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Review

## Gain- and loss-of-function in chick embryos by electroporation

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### Abstract

It remained very difficult to manipulate gene expression in chick embryos until the advent of *in ovo* electroporation which enabled the induction of both gain-of-function, and recently loss-of-function, of a gene of interest at a specific developmental stage. Gain-of-function by electroporation is so effective that it has become widely adopted in developmental studies in the chick. Recently, it became possible to induce loss-of-function by introducing an siRNA expression vector by electroporation. In this review, the methods of electroporation for gain-of-function and for loss-of-function by siRNA are discussed.

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### 1. Introduction

The chick embryo has been a cornerstone of experimental embryology since it is easily accessible for manipulation during embryogenesis, and this model system has contributed greatly to our understanding of tissue interactions during development. In particular, transplantation experiments between chick and quail have enabled close monitoring of the fate of transplanted cells, establishing both the migratory pathways and the repertoire of neural crest cell derivatives (Le Douarin, 1982, 2000). However, gene targeting experiments have been difficult in the chick, because the embryos remain in the oviduct and uterus for the first 24 h of embryogenesis, which has limited analyses of gene function, and led to a gradual decrease in interest in this developmental model.

Muramatsu et al. (1997) showed that electroporation could be used to induce misexpression of certain genes in chick embryos. We subsequently established the conditions for the routine use of *in ovo* electroporation for efficient ectopic expression of genes in chick embryos (Funahashi et al., 1999; Nakamura et al., 2000). Recently, gene silencing by the introduction of an siRNA expression vector by electroporation has become possible (Katahira and Nakamura, 2003). Here, we review the application of

these methods for the misexpression and silencing of a gene of interest in the chick embryo.

### 2. Gain-of-function by *in ovo* electroporation

#### 2.1. Procedure for *in ovo* electroporation

Application of an electric field to cells can induce the reversible opening of pores in the cell membrane. To limit damage to the embryos, we use repeat charging of low voltage rectangular pulses.

We routinely use the pMiwIII expression vector, which is a modified version of pMiwSV that has a Rous sarcoma virus (RSV) enhancer and a  $\beta$ -actin promoter (Suemori et al., 1990; Wakamatsu et al., 1997) with an additional multicloning site (Matsunaga et al., 2000). The pRc/CMV vector (Invitrogen Co., Carlsbad, CA, USA), which has a cytomegalovirus (CMV) enhancer, also works well. These vectors consistently give transient expression for at least 72 h. For long-term misexpression, the RCAS provirus vector is more effective. If a virus vector is electroporated into virus-resistant embryos, misexpression is restricted to the descendants of the transfected cells, which enables us to trace the cell lineage (Sugiyama and Nakamura, 2003).

For the transfection into the neural tube of stage 10 embryos, the vitelline membrane is cut to allow DNA

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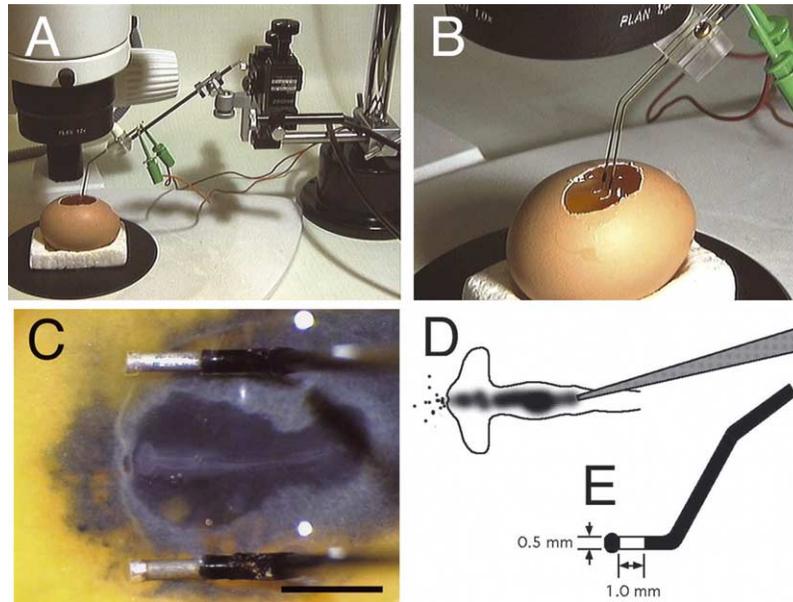


