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Conditional gene knock-down by CRE-dependent short interfering RNAs

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Short interfering RNAs (siRNAs) are short (21–23 nt) double-stranded RNAs that direct the sequence-specific degradation of corresponding mRNAs, resulting in suppression of gene activity. siRNAs are powerful tools for gene functional analysis in mammals. Chemically synthesized siRNAs permit transient gene repression but preclude inhibition of stable gene products as well as long-term phenotypic analyses. Permanent gene suppression can be achieved by transcribing siRNAs as stem–loop precursors from Pol III promoters. This approach, however, has a major limitation: inhibition cannot be controlled in a time- or tissue-specific manner. Thus, the approach cannot be applied to genes essential for cell survival or cell proliferation. To overcome these limitations, we have designed a CRE–*lox*-based strategy that allows one to repress gene activity in a time-dependent manner in cells, and in a time- or tissue-dependent manner in animals. Our approach promises to improve dramatically the procedures for functional genetics in mammals.

INTRODUCTION

RNA interference is a powerful strategy for gene inhibition in a variety of organisms (Sharp, 2001). In plants or in the worm *Caenorhabditis elegans*, the procedure is to introduce a double-stranded RNA corresponding to the sequence of the target mRNA into the cells. This double-stranded RNA is cleaved into short interfering RNAs (siRNAs) by a protein complex that includes the RNase Dicer (Bernstein *et al*, 2001). siRNAs are double-stranded RNA sequences 21–23 nucleotides (nt) long with a 3′ protruding tail of 2 nt (Zamore *et al*, 2000; Elbashir *et al*, 2001a). The antisense strand of siRNA (Martinez *et al*, 2002) guides another complex of proteins, the RISC complex (Hammond *et al*,

2000), towards the target mRNA, and induces its cleavage at a position corresponding to the centre of the siRNA sequence (Elbashir *et al*, 2001b,c). In mammalian cells, RNA interference can be triggered by short synthetic double-stranded RNA sequences mimicking the siRNAs generated by Dicer cleavage in lower organisms, without inducing the interferon response that is observed with long double-stranded RNAs in most cell types (Elbashir *et al*, 2001a). Synthetic siRNAs, however, have a short half-life in cells, and consequently their effect is transient. siRNAs can be constitutively transcribed as stem–loop precursors by the Pol III polymerase, and stable cell lines can be established in which the siRNA is expressed in a constitutive manner (Brummelkamp *et al*, 2002b; Donze & Picard, 2002; Miyagishi & Taira, 2002; Yu *et al*, 2002). The siRNA cassette can be inserted into a viral vector, allowing the transduction of a high proportion of cells in a population, including in cells that are otherwise difficult to transfect such as primary cell cultures (Brummelkamp *et al*, 2002a; Rubinson *et al*, 2003; Stewart *et al*, 2003). However, genes that are essential for cell survival or cell proliferation cannot be studied in this manner because their inhibition prevents the establishment of cell lines. Thus, there is a great need for a system that permits full exploitation of this strategy. Pol III promoters are very sensitive to sequence modifications, and it therefore appeared difficult to insert a binding site for an inducible factor such as tetracycline. Furthermore, to avoid undesired inhibition of target genes during the establishment of stable cell lines, it is of prime importance that the background expression of siRNA is kept to minimal levels. For these reasons, we have designed a CRE–*lox*-based approach, in which the formation of the stem–loop siRNA precursor is dependent on the expression of the CRE recombinase (Dale & Ow, 1991). We demonstrate a CRE-dependent production of siRNA, which is accompanied by a CRE-dependent inhibition of a target gene. This strategy should prove highly useful for functional genetics in tissue culture as well as in animals.

