genesis 37:91–101 (2003)

ARTICLE

Microbubble-Enhanced Sonoporation: Efficient Gene Transduction Technique for Chick Embryos

Sho Ohta,[1] Kentaro Suzuki,[1] Katsuro Tachibana,[2] and Gen Yamada[1]*

[1]Center for Animal Resources and Development (CARD) and Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan

[2]Department of Anatomy, Fukuoka University School of Medicine, Fukuoka, Japan

Received 25 April 2003; Accepted 7 August 2003

Summary: The gene transduction technique is a useful method to study gene functions that underlie vertebrate embryogenesis. In this study, a new gene transduction technique is reported using microbubble-enhanced sonoporation (hereafter referred to as sonoporation) to achieve ectopic and transient gene expression for several embryonic organs including embryonic chick limb bud mesenchymes. The technique proposed in this study has the advantages of 1) relatively simple gene transduction procedures, and 2) efficient exogenous gene transduction and expression with lower damages to embryos. Green fluorescent protein (GFP) or LacZ was misexpressed in limb bud mesenchymes by sonoporation, with the introduced expression transiently detected in the injected sites. Most of the transduced chick embryos survived without showing significant embryonic abnormalities or cell death after sonoporation. To demonstrate its efficacy for assessing the effect of transient gene transduction, the *Shh* (*sonic hedgehog*) was transduced into the developing chick limb bud. The transduced limb bud displayed limb malformations including partial digit duplication. Advantages and possible future applications in relation to this method are discussed. genesis 37:91–101, 2003.

Key words: sonoporation; ultrasound; gene expression; limb bud; *Shh* (*sonic hedgehog*); gene transduction

INTRODUCTION

Gene transduction is a widely utilized method to analyze gene functions underlying vertebrate embryogenesis. Many different methods/protocols have been invented to transduce and express genes exogenously in embryonic tissues or in adult organs (Yamada *et al.*, 1997; Katahira *et al.*, 2000; Yasugi and Nakamura, 2000; Timmer *et al.*, 2001). For each gene introductory procedure, there are several advantages and disadvantages among several experimental parameters.

Nonviral type gene transduction techniques, e.g., lipofection and electroporation, can transduce genes for target tissues based on the physicochemical nature of the mechanisms of gene introduction (Araki and Nakamura, 1999; Yasuda *et al.*, 2000; Yasugi and Nakamura, 2000; Takahashi *et al.*, 2002).

Electroporation has been widely utilized for transient gene introduction because it does not require viral vector constructions or viral preparation, including packaging. However, cell anomalies or tissue damages after electroporation have often been observed. Such damages appear to be inevitable due to the physicochemical procedure of electroporation which applies an electric field to the cells or target organs. For transducing exogenous genes into cell lines, e.g., ES cells, such cell damage has been deemed moderate because of the advantages of applying electric fields to the dispersed cells rather than to the tissue mass. Furthermore, an inability or inefficiency to transduce genes into embryonic tissues such as limb mesenchymes has been recognized. A recent publication suggested the utility of a modified electrode which could be used for mesenchymal gene transduction to improve such inefficiency (Oberg *et al.*, 2002). Other than electroporation, it has been demonstrated that gene transduction using recombinant retrovirus or adenovirus techniques are useful for analyzing gene functions in embryos (Kengaku *et al.*, 1998; Rodriguez Esteban *et al.*, 1998; Pizette and Niswander, 1999; Iba, 2000). Although such recombinant viral vectors can effectively introduce genes into chick embryos, other disadvantages have been recognized. Limitations in terms of the length of the cloned gene fragments for retroviral vectors due to several mechanisms of viral replication and packaging have been recognized (Morgan and Fekete, 1996). Also, exogenous genes could be introduced predominantly into actively proliferating

* Correspondence to: G. Yamada, Center for Animal Resources and Development (CARD) and Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Honjo 2-2-1, Kumamoto 860-0811, Japan.
E-mail: gen@kaiju.medic.kumamoto-u.ac.jp

Contract grant sponsors: the Ministry of Education, Culture, Sports, Science and Technology (Grant-in-Aid for 21st Century COE Research), General Promotion of Cancer Research in Japan (Grant-in-Aid for Scientific Research on Priority Areas (A)).

DOI: 10.1002/gene.10232

cells in the case of retroviral infection because infection/integration requires active cell mitosis. Vector DNA construction, preparation, and increasing the viral titer of recombinant retroviruses often requires considerable time. Adenoviruses have also been also used for efficient exogenous gene transduction, and often display different gene introduction frequencies depending on the cells or tissue types (Tomko *et al.*, 2000).

A method termed microbubble-enhanced sonoporation (sonoporation) has been introduced as a highly efficient method for transient gene transduction, originally used for adult blood vessels (Taniyama *et al.*, 2002a). This technique has been mainly utilized in studies of gene therapy, e.g., for vascular gene transduction in adult organs (Lawrie *et al.*, 2000; Shohet *et al.*, 2000; Unger *et al.*, 2000; Taniyama *et al.*, 2002b). Although ultrasound treatment itself can enhance exogenous gene transduction (Kim *et al.*, 1996; Wasan *et al.*, 1996; Newman *et al.*, 2001), the frequency of gene introduction can be further enhanced in the presence of echo-contrast microbubbles both in vitro and in vivo (Koch *et al.*, 2000). It has been previously reported that microbubbles included in echo-contrast agents significantly increase the efficiency of drug uptake and gene transduction into cells by sonoporation (Bao *et al.*, 1997; Tachibana and Tachibana, 2001). Such experiments are based on observations that echo-contrast agents, such as albumin-microbubbles, accelerate thrombolysis through exposure to ultrasound energy. In such conditions, thrombolysis is mediated by a microjet-stream emanating from a microbubble rupture generated by ultrasound exposure (Tachibana and Tachibana, 1995). Following various experimental modifications, ultrasound-mediated microbubble rupture has been proposed as a technique for introducing drug uptake and exogenous gene transduction in vitro (Ay *et al.*, 2001; Feril *et al.*, 2003) and in vivo (Anwer *et al.*, 2000). Although the exact mechanism underlying efficient drug uptake and gene transduction remains incompletely understood, the rapid collapse of microbubbles during sonoporation has been considered to play a major role in drug/gene delivery into cells. It has also been speculated that the presence of microbubbles can significantly reduce the threshold of acoustic cavitation production upon ultrasound exposure in a target site.

Endoh *et al.* showed that the *GFP* (green fluorescence protein) gene injected with a microbubble solution into the amniotic cavity was introduced into late-staged embryonic skin of a mouse by sonoporation. Based on electron microscope examination, the introduced mouse embryos displayed microholes in the cutaneous surface but still maintained their normal cell morphology within 24 h of the treatment (Endoh *et al.*, 2002). Thus, it has been speculated that ultrasound exposure with microbubbles might generate transient cellular microholes on the surface of target organs, thereby efficiently introducing genes into the operated sites.

However, application of sonoporation has so far been predominantly performed on nonembryonic tissues. To test the efficacy of gene transduction with sonoporation for application in embryology, we transduced GFP or LacZ expression vectors into the limb bud mesenchyme of chick embryos and examined the resulting expression. The introduced gene expression was monitored and expression was successfully detected in tissues, e.g., limb bud mesenchymal cells. We were able to show that sonoporation achieves efficient gene transduction into chick limb bud mesenchyme with relatively simple experimental procedures. When *Shh* (*sonic hedgehog*) was misexpressed in the anterior region of limb bud mesenchyme by sonoporation, the transduced limb bud displayed prominent digit duplication phenotypes suggesting the utility of this procedure for analyzing various aspects of organogenesis during vertebrate development. Examination of various parameters and experimental conditions for sonoporation to transduce exogenous genes into embryos was performed and is discussed.

RESULTS

Gene Transduction Utilizing Microbubble-Enhanced Sonoporation

Application of sonoporation procedures to chick embryos are composed mainly of two steps, i.e., DNA-microbubble mixture injection and exposure to ultrasound (Fig. 1a–d). A mixture containing DNA and microbubbles (typically 0.25 μl, depending on the target site) was first injected into developing chick limb bud mesenchyme to evaluate the introduction efficiency into developing chick limb buds (Fig. 1a,b). Immediately after injection, ultrasound irradiation was performed utilizing an ultrasound probe placed adjacent to the injected limb bud (Fig. 1c,d). GFP or LacZ expression was detected to monitor the gene transduction efficiency after several hours, e.g., 12 h for the case of Figure 2. Thus, the whole procedure could be performed in a few minutes, mainly ultrasound exposure time, after rapid microinjection of the DNA-microbubble mixture.

