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Cytoplasmic requirement for peroxisome biogenesis in Chinese hamster ovary cells

(organellar assembly/somatic cell genetics/cytoplasmic inheritance)

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ABSTRACT **Hybrids constructed by fusion of wild-type Chinese hamster ovary cells (CHO-K1) to peroxisome-deficient CHO mutants (ZR-78.1) contain normal peroxisomes, demonstrating that the mutation(s) are recessive. "Nuclear hybrids" prepared by fusion of CHO-K1 karyoplasts to mutant ZR-78.1 occasionally fail to regain intact peroxisomes (≈1/300 cells). These peroxisome-deficient nuclear hybrids closely resemble the original mutant cells by biochemical criteria, but their modal chromosome number is 36–38, the same as that of CHO hybrids generated from intact cells. When the peroxisome-deficient nuclear hybrids are fused to wild-type cytoplasts, a fraction of the fusion products (at least 70%) continue to propagate normal peroxisomes indefinitely. Peroxisome biogenesis cannot be reinitiated in cells of mutant ZR-78.1 by fusion to wild-type cytoplasts. Our results suggest that a wild-type nucleus by itself is necessary but not sufficient for restoration of normal peroxisome biogenesis and that a cytoplasmic component of wild-type cells, possibly a normal peroxisome, is also required.**

A typical animal cell, such as a Chinese hamster ovary (CHO) cell fibroblast, contains ≈100 peroxisomes (1, 2). The structural simplicity and apparent nonessentiality of intact peroxisomes for the growth of animal cells in tissue culture (3) render them uniquely suited for investigations of membrane biogenesis. Peroxisomes were postulated to arise by budding off from the endoplasmic reticulum (4, 5), but numerous attempts to provide direct evidence for this model were unsuccessful (2). It is now known that peroxisomal membrane and matrix proteins are synthesized on cytoplasmic polyribosomes and that these proteins are imported post-translationally into existing peroxisomes (2, 6, 7). Under conditions of extensive peroxisome proliferation induced by certain chemicals, peroxisome budding is accompanied by the formation of a "peroxisomal reticulum" (6) that gives rise to individual peroxisomes and disappears once proliferation has ceased. In view of these data, it has been proposed that peroxisomes may form by budding or fission of existing peroxisomes and not by a *de novo* pathway (8).

If peroxisomes in somatic cells arise only by fission of preexisting peroxisomes, then a cell that has lost its peroxisomes as the result of a nuclear mutation might not be able to regenerate them after correction of the genetic lesion (i.e., by DNA transfection or by introduction of a wild-type nucleus). A "seed" peroxisome, or at least some sort of import-competent peroxisome precursor, might also be needed. Experimentally, this possibility might be revealed as a cytoplasmic requirement for the reinitiation of peroxisome biogenesis.

To approach this issue, we have fused CHO mutant cells deficient in peroxisomes (3) to wild-type CHO karyoplasts. The resulting nuclear hybrids, selected by their resistance to antibiotics, occasionally failed to regain peroxisomes. We demonstrate that these peroxisome-deficient nuclear hybrids can regain the capacity to propagate intact peroxisomes indefinitely when fused to wild-type cytoplasts.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (≈110 TBq/mmol) and [2-^{14}C]ethanolamine (18.6 MBq/mmol) were from Amersham. Dihydroxyacetone [^{32}P]phosphate was synthesized enzymatically (9). Palmitoyl-CoA was from PL-Biochemicals, titanyl sulfate was from Chemtech (Hayward, CA), and 12-(1′-pyrene)-dodecanoic acid (P12) was from Molecular Probes. Bio-Safe II liquid scintillation cocktail was from RPI (Mount Pleasant, IL). Silica gel 60 TLC plates (E. Merck) were from American Scientific Products (McGraw Park, IL). Polyethylene glycol (PEG) was from Fisher. Mercuric chloride was from J. T. Baker. Other reagents were from Sigma.

