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A hybrid CMV-H1 construct improves efficiency of PEI-delivered shRNA in the mouse brain

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ABSTRACT

RNA-interference-driven loss of function in specific tissues *in vivo* should permit analysis of gene function in temporally and spatially defined contexts. However, delivery of efficient short hairpin RNA (shRNA) to target tissues *in vivo* remains problematic. Here, we demonstrate that efficiency of polyethylenimine (PEI)-delivered shRNA depends on the regulatory sequences used, both *in vivo* and *in vitro*. When tested *in vivo*, silencing of a luciferase target gene by shRNA produced from a hybrid construct composed of the CMV enhancer/promoter placed immediately upstream of an H1 promoter (50%) exceeds that obtained with the H1 promoter alone (20%). In contrast, in NIH 3T3 cells, the H1 promoter was more efficient than the hybrid construct (75 versus 60% inhibition of target gene expression, respectively). To test CMV-H1 shRNA efficiency against an endogenous gene *in vivo*, we used shRNA against thyroid hormone receptor α1 (TRα1). When vectorized in the mouse brain, the hybrid construct strongly derepressed CyclinD1-luciferase reporter gene expression, CyclinD1 being a negatively regulated thyroid hormone target gene. We conclude that promoter choice affects shRNA efficiency distinctly in different *in vitro* and *in vivo* situations and that a hybrid CMV-H1 construct is optimal for shRNA delivery in the mouse brain.

INTRODUCTION

RNA interference (RNAi) is now a well-described mechanism by which a double-stranded RNA (dsRNA) leads to the sequence-specific inhibition of its homologous gene. First described in plants (1), it has since been applied to numerous invertebrate and vertebrate models (2). In mammalian cells, the introduction of long dsRNAs into mammalian cells activates protein kinase PKR and RNase L, leading to an interferon response and hence to the non-specific extinction of genes resulting in cell death (3,4). This non-specific effect of long dsRNAs into mammalian cells can be bypassed by using small RNA duplexes of 19–21 nt, which are sufficient to trigger specific RNAi in mammalian cells without activating the interferon response (5).

This highly efficient and specific technology opens up a broad spectrum of experimental and therapeutic possibilities (6,7). However, the lack of suitable delivery systems for short interfering RNAs (siRNAs) *in vivo* has hampered the advance of such applications.

To date two main methods have been developed to obtain sufficient intracellular levels of RNAi for gene silencing in mammals *in vivo*. The first method involves direct vectorization of siRNA into the target cell. The alternative is to vectorize plasmids containing short hairpin RNA (shRNA) cassettes, which leads to shRNA transcription, followed by Dicer-dependent loop cleavage and siRNA production. Most often, the promoters used to direct the expression of the shRNAs are H1 (8) and U6 (9–11) or CMV (12). So far these promoter systems have been tested with a variety of viral delivery systems

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including adenoviruses (12,13), retroviruses (14) and lentiviruses (15). However, there are only few reports of their use with non-viral delivery of shRNA-carrying plasmids, neither *in vitro* nor *in vivo*.

We previously demonstrated that cationic lipids could provide efficient delivery of siRNAs into the brain of newborn mice brains, producing >80% inhibition of an exogenous gene with only 5 pmol of siRNAs (16). However, generally speaking cationic lipoplexes are less efficient than polyplexes for plasmid vectorization *in vivo*. For instance in the adult mammalian brain, the cationic polymer polyethylenime (PEI), and particularly linear 22-kDa PEI (L-PEI), easily outclasses other lipid- or polymer-based vectors (16) [for review see (17)]. We thus chose to use PEI vectorization to evaluate the capacity of two different constructs: either H1 alone or a hybrid CMV enhancer and minimal promoter/H1 promoter construct (hereafter called the CMV/H1 construct). Each construct was tested for its capacity to direct the expression of shRNA-carrying plasmids *in vitro* and *in vivo* in the brains of newborn and adult mice.

We show that, when tested *in vitro* on two different cell lines, the efficiency of target silencing is independent of the promoter used. In contrast, when tested *in vivo* in either the developing or the adult brain, the efficiency of the shRNAs depends markedly on the promoter used to drive production. We found that the H1-shRNA construct provided only a slight decrease in luciferase target gene expression. However, surprisingly, we found that the hybrid CMV-H1 construct directing shRNA production provided a significant inhibition of the co-transfected luciferase gene in both *in vivo* situations tested.

Further, we tested the efficiency of a shRNA directed against endogenous thyroid hormone α1 (shTRα1) in cell cultures and in the newborn mouse brain. We first demonstrated that the shTRα1 efficiently inhibits the expression of endogenous TRα1 in cell culture. Then we show that the presence of the shTRα1-carrying plasmid in the newborn mouse brain strongly increases the expression of a co-transfected CyclinD1-luciferase reporter gene, CyclinD1 being a negatively regulated thyroid hormone target gene in our experimental paradigm. These data demonstrate that shRNAs under the control of a hybrid CMV-H1 construct efficiently inhibit endogenous gene expression *in vivo*, and that this technology can be used for further gene function analysis in the brain of newborn mice.

METHODS

Plasmid constructions and siRNAs

The *Renilla reniformis* (RLluc)- and *Photinus pyralis* (PPluc)-luciferases carrying plasmids are respectively pRL-CMV (Promega) and a pGL2-basic vector with a cytomegalovirus (CMV) promoter inserted in the multiple cloning site referred in the following as pGL2-CMV. The shRNA sequence directed against pGL2 luciferase (shLuc in the text) is the following: 5′CGTACGCGGAATACT TCGATTCAAGAGATCGAAGTATTCCGCGTACG3′. The H1-shLuc construction based on a pSUPERbasic backbone (8) was kindly provided by Dr A Harel-Bellan (Institut André Lwoff, Villejuif, France).

