Ile-tRNA synthetase (*19*). This is the longest synthetase polypeptide sequenced and, together with the lack of repeats for the other sequenced synthetases, suggests that gene duplications did not play a role in the evolution of these enzymes.

The amino acid sequence homology between Ile- and Met-tRNA synthetases is remarkable in view of the lack of homology between other synthetases. Wetzel pointed out that, based on several correlations, Ile- and Met-tRNA synthetases are part of a small subfamily of synthetases that may have an evolutionary relation that is closer than average (*20*). Sequences of the valine and leucine enzymes are of special interest in this regard.

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24. Supported by grant GM15539 from the National Institutes of Health and by the Medical Research Council of Canada (G.A.M.). T.W. was an American Cancer Society postdoctoral fellow (1981–1983). We thank H. Wu for helpful conversations.

5 June 1984; accepted 11 October 1984

Inability of Mouse Blastomere Nuclei Transferred to Enucleated Zygotes to Support Development in Vitro

Abstract. *More than 90 percent of enucleated one-cell mouse embryos receiving pronuclei from other one-cell embryos successfully develop to the blastocyst stage* in vitro. *In this investigation, nuclei from successive preimplantation cleavage stages were introduced into enucleated one-cell embryos and the embryos were tested for development* in vitro. *Although two-cell nuclei supported development to the morula or blastocyst stage, four-cell, eight-cell, and inner cell mass cell nuclei did not. The inability of cell nuclei from these stages to support development reflects rapid loss of totipotency of the transferred nucleus and is not the result of simultaneous transfer of membrane or cytoplasm.*

Ever since it was found that transplantation of blastula nuclei to enucleated *Rana pipiens* zygotes can result in normal embryogenesis (*1*), the ability of nuclei from various sources to support development has been a subject of investigation. Thus, when it was shown that endodermal nuclei support the development of fertile adults from ultraviolet-irradiated *Xenopus laevis* eggs (*2*), it was concluded that early embryonic nuclei remain unrestricted in their developmental potential in amphibians. The developmental potential of nuclei from later stage embryos and adults is less certain. Although several investigators have reported characteristic developmental abnormalities associated with transplantation of nuclei from differentiated tissues into *R. pipiens* zygotes (*3*), some nuclei from tadpole intestinal epithelium retain the ability to support complete development (*4*). Thus it remains unclear whether embryonic development and differentiation are accompanied by a restriction in nuclear potential to support normal development (*5*).

More recently, this restriction in mammals has become amenable to experimental analysis. It has been reported (*6*)

Table 1. Development of mouse embryos with transplanted nuclei. Embryos were obtained from spontaneous inter se matings of ICR (Swiss albino) or C57BL6/J mice. Embryo isolation and culture in vitro and the isolation of single ICM cells from immunosurgically obtained ICM's (*14*) were as previously described (*8*), as were enucleation and nuclear transfer. One-cell embryos were incubated in Whitten medium (*15*) containing cytochalasin B (5 μg/ml; Sigma) and demecolcine (0.1 μg/ml; Sigma) for 15 to 30 minutes before microsurgery. Enucleation of both pronuclei was performed and karyoplasts obtained from one-, two-, four-, or eight-cell embryos also exposed to enucleation medium were fused with enucleated one-cell embryos by using inactivated Sendai virus. Embryos having ICM cell nuclei were obtained by similarly fusing single ICM cells with enucleated one-cell embryos. All manipulations were performed at room temperature with a Leitz hanging-drop oil chamber. Embryos that underwent fusion were washed and cultured for 5 days in drops of Whitten medium containing 100 μ*M* disodium EDTA (*16*) under silicone oil in an atmosphere of 5 percent O_2, 5 percent CO_2, and 90 percent N_2. The proportion of embryos surviving the removal of both pronuclei was 99 percent (481 of 486).

