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Original Article

Nuclear transplantation from stably transfected cultured cells of *Xenopus*

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ABSTRACT By nuclear transplantation we have generated embryos from enucleated *Xenopus* eggs and nuclei of stably transfected *Xenopus* cell lines. We have devised a novel method of transplantation in which cell permeabilization is controlled by a temperature effect on streptolysin O-treated cells. This method is easier and quicker to operate than the conventional cell rupture technique. Single nuclei from cell lines transfected with the *lacZ* reporter gene were transplanted to *Xenopus* eggs in which the egg nuclei were destroyed by UV irradiation. We show that the *lacZ* transgene is transmitted from donor cells to nuclear transplant embryos. Expression of the *lacZ* transgene has been controlled by the elongation factor 1-α promoter (Krieg *et al., Dev. Biol. 133:* 93-100, 1989). In the nuclear transplant embryos, β-galactosidase transcripts are expressed at the expected time of development, that is after the mid-blastula transition. In addition, we show that early embryo-specific genes, not expressed in cultured cells, are normally activated in nuclear transplant embryos. Therefore, expression of these genes can be used to monitor the effects of transfected test genes. Although most of the nuclear transplant embryos do not develop beyond the gastrula stage, explants of equatorial tissue from these embryos can undergo differentiation characterized by the expression of muscle and notochord markers. The use of nuclear transplantation, as described here, provides a means of avoiding the mosaic expression of DNA or mRNA injected into *Xenopus* eggs.

KEY WORDS: *Xenopus, nuclear transplantation, cultured cells, transgenic*

Introduction

Our current understanding of early development has been greatly enhanced by the over-, under-, or ectopic expression of cloned genes in embryos. Within the vertebrates, amphibian embryos have many advantages for embryological analysis, but have largely resisted attempts to obtain a well controlled and uniform expression of introduced genes. Messenger RNA injected into fertilized eggs is very efficiently translated (Gurdon *et al.*, 1974), but is quite unevenly distributed among blastula cells (Colman and Drummond, 1986; Harvey and Melton, 1988). DNA injected into eggs is distributed and expressed in an erratic and mosaic pattern (Etkin *et al.*, 1984; Etkin and Pearman, 1987) and cells will contain variable numbers of introduced genes in different chromosomal locations. As a result of these limitations, cells in different regions of an embryo may differ largely in the magnitude of gene expression resulting from mRNA or DNA injection. It will be very desirable, in future, to take full advantage of experiments in which genetic interactions are worked out by overexpressing one gene and observing its effect on the function of other genes, as has been done in *Drosophila* (for example, see Noordermeer *et al.*, 1992, 1994). It is therefore an important aim to obtain a uniform distribution and quantitatively controlled overexpression of genes introduced into *Xenopus* embryos in order to combine the precision of genetic analysis with the embryological advantages of *Xenopus*.

With this aim in mind, we have explored the use of nuclear transplantation as a route towards obtaining a uniform distribution and controlled expression of introduced genes. In previous work we and others have been able to successfully transplant nuclei from amphibian cell cultures to enucleated eggs (Gurdon and Laskey, 1970a,b; Laskey and Gurdon, 1970; Kobel *et al.*, 1973; Gurdon *et al.*, 1975; Von Beroldingen, 1981). In more recent work, Kroll and Gerhart (1994) have transplanted transfected cultured cell nuclei to nucleated and enucleated *Xenopus* eggs. It is noticeable from their results that nuclei transplanted to non-enucleated unfertilized eggs gave rise to embryos only 12% of which expressed the transfected gene, and that the extent of transfected gene expression was variable. Conversely, nuclear transplant embryos derived from enucleated eggs gave a higher percentage and much more uniform

Abbreviations used in this paper: EF-1α, elongation factor 1-α; MBT, mid-blastula transition; PCR, polymerase chain reaction; SLO, streptolysin O; UV, ultraviolet; X-gal, bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

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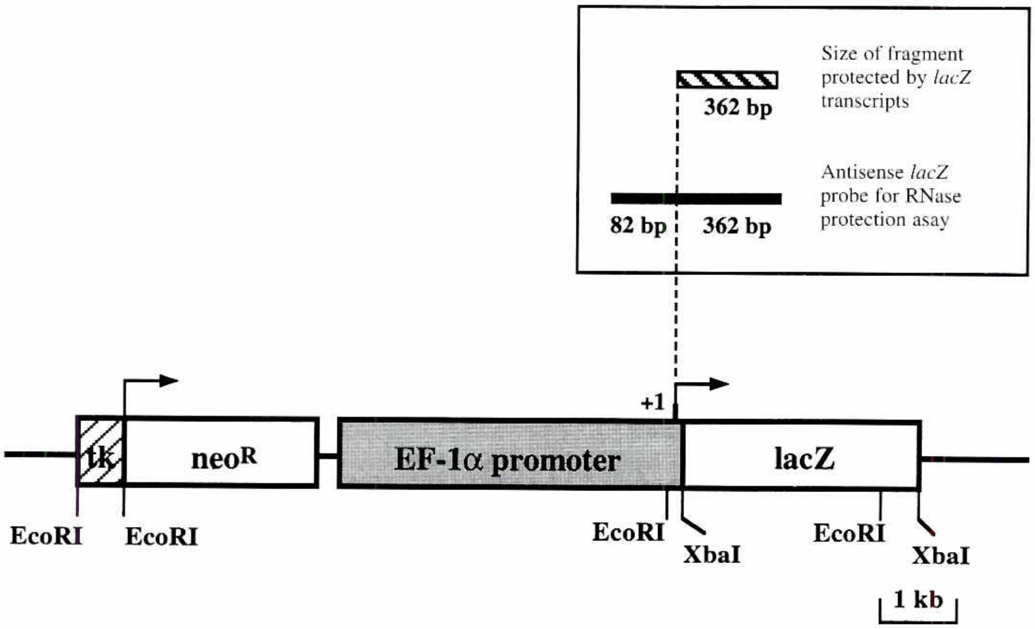

Fig. 1. Structure of the *Xenopus* ptkneo-EF1lacZ plasmid construct. *Expression of the lacZ gene is controlled by the 4.5 kb 5' upstream sequence of the Xenopus elongation factor 1-α gene (Krieg et al., 1989). The 4.5 kb fragment includes the 5'-untranslated region (+1 to +37) of the EF-1α gene. The neomycin resistance gene (neo^R) is driven by the HSV thymidine kinase (tk) promoter. Plasmid DNA was transfected into Xenopus XL177 cell line by electroporation. The upper panel shows the design of the antisense lacZ probe for RNase protection assay. Probes protected by lacZ transcripts give rise to a band of 362 bp.*

expression. For this reason, and to avoid uncertainties due to the unknown composition of a fusion of egg and transplanted nuclei, we have carried out all our experiments with enucleated eggs.

In this report, we describe three significant advances towards the long term objective outlined above. First, we describe a new technique which greatly eases the manipulative demands of transplanting nuclei from cultured cells. Second, we demonstrate more fully than has been done so far, the uniformity of transmission of transfected genes among cells in a nuclear transplant embryo. Third, we show that early embryo-specific genes, not expressed in cultured cells, are activated in nuclear transplant embryos. These genes provide essential markers of early gene activity in normal embryos and can therefore be used to monitor the genetic effects of transfected genes.