Experimental parameters, including duty cycle (On cycle/Off cycle; pulse repetition frequency), intensity, and exposure time of the ultrasound irradiation were examined to achieve better gene transduction efficiency for chick limb bud (Fig. 2). In the case of limb buds, it was found that an ultrasound intensity of less than 1.0 W/cm^2 achieved only a very low level of exogenous gene expression judged by GFP expression (Fig. 2a). An ultrasound irradiation duration time of less than 30 sec also resulted in less exogenous gene expression (Fig. 2c). Although more analyses are required to examine several parameters, gene transduction for developing limb buds appeared to require an ultrasound intensity of more than 1.0 W/cm^2 and an irradiation time longer than 30 sec (Fig. 2a–d). It was found that gene transduction under conditions of 2.0 W/cm^2 with 60 sec of irradiation resulted in a high frequency of gene transduction into chick limb buds (Fig. 2d).

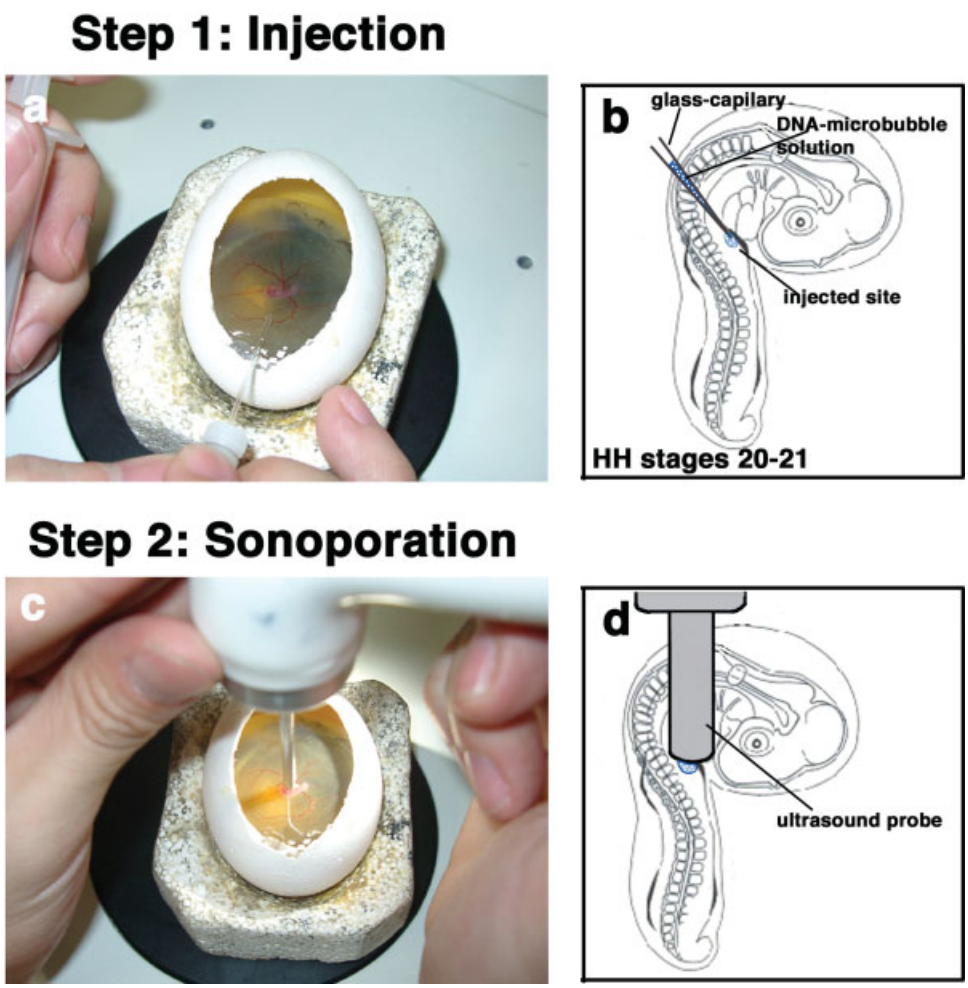

FIG. 1. Procedures for sonoporation-mediated gene transduction. Procedures consist of two steps. **a,b:** DNA-microbubble mixture was injected into chick limb bud at HH stages 20–21. **c,d:** Immediately after injection, ultrasound irradiation was performed with the ultrasound probe placed adjacent to the injected site (introduction into the chick forelimb bud is illustrated in **d**).

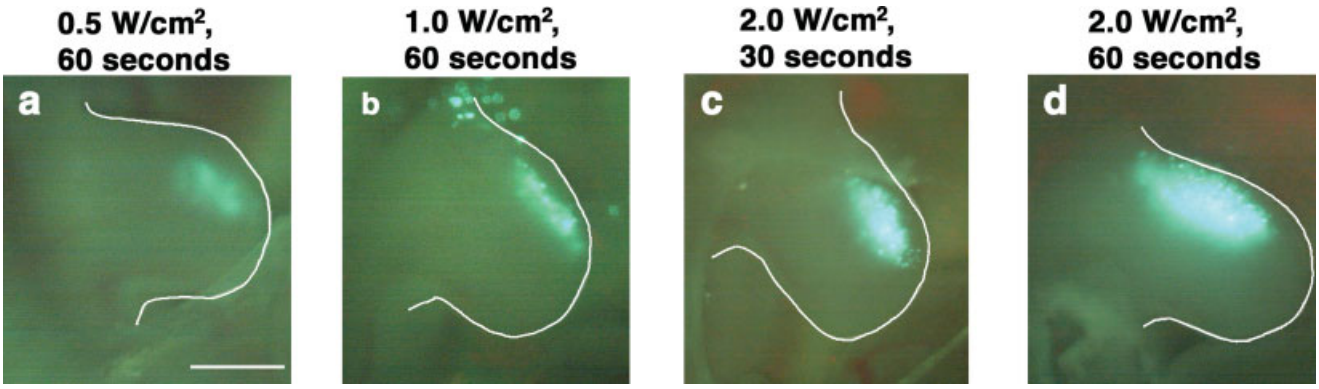

FIG. 2. Examination of gene transduction conditions by sonoporation into chick limb buds with GFP expression vector. **a–c:** Exogenous gene expression was not optimal with ultrasound intensities less than 1.0 W/cm^2 or exposure time less than 30 sec. **d:** 2.0 W/cm^2 with 60 sec of exposure resulted in efficient gene transduction. All conditions were performed with a 20% duty cycle. Scale bar = 1 mm.

Histological Analysis of the Site of Gene Expression in Transduced Chick Limb Mesenchymes

To examine gene transduction efficiency in chick limb bud mesenchyme by sonoporation, expression of LacZ was examined histologically. LacZ staining was performed after 12 h of transduction in chick limb buds at HH stages 20–21. The injected region displayed prominent LacZ staining with clustered LacZ-positive cells scattered at the injected site in the limb mesenchyme (Fig. 3a,b). Estimation of gene transduction efficiency was calculated to be ~20% of the injected site in the

