intact, non-recombined transgene yields a PCR fragment of 3540 bp, whereas the recombined molecule, after excision of the Cre-ER^T sequence, gives a 675 bp band.

Immunohistochemistry. BHK cells microinjected with the plasmid containing the PGK-loxP-Cre-ER^T-loxP-EGFP transgene were grown on sterilized coverslips for 24 h and fixed by adding 4% paraformaldehyde for 10 min. They were then permeabilized with 0.2% Triton X-100, washed in phosphate-buffered saline and incubated for 1 h with a rabbit polyclonal anti-Cre antibody (BabCO, catalog no. PRB-106C) at 1/300 dilution and for 1 h with a goat anti-rabbit IgG–FITC conjugate. Before mounting, cells were counterstained with Evan's Blue (0.03%). Pictures were taken using a Zeiss Axiophot Epifluorescence microscope.

RESULTS

Construction of the loxP-flanked Cre-ER^T gene

An EGFP-encoding transgene driven by a PGK promoter was prepared so as to contain, between the promoter and the coding sequence, a loxP-flanked Cre-ER^T gene (Fig. 1; see Materials and Methods). Due to basal transcription of the Cre-ER^T gene in transformed bacteria and because the transgene was accessible to the Cre fusion protein, the plasmid DNA preparations we obtained contained, together with the full-length non-recombined plasmid (PGK-loxP-Cre-ER^T-loxP-EGFP, Fig. 1A), a proportion of plasmid molecules in which the loxP-flanked Cre-ER^T sequences had been excised (PGK-loxP-EGFP, Fig. 1B). The two plasmid preparations were purified by *Eco*47III digestion and electrophoresis (see Materials and Methods).

The loxP-flanked Cre-ER^T gene blocks transcription of a downstream coding sequence (EGFP)

The plasmids, containing either the PGK-loxP-Cre-ER^T-loxP-EGFP or PGK-loxP-EGFP transgenes (Fig. 1) and a SV40 promoter-driven neomycin resistance gene, were introduced into BHK cells by direct nuclear microinjection. The cells were

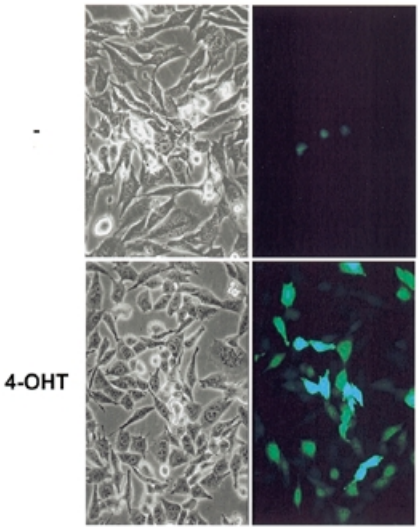

Figure 2. Expression of EGFP in BHK cells transfected with a PGK-loxP-Cre-ER^T-loxP-EGFP transgene depends on exposure to 4-OHT. The same fields of cells have been photographed under phase contrast (left) and UV (right) illumination.

maintained in the presence of G418, to select for cells expressing the injected transgene. In the absence of 4-OHT, the loxP-Cre-ER^T-loxP cassette efficiently blocked transcription of the EGFP gene, however, 10 ± 3.14% (mean ± SD, $n = 4$) of the microinjected cells spontaneously expressed EGFP as quantitated by FACS analysis (Figs 2A and B and 3). This proportion remained unchanged throughout the period of study (10 day cultures with repeated series of cell freezing and thawing). Others using double transgene systems *in vitro* (10) have also reported a low level of 4-OHT-independent Cre-ER^T recombinant activity.

