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Original Article

Embryonic expression of ß-actin-lacZ hybrid gene injected into the fertilized ovum of the domestic fowl

MITSURU NAITO[1]*, KIYOKAZU AGATA[2¤], KATSUMASA OTSUKA[3], KATSUTOSI KINO[3], MOTOYOSHI OHTA[3], KAZUO HIROSE[3], MARGARET M. PERRY[4] and GORO EGUCHI[2]

[1]*National Institute of Animal Industry, Tsukuba Norindanchi, Ibaraki, Japan,* [2]*National Institute for Basic Biology, Myodaiji-cho, Okazaki, Japan,* [3]*Aichi-ken Agricultural Research Center, Nagakute, Aichi, Japan and* [4]*AFRC Institute of Animal Physiology and Genetics Research, Roslin, Midlothian, United Kingdom*

ABSTRACT An experiment was carried out to investigate the expression of cloned DNA injected into the germinal disc of the chick fertilized ovum. The ß-actin-lacZ hybrid gene, MiwZ, was injected, in the closed circular form, into the cytoplasm of the germinal disc at the single-cell stage. The embryos were cultured *in vitro*, then in recipient eggshells up to day 4 of incubation. The survival rate of the embryos at day 4 was 42% (55/130), and the rate of embryos expressing MiwZ was 64% (35/55). Twenty-two embryos expressed the MiwZ in both embryonic and extraembryonic tissues, while the remainder expressed the MiwZ in only extraembryonic tissues. Mosaic expression was observed in most of the embryos expressing MiwZ in embryonic tissues. Expression throughout all tissues of the embryo including blood cells occurred in one case. In this case, the injected DNA was assumed to have integrated at an earlier stage. The results indicate that it is now possible to investigate the promoter activities of introduced exogenous genes as well as the effect of introduced genes on embryogenesis in early chick embryos. This technique may also facilitate the production of transgenic chicks.

KEY WORDS: *chick, germinal disc, microinjection, lacZ*

Introduction

Perry (1988) developed a complete culture system for the chick embryo from a single cell to hatching. Recently, Naito *et al.* (1990) have improved this method and obtained a much higher rate of hatching (34.4%) of fertilized ova grown in culture. If cloned genes can be introduced into single-cell stage embryos and the recipients are developed *in vitro*, this will prove a powerful method for analyzing embryogenesis at the molecular level.

Various methods of gene transfer to chicks have been employed in attempts to obtain transgenic chicks. Transgenic chicks have been produced successfully using retroviruses as a vector (Souza *et al.*, 1984; Shuman and Shoffner, 1986; Salter *et al.*, 1986, 1987; Hippenmeyer *et al.*, 1988; Bosselman *et al.*, 1989; Crittenden *et al.*, 1989; Salter and Crittenden, 1989). This method, however, has disadvantages such as a limitation in size of the inserted genes and difficulties in preparation of high-efficiency infectious viruses. Petitte *et al.* (1990) have succeeded in developing a technique for blastodermal cell transfer and attempted to produce transgenic chicks from blastodermal chimeras (Gibbins *et al.*, 1990). Sang and Perry (1989) microinjected the bacterial CAT gene into the fertilized ovum at the single-cell stage and analyzed the fate of the injected DNA during development. They reported that some of the DNA was lost after injection, but that the remainder was replicated approximately 20-fold during the first 24 h of development. Between day 1 and day 7 in culture, the DNA was gradually lost and diluted out as the embryos developed. No evidence for chromosomal integration of the exogenous DNA was obtained, suggesting that the plasmid DNA persisted episomally. These observations suggest that the microinjection method may not be appropriate for the efficient production of transgenic chicks, but could be suitable for analyzing the expression and the effect of microinjected genes during early development of chick embryos. Injected DNA may be expressed extrachromosomally without position effect during the dynamic morphogenetic events occurring in the first 3 days of chick development. To examine whether microinjected DNAs are efficiently expressed in early embryos, we introduced the *E. coli* ß-galactosidase gene recombined with the chicken ß-actin promoter/enhancer and the RSV enhancer element into fertilized eggs, and obtained the expression of the exogenous gene at high frequency.

