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Enucleation and reconstruction of interferon-producing cells

(L cells/Sendai virus)

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ABSTRACT **Enucleation of L cells leads to loss of the capacity to produce interferon, showing that the cell nucleus is essential for interferon formation. However, when the cells were enucleated while interferon formation was proceeding, the cytoplasts were capable of continuing to synthesize interferon by a process shown to be protein synthesis, showing that the interferon messenger RNA leaves the nucleus after synthesis. Reconstructed cells were obtained by Sendai virus fusion of karyoplasts and cytoplasts. Such reconstructed cells were capable of producing at least as much interferon (43 interferon units/10^4 nucleated cells) as control cells (31 interferon units/10^4 nucleated cells).**

Nearly all mammalian and avian cells produce interferon in response to viruses or double-stranded polyribonucleotides. The process, which is only partially understood, involves activation of a cellular interferon gene, followed by transcription and translation of the mRNA. The scheme, initially formulated as a result of work with metabolic inhibitors (1), has recently been strengthened by two findings: (*i*) that specific chromosomes are essential for interferon production, presumably because they carry the structural gene for interferon (2, 3), and (*ii*) that the interferon mRNA, present in induced cells, can be translated in heterologous tissue culture cells, *Xenopus* oocytes, and cell-free systems (4–7). Little is known about the detailed mechanisms involved, particularly the way in which treatment with virus or polyribonucleotide activates the cellular gene system for interferon. Nor is it known how the process is modulated by priming with interferon (8), by superinduction with metabolic inhibitors (9), or by repeated treatment of the cells with inducer—the hyporesponsive process (10). All these processes are likely to involve subtle nuclear–cytoplasmic interactions, and the enucleation technique using centrifugation in the presence of cytochalasin B (CB) (11) provides a powerful tool for investigating the roles of nucleus and cytoplasm in the control of cellular processes. In particular, it is now possible to reconstruct viable cells by fusing cytoplasts (enucleated cells) to karyoplasts (nucleated cellular fragments) with Sendai virus (12), the cell fragments being derived from the identical or from genetically distinguishable cell parents (13). We have used the techniques of cell enucleation and reconstruction to study the role of the nucleus during interferon formation and to prepare reconstructed cells capable of making interferon.

MATERIALS AND METHODS

Cells and Viruses. Mouse L cells were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum (FCS). They were shown to be mycoplasma-free by the uridine phosphorylase method (14). Newcastle disease virus (NDV) (strain B1) was received from T. C. Merigan, Division of Infectious Diseases, Stanford University, and was grown in fertile hen eggs. Ultraviolet-irradiated NDV (UV-NDV) was prepared by irradiating 1-ml aliquots of virus with 720 ergs (72 μJ)/mm^2 from a General Electric germicidal lamp (G30TS) after overnight dialysis of the virus against phosphate-buffered saline. Infectivity, as measured by plaque assay in primary chick embryo cells (15), fell from 1.1×10^9 plaque-forming units (PFU)/ml to 7×10^4 PFU/ml as a result of this treatment. NDV (strain La Sota), received from G. Poste, Roswell Park Memorial Institute, Buffalo, NY, and Sendai virus, received from D. Kingsbury, St. Jude Children's Research Hospital, Memphis, TN, were also grown in fertile hen eggs. These viruses were inactivated by ultraviolet light as described above except that the Sendai virus received 7200 ergs (720 μJ)/mm^2. Since neither of these viruses formed plaques in chick or L cells, they were titrated by a hemagglutination assay using serial 1:2 dilutions of virus with an equal volume of 0.5% chick embryo red cells. Semliki Forest virus was grown in chick embryo cells in suspension as described (16).

Preparation and Assay of Interferon. Interferon was induced either with NDV or UV-NDV by treatment of L cells with virus for 1 hr at 37° followed by washing with minimum essential medium plus 2% FCS and incubation in the same medium at 37° before the fluids were harvested 24 hr later.

Samples for interferon assay were dialyzed for 5 days against a pH 2 buffer to destroy virus before overnight dialysis against Earle's buffered salt solution and assay.

