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2003-08-22

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# An enhanced U6 promoter for synthesis of short hairpin RNA

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Received May 20, 2003; Revised and Accepted June 25, 2003

## ABSTRACT

Short hairpin RNAs (shRNAs) transcribed by RNA polymerase III (Pol III) promoters can trigger sequence-selective gene silencing in culture and *in vivo* and, therefore, may be developed to treat diseases caused by dominant, gain-of-function type of gene mutations. These diseases develop in people bearing one mutant and one wild-type gene allele. While the mutant is toxic, the wild-type performs important functions. Thus, the ideal therapy must selectively silence the mutant but maintain the wild-type expression. To achieve this goal, we designed an shRNA that selectively silenced a mutant Cu,Zn superoxide dismutase (SOD1<sup>G93A</sup>) allele that causes amyotrophic lateral sclerosis. However, the efficacy of this shRNA was relatively modest. Since the allele-specific shRNA has to target the mutation site, we could not scan other regions of SOD1 mRNA to find the best silencer. To overcome this problem, we sought to increase the dose of this shRNA by enhancing the Pol III promoter. Here we demonstrate that the enhancer from the cytomegalovirus immediate-early promoter can enhance the U6 promoter activity, the synthesis of shRNA and the efficacy of RNA interference (RNAi). Thus, this enhanced U6 promoter is useful where limited choices of shRNA sequences preclude the selection of a highly efficient RNAi target region.

## INTRODUCTION

RNA interference (RNAi) can mediate sequence-selective suppression of gene expression in a wide variety of eukaryotes by introducing short RNA duplexes (small interfering RNAs or siRNAs) with sequence homologies to the target gene (1,2). Furthermore, short hairpin RNAs (shRNAs) transcribed *in vivo* under the control of RNA polymerase III (Pol III) promoters can trigger degradation of corresponding mRNAs similar to

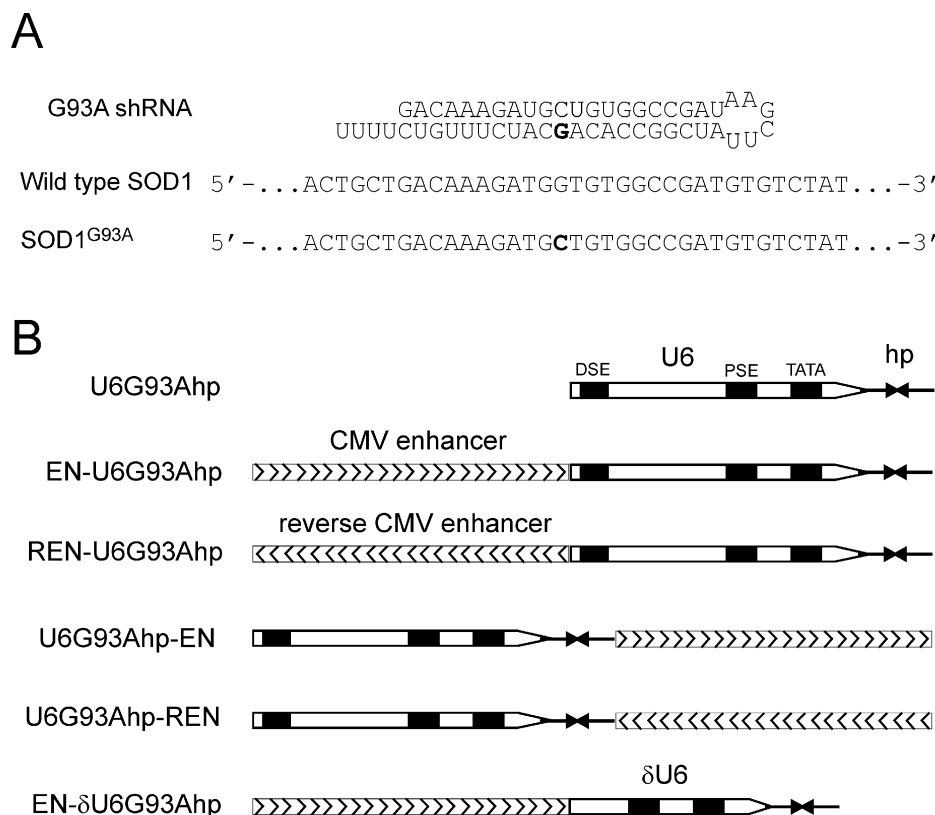
siRNAs and inhibit specific gene expression (3–11). Constructs that synthesize shRNA have been incorporated into viral vectors and these vectors can mediate RNAi in culture as well as *in vivo* (12–16). Therefore, Pol III–shRNA constructs may be developed to mediate long-term silencing of dominant, gain-of-function mutant genes that cause diseases.

