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Gene silencing in chick embryos with a vector-based small interfering RNA system

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In this paper, the use of vector-based RNA interference (RNAi) to specifically interfere with gene expression in chick embryos is reported. *In ovo* electroporation was carried out to transfer a small interfering RNA (siRNA) expression vector into chick embryos. *En2* was chosen for the target gene because the family gene, *En1*, is expressed in a similar pattern. Four sets of 19-mer sequences were designed with the *En2* open reading frame region connected to a sequence of short hairpin RNA (shRNA), which exerts siRNA effects after being transcribed, and inserted into pSilencer U6-1.0 vector. *En2* and *En1* expression were suppressed by the siRNA whose sequence completely matched *En2* and *En1*. Suppression occurred when the siRNA sequence differed by up to two nucleotides from the target sequence. The sequence that differed by four nucleotides from the target gene did not show siRNA effects. One set that completely matched the *En2* target did not show siRNA effects, which may be due to location of the siRNA in the target gene. Thus, multiple sets of shRNA must be prepared if we are to consider. This system will greatly contribute to the analysis of function of genes of interest, because the target gene can be silenced in a locally and temporally desired manner.

Key words: chick, *engrailed*, *in ovo* electroporation, RNAi, shRNA, siRNA.

Introduction

In the field of experimental embryology, chick embryos have been used as a model animal because of accessibility to the embryos. Since *in ovo* gene transfer by electroporation was developed (Muramatsu *et al*. 1997; Ogino & Yasuda 1998; Funahashi *et al*. 1999), we can analyze the function of a gene of interest conventionally. But in chick embryos, it is still difficult to knock down function of certain genes, because it is not possible to make mutant animals by gene targeting. In some studies, antagonistic constructs have been used (Araki & Nakamura 1999; Matsunaga *et al*. 2000), but this method is limited to certain kinds of molecule. Morpholino antisense oligonucleotides have recently been used in chick embryos, and have been shown to be efficient for gene silencing (Kos *et al*. 2001; Kos *et al*. 2003; Sugiyama & Nakamura 2003). We can transfer fluorescein-conjugated morpholino antisense oligonucleotides by electroporation, but because these molecules interfere with translation, we need antibody against the molecule to evaluate the results precisely.

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Powerful gene silencing by double-stranded RNA, known as RNA interference (RNAi), was developed in *Caenorhabditis elegans* and *Drosophila* (Fire *et al*. 1998; Kennerdell & Carthew 1998). Double-stranded RNA molecules of 21 bp, termed small interfering RNA (siRNA), also have gene-silencing activity in mammalian cells (Caplen *et al*. 2001; Elbashir *et al*. 2001a; Harborth *et al*. 2001). Recently, it was shown that short hairpin forming 45–50-mer RNA (shRNA) that is complementary to the gene of interest has RNAi effects (Svoboda *et al*. 2001; Brummelkamp *et al*. 2002; Paddison *et al*. 2002; Paul *et al*. 2002; Sui *et al*. 2002; Yu *et al*. 2002). An siRNA expression vector for mammalian cells has been developed, and gene silencing by transfecting cultured cells with the expression vector has been reported (Svoboda *et al*. 2001; Brummelkamp *et al*. 2002; Lee *et al*. 2002; Miyagishi & Taira 2002; Paddison *et al*. 2002; Paul *et al*. 2002; Sui *et al*. 2002; Yu *et al*. 2002).

We wondered if vector-based siRNA could be realized by *in ovo* electroporation in chick embryos. Because siRNA is reported to exert its effects by degrading mRNA in an RNase III-dependent manner, the effect could be conveniently detected by *in situ* hybridization. To confirm the sequence specificity and the site dependency of siRNA for the target gene, we tested the *En2* gene, because the *engrailed* family *En1* is expressed in a similar expression pattern to

En2 in the midbrain–hindbrain (Gardner *et al.* 1988; Patel *et al.* 1989). Thus, *En1* would be a good control.

