Isolation and Characterization of Chinese Hamster Ovary Cell Mutants Defective in Assembly of Peroxisomes

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Abstract. We made use of autoradiographic screening to isolate two Chinese hamster ovary (CHO) cell mutants deficient in peroxisomal dihydroxyacetonephosphate acyltransferase, a key enzyme for the biosynthesis of ether glycerolipids such as plasmalogens. Morphological analysis revealed no evidence of peroxisome in these mutants. Catalase was as active as in the normal cells but was not sedimentable. Pulse–chase radiolabeling experiments and cell-free translation of RNA demonstrated that acyl-CoA oxidase, the first enzyme of the peroxisomal β-oxidation system, was synthesized as the 75-kD form but was not converted to 53- and 22-kD mature components that were present in the wild-type CHO cells; rather, degradation was apparent. Peroxisomal thiolase was synthesized as in normal cells but remained as a larger, 44-kD precursor, whereas maturation to the 41-kD enzyme was detected in the wild-type cells. The peroxisomal 70-kD integral membrane protein was also equally synthesized, as in the wild-type cells, and was not degraded. These results suggest that assembly of the peroxisomes is defective in the mutants, whereas the synthesis of peroxisomal proteins appears to be normal. Cell-fusion studies revealed that the two mutants are recessive to the wild-type CHO cells and belong to different complementation groups. Thus, these mutants presumably contain different lesions in gene(s) encoding factor(s) required for peroxisome assembly.

THE peroxisome is an ubiquitous intracellular organelle present in almost all, if not all, eukaryotes. It is classically defined as a subcellular organelle containing catalase and at least one H_2O_2-producing oxidase (de Duve and Baudhuin, 1966). Peroxisome functions in the catabolism of a wide variety of substrates such as fatty acid, D-amino acid, L-α-hydroxy acids, uric acid, and polyamine. Several human genetic disorders with evidence of absence of peroxisome are linked to various biochemical dysfunctions. Hence, the peroxisome plays crucial metabolic roles, including the catabolism of very long chain fatty acids by β-oxidation system, biosynthesis of ether-linked glycerolipids such as plasmalogens, metabolism of cholesterol and phytanic acid, and synthesis of bile acids (Schutgens et al., 1986; Moser, 1987). Among these disorders, cerebrohepatorenal syndrome (Zellweger syndrome) is a typical, severe disease (Goldfischer and Reddy, 1984; Zellweger et al., 1988).

Many lines of biochemical and morphological evidence are consistent with the idea that peroxisomes are formed by division of preexisting peroxisomes after posttranslational import of newly synthesized proteins (see Lazarow and Fujiki, 1985; and Borst, 1986 for review). Peroxisomal proteins, including membrane polypeptides, are synthesized on free polyribosomes in the cytosol, mostly at the final sizes. Posttranslational import of several proteins into peroxisomes has been reproduced in vitro (Fujiki and Lazarow, 1985; Imanaka et al., 1987; Small and Lazarow, 1987; Miyazawa et al., 1989). Targeting signal(s) in in vivo and in vitro import have been noted for peroxisomal enzymes such as luciferase (Gould et al., 1987, 1988) and acyl-CoA oxidase (AOx)[1] of rat liver (Miyazawa et al., 1989) as well as *Candida tropicalis* (Small et al., 1988). The topogenic signal identified for several enzymes resides at the extreme COOH terminus and comprises the sequence -Ser-Lys-Leu-COOH (Gould et al., 1988; Miyazawa et al., 1989).

Zoeller and Raetz (1986) isolated CHO cell mutants deficient in dihydroxyacetonephosphate acyltransferase (DHAP-ATase), a peroxisomal key enzyme in the synthesis of ether-linked glycerolipids such as plasmalogens (Hajra and Bishop, 1982). In these mutants, catalase, a matrix enzyme of peroxisome is fully active but not particle bound. Although the mutants have not yet been fully characterized morphologically and biochemically, they may be defective in peroxisomes, as noted in biopsied tissues and in fibroblasts from patients with Zellweger syndrome (Wanders et al., 1988). To investigate mechanisms related to the biogenesis of peroxisomes, at molecular and cellular levels, a mutant cell deficient in peroxisomal assembly would be useful. It would also serve as a somatic cell model system for studying human peroxisome

1. *Abbreviations used in this paper*: AOx, acyl-CoA oxidase; DHAP-ATase, dihydroxyacetonephosphate acyltransferase; EMS, ethyl methanesulfonate; HAT, hypoxanthine/aminopterin/thymidine; 70 and 22 IMP, 70- and 22-kD integral membrane proteins, respectively.

deficiency diseases such as Zellweger syndrome. We report here the isolation and characterization of two cell mutants that resemble the fibroblasts of Zellweger patients and belong to different complementation groups.