Abbreviations used in this paper: RSV, raus sarcoma virus; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; ES cell, embryonic stem cell.

***Address for reprints**: National Institute of Animal Industry. Tsukuba Norindanchi, P.O. Box 5, Ibaraki 305, Japan. FAX: 298-38-8606.

¤Present address: Department of Life Science, Himeji Institute of Technology, Harima Science Park City, Hyogo, Japan

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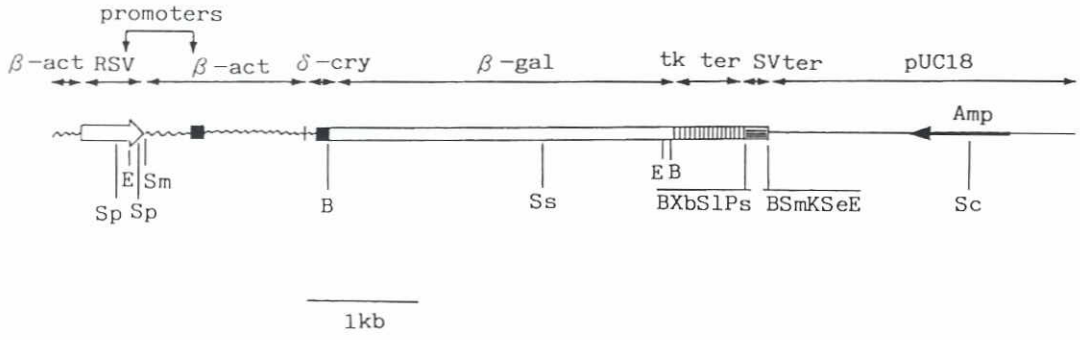


Fig. 1. Structure of the recombinant plasmid, MiwZ. *ß-actin (ß-act) and δ-crystallin (δ-cry) sequences are shown by wavy lines and their exon sequences by the solid boxes. Bacterial sequences (ß-gal) are indicated by open box, HSV-tk sequences by box with vertical stripes, SV40 sequences by box with horizontal stripes, the RSV LTR sequences by the open arrow, and plasmid vector sequence by the horizontal line. Promoters and termination signals (tk ter and SV ter) are also indicated. Restriction sites: B, BamHI; E, EcoRI; K, KpnI; Ps, PstI; Sc, ScaI; Sl, SalI, Sm, SmaI; Sp, SphI; Ss, SstI; Xb, XbaI.*

Results

Introduction of the MiwZ by oblique or vertical injection

The pronuclei in the central region of the germinal disc are masked by yolk spheres and could not be identified by microscopy. For the preliminary experiments, the micropipette was inserted obliquely into the central site of the germinal disc and DNA was injected into the cytoplasm of fertilized ova, followed by up to 4 days of embryo culture. Expression of the MiwZ was detected in most cultures, but the area of the expression of MiwZ was spread in a radial manner from the injection site at the blastoderm stage (Fig. 2A). In 3-day-old embryos, expression of the MiwZ was observed frequently in the extraembryonic tissues in a radial manner as in Fig. 2B, but no embryonic tissue expression was observed in any of the cultures. These results suggest that injected DNAs do not diffuse through the cytoplasm of fertilized ova, and that only the cells derived from the injected region incorporate exogenous DNA into the nuclei and express MiwZ. Thus, in the next experiments, the micropipette was inserted vertically into the center of the germinal disc, in order to inject DNAs into that part of the cytoplasm which contributes to embryonic tissues and germ cells (Ginsburg and Eyal-Giladi, 1987). As expected, embryonic tissue expression of the MiwZ was observed in some cultures by vertical injection.