Nuclear donor	Recipient	Number of embryos fused	Development			Percent*
			Submorula	Morula	Blastocyst	
Zygote	Enucleated zygote	21 of 21	1	0	20	95
Two-cell stage	Enucleated zygote	151 of 174	123	9	19	19
Four-cell stage	Enucleated zygote	81 of 84	77	4	0	5
Eight-cell stage	Enucleated zygote	111 of 116	111	0	0	0
ICM	Enucleated zygote	84 of 101	84	0	0	0
None	Nonenucleated zygote†		27	22	182	88
None	Nonenucleated zygote‡		1	0	11	92

*Percentage of embryos developing to morula or blastocyst stage. †Nonenucleated (control) zygotes were used in parallel with each series of experiments. ‡Nonenucleated (control) zygotes exposed to enucleation medium and Sendai virus.

that nuclei from inner cell mass (ICM) cells injected into fertilized one-cell mouse embryos from which pronuclei are subsequently removed can support development through the preimplantation stages, whereas nuclei from trophectoderm cannot. Results consistent with a divergence in the developmental potential of ICM and trophectoderm nuclei were also obtained by transferring nuclei into intact one-cell embryos: zygotes receiving ICM cell nuclei developed into tetraploid blastocysts, whereas those receiving trophectoderm nuclei did not (*7*). In addition, small numbers of enucleated zygotes injected with ICM nuclei developed to term (*6*).

Using our newly developed nuclear transfer procedure, which relies on karyoplast fusion rather than direct microsurgical injection (*8*), we introduced nuclear karyoplasts from one-, two-, four-, and eight-cell embryos and ICM cells into enucleated one-cell embryos and monitored the resultant embryos for development in vitro. As previously reported (*8*), nearly all enucleated zygotes that fused with zygote karyoplasts developed to blastocysts in vitro: however, the rate of successful development decreased sharply when nuclei from subsequent developmental stages were transferred. Only 19 percent of enucleated zygotes containing two-cell nuclei developed to the morula or blastocyst stage (Table 1). Of 81 enucleated embryos receiving four-cell nuclei, only four developed beyond the two-cell stage. These four embryos formed abnormal morulae that did not undergo organized cavitation. Only 1 of 111 enucleated embryos receiving eight-cell nuclei and 1 of 84 embryos receiving ICM nuclei developed beyond the two-cell stage. Both embryos were arrested at the three- or four-cell stage. Approximately 90 percent of the control embryos, with or without exposure to enucleation media and Sendai virus to determine any harmful effects of the various media or of exposure to room temperature, developed to the morula or blastocyst stage (Table 1).

Table 2. Development of nonenucleated zygotes to which nuclei or cytoplasm from embryos of different developmental stages were transferred. The introduction of nuclei into nonenucleated one-cell embryos was performed as described in the legend to Table 1. Similarly, membrane-bound cytoplasmic vesicles 15 to 20 μm in diameter, obtained from successive preimplantation-stage embryos, were fused with nonenucleated zygotes in a manner identical to that used for karyoplast fusion.

Donor stage	Recipient	Development			Percent*
		Sub-morula	Morula	Blastocyst	
		Nuclear donor			
Two-cell	Zygote	7	4	23	79
Four-cell	Zygote	7	1	5	46
Eight-cell	Zygote	22	2	5	24
ICM	Zygote	11	4	1	31
		Cytoplasm donor			
Two-cell	Zygote	2	1	5	75
Four-cell	Zygote	0	0	7	100
Eight-cell	Zygote	2	2	14	88
No donor	Zygote†	3	2	40	93

*Percentage of embryos developing to morula or blastocyst stage. †Nonenucleated (control) zygotes were used in parallel with each series of experiments.

To determine whether the effect on development was due to the transferred nucleus itself or whether the transferred cytoplasm also plays a role in reducing developmental potential, karyoplasts and cytoplasts from different embryonic stages were fused with nonenucleated zygotes. Seventy-five and 50 percent of the embryos that fused with karyoplasts from two-cell and four-cell stages, respectively, developed to the morula or blastocyst stage, whereas less than one-third of the embryos that fused with karyoplasts from older embryos developed normally (Table 2). Nearly all zygotes that fused with cytoplasts developed to blastocysts, as did the controls (Table 2). It appears that neither the cytoplasm nor the fusion process per se affects the developmental potential of the zygote, although the presence of nuclei from older embryos is inhibitory.