Cell Lines and Medium. CHO-K1 cells were obtained from the American Type Culture Collection (CCL-61). The isolation of ZR-78, a CHO mutant strain deficient in peroxisomes, has been described (3). Strain ZR-78.1 is an *HPRT*$^-$ and ouabain-resistant subclone of ZR-78. CHO-K1 was routinely grown in Ham's F12 (F12) medium (GIBCO) supplemented with 10% (vol/vol) fetal bovine serum (HyClone), streptomycin (100 μg/ml), and penicillin-G (100 units/ml) in an atmosphere of 5% CO_2/95% air. Mutant ZR-78.1 was cultured in growth medium containing 1 mM ouabain. Cell hybrids and nuclear hybrids were grown in HAT medium (100 μM hypoxanthine/10 μM aminopterin/20 μM thymidine) (10) containing 1 mM ouabain (HAT/ouabain).

Enzyme Assays of Cell Extracts and Determination of Plasmalogen Content. Cells were harvested prior to confluence, resuspended in buffer containing 0.25 M sucrose, 25 mM Tris·HCl (pH 7.4), 0.5 mM dithiothreitol, 1.0 mM EDTA, and 0.02% sodium azide, and sonicated briefly as described (3). Dihydroxyacetone phosphate acyltransferase (DHAP-acylTase) was assayed at pH 5.5 (3, 9). Subcellular fractions were prepared as described (3). Catalase assays (11) were performed as modified by Zoeller and Raetz (3). Protein was determined by the method of Lowry *et al.* (12). Plasmalogen content of cells radiolabeled with [2-^{14}C]ethanolamine was determined using a two-stage one-dimensional TLC system, designed to separate phosphatidylethanolamine and plasmenylethanolamine (13).

Cell Fusion, Hybrid Selection, and Chromosome Analysis. Approximately 9×10^5 CHO-K1 and 9×10^5 ZR-78.1 cells were mixed and plated in growth medium to form a confluent monolayer in a 35-mm tissue culture dish. After overnight

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Abbreviations: DHAPacylTase, dihydroxyacetone phosphate acyltransferase; P12, 12-(1′-pyrene)dodecanoic acid.

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incubation at 33°C, fusion was induced by a 1-min exposure to 50% (wt/wt) PEG (M_r, 3350) as described (14). The day after fusion, cells were harvested using trypsin and replated into four 100-mm tissue culture dishes in growth medium at 37°C. Forty-eight hours after fusion, the medium was aspirated and replaced with HAT/ouabain (the selective medium). Control cells were cocultured in HAT/ouabain but not exposed to PEG. Giemsa-stained metaphase chromosome spreads were prepared as described (15). A minimum of 25 spreads were counted for each cell type.

Assay of Peroxisomal DHAPacylTase in Colony Replicas. Approximately 500–2000 cells were plated per 100-mm tissue culture dish in growth medium. After 24 hr at 37°C, the cells were overlaid with HD-17 polyester cloths, as described (3, 16). Colonies were allowed to grow into the polyester cloths at 37°C, with medium changes every 3 or 4 days. After 10 days, polyester cloths containing colonies were removed, washed in isotonic phosphate-buffered saline (PBS, pH 7.4), blotted dry, and frozen to lyse the cells. Cells in master dishes were given fresh medium and stored at 28°C. Peroxisomal DHAPacylTase activity of the immobilized colonies was assayed at 37°C, as described for filter paper replicas (3), except that the volume per assay was increased to 2 ml.

Enucleation. Cells were enucleated by centrifugation through a discontinuous Ficoll density gradient containing cytochalasin B (10 μg/ml) (17) with modifications. Gradients consisted of Ficoll step fractions [2 ml at 25% (vol/vol), 2 ml at 18%, 0.5 ml at 16%, 0.5 ml at 15%, and 2 ml at 12.5%] in F12 medium without serum but containing cytochalasin B (10 μg/ml). Cells were applied to each gradient in 3 ml of 10% Ficoll containing cytochalasin B. After centrifugation for 60 min at 25,000 rpm in a SW 41 Ti rotor at 32°C, cytoplasts were harvested from the 15–16% Ficoll region by using a Pasteur pipette. Karyoplasts were harvested from the interface of the 18% and 25% Ficoll layers. Whole-cell contamination of each fraction was estimated by counting the number of colonies present 6 days after plating at 37°C.