RESULTS AND DISCUSSION

Our strategy for CRE-dependent production of siRNAs is described in Fig 1. The siRNA sequence is under the control of a Pol III promoter (H1), as described previously (Brummelkamp *et al*,

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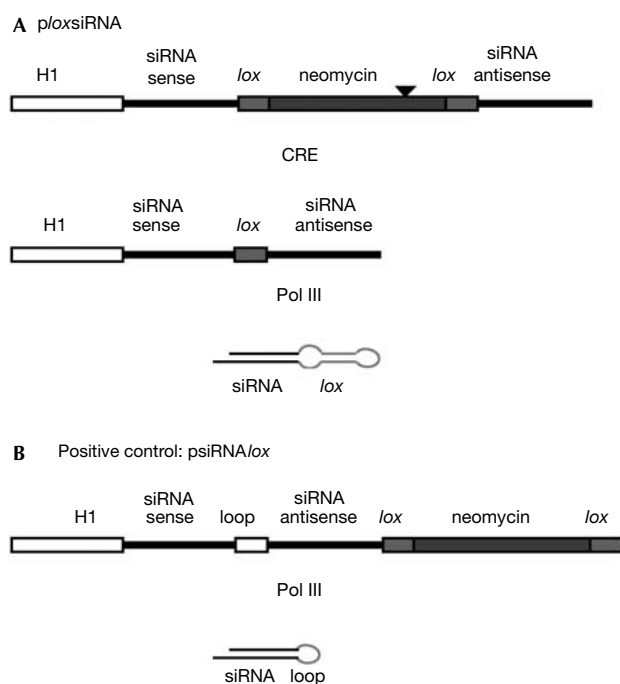E-mail: ahbellan@vjf.cnrs.fr

Fig 1 | Strategy for siRNA production and plasmids used in this study. Black arrowhead: position of the Pol III stop codon in p*lox* siRNA.

Fig 2 | CRE-dependent production of siRNA. COS cells were transfected with the indicated vectors, and RNAs were analysed using a probe complementary to the siRNA antisense strand. (**A**) A short and a long exposure of the same northern blot are shown. The positions of the precursors and processing products are indicated on the left, and the positions of an RNA ladder as well as that of cellular noncoding RNAs (U6 and Let-7) are indicated on the right; the asterisk (*) indicates bands that we interpret as corresponding to read-through products or processing intermediates. (**B**) The blot was hybridized with a U6 probe (U6) or RNA was stained with ethidium bromide (EtBr) to control for the amount of total RNA in each lane.

2002b). The sense strand is separated from the antisense strand by a neomycin cassette that is flanked by two *lox* sites. In the absence of CRE, a stretch of four T's in the neomycin cassette (black arrowhead in Fig 1A) terminates Pol III transcription, thereby preventing transcription of the antisense strand and resulting in an incomplete transcript. In the presence of CRE, recombination eliminates the neomycin cassette, allowing the read-through of the entire siRNA. The remaining intercalated *lox* sequence acts as a loop, which is necessary for formation of the siRNA and proper processing of the stem–loop precursor (Brummelkamp *et al*, 2002b). The strategy was tested using green fluorescent protein (GFP) as the target reporter gene with a previously described siRNA sequence (Brummelkamp *et al*, 2002b). A plasmid in which the entire siRNA was inserted upstream from the *lox* neomycin cassette, allowing the constitutive expression of siRNA, was used as a positive control; the backbone vector (pLox) was used as a negative control. Expression and processing of the stem–loop precursor was monitored by northern blot (Fig 2). As expected, a precursor of approximately 50 nt was observed for the constitutive expression vector psiRNA*lox*, and this precursor was processed into a 20 nt species. Formation of these products did not require the expression of the CRE recombinase, as expected. With p*lox*siRNA, in the absence of CRE, no product could be detected in the range of sizes analysed. In the presence of CRE, however, a major band corresponding to the 90 nt precursor, and a 20 nt processing product could be detected. The level of this stem-*lox* precursor was lower than that observed with the constitutive vector (compare siRNA *lox* and *lox* siRNA in Fig 2A). This is probably due to the kinetics of accumulation of the precursor, which requires a recombination event that is not needed for the constitutive vector. However, the ratio between stem–loop
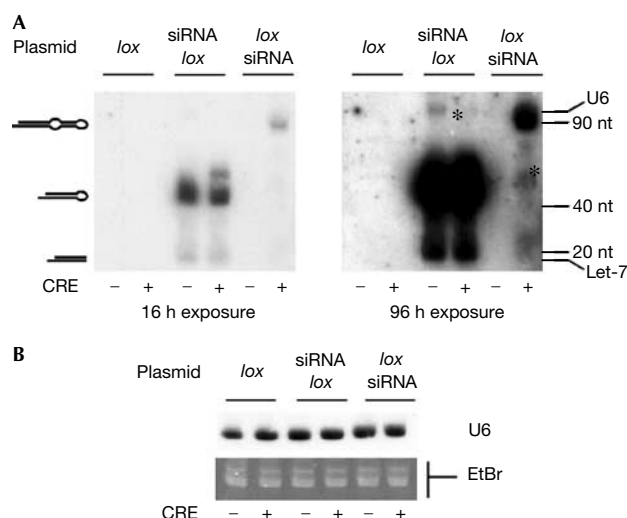
precursor and product appeared to be roughly similar for the two plasmids, suggesting that the *lox* sequence did not significantly impair precursor processing.

