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Tet-on inducible system combined with *in ovo* electroporation dissects multiple roles of genes in somitogenesis of chicken embryos

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Abstract

The *in ovo* electroporation technique in chicken embryos has enabled investigators to uncover the functions of numerous developmental genes. In this technique, the ubiquitous promoter, CAGGS (CMV base), has often been used for overexpression experiments. However, if a given gene plays a role in multiple steps of development and if overexpression of this gene causes fatal consequences at the time of electroporation, its roles in later steps of development would be overlooked. Thus, a technique with which expression of an electroporated DNA can be controlled in a stage-specific manner needs to be formulated. Here we show for the first time that the tetracycline-controlled expression method, "tet-on" and "tet-off", works efficiently to regulate gene expression in electroporated chicken embryos. We demonstrate that the onset or termination of expression of an electroporated DNA can be precisely controlled by timing the administration of tetracycline into an egg. Furthermore, with this technique we have revealed previously unknown roles of RhoA, cMeso-1 and Pax2 in early somitogenesis. In particular, cMeso-1 appears to be involved in cell condensation of a newly forming somite by regulating Pax2 and NCAM expression. Thus, the novel molecular technique in chickens proposed in this study provides a useful tool to investigate stage-specific roles of developmental genes.

Keywords: Tet-on; Tet-off; *In ovo* electroporation; Somitogenesis; RhoA; cMeso-1; Pax2; NCAM; Cell condensation

Introduction

The chicken embryo has long been a powerful model in modern developmental biology for two major reasons. First, the tissue transplantation technique to make chicken–quail chimeras, in which a donor tissue can unambiguously be distinguished from the host embryo, has made a huge contribution to studying cell lineages and the mechanisms of tissue interactions (Le Douarin and Kalcheim, 1999). Second, the *in ovo* electroporation technique, developed in Nakamura's laboratory (Funahashi et al., 1999), has enabled investigators to genetically manipulate cells and tissues in a stage- and tissue-specific manner. Moreover, the *in ovo* electroporation technique can be combined with classical tissue transplantations, thus making chick embryos a most powerful and unique model animal (Stern, 2005). In most cases of *in ovo* electroporation, the ubiquitously active promoter CAGGS has been used, which contains the CMV and chicken beta-actin promoter (Niwa et al., 1991). This promoter becomes active soon after electroporated into cells (Momose et al., 1999).

During development, it is well known that the same set of genes is used repeatedly in different contexts. This notion is very obvious in somitogenesis. The somites are transient structures that are regularly aligned on both sides of the midline along the anteroposterior axis of the body. Somitic precursors originate from the early mesoderm, which forms in amniote embryos by ingression from the epiblast along the primitive streak. Soon after the ingression, the presomitic mesoderm (PSM) becomes visible as a pair of strips located on both sides

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of the midline. As the PSM undergoes morphological segmentation, the "segmentation clock" operates in unsegmented PSM, where oscillatory expression of a set of segmentation genes is seen. A central feature of the segmentation clock is Notch signaling (Giudicelli and Lewis, 2004). Importantly, the Notch signaling is also critical for the morphological segmentation, the event following the clock (Sato et al., 2002). Thus, Notch signals are repeatedly used in multiple steps ranging at least from the formation of PSM to the final step of the segmentation. It is therefore important to understand the role of such genes in each of different steps in the development. In general, however, if a gene manipulation at an earlier stage in development causes fatal or severely dysmorphic effects, other possible roles of the gene at later stages would be overlooked.

We have indeed experienced such problems in the course of molecular investigations of somitogenesis. We previously attempted to study the roles of cMeso-1 (Buchberger et al., 1998) and Pax2 (Suetsugu et al., 2002) in boundary formation since the genes are expressed in a stripe pattern at the next-forming boundary in the anterior PSM (see also below, Figs. 4 and 5). For this purpose we had electroporated a plasmid containing CAGGS-cMeso-1 or CAGGS-Pax2 into the ingressing mesoderm, the technique widely used for genetic manipulation of PSM in chickens (Nakaya et al., 2004; Sato et al., 2002). However, none of these constructs gave rise to successful transgenesis of PSM probably because the electroporated cells with these activated genes failed to correctly ingress from the primitive streak (more details described in the present study). Thus, we have been unable to analyze the roles of these genes in the somitogenesis.

