templates leads to an efficient incorporation of both G and C for a 5.4:1 template, but a very low efficiency for a 1.9:1 template. This reduced efficiency is probably due to the extensive and stable self-structure of poly(CG) templates containing a substantial proportion of G.

The few experiments with more complex templates indicate that a wide variety of behaviors is to be anticipated. It is encouraging that only templates containing all four bases facilitate the incorporation of all four bases into product with reasonable efficiency.

We anticipate that even relatively short oligonucleotide sequences will display an enormous diversity in their nonenzymatic template chemistry. Templates will differ in the efficiencies with which they initiate, the rates at which they propagate, the probabilities that they terminate prematurely, and so on. This diversity corresponds, at the level of molecular structure, to the phenotypic differences in fecundity that distinguish organisms, and must make a substantial contribution to the "variation" on which natural selection at the level of molecular replication acts. It seems possible that an extension of our scheme, or of a scheme similar to it, might make it possible to study natural selection in a nonenzymatic system.

The relevance of our results to discussion of the origins of life is necessarily indirect, since it is unlikely that 2-methylimidazole was abundant on the primitive Earth. Nonetheless, the demonstration of a relatively simple reaction in which preformed copolymer templates containing all four of the natural bases direct the synthesis of their complements is significant. It makes it more plausible that the replication of polynucleotides was important at a very early stage in the evolution of life.

T. INOUE
L. E. ORGEL
Salk Institute for Biological Studies, San Diego, California 92138

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1. Abbreviations: A, adenosine; C, cytidine; G, guanosine; U, uridine; pN(N is A, C, G, or U), nucleoside 5′-phosphate; pNp, 5′-phosphonucleoside 2′(3′)-phosphate; 2-MeImpN, (nucleoside 5′-phosphor)-2-methylimidazolide.
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Gene Reactivation in Erythrocytes: Nuclear Transplantation in Oocytes and Eggs of *Rana*

Abstract. *Adult erythrocyte nuclei of* Rana, *transplanted and incubated in the cytoplasm of maturing oocytes, direct matured oocytes to form swimming tadpoles. These results demonstrate that nuclei of noncycling and terminally differentiated erythrocytes contain the genes to specify tadpole development, and conditioning these nuclei in the cytoplasm of oocytes leads to a widespread reactivation of dormant genes.*

It is now well established that cell specialization is achieved by genetic control. However, it is not known whether the phenotypes of specialized cells are controlled mainly by irreversible alterations in the DNA or by highly stabilized changes in the DNA or chromatin proteins that are potentially reversible. The most rigorous test of this question has been nuclear transplantation in amphibians. Young embryonic nuclei transplanted into enucleated eggs promote the development of the eggs into normal larvae and sexually mature frogs, thus demonstrating genetic totipotency (*1, 2*). Adult nuclei from specialized somatic cell types [lymphocytes (*3*), skin (*4*), and erythroblasts (*5*)] and from germ cells [spermatogonia (*6*)] direct development only to early larval stages and therefore exhibit genetic pluripotency.

Since adult germ cell nuclei by definition are genetically totipotent, but fail to promote normal development of enucleated eggs (*6*), the components in egg cytoplasm apparently have not met the requirements of nuclei from specialized cell types. However, the cytoplasm of oocytes does contain factors capable of transforming oocyte and embryonic nuclei into functional pronuclei (*7*). Therefore, we suggested earlier that these factors might induce nuclei from specialized cells to reveal enhanced genetic potential (*2, 7*).

We now report on the developmental potential of nuclei from noncycling and terminally differentiated adult erythrocytes. Whereas stimulated lymphocytes (*8*), cultured skin cells (*9*), and erythroblasts (*10, 11*) are synthesizing RNA and proteins, erythrocytes are extensively dormant in this respect (*10, 11*). We show that erythrocyte nuclei, after sequential exposure to the cytoplasm of oocytes and eggs, direct the formation of swimming tadpoles. If, however, they are injected directly into eggs, development does not proceed beyond the early gastrula stage. These results demonstrate that (i) adult erythrocyte nuclei contain the genes to specify tadpole development and (ii) conditioning erythrocyte nuclei in the cytoplasm of oocytes leads to a widespread reactivation of dormant genes.