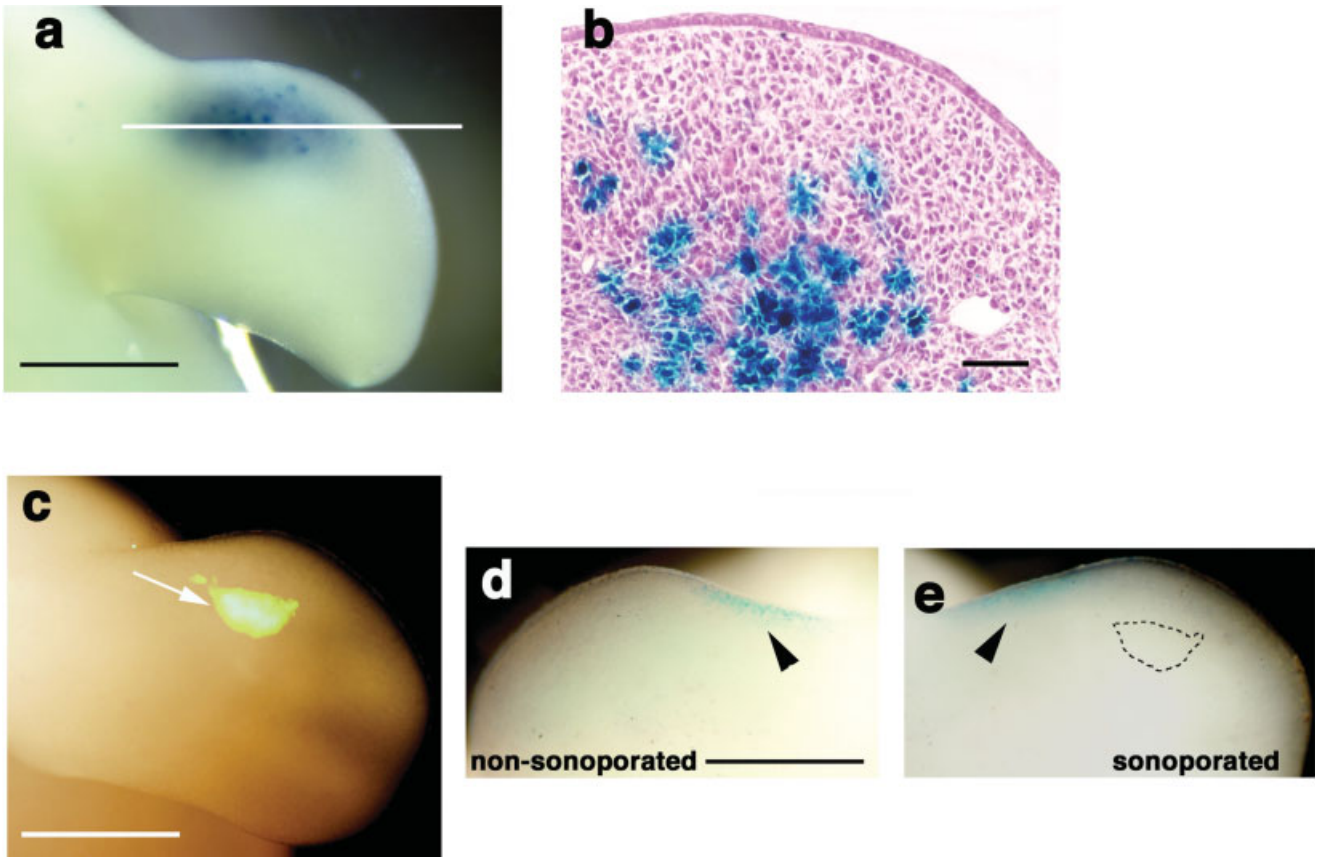

FIG. 3. Histological analysis of transduced chick limb buds. **a:** pEBActN-LacZ was introduced into limb buds (at HH stages 20–21) by utilizing sonoporation. LacZ expression was detected in anterior limb buds after 12 h. The white line in a represents the position of a section in **b**. **b:** In the transverse section of a transduced limb bud, LacZ-positive cells were detected in the mesenchyme. **c–e:** Examination of the cell death after sonoporation. After performing sonoporation with the GFP expression vector in the anterior chick limb bud at HH stage 24–25 (transduced site was shown by white arrow in **c**), cell death was examined by Nile blue staining after 12 h. The operated site of the embryo did not manifest cell death (dotted circle region in **e**). Note the endogenous cell death in the anterior necrotic zone of the limb (shown by black arrowheads in **d,e**). **d:** The control side of the non-sonoporated limb. Scale bar = 1 mm in **a,c,d,e** and 50 μm in **b**.

limb mesenchyme based on the percentage of LacZ-positive cells among the average total cells included in the injection site after such a volume of injection. This suggests that injection into the limb bud mesenchyme followed by sonoporation achieved efficient gene transduction.

To examine the extent of damage and deterioration of tissues subjected to sonoporation, cell death was examined by Nile blue staining 12 h after sonoporation (Fig. 3c–e). No significant cell death signals were detected for such limb specimens (Fig. 3e).

Kinetics of Exogenously Introduced GFP Expression by Sonoporation In Ovo

In order to evaluate the efficacy of gene transduction by sonoporation, we introduced a GFP expression vector into chick embryonic limb buds to assess the kinetics of the introduced gene expression (introduced at HH stages 20–21). GFP expression was detected 3 h after sonoporation (Fig. 4a,e), becoming stronger after 6 h (Fig. 4b,f), reaching a maximum level after 12 h (Fig. 4c,g). Although GFP expression gradually decreased (Fig. 4d,h), the expression was still detected at 48 h (occasionally at 72 h) after sonoporation (data not shown). In gene transduction by electroporation, it has been reported that a GFP expression vector with similar promoter activities enables exogenous gene expression a few hours after the transduction for a similar duration.

The survival rate for sonoporated embryos was ~96% during a period of 24–48 h after operation (n = 48 out of 50 trials; embryos sonoporated at HH stage 20–21). All surviving chick embryos displayed GFP expression under the above experimental conditions (a total of 48 embryos). Based on the high survival rate of the sonoporated embryos, further assessment of surviving embryos after a longer incubation period was performed to examine the influence on late embryogenesis. Approximately 60% of the sonoporated embryos survived for 7 days after the operation and reached HH stage 35–36 (sonoporated at HH stage 20–21). The sonoporated embryos showed no morphological abnormalities, indicating that gene transduction using sonoporation caused little damage to embryos.

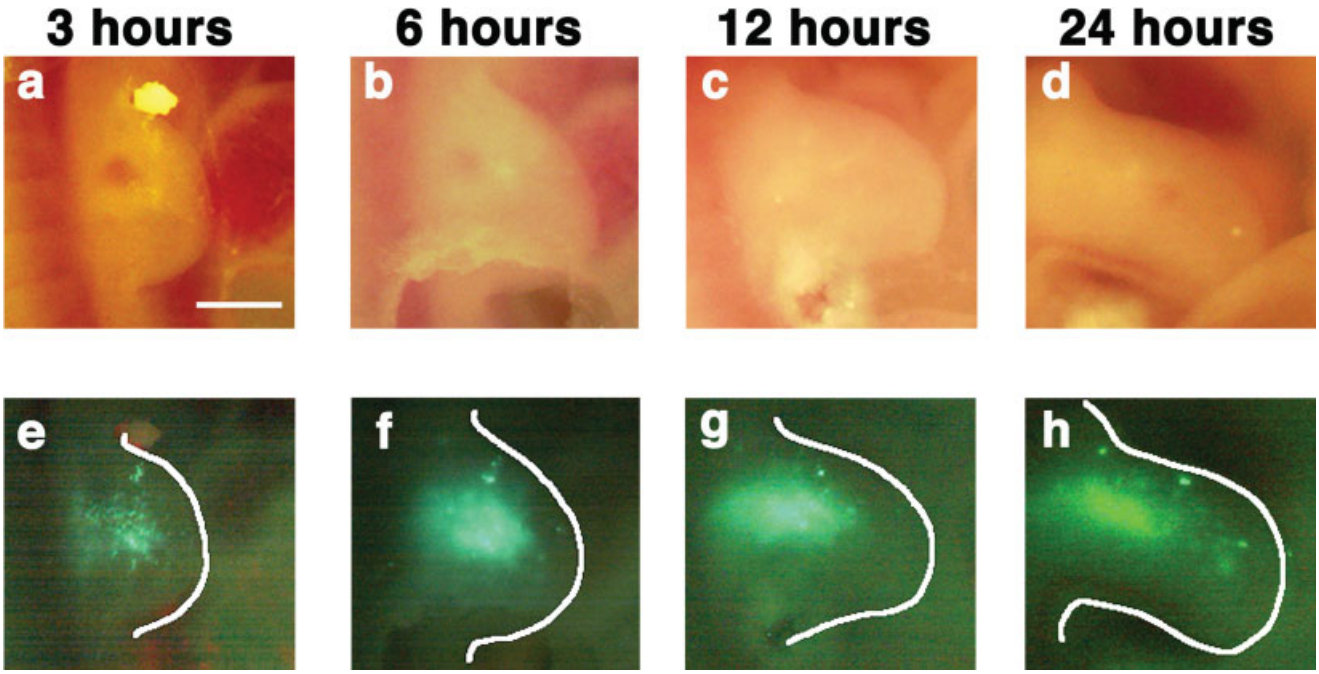

FIG. 4. Time course of GFP expression after sonoporation. pEGFP was transduced into chick limb buds by utilizing sonoporation at HH stage 20–21. Brightfield photos of the transduced limbs are shown in **a–d**. **a,e:** Three hours after transduction, GFP expression was detectable in the limb bud mesenchyme. The level of GFP expression progressively increased (**b,f**), reaching a plateau after 12 h of gene transduction (**c,g**). **d,h:** The level of GFP expression decreased after 24 h of transduction. Scale bar = 1 mm.