Fig. 1. In ovo electroporation. A pair of electrodes held by a manipulator (A) are inserted from a window opened on the shell (B). The electrodes are placed on the vitelline membrane overlying the embryo (C), and a 50 ms, 25 V pulse is charged five times. The entire procedure is carried out under a dissection microscope. Plasmid solution is injected into E1.5 (HH stage 10) chick neural tube (D) prior to the pulse charge. Dimensions of the electrode are shown schematically in (E). Most part of the electrode is insulated (black in figure) so that only the tip is exposed (white area). Bar: 2 mm, (C) (from Funahashi et al., 1999).

solution to be injected into the central canal with a micropipette (Fig. 1D). The embryos are easier to observe if Indian ink (Rotring) diluted 4- to 5-fold with Hanks' buffered saline solution is injected underneath the embryo (Fig. 1C).

A pair of electrodes (0.5 mm in diameter, 1 mm exposure, Unique Medical Imada) are set on a micromanipulator (MN-151; Narishige, Fig. 1A–E), and placed on the vitelline membrane 4 mm apart. Then a rectangular pulse of 50 ms, 25 V is charged 3–5 times. It is possible to restrict

transfection to very small region of the embryo by using a tungsten electrode as a cathode, placing it in the lumen of the neural tube, and applying a 25 ms, 7–8 V pulse that is charged twice (Sugiyama and Nakamura, 2003).

## 2.2. Expression of transfected DNA

The negative charge on DNA means that only the tissue at the anode side is transfected (Fig. 2A–D). Co-electroporation with a vector encoding *lacZ* reporter or GFP makes

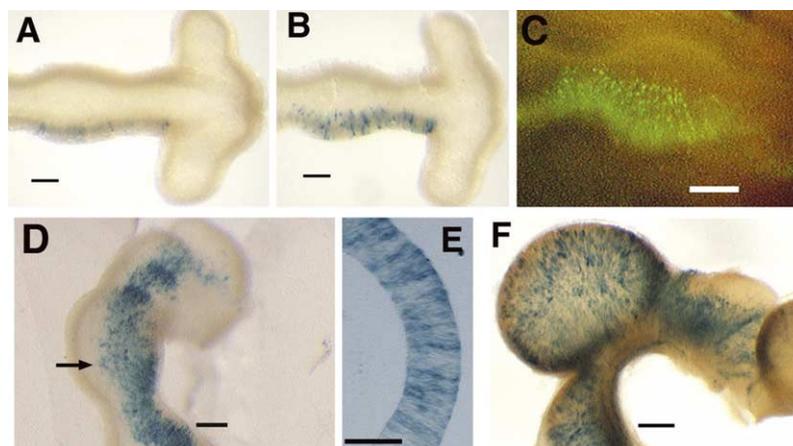


Fig. 2. Efficiency of electroporation. The efficiency of in ovo electroporation can be evaluated by co-injecting *lacZ* expression vector (pMiwZ) or *GFP* expression vector (pEGFP-N1). The translation product of *LacZ* is already recognizable 2 h after electroporation (A), and becomes stronger at 3 h (B). (C) Nine hours after electroporation with *GFP* vector. The efficiency of transfection can also be evaluated in ovo with *GFP* vector. In the transfection zone, 24 h after electroporation (D, E), more than half of the cells express *lacZ*. The expression is transient, but *lacZ* expression is still strong 72 h after electroporation (F). Arrow in D indicates the section in E. Scale bars 200 μm, (A, B, C, D, F); 50 μm, (E) (from Funahashi et al., 1999).

it possible to visually assess the efficiency of transfection of the gene of interest (Fig. 2C; Momose et al., 1999). The time course of expression of the introduced gene was monitored by  $\beta$ -galactosidase activity derived from the pMiwZ vector which encodes *lacZ* (Suemori et al., 1990).  $\beta$ -Galactosidase activity could be detected by 2 h after electroporation (Fig. 2A), increased in the following hour (3 h after electroporation, Fig. 2B), and reached peak activity around 20 h after electroporation (Fig. 2D). More than 50% of cells were transfected at peak (Fig. 2E). Reporter activity decreased slowly after the peak, but high  $\beta$ -galactosidase activity could still be detected at 72 h after electroporation (Fig. 2F).

