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The transplantation of nuclei from single cultured cells into enucleate frogs' eggs

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SUMMARY

Nuclei from a monolayer of cultured epithelial cells have been transplanted singly to enucleated unfertilized eggs of *Xenopus laevis*.

About three-quarters of the injected eggs failed to cleave or cleaved abortively. Most of the remainder formed partial blastulae. Less than 1 % reached postneurula stages of development.

Nuclei from first-transfer blastulae were used for serial transplantation. The nuclei of partial blastulae promoted normal or nearly normal development more often than those of complete blastulae. Several clones were obtained which included nearly normal tadpoles with apparently normal muscle, nerve, blood, and other specialized cell types. Three tadpoles commenced feeding and have completed metamorphosis into young frogs.

To prove that the donor- and not egg-nucleus participated in the formation of nuclear-transplant tadpoles, genetically marked 2-*nu* and 1-*nu* diploid nuclei were transplanted to 2-*nu* eggs; the resulting tadpoles had the nucleolar number characteristic of donor, not egg, nuclei. Chromosome counts and nuclear diameter measurements on the epidermal cells of these tadpoles revealed the presence of diploid mitoses and the absence of haploid nuclei.

The behaviour of egg and donor nuclei immediately after nuclear transfer was determined by tracing the early cleavage pattern of individual eggs and by following, autoradiographically, the fate of donor nuclei whose DNA had been previously labelled with ^{3}H-thymidine.

These experiments show that the serial transplantation of nuclei from monolayer cell cultures can lead to sufficiently normal nuclear-transplant embryo development to provide a useful test of the genetic properties of the donor cell line.

INTRODUCTION

When the transplantation of a living cell nucleus to an enucleate egg is followed by normal or nearly normal development, an opportunity is provided to investigate the presence and expression of nuclear genes in a way not currently possible by other experimental procedures. This is because the expression of a much greater range of genes is required for development and cell differentiation than is needed for other kinds of cell function, such as the growth and division of a single cell line. A limitation of nuclear transfer experiments is that only a very small proportion of nuclei from a specialized animal tissue support normal development after transplantation (reviews by Gurdon, 1963; King, 1966). Since most adult organs contain a mixture of cell types, it is hard to define the characteristics of those few cells whose nuclei subsequently support development. This

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difficulty seriously restricts the widespread application of nuclear transplantation to the genetic analysis of specialized cells, but would be overcome if nuclei could be successfully transplanted from single cultured cells. Not only can cultured cells be cloned to provide a population which is homogeneous in cell type, but they can also be made relatively homogeneous in growth and metabolism, and can be synchronized in respect of the cell cycle. Thus if only a very small percentage of nuclei from a homogeneous population of cells should support embryonic development after transplantation, the characteristics of the particular cells from which the successfully transplanted nuclei were taken could be stated with certainty.

We know of only one previous attempt to transplant nuclei from cells cultured *in vitro* as a monolayer—that of King & DiBerardino (1965) using cells prepared from the renal adenocarcinoma of *Rana pipiens*. Sixteen complete blastulae were obtained from 90 first or serial transfers of nuclei from such cells, but none developed beyond the gastrula stage. The experiments which we now describe were carried out on monolayer cultures of *Xenopus laevis* epithelial cells. Special attention has been devoted to the provision of evidence that participation of the host egg nucleus was not involved in the formation of the most normal nuclear-transplant tadpoles obtained.

MATERIALS AND METHODS

Donor cells. Monolayer cell cultures were prepared from stage 40 (Nieuwkoop & Faber, 1956) swimming tadpoles by finely mincing the material and incubating the fragments at 25 °C in a 0·5 % solution of trypsin (Difco 1:250) in 88 mM-NaCl, 2 mM-KCl, 2·4 mM-$NaHCO_3$ and 15 mM-Tris, adjusted to pH 7·8 by HCl. Trypsin treatment was stopped by addition of an equal volume of growth medium. This consisted of: 67 parts of Leibovitz L-15 medium (Leibovitz, 1963) as used by Balls & Ruben (1966) for *Xenopus* cell cultures, 23 parts of glass-distilled water, and ten parts of foetal bovine serum. Cells were dispersed by mechanical agitation, harvested by centrifugation, washed and plated in medical flat bottles at 5×10^5/ml. Cultures were maintained at 25 °C in free gas exchange with the atmosphere. Microbial contamination was eliminated by including in the medium 100 i.u./ml benzylpenicillin sodium salt; 70 μg/ml gentamicin sulphate, and 20 i.u./ml mycostatin, a combination found to suppress effectively growth of micro-organisms (Laskey, 1970). Medium was changed every 3 to 4 days and cultures were divided every 7 days.

When cultures were prepared in this way, most of the cells were of epithelial morphology (Fig. 3A) having a mean division cycle of 36 h. For most of the experiments described here, cultures were established from animals heterozygous for the anucleolate mutation (Elsdale, Fischberg & Smith, 1958).

Donor cells were prepared for nuclear transplantation by brief incubation in the trypsin-saline solution described above, which detached them from their

substrate. The cell suspension was washed twice in the same solution without trypsin and retained in this solution for nuclear transplantation. The merits of this and other cell dissociation procedures for the purpose of nuclear transplantation are discussed in the next paper (Gurdon & Laskey, 1970).

Recipient eggs. Unfertilized eggs were obtained from wild-type females by the usual procedure of injecting mammalian gonadotrophic hormone (Pregnyl, Organon Laboratories). Eggs were exposed to ultraviolet irradiation at their animal pole in order to inactivate the egg chromosomes according to the procedure previously found to be successful (Gurdon, 1960*a*).

Nuclear transplantation. Of the two methods which we have found suitable for transplanting nuclei from single cultured cells (Gurdon & Laskey, 1970), we have used, for all experiments described here, the detached cell method. This employs a stereomicroscope and low power micromanipulator to suck isolated cells into a tight-fitting micropipette. The amount of donor cell cytoplasm injected into each recipient egg is about $2/10^6$ of the egg volume and is assumed to have no effect on development.

Culture of nuclear-transplant embryos. After receipt of a transplanted nucleus, recipient eggs were cultured for 3–8 h at 19 °C in the following solution, based on that of Barth & Barth (1959): 88 mM-NaCl, 2·0 mM-KCl, 0·33 mM-Ca $(No_3)_2$, 0·41 mM-$CaCl_2$, 0·82 mM-$MgSO_4$, 2·4 mM-$NaHCO_3$, 10 μg/ml of both streptomycin sulphate and benzylpenicillin sodium salt, and 15 mM-Tris-HCl to bring the pH of the whole solution to 7·6. At some stage within this period the medium was replaced with a tenfold dilution of the above solution and the embryos were maintained in this as long as they lived.

Developmental stage numbers are those of Nieuwkoop & Faber (1956).

Nucleolar and chromosome counts. When a nuclear-transplant embryo seemed unlikely to develop further, a fragment was squashed and the number of nucleoli per nucleus was counted by scoring at least 50 nuclei under the phase-contrast microscope. In the case of tadpoles, the tail was fixed for cytological examination, and the rest of the body was prepared for chromosome counting by conventional methods. The tissue was incubated for about 4 h in a solution of 10^{-4}M colchicine, and 15 mM-Tris-HCl, pH 7·6. This was followed by fixation for 10–15 min in 3:1 ethanol/acetic acid, staining for 1 h in aceto-orcein (2 % orcein in 50 % glacial acetic acid), and by appropriate squashing to spread the chromosomes.