Results

Production of stably transfected Xenopus cell lines

To obtain a consistent source of donor nuclei for transplantation, we transfected the *Xenopus* cell line (XL177) (Miller and Daniel, 1977; Ellison *et al.* 1985) with a plasmid that carries the *lacZ* reporter gene and the neomycin resistance gene (Fig. 1). The *lacZ* gene is under the control of the constitutively active *Xenopus* elongation factor 1-α (EF-1α) promoter. This promoter is activated at the mid-blastula transition (MBT) (Newport and Kirschner, 1982a,b) in a non-tissue specific manner (Krieg *et al.*, 1989).

To determine suitable conditions for transfection in *Xenopus* cells, different transfection methods such as calcium phosphate, lipofection and electroporation, were tested. Transfection of XL177 cells was carried out by electroporation (Potter *et al.*, 1984), which we found to be 10 times more effective than the calcium phosphate co-precipitation method. After selection with the antibiotic geneticin, resistant clones were stained with X-gal to assay the level of *lacZ* expression. Seven clones contained blue cells and were therefore *lacZ* positive. However, the percentage of blue cells in the clones varied from 0.5-2%. Heterogeneous expression of the *lacZ* gene in mammalian clonal cell lines has been reported previously (MacGregor *et al.*, 1987). The fact that not all of the cells from a neomycin resistant clone stained blue may be explained in two ways. One is that the "clone" is heterogeneous and contains some cells that carry the *lacZ* transgene and others that do not. A second possibility is that all cells carry the *lacZ* transgene, but most express *lacZ* at too low a level to be revealed by X-gal staining. To distinguish between these possibilities, two neomycin resistant clones were subjected to limiting dilution. If the first hypothesis is true, we would expect to obtain many clones with no staining as well as a few in which every cell stains blue. In fact, we found that all clones that stained blue after limiting dilution still contained only 1% of cells positive for *lacZ* (data not shown). We conclude that

TABLE 1

SURVIVAL OF NUCLEAR TRANSPLANT EMBRYOS DERIVED FROM ENUCLEATED *XENOPUS* EGGS

lacZ clone	Nr. of nuclear transplants	No cleavage or abortive cleavage	Partial cleavage	Complete blastulae	Gastrulae
1.18	264	210 (79%)	34 (13%)	20 (8%)	10 (4%)
2.1	582	463 (80%)	70 (12%)	49 (8%)	28 (5%)
2.14	1609	1199(75%)	167(10%)	243 (15%)	87 (5%)
2.5	114	84 (74%)	21 (18%)	9 (8%)	6 (5%)
Total	2569	1956 (76%)	292 (11%)	321 (13%)	131 (5%)

Recipient eggs were enucleated by UV irradiation immediately prior to transplantation. Donor nuclei were obtained from four different clones of *lacZ* expressing *Xenopus* cell lines (1.18, 2.1, 2.14 and 2.5). Streptolysin O was used for membrane-lysis of the donor cells. With this method, 13% of the total transplants reached blastula stage and 5% of the total reached gastrula stage.

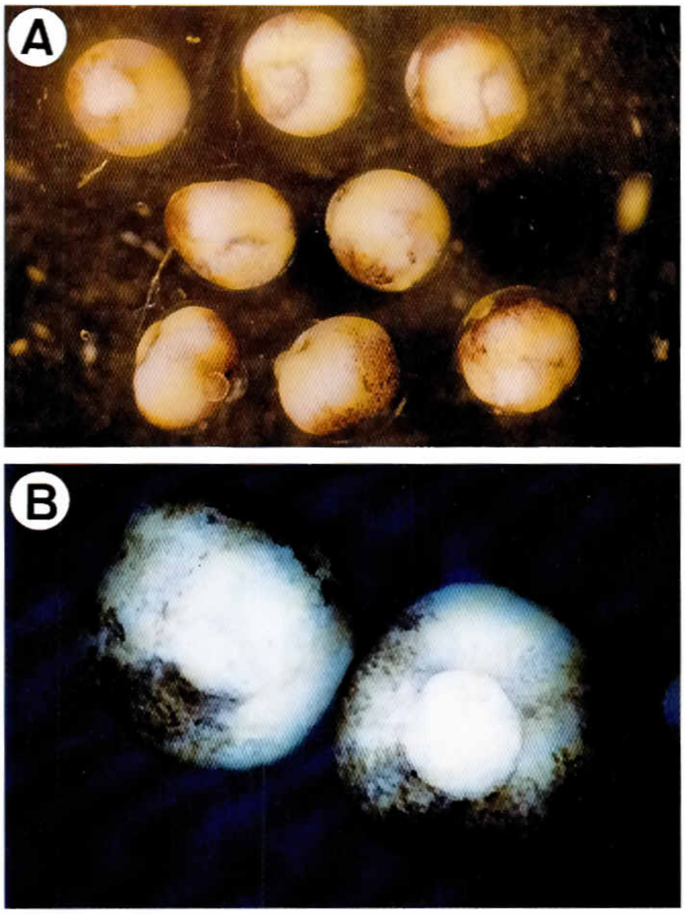

Fig. 2. Nuclear transplant embryos at gastrula stages. (A) *Nuclear transplant embryos derived from lacZ expressing cell line.* **(B)** *Close up of two gastrula stage nuclear transplants. The embryos show normal blastopore lips and yolk plugs.*

the transfected cell lines are heterogeneous in *lacZ* expression, but not in genetic composition, a view supported by the fact that different intensities of staining are observed among positive cells within a single clone. All the following experiments used nuclei from the genetically homogeneous stably transfected cell lines, obtained from limiting dilution.

Nuclear transplantation using streptolysin

In *Xenopus*, the traditional method of nuclear transplantation involves mechanical rupture of the cell membrane by the use of glass pipettes with an internal diameter just a little smaller than that of the dissociated cells. When a cell is sucked into a pipette, the cell membrane is broken and its nucleus can then come into direct contact with the host cell cytoplasm. Although this method works nicely with dissociated embryonic cells, it is technically demanding to apply the same method to long-term cultured cells because of their small size. In earlier work with oocytes, Gurdon (1976) introduced the use of lysolecithin as a way of chemically permeabilizing cells for nuclear transplantation. For the work to be described, we have adopted the use of streptolysin O (SLO), a bacterial cytolysin which forms pores on the cell membrane as it polymerizes and the polymerization process is temperature dependent (Ahnert-Hilger *et al.*, 1989). Compared to lysolecithin, it has the advantage that cells preincubated in SLO can be permeabilized whenever desired by simply raising the temperature. In addition, it has been used extensively for the preparation of intact Hela cell nuclei (Leno *et al.*, 1992). We have tested the developmental capacity of tailbud endodermal cells after SLO treatment by nuclear transplantation. We are able to obtain swimming tadpoles that survived until stage 35 from SLO-treated endodermal cells (data not shown). This result suggests that SLO has no adverse effect on the development of nuclear transplant embryos.

Using SLO to transplant nuclei from the *lacZ* transfected cultured cells to enucleated eggs, we obtained nuclear transplant embryos that can be classified into three groups (Table 1). After transplantation, the majority of the embryos (76%) fell into the first group showing either no cleavage or abnormal cleavage, and development stopped after a few cell divisions. 11% of transplanted eggs displayed the partial cleavage phenotype, where cleavage occurs in only one of the two-cell blastomeres. Such partially-cleaved embryos have been successfully used for serial nuclear transplant experiments (e.g. Gurdon *et al.*, 1975), and seem to result from a difference in cell division rate between the slowly dividing transplanted nucleus and the rapidly-dividing embryos. The remaining nuclear transplant embryos (13%) underwent complete cleavage, and resembled morphologically normal blastulae. Of these, most failed to gastrulate properly though some appeared normal up to stage 10. The remainder (5% of initial transplants) developed to the late gastrula stage, or in rare cases to early neurulae. These had formed normal blastopore lips and yolk plugs (Fig. 2A,B) and are morphologically normal.