The level of expression of a transgene introduced by electroporation depends on the concentration of the DNA solution (Momose et al., 1999). Sato et al. (2001) took advantage of this and performed a semi-quantitative analysis of the effects of *Fgf8* misexpression on midbrain, hindbrain development. When *Fgf8a* expression vector was electroporated at a concentration of 1  $\mu\text{g}/\mu\text{l}$ , the presumptive diencephalon changed fate to differentiate into the tectum. When *Fgf8b* expression vector was electroporated at a concentration of 1  $\mu\text{g}/\mu\text{l}$ , the presumptive midbrain changed fate to become cerebellum. Surprisingly, electroporation of *Fgf8b* expression vector at a 100-fold lower concentration induced similar phenotypic effects to *Fgf8a*. In another words, *Fgf8b* could exert 100-fold stronger signal than *Fgf8a*. It was concluded that the type difference could be attributable to the difference in the intensity of the signal.

### 2.3. Application of in ovo electroporation

Electroporation is so effective that it opens a wide range of new possibilities in the study of development in the chick. Misexpression can be induced by direct electroporation of the replication-competent retrovirus RCAS vector (Takeuchi et al., 1999; Yasuda et al., 2000; Sugiyama and Nakamura, 2003). This obviates the need for preparation of high titres of virus particles, and, in virus-competent embryos, expression of the transgene is restricted to the descendents of the transfected cells, which enables cell lineage analysis. In addition, while electroporation was difficult in very early stage embryos, the use of flat electrodes and a new culture system has made it possible to transfer genes to embryos at the primitive streak stage (Yasuda et al., 2000; Kobayashi et al., 2002).

## 3. Loss-of-function by in ovo electroporation

### 3.1. General remarks

In ovo electroporation revived interest in the chick embryo as a model for the study of developmental biology. While overexpression of a target gene or expression of an ectopic gene can provide important information, it remained

difficult to silence the expression of a specific gene. However, recently morpholino antisense oligonucleotides, that have proven very effective in zebrafish embryos, have been shown to efficiently silence gene expression in chick embryos (Kos et al., 2001, 2003; Sheng et al., 2003; Sugiyama and Nakamura, 2003). Morpholino oligonucleotides must be conjugated with FITC (fluorescein isothiocyanate) to give a positive charge before they can be transferred by electroporation. Since the morpholino antisense oligonucleotides interfere with translation, we need antibody against the molecule for evaluation.

Double-stranded RNA (dsRNA) was shown to trigger homologous gene silencing by a mechanism termed RNA-mediated interference (RNAi), and has proven to be a powerful gene silencing system in *Caenorhabditis elegans* and *Drosophila*. In mammals, however, dsRNA provokes a strong cytotoxic response (Baglioni and Nilsen, 1983; Williams, 1999) so the RNAi method is not applicable in higher vertebrates. This toxicity is non-specific and can be circumvented by use of synthetic short 21- to 22-nucleotide (nt) interfering RNAs (siRNA) (Elbashir et al., 2001a,b). Recently it was shown that a short hairpin dsRNA (shRNA) exerts RNAi effects (Svoboda et al., 2001; Brummelkamp et al., 2002; Paddison et al., 2002; Paul et al., 2002; Sui and Soohoo, 2002; Yu et al., 2002). shRNA is processed by the cellular machinery into dsRNA to exert RNAi effects. A vector has been developed for shRNA expression, which has an RNA polymerase III promoter (the U6 or H1 promoter) (Svoboda et al., 2001; Brummelkamp et al., 2002; Miyagishi and Taira, 2002; Paddison et al., 2002; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002). Gene knockdown by vector-based siRNA introduced by in ovo electroporation in chick embryos has been performed (Katahira and Nakamura, 2003) and was shown to be effective for the study of gene expression cascades (Kimura et al., 2004).