Purification of Karyoplasts and Formation of Nuclear Hybrids. The karyoplast fraction was plated in a 100-mm tissue culture dish containing 15 ml of F12 medium. After 3 hr at 33°C, whole cells and large karyoplasts had attached to the dish. Small karyoplasts (containing a minimal amount of cytoplasm) were removed with the supernatant medium. Small karyoplasts were mixed with an equal number of ZR-78.1 cells (≈6×10^6 total particles) and plated in a 60-mm dish precoated with poly(L-lysine) (16). After 6–16 hr at 33°C, fusion was induced as described above. Nuclear hybrids were selected in HAT/ouabain medium 48 hr after fusion.

Selection of Cytoplast Fusion Products by Using Polystyrene Beads. Prior to enucleation, CHO-K1 cells were loaded with 1-μm polystyrene beads (Polysciences) to achieve 20–30 beads per cell, as described (18). Bead-containing cells were enucleated as described above. Bead-containing cytoplasts were mixed with 1.5×10^5 cells in a cytoplast/cell ratio of 20:1 and plated to form a confluent monolayer in a 35-mm dish. Cytoplasts were then fused to cells using PEG as described above.

On the following day, the fusion mixture was harvested using trypsin, and ≈20% of the cells were replated into ten 96-well microtiter dishes in F12 medium at 10–20 cells per well. Plating cells at low density facilitated the visual identification of fusion products (bead-containing cells). Approximately 24 hr after replating, bead-containing cells were observed in 15–20 microtiter wells. The cells from these wells were harvested with trypsin and replated a second time into new microtiter dishes at ≈1 cell per well. After 1 day, bead-containing cells (now separated from their nonbeaded counterparts) were switched to HAT/ouabain medium, grown for 5 days, harvested, pooled, cloned onto polyester filters, and assayed for the presence of peroxisomal DHAPacylTase.

Quantitation of P12/UV Resistance of Cell Lines. To assess the resistance of the various strains to photosensitized oxidation, cells were seeded in 96-well microtiter dishes in 0.2 ml of growth medium at 2×10^3 cells per well and allowed to attach overnight at 33°C (19, 20). On the following day, the medium was aspirated from the wells and 0.2 ml of growth medium containing increasing concentrations of P12 was added to the wells, and the cells were incubated for 20 hr at 37°C. Next, the medium was removed by aspiration. The cells were washed once with growth medium, received 0.2 ml of fresh medium, and were immediately irradiated by illumination for 2 min with two long-wavelength UV fluorescent lamps (15 W, Sylvania F1ST8 blacklight blue bulb) placed underneath at a distance of 2 cm (21). A 3-mm glass plate was positioned between the tissue culture dish and the UV source to eliminate residual UV-irradiation with wavelengths <300 nm (21). The intensity at 365 nm was ≈3000 μW/cm² when measured through the plastic dish with a 365-nm Blak-Ray UV meter (Ultraviolet Products, San Gabriel, CA). After irradiation, the plates were maintained for 3–4 days in the incubator prior to quantitation of surviving cells using Crystal violet staining (19, 20).

Selection of Cytoplast Fusion Products by P12/UV Enrichment for Peroxisome-Positive Cells. As an alternative method of selecting peroxisome-containing cells, peroxisome-deficient nuclear hybrids were cultured in HAT/ouabain medium for 3–4 days after PEG-induced fusion to cytoplasts (see above), harvested, and replated at 7×10^5 cells per 35-mm dish. The following day, the cells received 2.5 μM P12 in growth medium and were incubated at 37°C for 18 hr (21). Next, the cells were washed once with medium, received fresh medium, and were exposed to long-wavelength UV as described above (21). The surviving cells were cultured for 3–5 days, harvested, replated in 100-mm dishes at ≈600 cells per dish, and overlaid with polyester cloths. After 10 days, the peroxisomal DHAPacylTase in the immobilized colonies was assayed as described above.