Table 2 summarizes previous results of nuclear transplantation using cultured cells. On average, 11% of the nuclear transplants underwent complete cleavage and reached a late blastu-

TABLE 2

SUMMARY OF PREVIOUS WORK ON NUCLEAR TRANSPLANTATION USING TRADITIONAL NARROW PIPETTE METHOD ON CULTURED-CELL NUCLEI

Source of cell culture	Nr. of nuclear transfers	% of partial or complete blastulae	% of complete blastulae	References
Adult kidney	789	26%	13%	Laskey and Gurdon, 1970
Adult lung	502	24%	12%	Laskey and Gurdon, 1970
Adult heart	273	22%	11%	Laskey and Gurdon, 1970
Adult testis	73	26%	13%	Laskey and Gurdon, 1970
Adult skin	1031	31%	16%	Laskey and Gurdon, 1970
Stage 40 tadpoles	3546	30%	3%	Gurdon and Laskey, 1970a
Differentiated adult skin cells	129	31%	9%	Gurdon *et al.*, 1975
Average	-	27%	11%	
lacZ-transfected XL177 cells	2569	24%	13%	Results presented in this paper

In earlier nuclear transfer experiments, on average, the percentage of partial or complete blastulae obtained was 27% and the percentage of complete blastulae was 11% depending on the source of donor nuclei.

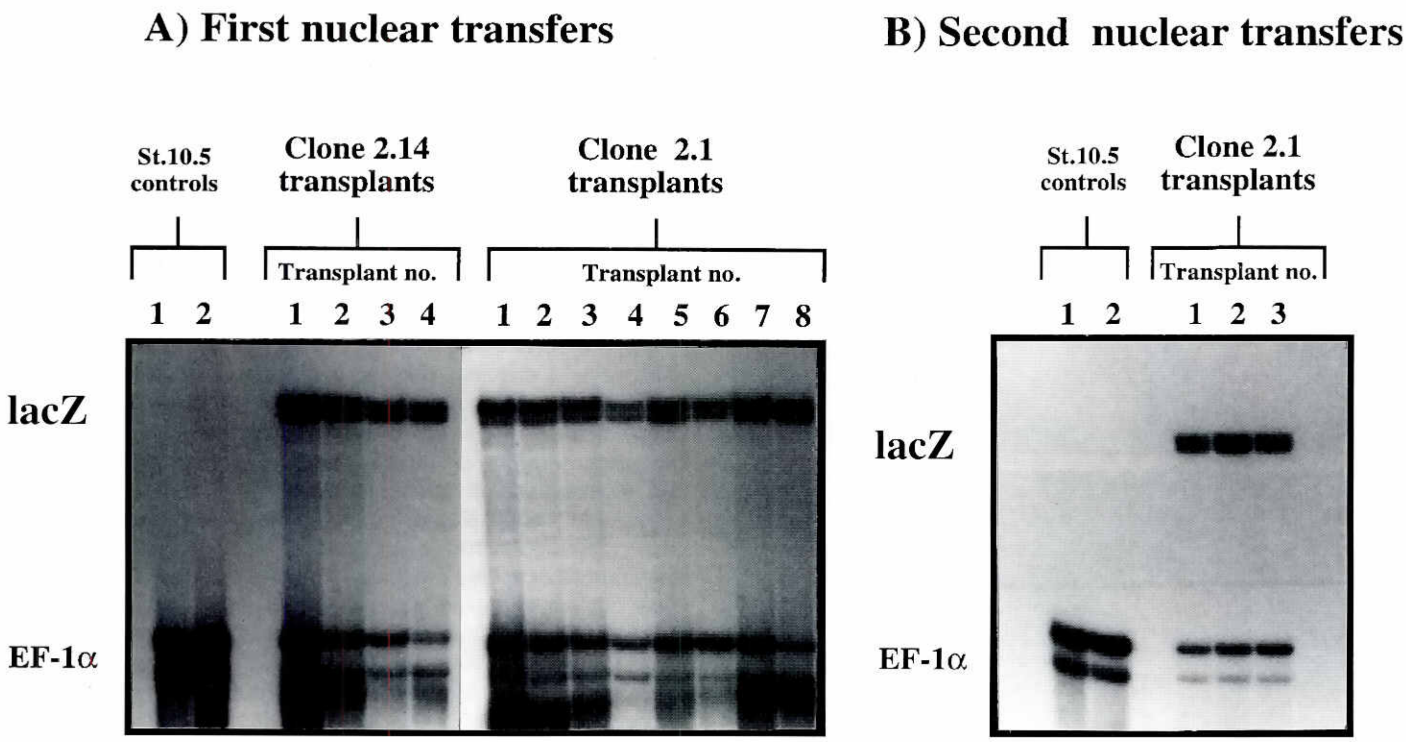

Fig. 3. Transmission of the *lacZ* transgene from single transplanted nuclei to nuclear transplants. *A PCR-detection assay was performed on DNA extracted from single nuclear transplant embryos. The PCR products were labeled with 1 μCi [α-^{32}P]dATP to enhance the sensitivity of detection. Blastula stage nuclear transplants derived from* **(A)** *lacZ expressing cell lines 2.1 and 2.14, or* **(B)** *dissociated cells from a first generation nuclear transplant; both showed a strong lacZ signal in the PCR assay. The presence of the lacZ signal in nuclear transplants indicates stable transmission of the lacZ transgene from donor nuclei to nuclear transplant embryos. Primers specific to the endogenous EF-1α gene served as internal controls for the PCR reaction.*

la stage. With the SLO method reported here, 13% of the nuclear transplants became complete blastulae. In conclusion, the SLO method of nuclear transplantation is as successful for transplanting cultured cell nuclei as the traditional narrow pipette method, and is far easier and quicker to operate.

Transmission of lacZ transgene from donor cells to nuclear transplant embryos

It is important to ensure that the nuclear transplant embryos we obtain from transfected cultured cells are indeed generated by activity of the transplanted nucleus, and that the transfected *lacZ* gene is present throughout the embryo. In whole nuclear transplant embryos, the transfected *lacZ* gene was not detected by X-gal staining. We therefore devised a PCR-based assay to detect the presence of *lacZ* gene in single embryos. We demonstrated that control blastula stage embryos, or enucleated eggs that were harvested immediately after receiving single nuclei from *lacZ* transfected cells, were both negative in the PCR assay (data not shown). Using this PCR-based method, we assayed blastula stage nuclear transplant embryos produced from two *lacZ* expressing *Xenopus* cell lines 2.1 and 2.14. In all 20 cases analyzed, we observed a positive signal from the *lacZ* transgene. Figure 3A shows representative results of the PCR assay on 12 first generation nuclear transplant embryos. The fact that *lacZ* DNA is observed in all of the samples analyzed, each one being derived from a single transplanted nucleus, indicates that all cells within each *lacZ* expressing cell line contain the *lacZ* transgene.