The efficiency of the siRNAs for gene inhibition was next assessed in transient transfection assays (Fig 3). As expected, the constitutive vector psiRNA*lox* inhibited GFP protein expression independently of CRE expression. In the case of the p*lox*siRNA plasmid, no inhibition was observed in the absence of CRE. In the presence of CRE, GFP was inhibited. The level of inhibition increased with time: after 48 h (Fig 3A), inhibition was only partial as compared with the psiRNA*lox* vector, in good correlation with siRNA accumulation (Fig 2); it was greater after 60 h (Fig 3B), although some GFP was still detectable. No inhibition was observed with a p*lox*siRNA vector directed against an irrelevant sequence (data not shown), indicating that the inhibition was gene specific.

Lack of complete inhibition is probably due to the fact that, whereas GFP accumulates soon after transfection, the siRNA product accumulates at a slower rate. Consistent with this hypothesis, GFP was undetectable in two-step transfections in which the siRNA was allowed to form for 24 h before transfection of the GFP expression vector (Fig 4). Taken together, these results indicate that *lox*-containing siRNAs formed from p*lox*siRNA, although the levels are not high (as monitored by northern blot), are indeed efficient for gene inhibition (Fig 2).

The effect of the siRNAs was analysed on a reporter gene expressed from an integrated sequence, using a cell line in which GFP expression is inducible. Cells were transfected with the
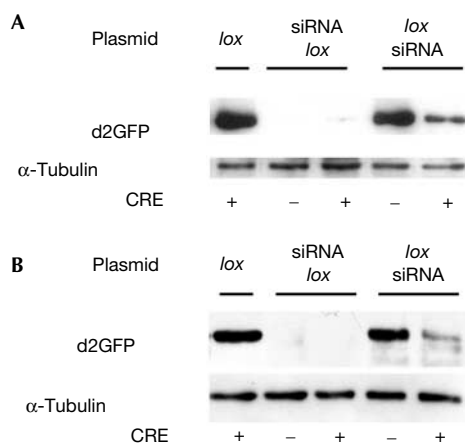
Fig 3 | CRE-dependent inhibition of GFP in transiently transfected cells. Cells were transfected as in Fig 2, and extracts were analysed 48 h (**A**) or 60 h (**B**) later by western blot using anti-GFP or anti-tubulin antibodies. These experiments were reproduced 2–6 times. d2GFP, unstable form of GFP.
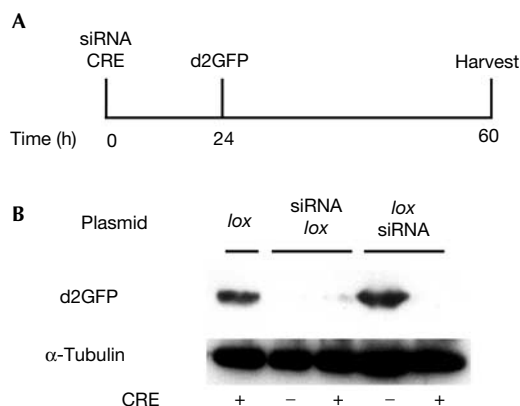