RESULTS

(A) *The development of nuclear-transplant embryos*

First transfers. The developmental fate of eggs which received single cultured-cell nuclei is summarized in Table 1. About three-quarters of all recipient eggs either fail to cleave at all, or show abortive cleavage in which the irregularly shaped blastomeres rarely contain nuclei. Most of the remaining eggs undergo partial cleavage in which half or more of the egg consists of apparently normal

blastomeres and the rest is uncleaved or abortively cleaved. Partially cleaved blastulae are unable to gastrulate normally, though the patch of regularly cleaved cells may persist for up to 24 h. Less than 5 % of all recipient eggs cleave entirely regularly to form complete blastulae. Of these, 90 % die as blastulae without developing further. A few gastrulate abnormally in such a way that axis formation is impaired by protruding yolk. Only a very small fraction (< 0·1 %) of all first-transfer embryos develop into normal or nearly normal tadpoles.

Table 1. *The development of eggs receiving transfers of single cultured-cell nuclei*

	Cleavage				
			Partial cleavage		
Total no. of nuclear transfers	Uncleaved	Abortive cleavage	½	¾	Complete cleavage
3546	1871	597	819	135	124
100 %	53 %	17 %	23 %	4 %	—
	70 %		27 %		3 %

	Post-blastula development			
Total no. of complete blastulae	Arrested as blastulae	Abnormal during gastrulation	Nearly normal tadpoles (stage 26–40)	Normal feeding tadpole
124	107	13	3	1
100 %	86 %	10 %	4 %	

These results were obtained in 12 different experiments involving the use of eggs from 22 different frogs.

The development of eggs which received cultured-cell nuclei is conspicuously less normal, on average, than that obtained with tadpole endoderm nuclei. However, the eggs of different frogs vary considerably in their capacity to support nuclear-transplant embryo development (Gurdon, 1960*b*), and a true comparison of the developmental capacity of nuclei from cultured and other cells requires that both kinds of nuclei be transplanted into recipient eggs of the same frog. Table 2 shows that, under these conditions, both cleavage and post-blastula development promoted by cultured-cell nuclei are much inferior to that promoted by swimming tadpole endoderm nuclei. In this experiment, the cultured-cell and tadpole nuclei gave typical results, and we conclude that a real difference exists in the developmental capacity of nuclei from these two kinds of cells.

Serial transfers. In earlier experiments with tadpole intestine nuclei, it was found that the development of serial nuclear-transplant embryos is often more normal than that expected of the first-transfer embryo whose nuclei were serially

transplanted (Gurdon, 1962). This effect can be understood if many first-transfer embryos consist of a mosaic of cells, some containing normal, and some abnormal, nuclei. By serial transplantation, a few embryos consisting only of cells with normal nuclei should be obtained, as explained in the Discussion. The cultured-cell, first-transfer embryos used for the following experiments included partial as well as complete blastulae, since the former gave good results in previous experiments with tadpole intestine nuclei (Gurdon, 1962).

Table 2. *The developmental capacity of cultured-cell nuclei compared to that of tadpole endoderm cell nuclei*

Source of nuclei	No. of nuclear transfers	Cleavage as % of total transfers			Later development as % of complete blastulae			
		Uncleaved and abortive	Partial	Complete	Arrested blastulae	Abnormal gastrulae	Abnormal tadpoles	Normal tadpoles
Cultured cells*	140	71	25	4	100	—	—	—
Stage 40 tadpole endoderm*	230	33	43	24	24	40	24	12
Previous results with stage 40 endoderm†	436	29	50	21	20	45	20	15

* The results recorded in the first two rows of the Table were obtained with eggs of four different females, the eggs of each female being used to an equal extent for transfers of cultured-cell and tadpole nuclei.

† The previous results with stage 39–41 tadpole endoderm nuclei are taken from Gurdon (1960*c*).

Table 3 compares the cleavage promoted by first and serial transfers of cultured-cell nuclei. The main conclusions are that the cleavage of serial-transfer embryos is much more normal than that of first-transfer embryos, and is as normal as that obtained from the nuclei of blastulae reared from fertilized eggs. The cleavage promoted by nuclei of partial and complete blastulae is similar.

The postblastula development of embryos obtained by serial transplantation from cultured-cell nuclei is summarized in Fig. 1, which shows the development of the most normal embryo contained in each of 70 serial-transfer clones prepared from the nuclei of partial and complete first-transfer blastulae. One important conclusion is that serial-transfer embryos develop much more normally than would the first-transfer embryo from whose nuclei they were prepared. This is particularly obvious in the case of partial blastulae, all of which would have died before the early gastrula stage. A second important, but unexpected, conclusion, illustrated in Fig. 1, is that the nuclei of partial blastulae promote significantly more normal development than those of complete blastulae. The interpretation of this result is considered in the Discussion.

Table 3. *Cleavage promoted by the serial transplantation of nuclei from partial and complete first-transfer embryos*

Source of nuclei	No. of embryos used as serial donors	No. of nuclear transfers	Cleavage as % of total transfers		
			Uncleaved and abortive	Partial	Complete
Cultured cells (first transfers)	—	627 (100 %)	75	22	3
Complete first-transfer blastulae*	5	290 (100 %)	5	66	29
Partial first-transfer blastulae*	6	310 (100 %)	16	57	27
Blastulae reared from fertilized eggs (from Gurdon, 1960*c*)	—	533 (100 %)	27	41	32

* All serial nuclear transfers were made from blastulae at stage 8, and into the eggs of two frogs, used equally for each serial donor.

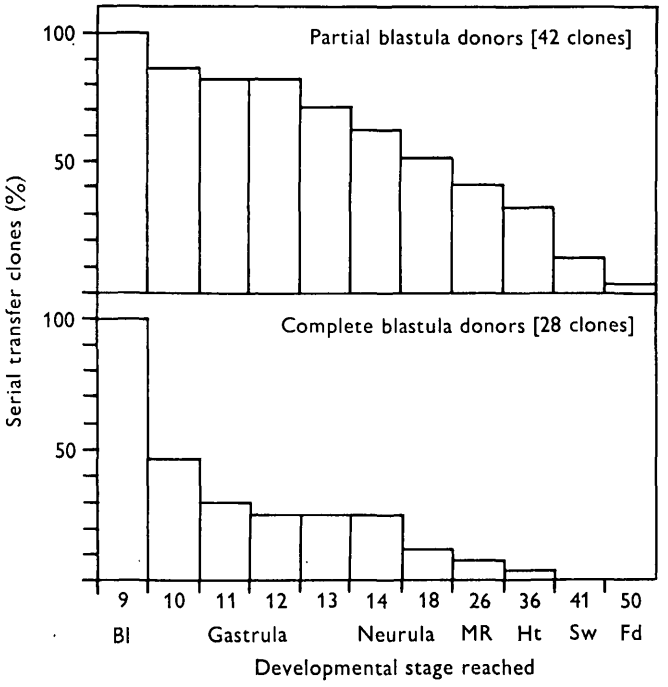

Fig. 1. The development of embryos in clones derived from serial nuclear transfers, showing the developmental stage reached by the most normal embryo in each clone. Each clone involved the transfer of about 50 nuclei from a partial or complete first-transfer embryo, which itself resulted from the transplantation of a single cultured-cell nucleus. Abbreviations: Bl, blastula; MR, muscular response; Ht, heart-beat; Sw, spontaneous swimming; Fd, feeding.