RESULTS

Analysis of Peroxisome-Deficient CHO Cells by Somatic Cell Fusion. As shown in Fig. 1*A*, colonies of wild-type CHO-K1 contain high levels of peroxisomal DHAPacylTase, as judged by colony autoradiography, but colonies of mutant ZR-78.1 (Fig. 1*B*) are profoundly deficient. When CHO-K1 cells are fused to cells of strain ZR-78.1, the hybrids (Fig. 1*C*) had wild-type DHAPacylTase activity, indicating that the mutation in ZR-78.1 is recessive.

Peroxisomal DHAPacylTase was quantitated in extracts (Table 1, experiment I). Single CHO-K1–ZR-78.1 hybrid cells, grown to late logarithmic phase, regained the full DHAPacylTase specific activity of wild-type cells, indicating that the mutation in ZR-78.1 is not codominant. CHO-K1–ZR-78.1 hybrids also regained normal amounts of particulate catalase and plasmenylethanolamine, confirming the restoration of peroxisome function.

The modal chromosome number of metaphase chromosome spreads of CHO-K1–ZR-78.1 hybrids is 38 (Table 1), approximately twice that of the parental cells. The modal chromosome number is the same whether it is determined after 25 or 144 cell divisions. When 10^4 or more CHO-K1–ZR-78.1 hybrids growing on HAT/ouabain medium are cloned onto polyester cloths and assayed for DHAPacylTase, ≈1 in 3.5×10^3 colonies appear as mutants, most likely because of an occasional chromosome segregation. Spontaneous mutants are not observed in 10^4 CHO-K1 colonies.

Isolation of Peroxisome-Deficient Nuclear Hybrids. Karyoplasts prepared from CHO-K1 cells can be fused to peroxi-

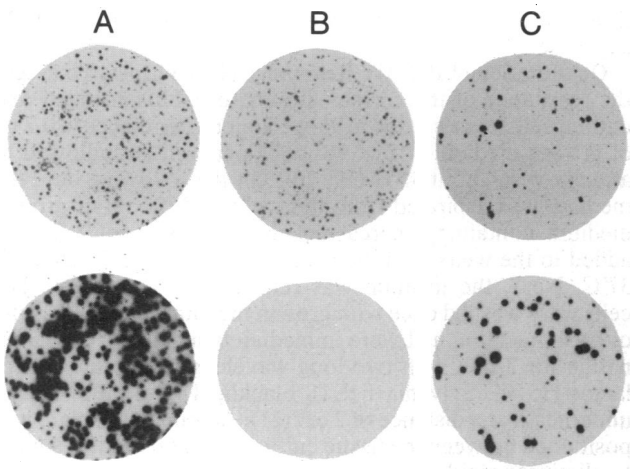

FIG. 1. Colony autoradiography of peroxisomal DHAPacylTase activity of wild-type cells, mutant cells, and cell hybrids. Cell hybrids were obtained by PEG fusion. The day after fusion, cells were harvested using trypsin and replated into four 100-mm dishes. Forty-eight hours after fusion, the medium was aspirated and replaced with HAT/ouabain (the selective medium). Peroxisomal DHAPacylTase activity was assayed on colonies grown into polyester cloths as described (3). After autoradiography, colonies on the polyester cloths were visualized with Coomassie blue. The figure shows the colonies stained with Coomassie blue (*Upper*) and the corresponding autoradiography (*Lower*). (*A*) CHO-K1 cells. (*B*) ZR-78.1 cells. (*C*) CHO-K1–ZR-78.1 cells.

some-deficient mutant cells (strain ZR-78.1), using essentially the same protocol employed for standard cell–cell fusions. Karyoplasts themselves are not viable, but the "nuclear hybrids" resulting from CHO-K1 karyoplast–ZR-78.1 fusion are able to grow in HAT/ouabain medium and can form colonies on polyester cloths. When assayed by colony autoradiography for peroxisomal DHAPacylTase, the incidence of peroxisome-deficient nuclear hybrids is ≈1 in 300 colonies (Fig. 2), 10-fold higher than in a population of cell–cell hybrids (see above).