Fig 4 | CRE-dependent inhibition of GFP in two-step transfections. (**A**) Experimental design. Cells were first transfected with ploxsiRNA, psiRNAlox or plox, together with the CRE expression vector pMC CRE or the empty vehicle vector pMC for the controls, and then again 24 h later with pCMV-GFP. (**B**) Western blot analysis. These experiments were reproduced twice.

siRNA- and CRE-expressing vectors, together with an RFP expression vector to distinguish transfected cells, and GFP expression was subsequently induced. GFP expression was observed in about 30% of the cells 24 h after induction, and in 60% of the cells 48 h after induction. Similar proportions were observed in control-transfected (RFP-positive) cells that received the plox empty vehicle vector (Fig 5A,B). In contrast, essentially no GFP expression was detected in transfected cells that received the constitutive psiRNAlox vector, irrespective of CRE expression. With the ploxsiRNA vector, a 'normal' proportion (around 30% at day 1 and 65% at day 2) of GFP-positive cells among the transfected cells was observed in the absence of CRE, but this proportion dropped to less than 5% in the presence of CRE.

CRE-dependent inhibition was also achieved with a stably integrated siRNA-expressing vector directed against an endogenous
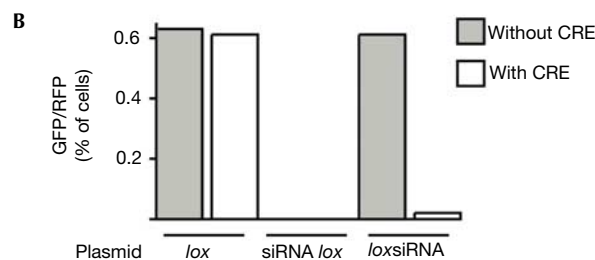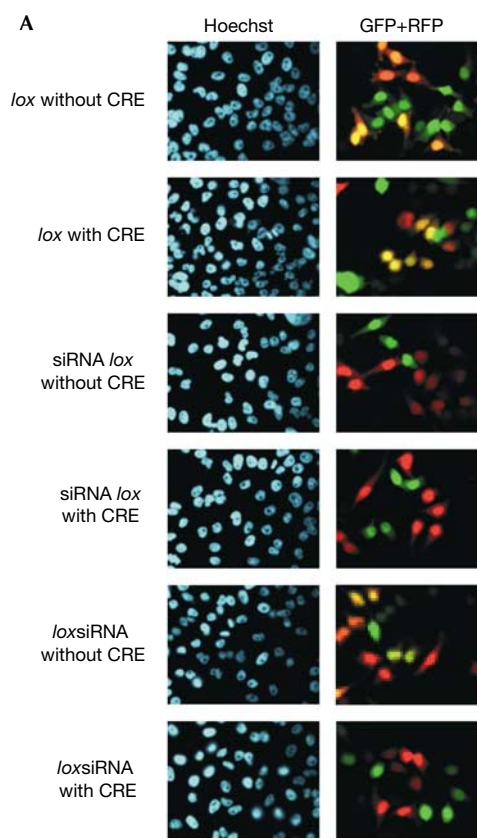