For the CMV-H1-shLuc construction, the H1-shLuc plasmid was digested by BamH1 and HindIII. The 280-bp insert containing the shLuc and the H1 promoter sequences was inserted into the luciferase-free pRL-CMV vector obtained after the digestion of pRL-CMV with PstI and XbaI. This digestion releases the *Renilla* luciferase gene sequence but conserves the full CMV enhancer and early promoter. The selected clones were sequenced using the CMV forward primer (MWG Biotech). One clone, clone 6 was found to carry both the shLuc sequence and the H1 promoter just following the CMV enhancer/early promoter (see scheme, Figure 1).

The CMV-shLuc construction was obtained by ligation of a synthetic double-stranded oligonucleotide shLuc carrying PstI and XbaI ends (Eurogentec) inside the PstI/XbaI sites of pRL-CMV.

Two 21-nt-long double-stranded siRNA targeted to firefly luciferases siLuc and siLucmt were synthesized according to the sequences previously described (5,16). For the inhibition of TRα1, we designed a 21-bp sequence directed against a specific sequence for TRα1 gene (siTRα1). The sequence of the siTRα1 was the following: siTRα1(up): 5′CGCUCUUCCUGGAGGUC UUTT3′; siTRα1(down): 5′AAGACCUCCAGGAAGA GCGTT3′. For the CMV-H1-shTRα1 construction, we designed a 100-bp palindromic sequence based on the siTRα1 sequence and containing an 8-bp loop (TCAAGAG). This 100-bp sequence was designed with Bsa1 and Not1 cohesive ends, which allowed its insertion inside the Bsa1/Not1 site of CMV-H1-shLuc plasmid where the shLuc sequence was deleted previously.

Preparation of complexes and gene transfer *in vivo*

All animal experiments were conducted in accordance with the principles and procedures described in Guidelines for Care and Use of Experimental Animals.

DNA was complexed L-PEI 22 kDa with a 6N/P charge ratio (where N = the positive charges carried by protonable amines of PEI and P = the negative charges carried by DNA phosphates). For this, 0.18 µl of L-PEI 100 mM were added per µg of DNA used. All solutions were prepared in 5% glucose.

The protocol of stereotaxic injection into the lateral ventricles of newborn mice brains was as described previously. Briefly, 2 µl of a solution containing 0.1 µg of each luciferase (i.e. pGL2-CMV and pRL-CMV) and 0.2–0.8 µg of either the shLuc-containing plasmid tested or a plasmid carrying an irrelevant shRNA under a CMV enhancer/promoter, were stereotaxically injected into the lateral ventricles of newborn mice brains. Here, 24–72 h post-transfection, the animals were dissected, the brains were removed and the dual luciferase assay was performed to detect the activity of *Photinus* (pGL2) and *Renilla* (pRL) luciferases separately. Five animals were injected per group, and the two hemispheres were analysed separately for reporter gene expression (i.e. $n = 10$ hemispheres per group). Each experiment was performed at

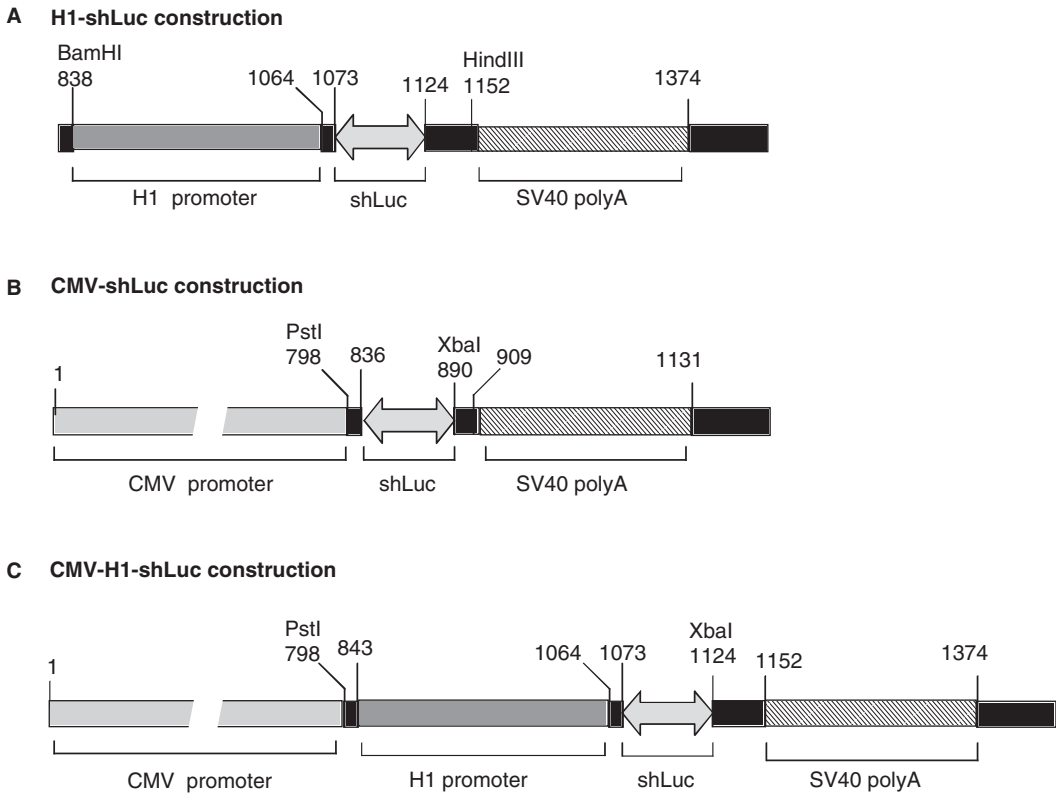

Figure 1. Scheme of the different promoter constructions driving shLuc. The three promoter constructs tested for driving-shLuc mediated inhibition after PEI-based transfection in the brain are shown. In (**A**) the H1 promoter alone; (**B**) CMV enhancer/promoter used alone and (**C**) a hybrid CMV-H1 promoter. In each construction, the shLuc contains a TTTTT sequence required for the H1-transcription arrest and is followed by a SV40 polyadenylation sequence transcription arrest for RNA pol II in CMV- and CMV-H1 promoter constructs.

least three times providing similar results. Means are shown ± SEM. Unpaired Student's *t*-test or a non-parametric Wilcoxon test were used for statistical comparison between the control and treated groups.