To determine whether nuclei from later stages are more susceptible to injury during transfer, we microsurgically removed both nuclei from two-cell embryos and introduced a single two-cell nucleus into each enucleated embryo. Of 22 such embryos, all incorporated the donor nucleus and 18 developed to the blastocyst stage (although, in approximately half the embryos, the two blastomeres remained unfused and thus only half the embryo cytoplasm participated in blastocyst formation). These results indicate that later stage nuclei are not

Table 3. Development of mouse embryos derived from enucleated and nonenucleated zygotes into which ICM nuclei were mechanically injected. Dissociated ICM cells were placed in hanging drops alongside nonenucleated one-cell embryos in Hepes-buffered Whitten medium without cytochalasin B and demecolcine. A single ICM cell was aspirated into an injection pipette with an outside diameter of 8 μm—sufficiently small to disrupt the cytoplasmic membrane but large enough to retain the integrity of the nuclear membrane (*6*). The pipette containing the ICM cell nucleus was advanced through the zona pellucida of a nonenucleated one-cell embryo but remained in the perivitelline space, indenting the cytoplasmic membrane. The egg membrane was penetrated by aspirating membrane and cytoplasm into the pipette. The loss of integrity of the cytoplasmic membrane, which could be seen inside the micropipette, was followed by sequential injection of the aspirated cytoplasmic contents and then the ICM cell nucleus. The pipette was carefully withdrawn and the surviving embryos were returned to the incubator. One to 6 hours after introduction of the nucleus, surviving embryos were placed into Whitten medium containing cytochalasin B (5 μg/ml) and demecolcine (0.1 μg/ml) for 15 to 45 minutes. The embryo was subsequently placed in a hanging drop of Hepes-buffered Whitten medium with cytochalasin B and demecolcine, a 12- to 15-μm enucleation pipette was advanced through the zona pellucida at the previous site of penetration, and the male and female pronuclei were removed (*8*). Surviving embryos were washed and cultured for 5 days in vitro. The plasma membranes of control embryos, which were injected with medium, were penetrated as described above and a small volume of Whitten medium was injected into the cytoplasm. A second group of control embryos was similarly injected with medium and incubated in enucleation medium. Membrane-bound embryo cytoplasm was then removed in an amount equal to or greater than that typically removed during enucleation.

Procedure	Number of embryos surviving	Development			Percent*
		Sub-morula	Morula	Blastocyst	
Nuclear injection and enucleation	72 of 231	72	0	0	0
Nuclear injection without enucleation	65 of 161	52	7	6	20
Medium injection without enucleation	44 of 124	2	6	36	95
Medium injection and cytoplasm removal	10 of 24	0	1	9	100
Unmanipulated controls		3	2	23	89

*Percentage of embryos developing to morula or blastocyst stage.

more susceptible to injury, but rather require a cytoplasmic environment consistent with their nuclear function.

Our results, which indicate that the potential of embryonic nuclei to support preimplantation development becomes restricted at the early cleavage divisions, contrast with the results of Illmensee and Hoppe (*6*). To eliminate the possibility that the karyoplast fusion technique itself, as opposed to mechanical injection of nuclei, underlies this discrepancy, we mechanically injected ICM cell nuclei into zygotes, some of which were subsequently enucleated (Table 3). Approximately one-third of the embryos survived the injection of ICM nuclei and enucleation; 40 percent of these embryos divided once and a few divided twice, but no embryo developed beyond the four-cell stage. This procedure itself is not harmful to development, since embryos injected with medium and then subjected to removal of cytoplasm developed at the same rate as control (unmanipulated) embryos (Table 3). Thus the results of karyoplast fusion or mechanical injection indicate that ICM cell nuclei are unable to support development of enucleated zygotes and that they reduce the development of nonenucleated zygotes into which they are introduced.