In diseases caused by a gain-of-function type of mutation, the mutant is toxic but the wild-type performs important functions. Therefore, the ideal therapy should selectively silence the mutant gene but maintain the wild-type gene expression. Although opinions vary (17–19), many experiments have shown that siRNAs and shRNAs can discriminate between mRNAs that differ at a single nucleotide and selectively degrade the perfectly matched mRNA, while leaving the mRNA with a single nucleotide mismatch unaffected (7,9,12,17,20). The discriminating siRNA or shRNA must include the altered nucleotide in its sequence and, in most instances, the optimal design places the altered nucleotide near or at the middle of the siRNA or shRNA. This limits the sequence selection in designing siRNA or shRNA around the site of mutation. Because the sequence of siRNA or shRNA greatly influences the efficacy of RNAi (18,21), the sequences surrounding the mutation site may not be optimal and could produce poor inhibitors of the mutant gene.

We have designed an shRNA-expressing construct controlled by a Pol III U6 promoter (22) to silence a mutant Cu,Zn superoxide dismutase (SOD1<sup>G93A</sup>) allele that causes amyotrophic lateral sclerosis (ALS), a fatal degenerative motor neuron disease (23). While testing the efficacy of this shRNA, we found it selectively inhibited the expression of a mutant SOD1<sup>G93A</sup> but did not affect SOD1<sup>WT</sup> (24). However, the efficacy of RNAi produced by this construct was relatively modest, which might affect the ultimate therapeutic efficacy. One way to overcome this problem was to increase the dose of the shRNA by enhancing the Pol III promoter activity. We realized that some snRNAs are synthesized by Pol II while others are synthesized by Pol III, and they share enhancer elements (25–30). Hence, a Pol II enhancer might be able to enhance the Pol III transcription. We tested this by placing the enhancer from the cytomegalovirus (CMV) promoter near the U6 promoter and demonstrated that this enhanced the U6

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**Figure 1.** Design of hairpin constructs against mutant SOD1<sup>G93A</sup>. **(A)** Nucleic acid sequence surrounding the mutation site of SOD1<sup>G93A</sup>. Notice the G (bold) in the antisense strand of the hairpin that matches with the C (bold) in SOD1<sup>G93A</sup> but mismatches with a G in SOD1<sup>WT</sup>. **(B)** Variations of the U6 promoter. U6G93Ahp, authentic U6 promoter with G93A hairpin; EN-U6G93Ahp, forward CMV enhancer placed 5' of the U6 promoter; REN-U6G93Ahp, reverse CMV enhancer placed 5' of the U6 promoter; U6G93Ahp-EN, forward CMV enhancer placed 3' of U6G93Ahp; U6G93Ahp-REN, reverse CMV enhancer placed 3' of U6G93Ahp; EN-δU6G93Ahp, forward CMV enhancer placed 5' of the crippled U6 promoter with DSE deletion.

promoter activity, increased the shRNA synthesis and strengthened the silencing of the target gene. This enhanced promoter may be broadly useful in similar situations in targeting other disease-associated mutants.