Excision of the Cre-ER^T gene is regulated by 4-OHT and allows expression of EGFP

The proportion of EGFP-expressing microinjected cells increased to 61 ± 13.3% ($n = 4$, $P < 0.006$) after exposure to 1 µM 4-OHT (Figs 2C and D and 3A). This level of expression was already reached 24 h after 4-OHT had been administered (Fig. 3B). A similar proportion of fluorescent cells was observed (62.9 ± 11.23%, $n = 4$) in cultures of cells that had been microinjected with the recombined transgene (i.e. PGK-loxP-EGFP, Fig. 3), independently of the presence or absence of 4-OHT. These observations were confirmed by PCR analysis of cellular DNA: cultures of BHK cells microinjected with PGK-loxP-Cre-ER^T-loxP-EGFP in the presence of 4-OHT resulted in near complete removal of the Cre-ER^T cassette (Fig. 4). The subcellular localization of Cre-ER^T protein was monitored using an anti-Cre antibody. Prior to 4-OHT treatment, anti-Cre staining was cytoplasmic (Fig. 5A); exposure to 4-OHT induced the migration of Cre-ER^T towards the nucleus (Fig. 5B), thus allowing recombination at loxP sites.

DISCUSSION

We have shown that an inducible recombinase gene can be used to control the activity of a gene placed downstream *in cis*.

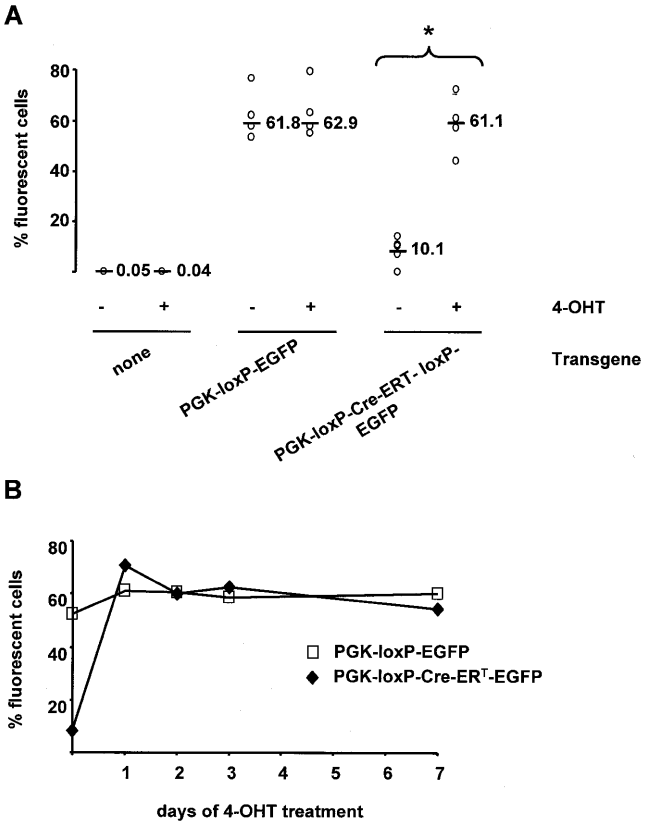

Figure 3. Control cells and cells microinjected with recombined (PGK-loxP-EGFP) or intact (PGK-loxP-Cre-ER^T-loxP-EGFP) transgenes were incubated in the absence (–) or presence (+) of 1 µM 4-OHT and the proportion of fluorescent cells was determined by FACS ($n = 4$ experiments, 10 000 cells counted in each series for every experimental group). (**A**) As expected, control non-transfected BHK cells, whether treated or not, were not fluorescent, whereas a large proportion of cells containing the recombined transgene (PGK-loxP-EGFP) fluoresced, independently of the presence of 4-OHT (61.8 ± 10.4 versus 62.9 ± 11.23%, $n = 4$, NS). In contrast, cells harboring the PGK-loxP-Cre-ER^T-loxP-EGFP transgene became fluorescent in similar proportions only after 4-OHT was added (10.1 ± 3.14 versus 61.1 ± 13.23%, $n = 4$, $^*P < 0.006$). This increase occurred in <24 h, the earliest time point analyzed after 4-OHT addition (**B**).

The advantages of this new approach, when compared with previous conditional transgenic systems (11–13) are 3-fold. (i) Both the inductive and effector elements are placed together within a single molecule of DNA, making experimental design simpler. (ii) There is no need for permanent administration of the inductive substance. (iii) It functions independently of the proliferative state of the cells in which the recombinase has been produced, in contrast to the HSV-TK/gancyclovir system (14–17), in which only dividing cells can be targeted (killed).