Induction of Additional Digit Formation by *Shh* Misexpression Using Sonoporation

In vertebrate limb buds, one of the embryonic signaling regions is located at the posterior region of the limb bud known as the zone of polarizing activity (ZPA; Saunders and Gasseling, 1968). Signaling emanating from the ZPA is involved in establishing the anteroposterior patterning for the limb as suggested by many studies, including ZPA grafts which induced mirror-image digit duplication upon transplantation into the anterior limb bud. Numerous functional studies have postulated *Shh* as one of the key signals in this process (Riddle *et al.*, 1993). To further analyze the consequences of gene transduction by sonoporation, we transduced the pCAGGS-*cShh* vector with CMV promoter and chick β-actin enhancer (Miyazaki *et al.*, 1989) into anterior limb bud mesenchyme with the GFP expression vector at HH stages 20–21. *Gli1* is one of the signaling genes of the *Shh* signaling pathway (Marigo *et al.*, 1996). We observed ectopic Gli1 expression at the injected site of the limb bud (Fig. 5b), which indicated ectopic activation of the *Shh* signaling pathway in the transduced limb bud mesenchymal region. The transduction efficacy was confirmed by GFP expression after 12 h of transduction (Fig. 6a), also examined by in situ hybridization for *Shh* after 24 h of transduction (data not shown). The pCAGGS-c*Shh* transduced limb bud showed digit duplication (Fig. 6c), with some phenotypic variations probably due to varying levels of *Shh* misexpression. The observed phenotypes varied from complete duplication of digit 3 to incomplete digit 2 formation (Fig. 6c; complete digit 3 duplication; n = 3/12, complete digit 2 duplication; n = 6/12, incomplete digit 2 formation; n = 3/12). Mirror-image digit duplications, which have been reported after ZPA grafts or high doses of SHH application (Tickle, 1981; Yang *et al.*, 1997), were not observed in the current experimental conditions. Judged by previous reports on the estimation of SHH levels for the degree of digit duplication, pCAGGS-*cShh* vector transduction by the current sonoporation conditions may be equivalent to the effects by the transplantation for ~35–79 ZPA cells (Tickle, 1981).

Exogenously Transduced GFP or LacZ Expression in Various Chick Embryonic Tissues by Sonoporation In Ovo

To assess the possibility of applying sonoporation for analyzing various gene functions, gene transduction into the branchial arch at HH stages 20–21, lateral plate mesoderm at HH stages 15–16, neural tubes at HH stages 10–11, and surface ectoderms of limbs at HH stages 21–22 were performed by a single round of injection and sonoporation (Table 1). Clusters of cells which express GFP were detected in the branchial arch and the lateral plate mesoderm after sonoporation into each mesenchyme similar to the trials for limb bud regions (Fig. 7a–d). Histological examination revealed prominent LacZ expression in the mesenchyme of the branchial arch and the lateral plate mesoderm. Sonoporation-mediated gene transduction into the neural tubes resulted

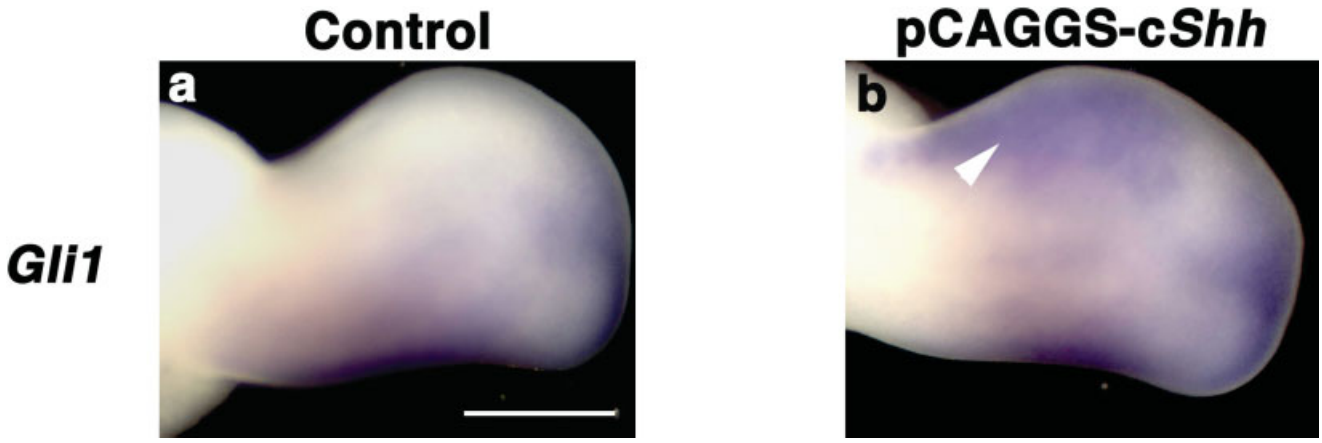

FIG. 5. Ectopic *Gli1* expression by introducing of c*Shh* expression vector (pCAGGS-c*Shh*). **a:** pEGFP was transduced into anterior limb buds at HH stage 20–21 as a control. **b:** pCAGGS-c*Shh* was transduced into anterior limb buds at HH stages 20–21. Ectopic *Gli1* expression was observed in the anterior limb bud only by pCAGGS-c*Shh* transduction after 24 h of the gene transduction. The endogenous *Gli1* expression in the posterior region and distal most region of the limb bud was also observed (**a,b**). Scale bar = 1 mm.

in scattered GFP expression along the neural tubes, probably due to the diffusion of the DNA-microbubble mixture along the lumen of the neural tube (Fig. 7e,f). Examination of the extent of the diffusion of the DNA-microbubble mixture in several injected sites were monitored (data not shown). Diffusion of the DNA-microbubble mixture in the lumen of neural tubes or inside limb mesenchyme was similar to the diffusion of an equal amount of dye (1% FastGreen solution) injection, indicating no enhancement or reduction of diffusion after mixing with the microbubble solution (data not shown). Electroporation, in contrast, enables electric field-dependent expression of the introduced gene, as typically observed for half-side specific expression in the neural tube lumen. Gene transduction by electroporation for developing neural tubes has been utilized to analyze various gene functions (Okafuji *et al.*, 1999; Nakamura and Funahashi, 2001).

Endoh *et al.* (2002) reported gene transduction into the cutaneous epidermis using sonoporation. To assess the applicability of sonoporation for the embryonic surface ectoderm, gene transduction was performed for limb ectoderm. After injecting a DNA-microbubble mixture adjacent to the limb ectoderm inside the amnionic cavity, ultrasound exposure was immediately performed. Scattered gene transduction at the surface of the limb ectoderm was observed (Fig. 7g,h). In sum, the current results indicate that sonoporation may be one of the possible experimental approaches to study gene functions during organogenesis.

Serially Transduced Exogenous Gene Expression in the Developing Limb

Because of the high frequency and low tissue damage after sonoporation, serial transduction of exogenous genes was performed for developing chick embryos. Given that a redundant genetic cascade may underlie various organogeneses, elucidating several gene functions in the same embryonic specimen is required. Thus, serial gene transduction procedures were performed utilizing several different types of approaches. Embryonic specimens were treated twice with sonoporation-mediated gene transduction (total 2 min of ultrasound exposure in the case shown in Fig. 8). After transducing the GFP gene into the anterior limb bud, transduction for the posterior limb bud region was performed immediately after the first gene transduction. As a result, specimens were processed for two DNA-microbubble mixture injections and sonoporations. Prominent two-sited GFP expression was observed after such procedures (Fig. 8). Such exposures did not result in embryonic damage or morphological alterations. The survival rates of the transduced embryos did not show significant deterioration after such serial gene transduction (survival rate 82% after 48 h of treatment; a total of 22 samples were treated).