Three peroxisome-deficient nuclear hybrids (LA-179, LA-290, and LA-577), derived from three independent karyoplast–ZR-78.1 fusions, were purified and characterized (Table 1, experiment II). As expected for a CHO–CHO hybrid (24), however, the modal chromosome numbers of LA-179, LA-290, and LA-577 were nearly tetraploid, and all three strains grew in HAT/ouabain medium.

We hypothesized that the frequent occurrence (≈1/300 colonies) of peroxisome-deficient nuclear hybrids (Fig. 2) might be due to some kind of cytoplasmic requirement for the reinitiation of peroxisome biogenesis in strain ZR-78.1, such as an intact "seed peroxisome" acting as an import-competent vesicle. The observation that ≈299 of 300 nuclear hybrids do have normal peroxisomes may be attributed to the fact that karyoplasts retain a rim of cytoplasm sufficient to contain a few mitochondria and as many as 15–20 peroxisomes (25).

Restoration of Peroxisome Biogenesis in Peroxisome-Deficient Nuclear Hybrids. If a wild-type nucleus by itself is not sufficient for the regeneration of normal peroxisomes in ZR-78.1, as suggested by the frequency of peroxisome-deficient nuclear hybrids, then introduction of normal peroxisomes into the cytoplasm of such hybrids might be necessary to restore peroxisomes. To approach this question, we fused wild-type cytoplasts to each of the three peroxisome-deficient nuclear hybrids and also to cells of strains ZR-78.1 and CHO-K1. After selection of the desired fusion products containing polystyrene beads, single cells were grown in HAT/ouabain medium (nuclear hybrids), ouabain medium (ZR-78.1), or F12 (CHO-K1) for ≈20 divisions to generate colony replicas on polyester cloth.

As shown in Fig. 3*A*, colony autoradiography of the purified peroxisome-deficient nuclear hybrid (LA-179) reveals the complete absence of DHAPacylTase, indistinguishable from mutant ZR-78.1 (Fig. 1). In contrast, ≈70% of the colonies derived from the fusion of bead-containing wild-type cytoplasts with LA-179 were peroxisome-positive (Fig. 3*B*). Similar results were also obtained with strains LA-577 and LA-290 (data not shown). Further purification by subcloning of individual peroxisome-positive colonies derived from the

Table 1. Peroxisomal markers of parental and hybrid strains

Exp.	Cells	DHAPacylTase activity, pmol per min per mg	Plasmalogen, % of total PE	Particulate catalase, units/mg	Modal chromosome number
I.	Original strains and standard cell hybrids				
	CHO-K1	235 ± 37	51 ± 2	19 ± 2	20
	ZR-78.1	8 ± 2	5 ± 1	2 ± 1	20
	CHO-K1–ZR-78.1	203 ± 24	50 ± 2	21 ± 2	38
II.	Peroxisome-deficient nuclear hybrids				
	LA-179	12 ± 2	9 ± 1	4 ± 1	36
	LA-290	6 ± 1	7 ± 1	4 ± 1	38
	LA-577	5 ± 2	8 ± 1	4 ± 1	37
III.	Peroxisome-deficient nuclear hybrids reconstituted with wild-type cytoplasts				
	LA-179-51	176 ± 35	47 ± 1	21 ± 2	36
	LA-290-52	168 ± 15	46 ± 1	19 ± 2	38
	LA-577-53	218 ± 31	46 ± 2	22 ± 1	37

For enzyme assays cells were harvested prior to confluence, washed in PBS, and resuspended in sucrose/Tris buffer (3). DHAPacylTase activity was assayed as described (22) at pH 5.5 in the presence of *N*-ethylmaleimide. Particulate and cytosolic fractions were prepared, and catalase assays were performed. To determine cellular plasmalogen content, cells were labeled with [2-^{14}C]ethanolamine, lipids were extracted using the method of Bligh and Dyer (23), and ethanolamine-containing phospholipids were separated using a two-stage one-dimensional TLC designed to separate phosphatidylethanolamine (PE) and plasmenylethanolamine (13). Metaphase chromosome spreads from cells at the 144th generation were prepared and stained with Giemsa (15). At least 25 spreads were counted to establish the modal chromosome number (15) for each cell type. All other data are average ± SD of triplicate assays.