Comparison of the embryos comprising serial nuclear-transfer clones reveals great variation in the developmental abnormalities observed not only *between* clones but also *within* each clone (Fig. 2). It has been pointed out previously (Briggs, King & DiBerardino, 1960; Gallien, Picheral & Lacroix, 1963; Hennen, 1963) that the developmental abnormalities of nuclear-transplant embryos are commonly associated with, and may be the result of, chromosomal

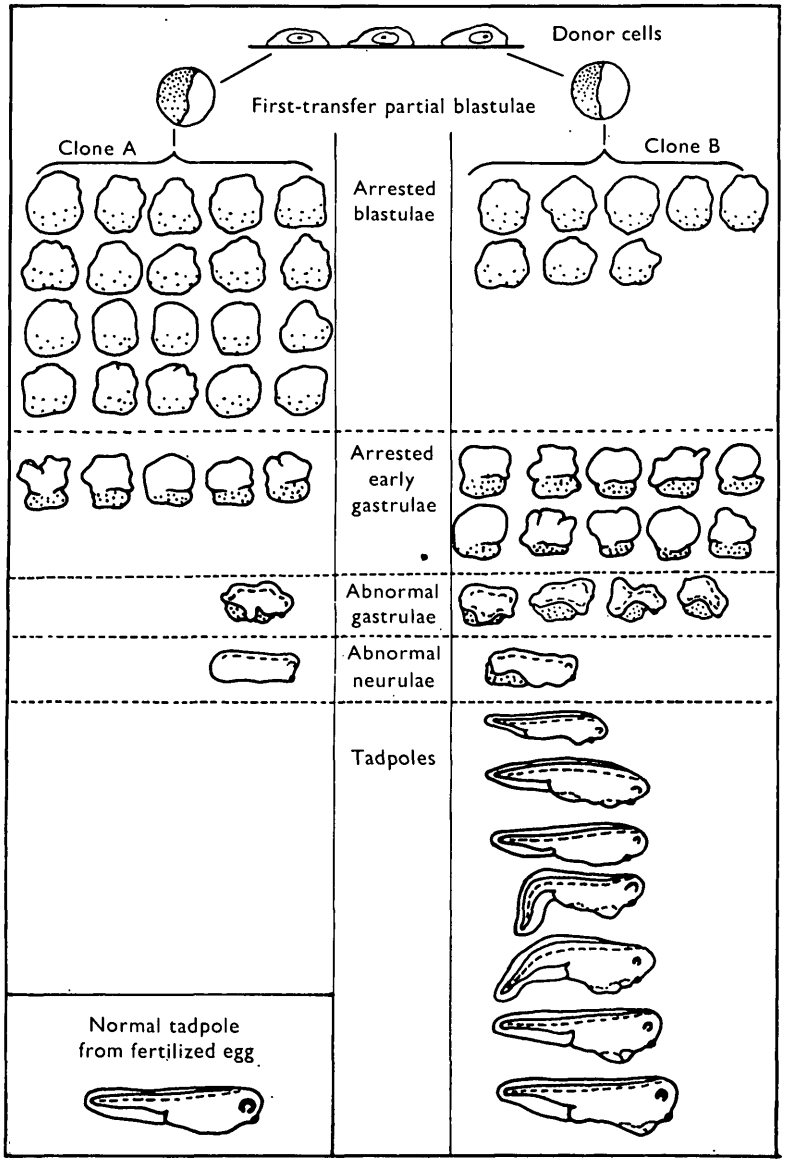

Fig. 2. Developmental variation within and between serial-transfer clones. About 70 nuclear transfers were made from two partial blastulae, themselves the result of first transfers from cultured cells. Recipient eggs from the same frog were used for both serial transfer clones.

abnormalities arising during the divisions which follow nuclear transfer. If chromosome losses take place during the early mitotic divisions of transplanted cultured-cell nuclei, the first-transfer blastulae which provide nuclei for serial transfers could contain, or sometimes consist of, cells with incomplete chromosome sets. In this case, the developmental variation within and between clones may be due to the variable chromosome content of donor cell nuclei in the first-transfer embryos.

The most normal development promoted by cultured-cell nuclei. The most normal nuclear-transplant embryos are of interest, partly because they place a lower limit on the extent to which it is possible to transplant cultured-cell nuclei without damage, and partly because they test the presence and expression of genes in the nuclei of cultured cells. We have obtained one first-transfer and two serial-transfer embryos which have passed successfully through metamorphosis (Fig. 3D). Until the age of sexual maturity, their capacity for gametogenesis is not known, but these frogs appear to contain normal cell types of all other kinds. The proportion of cultured-cell nuclear transfers which pass metamorphosis is extremely small, and this cannot at present be regarded as a typical result of these experiments. On the other hand we have quite commonly obtained nearly normal swimmming tadpoles in serial-transfer clones (Figs. 2, 3B). These possess functional muscle and nerve cells, circulating red blood cells, and many other specialized cell types present in normal tadpoles at this stage of development. Evidently these tadpoles contain actin, myosin, haemoglobin and other kinds of molecules not synthesized at a detectable level by cultured epithelial cells nor present in egg cytoplasm. In our experience, about one out of every four serial-transfer clones prepared from the nuclei of partial blastulae contain at least one tadpole which reaches the muscular response and heart-beat stage of development.

Although the interpretation of the abnormal development of nuclear-transplant embryos is full of uncertainty (Gurdon, 1963), we feel justified in commenting on the surprising frequency with which serial nuclear-transplant tadpoles develop entirely normally to the hatching stage (36), but then reveal a number of abnormalities which seem to affect the organization of tissues rather than the differentiation of particular cell types; such defects include twisted or mis-shapen tails, incomplete optic cups, abnormal coiling of the intestine, etc. Furthermore, it is not uncommon to obtain serial-transfer clones in which several tadpoles display similar defects (e.g. Fig. 2, clone B). Although the cause of these abnormalities is not known, we have not yet been able to exclude the possibility that this is the most normal development which can be supported by the cultured-cell nuclei used in these experiments. The three metamorphosed frogs were obtained from nuclei of a cell culture very soon after it was first established, and the older cultures, used for subsequent experiments, could have undergone minor changes of karyotype which would make it impossible to obtain entirely normal frogs by transplantation of their nuclei.