Cotransduction of GFP and LacZ Expression Vector Into Chick Limb Ectoderm

Examination of the tissues after cotransduction of two expression vectors for the *GFP* and *LacZ* genes revealed that a significantly high rate of the GFP-expressing tissue regions coexpressed the *LacZ* gene in the case for ectodermal sonoporation (Fig. 9). Sectioning through the domains expressing exogenous genes revealed limb ectoderm tissues which coexpress two exogenous genes upon such gene transduction by sonoporation (data not shown). Although further analyses are necessary, relatively high frequencies of coexpression of the transduced genes in the target tissues may reflect the localized exposure of the ultrasound at the local tissue regions adjacent to the ultrasound probe.

DISCUSSION

Feasibility of the Gene Transduction Procedure by Sonoporation

In this article, a new transient gene transduction procedure, sonoporation, was introduced. Generally, tran-

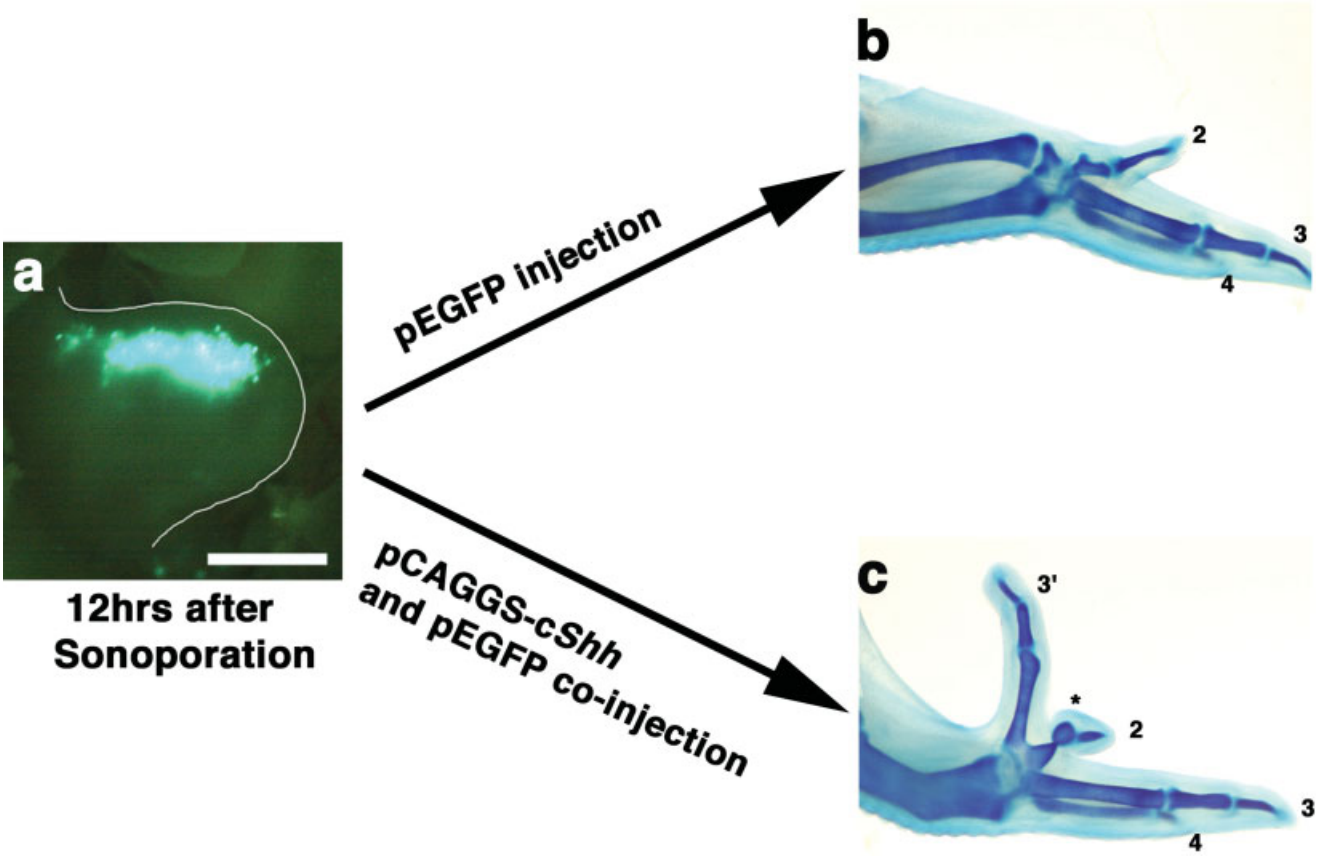

FIG. 6. Additional digit formation as a result of ectopic c*Shh* expression. **c:** Additional digits were observed upon transducing pCAGGS-c*Shh* into anterior chick limb buds. **a:** pCAGGS-c*Shh* and pEGFP were coexpressed to monitor gene transduction efficiency. GFP expression was detected in anterior limb buds. **b:** Normal wing formation 12 h after sonoporation-mediated pEGFP transduction as a control. **c:** Digit 3 duplication with truncated digit 2 formation. The asterisk shows a small cartilaginous element as previously reported (Drossopoulou *et al.*, 2000). Scale bar = 1 mm.

Table 1
Parameters of Gene Transduction Into Various Chick Tissues

Transduced sites and stages (HH stages)	DNA solution (μl): micro-bubbles (μl) (injection volume)	Ultra sound exposure Time (seconds)	Survival rates 48 hours after sonoporation
Branchial arch (HH stages 20–21)	20 (μl): 10 (μl) (0.25 μl)	60 seconds	91% (11/12)
Lateral plate mesoderm (HH stages 15–16)	20 (μl): 20 (μl) (0.25 μl)	60 seconds	80% (8/10)
Neural tube (HH stages 10–11)	20 (μl): 20 (μl) (0.2 μl)	10 seconds	50% (5/10)
Ectoderm of limb (HH stages 21–22)	20 (μl): 20 (μl) (1.0 μl)	10 seconds	100% (15/15)

The parameters for gene transduction into various chick embryonic tissues are summarized. Gene transduction into the branchial arch, lateral plate mesoderm, and ectoderm of limbs were performed with an ultrasound intensity of 2.0 W/cm^2 and duty cycle 20%. The concentration of DNA solution was 1.0 μg/μl. Survival rates were calculated among chick embryos during the 48 h after the operation.

sient gene transduction procedures are required to meet several experimental conditions for various gene transductions. Electroporation has been claimed to possess the advantage of requiring relatively simple experimental procedures (Yasugi and Nakamura, 2000). As a comparison, sonoporation also requires simple procedures, as shown in this study. Because manipulating electric fields by setting and positioning two electrodes is necessary, it could be said that the implementation of sonoporation may require even simpler setting of the ultrasound probe to the target tissue (Fig. 1c,d). A comparison of the efficacies of exogenously introduced