DNA concentration, embryonic development and MiwZ expression

The results of the expression of MiwZ injected vertically into the germinal disc are shown in Table 1. When the DNA concentration was 1.0 mg/ml, only 1 out of 62 manipulated embryos survived on day 4 of incubation. Development of most embryos was arrested at the early blastoderm stage. High doses of DNA may be very toxic for chick embryos, as found for *Xenopus* embryos (Gurdon, 1974). On the other hand, when the DNA concentration was 0.1 mg/ml, the survival rate of the manipulated embryos was 42% (55/130) on day 4 of incubation. In 64% (35/55) of them the MiwZ was expressed in the embryonic or extraembryonic tissues. Embryonic expression of the MiwZ was detected in 22 (40.0%) out of the 55 embryos surviving on day 4 of incubation, and in the remaining 13 embryos (23.6%) the MiwZ expressed in the extraembryonic tissues only.

MiwZ expression in the embryonic tissues

Fig. 4A shows an embryo cultured for 4 days after injection of water. The embryo developed normally, and no endogenous ß-galactosidase activity was detected at this stage by our histochemical procedure. Embryos shown in Figs. 3A, 3B and 3C, cultured for 3 or 4 days, show expression of the MiwZ in the embryonic as well as extraembryonic tissues, but the expression pattern is mosaic. Fig. 3A illustrates a 3-day embryo showing expression of the MiwZ in the embryonic and extraembryonic tissues. The MiwZ is expressed almost throughout the embryos, but in a mosaic manner. A radial staining pattern in each somite and a columnar staining pattern in the neural tube are observed (see insert). Figs. 3B and 3C illustrate other embryos incubated for 4 days. In the embryo shown in Fig. 3B, a columnar staining pattern of the neural tube is clear. In Fig. 3C, the expression of MiwZ can be mainly recognized in the heart and neural tube. Table 2 shows the site of expression of MiwZ in the 22 embryos incubated for 4 days. The incidence of expression was higher in the neural tube (64%) than in other organs and tissues. Fig. 4B shows an intensely stained embryo incubated for 4 days. This was the only case in which the expression of MiwZ occurred throughout the embryo, probably in all cell types including some blood cells.

Discussion

In this study, we succeeded in obtaining expression of the MiwZ in both embryonic and extraembryonic tissues by injecting the

TABLE 1

EXPRESSION OF MiwZ IN CHICK EMBRYOS AT 4 DAYS FOLLOWING INJECTION INTO THE CHICK GERMINAL DISC

DNA concentration	Number of eggs manipulated	Number (%) of embryos on day 4	Number of embryos (%) showing expression of MiwZ			
			No expression	Extraembryonic tissues only	Part* of embryo	Whole* embryo
1 mg/ml	62	1 (1.6)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)
0.1 mg/ml	130	55 (42.3)	20 (36.4)	13 (23.6)	21 (38.2)	1 (1.8)