Fig 5 | Inhibition of an integrated GFP transgene. HeLa 1002 cells were transfected as in Fig 2, and treated with Dox to induce GFP expression 72 h later for 24 h (**A**) or 48 h (**B**). (**A**) Microphotographs of cells after 72 h of Dox treatment. (**B**) Proportion of GFP-positive cells among the transfected, RFP-positive cells. These experiments were reproduced three times.

gene. A ploxsiRNA directed against p53 designed as in Fig 1, or the plox empty vector, was transfected into U2OS cells; stable clones were established using neomycin as a selectable marker. Selected clones were then transfected with the CRE-expressing vector or the empty vehicle vector as a control, and selected using a different selectable marker (hygromycin). CRE-dependent p53 inhibition was achieved in about 50% of the clones with the ploxsiRNAp53 vector; three examples of such clones are shown in Fig 6. No inhibition was observed when the ploxsiRNAp53 clones received the pMC empty vector, or in any of the clones stably transfected with the plox empty vector. This result demonstrates that this approach can be used to establish stably transfected cell lines in which a target endogenous gene can be downregulated at will.
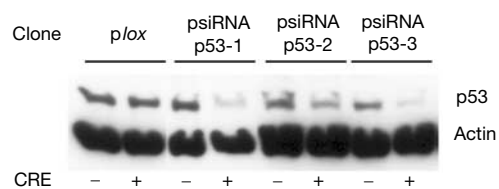
Fig 6 | Inhibition of an endogenous gene from an integrated construct. U2OS cells with integrated p*lox* or p*lox*siRNA as indicated were transfected with pMC CRE or pMC. p53 expression was monitored 4 weeks later.

Finally, the effect of siRNA was also monitored *in vivo*, after electroporation of the plasmids in muscles. The tibialis anterior (TA) muscles of mice constitutively expressing GFP in a ubiquitous manner were electroporated with both the siRNA- and CRE-expressing vectors, along with a nuclear LacZ expression vector to label the nuclei of transfected fibres. The electroporated muscles were examined 12 days later. The combination of the CRE-expressing plasmid and the p*lox*siRNA GFP vector resulted in a marked decrease of GFP in transfected fibres, whereas expression of CRE in the presence of the p*lox* control vector, or transfection of the p*lox*siRNA vector in the absence of CRE, did not affect GFP expression in transfected fibres (Fig 7). These results indicate that our CRE–*lox*-based strategy can also downregulate gene expression in live animals.

Taken together, these results indicate that efficient gene inhibition can be induced using the CRE–*lox* system in a simple and convenient manner. Such a system is an absolute requirement when analysing the function of essential genes in cells, and it should facilitate high-throughput functional genetics. It is also greatly needed for functional genetics in animals. The analysis of both the expression of the CRE-dependent siRNAs and the inhibition of a target gene demonstrated an essential advantage of our approach, which is the total absence of background expression in the absence of CRE.

In conclusion, this strategy allows one to tackle the functional analysis of essential genes. CRE can be expressed in an inducible or in a tissue-specific manner in animals, allowing one to achieve time-dependent and/or tissue-specific gene inactivation. In that sense, our approach is complementary to the Tet-inducible system that was published by van de Wetering *et al* (2003). Functional analysis of mammalian genomes should be made significantly easier.