Materials and Methods

Design of shRNA sequences

We designed shRNA to interfere with *En2* expression, referring to technical information of Ambion (Austin, TX, USA) and NipponBioService (Asaka, Saitama, Japan). Four sets of 19-mer oligonucleotides, immediately downstream of an AA dinucleotide, were selected from the chick *En2* open reading frame (ORF) sequence (Fig. 1a) and were named En2-150, En2-582, En2-648 and En2-846, according to the number from the first base of the start codon (ATG). En2-150, in the engrailed homology region (EH) 1 domain, was completely homologous to the corresponding part of the *En1* sequence (Logan *et al.* 1992). En2-648, in the homeodomain, differed by two nucleotides from the corresponding part of the *En1* sequence; En2-582, in the EH3 domain, differed by six nucleotides from the corresponding part of the *En1* sequence; and En2-846 differed by eight nucleotides from the corresponding part of the *En1* sequence. An oligonucleotide in which four nucleotides were substituted from En2-648 was designed, and designated as En2-648s4 (Fig. 1b). We confirmed that selected oligonucleotide sets did not have homology to any other genes by a BLAST search, so that they would not interfere with other genes.

The oligonucleotides were synthesized and column-purified at NipponBioService. The 19-mer sense siRNA sequence and antisense siRNA sequence were linked with a nine nucleotide spacer (TTCAAGAGA) as a loop. Six T bases and 6 A bases were added as a termination signal to the 3′ end of the forward oligonucleotides and 5′ end of the reverse oligonucleotides, respectively. Then four nucleotides corresponding to the *Eco*RI (AATT) and *Apa*1 (GGCC) restriction sites were added to the 5′ and 3′ end of the reverse oligonucleotides, respectively (Fig. 1c).

Selection of a clone

Forward and reverse oligonucleotides were incubated in annealing buffer (100 mM K-acetate, 30 mM HEPES-KOH (pH 7.4) and 2 mM Mg-acetate) for 3 min at 90°C, followed by incubation for 1 h at 37°C. The annealed DNA for siRNA was ligated with linearized pSilencer1.0-U6 siRNA expression vector (Ambion) at *Apa*1 and *Eco*RI sites. After transfection, many clones were picked and sequenced from both sides. Many clones contained no insert or mutated insert, and some inserts were hard to sequence because they formed very complex structures. To improve sequencing, we added dimethylsulfoxide (DMSO) up to 5% in

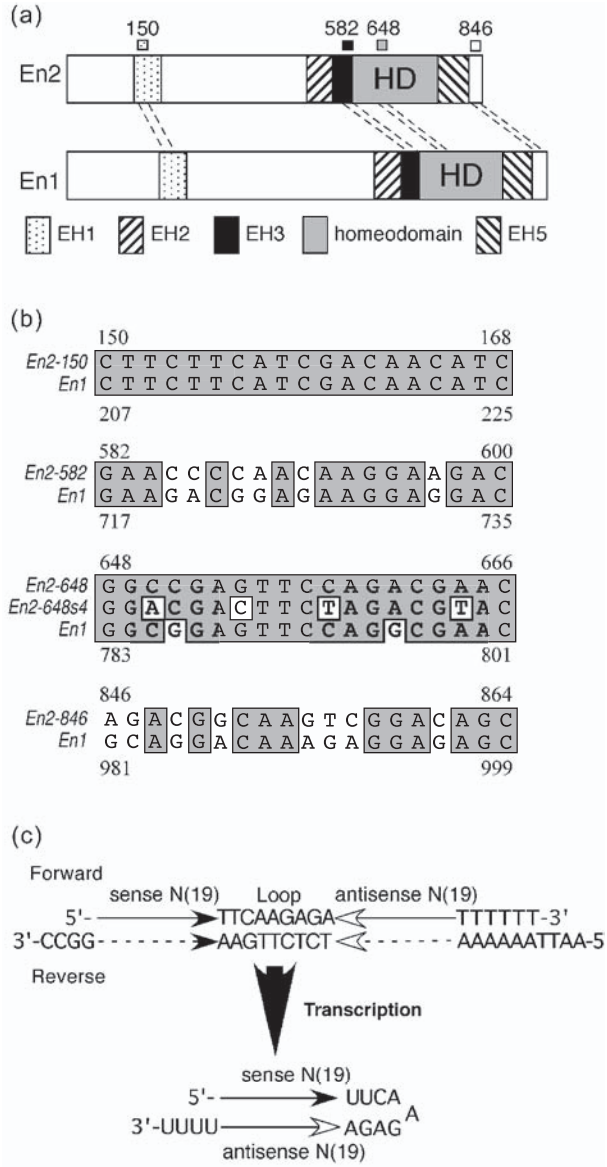

Fig. 1. Design of vector-based *En2* short hairpin RNA (shRNA). (a) Location of the small interfering RNA (siRNA) in the *En2* open reading frame (ORF) and the corresponding sequence of *En1* in the ORF. The number above the selected sequences for siRNA indicates the initial number in the ORF sequence. Specific domains, such as EH1, EH2, EH3, EH5 and homeodomain, are depicted as boxes. (b) Detailed sequences for siRNA and their sequences as they correspond to *En1*. Oligonucleotides for siRNA were named En2-150, En2-582, En2-648 and En2-846, according to the number from the first base of the start codon (ATG). (c) Design of shRNA. Sense and antisense sequences are linked with the loop sequence, and six T bases were added to the 3′ end of the forward strand. The upper strand is the forward oligonucleotide and the lower strand is the reverse oligonucleotide. The predicted secondary structure of the shRNA transcript is depicted at the bottom.