For analysis of CyclinD1 expression, we used a luciferase reporter gene under the control of a CyclinD1 promoter. In each group, 0.5 μg of CyclinD1-luciferase plasmid was injected per hemisphere, along with either 0–200 nM of siTRα1, or 0.2–0.5 μg of CMV-H1-shTRα1 plasmid. Note that the total amount of nucleic acids transfected was constant in each experiment, as appropriate quantities of siGFP (siRNA directed against green fluorescent protein) were added bringing the final siRNA concentration in all cases to 200 nM. Likewise, in the experiments using CMV-H1-shTRα1, the total amount of plasmids injected was 1 μg per hemisphere. The irrelevant plasmid used to complete the total amount of nucleic acids in the different groups of the shTRα1 experiment was the empty pcDNA3 (Invitrogen). In these experiments, 10 animals were injected per group and the two hemispheres were quantified separately for reporter gene expression (i.e. $n = 20$ per group). Each experiment was performed at least twice providing similar results. Means ± SEM are shown. Statistical analysis was done using unpaired Student's *t*-test or a nonparametric Wilcoxon test for comparison between the control and treated groups.

Cell culture studies

Human carcinoma cells (HeLa, HeLaX1/5 and HeLa2053) and mouse fibroblastic cells (NIH 3T3) were cultured in complete growth medium (Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal calf serum (FCS), 100 μg/ml penicillin and streptomycin) at 37°C with 5% CO_2. One day prior to transfection, 20×10^4 cells were plated in 48-well plates. The next day, the medium was replaced by reduced serum medium (Opti-MEM, Invitrogen). Each well was incubated with a 100 μl transfection mix containing pRL-CMV and pGL2-CMV (both at 30 ng), and 0.37 μl Lipofectamine 2000 reagent (Invitrogen), either with 250 ng pshRNA plasmids or 70 ng siRNAs in 400 μl Opti-MEM for 4 h. HeLaX1/5 cells, which stably expressed firefly luciferase protein under the control of TetOFF-inducible promoter (18) was co-transfected similarly using shRNA carrying plasmids (250 ng) and pRL-CMV (0.1 μg). After the incubation period, the transfection was stopped by adding 500 μl of complete medium with 20% FCS. After 24 h incubation, cells were washed with PBS and lysed by adding passive lysis buffer (Promega). An aliquot of the lysate was assayed for protein concentration (BioRad) and for luciferase activities using Dual luciferase kits (Promega). The firefly luciferase activity was then divided by the *Renilla* luciferase activity in order to normalize for

transfection efficiencies of HeLa and NIH 3T3. All experiments were done in triplicate.

For siTRα1 transfections, the same protocol was adapted to transfect siTRα1 into NIH 3T3 cells stably expressing both TRα1 and TRα2 isoforms. Three concentrations of siTRα1 were tested ranging from 5 to 100 nM (500 µl/well). In this experiment, the transfection vector used was jetSI/DOPE, using the complexation protocol described previously (Preparation of complexes and gene transfer *in vivo* section). One day post-transfection, cells were harvested and total RNAs extracted. For all experiments, reverse transcription was performed with MMLV-RT (Invitrogen) using 1 µg of total RNA mixed with 500 ng of hexamer oligonucleotides according to the manufacturer's instructions. Here, 1% of cDNAs was used for quantitative PCR (Q-PCR) using specific primers (as listed below) and SYBR-Green PCR kit (Qiagen). Q-PCR reactions were performed at least three times in duplicate on a DNA engine Opticon system (MJ Research). Each point was repeated three times (i.e. three wells for each condition) and each well was measured in triplicate. Data were analysed by normalizing TRα1 and TRα2 expression to that of a control gene 36B4. The sequences of the oligonucleotides used are as follows: TRα1 forward: 5′CAGAGGGTGTGCGGAGCTGGT3′; TRα1 reverse: 5′CCTGTCCAAGGGCTGGAGGGT3′; TRα2 forward: 5′GCATGTTGTTCAGGGTCCGCAG GT3′; TRα2 reverse: 5′GGGCTCTTCGGGCTCTGGT GCT3′; 36B4 forward: 5′ACCTCCTTTCCAGGCTTT3′, 36B4 reverse: 5′CCCACCTTGTCTCCAGTCTTT3′. Specificity of the amplification was optimized by determining melting curves of the amplicons.