### 3.2. siRNA design

Some rules for designing the siRNA sequence have been proposed (Elbashir et al., 2001a,b; Semizarov et al., 2003; Jackson et al., 2003; Khvorova et al., 2003). However, it is likely that we still may have to design plural siRNAs to efficiently silence target gene expression. The proposed rules are as follows: Choose target sequences that have 3' TT overhangs and start with AA or AN. Select the target region from a given cDNA sequence beginning 50–100 nt downstream of the start codon. It is recommended to avoid the UTR region, because regulatory proteins that bind the UTR region may interfere with binding of siRNA (Levenkova et al., 2004). The GC content should be kept within the 40–70% range (Semizarov et al., 2003), and more than three guanines in a row should be avoided. However, some findings have contradicted these rules (Jarvis and Ford, 2001; Katahira and Nakamura, 2003), so more study is needed to better define the best parameters for

designing a siRNA sequence. A BLAST search should be carried out to avoid cross reaction with non-target mRNA.

Several steps are required for RNAi to function; RISC (RNA-induced silencing complex) formation, RNA-duplex unwinding, strand selection and degradation of the target mRNA. For effective duplex unwinding and strand selection, an siRNA duplex in which the 5' antisense terminal displays a lower internal stability has been shown to be effective (Khvorova et al., 2003). The internal stability signature of the RNA duplex is measured by minimum free energy of pentamer subsequences, and is calculated using the nearest neighbor method (Freier et al., 1986). Some companies support siRNA design (Genscript, Ambion, Japan BioService, etc.) (Wang, 2004).

### 3.3. Effects of siRNA

We show an example of the use of siRNA introduced by *in ovo* electroporation to target En2 expression in chick embryos (Fig. 3A; Katahira and Nakamura, 2003). We designed four candidate siRNA sequences (Fig. 3B). The 19mer sense and antisense sequence were linked to a nucleotide spacer (TTCAAGAGA) as a loop (Fig. 3C). Six T bases were added as a termination signal to the forward strand (Fig. 3C). After adding EcoRI and ApaI restriction sites to the 5' and 3' end of the reverse strand (Fig. 3C), respectively, the oligonucleotide was inserted in the pSilencer-U6 expression vector (Ambion). The transcripts generated by this vector can form stem loop hairpin structures and be processed to double-strand siRNA (Fig. 3C). Electroporation of the vector was carried out in the midbrain–hindbrain region where En2 mRNA and protein are expressed (Fig. 4C,F,I,L). En2-150 (Fig. 4A,B), En2-582 (Katahira and Nakamura, 2003) (Fig. 4G,H) and En2-648 (Fig. 4G,H) effectively degraded endogenous En2 mRNA. En2-150 completely matches the corresponding sequence of En1 (Fig. 3B), and effectively interfered with En1 expression (Fig. 4D,E), which is expressed in an overlapping manner to En2. En2-648, which mismatches the corresponding En1 sequence by two nucleotides, weakly interfered with En1 expression (Fig. 3B, 4J,K). En2-648s4, in which four nucleotides were substituted, did not affect En2 expression. These results indicate that siRNA can exert its effects, even with a difference of up to two nucleotides from the target sequence. En2-846 did not show any siRNA effect.

We measured the thermodynamics of the binding of these siRNAs to examine if the proposition of Khvorova et al. could be applicable (Fig. 5). The model fundamentally predicts the efficacy of the designed siRNA. Of the four siRNAs against En2, En2-150 displayed a lower stability at the 5' antisense terminus than the 3' terminus, and exerted siRNA effects as expected. However, En2-846 showed higher stability at the 5' antisense terminus, and was not functional as siRNA. The thermodynamic pattern of En2-582 and En2-648 does not provide any information