the reaction mixture. We used a clone that does not contain mutation.

In ovo *electroporation*

Fertile chick eggs obtained from a local farm were incubated at 38°C. They were staged according to Hamburger and Hamilton (1951). *In ovo* electroporation was carried out to transfect the plasmid to stage 10 chick embryos as previously reported (Funahashi *et al*. 1999; Nakamura *et al*. 2000). Green fluorescence protein (GFP) expression vector (pEGFP-N1; Clontech, Palo Alto, CA, USA) was co-electroporated with siRNA constructs to check efficiency. Plasmid solution was 1–2 μg/μL. As described previously (Funahashi *et al*. 1999; Nakamura *et al*. 2000), transfection occurs on the hemilateral side of the neural tube, and the other side serves as a control.

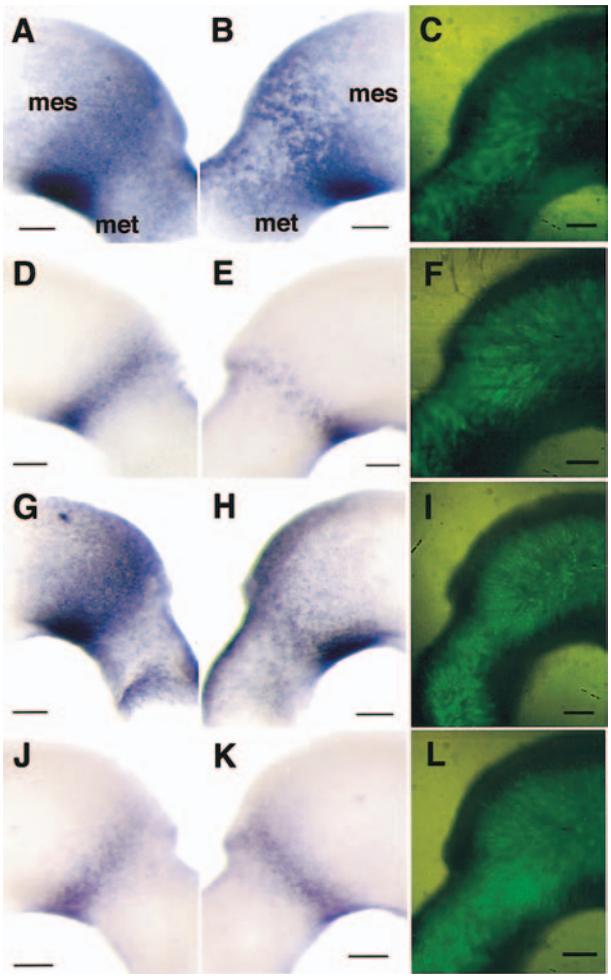

Fig. 2. Electroporated short hairpin RNA (shRNA) constructs showed RNA interference (RNAi) effects in the midbrain–hindbrain region 24 h after electroporation. Whole-mount *in situ* hybridization for *En2* (A,B,G,H) and *En1* (D,E,J,K). Green fluorescent protein (GFP) fluorescence shows the transfected sites (C,F,I,L). Photographs on the same row are from the same embryo. En2-150 completely matched the corresponding sequence of *En1*, and it suppressed both *En2* (A,B) and *En1* (D,E) expression. En2-582 suppressed *En2* expression, but reduction of *En2* mRNA was weak (H). This may be due to the fact that En2-582 contained four consecutive C bases, or to less accessibility of small interfering RNA (siRNA) to the En2 mRNA because of the complex secondary structure of the mRNA. *En1* was not reduced by En2-582 (K), which may be due to the fact that En2-582 differed by six nucleotides from the corresponding *En1* sequence. Control side (A,D,G,J). Experimental side (B,E,H,K). mes, mesencephalon; met, metencephalon. Bars, 100 μm.