## MATERIALS AND METHODS

### Plasmid construction

The SOD1<sup>G93A</sup>GFP fusion plasmid was constructed as described before (24). Briefly, mutant human SOD1<sup>G93A</sup> cDNA was PCR cloned between the PmlI and PstI sites of pCMV/myc/mito/GFP (Invitrogen). This cloning step deleted the mitochondrial targeting sequence. U6G93Ahp was constructed as described (6). Similarly, U6misG93A was created using the sequence GACAAAGCTGCTGTATCGGCT (sense strand), which contains five mismatched nucleotides (bold) against the SOD1<sup>G93A</sup> mutant. The CMV enhancer was PCR cloned from the pDsRed2-N1 vector (nucleotides 1–484; Clontech) and inserted either upstream between KpnI and NheI or downstream between NotI and SacI sites of U6G93Ahp.

### Cell culture and transfection

Human embryonic kidney cell line 293 was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented

with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Twenty-four hours before transfection, cells (70–90% confluency) were detached by trituration, transferred to 6-well plates and cultured in 10% FBS-containing medium without antibiotics. The cells were transfected with 4 µg of the target vector SOD1<sup>G93A</sup>GFP and 8 µg of each of the hairpin vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The transfection efficiency was ~95% in all experiments. After 24 h, the culture medium was changed to DMEM supplemented with 10% FBS and antibiotics. At 40 h after transfection, the cells were harvested and quickly frozen in liquid nitrogen.

### Measurement of GFP fluorescence intensity

The harvested cells were lysed in ice-cold reporter lysis buffer (Promega) containing protease inhibitors (complete, EDTA-free, 1 tablet/10 ml buffer; Roche Molecular Biochemicals). The lysate was cleared by centrifugation at 16000 g and 4°C for 10 min. The total protein in the cleared lysate was measured using the BCA assay (Pierce, Rockville, IL). The total protein concentration in each sample was adjusted to 0.5 mg/ml with the reporter buffer. Fluorescence of GFP in 140 µl of sample was measured by fluorescence spectroscopy (Photon Technology International) with excitation at 460 nm and recording from 480 to 600 nm. The spectrum peak was

detected at 502 nm, representing the fluorescence intensity of GFP. Fluorescence in the untransfected lysate was measured as background and subtracted from measurements of the transfected lysates.

### Western blots

Twenty micrograms of total protein were resolved on a 12% SDS-PAGE gel and transferred onto GeneScreen Plus membrane (Perkin Elmer). The membrane was incubated sequentially with a sheep anti-SOD1 (BioDesign) and horseradish peroxidase-labeled goat anti-sheep IgG (Amersham). The protein bands were visualized using SuperSignal kit (Pierce) and Kodak Digital Image Station 440CF.

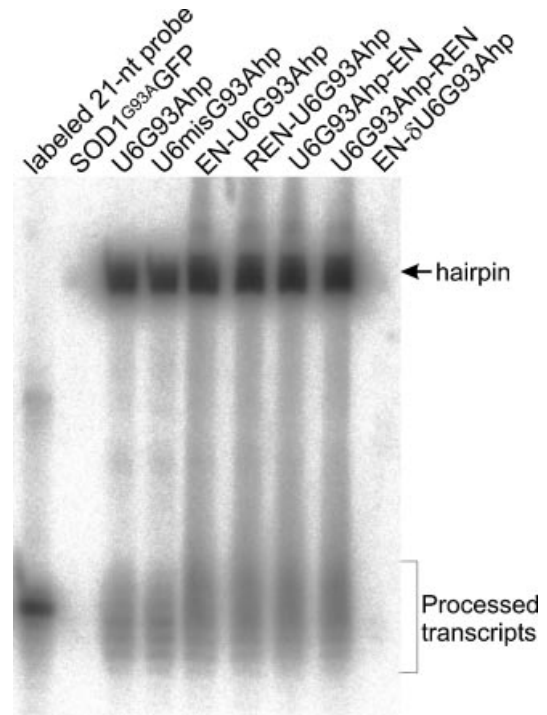
### Northern blot analysis

Cellular RNA was isolated with TRI reagent (Sigma). Twenty micrograms of total RNA were fractionated on a 15% polyacrylamide gel and transferred to Hybond<sup>TM</sup>-N<sup>+</sup> membrane (Amersham). The membrane was probed with <sup>32</sup>P-labeled synthetic RNA oligonucleotide complementary to the antisense strand of the G93A hairpin.