Our experimental strategy bears a resemblance to a report in which a testes-specific promoter-driven loxP-flanked Cre recombinase transgene was used to obtain a 'self-induced' deletion of DNA sequences in the male germline of transgenic mice (18). We have now introduced an SV40-derived intron in the coding region of Cre, as described (18), in order to avoid spontaneous recombination of the loxP-flanked Cre-ER^T in competent bacteria (see Results).

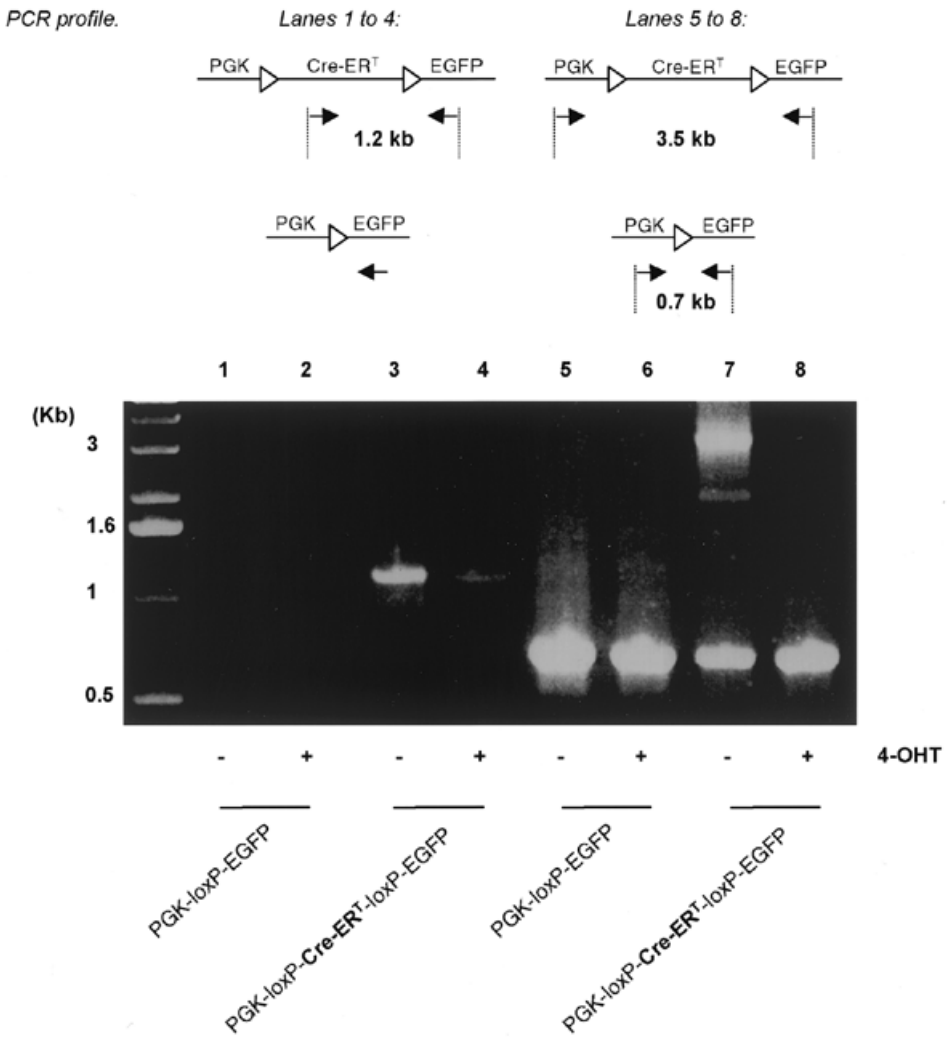

Figure 4. PCR analysis of genomic DNA extracted from transfected BHK cells. Either a PGK-loxP-EGFP (lanes 1, 2, 5 and 6) or a PGK-loxP-Cre-ER^T-loxP-EGFP (lanes 3, 4, 7 and 8) transgene was used and the cells cultured either in the absence (lanes 1, 3, 5 and 7) or presence (lanes 2, 4, 6 and 8) of 1 µM 4-OHT. Two types of PCR were performed: (i) using one oligonucleotide located between the two loxP sites (lanes 1–4); (ii) using two oligonucleotides flanking the loxP-Cre-ER^T-loxP cassette (lanes 5–8). The 1266 and 3540 bp long fragments are diagnostic of the non-recombined Cre-ER^T-containing transgene (lanes 3 and 7, respectively). Using two oligonucleotides flanking the loxP sites (see Materials and Methods) only a fragment of 675 bp is obtained after recombination (lane 8) or from cells bearing the PGK-loxP-EGFP transgene (lanes 5 and 6). In 4-OHT-treated cells bearing the PGK-loxP-Cre-ER^T-loxP-EGFP transgene failure to amplify the 1266 and 3540 bp fragments is evidence of recombination (lanes 4 and 8). Triangles represent the loxP sequences. Small arrows indicate the relative positions of the oligonucleotides, as described in Materials and Methods.

We have shown that Cre-ER^T protein was cytoplasmic in the absence of 4-OHT, but nuclear when this anti-estrogen is present, as suggested (6,7). It is thus reasonable to propose that Cre-ER^T remains in the cytoplasm bound by heat shock proteins until ligand binding (6,7), then the Cre-ER^T is transferred to the nucleus thanks to the internal nuclear localization signal of Cre (19).