Blood, obtained by cardiac puncture of adult *Rana pipiens* (four females and seven males), was collected in heparinized tubes and centrifuged at 500*g* for 15 minutes. The supernatant and buffy coat were removed, and the erythrocyte pellet was suspended in Ringer solution. Blood smears prepared from these suspensions contained 99.95 percent erythrocytes (range, 99.8 to 100 percent; 1000 cells per frog) (Fig. 1A). The rarely occurring contaminating white cells were identified as nonhemoglobin cells with the Leitz microscope (magnification, ×100) at the time of nuclear transfer. Therefore, only erythrocytes were used for donor cells.

Three to eight cells suspended in Ringer solution were aspirated into a glass micropipette (inner diameter, 25 μm), expelled into distilled water, and immediately aspirated back into the pipette, where the cell membranes ruptured. The nuclei were then injected near the equator into oocytes in the stage of first meiotic metaphase (*7*). Approximately 24 hours later, when the oocytes completed maturation in vitro (18°C), they were activated by being pricked with a glass microneedle and the second black dot, indicative of the egg nucleus at second meiotic metaphase, was removed microsurgically. In another series of experiments, erythrocyte nuclei were injected directly into enucleated eggs (*12*). The type of development from the two series of hosts was compared.

Of the eggs injected with erythrocyte nuclei, 7 percent formed partially cleaved blastulas (Table 1). Most of

these blastulas were used as donors for transfer into a second series of enucleated eggs. In the retransplantation series, a single nucleus was implanted into each recipient egg. Although 49 percent of these eggs cleaved into blastulas, only a few attained early stages of gastrulation. However, when erythrocyte nuclei were injected into oocyte hosts that later matured in vitro, 16 percent cleaved into blastulas, and 3 percent developed into postneurula embryos. In the nuclear transfers from these first-generation blastulas into enucleated eggs, 63 percent of the hosts formed blastulas. Fifty percent of the eggs formed gastrulas, 45 percent formed neurulas, 24 percent formed primitive organ systems in postneurula embryos, and 10 percent developed into swimming tadpoles.

There are two reasons for performing retransplantation experiments from first-generation blastulas. First, partially cleaved blastulas cannot proceed through normal morphogenesis. Second, both partial and complete blastulas frequently are chromosomal mosaics; that is, some cells within individual nuclear transplants consist of the apparently normal karyotype, while others have abnormal karyotypes (*13*). Therefore, random selection of donor nuclei for transplantation into second-generation hosts leads to clones whose members express different developmental potentials. The farthest development displayed by members of a clone indicates the best estimate of the genetic potential of the original donor nucleus.

Twelve clones were produced in the oocyte-egg host series (Table 1). Eight clones (67 percent) contained members that proceeded to postneurula stages, and six (50 percent) of these clones derived from complete blastulas contained members that attained the swimming larval stages (Table 2). The combined data derived from original and clonal experiments totaled 46 postneurula embryos, most of which displayed muscular and nerve function, and 18 of which developed into functional tadpoles. As judged from the original population of 130 nuclei tested, at least 11 (8.5 percent) adult erythrocyte nuclei can program for postneurula development, and at least 6 (4.6 percent) can direct the formation of swimming tadpoles. However, only 57 percent of the blastulas were cloned in retransplantation experiments because the rest could not be accommodated within the time span of the experiment. Among the nine remaining blastulas, two developed into neurulas and three became postneurulas. It is possible that at least the five embryos that attained neurula and postneurula stages contained nuclei that would have expressed greater potential after retransplantation.

Table 1. Developmental potential of adult erythrocyte nuclei.

Host	Transfer generation	Injected hosts (No.)	Stage of development of injected host					
			Blastula* (No.)		Gastrula (No.)	Neurula (No.)	Postneurula (No.)	Larva (No.)
			Partial	Complete				
Egg	First	86	1, 5† (7%)					
Egg	Second	76	22 (49%)	15	5 (7%)			
Oocyte	First	130	6, 3† (16%)	3, 9†	5 (4%)‡	5 (4%)‡	3 (3%)‡	
Egg	Second	181	23 (63%)	91	90 (50%)	82 (45%)	43 (24%)	18 (10%)

*All blastulas displayed regular cleavage, indicating that only one erythrocyte nucleus and its centrioles were functioning. †Blastulas used for donor nuclei for second transfer. ‡Based on 118 injected hosts, since 12 blastulas were used for second transfer.