METHODS

Plasmid construction. P*lox* was constructed by inserting into the p*lox*Neo plasmid the Pol III promoter H1 as an *Nhe*1–*Xba*1 insert. The sequences corresponding to the siRNA sense and antisense strands were introduced as synthetic oligonucleotides using, respectively, the *Xba*I site or the *Bam*H1 and *Kpn*1 sites. Sequences were CTAGC CCC GCAAGCTGACCCTGAAGTTCAT T (sense) and GATCC ATGAACTTCAGGGTCAGCTTGC TTTT GGTACC TAGA CCC (antisense) for GFP, and GCATGAACCGGAGGCC CAT T (sense) and GATCC ATGGGCCTCCGGTTCATGC (antisense) for p53.
Transfection. COS-7 cells were transfected with 500 ng of CMV-GFP or CMV-d2GFP (Clontech), 8 μg of pMC CRE (Gu *et al*, 1993) (or pMC backbone for the negative controls), and 4 μg of
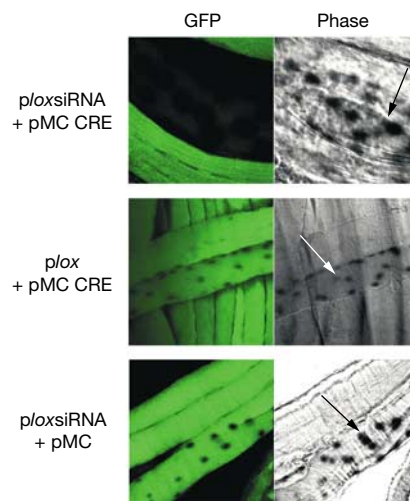

Fig 7 | CRE-dependent inhibition of GFP *in vivo*. The TA muscles of GFP transgenic mice were electroporated with the indicated plasmids along with an MCK-LacZ to detect transfected fibres, and analysed 12 days later for LacZ and GFP expression as described in the Methods section. The arrows point to LacZ-positive nuclei in a transfected fibre.

p*lox*siRNA, psiRNA*lox* or p*lox* using Polyfect (Qiagen). In some experiments, cells were transfected twice, first with 2 μg of pMC CRE or pMC backbone and 1 μg of p*lox*siRNA, psiRNA*lox* or p*lox* and 24 h later with 500 ng of CMV-d2GFP. Under the conditions that we have used, about 80% of the cells scored positive for GFP fluorescence. In some experiments, 300 ng of CMV luc or CMV-RFP (a kind gift from Michel Kress) was included. HeLa 1002 cells (a cell line derived from HeLa with an integrated GFP transgene inducible by doxocycline) were transfected with siRNA- and CRE-expressing plasmids as described above. Cells were treated with 1 μg/ml of doxocycline 72 h later for 24–72 h, before examination under an Axiovert fluorescence microscope.
Western blot. Extracts were analysed 60 h after transfection using standard procedures.
Northern blots. Total RNA (40 μg) was loaded onto a 15% denaturing polyacrylamide gel. RNAs were transferred onto nitrocellulose membranes by electrotransfer (45 min at 390 mA) in TBE buffer. The probe was a ^{32}P-labelled antisense sequence corresponding to a 22 nt GFP target (5′CTAGAATGAACTTCAG GGTCAGCTTGCGGT3′).
Muscle electroporation. The MCK–nlsLacZ construct contains the coding sequence of nuclear β-galactosidase under the control of the muscle creatine kinase promoter.

Actin-GFP transgenic mice (5–10 weeks old) (Ikawa *et al*, 1998) were anaesthetized with 300 μl of 0.05% xylazine–1.7% ketamine in 0.9% NaCl. After skin incision, 8 μg of DNA containing 3 μg of CRE and/or siRNA expression vector and 2 μg of MCK–nlsLacZ were injected into the TA muscle using a 1 ml syringe with a 27-gauge needle. Caliper electrode plates (Q-biogen, France) were immediately applied on each side of the muscle, and a series of eight electrical pulses (2 Hz, 20 ms each) was delivered with a standard square-wave electroporator (ECM 830, Q-biogen). Electrical contact was ensured by shaving and conductive gel application.

At 12 days after injection, TA muscles were dissected and fixed for 30 min with 4% paraformaldehyde in phosphate-buffered saline (PBS) and incubated for 2–3 h in 0.4 mg/ml 5-bromo-4-chloro-indolyl-β-D-galactoside, 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6$ and 2 mM $MgCl_2$ in PBS at 37 °C for LacZ staining. LacZ-positive regions were further dissected under a microscope. Fluorescent and phase contrast images were acquired on a Zeiss confocal microscope (LSM510, Zeiss).

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