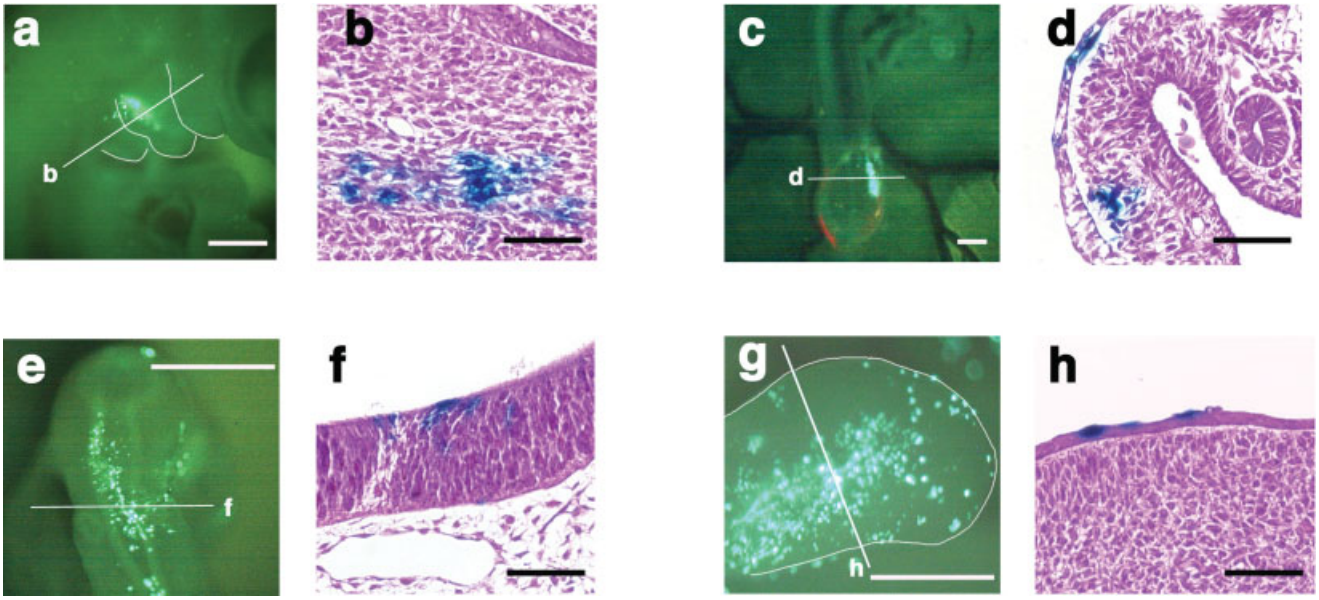

FIG. 7. Gene transduction into various chick embryonic tissues by sonoporation with GFP or LacZ expression vector. **a:** pEGFP transduction into the branchial arch at HH 20–21. GFP expression was detected in the proximal and posterior part of the second branchial arch 12 h after gene transduction. **b:** LacZ expression in the branchial arch mesenchyme is shown by the white line in **a**. **c:** GFP expression in lateral plate mesoderm 10 h after gene transduction. **d:** LacZ expression in the lateral plate mesoderm region and surface ectoderm is shown by the white line in **c**. **e:** GFP expression in a neural tube 12 h after gene transduction. **f:** LacZ expression in the neuroectoderm is shown by the white line in **e**. **g:** GFP expression in limb ectoderm 12 h after gene transduction. **h:** LacZ expression in limb ectoderm is shown by the white line in **g**. Scale bar = 1 mm.

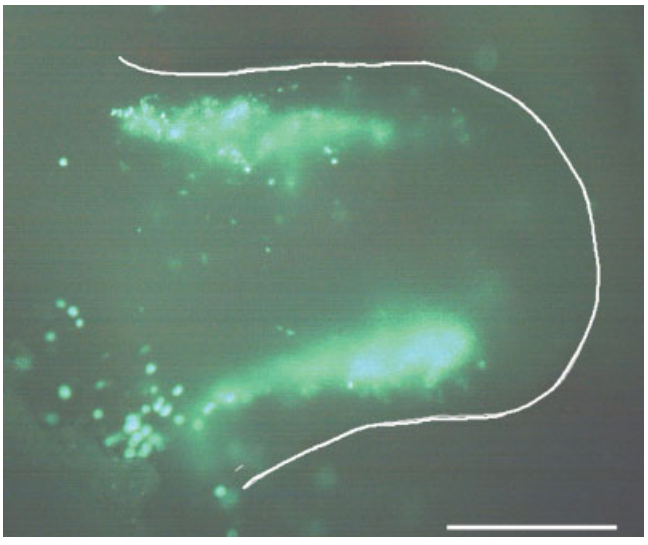

FIG. 8. Serial gene transduction into the developing limb. pEGFP was introduced first to the anterior limb mesenchyme. A second gene introduction into the posterior limb mesenchyme was performed immediately after the initial transduction. GFP expression was detected after 12 h of the transduction at both sites. Scale bar = 1 mm.

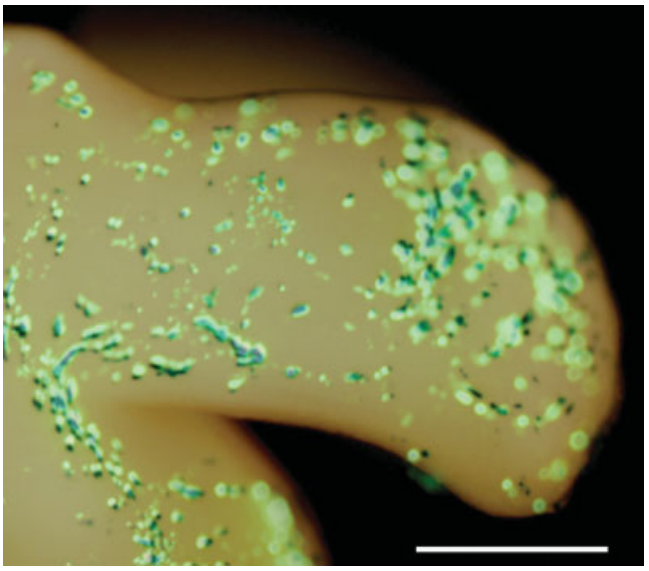

FIG. 9. Cotransduction of GFP and LacZ expression vector into chick limb ectoderm. Examination of the limb ectodermal tissues by GFP and LacZ staining after cotransduction of two expression vectors for GFP gene and LacZ genes. Equal amounts of each DNA solution were mixed to make 1.0 μg/μl solution. After cotransduction of the GFP and LacZ expression vector, GFP expression was first monitored. Subsequently, LacZ expression was stained after fixing the limb bud. The two images were photo-processed and shown as overlaid images.

gene expression profiles for several organs revealed some characteristic tendencies of sonoporation. Gene transduction into the neural tube and limb ectoderm often resulted in dispersed gene expression, possibly due to diffusion of the microinjected microbubbles before ultrasound exposure. Examination of the extent of the diffusion for the injected DNA-microbubble mixture was monitored (data not shown). Diffusion of the DNA-

microbubble mixture in the lumen of neural tubes or inside limb mesenchyme was similar to the diffusion of injected dye (1% FastGreen solution; data not shown). Gene transduction into branchial arches and limb bud mesenchyme resulted in rather locally restricted gene expression after the transduction. Because the sonoporation procedure included microinjection as part of the procedure accompanying possible diffusion, these results may imply that this procedure could be applicable for mesenchymes with less cavitated structures. The efficacy of gene transduction might also depend on the nature of the cells in different regions, because neuroectoderm and limb ectoderm cells tend to express exogenous genes after a relatively short period of exposure to ultrasound (Table 1). Such regional differences including several ectodermal regions and mesenchymal regions should be further studied with different conditions for sonoporation. As part of such differences of gene transduction efficacy, it will be important to regulate the state of diffusion or the retention of injected solution at the operated site. Our previous results utilizing a higher concentration of injected DNA, which increased the viscosity of the DNA-microbubble mixture, failed to increase gene transduction efficiency in neural tubes (data not shown). Increasing viscosity of the injected mixtures might also have affected several physicochemical characteristics of cavitation and the disruption of the microbubbles generated by ultrasound exposure, thus possibly affecting the frequency of gene transduction.

Gene transduction efficiency mediated by sonoporation could be affected by several parameters, including selection of the stages and target regions of embryos and the physicochemical nature of the injected solutions, which may require further analyses.

Sonoporation-Mediated Gene Transduction Into the Developing Limbs

The current study revealed that the developing limbs of chick embryos may be one of the suitable target tissues for molecular developmental analysis by sonoporation. The developing limb bud has been investigated as one of the major developmental systems for organogenesis (Izpisua Belmonte *et al.*, 1991; Pizette *et al.*, 2001; Kardon *et al.*, 2002; Tanaka *et al.*, 2002; Trelles *et al.*, 2002). Chick embryos have been investigated in such researches with electroporation or retrovirus-mediated gene expression. Recently, gene transduction by electroporation utilizing various expression vectors has been successfully performed to study gene functions in vivo (Takeuchi *et al.*, 1999). We performed sonoporation-mediated gene transduction utilizing an expression vector, e.g., with the pCAGGS vector containing the chick *Shh* gene. Transduction of *Shh* induced ectopic *Gli1* expression in the anterior limb bud, suggesting that sonoporation may be useful for ectopic gain-of-function studies for analyzing gene cascade during organogenesis. In addition to the alteration of the marker gene expression upon *Shh* transduction, it resulted in limb dysmorphogenesis, e.g., duplication of digit 3. It is known that an average of 79 ZPA cells grafted in the anterior limb bud mesenchyme yielded digit 3 duplication (Tickle, 1981). Although the exact extent of the misexpressed SHH level remains unclear, the sonoporated limb bud specimen may represent a digit duplication plausibly overexpressing a similar level of SHH. This result suggests that sonoporation-mediated gene transduction may be applicable to various molecular developmental analyses.