## RESULTS AND DISCUSSION

The shRNA against SOD1<sup>G93A</sup> (G93Ahp) contains a stem that is homologous to SOD1<sup>G93A</sup> mRNA but has a mismatched nucleotide with SOD1<sup>WT</sup> at the middle of the stem (Fig. 1A). When transfected into cultured cells, this shRNA selectively inhibited the expression of SOD1<sup>G93A</sup> but did not affect the expression of SOD1<sup>WT</sup> (24) (also see below). However, the RNAi efficacy of this shRNA was relatively poor (see below). We therefore sought to increase the potency of this shRNA by increasing its expression. We modified the U6 promoter by placing the enhancer from the CMV immediate-early promoter near the U6 promoter, either upstream or downstream from U6G93Ahp and in either forward or reverse orientation (Fig. 1B).

We co-transfected each of the seven constructs containing various combinations of U6 promoter, G93Ahp and CMV enhancer, with a target construct that encoded a SOD1<sup>G93A</sup> and GFP fusion protein (SOD1<sup>G93A</sup>GFP), into human 293 cells. Northern blot analysis demonstrated that addition of the CMV enhancer near U6G93Ahp in all four configurations (Fig. 1) increased the expression of G93Ahp (Fig. 2). Deletion of the distal sequence element (DSE), an obligatory component of the U6 promoter (25–28), abolished expression of G93Ahp even in the presence of the enhancer (Fig. 2). Quantification of SOD1<sup>G93A</sup>GFP expression by GFP fluorescence using a fluorometer (31) showed that, compared with SOD1<sup>G93A</sup>GFP alone transfection (Fig. 3A and B, 1), G93Ahp produced by the unmodified U6 promoter (U6G93Ahp) inhibited SOD1<sup>G93A</sup>GFP expression modestly (Fig. 3A and B, 2). Attaching the CMV enhancer in all four configurations to U6G93Ahp (Fig. 1B) enhanced the inhibition of SOD1<sup>G93A</sup>GFP expression to a similar degree (Fig. 3A and B, 3–6). Deletion of the DSE abolished the inhibition of SOD1<sup>G93A</sup>GFP expression (Fig. 3A and B, 7). Finally, U6 promoter directed synthesis of mismatched shRNA did not show any inhibitory activity towards the target gene (Fig. 3A and B, 8).