*Embryonic and extraembryonic tissue expression

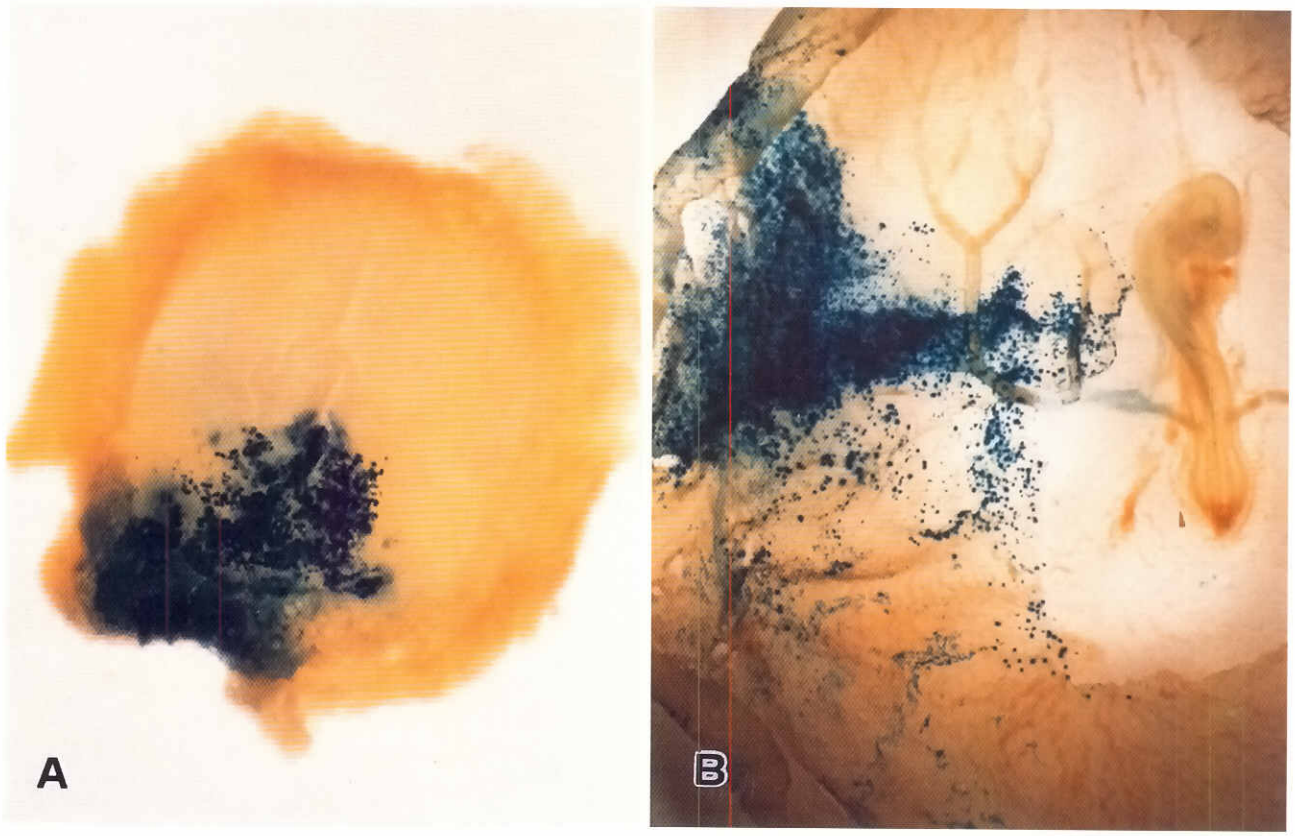
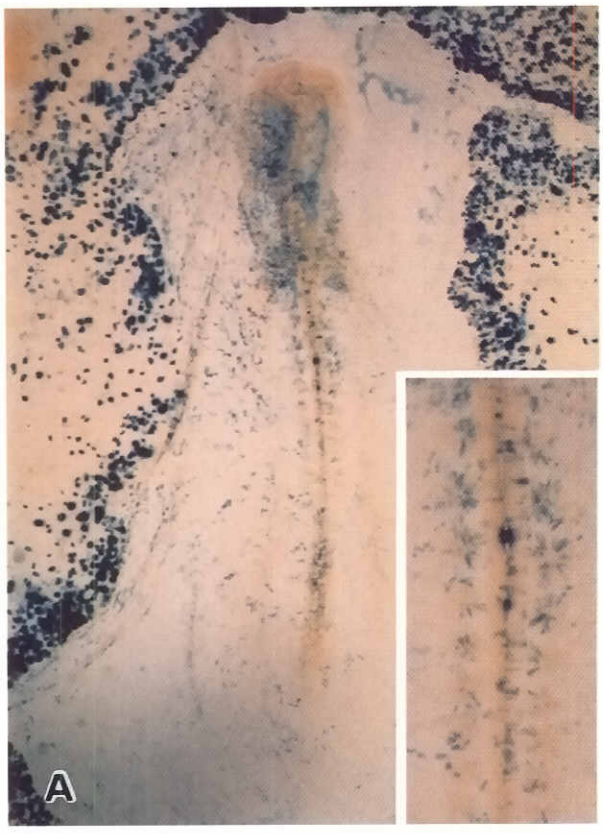
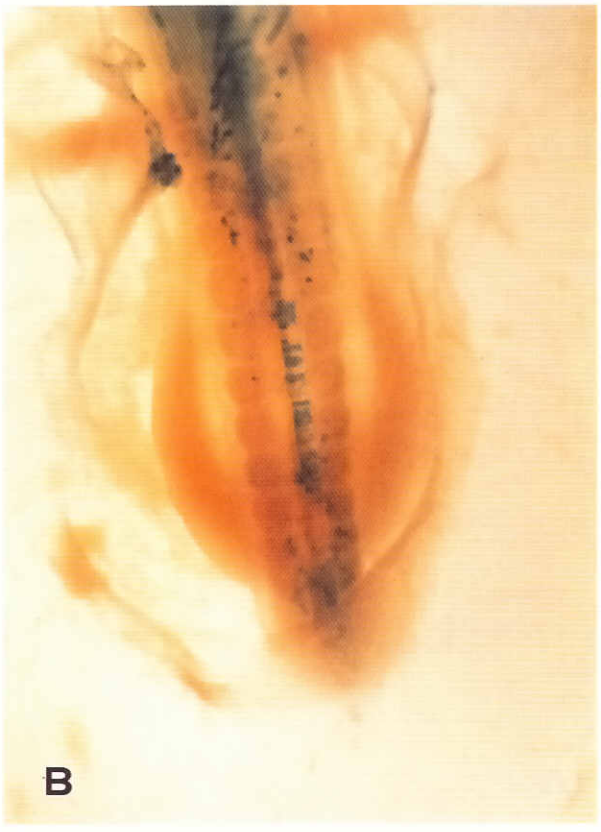