Three types of controls were performed to evaluate the quality of the gametes, parthenogenetic activity, and enucleation efficiency. Among the 808 inseminated eggs whose sibling eggs were used for enucleated hosts, 92 percent developed into normal tadpoles, attesting to the high quality of the eggs and sperm. Oocytes that had completed maturation in vitro and mature uterine eggs (total, 4336) were activated; these underwent abortive cleavages and none developed into blastulas, demonstrating that development of the nuclear transplants was not due to parthenogenesis. In another group of activated oocytes and eggs (total, 258) the black dot (host nucleus) was removed surgically; none of these displayed puckers or furrows of the egg surface within the first 5 hours after activation, and on the following day they remained uncleaved, indicating successful removal of the host nucleus.

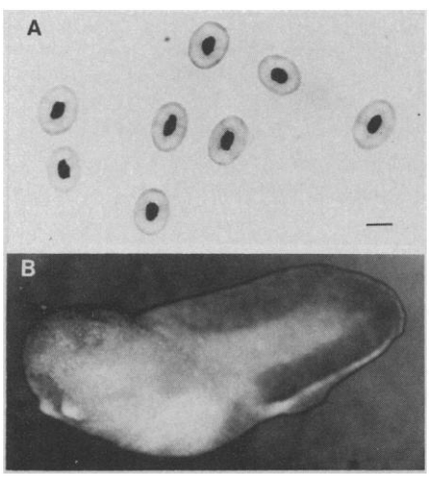

Fig. 1. (A) Adult erythrocytes from blood smear stained with hematoxylin-eosin. Other cells from this population were used for nuclear donors. Scale bar, 10 μm. (B) Clonal nuclear transplant tadpole derived from adult (male) erythrocyte nucleus. Photographed at stage 25 (11 days old). Body length is approximately 3.8 mm. The tail tip was cut 5 days earlier for chromosome study. Note eye, regressing suckers, and closure of operculum fold. See clone 7 in Table 2 for additional information.

In addition, the transplants were examined cytologically to determine nucleolar and chromosome numbers, and the exovate formed at the time of enucleation was examined for the presence of the host nucleus (Table 2). Nuclear transplants that initiated cleavage at 3 to 3½ hours after activation were diploids, and those that were delayed one interval were tetraploids. Estimates of epidermal cell size on living embryos revealed them to be uniformly diploids and tetraploids, respectively. If the host nucleus was responsible for development and the egg cleaved on time or was delayed one cleavage interval, the embryos would be haploid and diploid, respectively. If the haploid host nucleus fused with the diploid transplanted nucleus, triploid or hexaploid individuals would result, depending on the time of initial cleavage. No such individuals were detected in our analyses. Finally, the egg nucleus was recovered in the exovates of seven clonal donors. In the eighth donor, the exovate was not processed for technical reasons; however, this embryo must have been derived from the transplanted diploid nucleus because it was delayed one cleavage interval and was tetraploid. Among the 43 progeny in the clones, the egg nucleus was recovered in 38 instances, and other members of the clones verified the authenticity of the origin of development of the other five.

The youngest swimming larvae [stage 20; two clones; stage seriation of Shumway (*14*)] had well-formed heads, trunks, and tails. In the head region, nares, mouth, and suckers were present. The larvae displayed heart beat and gill circulation. Internally, they had differentiated

brains, spinal cords, ears, eyes, and internal nares. The guts had formed mouth, pharynx, and mid- and hindgut regions. Well-formed notochords were present, as well as hearts, pronephric tubules, and somites. Externally, older larvae (stage 22–23$^+$; three clones) swam vigorously, the hearts beat regularly, and peripheral blood circulated through the capillaries of surface tissue of the body and tail. They had well-formed eyes, and the mouths were open. The operculum folds were closed over the right gills, and the intestines had undergone some degree of coiling. Internally, the brains and spinal cords had differentiated into gray and white cellular components. The eyes had formed lenses and displayed neural and pigmented retinas. The guts had fashioned esophagi, stomachs, and livers and had undergone some degree of intestinal coiling and formed hindguts. Cartilage was present in the mouth region. All other structures present in the nuclear transplants at stage 20 were considerably more advanced in these older larvae. The oldest stage tadpole (stage 25$^+$; one clone) displayed larval pigment and more extensive coiling of the intestine. The operculum fold was completely closed (Fig. 1). Although this tadpole attained the feeding stage, it failed to feed, became moribund 2 days later, and was fixed for cytological study.