RESULTS AND DISCUSSION

Our objectives in this study were to compare the capacities of different constructs to direct efficient production of shRNA *in vitro* and *in vivo* in the mammalian brain. The expression cassettes of shRNA delivery vectors typically exploit RNA polymerase III (Pol III) promoters (8–11), (19–20), whilst some authors have suggested that use of a Pol II promoter can be used successfully in certain cell contexts using non-viral (21) or lentiviral vectors (22). However, the use of Pol II promoters for shRNA production is subject to certain constraints. For instance, some authors have demonstrated a need for both a very short distance (6 bp) between the Pol II promoter and the shRNA sequence as well as a short polyadenylation signal (21). Others have shown that the presence of an intron between the Pol II promoter and the shRNA sequence is necessary for efficient production (22). These constraints do not apply to Pol III constructs. We therefore tested the efficiency of a Pol III construct (H1) in different *in vivo* and *in vitro* situations. However, we found that the H1 promoter alone was not very efficient *in vivo*. So we also tried a hybrid CMV enhancer minimal promoter/H1 promoter construct (CMV/H1 construct). We chose this strategy because this enhancer had previously been shown to increase promoter efficiency *in vivo*, such as for the PDGF beta promoter (23) or to increase U6-mediated transcription of shRNAs in plasmids (24). Other fusion promoters have also been described to increase the shRNA production efficiency by recombinant SV40 derivative viruses (25) or by lentiviral vectors (26). In our experimental set up, a construct with CMV enhancer/promoter (CMV-shRNA) alone was used as a control for the enhancement of the H1 promoter, knowing that in our construct the CMV promoter was too far from the hairpin to be efficient in producing shRNA, as shown by Xia *et al.* (12). Given that one of the major advantages of using an shRNA approach is that it should be able to be exploited to provide tissue-specific and developmental-stage-specific gene knockdown, we examined the efficiencies of these constructs in the context of the developing and the adult mouse brain.

Design of different promoter constructs driving the transcription of shLuc

Three different promoter constructs were tested for the production of shLuc (Figure 1). The first was a classic H1 promoter transcribing shLuc from a pSUPER vector backbone (8). In this construction, the type III RNA polymerase synthesizes the shRNA and is stopped by a five-T stretch at the 3′ end of the shRNA (Figure 1a). In this H1-shLuc construct, the first nucleotide of the shLuc sequence is located 30 bp from the TATA box of the H1 promoter (27). Second, in order to improve H1-mediated production of shRNAs, we also constructed a CMV-H1 hybrid construct driving the production of the shRNA. In this construction, the full enhancer and promoter sequence of CMV has been integrated upstream of the H1 promoter (CMV-H1-shLuc). Finally, as a control to validate that in the CMV-H1 construct, the production of the hairpin is generated by the H1 promoter and not by the CMV promoter, we designed a CMV-shLuc plasmid where the CMV enhancer/promoter is too far from the hairpin sequence to allow efficient shRNA production. We thus did not expect the CMV-shLuc plasmid to provide any inhibition of the target luciferase (Figure 1c).

In cell culture, both H1-shLuc and CMV-H1-shLuc trigger efficient target gene knockdown

We tested the efficiency of H1-shLuc to trigger the inhibition of a co-transfected luciferase target gene by RNAi in a mouse fibroblastic cell line (NIH 3T3), as this construct has been shown to be effective in certain culture conditions (28). We then compared the H1-shLuc efficiency to that of the CMV-H1-shLuc in the same cultures.

As described in the Methods section, two luciferase reporter genes (pGL2-CMV and pRL-CMV at 30 ng each) were co-transfected in NIH 3T3 cells (Figure 2a), along with the different constructions described in Figure 1. Quantification of luciferase expression revealed strong inhibition efficiency for both H1-shLuc and CMV-H1-shLuc (0.25 µg–100 µl transfection mixture). This 80% inhibition of the target gene with H1- and CMV-H1-shLuc was stronger than obtained with siLuc used at 70 ng (Figure 4a). Similar results were obtained using HeLa cells instead of NIH 3T3 cells

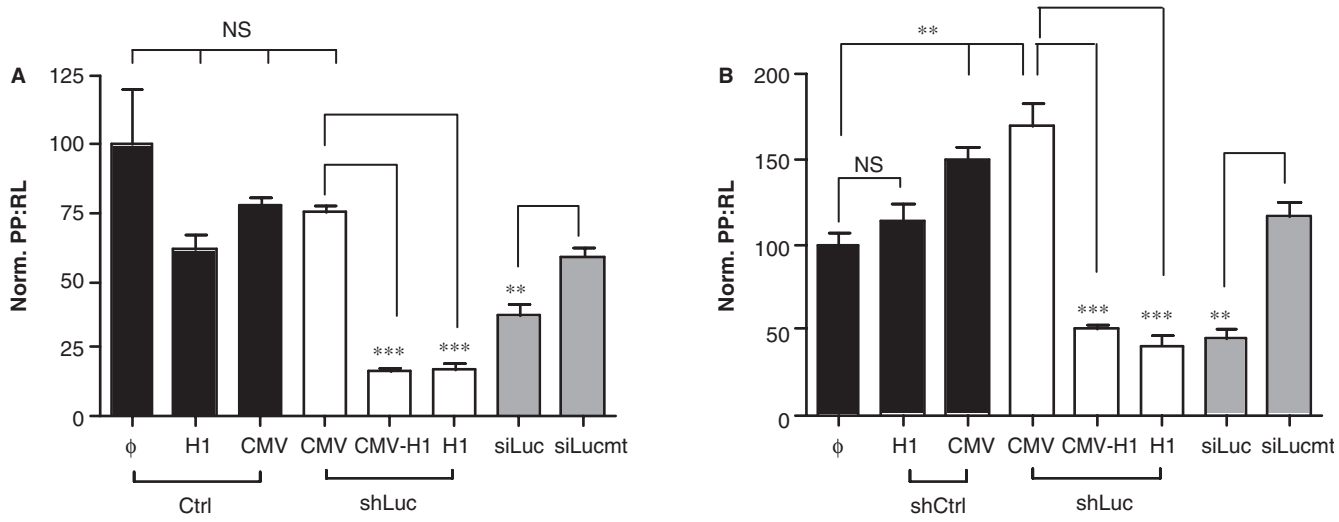

Figure 2. H1- and CMV-H1-shLuc show equal efficiency in cell cultures. (**A**) Plasmids containing shLuc (white bars) or control sequences (black bars) or siRNAs against PP luciferase (grey bars) were transfected in NIH 3T3 cells as described in the methods section, along with two reporter genes (pGL2-CMV and pRL-CMV). Only H1-shLuc and CMV-H1-shLuc lead to an inhibition, like siLuc. (**B**) HeLa X1/5 cells stably expressing firefly luciferase were transfected by the different plasmid constructions described in (a) or with siRNAs. As in NIH 3T3 cells, only H1-shLuc, CMV-H1-shLuc and siLuc provided significant inhibition of the endogenous luciferase expression. Means ± SEM are shown. NS = 'not significant'; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ ($n = 3$ samples per group). This graph is for a single representative experiment, which was repeated three times giving similar results.