Materials and Methods

Materials

Catalase was purified from rat liver according to Leighton et al. (1969); AOx and bifunctional protein were generous gifts from Dr. T. Hashimoto (Shinshu University, Matsumoto, Japan), 70-kD integral membrane protein (70 IMP; Fujiki et al., 1982) was purified from peroxisomal membranes exactly as described for the isolation of 22-kD integral membrane protein (22 IMP; Fujiki et al., 1984). Anti-catalase, anti-AOx and anti-70 IMP antisera were, respectively, raised in rabbits by conventional subcutaneous injection. Anti-bifunctional protein antibody was a gift from Dr. T. Hashimoto. Growth medium, Ham's F12 medium, and FCS were purchased from Gibco Laboratories (Grand Island, NY). Glycerol kinase (*Candida mycoderma*), hypoxanthine/aminopterin/thymidine (HAT) supplement, and Pansorbin were from Boehringer-Mannheim (Mannheim, FRG). Ouabain, dihydroxyacetonephosphate, and palmitoyl-CoA were from Sigma Chemical Co. (St. Louis, MO). 6-thioguanin and dihydroxyacetone were purchased from Nacalai Tesque Co. (Kyoto, Japan). Ethyl methanesulfonate (EMS) was from Aldrich Chemical Co. (Milwaukee, WI). Digitonin, ATP, *N*-ethylmaleimide (NEM), and polyethylene glycol 6000 were from Wako Chemicals (Osaka, Japan). 3,3'-diaminobenzidine tetrahydrochloride (DAB) was from Kanto Chemicals (Tokyo, Japan). [^{35}S]Methionine (>1,000 Ci/mmol), [^{125}I]protein A (>30 mCi/mg), and γ-[^{32}P]ATP (>5,000 or ~3,000 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL). Enlightning, an autoradiography enhancer, was from New England Nuclear (Boston, MA).

Cell Culture and Isolation of Mutants

CHO-K1 cells were obtained from Dr. M. Imada of our institute and were grown in Ham's F12 medium supplemented with 10% (vol/vol) FCS under 5% CO_2/95% air. Cells were passaged using trypsin-EDTA, mutagenized for 16 h with EMS (400 μg/ml), grown for 3–4 d, and stored frozen. These mutagenized cells were plated at ~250 cells per 100-mm dish. After incubation for 24 h, the cells were overlaid with filter paper (No. 50; Whatman Inc., Clifton, NJ) and further maintained for 10–15 d at 37°C. Mutants defective in peroxisomal DHAP-ATase were isolated by an autoradiographic screening procedure according to Zoeller and Raetz (1986), except that screening was done at 37°C. Putative mutants were subjected to another cycle of colony screening and further purified by limiting dilution, without screening.

Enzyme Assays

DHAP-ATase assay was performed as described (Zoeller and Raetz, 1986), using ^{32}P-labeled DHAP and palmitoyl-CoA, by the method of Schlossman and Bell (1976). Catalase was assayed as described (Baudhuin et al., 1964).

Latency of Catalase

Cells were harvested by trypsinization and washed twice, sequentially, with growth medium and 0.25 M sucrose/10 mM Hepes-NaOH, pH 7.4. The cells were suspended at 1–1.5 × 10^6 cells/ml in 0.25 M sucrose/10 mM Hepes-NaOH, pH 7.4 and incubated on ice for 6 min in the same solution but containing different concentrations of digitonin (Wanders et al., 1984); the activity of catalase was measured as described above, except that the assay mixture contained 0.25 M sucrose.

Morphological Analysis

Cytochemical Analysis. Cells were fixed for 60 min at room temperature with 4% paraformaldehyde/1% glutaraldehyde/0.01% $CaCl_2$/0.1 M cacodylate buffer, pH 7.4, and incubated for 1 h at 37°C in the dark in medium containing 5 mM DAB/0.1 M glycine-NaOH, pH 10.5/0.15% H_2O_2; the cells were postfixed for 1 h at 0°C with 1% osmium tetroxide followed by reduction with potassium ferrocyanide, dehydrated in ethanol and propylene oxide, and then embedded in Epon 812. Ultrathin sections were cut on an ultratome (LKB Instruments, Gaithersburg, MD), counterstained briefly with 40 mM lead citrate, and examined under a Hitachi H-600 electron microscope (Hitachi Ltd., Tokyo).

Immunocytochemical Analysis. Cells were fixed in a culture plate for 2 h at room temperature with 4% paraformaldehyde/0.01% $CaCl_2$/0.15 M cacodylate buffer, pH 7.4, and treated for 15 min at ambient temperature with PBS containing 0.05% Triton X-100. The cells were incubated for 2 h at room temperature with rabbit anti-rat liver catalase antiserum. After washing three times with PBS, the cells were incubated for 30 min at ambient temperature with horseradish peroxidase-labeled goat anti-rabbit IgG antibody followed by reaction with DAB and 0.01% H_2O_2 in 0.1 M cacodylate buffer, pH 7.4. The cells were then examined under an Olympus BH-2 microscope.