Previous tests of adult erythroid nuclei in *Xenopus* eggs did not result in the extent of development that we report. The oldest stage obtained with adult erythrocyte nuclei was early gastrulation (*5*), comparable to our results in *Rana* eggs. Adult erythroblast nuclei in the best cases promoted the development of late-stage postneurulas to initial-stage larvae (*5*). In one case each from nuclei of adult lymphocytes (*3*) and skin (*4*), a tadpole was obtained approximately equivalent to our stage 22–23 tadpoles. However, the genome of erythrocyte nuclei is much more extensively dormant than are the genomes of nuclei of specialized somatic cells previously tested (*8–11*). Although we cannot evaluate exactly the role of maternal templates in the development of nuclear transplants, the contribution made by the transplanted somatic nucleus is significant because eggs lacking a functional nucleus do not develop beyond the blastula stage (*15*).

The molecular mechanisms responsible for this gene reactivation in erythrocyte nuclei involve in some manner the ability of noncycling erythrocyte nuclei to undergo DNA replication again. Only a few adult erythrocyte nuclei transplanted into eggs initiate a small degree of DNA synthesis, but if they are injected into diplotene oocytes and are incubated through meiotic maturation, over 75 percent of the nuclei synthesize significant amounts of DNA in activated eggs (*11*). We have previously hypothesized that molecular components in maturing oocytes induce a remodeling of chromatin proteins and prepare repressed chromatin to respond to the factors in activated eggs that induce DNA synthesis (*2, 7, 11*). The precise mechanisms for this gene reactivation remain to be elucidated. Nevertheless, the formation of tadpoles attests to the fact that a significant portion of the DNA of dormant erythrocyte nuclei is reactivated and replicated many times with a high degree of fidelity. Furthermore, the DNA is capable of widespread RNA synthesis required for the protein synthesis necessary to specify the cell, tissue, and organ types present in functional tadpoles. Whether complete gene reactivation is possible is the focus of future studies. In the meantime this widespread reactivation of dormant genes suggests the future feasibility of controlling dormant genes in biomedical problems of regeneration, aging, and cancer.

Table 2. Cytological verification of postneurulas and larvae derived from adult erythrocyte nuclei. Three additional postneurulas derived from original transplantation experiments cleaved on time and were diploids.

Clone	Blastula retransfer donor		Progeny				
	Recovered egg nucleus	Chromosome number*	Stage	No.	Recovered egg nucleus	Nucleolar number†	Chromosome number*
1	+	2N	17–18$^-$	4	+	2N	
			20	3	+	2N	2N
2	+	2N	16–17	2	+	2N	
			19	1	+		
			19	1	−	2N	
			22–23	2	+	2N, 4N‡	2N, 4N‡
			23	1	+		2N
3	+	2N	17–19	4	+	2N	
			23$^+$	2	+	2N	2N
4	−	4N‡	16–18	5	+	4N, 8N‡	
			16	1	+		
			16	1	−		
			20	3	+	4N	
			20	1	−	4N	
			20	1	−		
5	+	2N	18–22	2	+	2N	
			23$^+$	1	+	4N‡	4N‡
6	+	4N‡	17$^+$	1	+	4N	
7	+	2N	18	2	+	2N	2N
			23–25$^+$	3	+	2N	2N
8	+	2N	16	1	−		
			18$^-$	1	+	2N	

*Nuclei containing 22 to 26 chromosomes were classified as diploid (2N) and those containing 44 to 52 chromosomes as tetraploid (4N). Chromosome number was determined in 3 to 14 cells per nuclear transplant. †Data for nucleolar number based on at least 50 nuclei located in various parts of each nuclear transplant. Ploidy is based on the maximum number of nucleoli (2N, 4N, and 8N equal 2, 4, and 8 nucleoli, respectively). ‡Delayed one interval in initiating first cleavage. In clone 4, one postneurula derived from a tetraploid donor nucleus was delayed in cleavage and was an octoploid.

MARIE A. DIBERARDINO
NANCY J. HOFFNER
Department of Physiology and Biochemistry, Medical College of Pennsylvania, Philadelphia 19129

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