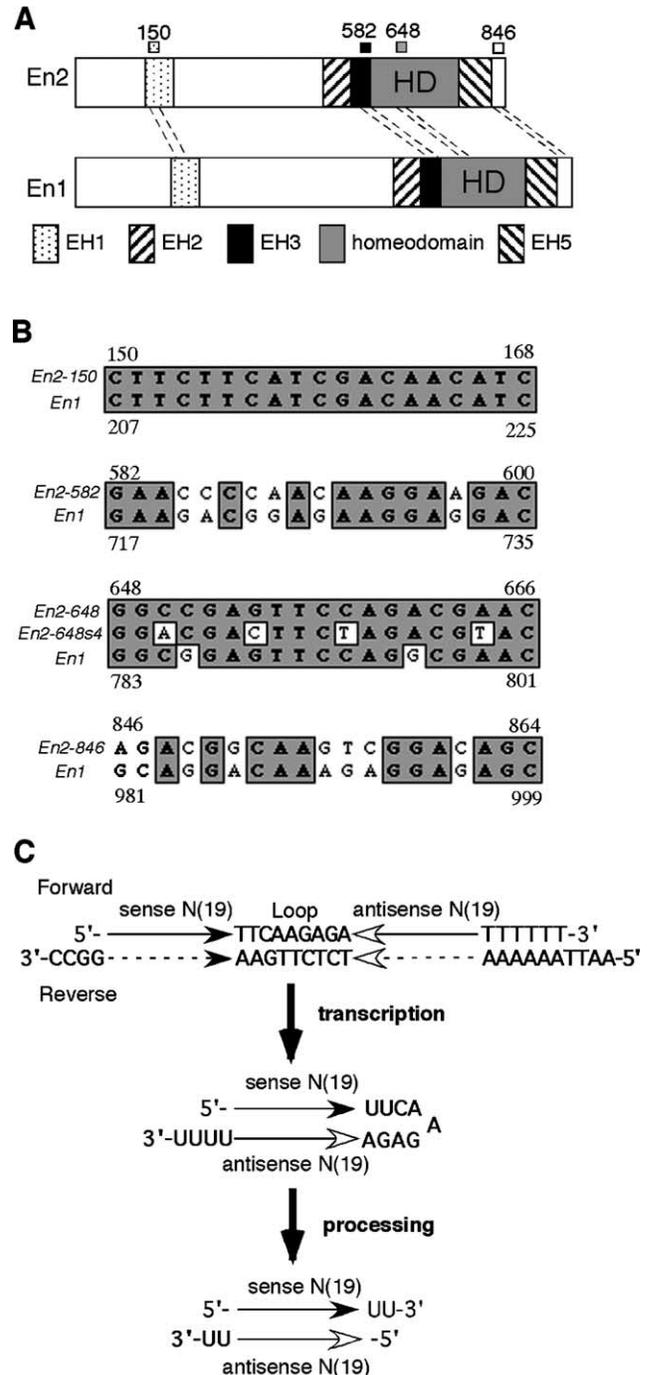


Fig. 3. Design of vector-based En2 siRNA. (A) Location of the siRNAs in En2 ORF and corresponding part of En1 in ORF. The number above the selected sequences for siRNA indicates the initial number in the ORF sequence. Specific domains, such as EH1, EH2, EH3, EH5 and homeodomain, are depicted as boxes. (B) Detailed sequence for siRNAs and their corresponding sequence on En1. siRNA oligonucleotides 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 the insert DNA for shRNA. Sense and antisense sequences are linked with the loop sequence, and six Ts are added to the 3' end of the forward strand. Upper strand is the forward oligonucleotides, lower strand is the reverse oligonucleotides. The transcripts may form a hairpin RNA (Middle), which may be processed to double strand siRNA (Bottom) (from Katahira and Nakamura, 2003).