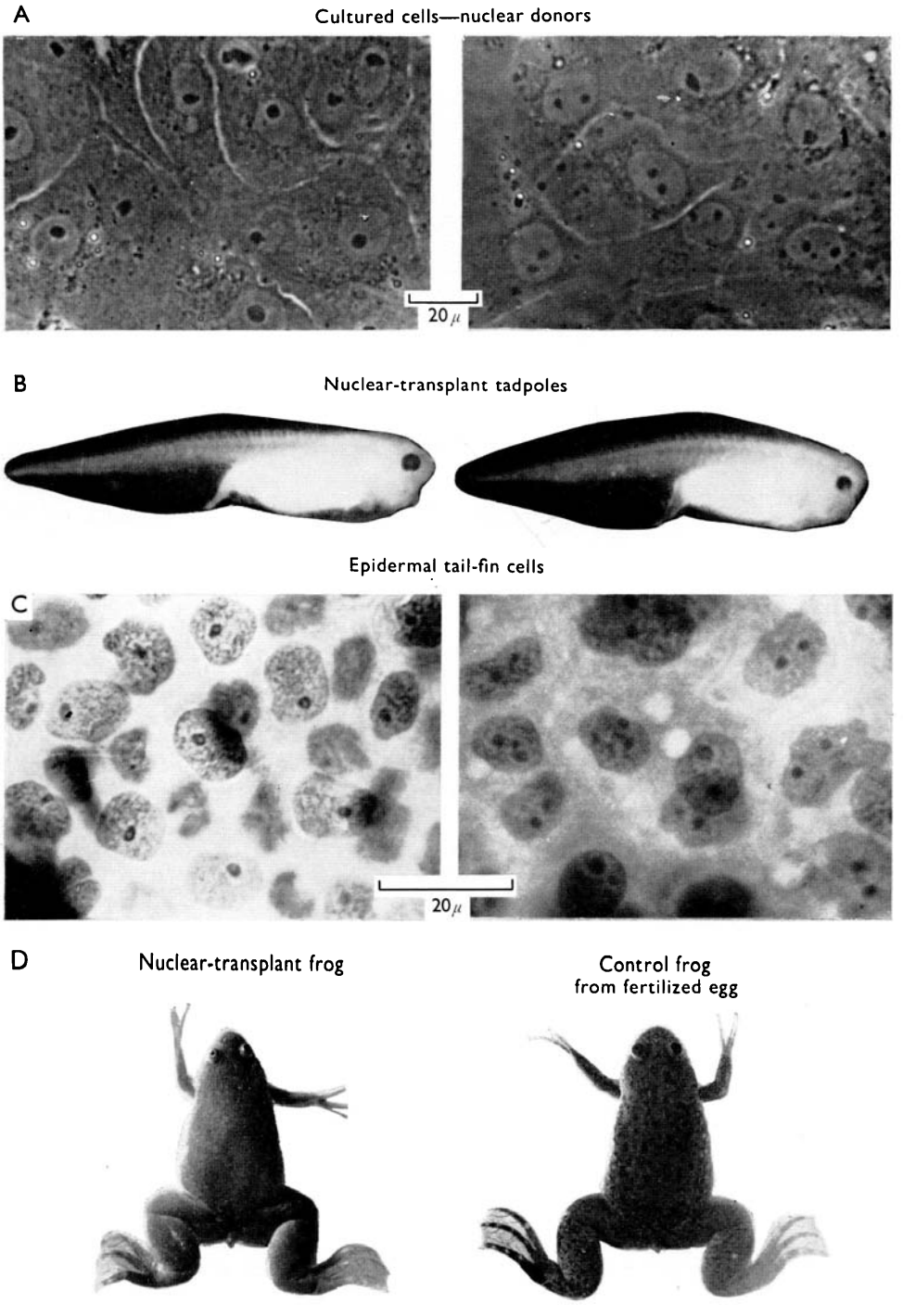

Fig. 3. (A) 1-*nu* and 2-*nu* cultured cells of the kind used to provide nuclei for transplantation. Phase-contrast photographs. (B) Nearly normal swimming tadpoles obtained by the serial transplantation of single cultured-cell nuclei. (C) 1-*nu* and 2-*nu* nuclei of epidermal cells of nuclear-transplant tadpoles. Whole mounts of fixed tail-fin. (D) Nuclear-transplant and control frogs, 3 months after metamorphosis.

(B) *Nucleolar and chromosome numbers of nuclear-transplant embryos*

The use of a nucleolar mutation to determine the nuclear origin of transplant embryos. In any experiment where significance is to be attached to the development of embryos which result from a very small proportion of all nuclei transplanted, it is essential to prove beyond doubt that the nuclei of the embryos concerned are the mitotic products of the transplanted nucleus and not of the egg nucleus; this is necessary because occasional errors in enucleation, by whatever method, are likely to occur. Direct proof of the nuclear origin of an embryo requires that the mitotic products of the transplanted nucleus are distinguishable from those of the host egg nucleus, a condition most easily satisfied by the use of a genetic difference between the donor and recipient strains. In these experiments, donor nuclei from cells heterozygous for the anucleolate mutation have been used, in the way described previously (Elsdale, Gurdon & Fischberg, 1960).

Table 4. *Chromosome numbers of nuclear-transplant tadpoles*

	Ploidy and range of chromosome numbers							
	Haploid		Diploid		Triploid		Tetraploid	
	18±2	18±5	36±2	36±5	54±2	54±5	72±4	72±10
	Nuclear-transplant tadpoles							
No. of mitoses scored	5	1	47*	68	7	1	2	5
Total mitoses in each ploidy class	6		115		8		7	
No. of tadpoles analysed	1		26		2		2	
	Cultured-cell lines							
No. of mitoses scored	—	—	8†	6	—	—	1‡	—
			14				1	

* This figure includes eight mitoses of 36±1, and two of exactly 36, found in nine different tadpoles.

† Includes two mitoses of 36±1, and four of exactly 36.

‡ One mitosis of approximately octoploid chromosome number was also found.

Chromosome preparations were made as described in Methods. The tadpoles analysed were contained in ten different serial clones, and were normal or nearly normal at stage 36. The haploid and diploid tadpoles had only one nucleolus/nucleus; the triploids and tetraploids had not more than two nucleoli/nucleus.

When 1-*nu* nuclei are transplanted to wild-type (2-*nu*) recipient eggs, the nuclei of a resulting diploid nuclear-transplant embryo should all have one nucleolus if its nuclei are products of the transplanted nucleus, but 30–60 % of them will have two nucleoli if they are diploid products of a recipient egg nucleus which escaped enucleation.

Chromosome numbers. The diploid chromosome number for *Xenopus laevis*

is 36 (Wickbom, 1945). The relatively high number of morphologically similar chromosomes has made it difficult to obtain exact determinations of chromosome numbers. This difficulty is greatly increased by the presence of yolk which resists squashing and therefore either prevents the even spreading of chromosomes, or provides channels through which loose chromsomes may be swept away from the others. For these reasons we have not attempted to make a detailed study of karyotypic abnormalities of nuclear transplant embryos. The results presented here were obtained in order to distinguish 1-*nu* haploids from 1-*nu* diploids, 2-*nu* diploids from 2-*nu* tetraploids, etc.

The counts made on 31 nuclear-transplant tadpoles are summarized in Table 4 and are clustered around the main ploidy classes. Several mitoses were found in which the chromosome number was very close, if not equal, to the exact diploid number of 36. These results allow us to conclude (i) that haploids can be clearly distinguished from diploids by chromosome counting, (ii) that all counts made from one tadpole fall into the same ploidy class, (iii) that the great majority of tadpoles have chromosome numbers similar to that of the donor cells, and are clearly not haploid.

It is unusual to find as many as one haploid and two triploid tadpoles in a sample of 31. Since all three were members of serial clones which also contained diploid tadpoles, they probably arose during serial transplantation by participation of an egg pronucleus which escaped the normally lethal effects of irradiation, an event which is known to happen only very rarely in most experimental series (Gurdon, 1960*a*). The tetraploid tadpoles must have arisen, in view of their nucleolar number, by a doubling of the chromosome complement immediately after nuclear transfer, as is known to happen in about 10 % of all transfers of blastula nuclei (Gurdon, 1959).

