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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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

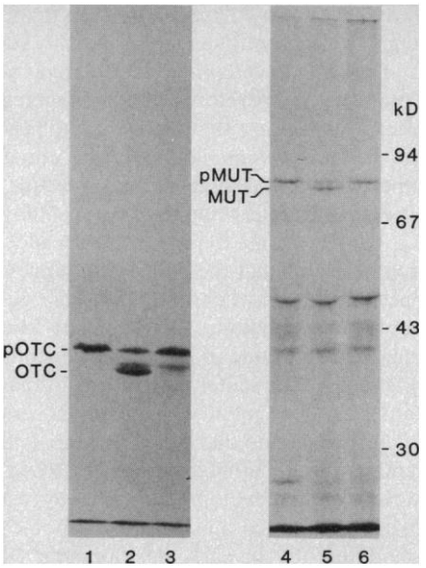

Fig. 1. Inhibition by RNase of conversion of mitochondrial protein precursors to mature forms. The assay for mitochondrial import and processing of precursor polypeptides has been described (*19*). Total polyadenylated [poly(A)$^+$] mRNA was isolated from rat liver and translated in vitro in a rabbit reticulocyte lysate system (Bethesda Research Laboratories) containing [^{35}S]methionine, after which protein synthesis was halted by addition of cycloheximide (to a final concentration of 30 μg/ml). The translation mixture was then incubated at 37°C for 10 minutes in the presence or absence of RNase A (Sigma type III-A, 100 μg/ml). To assay for posttranslational protein import into mitochondria, translation mixture was incubated with an equal volume of intact rat liver mitochondria (4 mg of protein per milliliter) in a buffer consisting of 2 m*M* Hepes, *p*H 7.4; 220 m*M* mannitol, 70 m*M* sucrose, 4 m*M* EGTA, 4 m*M* $MgCl_2$, 4 m*M* ADP, and 20 m*M* glutamate, for 60 minutes at 27°C. Analysis of labeled products (*9, 19*) consisted of immunoprecipitation with antiserum to OTC (lanes 1 to 3), or with antiserum to MUT (lanes 4 to 6), and identification by fluorography after electrophoresis on gels of polyacrylamide (9 percent) in the presence of sodium dodecyl sulfate. Lanes 1 and 4, translation mixture (carried through mock incubation without added RNase or mitochondria); lanes 2 and 5, control import (translation mixture incubated without RNase and with added mitochondria); lanes 3 and 6, RNase-treated (translation mixture treated with RNase and incubated with added mitochondria).

these factors are required is unknown. Several newly synthesized precursors appear as complexes much larger than their monomeric forms in chromatographic studies (*2, 6*). It is possible that this reflects the interaction of precursors with a cytoplasmic recognition element. Targeting of secreted, lysosomal, and membrane proteins is mediated by an RNA-containing signal recognition particle, which binds amino-terminal signal sequences and delivers polysomes synthesizing these proteins to the endoplasmic reticulum (*7*). The possibility that a similar particle might function in recognition and targeting of newly synthesized proteins destined for mitochondria is the subject of this report.

As demonstrated previously (*8–10*), the mitochondrial matrix enzymes ornithine transcarbamylase (OTC) and methylmalonyl CoA mutase (MUT) are synthesized in vitro as precursor polypeptides pOTC (Fig. 1, lane 1) and pMUT (Fig. 1, lane 4) which are imported and processed (Fig. 1, lanes 2 and 5) on incubation with mitochondria isolated from rat liver. To determine if a cytoplasmic RNA component was involved in this import pathway we treated the reticulocyte lysate translation mixture with ribonuclease (RNase). Treatment with RNase A (100 μg/ml) resulted in a nearly complete inhibition of conversion of each precursor to its mature counterpart (Fig. 1, lanes 3 and 6). Similar results were obtained with the precursor subunits of four other matrix enzymes: medium chain acyl-CoA dehydrogenase, long chain acyl-CoA dehydrogenase, isovaleryl-CoA dehydrogenase (*11*), and the α and β subunits of propionyl CoA carboxylase. The nature of the intermediate-sized protein seen in OTC processing reactions (Fig. 1, lanes 2 and 3) is unclear at present and has been discussed elsewhere (*3, 9, 12*).