Interferon was assayed by a micromodification of the viral RNA reduction assay (17) in which serial 0.5 $\log_{10}$ dilutions of interferon were incubated overnight with a monolayer of 10^5 L cells in small glass vials before challenge with Semliki Forest virus (1×10^7 PFU) in the presence of actinomycin D (1 μg/ml) and addition of [^{3}H]uridine (final, 1 μCi/ml) 2 hr later. Incorporation of isotope was terminated 3 hr later by withdrawal of fluids, washing with ice-cold 0.9% NaCl, ice-cold 5% trichloroacetic acid (twice), and 95% EtOH. The dried cell sheets were dissolved in 100 μl of Soluene (Packard Instrument Co. Ltd.,) (diluted 1:3 with toluene) before addition of 2 ml of scintillation fluid and measurement of radioactivity. The interferon titer was defined as the reciprocal of the dilution causing a 50% depression in virus RNA synthesis. The results are expressed in international reference research units; all assays contained an internal standard.

Cell Enucleation and Reconstruction. Cells were enu-

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Abbreviations: FCS, fetal calf serum; CB, cytochalasin B; NDV, Newcastle disease virus; UV-NDV, ultraviolet-inactivated Newcastle disease virus; PFU, plaque-forming unit; HAU, hemagglutinating unit.

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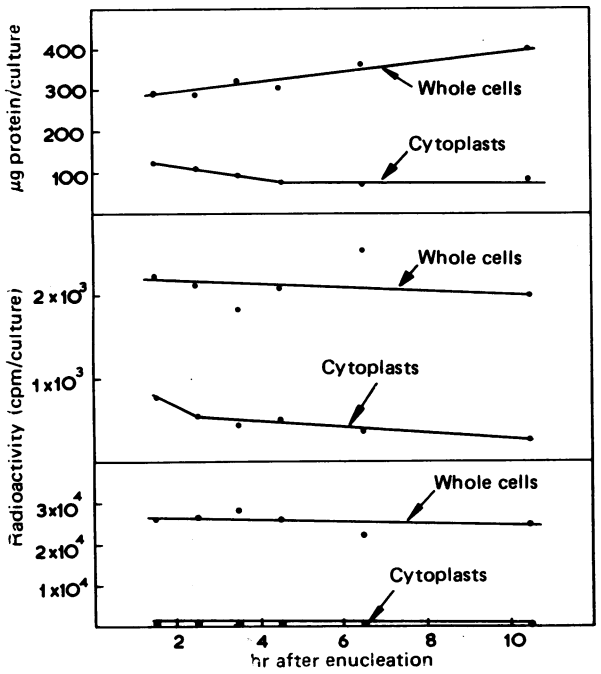

FIG. 1. Total protein content (*Top*) and radioactive incorporation of [^{3}H]leucine (*Middle*) and [^{14}C]uridine (*Bottom*) into acid-insoluble material of whole cells and cytoplasts at different times after enucleation. L cells (8 × 10^5/25-cm^2 flask) were pulsed for 1 hr with a mixture of isotopes and then processed as described in *Materials and Methods*. The radioactive incorporation into cytoplasts has been corrected for any loss in cell numbers during enucleation.

cleated in 25-cm^2 flasks by method A described by Veomett *et al.* (18). They were reconstructed by adding excess karyoplasts to a monolayer of cytoplasts and using Sendai virus to cause fusion. The karyoplast pellet in each 25-cm^2 flask was carefully resuspended in a small volume of the growth medium containing CB, the pooled karyoplasts were centrifuged at 500 rpm for 7 min, and the pellet was washed twice with balanced salt solution before resuspension in balanced salt solution (0.6 ml per fusion reaction). Meantime, the cytoplast monolayers were allowed to recover from the effects of CB treatment by incubation in minimum essential medium + 10% FCS for 30–45 min at 37°. The medium was removed, ice-cold balanced salt solution was added (2.5 ml per flask), and the flask was put at 4° for 20 min.

The balanced salt solution was then removed and 1.0 ml of UV-inactivated Sendai virus in the salt solution was added [200 hemagglutinating units (HAU)/ml]. The flasks were placed at 4° for a further 20 min. Control flasks were treated with an equal amount of Sendai virus that had been treated with 0.5% β-propiolactone, so that it no longer caused cell fusion. After this, the virus was removed and the monolayers were washed twice with ice-cold balanced salt solution (5 ml per flask) with vigorous shaking of the flask with each wash. The karyoplasts (0.6 ml of the washed suspension containing karyoplasts from 12 flasks) or control fluid (balanced salt solution) was then added and the flasks were rocked and put at 4° for a further 20 min, the flasks being rocked every 5 min. Then 2.2 ml per flask of minimum essential medium plus 10% FCS warmed to 45° was added, and the flasks were incubated at 37° for 45 min. The flasks were then shaken vigorously, the medium was removed, and the monolayers were washed twice with minimum essential medium + 10% FCS at 37° (5 ml per flask) with vigorous shaking during each wash. Finally, 4 ml of medium plus 0.2 ml of NDV (strain La Sota, 2500 HAU/ml) was added and the flasks were incubated overnight at 37°. The fluids were removed, dialyzed at pH 2 for 5 days, and assayed for interferon. The cell sheets were stained with crystal violet, and the percentage of nucleated cells and number of cells per flask were estimated by counting with a microscope at a magnification of ×100.