Fig. 2. Expression of the MiwZ by oblique injection. *The MiwZ was injected obliquely into the germinal disc of the fertilized ovum at the single-cell stage. Expression of the MiwZ was analyzed after 1 day* **(A)** *and 3 days* **(B)** *of incubation.*

corresponding DNA into the cytoplasm of the chick germinal disc at the single-cell stage. This technique enables us to investigate the promoter activities of introduced genes and also the effect of introduced genes, the products of which are expected to regulate chick embryogenesis at the molecular level. In this study, we obtained one promising case in which the MiwZ was expressed throughout the embryo (Fig. 4B). In this case the injected DNA might have been incorporated into the nucleus at the single-cell stage, and the DNA integrated in the host chromosome at an early stage of development. This suggests that it may be possible to produce transgenic chicks by the method of injecting DNA into the cytoplasm of the germinal disc at the single-cell stage. Most of the embryos expressing the MiwZ in this study were mosaic. This kind of mosaicism is also observed in *Drosophila* and *Xenopus* when DNA is microinjected into the cytoplasm (Steller and Pirrotta, 1985; Etkin and Pearman, 1987). The oblique injection experiment indicated that injected DNA does not diffuse throughout the cytoplasm of the germinal disc (Figs. 2A-B). If DNA is injected at a site far from the first cleavage plane, it will not be distributed in all of the cells of the embryo. In such a case DNA might be introduced only into one of the two daughter cell nuclei after the first cleavage or a small population of cells at a later stage, so that the distribution of DNA would become mosaic in the embryos or extraembryonic tissues. In order to minimize this kind of mosaicism, the micropipette was inserted «vertically» into the central site of the germinal disc close to the pronuclei. Since the cytoplasm of the chick germinal disc is opaque and the thickness of the germinal layer is about 100 μm (Kochav *et al.*, 1980), it is difficult to position the tip of the micropipette in the germinal layer. Sang and Perry (1989) marked the micropipette at a distance of 150-200 μm and 2.0-2.5 mm from the tip to determine the depth of penetration into the cytoplasm and the volume of DNA, respectively. By their method, it seems difficult to inject DNA into the germinal layer close to the upper surface. It may be important to inject DNA into the central part of the germinal layer close to the upper surface because embryonic tissues and germ cells may originate from the central zone of the area pellucida (Ginsburg and Eyal-Giladi, 1987). In order to overcome the difficulty of injecting DNA into the germinal layer, a continuous flow of solution from the micropipette was maintained while the micropipette was inserted vertically into the central area of the germinal disc. This method ensured that some of the DNA was placed in the cytoplasm of the central area of the germinal layer close to the upper surface. Although the volume of DNA flowing out from the micropipette was not constant, injection into the germinal layer in many of the manipulated eggs appeared to be successful, because the expression of MiwZ was detected in about 60% of the cultures surviving on day 4 of incubation.