In situ *hybridization and immunohistochemistry*

Specimens were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). In embryos at a later stage than 14, the epidermal and mesenchymal tissues were removed from embryos, and the neural tube was exposed. Whole-mount *in situ* hybridization was carried out as previously described by Bally-Cuif *et al*. (1992), except that the hybridization and wash were carried out at 65°C rather than at 70°C. Digoxigenin (DIG)-labeled antisense RNA probe was used. Alkaline phosphatase (ALP)-conjugated anti-DIG (Roche, Mannheim, Germany) was used for detection, with the color substrates 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). After whole-mount *in situ* hybridization, embryos were immunostained with anti-En2 monoclonal antibody 4D9 (Patel *et al*. 1989) as a primary antibody, and horse radish peroxidase (HRP)-conjugated antimouse antibody (Jackson, West Grove, PA, USA) as a secondary antibody (Funahashi *et al*. 1999). NBT/BCIP color was removed to detect only the HRP immunostaining by incubating in N,N-dimethylformamide (DMF) at 55°C for 3 h.

En2 antisense probe was a 687 bp fragment (–130–457). *En1* antisense probe was a 590 bp fragment (– 38–552), as described previously (Itasaki & Nakamura 1992).

Results

Gene silencing by electroporation with shRNA expression plasmids

To explore the conventional RNA interference system in chick embryos, we attempted to apply electroporation to transfect plasmids that encode shRNA. We selected *En2* for silencing, because *En1* could be used as a control. Four sets of 19-mer oligonucleotides that are in the *En2* ORF were designed: En2-150, which completely matches the corresponding sequence of *En1*; En2-648 and En2-582, which differ

by two and six nucleotides, respectively, from the corresponding sequence of *En1*; and En2-846, which differs by eight nucleotides from the corresponding sequence of *En1* (Fig. 1a,b).

Expression vector of GFP was co-electroporated to assess the transfection site of the shRNA. shRNA was transcribed by RNA polymerase III, and GFP was transcribed by RNA polymerase II. Because both plasmids assure ubiquitous expression, GFP-expressing cells were regarded as shRNA-transfected cells.

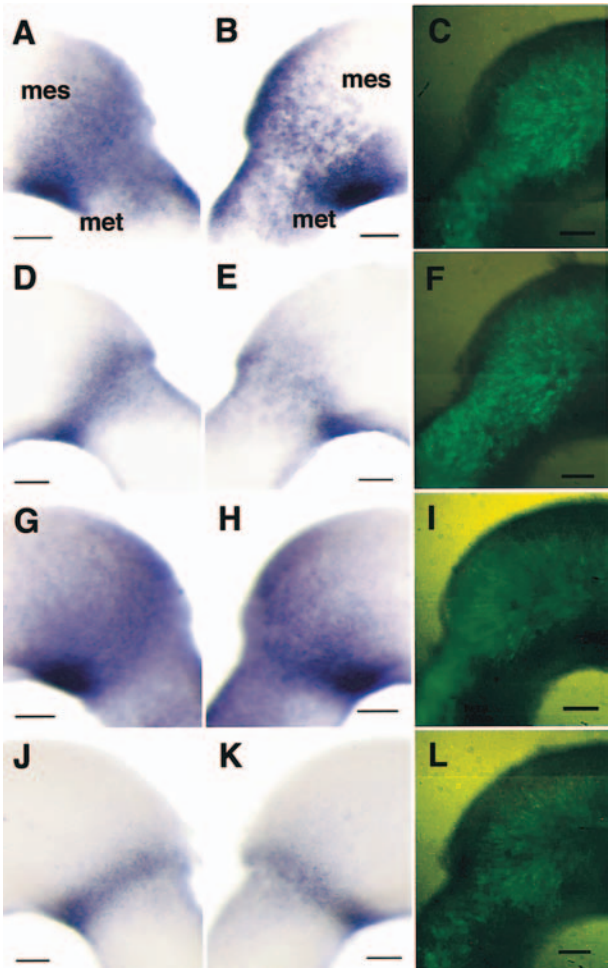

Fig. 3. Sequence specificity of the RNA interference (RNAi) effects in the midbrain–hindbrain region 24 h after electroporation. Whole-mount *in situ* hybridization for *En2* (A,B,G,H) and *En1* (D,E,J,K). Green fluorescent protein (GFP) fluorescence shows the transfected sites (C,F,I,L). Photographs on the same row are from the same embryo. En2-648 suppressed *En2* as clearly as En2-150 (B), and suppressed *En1* weakly (E). This result indicates that short hairpin RNA (shRNA) could interfere with the sequence that differed by two nucleotides from the target sequence (Fig. 1B). En2-648s4, a four nucleotide substitution from *En2* and six nucleotide substitution from *En1*, did not affect *En2* and *En1* expression (H,K). Control side (A,D,G,J). Experimental side (B,E,H,K). mes, mesencephalon; met, metencephalon. Bars, 100 μm.