Incorporation of Radioactive Precursors into Whole Cells and Cytoplasts. Cells, or cytoplasts, were incubated with the precursor for the times stated in the text before removal of medium. They were washed three times with ice-cold 0.9% saline, three times with ice-cold 5% trichloroacetic acid, and once with 95% ethanol. The cell sheets were dissolved in 0.25 M NaOH before neutralization, addition of scintillation fluid, and measurement of radioactivity. The protein content of the different preparations was determined according to Lowry *et al.* (19), with bovine serum albumin as standard.

RESULTS

Cell Enucleation and Characterization of Cytoplasts. Enucleation of L cells by centrifugation in the presence of 10 μg of CB per ml gave 99.6% enucleation (average of four determinations, range 99.4–99.75%), as judged by counting of stained monolayers after enucleation, or 98.1% enucleation, as judged by labeling the cell sheets with [^{3}H]thymidine for 2 hr before enucleation, followed by determination of radioactivity before and after enucleation. Trypsinization and counting of the cells before and after enucleation showed that cell recovery was 91% (average of four determinations, range 87–94%). The synthetic activity of the cytoplasts was measured by comparison of the incorporation of [^{3}H]thymidine, [^{14}C]uridine, and [^{3}H]leucine into acid-insoluble material in cytoplasts and in whole cells. The results (Fig. 1) showed that slightly over half the cell protein was lost during enucleation and that the cytoplasts had only about 25% of the protein synthetic activity of the whole cells, but that this activity was only slightly diminished after 10 hr of incubation in growth medium. The cytoplasts were effectively unable to incorporate [^{14}C]uridine or [^{3}H]thymidine (data not shown). Measurement of the trichloroacetic acid-soluble pools of the cytoplasts showed that they decreased to 68% of controls ([^{3}H]leucine) or to 50% of controls ([^{14}C]uridine). Correction of the protein synthetic activity of the cytoplasts for this reduction in the pool size raised the activity to 37% of the controls.

Production of Interferon in Whole Cells and Cytoplasts. Infection of L cells with NDV (B1 strain) led to limited virus multiplication; the product consisted mainly of noninfective particles, as shown by the lower PFU/HAU ratio ($\log_{10}$ 4.45) of the product compared with that of egg-grown virus ($\log_{10}$ 5.74). Interferon was produced in whole cells in high yield (Fig. 2 *left*), but there was also a virus-mediated cytopathic effect, first visible at 14 hr after infection.

Since we did not wish to study interferon production in cells, or cytoplasts, showing visible cytopathic effects, we used UV-NDV, which produced a slightly lower yield of interferon with very similar kinetics without any virus multiplication (Fig. 2 *right*). When cells were enucleated either immediately before or immediately after infection with NDV or UV-NDV (strain B1), no interferon was formed (Table 1), showing that the nucleus was essential for interferon formation. Enucleation of the cells was responsible for the reduction in interferon yield since neither centrifugation of the cells in the absence of CB nor treatment of the cells with CB in the absence of centrifugation affected the yield of interferon (data not shown).

Enucleation during Interferon Production. Experiments with actinomycin, which inhibits interferon production because