In the present experiment, we injected the closed circular form of DNA in order to investigate the transient expression of the DNA, because circular form DNA seems to resist digestion with DNase in





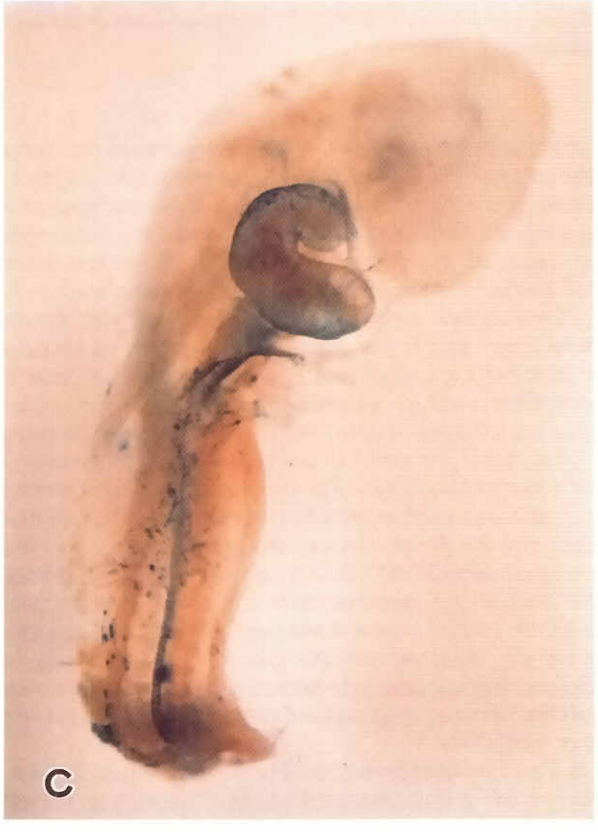


Fig. 3. Mosaic expression of the MiwZ in the embryos. *The MiwZ was injected vertically into the germinal disc of the fertilized ovum, and incubated for 3 days* **(A)** *and 4 days* **(B, C).** *In Fig. 3A, the middle part of neural tube and somites is enlarged in the insert (lower right corner).*