(data not shown). The control CMV-shLuc did not provide inhibition of PP:RL ratio. Even though the CMV-shLuc construct was conceived so as not to produce hairpins we tried to verify its eventual ability to synthesize hairpins by northern blotting. Using northern blot protocols adapted for small size RNA inoculated from transfected 3T3 cells, we found that the CMV-H1-shLuc construct produced a visible 60-nt hairpin sequence, whereas no signal of a functional hairpin detection with the CMV-shLuc construction was seen (data not shown).

The same inhibition of an endogenous luciferase was obtained with H1-shLuc or CMV-H1-shLuc on HeLa cells stably expressing firefly luciferase, HeLa X1/5 (Figure 2b) and HeLa 2053 cell lines which expressed PPluc under the control of a tetracycline responsive promoter (Tet-ON) (data not shown). As shown in Figure 2, we observed a strong inhibition of PP:RL ratio in the presence of H1- or CMV-H1-shLuc, both constructs being able to inhibit endogenous gene expression.

H1-shLuc provides only limited inhibition of target gene expression in the newborn mouse brain

The first construction tested *in vivo* was the H1-shLuc plasmid, which was tested in the newborn mouse brain model. This model provides a useful paradigm for assessing the performance of vectors and plasmids in an *in vivo* context (29,30). After co-transfection of this construction along with target (PPluc) and control (RRluc) luciferase reporter genes in the lateral ventricles of newborn mice, we found that the H1-shLuc-driven inhibition of PPluc was very limited (Figure 3a). In fact, inhibition of target gene expression never exceeded 25% at any time point tested (from 16 to 72 h) (Figure 3b).

This result contrasts with the high efficiency of pSUPER-p53 as first described by Brummelkamp *et al.*, where an H1-mediated transcription of a p53-targeting shRNA leads to the strong knockdown of target gene expression in cell culture (8). Thus, it is worth noting that in our experiments in HeLa cells, the PP:RR ratio was decreased by >80% in the presence of H1-shLuc, a level that is ~2.5 times greater than the inhibition obtained in the mouse brain (25%) with the identical construction. This divergence suggests that the efficiency of shRNA-carrying plasmids in terms of target gene inhibition is cell context-dependent and supports the idea that each tissue and condition (*in vivo* versus *in vitro*) requires optimization for the use of shRNAs-triggered RNAi. Two hypotheses could explain this difference in the efficiency of H1-shRNAs in the mouse brain and in cell cultures. The first could be a lower copy number of shRNAs produced by H1 in the brain compared to cell cultures. The second possibility could be the lower efficiency of Dicer-induced processing of the hairpin in the brain that would lead to less efficient production of shRNA molecules for RNAi triggering.

The hybrid CMV-H1 construct provides the best inhibition of target gene expression in the mammalian brain

We next tested the CMV-H1-shLuc construction in the newborn mouse brain and found that it triggered >50% inhibition of PP:RRluc ratio (Figure 4a). The maximal inhibition obtained with this hybrid promoter construction was observed at 50 h post-transfection (Figure 4b) and was still visible at 72 h post-transfection (data not shown).