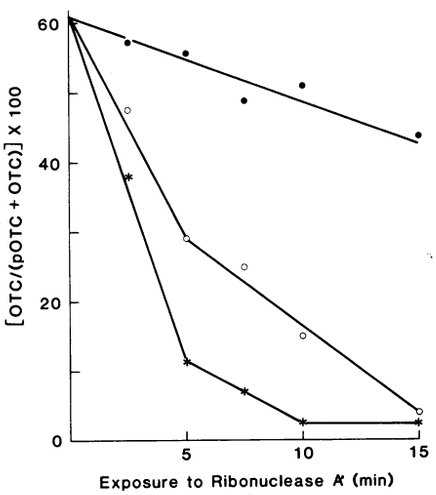

Fig. 2. Titration of RNase inhibition of mitochondrial protein import. Translation mixture (see Fig. 1) was incubated at 37°C with 0 μg/ml (●), 10 μg/ml (○), and 100 μg/ml (*), respectively, of RNase A. At the times indicated, portions (15 μl) were removed and used in mitochondrial protein import assays (see legend to Fig. 1). The bands corresponding to pOTC and OTC were quantified by densitometric scanning. The amount of the intermediate OTC species was not affected by either RNase treatment or period of incubation at 37°C and was ignored in calculation of quantitative conversion.

To characterize this inhibitory effect further, we used the conversion of pOTC to OTC as the assay for import, and RNase A (*13*) as inhibitor. The inhibitory effect was dependent on both time of incubation at 37°C and concentration of RNase (Fig. 2). Whereas incubation of the translation mixture alone at 37°C led to a slow decline in fractional conversion of pOTC to OTC, the decline was more rapid and the final amount of pOTC converted to OTC was lower in the presence of RNase. An essentially complete inhibition was achieved by incubation with RNase A (100 μg/ml) for 10 minutes at 37°C. Unless otherwise stated, this was the standard RNase treatment employed in subsequent experiments. This RNase concentration (5000 unit/ml) is high when compared to those typically used to deplete endogenous messenger RNA (mRNA) from translation systems (nuclease for 5 minutes, 20 unit/ml, at 25°C), but is comparable to those needed to attack an RNA protected by association with proteins (*7, 14*).

Import of pOTC into mitochondria was not inhibited when samples were treated with RNase at 27°C (Fig. 3A, lane 4). Many ribonucleoprotein structures are stabilized by Mg^{2+} and chelation of the divalent cation has been shown to produce unfolding of these particles, making them more susceptible to nucleolytic attack (*15*). Inhibition of pOTC import by RNase was observed at 27°C when divalent cations were removed by addition of EDTA (Fig. 3A, lane 5). Thus, the RNase-sensitive component required for mitochondrial protein import exhibits characteristics of an RNA bound in a ribonucleoprotein complex.

In order to determine whether the inhibitory effect was specific to RNase A (which cleaves at pyrimidine nucleotides) we also employed RNase T_1 (which cleaves at guanine residues) (Fig. 3B). Under experimental conditions in which RNase A was markedly inhibitory (Fig. 3B, lane 1), RNase T_1 exerted little effect (Fig. 3B, lane 2). When RNase T_1 (5000 unit/ml) was added in the presence of EDTA (Fig. 3B, lane 3), or when the RNase T_1 concentration was increased to 100,000 unit/ml (Fig. 3B, lane 5), however, marked inhibition of import was noted.

Under standard conditions, in vitro import and processing of OTC by mitochondria led to a loss of pOTC from the extramitochondrial supernatant (Fig. 3C, lane 4) with a concomitant increase of mature OTC sedimenting with the mitochondrial fraction (Fig. 3C, lane 5). After incubation of translation mixture with

RNase A, however, most of the immunoprecipitable OTC was found in the supernatant (Fig. 3C, lane 2); very little sedimented with mitochondria (Fig. 3C, lane 3). The mitochondria did not contain significant amounts of pOTC either bound to the outer membrane but not translocated [as can occur when mitochondrial uncouplers are used to dissipate the electrochemical potential across the mitochondrial inner membrane (*16*)] or translocated but not cleaved to OTC [as can occur when *o*-phenanthroline is used to inhibit the matrix protease (*17*)]. These findings indicate that RNase is acting to impair the binding of precursors to mitochondria, rather than interfering with such mitochondrial events as transmembrane translocation or proteolytic processing.