To check directly that the *lacZ* transgene is present in each cell of the nuclear transplant embryos, we carried out a serial transplantation experiment. First transfer embryos generated from cell line 2.1 or 2.14 were dissociated and single blastula cells from different regions (animal or vegetal region) were used to provide nuclei for serial transplantation into enucleated recipient eggs. If the first transfer embryo contains the *lacZ* transgene in every cell, all of the second transfer embryos should show a *lacZ* signal in the PCR assay. A positive signal for *lacZ* was observed for all 26 of the second transfer embryos analyzed. Figure 3B shows the result of PCR from three of these second generation nuclear transplants using dissociated cells from first transfer embryos. From this result, it is likely that the first transfer embryos contain the *lacZ* transgene in every cell, a point not directly tested in previous work.

Therefore, we conclude that the *lacZ* transgene is present in the *lacZ* expressing cell lines and also in the first transfer embryos. In addition, the *lacZ* transgene is transmitted to cleavage nuclei during the course of subsequent nuclear transplantation.

Expression of the lacZ transgene in nuclear transplants

When X-gal or antibody staining was used to detect the *lacZ* transgene activity in gastrula stage transplant embryos, no signal was observed. We suppose that is due to too low a level of

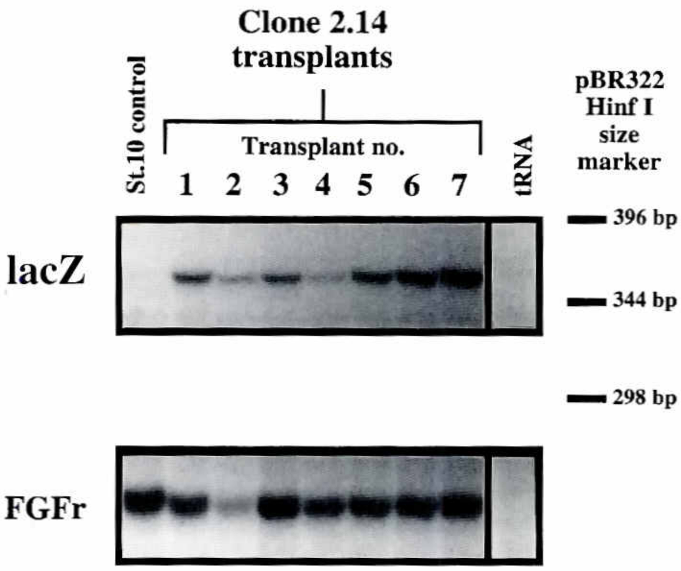

Fig. 4. Expression of a *lacZ* transgene in nuclear transplants. *An RNase protection assay was performed on gastrula stage nuclear transplants derived from the lacZ expressing cell line 2.14. Transplant numbers 1, 2, 3 and 4 were harvested at stage 10.25 and numbers 5, 6 and 7 at stage 10.5. All samples gave a protected fragment of expected size (362 bp) indicating the presence of lacZ transcripts rather than DNA. FGF receptor was used as a loading control. The pBR322 Hinf I size marker only applies to the upper panel.*

lacZ enzyme activity or protein generated in the nuclear transplant embryos. We consider it important to determine whether the transfected gene is successfully transcribed.

An anti-sense RNA probe that covers part of the EF-1α promoter region and part of the *lacZ*-coding region (Fig. 1) was used in an RNase protection assay to discriminate between DNA and *lacZ* transcripts according to the size of the protected fragment (444 bp for DNA, 362 bp for *lacZ* transcripts). With this probe, we detected a protected signal from gastrula stage nuclear transplant embryos (Fig. 4). The protected fragment is of the size expected for *lacZ* transcripts (362 bp). No *lacZ* expression was detected in early blastula stage nuclear transplant embryos (data not shown). As a reference point for the onset of zygotic transcription (MBT), we also assayed for the level of zygotic EF-1α transcripts (Fig. 6). We found that zygotic EF-1α expression was associated with *lacZ* expression. Therefore, these results indicate that the *lacZ* transgene is expressed in nuclear transplant embryos and that its expression is activated after the MBT.

Furthermore, we wished to determine whether the *lacZ* transgene is expressed in all cells of nuclear transplant embryos. By using *in situ* hybridization against *lacZ* transcripts on sections, we found that expression of the *lacZ* transgene is present in all regions of 11 late blastula or gastrula transplant embryos tested. No staining is detected in control embryos (Fig. 5A). Figure 5B shows a section from a nuclear transplant embryo derived from cell line 2.14. In general, 65-80% of cells of the transplant embryos show staining at detectable level. *lacZ* staining is seen as a ring around the nuclei and is more clearly shown in an enlarged view of a separate section (Fig. 5C,D). A variable intensity of staining is found among the positive cells, as was also observed for X-gal staining of cultured cells derived from a single clone. Additionally, *lacZ* staining was also seen in every serial nuclear transplants (data not shown).

From these results, we conclude that the *lacZ* transgene is expressed as transcripts at the expected time of development (i.e. MBT) and also that the transgene is expressed in all regions of the nuclear transplant embryos.

Expression of early genes in cultured cell nuclear transplant embryos

The principal aim of the present nuclear transplantation experiments is to eventually work out gene interactions in early development by analyzing the overexpression of genes in nuclear transplant embryos. The consequence of overexpression is best accessed by monitoring genes normally activated in early development. We must therefore determine whether embryos obtained from enucleated eggs and transplanted cultured cell nuclei will express genes characteristic of normal development. Although a minority of cultured cell nuclear transplant embryos reach neurula stage, this does not prove that early genes are activated normally either in these embryos or in the great majority of such embryos that do not develop normally. We therefore need to determine patterns of endogenous gene expression in such embryos when the normality of their further development is not evident. We have chosen four genes for analysis, namely, *Xbrachyury (Xbra)* (Smith *et al.*, 1991), *goosecoid (gsc)* (Cho *et al.*, 1991), *Xwnt-8* (Christian *et al.*, 1991) and *Mix.1* (Rosa, 1989), all of which are transcribed in early *Xenopus* development. In normal embryos, these genes are activated after the mid-blastula transition (MBT), a stage marked by the zygotic activation of the EF-1α gene. In an early gastrula, *Xbra* is expressed in all presumptive mesoderm cells (Smith *et al.*, 1991), *gsc* in the dorsal lip (Spemann organiser) region (Cho *et al.*, 1991) and *Xwnt-8* in ventral and lateral mesoderm (Christian *et al.*, 1991; Smith and Harland, 1991; Christian and Moon, 1993). *Mix.1* is expressed in prospective endoderm and in part of the future mesoderm (Rosa, 1989). None of these genes are transcribed in the *lacZ* expressing 2.14 cell line used to provide donor nuclei for transplantation (Fig. 6). Unfertilized eggs do not contain maternal transcripts of these genes (Fig. 6) except for a very low level of *Xbra* (Smith *et al.*, 1991). We found that *gsc, Xwnt-8* and *Mix.1* are activated in all 7 gastrula stage nuclear transplant embryos derived from cell line 2.14 (Fig. 6). Xbra mRNA is detected in 4 out of 7 nuclear transplant embryos (Fig. 6). This could be due to a lack of normal cell-cell interactions in some of the nuclear transplant embryos because it has been shown that *Xbra* expression is dependent on cell-cell aggregation (Lemaire and Gurdon, 1994). In addition, zygotic EF-1α transcripts are also present in the same samples indicating the onset of zygotic transcription in nuclear transplant embryos. Therefore, these results demonstrate that the expression of embryo-specific genes such as *Xbra, gsc, Xwnt-8* and *Mix.1* is activated in nuclear transplant embryos.