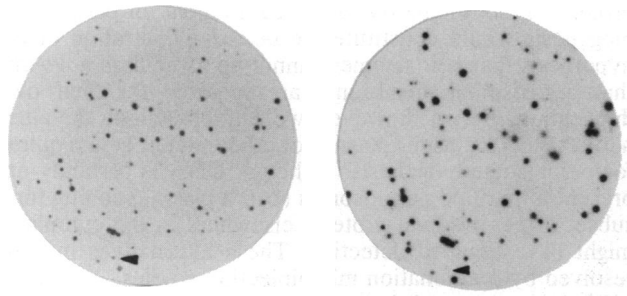

FIG. 2. Identification of a peroxisome-deficient nuclear hybrid using colony autoradiography. Wild-type karyoplasts were prepared, purified, and fused to an equal number of ZR-78.1 mutant cells. After selection of nuclear hybrids in HAT/ouabain medium, single cells were grown clonally and assayed for peroxisomal DHAPacylTase activity in colony replicas. The figure shows colonies of nuclear hybrids stained with Coomassie blue (*Left*) and the corresponding autoradiography (*Right*). In this example, a peroxisome-deficient nuclear hybrid (arrowhead), having no DHAPacylTase activity, was identified. Several peroxisome-deficient nuclear hybrids (isolated in separate experiments) were obtained by this procedure.

above fusions gave rise to strains LA-179-51, LA-290-52, and LA-577-53, termed "reconstituted" hybrids (Table 1, experiment III). As indicated, these clones regain nearly normal levels of particulate catalase, plasmenylethanolamine, and DHAPacylTase (Table 1, experiment I). The biochemical and phenotypic properties of the reconstituted nuclear hybrids are stable for at least 144 generations.

When CHO-K1 cytoplasts are fused to ZR-78.1 cells, no peroxisome-positive colonies are detected (Fig. 3*C*). This finding is consistent with the view that there is a nuclear mutation in ZR-78.1 (3). The absence of peroxisome-positive colonies in Fig. 3*C* also eliminates the possibility that the few intact CHO-K1 cells contaminating our cytoplast preparations are responsible for the peroxisome-positive colonies shown in Fig. 3*B*. (Contamination of cytoplasts with viable CHO cells is 1 in 10^4–10^5.) Cytoplasts generated from mutant cells (ZR-78) did not restore peroxisomes to LA-577 or LA-290 (data not shown). Fusion of wild-type cytoplasts to wild-type cells had no effect on the wild-type level of DHAPacylTase (Fig. 3*D*).

An Alternative Procedure for Demonstrating Peroxisome-Containing Progeny in CHO-K1 Cytoplast–LA-577 Fusion Mixtures. CHO cells incorporate P12 into membrane lipids (21). Exposure of P12-labeled cells to long-wavelength UV irradiation killed cells. CHO mutants deficient in plasmalogen biogenesis and peroxisome assembly are hypersensitive

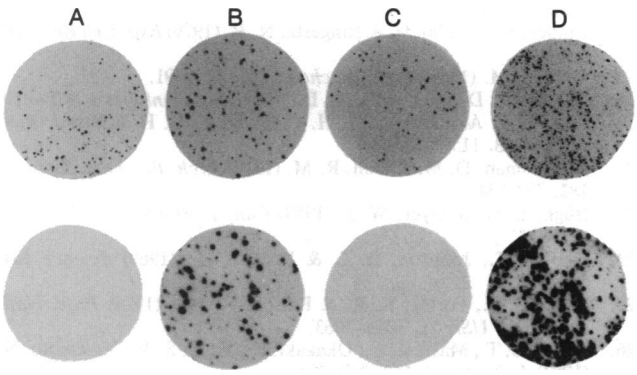

FIG. 3. Colony autoradiography of peroxisomal DHAPacylTase activity in various cell lines and fusion products. The figure shows the colonies stained with Coomassie blue (*Upper*) and the corresponding autoradiography (*Lower*). (*A*) Peroxisome-deficient nuclear hybrid (LA-179). (*B*) LA-179 fused to wild-type cytoplasts. (*C*) ZR-78.1 fused to wild-type cytoplasts. (*D*) CHO-K1 fused to wild-type cytoplasts.

to P12/UV treatment, making it possible to select peroxisome-containing cells from a large population of peroxisome/plasmalogen-deficient cells (21).