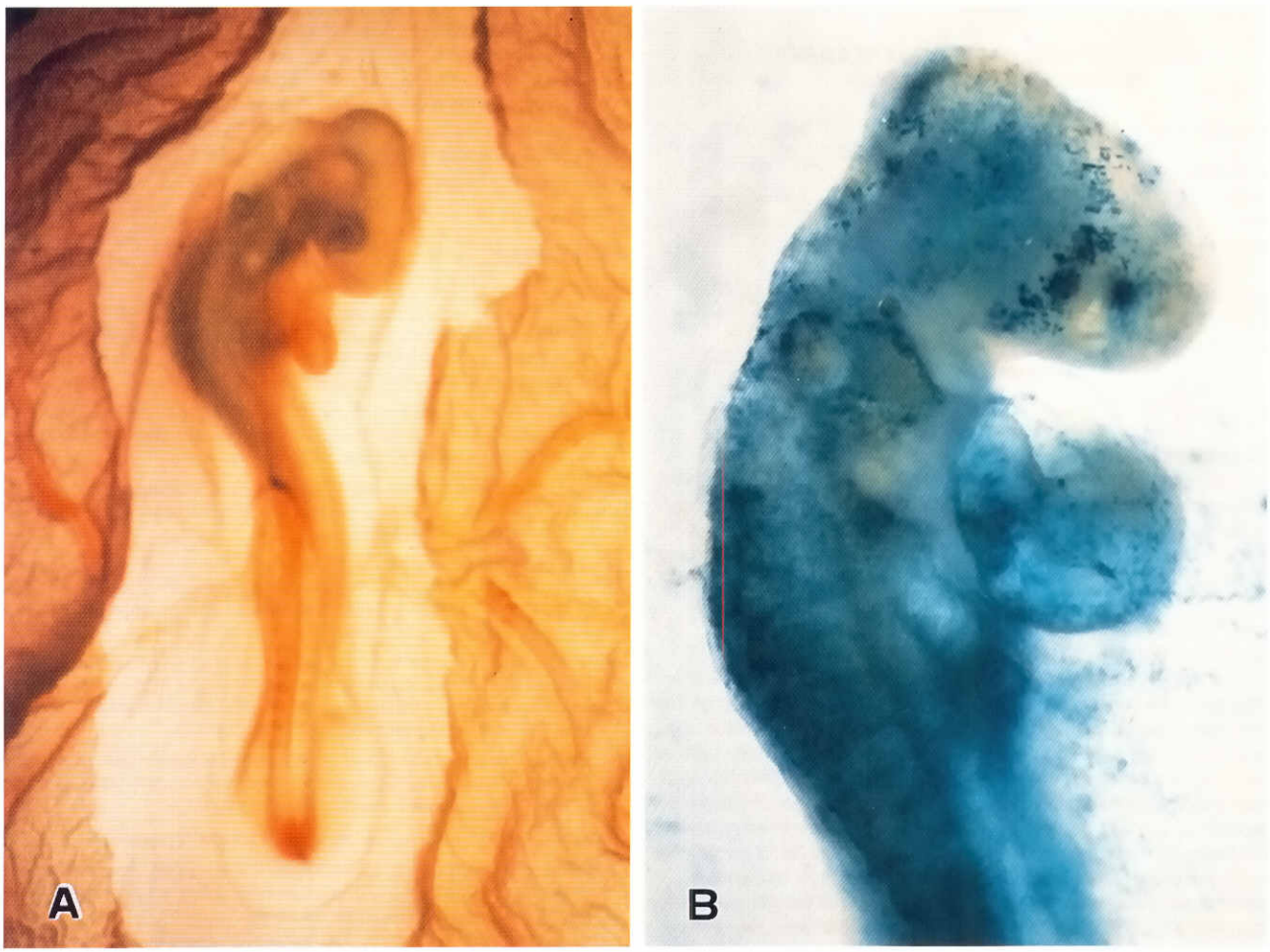


Fig. 4. Expression of the MiwZ throughout the embryos. *The MiwZ was injected into the germinal disc of the fertilized ovum and incubated for 4 days* **(B)**. *A control embryo was incubated for 4 days and stained* **(A)**.

the cytoplasm (Fu *et al.*, 1989) and to be maintained extrachromosomally to a later stage. Sang and Perry (1989) reported that the linear DNA molecules injected into the germinal disc are ligated rapidly after injection to form random concatamers. However, they did not obtain any evidence that injected DNA is incorporated into the host chromosomes from analysis of the fate of injected DNA in 253 embryos. In the present experiment, we have only analyzed expression of the injected DNA but not its fate. However, the injected DNA is assumed to have been integrated at an earlier stage in at least one case (Fig. 4B). This suggests that some closed circular DNA molecules could be incorporated into the host chromosomes, and further analysis of the fate of DNA injected by our method is planned.