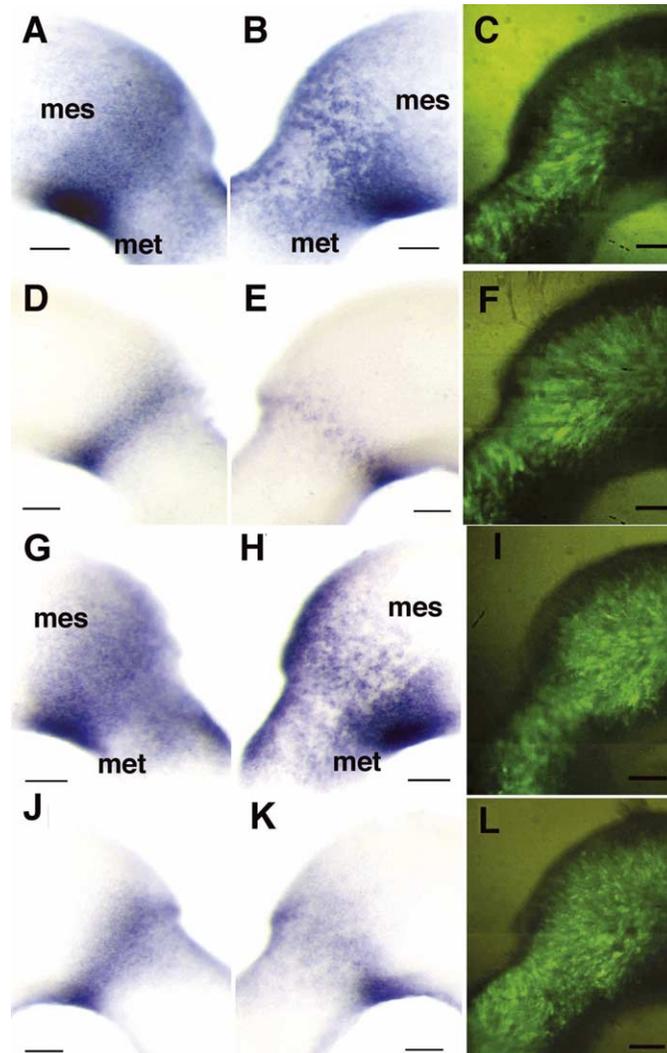


Fig. 4. Electroporated shRNA constructs displayed RNAi effects in midbrain–hindbrain region at 24 h after electroporation. Whole-mount in situ hybridization for *En2* (A, B and G, H) and *En1* (D, E and J, K). GFP fluorescence shows the sites of transfection (C, F, I, L). Photographs on the same row are from the same embryo. En2-150 completely matches the corresponding sequence of *En1*, and it suppressed both *En2* (A, B) and *En1* (D, E) expression. En2-648 suppressed *En2* to the same extent as En2-150 (H), and weakly suppressed *En1* (K). This result indicates that siRNA could interfere with a sequence with a two nucleotide mismatch (Fig. 3B). Control side (A, D, G, J). Experimental side (B, E, H, K). Scale bars: 100  $\mu$ m, mes: mesencephalon, met: metencephalon (from Katahira and Nakamura, 2003).

about their efficacy, but they were effective siRNA. So clearly it is important to avoid sequences that show higher stability at the 5' antisense terminus.

We evaluated the functional effects of the En2-150 expression vector (Fig. 6). There was a slight reduction in the RNA signal detected by in situ hybridization by 6 h after electroporation (Fig. 6B), and by 12 h after electroporation, there was marked reduction of RNA signal (Fig. 6E). Immunostaining with anti-En2 antibody indicated that the knockdown effects of this vector were not detectable at the protein level at 6 h after electroporation (Fig. 6C), but by 12 h after electroporation, there was a decrease in protein levels (Fig. 6F).

The probe for En2 does not cover the same region as En2-582 or En2-648 (the probe we used is 130–457), but after electroporation of these siRNA vectors, in situ

hybridization showed degradation of En2 mRNA, indicating that that siRNA induces digestion of the target mRNA.

#### 4. Conclusion

It is now possible to induce gain-of-function of a gene of interest in chick embryos using electroporation, and this method is so effective that it has rapidly become a major tool for developmental studies in the chick. Electroporation of carefully designed siRNA vectors now provides an efficient method to induce loss-of-function in the chick, and functions via degradation of the target mRNA. Both gain-of-function and knockdown effects can be induced at specific locales within the embryo. Furthermore, it is