The availability of the P12/UV-resistance phenotype, coupled with the use of colony autoradiography, facilitated the confirmation of the experiment of Fig. 3, in which the cytoplast cell fusion products were identified by the presence of polystyrene beads. First, the sensitivity of LA-577 (peroxisome-deficient nuclear hybrid) and mutant ZR-78.1 to P12/UV treatment was compared to that of CHO-K1. As shown in Fig. 4, the peroxisome-deficient nuclear hybrid was just as sensitive to P12/UV treatment as was ZR-78.1. At 2.5 μM P12, there was 99% killing of LA-577 and ZR-78.1 but only 40% killing of CHO-K1.

Thus, we anticipated that restoration of normal peroxisomes to LA-577 by fusion to CHO-K1 cytoplasts should restore resistance to P12/UV killing. LA-577 cells were fused to CHO-K1 cytoplasts in the presence of PEG, as described above, but the selection of bead-containing cells was omitted. Instead, survivors were grown in HAT/ouabain medium, and after ≈4 days the culture was divided into two portions, one of which was exposed to a single cycle of P12/UV selection. The other half of the culture remained untreated and was grown separately. Next, cells were seeded from each culture at a low density, grown onto polyester filters, and assayed for DHAPacylTase. As shown in Table 2, ≈1% of the colonies derived from the fusion mixture that was not subjected to P12/UV treatment was positive for DHAPacylTase, in agreement with the results of the bead-selection protocol. The frequency of DHAPacylTase-positive colonies among the progeny of the P12/UV-enriched culture was ≈18% (Table 2).

When ZR-78.1 cells were fused to wild-type cytoplasts, grown in ouabain medium, and analyzed as above, no peroxisome-positive colonies were detected, either with or without P12/UV enrichment (Table 2).

DISCUSSION

Two key observations support the existence of a cytoplasmic requirement for peroxisome biogenesis in cultured CHO cells. (*i*) The incidence of peroxisome-deficient nuclear hybrids, derived from the fusion of wild-type karyoplasts to peroxisome-deficient mutant cells, is too high (≈1/300 col-

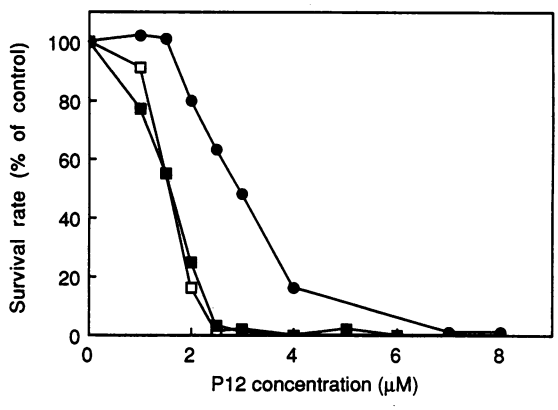

FIG. 4. Sensitivity of CHO wild-type, peroxisome-deficient mutant, and peroxisome-deficient nuclear hybrid cells to P12/UV killing. Cells (2×10^3 cells per well in 96-well microtiter dishes) were incubated at 37°C for 20 hr in 0.2 ml of growth medium containing P12 as indicated. Next, the cells were washed once with medium, and they received fresh growth medium prior to a 2-min irradiation with long-wavelength UV. The cells were allowed to grow for another 3–4 days, washed with PBS, and stained for quantitation using Crystal violet. The survival rate is the percentage of the absorbance measured in each well relative to control UV-exposed cells not labeled with P12. Each point is the average of three determinations. ●, CHO-K1; □, ZR-78; ■, LA-577.