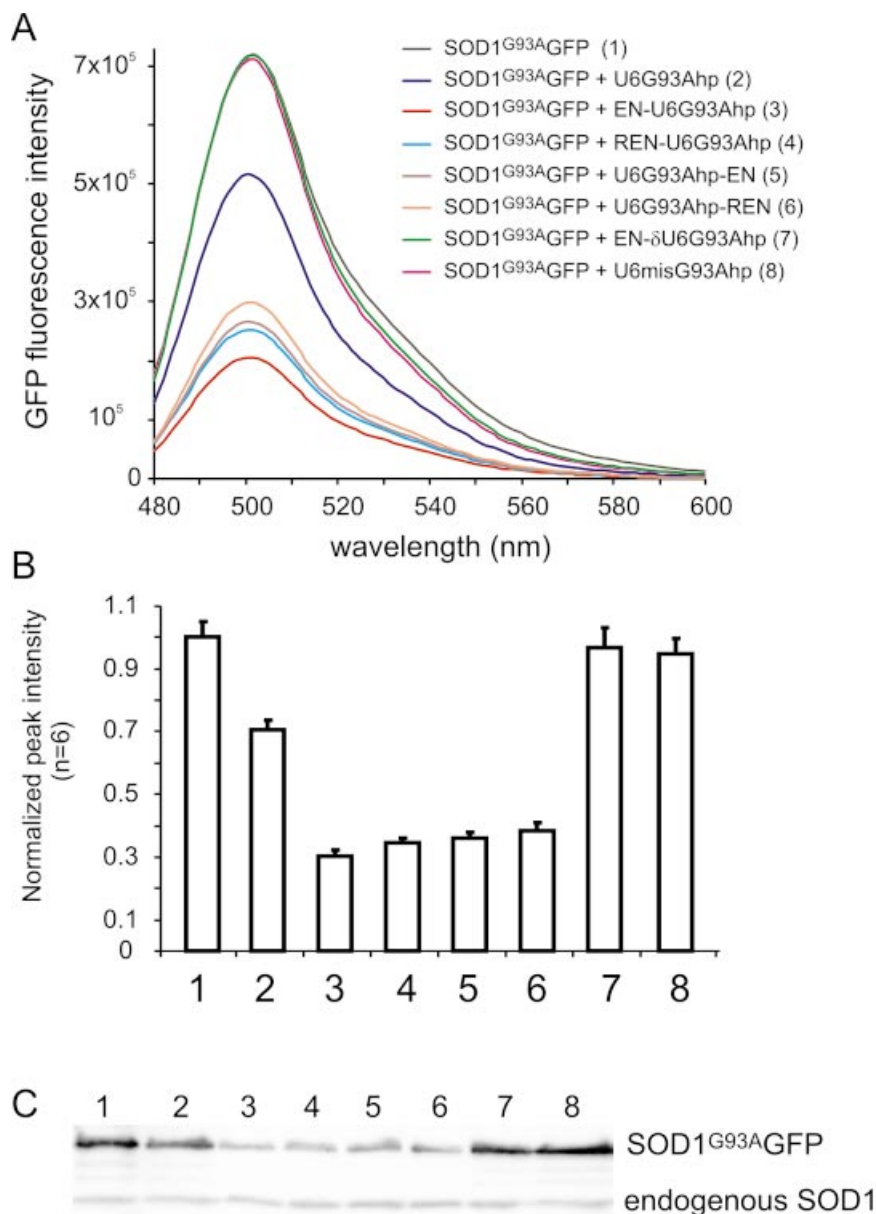
**Figure 2.** Northern blot detecting the G93Ahp transcripts. Total RNA was extracted from human 293 cells doubly transfected with SOD1<sup>G93A</sup>GFP and various U6G93Ahp constructs (Fig. 1B). G93Ahp was detected using a <sup>32</sup>P-labeled 21 nt RNA probe complementary to the antisense strand of the hairpin stem. Note the enhanced expression of the shRNA in cells transfected with the U6G93Ahp constructs containing the CMV enhancer.

Western blot using a polyclonal anti-SOD1 antibody confirmed the above finding and, furthermore, showed that the enhanced synthesis of G93Ahp only inhibited SOD1<sup>G93A</sup>GFP expression but did not affect the endogenous human SOD1 levels (Fig. 3C), indicating that the high levels of G93Ahp expression do not affect the specificity of G93Ahp for the mutant SOD1<sup>G93A</sup>.

Our results demonstrate that the CMV enhancer can enhance U6 promoter activity and increase the production of shRNA. This modified promoter may be useful where limited choices of shRNA sequences preclude the selection of a highly efficient RNAi target region and, therefore, could be used for selective inhibition of mutant gene expression *in vitro* and *in vivo* and to develop therapies for diseases caused by dominant, gain-of-function gene mutations.

### ACKNOWLEDGEMENTS

We thank Dr Guiliang Tang for teaching us northern blotting, Drs Phillip Zamore and Theresa Zhang for critically reading the manuscript and members of the Xu laboratory for advice and support. This work was supported by grants from the ALS Association, NINDS (RO1 NS41739 and NS35750) and The Robert Pachard Center for ALS Research at Johns Hopkins to Z.S.X. The contents of this report are solely the responsibility of the authors and do not necessarily represent the official views of the NINDS.



**Figure 3.** CMV enhancer increases the inhibition of target gene expression. (A) Fluorometer measurement of GFP fluorescence in lysates from the 293 cells transfected with SOD1<sup>G93A</sup>GFP and various U6G93Ahp constructs (Fig. 1B). (B) Average peak GFP fluorescence intensity from the six independent experiments shown in (A). The numbers on the x-axis correspond to the numbers in the legend to (A). Error bars indicate SEM. (C) Immunoblot of SOD1. The lane numbers correspond to the numbers in the legend to (A).

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