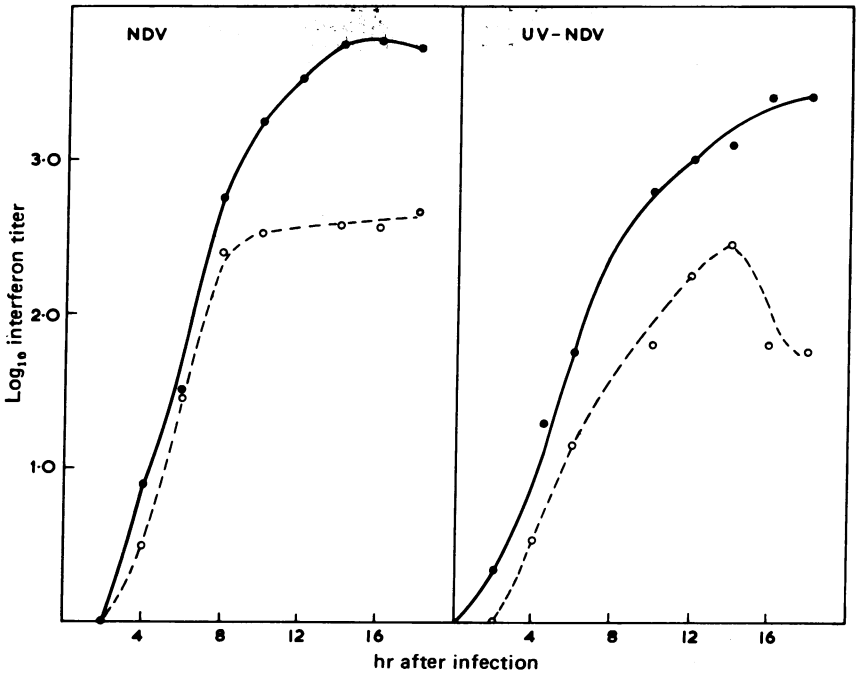

FIG. 2. Production of interferon by (*Left*) NDV and (*Right*) UV-NDV in L Cells. ●, Extracellular; ○, intracellular.

of its inhibitory effect on transcription, have shown that when actinomycin was added at increasingly later periods after the induction of interferon synthesis, it had a decreasingly inhibitory effect on subsequent interferon production. This has been interpreted as being due to the formation of a stable mRNA for interferon, although other interpretations are possible (see ref. 1). The enucleation technique provided a direct way to examine the role of the cell nucleus at different times during interferon production, since enucleation was effectively complete and produced cytoplasts that continued protein synthesis for at least 10 hr. In addition, the yield of interferon was high enough to enable the rate of interferon formation to be measured over quite short periods.

Cells were treated with UV-NDV and enucleated at different times thereafter, and the subsequent rate of interferon production was followed. Parallel, nonenucleated cultures were treated with 1 μg of actinomycin per ml for 1 hr at the same time as the enucleation (a treatment shown in separate experiments to completely inhibit interferon production when the actinomycin was added immediately after virus infection), while other untreated cultures served as controls. The results (Fig. 3) showed that both cytoplasts and actinomycin-treated cells continued to produce interferon for several hours after enucleation or actinomycin treatment. Comparison of the results obtained at the three different times shows that enucleation, and to a lesser extent actinomycin treatment, at 8 hr after infection caused a reduction in the rate of interferon production. However, the effect of enucleation was less at 10 hr and much less at 13 hr after infection. The decreasing effect of enucleation or actinomycin treatment on interferon production with longer times is probably due to the effective completion of the synthesis of the interferon messenger RNA by 13 hr after infection. This interpretation was confirmed by a direct experiment (Table 2) in which the effect of actinomycin on the yield of interferon between the time of addition and 24 hr was measured. The results (Fig. 2) showed that 10, 20, and 60% of the control amount of interferon was produced when actinomycin was added at 8, 10, and 13 hr, respectively, after induction. At the two earlier times the effect of enucleation on the rate of production was more marked that that of actinomycin. This was probably because, at these times, more of the interferon messenger than at later times is being processed in the nucleus or is perinuclear and is lost from the cell in the karyoplast. It is also clear from the figure that the rate of interferon production first rises, as would be expected early in the production cycle, and then falls, and that the rate of fall is very similar in control, actinomycin-treated, and enucleated cells.

Two other experiments showed that interferon production in cytoplasts was the result of protein synthesis. In the first, cycloheximide (100 μg/ml) was added to control, enucleated, and actinomycin-treated cultures immediately after treatment

Table 1. Effect of enucleation on interferon formation

Exp.	Treatment	Interferon yield, $\log_{10}$ units
1	NDV-infected control	3.95
	Enucleated, then infected	0.90 (0.1%)
	Infected, then enucleated	1.45 (0.3%)
2	UV-NDV-infected control	3.50
	Infected, then enucleated	1.85 (2.2%)