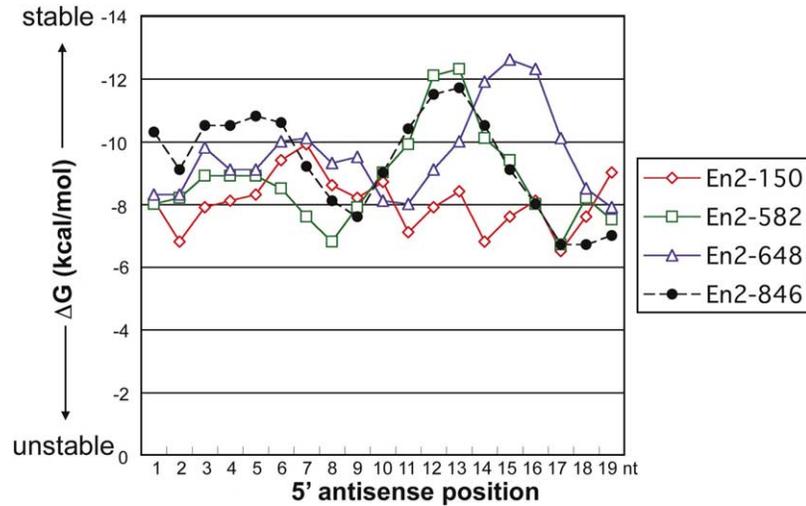


Fig. 5. Thermodynamic and functional characterization of siRNA Functional siRNAs (solid line, En2-150, En2-582, En2-648), and non functional siRNA (dashed line, En2-846). En2-846 has a stable 5' antisense terminus and did not exert any siRNA effects as predicted from the proposal of Khvorova et al. (2003). En2-150 has an unstable 5' antisense terminus, and functioned as siRNA, as predicted by Khvorova et al. (2003). En2-582 and En2-648 functioned as siRNA, though they do not have an unstable 5' antisense terminus. Abcissa: the position from the antisense 5' end, ordinate: minimal free energy ( $\Delta G$ ) of pentamer sequence. The larger the number the more stable the molecule.

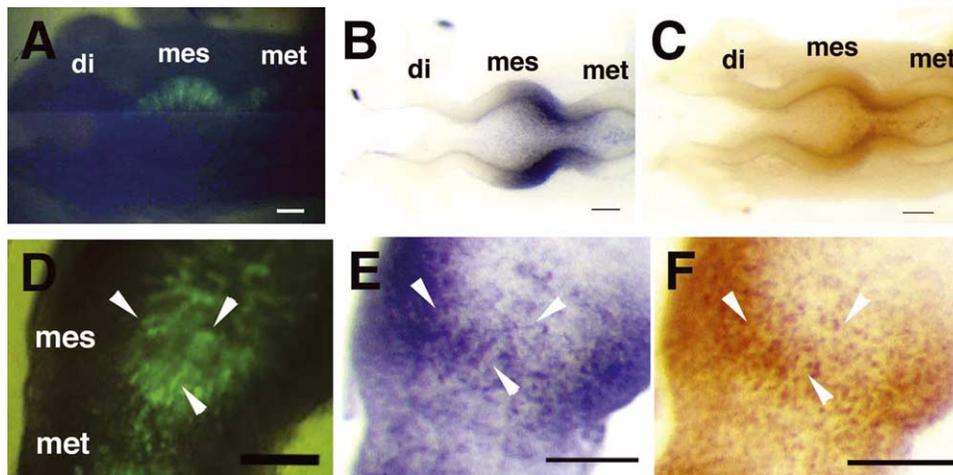


Fig. 6. Time course of siRNA effects on En2. Dorsal view of an embryo 6 h after electroporation (A–C). View from the experimental side of another embryo at 12 h after electroporation (D–F). *En2* mRNA levels were reduced by 6 h after electroporation comparing with the control side (B). At this time, there were no changes in En2 protein levels (C). siRNA expression sites were indicated by GFP (D). *En2* mRNA (E) and protein (F) were reduced clearly by 12 h after electroporation. Corresponding white arrow head indicates the same place (D, E, F). Color for whole-mount in situ hybridization (blue) was destained after HRP immunostaining (brown) (C, F). Scale bars, 100  $\mu$ m, di: diencephalons, mes: mesencephalon, met: metencephalon (from Katahira and Nakamura, 2003).

possible to trace the cell lineages affected. These methods are expected to revive the use of the chick as a model in developmental biology.

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