Electroporation with En2-150 expression vector interfered with expression of both *En2* and *En1* (n = 11 and n = 9, respectively; Fig. 2A–F). It is difficult to compare the degree of interference between *En2* and *En1*, because the expression pattern of *En1* (Fig. 2D) and *En2* (Fig. 2A) are a little different from each other. However, it appeared that En2-150 interfered similarly with *En2* and *En1*.

Electroporation with En2-582 interfered with *En2* expression (n = 3) in a somewhat different manner from that by En2-150 (Fig. 2B,H). Repression by En2-582 seemed weaker than by En2-150, which may be due to the fact that En2-582 contains four consecutive C bases (the manufacturer of oligonucleotides recommends to avoid more than three consecutive C bases). Alternatively, it may be due to the fact that siRNA may be less accessible to the target gene because of the complex secondary structure in this region of the mRNA (Leirdal & Sioud 2002; Miyagishi & Taira 2002). En2-582 differed by six nucleotides from the corresponding *En1* sequence, and did not affect *En1* expression (n = 3; Fig. 2J–L).

Electroporation with En2-846 did not interfere with *En2* expression (n = 6), which indicates that the matching site of En2-846, near the C-terminal end of the *En2* ORF, may be located in the complex secondary structure of the mRNA. Therefore, En2-846 could not access the target mRNA. *En1* expression was not affected by En2-846 either (n = 6, data not shown).

Specificity of gene silencing by shRNA

The specificity of the shRNA sequence was tested by En2-648 and En2-648s4. En2-648 differs by two nucleotides from the corresponding sequence of *En1*. Four nucleotides were substituted in En2–648s4. Consequently, En2-648s4 differs by four nucleotides from *En2* and six nucleotides from *En1* (Fig. 1b).

En2-648 silenced *En2* expression as did En2-150, as was assessed by *in situ* hybridization (Figs 2A–C, 3A–C). *En1* expression was also affected, but the degree of interference of *En1* was less than that of *En2* (Fig. 3D–F). The results indicate that shRNA could interfere with a sequence that differed by two nucleotides from the target gene.

En2-648s4, in which four nucleotides were substituted from the *En2* sequence, did not interfere with *En2* expression (n = 3; Fig. 3G–I). En2-648s4, which differed by six nucleotides from the target sequence, did not repress *En1* expression (n = 3; Fig. 3J–L). In summary, shRNA could affect sequences that differed by two nucleotides from the target sequence. Sequences that differed by more than four nucleotides could not be affected.

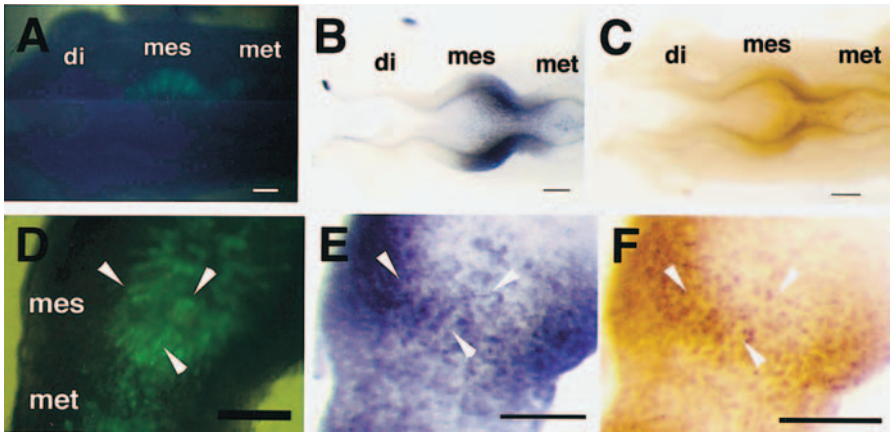

Fig. 4. Time-course of small interfering RNA (siRNA) effect on *En2*. Dorsal view of an embryo 6 h after electroporation (A–C). View from the experimental side of an embryo 12 h after electroporation (D–F). The *En2* mRNA level was reduced by 6 h after electroporation (compared with the control side; B). At this time, the En2 protein could not be detected (C). Short hairpin RNA (shRNA) expression sites are indicated by green fluorescent protein (GFP; D). *En2* mRNA (E) and protein (F) were clearly reduced by 12 h after electroporation. The corresponding white arrow head indicates the same location in (D), (E) and (F). Color for whole-mount *in situ* hybridization (blue) was destained after horse radish peroxidase immunostaining (brown; C,F). di, diencephalon; mes, mesencephalon; met, metencephalon. Bars, 100 μm.