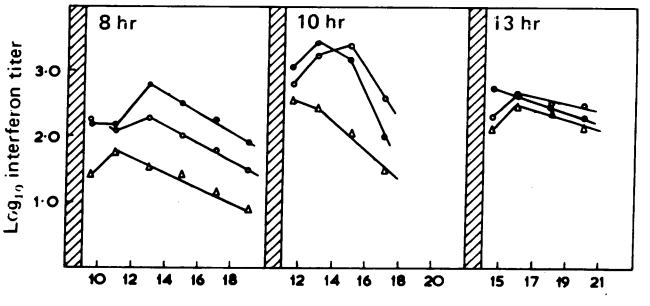

FIG. 3. The rate of interferon formation when cells infected with UV-NDV were enucleated at 8, 10, and 13 hr after infection (△), or treated with actinomycin D (1 μg/ml) for 1 hr at the same time (○) compared with controls (●).

Table 2. Effect of addition of actinomycin on interferon (IF) formation in L cells

Time, hr	Yield of IF at time point*	Yield of IF between time point and 24 hr*	
		In control	In presence of actinomycin
0	0	3.90	<1.0
2	0.5	3.90	1.25
4	1.25	4.15	2.15
6	1.70	3.95	2.75
8	2.60	3.95	2.70
10	3.20	3.65	2.90
12	3.45	3.75	3.20
14	3.75	3.70	3.65

Actinomycin (1 μg/ml) in minimum essential medium + 2% FCS was added at the times stated.
* In $\log_{10}$ units.

and the rate of interferon production was compared to that in parallel cultures not treated with cycloheximide. Table 3 shows that cycloheximide decreased the rate of interferon production in all three cultures at very similar rates, indicating that protein synthesis was required in each case. The lack of effect of cycloheximide during the first hour of treatment may be due to the time taken for the completed polypeptide to be glycosylated, since it is unlikely that cycloheximide will have any effect on the glycosylation process. A second experiment was designed to test the possibility that the cytoplasts did not synthesize interferon but merely released material present in a large internal pool. Measurement of the intracellular levels of interferon in control, enucleated, and actinomycin-treated cells showed that the levels were low (6–10% of the extracellular levels), and that this figure was very similar for all three sets of cultures at three different times after enucleation or actinomycin treatment (data not shown).

Reconstruction of Interferon-Producing Cells. Sendai virus-induced fusion of cytoplasts and karyoplasts leads to the formation of nucleated cells capable of cell division (12). In order to use this technique for the study of nuclear–cytoplasmic relationships in interferon-producing cells it was necessary to obtain a high yield of reconstructed cells and to reduce, as far as possible, whole cell contamination of both cytoplasts and karyoplasts. Yields of reconstructed cells varying from 3 to 8% could be obtained by careful attention to the details of the fusion procedure. Photomicrographs of marked microscopic fields confirmed that enucleated cells had fused with karyoplasts to produce nucleated cells. Whole cell contamination of the cytoplasts was estimated by measuring the yield of interferon from cytoplasts that had not been treated with karyoplasts and comparing the yield with that from whole cells. It was assumed that any interferon produced was due to remaining whole cells. Whole cell contamination of the karyoplasts was measured by adding an overinactivated preparation of Sendai virus that was incapable of inducing fusion to the cytoplast/karyoplast mixture and measuring the yield of interferon. Since any whole cells present in the karyoplast preparation would still attach to the plastic support and yield interferon, any increase of interferon yield compared with that of cytoplasts alone was due to the presence of nucleated cells in the karyoplasts. Table 4 shows the results of four consecutive reconstruction experiments. Comparison of column 7 with columns 3 and 5 showed that fusion of karyoplasts and cytoplasts with Sendai virus led to an approximately 10-fold increase in the number of nucleated cells, while comparison of column 8 with columns 4 and 6 showed that this was accompanied by a similar increase in interferon

Table 3. Effect of cycloheximide (100 μg/ml) on interferon production in enucleated, actinomycin-treated, and control cells

Time, hr	Cyclo-heximide	Interferon yields, $\log_{10}$ units		
		Enucleated	Actinomycin-treated	Control
11–12	–	2.15	2.80	3.05
	+	1.95	2.90	3.15
12–14	–	2.75	3.60	3.75
	+	1.10	1.85	2.15
14–16	–	2.40	3.75	3.50
	+	0.30	1.60	1.55

The cells were enucleated or treated with actinomycin at 10 hr after infection, and cycloheximide was added immediately afterwards. Interferon samples were then harvested at the times shown.

yields. Calculation of the interferon yield per viable cell gave a figure of 31 units/10^4 cells (range 23–39) for the control and 43 units/10^4 cells (range 30–59) for the reconstructed cells, showing that the reconstructed cells produced at least as much interferon per cell as the original cells. It was not possible to determine whether karyoplasts as such were capable of interferon production since they rapidly lost their integrity on incubation at 37°, becoming readily permeable to trypan blue.