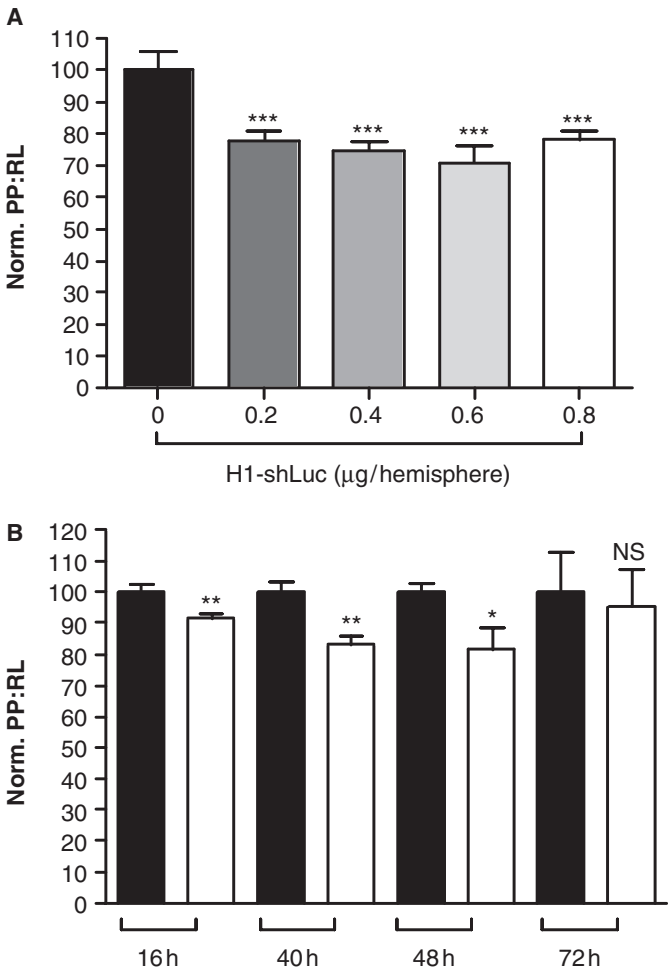

Figure 3. H1-shLuc provides only limited inhibition of a co-transfected luciferase reporter gene *in vivo*: dose dependence and time course. (**A**) Two luciferase reporter genes-containing plasmids (pGL2-CMV and pRL-CMV) were co-transfected by stereotaxic injection into the lateral ventricles of newborn mice brains, along with H1-shLuc construction at different concentrations (grey bars) ranging from 0.1 to 0.4 µg/µl, or with an irrelevant H1-shRNA as a control (black bar). The sequence of the shLuc hairpin is directed against its target gene PP-luc and has no homology with RL-luc, which serves as a control for transfection. The graph represents the normalization of PP-luc against RL-luc expression. In the presence of H1-shLuc at all doses tested, we observed a decrease of PP:RL ratio of ~25%. (**B**) The time course of H1-shLuc efficiency shows that inhibition of target gene expression does not vary between 16 and 48 h. At 72 h, no significant inhibition is found. Black and white bars correspond to 0.86 µg/hemisphere of irrelevant H1-shRNA and H1-shluc constructions, respectively. Means ± SEM are shown. NS = 'not significant'; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. $n = 10$ per group.

However, when tested in the adult mouse brain, the level of inhibition was not as strong as that observed in the newborn brain. In this situation, the CMV-H1-shLuc construct provided ~25% inhibition of PP:RRluc ratio compared to the control group. This inhibition was constant from 48 to 96 h post-transfection (Figure 4c). Note that in the adult mouse brain, the presence of the H1-shLuc construction did not produce any significant inhibition of its target luciferase gene (Figure 4c grey columns). So in the newborn and in the adult mouse brain context, the presence of the CMV enhancer upstream of the H1 type III promoter driving the synthesis of a hairpin sequence provides a significant improvement in terms of inhibitory effect induced by the shRNA.

Whilst this work was in progress, a paper appeared describing the use a PolIII promoter appended to a CMV enhancer (31), which is similar but distinct from our CMV-H1-shLuc construction, in which the entire CMV enhancer/promoter is located upstream of the H1-promoter. A further difference is that Ong *et al.* (2005) used the hybrid promoter first in plasmid constructs, *in vitro*, then in baculovirus-mediated infection of shRNAs *in vivo* in rat brains. In contrast, our results are obtained using non-viral constructs both *in vitro* and *in vivo*. Thus, given the generally greater facility of creating and using non-viral constructs, our findings that the hybrid promoter is optimal *in vivo* with plasmid-based constructs will open up new possibilities for applying shRNA technology in new settings.

CMV-shLuc is inefficient in both the newborn and in the adult mouse brain

In order to test whether the increased inhibitory efficiency of the CMV-H1 hybrid promoter was due to the CMV enhancer/promoter alone, we injected CMV-shLuc along with the two luciferase reporter genes in the brains of newborn mice. We found no inhibition of the target gene in the CMV-shLuc groups at any dose tested, compared to the control groups (Supplementary Figure 1). This construction was also tested in the adult mouse brain, where no inhibition was visible (data not shown). This result supports the idea that in the CMV-H1 hybrid promoter, the CMV only plays the role of an enhancer, and that the transcription of the shRNA is mediated by the type III promoter H1 alone and not by the CMV enhancer/promoter [as observed in (31)]. The position of the CMV promoter start site, >100 bp from the hairpin sequence, explains the lack of efficient shRNA production, as demonstrated by Xia *et al.* (12).

SiRNA or shRNA knockdown of thyroid hormone receptor α1 relieves T3-dependent repression of CyclinD1-luciferase transcription

After having demonstrated that the presence of a CMV enhancer/promoter upstream of an H1 promoter strongly increases the inhibition efficiency of shRNAs, we exploited this technology to follow physiological regulations in the mouse brain *in vivo*. Previous studies revealed both the role of thyroid hormones (TH) in NSC cycling *in vivo* (32), and the specific role of TRα1 as the mediator of this regulation. We hypothesized that the action of T3 on NSC cycling may implicate CyclinD1 and assessed whether this regulation was mediated or not by TRα1.

Indeed, CyclinD1 is known to be a T3-regulated gene. But T3-dependent regulations can vary as a function of tissue and developmental stage. For instance, T3 up-regulates CyclinD1 in the pancreas (33) and liver (34), while T3 represses CyclinD1 transcription in neuroblastoma cells (35). In order to assess CyclinD1 regulation in neurogenic areas of the brains of newborn