We next asked whether embryo-specific genes are activated in the correct regions of cultured cell nuclear transplant embryos, that is whether these genes show correct spatial regulation. *In situ* hybridization was performed on sections of embryos using an anti-sense RNA probe against *Xbra*. Indeed, we found that

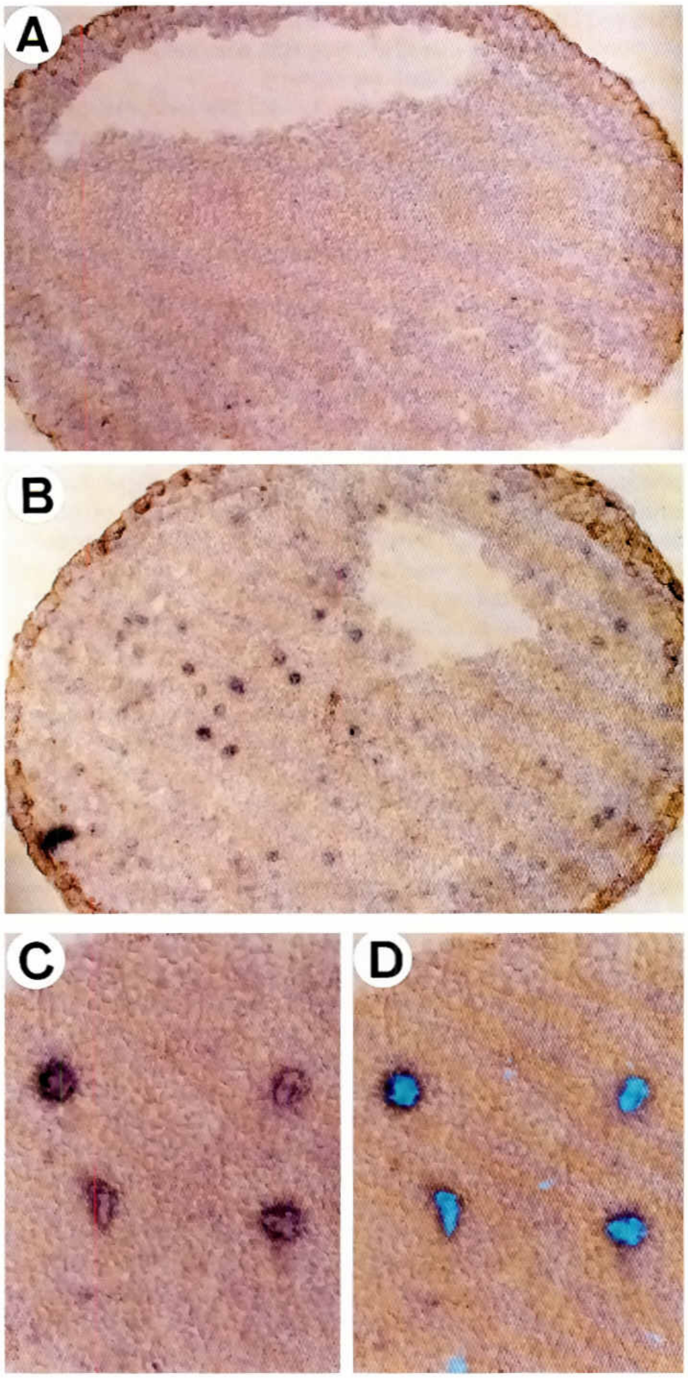

Fig. 5. *In situ* hybridization of nuclear transplant embryos for *lacZ* expression. *In situ hybridization was performed on sections of control embryos* **(A)** *and nuclear transplants derived from cell line 2.14* **(B)** *by using a digoxygenin-labeled anti-sense lacZ probe. The level of the lacZ transgene expression is variable among the positively-stained cells.* **(C)** *Enlarged view of a separate section to show the perinuclear location of lacZ mRNA. The same section is shown under both normal illumination and UV* **(D)** *to reveal nuclei stained with Hoechst.*

Xbra is expressed in the invaginating mesoderm region in three early gastrula nuclear transplant embryos tested (Fig. 7B) similar to that observed in normal control embryos (Fig. 7A).

We conclude from these results that embryos derived from transplanted nuclei of cultured cells can activate all of the early genes we have tested at the normal stage of development and in the correct region. These embryos therefore undergo gene activation which can thereby be used for testing the function of transfected genes.

Expression of late mesoderm-markers in tissue explants derived from nuclear transplant embryos

Many tissue specific genes of embryos are not expressed until after gastrulation, and therefore not within the life span of most of our cultured cell nuclear transplant embryos. To obtain expression of such genes, we have cultured explants of nuclear transplant embryos with the expectation that these would survive longer than whole embryos, and so permit terminal differentiation markers to be expressed. The notochord-specific antibody MZ15 (Smith and Watt, 1985) and muscle-specific antibody 12/101 (Kintner and Brockes, 1984) were used. It has been shown that explants derived from the dorsal and dorso-lateral marginal zones of early gastrulae differentiate into notochord (Slack and Forman, 1980) and muscle (Keller, 1976), respectively. We therefore dissected 14 nuclear transplant embryos into animal, equatorial and endoderm pieces at the early gastrula stage and cultured these as explants. The tissue explants can survive at least until the time when control embryos from fertilized eggs reach stage 26. At that time, the explants were fixed and sectioned, and double antibody-staining was performed. We showed that equatorial explants were positive for the muscle marker in 12 out of 14 cases. The notochord marker which requires the most dorsal mesoderm was seen in 7 out of 14 cases. Figure 8A,B shows a section of equatorial explant derived from a control embryo and nuclear transplant embryo, respectively.

To conclude, when parts of cultured cell nuclear transplant embryos are cultured as explants, they can survive until the equivalent of the tailbud stage. They are also able to express muscle and notochord markers in the correct germ layer and therefore are able to activate cell-type specific differentiation genes.

Discussion

In an attempt to establish a system for producing transgenic *Xenopus* embryos, we have generated stably transfected *lacZ* expressing cell lines and have transplanted nuclei from these transfected cells into enucleated eggs. In this system, we have demonstrated the advantages of using SLO as the permeabilizing agent for transplanting nuclei from cultured cells. We have shown the uniformity of transmission of the *lacZ* transgene among nuclear transplant embryo cells. In addition, we have demonstrated that early embryo-specific genes, not expressed in cultured cells, are activated at the normal stage in nuclear transplant embryos.

Permanent cell lines as a source of donor nuclei

DNA injection into fertilized *Xenopus* eggs was first used many years ago (Gurdon, 1974) and has been useful for creating transgenic frogs (Etkin and Roberts, 1983; Etkin *et al.*, 1984). However, in contrast to organisms in which heritable transgenesis can be readily achieved (*Drosophila* and mice), injected DNA in *Xenopus* yields mosaic results. The proportion of cells containing injected DNA, the number of copies of the transgene per nucleus, and the site of integration are all uncontrolled and therefore likely to be variable. These difficulties can be overcome by transplanting nuclei from stably transfected cultured cells.