Nucleolar numbers. In most experiments 1-*nu* nuclei were transplanted to eggs of wild-type females. The occurrence of 1-*nu* frogs in wild populations is very low (Blackler, 1968; L. Miller, personal communication: of 55 recently imported frogs whose nucleolar condition was examined none were 1-*nu*). However, we have tested the nucleolar condition of 'recipient' female frogs in three serial-transfer experiments which yielded nearly normal tadpoles of 1-*nu* diploid constitution. Some of the eggs laid by the 'recipient' females used for first and serial transfers were artificially fertilized. Of 18 first- and serial-transfer 'recipient females' tested in this way, none was 1-*nu*.

Table 5 relates the nucleolar condition of transplant embryos to the extent of their developmental abnormality. Of the two more normal groups, tail-bud and swimming tadpoles, most were 1-*nu* and 2-*nu*, and some of these were shown by chromosome counts to be diploid and tetraploid respectively. Among the more abnormal transplant-embryos there is a marked increase in the proportion of 2-*nu* and 0-*nu* embryos. The chromosome number of these embryos was not determined, but we think it possible that 0-*nu* embryos are derived from nuclei which had suffered the loss of some chromosomes (including the single chromo-

some bearing a nucleolus organizer) during the formation of a first-transfer blastula; such nuclei would therefore be particularly likely, being aneuploid, to promote abnormal development after serial transplantation.

Table 5. *Nucleolar numbers of transplant-embryos prepared from 1-*nu *nuclei*

Developmental condition of embryos	No. of embryos classified by maximum number of nucleoli/nucleus				
	0-*nu*	1-*nu*	2-*nu*	3-*nu*	4-*nu*
Abnormal early gastrulae (stage 10–11)	25	27 (36 %)	21	1	—
Abnormal late gastrulae and neurulae (stage 12–16)	6	29 (56 %)	18	—	—
Abnormal tail-bud tadpoles (stage 26–33)	3	21 (73 %)	4	1	—
Normal or nearly normal swimming tadpoles with heart-beat (stage 36+)	—	27* (85 %)	4†	1	—

* Includes ten tadpoles of diploid or near-diploid chromosome number.
† Includes two tadpoles of near-tetraploid chromosome number.
Chromosome numbers of other tadpoles and embryos were not determined.

Table 6. *Nucleolar numbers of tadpoles prepared from 1-*nu *and 2-*nu *nuclei transplanted to the same sample of recipient eggs*

Nucleolar no. of donor nuclei	No. of tadpoles classified according to maximum no. of nucleoli/nucleus		
	1-*nu*	2-*nu*	4-*nu*
1-*nu*	50*	17†	—
2-*nu*	—	22*	3†

* Chromosome counts showed 18 of these tadpoles to be diploid or near-diploid.
† Five of these embryos were near tetraploid. The chromosome numbers of other tadpoles were not determined.
The tadpoles included in this Table were contained in ten different serial-transfer clones. 1-*nu* and 2-*nu* nuclei were always transplanted to recipient eggs of the same 2-*nu* frogs, though different frogs were used to supply eggs for first and serial transfers.

We have considered one further uncertainty about the interpretation of experiments involving the use of a nucleolar marker. Conceivably the ultraviolet irradiation of the animal pole of an unfertilized egg could sometimes lead to the inclusion in the egg of *all* chromosomes on the second metaphase spindle (second polar body and egg pronucleus), as well as an inactivation of a nucleolus organizer. If the nucleolus organizer region of a chromosome set were par-

ticularly u.v.-sensitive, the nucleus of those eggs which received an unusually low dose of irradiation might be most likely to participate in development and could become 1-*nu* while remaining diploid. We have tested this possibility by transplanting 1-*nu* and 2-*nu* nuclei into the eggs of the same female (Table 6). 1-*nu* nuclei led to the formation of 1-*nu* and a few 2-*nu* embryos, whereas 2-*nu* nuclei yielded only 2-*nu* and 4-*nu* embryos. It is important to note that 1-*nu* embryos were not obtained from 2-*nu* nuclei. The chromosome counts that were made conformed to expectation as indicated in the Table.

Table 7. *Nuclear diameters of transplant-tadpoles and controls in* μ

	Ploidy			
	1N	2N	3N	4N
Theoretical values based on diploid*	7·49	10·70	13·16	15·30
Tadpoles reared from fertilized eggs†	7·29 ± 0·62	10·76 ± 0·75	13·11 ± 1·02	15·16 ± 0·84
Nuclear-transplant tadpoles‡	—	10·67 ± 0·75	—	—

* These values were calculated on the assumption that the flattened tail-fin nuclei of all ploidies have the same depth (smallest dimension), and that nuclear volume is directly proportional to chromosome number. Haploid, triploid, and tetraploid values were calculated in this way from an average diameter of 10·7 μ for diploid nuclei.

† Each value represents the average of 30 nuclei.

‡ Average of 180 nuclei in ten different tadpoles.

The values shown in the Table refer to the average of the two greatest diameters of nuclei, ± standard deviation, expressed in μ. Measurements were made on epithelial cell nuclei in whole mounts of tails fixed in Bouin's fluid and stained with Mayer's haemalum.

In conclusion, the combination of chromosome and nucleolar counts seem to us to prove beyond reasonable doubt that the transplanted nuclei have participated in the development of nuclear-transplant tadpoles.

The homogeneity of chromosome numbers within a nuclear-transplant tadpole. We consider here the possibility that nuclear-transplant tadpoles might consist of a mixture of cells, some with nuclei derived from the egg nucleus and others with nuclei derived from the transplanted nucleus. If the egg nucleus had become diploid, its mitotic products with two nucleoli would be easily seen in a predominantly 1-*nu* tadpole, but none were observed and the presence of diploid egg nuclei is therefore excluded.

Haploid nuclei derived from the egg nucleus would have only one nucleolus. The absence of any haploid chromosome sets from the 115 metaphases counted in 26 1-*nu* diploid (or near diploid) embryos (Table 4) argues against the presence of products of the egg nucleus in 1-*nu* diploid nuclear-transplant embryos.