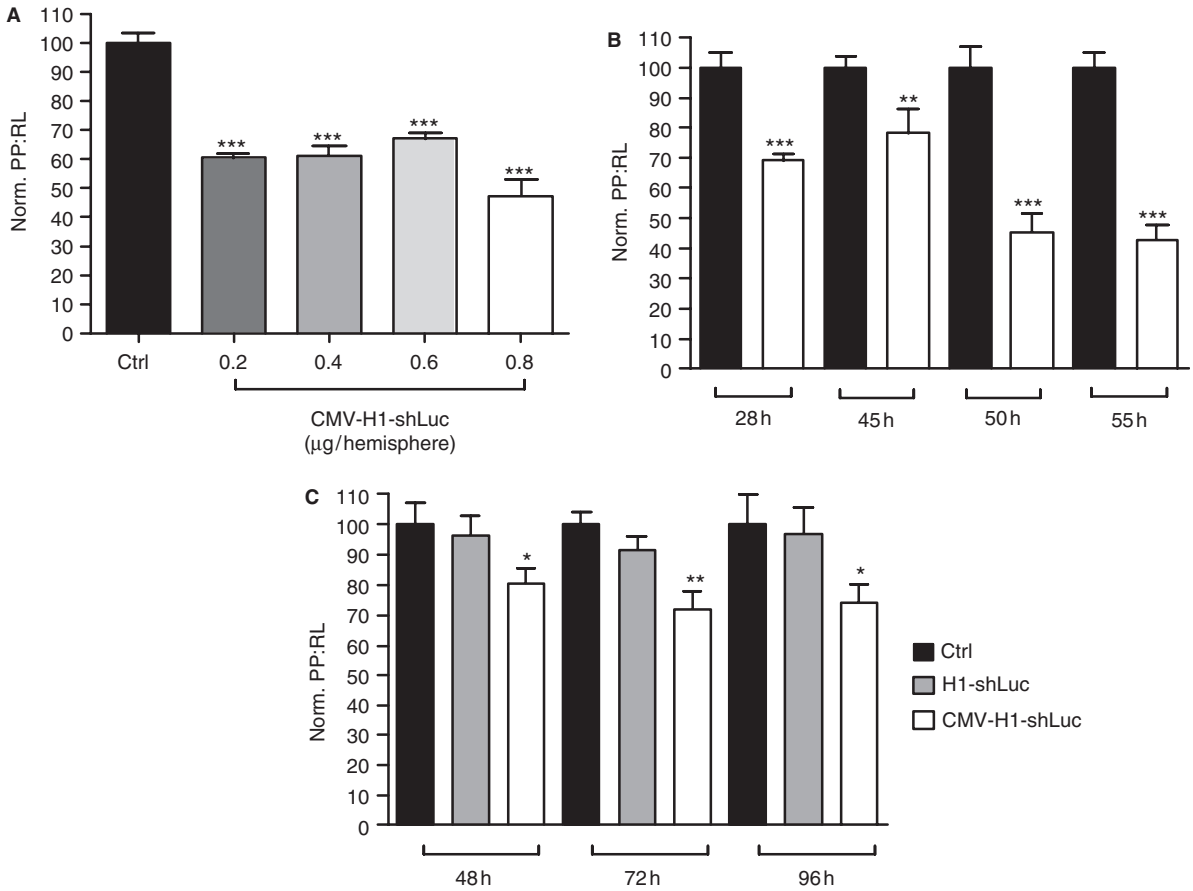

Figure 4. A CMV-H1 hybrid construct driving shLuc provides enhanced inhibition of a co-transfected target gene in the newborn (a and b) and in the adult (c) mouse brain. (**A**) Dose dependence of CMV-H1-shLuc efficiency. The inhibition efficiency of CMV-H1-shLuc was tested at different doses ranging from 0.1 to 0.4 μg/μl. After co-transfection of pGL2-CMV and pRL-CMV along with 0.4 μg/μl of CMV-H1-shLuc (i.e. 0.8 μg/hemisphere), we observed up to 50% inhibition of the targeted luciferase expression. This level of inhibition was obtained at 48 h post-transfection. (**B**) Time course efficiency of CMV-H1-shLuc. Significant inhibition of the target gene with 0.4 μg/μl of CMV-H1-shLuc was seen at all times tested. The maximal level of inhibition (50%) was seen at 50 h post-transfection. (**C**) In the adult brain, H1-shLuc provided no inhibition of PP:RL ratio (grey bars) compared to controls (black bars). CMV-H1-shLuc leads to 25% inhibition of the target gene at 72 h post-transfection (white bars) and up to 112 h post-transfection (data not shown). Means ± SEM are shown. NS = 'not significant'; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. $n = 10$ injected hemispheres per group.

mice, a CyclinD1-luciferase reporter gene was injected into the lateral ventricles of hypothyroid animals. Luciferase expression of the reporter gene was followed 18 h after T3 treatment compared to controls (Figure 5b). We observed a significant decrease of CyclinD1-luciferase expression in T3-treated animals. Thus, CyclinD1 transcription is negatively regulated in neurogenic areas lining the lateral ventricles of newborn mice brains.

In order to assess whether TRα1 is involved in this T3-mediated repression of CyclinD1 expression, we designed siRNAs directed against TRα1, and checked their efficiency and their specificity on 3T3 culture cells stably expressing TRα1 and TRα2 (Figure 5a). Using quantitative real-time RT-PCR, the siTRα1 designed was shown to specifically inhibit the expression of TRα1 and did not affect the expression of TRα2 at any dose tested (Figure 5a). By co-transfecting these siTRα1 along with the CyclinD1-luciferase reporter gene in the brain of euthyroid newborn mice, we observed a significant and dose-dependent increase in CyclinD1-luciferase expression (Figure 5c). The same result was obtained when using plasmids coding for shTRα1 under the control of a CMV-H1 construct: we observed a significant up-regulation of CyclinD1-luciferase expression in CMV-H1-shTRα1-injected mice brains (Figure 5d).

These data suggest first, that PEI-vectorized shRNA technology can be used to examine physiological regulations *in vivo* and second, that TRα1 may play a role in the regulation of CyclinD1 by thyroid hormones. Note that we observed the same increase in CyclinD1-luciferase expression following co-injection of either 200 nM of siTRα1 (Figure 5c) or by 0.5 μg of CMV-H1-shTRα1 plasmid per hemisphere (Figure 5d).