We have considered two explanations that would render these in vitro findings trivial or artifactual. The first possible explanation, that a contaminating protease (rather than RNase itself) is interfering with import, is refuted by three observations. (i) Five different preparations of RNase (*18*), including a preparation which was boiled for 30 minutes, were inhibitory; (ii) detailed comparison of [^{35}S]methionine-labeled polypeptides from RNase-treated and control translation mixtures revealed identical electrophoretic patterns; and (iii) import of pOTC was not inhibited when RNase treatment was performed in the presence of a human placental inhibitor of RNase (RNAsin; Promega Biotec). The second possible explanation, that RNase is acting directly or indirectly against mitochondria (rather than against a component of the translation mixture), was excluded by the results of experiments that depended on our finding that the inhibitory effect of RNase is observed at 37°C but not at 27°C. When labeled translation mixture was treated with RNase for 10 minutes at 37°C, brought to 27°C, and mixed with an equal volume of unlabeled translation mixture, subsequent mitochondrial import of labeled pOTC was markedly inhibited (Fig. 3D, lane 2). Import was unaffected when the experiment was reversed (RNase treatment of unlabeled translation mixture was followed by mixing at 27°C with labeled translation mixture) (Fig. 3D, lane 1). If RNase treatment was generating a mitochondrial poison, import should have been impaired in both mixing experiments. Another set of mixing experiments demonstrated that the RNA required for import of precursor proteins into mitochondria is a component of the reticulocyte lysate. When fresh reticulocyte lysate was added to an RNase-treated translation mixture, restoration of OTC import was observed (Fig. 3D, lane 3). When this lysate was treated with RNase, however, no restoration of precursor import was noted (Fig. 3D, lane 4).

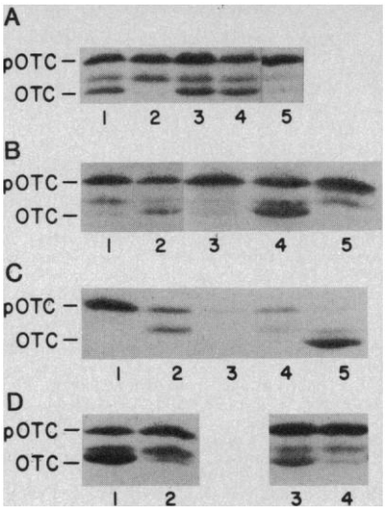

Fig. 3. RNase inhibition of mitochondrial import of precursor proteins. Only the OTC region of the gel is shown. (A) Effect of temperature and EDTA. Lane 1, control import; lane 2, RNase treatment at 37°C; lane 3, control import (mock incubation without RNase carried out at 27°C); lane 4, RNase treatment at 27°C; lane 5, EDTA and RNase treatment at 27°C (translation mixture brought to 5 m*M* EDTA prior to addition of RNase). (B) Effect of RNase T_1. Treatment with: lane 1, RNase A (100 μg/ml, 5000 unit/ml); lane 2, RNase T_1 (5000 unit/ml, Bethesda Research Laboratories); lane 3, 5 m*M* EDTA and RNase T_1 (5000 unit/ml); lane 4, 5 m*M* EDTA; lane 5, RNase T_1 (100,000 unit/ml). (C) Isolation of mitochondrial and extramitochondrial fractions. Lane 1, translation mixture. After mitochondrial protein import assay, samples were separated by centrifugation into mitochondrial pellets and extramitochondrial supernatants. Lane 2, RNase-treated supernatant; lane 3, RNase-treated mitochondrial fraction; lane 4, control supernatant; lane 5, control mitochondrial fraction. (D) Mixing experiments. Portions of translation products of rat liver total poly(A)$^+$ mRNA were incubated at 37°C for 10 minutes in the presence or absence of RNase, brought to 27°C, and used in mitochondrial import assays. Lane 1, [^{35}S]methionine-labeled translation mixture (15 μl) and RNase-treated unlabeled translation mixture (15 μl); lane 2, RNase-treated labeled translation mixture (15 μl) and unlabeled translation mixture (15 μl). In lanes 3 and 4, RNase-treated labeled translation mixture (15 μl) was supplemented with either 5 μl of untranslated rabbit reticulocyte lysate (lane 3) or 5 μl of RNase-treated, untranslated reticulocyte lysate (lane 4).