Possible Advantages of Sonoporation as a Gene Transduction Method

The current study revealed that embryonic toxicities were observed to be significantly low after sonoporation. In fact, no significant cell death was observed after sonoporation treatment of embryonic limbs. For decades, ultrasound exposure-assisted diagnosis has been widely utilized for fetal diagnosis. As for microbubble solutions per se, they have also been widely utilized for blood vessel contrasting diagnosis. Because sonoporation-mediated gene transduction is composed of procedures including injection of DNA-microbubble mixtures and subsequent ultrasound exposures, it is expected to give cause a small amount of tissue damage after the operation. In fact, it was confirmed that it could be one of the suitable gene transduction methods manifesting small amounts of embryonic damage. The current study has shown that gene transduction into several embryonic organs is feasible. Judged by the simplicity and small amount of damage caused by the procedure, this method could also be utilized in combination with other gene transduction methods. In fact, this procedure may be regarded as one of the selectable strategies for analyzing gene cascades, because it enables serial gene transductions. Although further analyses are necessary, the relatively high frequency of coexpression of the transduced genes may reflect the localized exposure of the ultrasound at the tissue regions adjacent to the ultrasound probe. Retroviral gene transfer has often been shown to be inappropriate for such purposes because of the nature of viral infection. Due to interference of the recombinant-virus-receptor interaction by the preceding viral infection, serial gene transduction by the same sort of retroviruses has been regarded as not useful due to the nature of viral infection.

Thus, although further technical improvement will be necessary, it may be possible to perform cotransduction of exogenous genes and also serial gene transductions into the target site by sonoporation.

In the current study, a 3 mm diameter ultrasound emission probe was utilized for gene transduction into embryos. Depending on the size of the target organ, the selection and modification of the size and shape of the ultrasound probes will be required. In addition to the 3 mm diameter, an ultrasound probe with a 1 mm diameter is now under evaluation for gene transduction efficiency in various embryonic organs (Ohta *et al.*, unpubl. results). Adoption of smaller and finer ultrasound probes might enable more restricted embryonic gene transduction with less tissue damage. Assessment of new kinds of microbubble solutions will also be useful because of the

potentially different physicochemical natures of the solutions. Some new microbubble solutions possess positively charged microparticles, which might be useful for stabilizing DNA upon gene transduction.

MATERIALS AND METHODS

Chick Embryos

Fertilized White Leghorn chicken eggs were purchased from Marui Farm, Co. (Japan). Eggs were incubated at 38.5°C and staged according to the Hamburger and Hamilton classification (Hamburger and Hamilton, 1951).

Plasmid Construction and Preparation

pEGFP-C1 was purchased from ClonTech (Palo Alto, CA). pEBActN-LacZ contains the coding region of LacZ as an insert of HindII/BamHIcDNA fragment of the *LacZ*, cloned into the pEBActNII expression vector (Saeki *et al.*, 1998). pCAGGS-*cShh* was constructed by inserting full-length chick *Shh* cDNA into the EcoRI site of the pCAGGS vector (Miyazaki et. al., 1989). Each plasmid was purified using a Qiagen (Chatsworth, CA) plasmid isolation kit. Plasmid DNA was dissolved in 10 mM Tris-HCl (pH 8.0) at a concentration of 1.0 μg/μl.

Preparation of DNA-Microbubble Mixture and Ultrasound Exposure

Optison (Mallinckrodt, San Diego, CA, purchased from Nepagene Co., Japan) was used for the microbubble solution. Optison is an albumin-shelled ultrasound contrast agent composed of perfluorocarbon-filled microbubbles with an average diameter of 3–4 μm ($5–8 \times 10^8$ bubbles/ml). Ten μl of a DNA solution, such as pEGFP-C1, pEBActNII-*LacZ*, or pCAGGS-c*Shh*, was diluted 2-fold with sterilized water and 10 μl or 20 μl of optison was added, as described previously (Endoh *et al.*, 2002). The DNA-microbubbles mixture was injected into various chick embryonic regions with a glass microneedle made from a glass capillary tube (GD-1.2; Narishige, Tokyo, Japan). The injected chick embryos were immediately treated with ultrasound irradiation utilizing an ultrasound probe (Sonitron 2000N, Rich Mar, Inola, OK, USA) with a diameter of 3 mm. Optimal ultrasound conditions are described in the text.

In Situ Hybridization for Gene Expression

Whole-mount in situ hybridization was performed with digoxigenin-labeled probes according to standard procedures. The probes were *Shh* (Riddle *et al.*, 1993), and *Gli1* (Marigo *et al.*, 1996).

LacZ Staining

pEBActNII-*LacZ* was injected to the anterior region of chick limb buds. After 12 h of gene transduction, embryos were rinsed several times with PBS and fixed with PBS containing 0.2% glutaraldehyde, 1% formalin, and 0.02% NP40 for 60 min at 4°C. They were washed four times with PBS for 20 min on ice, incubated at 49.5°C for 50 min, and stained with a solution containing 20 mg/ml X-gal. Stained embryos were placed in Tissue Tek (Sakura Finetek, Torrance, CA), embedded with paraffin, and sections (5 μm) were counterstained with hematoxylin and eosin.

Skeletal Staining

Chick embryos (HH stages 35–36) were fixed with 4% PFA overnight at 4°C, and then stained for 5 h with Alcian blue solution (0.02% Alcian blue dissolved in a mixture of 70% ethanol and 30% glacial acetic acid). After staining, embryos were cleared with 1% KOH and stored in 1% KOH with 50% glycerol.

ACKNOWLEDGMENTS

We thank Drs. Andrew McMahon, Richard R. Behringer, Evan Unger, Denis Duboule, Juan-Carlos Izpisua Belmonte, Lee Niswander and Cliff Tabin for reagents and/or suggestions, and Shiho Kitagawa for assistance. We also thank the unidentified reviewers.

LITERATURE CITED

Anwer K, Kao G, Proctor B, Anscombe I, Florack V, Earls R, Wilson E, McCreery T, Unger E, Rolland A, Sullivan SM. 2000. Ultrasound enhancement of cationic lipid-mediated gene transfer to primary tumors following systemic administration. Gene Ther 7:1833–1839.

Araki I, Nakamura H. 1999. Engrailed defines the position of dorsal di-mesencephalic boundary by repressing diencephalic fate. Development 126:5127–5135.

Ay T, Havaux X, Van Camp G, Campanelli B, Gisellu G, Pasquet A, Denef JF, Melin JA, Vanoverschelde JL. 2001. Destruction of contrast microbubbles by ultrasound: effects on myocardial function, coronary perfusion pressure, and microvascular integrity. Circulation 104:461–466.

Bao S, Thrall BD, Miller DL. 1997. Transfection of a reporter plasmid into cultured cells by sonoporation in vitro. Ultrasound Med Biol 23:953–959.

Drossopoulou G, Lewis KE, Sanz-Ezquerro JJ, Nikbakht N, McMahon AP, Hofmann C, Tickle C. 2000. A model for anteroposterior patterning of the vertebrate limb based on sequential long- and short-range Shh signalling and Bmp signalling. Development 127: 1337–1348.

Endoh M, Koibuchi N, Sato M, Morishita R, Kanzaki T, Murata Y, Kaneda Y. 2002. Fetal gene transfer by intrauterine injection with microbubble-enhanced ultrasound. Mol Ther 5:501–508.

Feril LB, Kondo T, Zhao QL, Ogawa R, Tachibana K, Kudo N, Fujimoto S, Nakamura S. 2003. Enhancement of ultrasound-induced apoptosis and cell lysis by echo-contrast agents. Ultrasound Med Biol 29:339–345.