However, there are also limitations to using cell lines in nuclear transplantation experiments. Firstly, the transfection efficiency of *Xenopus* cell lines is relatively low. Secondly, there is only a limited number of *Xenopus* cell lines available and they are mostly aneuploid. We believe that the reason why most of our nuclear transplant embryos do not develop beyond the late gastrula stage is probably due to the aneuploid nature of the cell line used (XL177).

Using the classical cell rupture method, *Xenopus* cell cultures have been used successfully in nuclear transplantation experiments (Gurdon and Laskey, 1970a,b; Laskey and Gurdon, 1970; Kobel *et al.*, 1973; Gurdon *et al.*, 1975). In this study, we have introduced the use of SLO to achieve permeabilization of donor cells. This method is far easier and quicker to operate than the classical method, when transplanting cultured cell nuclei.

Enucleated or non-enucleated eggs as nuclear recipients

The reason for using enucleated eggs in our experiments is to generate embryos solely derived from transplanted nuclei. In this way, we can be sure that every cell contains a nucleus with a transfected gene. In fact, uniform transmission of the *lacZ* transgene among cells have been shown by the PCR analysis of second generation nuclear transfer embryos. If non-enucleated eggs are used, it has to be assumed that every blastula cell has retained a copy of the donor nucleus in addition to a mitotic product of the egg nucleus; furthermore subsequent gene expression may be complicated by interactions between donor and egg chromosome sets.

In a recent publication, Kroll and Gerhart (1994) reported the generation of transgenic *Xenopus* embryos from transfected cell lines by nuclear transplantation. In their study, they tested both enucleated and non-enucleated eggs. They found the main difference between the two types of recipient eggs to be the survival of transplant embryos and the extent of variable expression between embryos. When enucleated eggs were used, the transplant embryos reached the neurula stage and not beyond. We believe it will be useful to analyse endogenous gene expression in nuclear transplant embryos so that their gene activities can be used to monitor the effects of transgenes in future studies. In this report, we are able to show that embryo-specific genes have been correctly activated in nuclear transplant embryos derived from the *lacZ*-transfected cell lines.

Expression of the lacZ transgene in nuclear transplants

We have detected *lacZ* transcripts by RNase protection and by *in situ* hybridization. By the latter method, we have been able to detect *lacZ* mRNA in 65-80% of cells in first transplant embryos, and these cells are distributed throughout the embryo. We suspect that the cells apparently negative for *lacZ* do in fact express the gene but too weakly to be detected. This is because heterogeneous *lacZ* expression was also observed in the *lacZ*-transfected cell lines after X-gal staining.

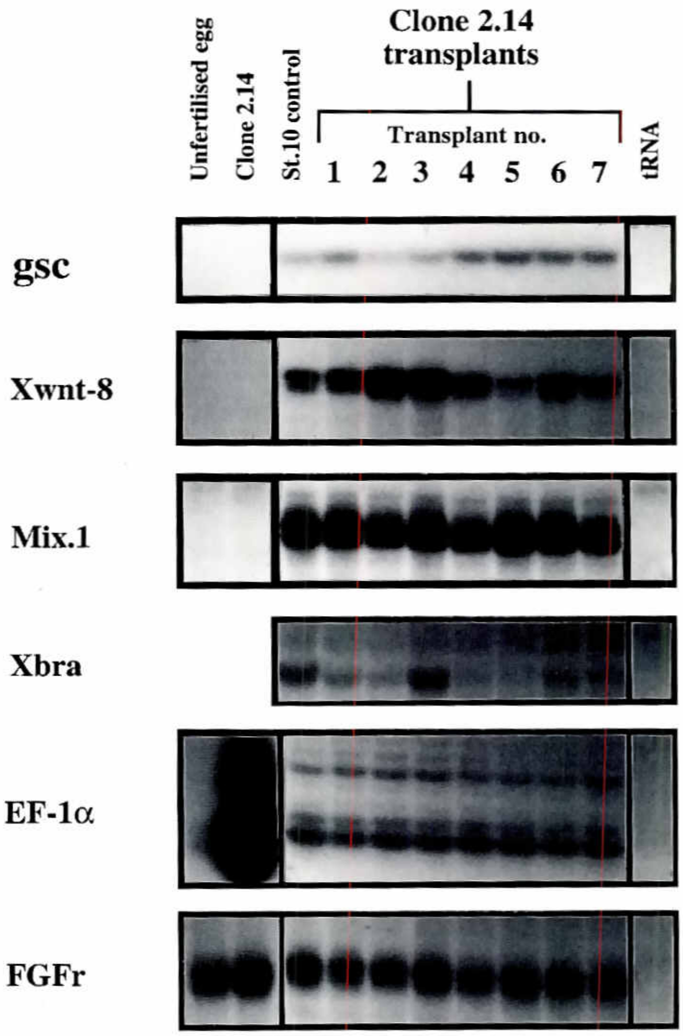

Fig. 6. Reprogramming of gene expression in nuclear transplants. *RNase protection assays were performed on the same batch of RNA samples prepared from nuclear transplants as in fig 4. The RNA probes used were specific to EF-1α, Xbra, gsc, Xwnt-8 and Mix.1. For gsc, Xwnt-8 and Mix.1, no transcripts were detected in unfertilized eggs or in lacZ expressing cell line 2.14 by this method. It has been shown that there is a low level of maternal Xbra transcripts in eggs (Smith et al., 1991). Xbra expression was not detected in cell line 2.14 (data not shown). The early genes EF-1α, Xbra, Xwnt-8 and Mix.1 are activated in the nuclear transplants. The increased level of endogenous EF-1α transcripts in embryos compared to unfertilized eggs indicates the activation of zygotic transcription. The expression of EF-1α, Xbra, gsc, Xwnt-8 and Mix.1 shows that zygotic transcription of early genes has been activated in transplanted cultured cell nuclei. Thus, the transplanted nuclei have been reprogrammed to adopt to a new pattern of transcription.*

We were not able to detect *lacZ* enzyme activity or *lacZ* protein by X-gal staining or antibody staining in nuclear transplant embryos. We believe that this is due to too low a level of expression of the *lacZ* transgene in the particular construct used for this work and may be improved in future work with stronger promoters.

In transgenic mice, discrepancies between the temporal and spatial expression pattern of transgenes and their endogenous