To circumvent these problems, we have now developed an electroporation technique with which we can conditionally manipulate expression of a transgene in a stage-specific fashion. For this purpose we applied to electroporated chicken cells the tetracycline (tet)-controlled expression method, a technique that has successfully been used to regulate gene expression in cultured mammalian cells and mouse embryos (Furth et al., 1994; Gossen and Bujard, 1992). Briefly, both the transcriptional activators, reverse tet-controlled transcriptional activator (rtTA) and tet-controlled transcriptional activator (tTA), act on the *cis*-element promoter, tetracycline responsive element (TRE) (Figs. 1A and 2A). rtTA binds TRE only in the presence of doxycycline (an analog of tetracycline; Dox) and activates transcription of the TRE-driven gene ("tet-on") (Fig. 1A). tTA, in contrast, binds to the TRE constitutively and activates the TRE-driven gene in the absence of Dox, whereas if added with Dox in a cell, tTA is released from TRE, leading to an inactivation of the TRE-driven gene ('tet-off") (Fig. 2A). Thus, one can control the onset or termination of expression of transgene by timing the Dox administration. Here we describe for the first time that both the tet-on and tet-off systems work efficiently in chicken embryos. Using this technique, we have revealed previously unknown roles of RhoA, cMeso-1 and Pax2 in somitogenesis. In particular, cMeso-1 appears to be involved in cell-condensation of a newly forming somite by controlling Pax2 and NCAM.

Materials and methods

Plasmids and constructs

The tetracycline-dependent transactivators, rtTA and tTA, DsRed2, short half-life EGFP (d2EGFP) and pBI-EGFP were purchased from Clontech. rtTA2^S-M2 was a gift from Dr. Hillen (Urlinger et al., 2000). rtTA, tTA, rtTA2^S-M2 or DsRed2 fragment was subcloned into pCAGGS expression vector (Momose et al., 1999; Niwa et al., 1991). A cDNA fragment encoding *RhoA* (a gift from Dr. Kaibuchi), *cMeso-1* (Buchberger et al., 1998) or *Pax2* (Okafuji et al., 1999) was inserted into blunt-ended *Mul*I site of pBI-EGFP.

Embryological manipulations

Embryonic stages were described according to Hamburger and Hamilton (1992). *In ovo* DNA electroporation was performed as previously described (Nakaya et al., 2004; Sato et al., 2002). Eighteen hours after electroporation when an embryo developed to st 12, 500 µl of HANKS' solution containing doxycycline was injected in-between the embryo and the yolk, followed by reincubation until the embryos were harvested.

Observation and detection of EGFP signals

Pictures of EGFP-positive embryos were acquired with a Nikon SMZ7500 stereomicroscope equipped with a Zeiss AxioCam HRc CCD camera. Exposure time, gain and magnification were left constant for each embryo. JPEG images were imported into Photoshop (image size was maintained constant for each picture during the same series of analyses).

For Western blotting and intensity quantification of EGFP signals, electroporated somites/PSM were dissected and treated in lysis buffer (50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 1% NP-40, 100 mM NaCl, 10 mg/ml leupeptin, 10 mg/ml aprotinin, 10 mM (*p*-amidinophenyl)-methanesulfonyl fluoride). Following centrifugation at 20,000×*g* for 4 min at 4 °C, the supernatant was suspended and then boiled for 10 min in Laemmli sample buffer. The eluates were subjected to SDS–PAGE followed by immunoblotting with mouse anti-GFP mouse monoclonal antibody (Clontech) and HRP-conjugated anti-mouse IgG antibody (Amersham). Signals for GFP protein were revealed with the ECL Advance Western Blotting Detection kit (Amersham). Densitometric analysis was performed using the luminous image analyzer LAS-3000 mini (Fuji Film).

Fig. 1. Inducible expression of electroporated DNA by the tet-on system. (A) A diagram showing the principle of tet-on system. *In ovo* electroporation of PSM is carried out at stage 8 as previously described (Sato et al., 2002). Soon after the electroporation, rtTA protein (yellow) is produced by CAGGS-driven cDNA. The rtTA protein can bind to TRE (*cis*-element shown in black) only after Dox (blue) is injected into an egg. CAGGS-driven DsRed2 (red) is constitutively produced. A cassette containing TRE is designed to transcribe two genes bidirectionally. The TRE cassette used in the experiments shown in this figure and also in Fig. 2 contained solely EGFP with the other transcription unit vacant (dotted arrow). (B) Dox-dependent induction of electroporated EGFP in early chicken embryos. The three plasmids shown in panel A were co-electroporated. At 3 h after Dox administration, signals of EGFP started to be observed. The lower panel is of a similar experiment but with rtTA2^S-M2, a modified protein of rtTA. (C) Western blotting analyses demonstrating an increase in EGFP signals. Embryos that showed similar yields of electroporation efficiency were collected at different time points after Dox administration. (D) Optimization of the dose of Dox to administer to an embryo. A Dox solution containing 250 ng was sufficient to induce TRE-EGFP. (E) The ratio of DNA amount at 1:2 between pCAGGS-rtTA2^S-M2 and pTRE-EGFP yielded more intense signals of EGFP compared to the ratio at 2:1.