The only remaining technical explanation for the discrepancy between our results and those of Illmensee and Hoppe (*6*) might lie in the enucleation method. We completely enucleated zygotes by removing intact pronuclei, whereas Illmensee and Hoppe achieved enucleation by the breakdown of pronuclei; persistence of pronuclei remnants in the embryos (*9*) may have allowed random functioning segments of the host genome to persist. Thus, only those embryos in which the proper qualitative and quantitative contribution of the host genome is serendipitously achieved would develop, explaining the low yield of embryos completing development (*6*).

It is now possible to address the question of why nuclei from early mouse embryos are unable to support development while nuclei from much older amphibian embryos are able to do so. The most reasonable explanation probably lies in the basic difference between development of mammalian and nonmammalian embryos. Embryonic development of nonmammalian species is, for a considerable length of time, independent of the transcriptional activity of the genome (*10*), while interference with transcription results in immediate arrest of mammalian development (*11*). Moreover, it is now clear that the embryonic genome in the mouse becomes active early in the two-cell stage, or even in the zygote, and continues stage-specific activity during development before implantation (*12*). Therefore, reprogramming after transfer into the zygote is impossible in the mammalian embryo, either inherently or because of lack of time, whereas the amphibian nucleus probably has sufficient time to reprogram. In addition, it seems that the concomitant presence of two unsynchronized, active genomes also inhibits development and that the magnitude of this effect depends on the developmental time interval separating the host and donor nuclei. Recent results indicating that both the male and female pronuclei must be present to ensure normal development (*13*) suggest that the maternal and paternal genomes act differently in early development. If such differential activity is essential, any transferred nucleus would have to be able to reprogram male- and female-specific genomic activity before normal development could proceed. It is very unlikely that such precise reprogramming, which might necessitate selective inactivation of various parts of the genome, can be achieved in a nuclear transfer experiment. Differential activity of maternal and paternal genomes (*13*), and the results presented here, suggest that the cloning of mammals by simple nuclear transfer is biologically impossible.

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17. This work was supported by grants CA-10815 and CA-25875 from the National Cancer Institute and by grants HD-12487 and HD-17720 from the National Institute of Child Health and Human Development. The thoughtful comments of Dr. B. B. Knowles are gratefully acknowledged.

25 July 1984; accepted 7 September 1984

RNA Required for Import of Precursor Proteins into Mitochondria

Abstract. *A cytoplasmic RNA moiety is necessary for posttranslational uptake of nuclear-encoded mammalian proteins destined for the mitochondrial matrix. Posttranslational addition of ribonuclease to a reticulocyte lysate-programmed cell-free translation mixture inhibited subsequent import of six different mitochondrial matrix enzyme precursors into rat liver mitochondria. The required RNA is highly protected, as indicated by the high concentrations of ribonuclease necessary to produce this inhibition. The dependence of the inhibitory effect on temperature, duration of exposure to ribonuclease, and availability of divalent cations is characteristic of the nuclease susceptibility of ribonucleoproteins. The ribonuclease-sensitive component was found in a 400-kilodalton fraction which contains the mitochondrial protein precursors.*

Most mitochondrial proteins are nuclear encoded, synthesized in the cytoplasm as precursors containing an amino-terminal peptide extension, and subsequently imported into the organelle. While many of the general features of the import process have been elucidated (*1, 2*), little is known concerning the mechanism by which proteins are specifically targeted to the mitochondrial outer membrane. These proteins are synthesized on membrane-free polyribosomes, rapidly transported to mitochondria (with a typical cytoplasmic half-life of 1 to 2 minutes), and posttranslationally imported (*1, 2*). Soluble cytoplasmic factors in a reticulocyte lysate are required for import of precursors into mammalian (*3, 4*) or yeast (*5*) mitochondria. However, the step in the import pathway at which