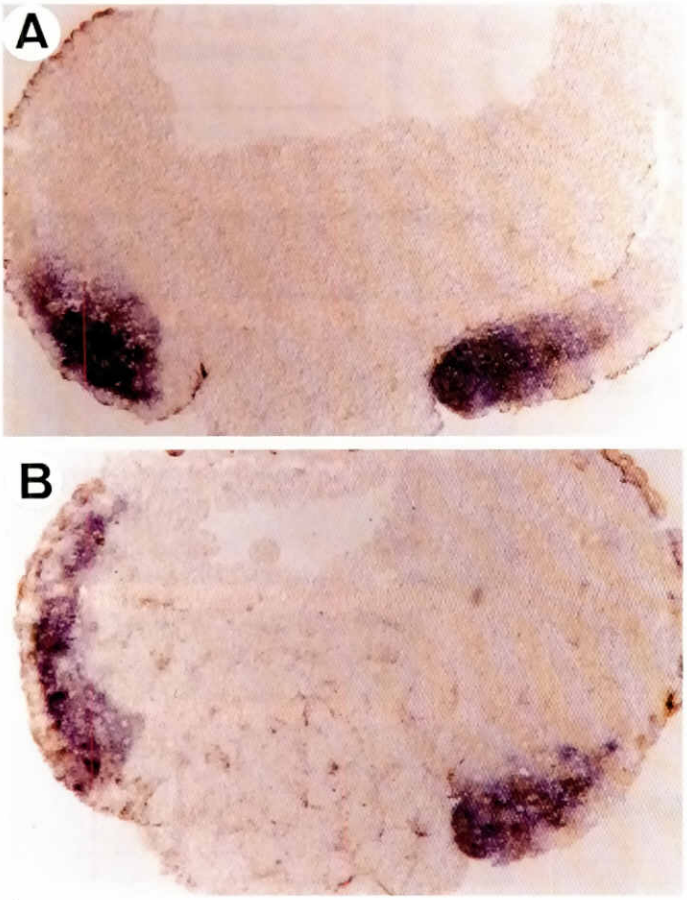

Fig. 7. *In situ* hybridization of nuclear transplant embryos using an Xbra probe. *In situ hybridization was performed on sections of control embryos* **(A)** *and gastrula stage nuclear transplant embryos derived from cell line 2.14* **(B)**, *using a digoxygenin-labeled anti-sense Xbra probe. Note the positive staining in region of the invaginating marginal zone. The dorsal side is to the right.*

counterpart have been reported (Beddington *et al.*, 1989; Kothary *et al.*, 1989). One of these authors suggested that this may be due to the use of a promoter region that did not contain all necessary regulatory elements. A similar argument may apply to our results with a low level of *lacZ* transcripts. As mentioned earlier, different levels of *lacZ* expression are observed within a clone of *lacZ* expressing cells. Therefore, the presence of the same number of copies of the transgene in the same chromosomal location does not necessarily correlate with a constant level of the *lacZ* transgene expression within a clonal population of cells.

Perspectives

The results from this paper show that, by means of nuclear transplantation, transgenic embryos displaying expression of a transgene can be successfully generated. Overexpression of exogenous genes in a spatially and temporally-regulated manner can be achieved by tissue-specific promoters (Kroll and Gerhart, 1994).

Although the use of an aneuploid cell line which has been in culture for many years seems to make it hard to obtain nuclear transplant embryo development beyond the gastrula stage, gastrula stage embryos will be valuable for several kinds of experiments involving genes expressed early in development. To cite one example, it would be very informative to overexpress an early regulatory gene in all cells of a late blastula, and hence to determine the extent to which it induces or represses genes involved in early development. It should be possible to extend the usefulness of the procedure we describe here by developing cell lines which can promote more advanced nuclear transplant embryo development than the line used here.

Materials and Methods

Transfection

A *Xenopus* epithelial cell line (XL177) derived from stage 40 tadpoles (Miller and Daniel, 1977; Ellison *et al.*, 1985) was used in this study. Cells were kept at 23°C in 61% Leibovitz-15, 10% fetal bovine serum and 1 mM L-glutamine. XL177 cells were transfected with the plasmid construct ptkneo-EF1lacZ by electroporation (Potter *et al.*, 1984). To prepare cells for electroporation, they were trypsinized, washed in ice-cold 70% PBS twice and resuspended at 5×10^6 cells/ml. Plasmid ptkneo-EF1lacZ DNA was added to 250 µl of cells to a final concentration of 100 µg/ml. The mixture was transferred to an ice-chilled cuvette (0.4 cm) and was kept on ice for 5 min. The settings of the Gene Pulser (Biorad) were as follows: 1000-1500 V, 25 µF, 400 ohm (time constant= 2 to 3 msec). The cuvette was returned to ice immediately after electroporation and 1 ml ice-cold culture medium was added. After 5 min on ice, the electroporated cells and debris were resuspended thoroughly and plated in a 25 cm^2 tissue culture flask for a recovery period of 48 h. Cells were then replated on two 10 cm dishes and selected with 800 µg/ml geneticin (Sigma) for 4 weeks. Clones were expanded and assayed for *lacZ* activity by X-gal staining.

X-gal staining

Cells in a monolayer were rinsed briefly with 0.1 M phosphate buffer (pH 7.3), fixed with 0.2% ice-cold glutaraldehyde (in 0.1 M phosphate buffer pH 7.3) for 5 min at 4°C. Staining solution containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 1 mM $MgCl_2$ in phosphate buffer (pH 7.3) and 0.04% freshly prepared X-gal (bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was overlaid onto cells. The color reaction was allowed to proceed overnight at 37°C.

Nuclear transplantation

Preparation of donor nuclei

Approximately 5×10^4 stably transfected XL177 cells were trypsinized, washed in lysis buffer [1xCa^{2+} free MBS (Gurdon, 1977) containing 10 mM EGTA] and pelleted by centrifugation for 5 minutes at 1,000 rpm. The cell pellet was then resuspended in 100 µl of ice-cold lysis buffer. Streptolysin O (SLO, Wellcome Diagnostics) was added to a final concentration of 0.5 units/ml. The cell suspension was kept on ice for 5 min to allow binding of SLO to the cell membrane. Four volumes of Ca^{2+}, Mg^{2+}-free 1xMBS containing 1% bovine serum albumin (Sigma) were added to the cell suspension. An aliquot of cells was diluted in one volume of the same buffer as above. Cells were then permeabilized by incubation at room temperature for 5 min and they were kept on ice until use. A trypan blue test was carried out to estimate the percentage of permeabilized cells after SLO treatment; this was usually 99%.

Preparation of recipient eggs

Freshly laid unfertilized eggs were collected from *Xenopus laevis* (Nasco) in high salt MBS (Gurdon, 1977). The UV irradiation procedure was as described in Gurdon (1977) with the modification that the second exposure to the Hanovia UV lamp was omitted. Eggs were arranged on a glass slide with the animal region facing up and exposed to a Mineralite UV lamp for 1 min, to destroy the female pronucleus and to soften the jelly coat. Transplantation of cultured cell nuclei was performed immediately after UV irradiation. In our experiments, the efficiency of enucleation was above 95% as determined by the androgenetic haploidy test (Gurdon, 1960).

Nuclear transfer

Immediately prior to transplantation, donor cells were transferred to an agarose lined Petri dish containing $1xCa^{2+}$, Mg^{2+} free MBS and 0.5% BSA. The nuclear transfer procedure was as described in Gurdon (1977). The inner diameter of the transplantation pipette was 2 to 3 times that of the cultured cells since mechanical rupturing was not necessary after SLO treatment. After transplantation, the eggs were transferred to 4% Ficoll 400 (Pharmacia) in 1xMBS and kept at 14°C. The medium was gradually changed to 1/10 MBS after 6-8 h.

PCR-detection assay

Cells or embryos were lysed in 25 µl PCR-lysis buffer (50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris, pH 8.3, 0.1 mg/ml gelatin, 0.45% Nonidet P40, 0.45% Tween 20 and 200 µg/ml Proteinase K) at 55°C for 1 h. The lysate was then heated at 95°C for 10 min. After 10 min of centrifugation, the supernatant was mixed with an equal volume of PCR mix (1xPCR buffer, 1 µCi [α-^{32}P]dATP, 10 nmoles each of dNTPs, 20 pmoles each of the primers). The PCR products given by the *lacZ* primers and EF1 primers (Rupp and Weintraub, 1991) are 263 bp and 221 bp, respectively. The sequence of the *lacZ* primers is as follows:

5'-TTGCTGATGCGGTGCTGATTACGAC-3' and
5'-GCGGTCAAAACAGGCGGCAGTAAGG-3'.