CONCLUDING REMARKS

The results presented here show that PEI-based delivery of shLuc to the newborn or the adult mouse brain is optimal when used with a plasmid construct bearing a hybrid CMV enhancer–H1 promoter. The system was most efficient in the newborn brain, reaching 50% inhibition

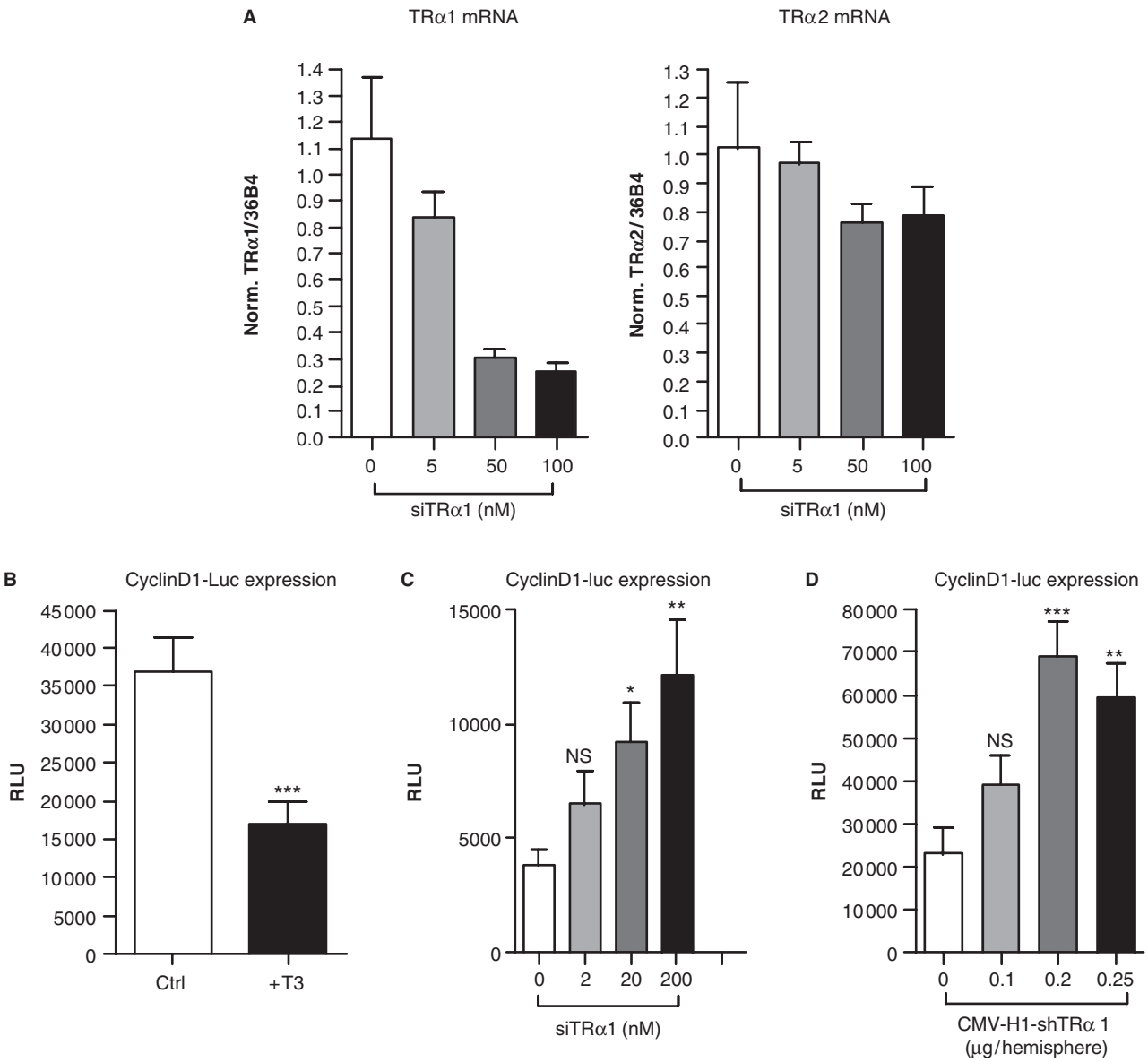

Figure 5. CyclinD1 transcriptional repression by T3 implicates thyroid hormone receptor α1 (TRα1). (**A**) Quantification of TRα1 and TRα2 mRNAs in 3T3 cells transfected by 5–100 nM of siTRα1 (500 µl per well). The total amount of TRα1 and TRα2 mRNA in each condition was normalized to that of 36B4 (a control gene) in the same group. Note that only TRα1, and not TRα2 expression, is inhibited by shTRα1 (**B**) CyclinD1-luciferase reporter gene expression *in vivo* is down-regulated in the presence of T3. CyclinD1-luciferase complexed by L-PEI was injected into the lateral ventricles of hypothyroid newborn mice. Then either a saline solution (NaCl 0.9%, control) or T3 (2.5 mg/kg body weight) was injected subcutaneously (see Methods section). Luciferase assays were performed 18 h later. T3 treatment induces a strong decrease in cyclinD1-luciferase expression compared to the control group. (**C**) Different doses (ranging from 2 to 200 nM) of siRNAs directed against TRα1 (siTRα1) were co-injected along with the CyclinD1-luciferase plasmid in the lateral ventricles of euthyroid newborn mice, inducing a strong activation of CyclinD1-luciferase expression. (**D**) The same experiment as described in (c) was performed by using plasmids coding for shTRα1 instead of siTRα1. The different doses of shTRα1-coding plasmid tested ranged from 0.1 to 0.25 µg/hemisphere. We observed a similar result to that seen with siTRα1, i.e. a strong de-repression of CyclinD1-luciferase following knockdown of TRα1. Means ± SEM are shown, $n = 20$ injected hemispheres per group. Each experiment was performed at least twice, providing similar results. NS = 'not significant'; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ ($n = 3$ samples per group).

of target gene expression. This level of inhibition is less than that obtained in the newborn mouse brain when siRNA against luciferase is delivered with cationic lipids (16). However, as cationic-lipid-delivered siRNA in the adult brain is inefficient (data not shown), we propose that this PEI-based delivery of shLuc under a hybrid promoter will become a useful tool for performing gene knockdown in the brain at different developmental stages. As this non-viral delivery technology combines high efficiency with the use of non-immunogenic components, it could also prove to be useful in terms of therapeutic applications. Indeed, the current demonstration that it can be used to dissect the role of different transcription factors in regulating specific genes, already opens up new possibilities for reverse

genetic studies focused on specific target tissues at defined developmental stages.

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Conflict of interest statement. None declared.

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