The repression of EGFP expression in cells microinjected with PGK-loxP-Cre-ER^T-loxP-EGFP in the absence of 4-OHT was highly significant, but incomplete. One possible explanation is that the EGFP gene may occasionally be translated as the result of skipping the Cre-ER^T STOP codon. However, PCR amplification using a pair of external oligonucleotides produced, in addition to the full-length 3.5 kb fragment, a 0.7 kb fragment indicative of recombination (Fig. 4, lane7). This may be due to cleavage of the ER^T portion of the fusion recombinase by intracellular proteases, as suggested by others (10). Using other inducible recombinases less vulnerable to possible leakage, such as those described in Zhang *et al.* (10), should overcome this limitation. This was not attempted in the present work, the aim of which is to report a new method rather than to provide its best variant.

This technique should be useful *in vivo*, in experimental animals or under clinical conditions of gene therapy, when irreversible activation of a transgene at a chosen time, bypassing the requirement for constant administration of the inducer molecule, may represent the most suitable approach. Conversely, an adaptation of this technique may be used to

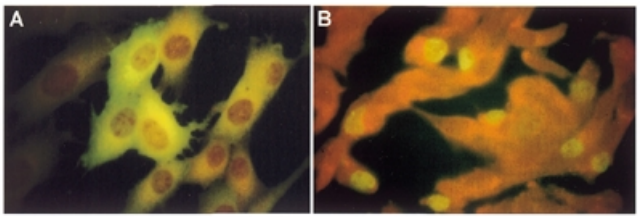

Figure 5. BHK cells microinjected with the PGK-loxP-Cre-ER^T-loxP-EGFP transgene were analyzed by immunohistochemistry with an anti-CRE antibody, either before (**A**) or after (**B**) exposure to 4-OHT (for 10 days). 4-OHT treatment results in translocation of CRE-ER^T protein from the cytoplasm to the nucleus.

Figure 6. Design of a 'self-excising' bi-cistronic construct which can be used to achieve the inducible and irreversible repression (excision) of a transgene of interest.

irreversibly inactivate the expression of a transgene at will. This could be achieved by loxP-flanking the Cre-ER^T gene together with the cDNA of interest, the latter sequence being located downstream of an internal ribosome entry site (20; Fig. 6).

In conclusion, we have shown that a segment of DNA capable of inducible self-excision, the loxP-Cre-ER^T-loxP cassette, can function as a rapid molecular switch to control the expression of a physically associated transgene. The simplicity and power of this approach make it particularly suitable for a variety of experimental designs involving the conditional expression of genes.

ACKNOWLEDGEMENTS

We are grateful to Mr Gregory Meichtry, Mr Nicolas Steiner and Mrs Gissela Flores for their technical help. This work was supported by grants from the Fondation Dinu Lipatti/Dr Henri Dubois Ferrière and the Fonds National Suisse pour la Recherche Scientifique (PNR 37, no. 4037 055105).

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