To define further the physical nature of the RNase-sensitive components, the labeled translation mixture was fractionated (Bio-Gel A-1.5m gel filtration column). The pOTC chromatographs as a complex, with a mass of ~400 kilodaltons (kD) and not as a monomer of 40 kD (Fig. 4A). The precursors of MUT and of the β subunit of another mitochondrial matrix enzyme, propionyl CoA carboxylase, whose monomer sizes are ~81 and ~62 kD, respectively, were also found to chromatograph as complexes of ~400 kD (data not shown). The high molecular weight fractions containing labeled pOTC were pooled and used for in vitro import assays. The pOTC from these fractions was imported very inefficiently into mitochondria (Fig. 4B, lane 1). This was expected, in light of

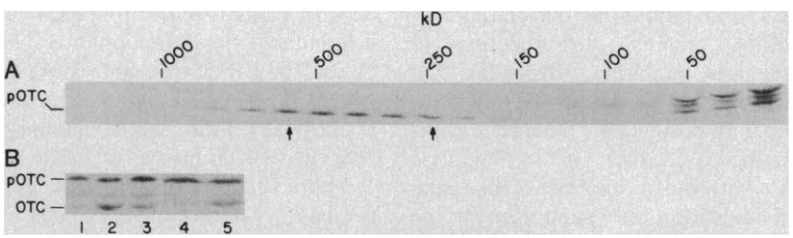

Fig. 4. (A) Gel filtration of in vitro synthesized pOTC. A post-ribosomal supernatant of the labeled cell-free translation mixture (Fig. 1) was prepared by centrifugation for 1 hour at 105,000*g* at 4°C. Portions (0.4 ml) were chromatographed on a Bio-Gel A-1.5m column (1 cm × 46 cm) in 10 m*M* Hepes, *p*H 7.4; 120 m*M* KCl, and 2 m*M* $MgCl_2$. Fractions of 0.55 ml were collected. Every other fraction was subjected to immunoprecipitation and analysis for OTC. Calibration of the column was performed with thyroglobulin, propionyl-CoA carboxylase, catalase, aldolase, and ornithine transcarbamylase as markers. We regard the pattern of bands (larger than pOTC) observed in the low molecular weight region of the column profile as contaminants since they were not consistently observed in fractions immune-precipitated for OTC. (B) RNase-sensitive component of lysate cochromatographs with pOTC. Fractions from Bio-Gel A-1.5m gel filtration containing pOTC [between arrows in (A)] were pooled and concentrated to ~200 μl (with 70 percent recovery of radioactivity) using Centricon 10 microconcentrators (Amicon). This 400-kD material was used in mitochondrial import and processing assays (Fig. 1). Lane 1, 400-kD pOTC (20 μl); lane 2, 400-kD pOTC (20 μl) and reticulocyte lysate (10 μl); lane 3, 400-kD pOTC (20 μl) and RNase-treated reticulocyte lysate (10 μl); lane 4, 400-kD pOTC (20 μl) treated with 5 m*M* EDTA and RNase for 10 minutes at 27°C and 10 μl of reticulocyte lysate treated with agarose-immobilized RNase and made RNase-free by centrifugation and removal of RNase; lane 5, as in lane 4 except that the 400-kD pOTC was incubated in the absence of RNase.