To confirm the absence of any haploid nuclei in a predominantly diploid

tadpole, we have taken advantage of the fact that the epidermal cells of tadpole tails are flattened to such an extent that the depth of nuclei of all ploidy values is very similar. Consequently the average diameter of tail-cell nuclei viewed at right angles to the fin surface differs considerably according to chromosome

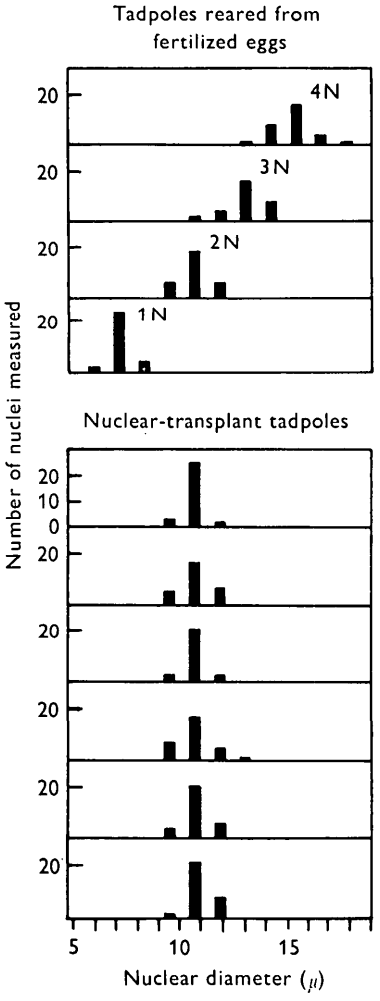

Fig. 4. Distribution of nuclear sizes in the tail-fin of tadpoles. Each histogram represents 30 nuclei. The diameter of a nucleus was taken as the average of its two greatest dimensions, that is, those seen when the tail-fin is viewed at right angles to its surface. The values given for tadpoles reared from fertilized eggs are based on measurements on five haploids, six diploids, two triploids, and two tetraploids. Among the nuclear-transplant tadpoles, each histogram represents measurements on one normal or nearly normal tadpole.

number (Table 7). The average diameter of nuclear-transplant tadpole nuclei is indistinguishable from that of diploid tadpoles reared from fertilized eggs (Table 7). The distribution of nuclear diameters in six nuclear-transplant tad-

poles is shown in Fig. 4 and fits well with what would be expected if all cells were diploid. Nuclei of haploid diameter were not detected in any of the tadpole tails examined (Fig. 4), though it is more likely than not that a nucleus of this size class would have been seen if as few as 1 % of all tadpole cells were haploid.

In conclusion, we believe that mitotic products of the egg nucleus have played no part in the development of the nuclear-transplant tadpoles obtained in these experiments.

(C) *The early cleavage of nuclear-transplant eggs*

The purpose of this section is to trace the events which immediately follow nuclear transfer and which lead to the formation of the blastulae whose nuclei were used for serial transplantation. This is the remaining link in the history of nuclear-transplant tadpoles from cultured cells, not so far documented. This has been established by following the morphological details of early cleavage and the fate of transplanted nuclei whose DNA had been previously labelled with [^{3}H]thymidine.

Early cleavage patterns. After following individually the behaviour of over 200 nuclear-transplant eggs provided by five different frogs, the great diversity of surface corrugations displayed by these eggs has been classified into the five main cleavage patterns illustrated in Fig. 5. The extent to which this classification is justified is seen in Table 8 which relates the pattern of early cleavage divisions to the type of embryo formed 10 h later when normal embryos have reached the blastula stage.

Nuclear-transplant eggs which fail to commence cleavage by the time normally fertilized eggs have cleaved twice generally remain uncleaved for several hours and then disintegrate (Fig. 5A–E). Of the eggs eventually classified as having undergone abortive cleavage, most undergo two or three cleavages which are often abnormal and characteristically delayed compared to normal (Fig. 5F–J). Eggs which eventually form half-cleaved blastulae form a normal 2 cell embryo at the usual time; after this, only one blastomere continues to cleave regularly, while the other remains uncleaved or undergoes abortive cleavage (Fig. 5K–O). Eggs in which the first two cleavage divisions are normal, but in which the subsequent divisions of one blastomere are abnormal, usually form $\frac{3}{4}$-cleaved blastulae (Fig. 5P–T). Almost all eggs, in which the first three or four cleavages are normal, form complete blastulae of normal appearance (Fig. 5U–Y), though minor irregularities arising through local, faulty divisions in mid-cleavage, would not always be noticed.

The fate of labelled donor nuclei. The donor cell population was continuously incubated in [^{3}H]thymidine for 36 h before nuclear transplantation, a procedure which resulted in about 70 % of all nuclei being labelled. Although most of the cells in the population have completed a cell cycle within 36 h, a significant proportion of cells in lines recently set up divide much more slowly. The incomplete labelling of the donor cell population affects the interpretation of

autoradiographic results in the following way. Any labelled nucleus must be the transplanted nucleus or one of its mitotic products. In any egg which contains both labelled and unlabelled nuclei, the unlabelled nucleus must be the egg nucleus. When all nuclei in an egg are unlabelled, these could be either egg- or transplant-nuclei.

The following sequence of events has been deduced from an examination of

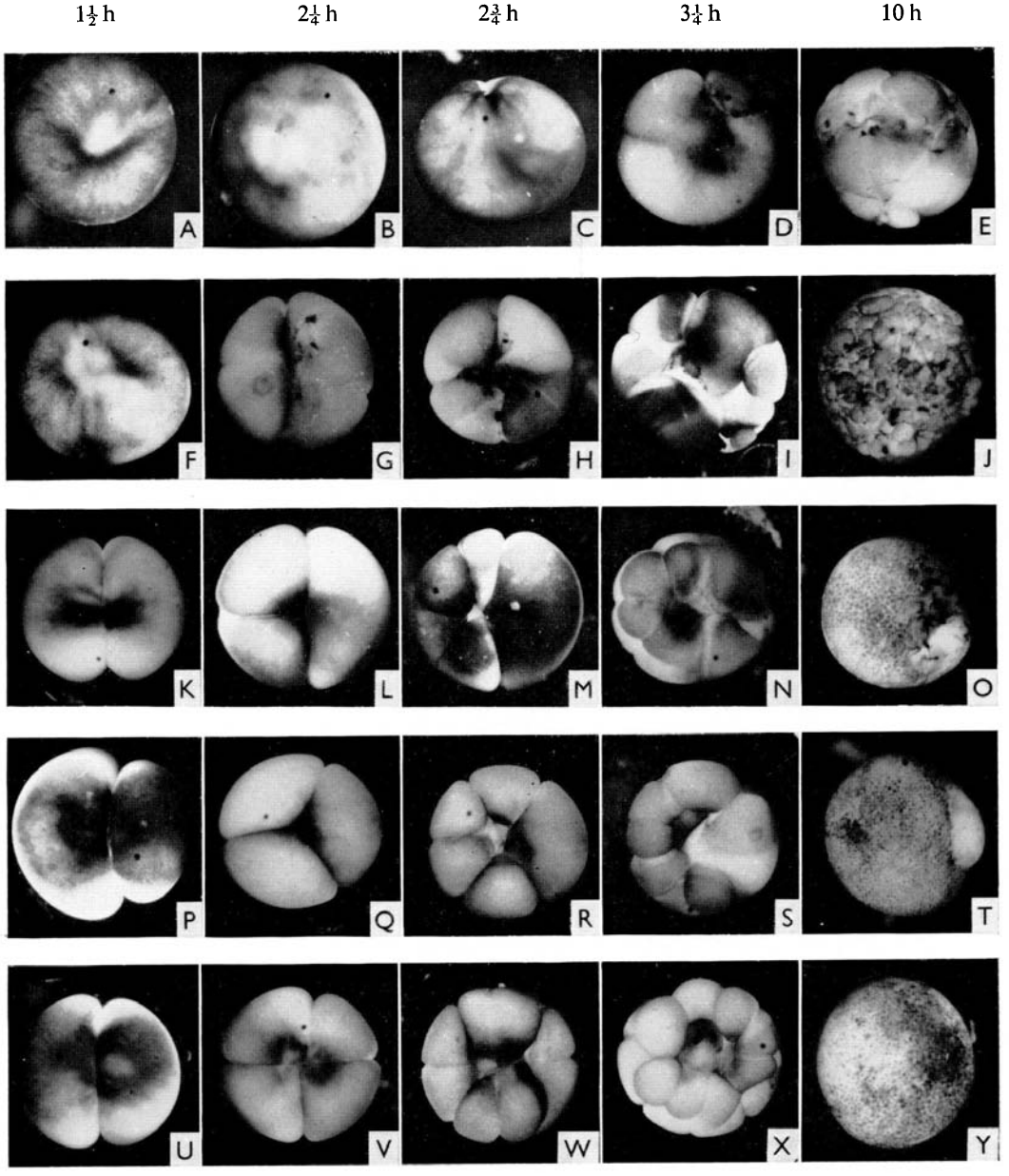

Fig. 5. Photographs of representative nuclear-transplant eggs taken at the stated times after nuclear transfer. Five main patterns of cleavage are illustrated. The magnification is such that the actual diameter of each egg is about 1·25 mm.