DISCUSSION

We have shown that enucleation of L cells leads to loss of the capacity to produce interferon. A similar result was obtained when human cells were enucleated before treatment with poly(rI·rC) (data not shown). The failure of enucleated cells to produce interferon shows that the cell nucleus is essential for interferon production. This confirms a conclusion reached as a result of the study of mouse–human hybrid cells (2, 3) and excludes, for example, the possible hypothesis that interferon is coded by a mitochondrial gene.

However, when the cells were enucleated while interferon formation was proceeding, the resulting cytoplasts were capable of continuing to synthesize interferon for several hours by a process that was shown to be protein synthesis. This is only the second report of continued synthesis of a cell-coded product in cytoplasts (20), and clearly demonstrates that the interferon mRNA is in the cytoplasm. Comparison of the effect of actinomycin treatment with that of enucleation at 8 hr after induction (a time when interferon production has just started) showed that enucleated cells subsequently produced interferon at a lower rate than did actinomycin-treated cells. However, at later times the difference was much smaller. The probable explanation of the difference is that the nuclear and perinuclear cytoplasmic material in the karyoplast contains more interferon mRNA at 8 hr after induction than at later times, suggesting movement of the mRNA away from the immediate nuclear region as messenger synthesis slows down. Comparison of the change in the rate of interferon production in enucleated cells with that in actinomycin-treated and in control cells on continued incubation showed that the rate decreased in each case with similar kinetics. The most likely explanation of this is that the interferon mRNA breaks down at similar rates, although whether this is a process that is specific for interferon mRNA and, in particular, whether this breakdown is the explanation of the hyporesponsive effect (10) is not known.

Reconstructed cells could be obtained by Sendai virus-induced fusion of karyoplasts and cytoplasts, and by careful attention to detail it was possible to raise the yield to 3–8%. This level was essential in order to distinguish interferon production due to reconstructed cells from that due to whole cell contamination of cytoplasts and karyoplasts.

Table. 4. Reconstruction of interferon (IF)-producing cells

Exp.	Whole cells		Enucleated cells (cytoplasts)		Reconstruction control (fusion using over-inactivated virus)		Reconstructed cells	
	No. of cells (1)	IF yield* (2)	No. of nucleated cells (3)	IF yield* (4)	No. of nucleated cells (5)	IF yield* (6)	No. of nucleated cells (7)	IF yield* (8)
1	4.0×10^6	4.20	1×10^4	1.80	1.2×10^4	2.30	2.7×10^5	2.95
	(100)	(100)	(0.25)	(0.4)	(0.3)	(1.0)	(6.5)	(5.7)
2	1.9×10^6	3.65	1.1×10^4	1.00	1.3×10^4	1.70	1.5×10^5	2.95
	(100)	(100)	(0.6)	(0.22)	(0.8)	(1.1)	(8)	(20)
3	2.6×10^6	3.90	1.3×10^4	0	2.1×10^4	1.50	9.4×10^4	2.45
	(100)	(100)	(0.5)	(0)	(0.8)	(0.4)	(3.6)	(3.6)
4	1.8×10^6	3.75	0.5×10^4	0.85	1×10^4	1.65	1.5×10^5	2.85
	(100)	(100)	(0.28)	(0.1)	(0.55)	(0.8)	(8.3)	(12.6)

Values are given per flask. The numbers in parentheses are the percentage of the respective whole cell control.
* In $\log_{10}$ units.

The yield of interferon per reconstructed cell was almost the same as that from the original culture, showing that cytoplasts, which themselves cannot undergo induction of interferon synthesis, can give normal yield after fusion with karyoplasts. The production of interferon is in fact a very sensitive procedure for monitoring production of a specific protein because of the very high specific activity [at least 2×10^8 units/mg of protein (21)] of interferon. The way is now open to prepare reconstructed cells from karyoplasts and cytoplasts from cells of a single species that have been treated in different ways (e.g., primed with interferon) or from different species to produce hybrid reconstructed cells.

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