Time-course of gene silencing

Effects of shRNA were detectable 6 h after electroporation by *in situ* hybridization (n = 6; Fig. 4A,B). At 6 h after electroporation, reduction of RNA signal was slight (Fig. 4B), and immunostaining on the same embryo showed that reduction in the protein was at an undetectable level (Fig. 4C).

By 12 h after electroporation, effects of shRNA became clear. *In situ* hybridization and immunostaining on the same embryo revealed that *En2* expression was silenced at the site where strong GFP fluorescence was observed (n = 6; Fig. 4D–F).

We followed the effects of En2-150 and En2-648 until 48 h after electroporation. Silencing could be detected until the stage we examined (n = 4, data not shown). Because *En2* expression becomes very weak after that stage, we did not follow its expression further.

Discussion

The present study has shown that *in ovo* transfection with shRNA expression vector effectively repressed target gene expression. We designed four sets of oligonucleotides that make hairpin loops and contain 19-mer forward and reverse sequences in the *En2* ORF. The oligonucleotides were inserted into pSilencer 1.0-U6, and transfected by electroporation into the midbrain–hindbrain region. Two siRNA, En2-150 and En2-648, effectively repressed *En2* expression, as assessed by *in situ* hybridization. This may indicate that shRNA caused mRNA degradation, because the probe for *in situ* hybridization is the N-terminal region, and does not cover the En2-648 region. In addition, repression of translation product of *En2* by shRNA was assessed by immunohistochemistry with anti-En2 antibody. Another siRNA, En2-582, also repressed *En2* expression, but its effect seemed weaker than that of En2-150 and En2-648. This may be due to the fact that En2-582 contained four consecutive C bases (not recommended by the manufacturer for siRNA), or due to less accessibility of siRNA because of the secondary structure of mRNA. The other siRNA, En2-846, did not affect *En2* expression. En2-846 matches the sequence very near to the C-terminal region of the *En2* ORF, and this site may be difficult for the siRNA to access because of the complex secondary structure of the target mRNA. Therefore, multiple sets of shRNA need to be prepared to silence the gene of interest (Jarvis & Ford 2001; Holen *et al*. 2002; Lee *et al*. 2002; Sørensen *et al*. 2003).

Some researchers have reported that even a one nucleotide mismatch of shRNA failed to suppress target gene expression (Elbashir *et al*. 2001a; Elbashir *et al*. 2001b; Brummelkamp *et al*. 2002; Yu *et al*. 2002; Zhang *et al*. 2003). Elbashir *et al*. (2001b) reported that substitution of only one nucleotide near the center of the siRNA abolished its effects, but that a two to four nucleotide substitution near the 3′ end of the siRNA did not affect its effects significantly. In the present study, shRNA that mismatched by two nucleotides suppressed gene expression. shRNA that differed by more than four nucleotides from the target gene did not exert RNAi effects.

It has been shown that silencing of the target gene can be detected by 4 h after application of synthesized siRNA to cultured cells (Byrom *et al*. 2002). In the present study, we transfected expression vector of *En2* shRNA to live chick embryos by electro-

poration. A slight decrease in *En2* mRNA could be detected by 6 h after electroporation. The results suggest that shRNA may have been transcribed rapidly to silence the target gene. Reduction in translation product was very subtle at 6 h after electroporation, but was clearly detected by 12 h after electroporation.

Realization of gene silencing in chick embryos has been long awaited. To date, morpholino antisense oligonucleotides have been used as a tool for gene silencing in chick embryos (Kos *et al*. 2001; Kos *et al*. 2003; Sugiyama & Nakamura 2003), but with this system, expensive antibodies against the gene products are needed to check if the genes have really been silenced or not. An shRNA system with an expression vector is very convenient to prepare and to apply to the embryo. This method will assure locally and temporally restricted gene silencing, and will greatly contribute to functional analysis of genes of interest.

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