Iba H. 2000. Gene transfer into chicken embryos by retrovirus vectors. Dev Growth Differ 42:213–218.

Hamburger V, Hamilton H. 1951. A series of normal stages in the development of the chick embryo. J Morphol 88:49–92.

Izpisua Belmonte JC, Tickle C, Dolle P, Wolpert L, Duboule D. 1991. Expression of the homeobox Hox-4 genes and the specification of position in chick wing development. Nature 350:585–589.

Kardon G, Campbell JK, Tabin CJ. 2002. Local extrinsic signals determine muscle and endothelial cell fate and patterning in the vertebrate limb. Dev Cell 3:533–545.

Katahira T, Sato T, Sugiyama S, Okafuji T, Araki I, Funahashi J, Nakamura H. 2000. Interaction between Otx2 and Gbx2 defines the organizing center for the optic tectum. Mech Dev 91:43–52.

Kengaku M, Capdevila J, Rodriguez Esteban C, De La Pena J, Johnson RL, Belmonte JC, Tabin CJ. 1998. Distinct WNT pathways regulating AER formation and dorsoventral polarity in the chick limb bud. Science 280:1274–1277.

Kim HJ, Greenleaf JF, Kinnick RR, Bronk JT, Bolander ME. 1996. Ultrasound-mediated transfection of mammalian cells. Hum Gene Ther 7:1339–1346.

Koch S, Pohl P, Cobet U, Rainov NG. 2000. Ultrasound enhancement of liposome-mediated cell transfection is caused by cavitation effects. Ultrasound Med Biol 26:897–903.

Lawrie A, Brisken AF, Francis SE, Cumberland DC, Crossman DC, Newman CM. 2000. Microbubble-enhanced ultrasound for vascular gene delivery. Gene Ther 7:2023–2027.

Marigo V, Johnson RL, Vortkamp A, Tabin CJ. 1996. Sonic hedgehog differentially regulates expression of GLI and GLI3 during limb development. Dev Biol 180:273–283.

Miyazaki J, Takaki S, Araki K, Tashiro F, Tominaga A, Takatsu K, Yamamura K. 1989. Expression vector system based on the chicken beta-actin promoter directs efficient production of interleukin-5. Gene 79:269–277.

Morgan BA, Fekete DM. 1996. Manipulating gene expression with replication-competent retroviruses. Methods Cell Biol 51:185–218.

Nakamura H, Funahashi J. 2001. Introduction of DNA into chick embryos by in ovo electroporation. Methods 24:43–48.

Newman CM, Lawrie A, Brisken AF, Cumberland DC. 2001. Ultrasound gene therapy: on the road from concept to reality. Echocardiography 18:339–347.

Oberg KC, Pira CU, Revelli JP, Ratz B, Aguilar-Cordova E, Eichele G. 2002. Efficient ectopic gene expression targeting chick mesoderm. Dev Dyn 224:291–302.

Okafuji T, Funahashi J, Nakamura H. 1999. Roles of Pax-2 in initiation of the chick tectal development. Brain Res Dev Brain Res 116:41–49.

Pizette S, Niswander L. 1999. BMPs negatively regulate structure and function of the limb apical ectodermal ridge. Development 126: 883–894.

Pizette S, Abate Shen C, Niswander L. 2001. BMP controls proximodistal outgrowth, via induction of the apical ectodermal ridge, and dorsoventral patterning in the vertebrate limb. Development 128: 4463–4474.

Riddle RD, Johnson RL, Laufer E, Tabin C. 1993. Sonic hedgehog mediates the polarizing activity of the ZPA. Cell 75:1401–1416.

Rodriguez Esteban C, Schwabe JW, Pena JD, Rincon Limas DE, Magallon J, Botas J, Belmonte JC. 1998. Lhx2, a vertebrate homologue of apterous, regulates vertebrate limb outgrowth. Development 125: 3925–3934.

Saeki Y, Wataya Kaneda M, Tanaka K, Kaneda Y. 1998. Sustained transgene expression in vitro and in vivo using an Epstein-Barr virus replicon vector system combined with HVJ liposomes. Gene Ther 5:1031–1037.

Saunders JW, Gasseling MT. 1968. Ectodermal-mesenchymal interactions in the origin of limb symmetry. In: Fleischmeyer R, Billingham RE, editors. Ectodermal-mesenchymal interactions. Baltimore: Williams & Wilkins. p 78–97.

Shohet RV, Chen S, Zhou YT, Wang Z, Meidell RS, Unger RH, Grayburn PA. 2000. Echocardiographic destruction of albumin microbubbles directs gene delivery to the myocardium. Circulation 101:2554–2556.

Tachibana K, Tachibana S. 1995. Albumin microbubble echo-contrast material as an enhancer for ultrasound accelerated thrombolysis. Circulation 92:1148–1150.

Tachibana K, Tachibana S. 2001. The use of ultrasound for drug delivery. Echocardiography 18:323–328.

Takahashi M, Sato K, Nomura T, Osumi N. 2002. Manipulating gene expressions by electroporation in the developing brain of mammalian embryos. Differentiation 70:155–162.

Takeuchi JK, Koshiba Takeuchi K, Matsumoto K, Vogel Hopker A, Naitoh Matsuo M, Ogura K, Takahashi N, Yasuda K, Ogura T. 1999. Tbx5 and Tbx4 genes determine the wing/leg identity of limb buds. Nature 398:810–814.

Tanaka M, Munsterberg A, Anderson WG, Prescott AR, Hazon N, Tickle C. 2002. Fin development in a cartilaginous fish and the origin of vertebrate limbs. Nature 416:527–531.

Taniyama Y, Tachibana K, Hiraoka K, Namba T, Yamasaki K, Hashiya N, Aoki M, Ogihara T, Yasufumi K, Morishita R. 2002a. Local delivery of plasmid DNA into rat carotid artery using ultrasound. Circulation 105:1233–1239.

Taniyama Y, Tachibana K, Hiraoka K, Aoki M, Yamamoto S, Matsumoto K, Nakamura T, Ogihara T, Kaneda Y, Morishita R. 2002b. Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle. Gene Ther 9:372–380.

Tickle C. 1981. The number of polarizing region cells required to specify additional digits in the developing chick wing. Nature 289:295–298.

Timmer J, Johnson J, Niswander L. 2001. The use of in ovo electroporation for the rapid analysis of neural-specific murine enhancers. Genesis 29:123–132.

Tomko RP, Johansson CB, Totrov M, Abagyan R, Frisen J, Philipson L. 2000. Expression of the adenovirus receptor and its interaction with the fiber knob. Exp Cell Res 255:47–55.

Trelles RD, Leon JR, Kawakami Y, Simoes S, Belmonte JC. 2002. Expression of the chick vascular endothelial growth factor D gene during limb development. Mech Dev 116:239–242.

Unger E, Metzger P 3rd, Krupinski E, Baker M, Hulett R, Gabaeff D, Mills J, Ihnat D, McCreery T. 2000. The use of a thrombus-specific ultrasound contrast agent to detect thrombus in arteriovenous fistulae. Invest Radiol 35:86–89.

Wasan EK, Reimer DL, Bally MB. 1996. Plasmid DNA is protected against ultrasonic cavitation-induced damage when complexed to cationic liposomes. J Pharm Sci 85:427–433.

Yamada G, Nakamura S, Haraguchi R, Sakai M, Terashi T, Sakisaka S, Toyoda T, Ogino Y, Hatanaka H, Kaneda Y. 1997. An efficient liposome-mediated gene transfer into the branchial arch, neural tube and the heart of chick embryos; a strategy to elucidate the organogenesis. Cell Mol Biol (Noisy le grand) 43:1165–1169.

Yang Y, Drossopoulou G, Chuang PT, Duprez D, Marti E, Bumcrot D, Vargesson N, Clarke J, Niswander L, McMahon A, Tickle C. 1997. Relationship between dose, distance and time in Sonic Hedgehog-mediated regulation of anteroposterior polarity in the chick limb. Development 124:4393–4404.

Yasuda K, Momose T, Takahashi Y. 2000. Applications of microelectroporation for studies of chick embryogenesis. Dev Growth Differ 42:203–206.

Yasugi S, Nakamura H. 2000. Gene transfer into chicken embryos as an effective system of analysis in developmental biology. Dev Growth Differ 42:195–197.