The profile of the PCR was as follows: 94°C, 1 min; 60°C, 1 min; 72°C, 1 min for 30 cycles. 2.5 units Taq polymerase (Boehringer) was used per reaction. The PCR product was electrophoresed through a 6% polyacrylamide-urea gel which was then exposed to a film for 2 to 3 days.

RNase protection assay

The *lacZ* probe was generated from pEF1-70 which contained a PCR fragment from ptkneo-EF1*lacZ* cloned into the *Eco*R I and *Hin*d III sites of pBluescript-SK. The PCR primers were:

EF1-70 5' TTGG GAATTC TGG ACT TGA TGA TGT C 3' (26-mer)
*Eco*R I

lacZ-360 5' TTT AAGCTT GAC CGT AAT GGG ATG GG 3' (26-mer)
*Hin*d III

The PCR product contained 82 bp of the EF-1α promoter, 35 bp of the 5' UTR of EF-1α and 327 bp of the 5' *lacZ*-coding region. To synthesize the antisense-RNA probe, pEF1-70 was linearized with *Pst* I and transcribed with T7 RNA polymerase. The protected fragments for *lacZ* transcripts and *lacZ* DNA were 362 bp and 444 bp respectively. Antisense-RNA probes for FGFr, Xbra, gsc and Mix.1 were prepared as in Lemaire *et al.* (1995) and for Xwnt-8 as in Lemaire and Gurdon (1994). Probes for EF-1α were prepared as in Sargent and Bennett (1990).

In situ hybridization

Embryos were fixed in MEMFA [0.1 M MOPS, pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$ and 3.7% formaldehyde (Hemmati-Brivanlou and Harland, 1989)] for 2 h, kept in methanol at -20°C and embedded in Histoplast:beeswax (98:2). Sections of 15 µm thickness were cut and dried on TESPA-treated slides. Subsequent steps were carried out as described by Lemaire and Gurdon (1994). The *lacZ* probes were prepared from placZ2-XS and placZ1-SR which were created by subcloning the *lacZ*-coding region as two *Xba* I-*Sac* I fragments into the pBluescript-SK vector. placZ2-XS contains a 2 kb fragment and placZ1-SR contains a 1.5 kb *Xba* I-*Sac* I fragment. In placZ1-SR, a deletion at the 3'-end was performed by EcoRI digestion and re-ligation. To synthesize the antisense-RNA probes, placZ2-XS was linearized with *Xba* I and transcribed with T3 polymerase and placZ1-SR was linearized with *Sac* I and tran-

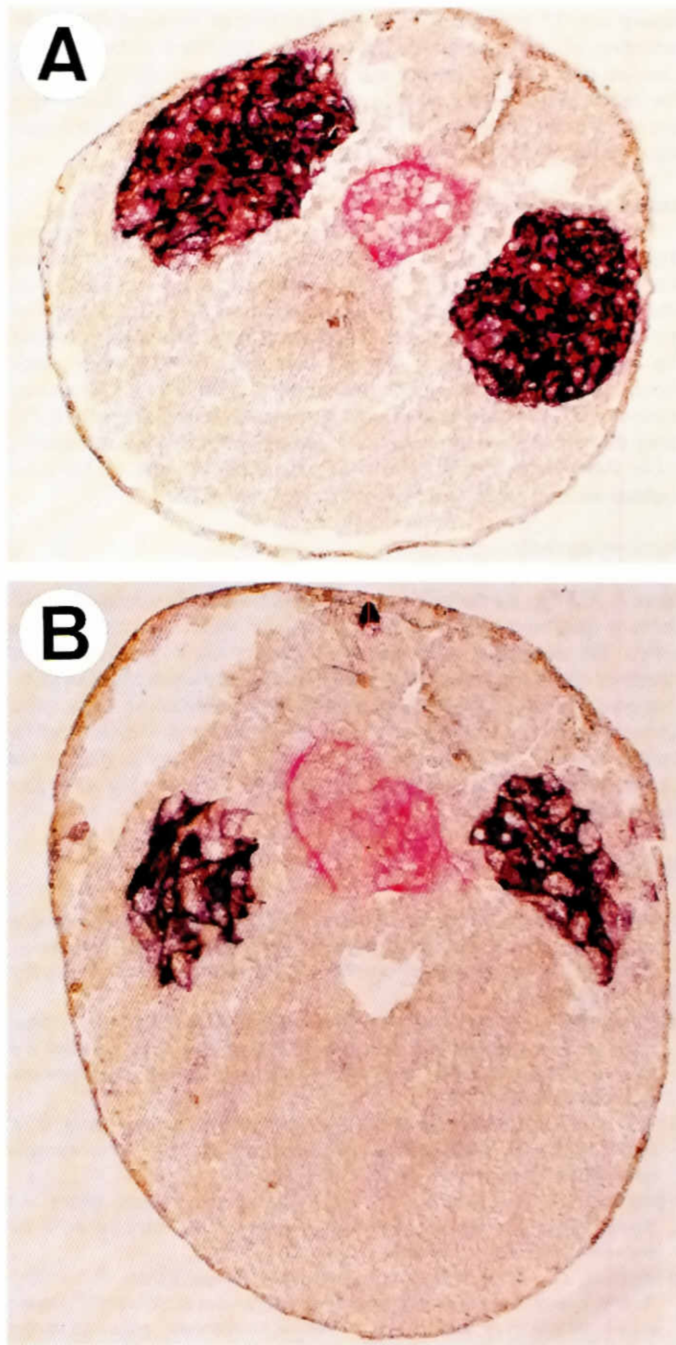

Fig. 8. Immunostaining of sections of equatorial explants derived from nuclear transplants. *The equatorial regions of an early gastrula control embryo* **(A)** *or of a nuclear transplant embryo derived from cell line 2.14* **(B)** *were explanted and cultured until sibling embryos from fertilized eggs has reached stage 26. The explants were sectioned and stained with the muscle-specific antibody 12/101 (dark blue), and with the notochord-specific antibody MZ15 (red). The section of the nuclear transplant embryo is positive for muscle and notochord as is the case of the control, indicating that mesodermal differentiation has occurred in the correct germ layer of nuclear transplant embryos.*

scribed with T7 polymerase. The two *lacZ* probes were used at a concentration of 0.5 µg/ml during hybridization. The Xbra probe was prepared as described in Lemaire and Gurdon (1994) and used at 1 µg/ml. The precipitating purple alkaline phosphatase substrate (Boehringer) was used for the color reaction. The sections were mounted in 90% glycerol and 10% PBS after Hoechst staining.

Immunohistochemistry

Tissue explants were fixed in MEMFA for 3-4 h when control embryos obtained from fertilized eggs reached stage 26. Samples were then kept in methanol at -20°C and embedded in Histoplast:beeswax. Muscle-specific 12/101 antibody staining was performed on 10 µm thick sections and subsequent steps were as described in Kato and Gurdon (1993). The substrates for the color reaction were 5-bromo-4-chloro-2-indolyl-phosphate (BCIP) and 4-nitro blue tetrazolium chloride (NBT). The same sections were doubled-stained with notochord-specific antibody MZ15 using the same protocol except that alkaline phosphatase substrate I, Vector Red (Vector Laboratories) was used. After Hoechst staining, the sections were mounted in 90% glycerol and 10% PBS.

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