The transcriptional activity of the MiwZ construct is very strong. Most positive cells are detected within 5-10 min of incubation in the X-gal solution. We have also obtained intense staining in all types of cultured cells of chick embryos transfected with MiwZ (data not shown). In mice, Suemori *et al.* (1990) obtained ES cells, in which MiwZ is uniformly expressed. All derivatives of these ES cells in chimeras expressed MiwZ. These data suggest that all cells incorporating MiwZ can express MiwZ, and that the mosaic expression may result from the non-uniform distribution of MiwZ, but not from differential on the promoter activity. However, in the present experiment the MiwZ expression tended to be exhibited by the extraembryonic membranes and neural tube (Table 2). It is supposed that this tendency may reflect the proliferation rate of cells, because DNA existing extrachromosomally is diluted out by proliferation.

Materials and Methods

Preparation of the fertilized ova

White Leghorn hens were artificially inseminated. The hens were killed by intravenous injection of sodium pentabarbitone (100-150mg/bird) at 2.75 h after oviposition of the preceding egg, and fertilized ova were obtained from the magnum of the oviduct. At this time the fertilized ovum is at the precleavage stage and the pronuclei are swelling. Syngamy takes place within 1 h (Perry, 1987). Ova were placed in glass jars of adequate size which contained approximately 5 ml of culture medium consisting of thin albumen and salt solution (3:2) according to the method of Perry (1988).

TABLE 2

SITE OF EXPRESSION OF MiwZ IN 4 DAY INCUBATED CHICK EMBRYOS

Expression site	Number of embryos*	Percentage
Neural tube	14	64
Ectoderm	8	37
Heart	5	23
Blood cell	5	23
Whole embryo	1	5

*Number out of 22 embryos that expressed the MiwZ

The gene to be introduced

The structure of the constructed plasmid, MiwZ, used in this experiment was previously described by Suemori *et al.* (1990) (Fig. 1). It contains *E. coli* ß-galactosidase (lacZ) gene under the control of RSV enhancer and chicken ß-actin gene promoter/enhancer. The circular form plasmid was suspended in distilled water and the concentration of DNA was 1.0 mg/ml or 0.1 mg/ml.

Microinjection of DNA

Micropipettes were made by pulling 1.0 mm siliconized microcapillary tubing. The tips were beveled down (20-25°) to an outside diameter of 10-15 µm. The micropipette was held by a micromanipulator (M-152, Narishige, Tokyo) and connected to a microinjector (IM-5B, Narishige, Tokyo) with a teflon tube (inside diameter 1 mm, outside diameter 2 mm). A pressure-lock gas syringe was connected to the mid point of the tube so that the pressure in the micropipette could be monitored. The tube was filled with distilled water and the micropipette containing DNA was filled with liquid paraffin in order to remove the air from the tube and the micropipette completely.

The micropipette was inserted into the central area of the germinal disc up to a depth of about 150 µm vertically or obliquely (30° from the vertical) through the albumen capsule and vitelline membrane. A continuous flow system was employed for DNA injection. The injection volume of the DNA was 10-50 nl.

Culture of eggs

The injected eggs were incubated individually for 24 h at 41.5°C in the glass jars sealed with plastic film. The embryos (yolks) were then transferred to recipient eggshells after removing the thick albumen and culture medium (Naito *et al.*, 1990). The shells were filled with thin albumen, and sealed with cling film secured by plastic rings and elastic bands (Perry, 1988). The reconstituted eggs were incubated for 3 days at 38°C and 60% relative humidity, with rocking through an angle of 90°. Embryos were fixed after 1, 3 and 4 days of incubation, and the expression of the injected DNA was analyzed. All procedures for DNA injection and embryo culture were carried out under sterile conditions.

Detection of gene expression

The expression of MiwZ was detected by a histochemical staining method. The embryos and extraembryonic tissues were removed from the yolk in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS(-)), and fixed with 1% glutaraldehyde in PBS(-) for 15-30 min. After washing three times with PBS(-), the samples were incubated for 2 h at 37°C with 0.05% 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal, Wako, Tokyo), 1mM-$MgCl_2$, 0.1% Triton X, 3mM-potassium ferrocyanide and 3mM-potassium ferricyanide in PBS(-).

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