Table 2. Peroxisomal DHAPacylTase activity in colonies derived from the fusion of mutant cells or peroxisome-deficient nuclear hybrids to wild-type cytoplasts, with or without P12/UV enrichment

Strain fused to CHO-K1 cytoplasts	P12/UV treatment	Number of DHAPacylTase-positive colonies	Total number of colonies on polyester
ZR-78.1	−	0	168
		0	167
ZR-78.1	+	0	184
		0	195
LA-577	−	2	144
		1	126
LA-577	+	20	110
		22	126

Bead-containing wild-type cytoplasts were prepared and fused to ZR-78.1 and LA-577 cells in the presence of PEG. The resulting hybrids were cultured for 3–4 days in HAT/ouabain selective medium. Next, half of the cells were replated and exposed to P12/UV treatment, whereas the other half remained untreated and were cultured separately. All cell samples were subsequently grown out at low density into polyester cloths prior to assaying peroxisomal DHAPacylTase activity in colony replicas.

onies) to be explained by chromosome segregation (≈1/3500 colonies). (*ii*) Peroxisome biogenesis is permanently restored in a fraction of clones derived from the fusion of peroxisome-deficient nuclear hybrids to wild-type cytoplasts but not from control fusions of the original mutants to the same wild-type cytoplasts.

The biochemical and phenotypic properties of the peroxisome-deficient nuclear hybrids (Table 1 and Fig. 4) are indistinguishable from those of the original mutant cells. However, the modal chromosome number of these hybrids is nearly tetraploid, just like that of conventional CHO–CHO hybrids (24). The observation that ≈299 of 300 nuclear hybrids still contain normal peroxisomes (Fig. 2) may be explained by the fact that karyoplasts retain a rim of cytoplasm, sufficient to encompass some peroxisomes. About 15% of the peroxisomal DHAPacylTase of intact cells is recovered with the karyoplasts. Presumably, only 1 out of 300 karyoplasts lack the critical cytoplasmic element (i.e., a peroxisome) required together with the nucleus for reinitiation of peroxisome biogenesis after fusion to mutant cells.

The feature that distinguishes the peroxisome-deficient nuclear hybrids (LA-179, LA-290, or LA-577) from the original peroxisome-deficient mutants is their ability to give rise to peroxisome-containing cells after fusion to wild-type cytoplasts. Two protocols (Fig. 3 and Table 2) were used to demonstrate the reappearance of normal peroxisomes among the progeny of fusions of wild-type cytoplast and peroxisome-deficient nuclear hybrids.

The absence of peroxisome-positive colonies among the fusion products of wild-type cytoplasts and ZR-78.1 cells demonstrates that viable wild-type cells, which contaminate wild-type cytoplasts at 0.01–0.001%, cannot account for the peroxisome-positive colonies that are observed in the experiments with the peroxisome-deficient nuclear hybrid. In addition, the reseeded peroxisome-deficient nuclear hybrids are still nearly tetraploid, showing that they have not acquired an additional wild-type nucleus.

Given the existence of a cytoplasmic requirement for reinitiation peroxisome biogenesis in CHO cells, it will be necessary to establish its identity. We favor the possibility that it will prove to be an intact peroxisome or, at least, a peroxisomal precursor capable of importing peroxisomal proteins. If so, the peroxisomal requirement for peroxisome biogenesis would constitute the best demonstration of the hypothesis that peroxisomes cannot be formed *de novo* and that peroxisomal membranes are required for their own duplication. As yet, however, we cannot exclude the alternative of a cytoplasmic plasmid or other nucleic acid required for peroxisome biogenesis. Although DNA is certainly not present in purified peroxisomes (26), a plasmid coding for a subset of peroxisomal proteins elsewhere in the cytoplasm might have escaped detection. These alternatives may be resolved by fractionation microinjection studies.

We have observed that mutant ZR-78.1 contains aberrant vesicles enriched in the major 69-kDa peroxisomal membrane protein (27), similar to the "peroxisomal ghosts" observed in Zellweger fibroblasts (28). Our results show that the putative "peroxisomal ghosts" present in mutant ZR-78.1 are not competent to restart normal peroxisome biogenesis in the presence of a wild-type nucleus.

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