recent findings that low molecular weight factors, probably proteins, are required for import of precursor proteins into mitochondria (*3–5*). Addition of unlabeled reticulocyte lysate to the pOTC-containing fractions substantially increased the import of OTC (Fig. 4B, lane 2). This stimulating activity of added lysate was abolished by prior treatment with trypsin (data not shown). Significantly, prior treatment of the added lysate with RNase did not abolish its import-stimulating activity (Fig. 4B, lane 3). Thus, while the 400-kD pOTC-containing fraction requires one or more additional protein factors for efficient import, it contains the RNase-sensitive component. Consistent with this interpretation is the finding that RNase treatment of both the precursor-containing 400-kD fraction and the added lysate was required to impair mitochondrial import of the fractionated material (Fig. 4B, lane 4). We conclude that the RNase-sensitive component either comigrates with or is part of the high molecular weight pOTC complex.

The results presented demonstrate that, in our cell-free reconstitution system, the posttranslational import of several mitochondrial matrix enzyme precursors by mitochondria is susceptible to inhibition by RNase. The mixing experiments (Fig. 3D) and the column fractionation experiments (Fig. 4) suggest that the RNase is not trivially generating a mitochondrial poison, but rather is acting on some as yet unidentified RNA component of the cytoplasm which copurifies with matrix enzyme precursors. Concentrations of RNase higher than those needed to degrade unprotected RNA's, and conditions that unfold protein-RNA complexes, are necessary to demonstrate inhibition, suggesting that the RNA involved must be tightly complexed with protein. We have been characterizing a subset of reticulocyte RNA's with the appropriate RNase susceptibilities, and are encouraged by the fact that 7*S*L RNA, known to be a necessary component of a cytoplasmic ribonucleoprotein (*7*), is one of these RNA species. Continuing studies with increasingly purified import systems should help elucidate the role of this putative ribonucleoprotein in the pathway of mitochondrial biogenesis.

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Persistence of the Entire Epstein-Barr Virus Genome Integrated into Human Lymphocyte DNA

Abstract. *The entire Epstein-Barr virus genome is integrated into Burkitt tumor cell DNA at the terminal direct repeat sequence of the virus. There is no homology between the GC-rich (G, guanine; C, cytosine) terminal repeat and the AT-rich (A, adenine; T, thymine) cell sequences with which it has recombined. More than 15 kilobases of cell DNA have been deleted and 236 base pairs are duplicated at one virus-cell junction site.*

Most of the adult population is infected with Epstein-Barr virus (EBV) which is latent in a small fraction of B lymphocytes. The EBV causes infectious mononucleosis and is thought to be an etiologic agent of Burkitt lymphoma and nasopharyngeal carcinoma (*1*). The entire 170-kbp (kilobase pair) virus genome persists in latently infected cells (*2, 3*). Frequently, such cells have many EBV episomes (*4*), which are formed by covalent joining of the terminal direct repeats at both ends of the linear virion DNA (*5*). Analysis of cells for integrated EBV DNA is complicated by the presence of these episomes. Attempts to demonstrate covalent linkage of viral and cell DNA by isopycnic centrifugation (*6, 7*) and cell hybridization (*8*) have yielded conflicting data. In situ hybridizations have demonstrated that most or all of the EBV genome is linked to chromosomal sites 4q25 of the IB4 cell line and 1p35 of the Namalwa cell line (*9*). Namalwa is a latently infected African Burkitt tumor cell line and contains only one copy of EBV DNA (*9*). The IB4 cell line, established by infection and transformation of normal fetal lymphocytes with EBV, contains several episomal copies of EBV DNA in addition to the integrated copy (*9, 10*). We now describe the organization of the integrated EBV DNA in Namalwa and IB4 cells and the nucleotide sequence of the Namalwa integration sites.

To investigate the organization of EBV DNA in Namalwa cells, cloned Eco RI fragments representing more than 90 percent of the EBV genome (Fig. 1) were hybridized to Eco RI fragments of Namalwa DNA. Most probes hybridized to a single Namalwa cell Eco RI fragment of the appropriate size for an EBV DNA fragment. Thus, the organization of these fragments appears to be similar to that of linear viral DNA and these fragments are unlikely to have recombined with cell DNA. The EBV Eco RI D fragment consists of the joined