eggs which contain at least one labelled nucleus. During the first hour after nuclear transfer, the injected nucleus and the egg pronucleus migrate towards the centre of the egg where they lie adjacent to each other in a relatively yolk-free region of cytoplasm (Fig. 6A, E, F). Nuclear division usually takes place at about 80 min after nuclear transfer at 19 °C. The irradiated egg nucleus has never been observed to enter mitosis, and usually disappears after reaching the spindle associated with the transplanted nucleus (Gurdon, 1960*a*), or after passing into one of the first two blastomeres (Fig. 6H). The transplanted nucleus sometimes

Table 8. *Cleavage history of nuclear-transplant embryos*

Early cleavage pattern	Late cleavage pattern No. of eggs classified according to pattern of cleavage at 12 h				
	Uncleaved	Abortive	½ cleaved	¾ cleaved	Complete
No cleavage at usual time of first two cleavages	70	10	—	—	—
First two cleavages delayed; later cleavages abnormal	10	15	2	—	—
First cleavage normal; later cleavages normal in only one-half of egg	—	3	39	14	—
First two cleavages normal; minority part of egg then cleaves abnormally	—	—	16	19	11
Cleavage normal as in fertilized eggs	—	—	—	1	29

Donor nuclei were taken from cultured cells. The cleavage pattern of each egg was recorded continuously during the first three cleavages, and then again at 12 h when normal eggs have reached the late blastula stage.

divides just before the first cleavage, as in normal eggs, or it may fail to divide and enter only one of the first two blastomeres (Fig. 6H). If its further divisions in one-half of the egg are normal (Fig. 6L, P) it will eventually form a half-cleaved blastula of the kind that has given the most satisfactory serial-transfer results. Direct evidence that half-cleaved blastulae originate in this way comes from eggs fixed as 3- to 5-cell embryos (Fig. 5L, M) which contain four clusters of nuclear vesicles in the small blastomeres (Fig. 6P). Except in eggs which contain only unlabelled nuclei, *all four* nuclear clusters are labelled (Fig. 6R, S), and the egg nucleus in the uncleaved half is unlabelled (Fig. 6Q).

It is not uncommon to observe chromatin, of transplant-nucleus origin, stretched between daughter nuclei, in the site of the last mitotic spindle (Fig. 6M–O). In such cases at least one of the daughter nuclei will lack some chromosomes or parts of chromosomes. Stretched chromatin may occur at any

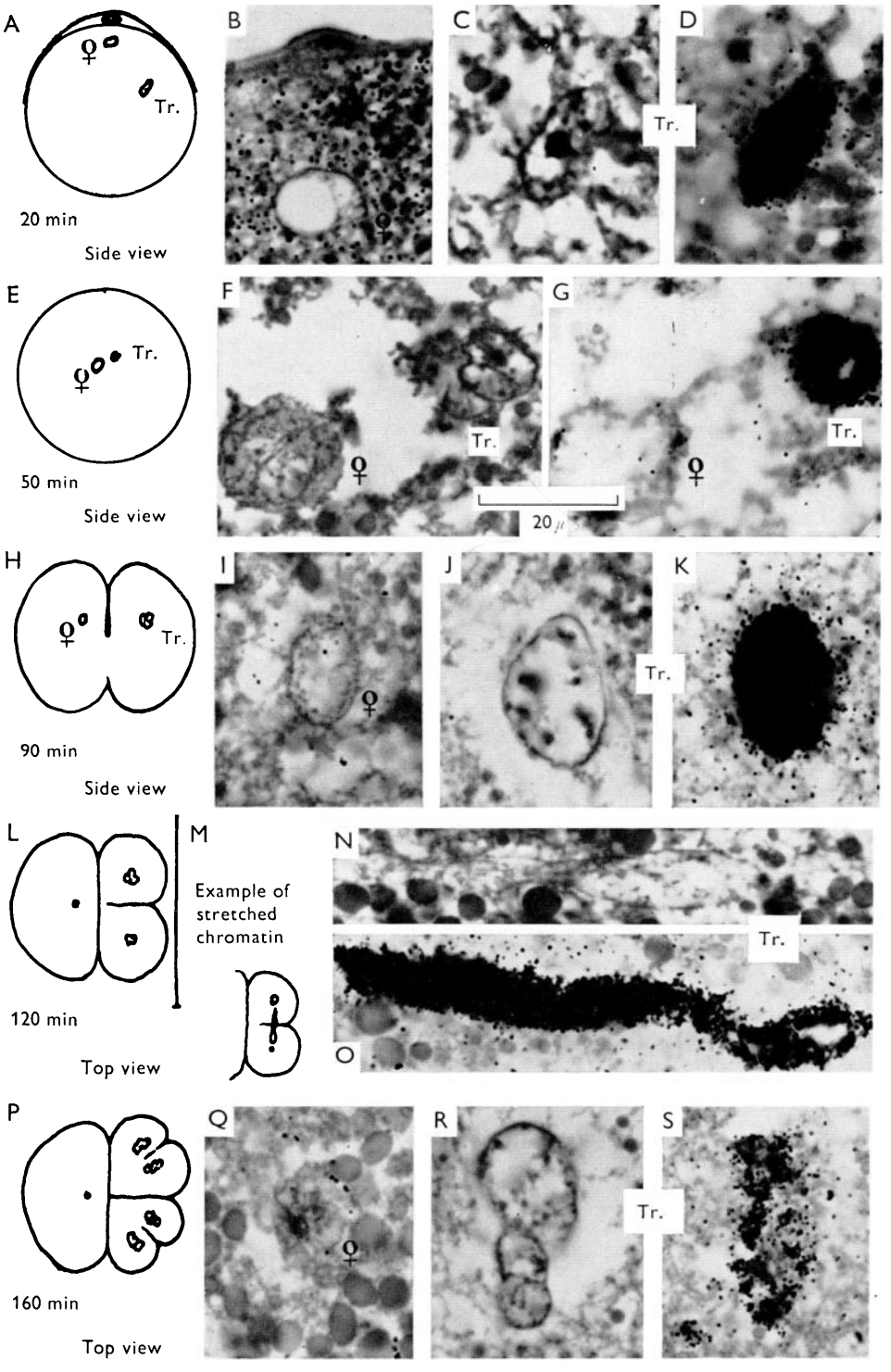

Fig. 6. Diagrams and photographs illustrating the behaviour of nuclei at various times after transplantation. The diagrams on the left show the sequence of events characteristic of eggs which form half-cleaved blastulae. B, G, I, Q are autoradiographs showing the unlabelled egg pronucleus (♀). C, F, J, N, R, show sections of transplant-nuclei (Tr.) before autoradiography and D, G, K, O, S, show sections of the same nuclei after autoradiography.

of the first divisions of the transplanted nucleus. It if happens at one of the first two divisions, most nuclei in the embryo will be abnormal, and we assume that such eggs undergo abortive cleavage, as observed in 10–20 % of all nuclear-transplant eggs.

About half of all eggs injected with nuclei fail to cleave at all (Table 1). This is not normally due to an early failure of the nuclear-transfer procedure, such as the removal of the donor nucleus during withdrawal of the injection pipette or the failure to break the donor cell wall. This is evident from the fact that we have found labelled nuclei free from the donor cell cytoplasm in 63 % of all examined eggs, a figure rather close to the 70 % of all donor nuclei which were labelled. The reason why so many of the nuclei exposed to egg cytoplasm should fail to promote early cleavage is not understood.

DISCUSSION

Proof that the irradiated egg nucleus does not participate in transplant-embryo development

There are several different ways in which it has been established that the cleavage and development of a recipient egg depends upon the introduction of a transplanted nucleus. As described elsewhere (Gurdon & Laskey, 1970), true cleavage of irradiated unfertilized eggs is not obtained if they are injected with saline solution, with an unbroken donor cell, or with many broken cells; a single broken cell, with an unbroken nucleus, is required. The use of labelled donor nuclei has shown how the mitotic products of the transplanted nucleus participate in early cleavage, and has shown that the irradiated egg nucleus does not do so in the same egg. The development of 1-*nu* diploid tadpoles from the transplantation of 1-*nu* diploid nuclei into 2-*nu* recipient eggs proves that mitotic products of the transplanted nucleus are primarily responsible for the more normal cases of transplant-embryo development. U.v.-irradiation of the metaphase chromosomes of an unfertilized egg does not result in the formation of a 1-*nu* diploid egg nucleus. The possibility that 1-*nu* nuclear-transplant tadpoles might contain a small proportion of nuclei derived from the egg nucleus was excluded by the absence of haploid nuclei and of 2-*nu* diploid nuclei. We consider these tests to show beyond reasonable doubt that the more normal nuclear-transplant embryos are populated entirely by mitotic products of the transplanted nucleus and therefore constitute a test of its genetic qualities.

The developmental capacity of nuclei from epithelial cell cultures

The fact that nuclei from cultured cells promote cleavage of recipient eggs much less successfully than tadpole endoderm nuclei is surprising, since the more rapid division rate of the former might be expected to suit them better for the rapid cleavage that follows nuclear transplantation. A failure commonly observed in transplanted cultured-cell nuclei is the non-disjunction of chromo-

somes during early mitoses, presumably on account of incomplete chromosome replication, as suggested previously on the basis of other nuclear transfer experiments (Briggs, Signoret & Humphrey, 1964). Severe cases of incomplete replication, with the subsequent formation of deficient daughter nuclei, would be expected to lead to abortive cleavages with many anucleate blastomeres; less severe cases might lead to the formation of apparently normal blastulae in which only a small part of the chromosome set is lacking. If only a very small amount of chromosome replication is incomplete, this might be expected to take place immediately after nuclear transfer, when the greatest change in rate of replication is required of a nucleus. In this case, a transplanted nucleus 'excused' from division at the time of the first egg cleavage might have a special opportunity to complete its first chromosome replication before nuclear division and subsequently to form normal daughter nuclei. Observation of early cleavage divisions and the results of serial transfers have shown that transplanted nuclei which fail to divide until the second division of the egg often support nearly normal development. This behaviour might well account for our finding that more normal development is promoted by the nuclei of partial blastulae than by those of complete blastulae.

The most normal nuclear-transplant tadpoles obtained in these experiments possessed functional and apparently normal muscle, nerve, blood and other cell types. The cell lines most extensively used for these experiments were grown from swimming tadpoles whose intestine and other endodermal tissues had been excised before mincing. Thus the only cells which might be regarded as embryonic at this stage had been removed and could not have contributed to the cell lines used as nuclear donors. The germ-cells must also have been eliminated since they are located in the endoderm at this stage (Nieuwkoop & Faber, 1956). We therefore believe that two major conclusions can be drawn from the experiments reported. First, cells derived from somatic tissue and cultured *in vitro* as a monolayer contain all genes necessary to promote the differentiation of the wide range of cell types present in a swimming tadpole, a conclusion in general conformity with that reached from the transplantation of intestinal epithelium cell nuclei (Gurdon, 1962). Second, and perhaps most important, our results show that nuclei can be transplanted from cultured cells in such a way as to provide a test of their genetic content.

RÉSUMÉ

Transplantation individuelle de noyaux de cellules en culture dans des oeufs énucléés de Xénope

Des noyaux de cellules épithéliales cultivées en couche monocellulaire, ont été transplantés individuellement dans des oeufs vierges énucléés de *Xenopus laevis*.

Les trois quarts environ des oeufs opérés ne se segmentent pas ou présentent des segmentations abortives. La plupart des germes restant ne forment que des blastulas partielles. Moins de 1 % dépassent la stade de la neurula.

Des noyaux de blastulas issues de cette première transplantation ont été utilisés comme donneurs pour des expériences de transplantation en séries successives. Les noyaux provenant de blastulas partielles sont, plus souvent que les noyaux issus de blastulas complètes, capables de promouvoir un développement normal ou presque normal. Dans de nombreux clônes on obtient des larves pratiquement normales, présentant tous les types cellulaires. Les cellules musculaires, nerveuses, sanguines ainsi que les autres types cellulaires spécialisés ne semble pas présenter d'anomalies. Trois larves ont pu se nourrir et subir les phénomènes de la métamorphose.

Afin de démontrer que le développement des larves issues de ces expériences de transplantation a bien été dirigé par le noyau greffé et non par le noyau propre de l'oeuf récepteur, un marqueur génétique a été utilisé: deux types de noyaux diploïdes, 2-*nu* et 1-*nu* ont été greffés dans les oeufs récepteurs 2-*nu*. Le nombre de nucléoles chez les germes issus de la transplantation, est celui qui caractérise le noyau donner, et non pas celui du noyau de l'oeuf récepteur. Le dénombrement des chromosomes et le mesure du diamètre des nucléoles effectués sur des cellules épithéliales des germes obtenus montre bien la présence des mitoses diploïdes et l'absence de noyaux haploïdes.

Le comportement du noyau greffé et du noyau de l'oeuf récepteur immédiatement après la transplantation a été déterminé pour chaque oeuf par l'étude du mode de clivage au cours des premières segmentations, et en suivant par autoradiographie le devenir du noyau donneur, dont l'ADN avait au préalable été marqué par la ^{3}H-thymidine.

Ces expériences montrent que la transplantation par séries successives de noyaux provenant de cellules cultivées en couche monocellulaire, peut conduire au développement d'embryons suffisament normaux, et constituer ainsi un test utile pour l'étude des propriétés génétiques de la lignée cellulaire donneuse.

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