Preparation of Cell Fractions

Cells were harvested by trypsinization, washed twice with growth medium and homogenized in 0.25 M sucrose/5 mM Hepes-KOH, pH 7.4/0.1% ethanol by 10 strokes of an Elvehjem-Potter homogenizer. A postnuclear supernatant was prepared, as described (Berthet and de Duve, 1951). A high-speed supernatant (cytosolic fraction) was prepared from the postnuclear supernatant by centrifugation at 100,000 *g* for 90 min. The pellet (particulate fraction) was resuspended in the homogenizing buffer.

Radiolabeling of Cells

Pulse-Chase Experiment. Cells growing in a 35-mm dish were pulse-labeled for 1 h with [^{35}S]methionine (0.1 mCi/ml) in methionine-free MEM supplemented with dialyzed FCS. The medium was removed and the cells were washed twice with HBSS and fed 2 ml F12 plus 10% FCS medium. At selected intervals, cells were washed twice with HBSS and lysed in 0.5 ml of 1% NP-40/0.1% SDS/10 mM Tris-HCl, pH 7.4/0.15 M NaCl. After centrifugation in an Eppendorf microfuge, the supernatants were subjected to immunoprecipitation with specific rabbit antisera, as described (Fujiki et al., 1984), except that 0.1% NP-40 was used instead of 1%.

Continuous Labeling. Cells were labeled at 37°C for 48 h in F12 plus 10% FCS medium containing [^{35}S]methionine (10 μCi/ml) with a change of medium at 24 h. Labeled cells were lysed and proteins were immunoprecipitated, as described above.

Cell-free Translation of RNA

RNA was isolated from wild-type and mutant CHO cells by the guanidium-thiocyanate method of Ullrich et al. (1977). Total RNA of normal rat liver was prepared from a postmitochondrial fraction, as described (Fujiki et al., 1985). Cell-free translation of RNA was carried out in a nuclease-treated rabbit reticulocyte lysate cell-free protein-synthesizing system with [^{35}S]methionine as label (Pelham and Jackson, 1976; Fujiki et al., 1984). Immunoprecipitation was done, as described (Fujiki et al., 1984).

Cell-Cell Fusion

For studies on genetic complementation, variants of mutant CHO cells (Z24 and Z65) resistant to both ouabain (Oua) and 6-thioguanine (TG) were isolated as follows (Kucherlapati et al., 1975): the mutant cells were mutagenized with EMS as described above, then selected for resistance to 60 μM 6-thioguanine. The TG-resistant (TG^r) cells were next screened for the mutant resistant to 3 mM ouabain, after treatment with EMS. Clones of variants (TG^{r}Oua^r) that were sensitive to HAT medium (F12 medium containing 5 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine supplemented with 10% FCS) were used for hybridization with the original sensitive cells (TG^{s}Oua^s). The TG^{r}Oua^r cell line and the cells to be fused were both plated for 24 h at each density of 2.5–3 × 10^5 cells in a 35-mm dish. After thoroughly removing the growth medium, the cells were treated with 1 ml of F12 medium containing 45% (wt/wt) polyethylene glycol and incubated for 1 min at room temperature, with gentle swirling (Sato et al., 1982). After washing four times with F12 medium, the cells were incubated at 37°C for 8 h in growth medium and replated in a 100-mm dish. For selection of hybrids, the cells were incubated in HAT medium containing 1 or 2 mM ouabain and maintained with several changes of medium until the control cells without polyethylene glycol-treatment were hardly viable (usually, 6–9 d). About 600–7,000 colonies were formed from one 35-mm dish. The entire population of cells on a dish was harvested by trypsinization.

Other Methods

SDS-PAGE was carried out according to Laemmli (1970), as described (Fujiki et al., 1982). Fluorography was performed with Enlightning. Immu-

Table I. DHAP-ATase Activity of Wild-Type and Mutant CHO Cells

	DHAP-ATase-specific activity		
	pmol/min per mg protein		
pH	5.5	7.4	7.4
N-Ethylmaleimide	(−)	(−)	(+)
CHO	285	248	155
Z24	7	307	12
Z65	1	237	9

Cells were lysed in an isotonic medium by sonication (10 s) and assayed for DHAP-ATase activity at pH 5.5 and pH 7.4 in the absence (−) or presence (+) of 5 mM NEM, as described (Zoeller and Raetz, 1986).

noblot was done according to the modified procedure (Fujiki et al., 1984) of Burnette (1981), except that 1% bovine skim milk was used for blocking instead of 1% bovine hemoglobin. Protein was determined by the method of Lowry et al. (1951) with BSA as the standard.

Results

Isolation of Mutants Defective in Peroxisomal DHAP-ATase

CHO-K1 cells treated with EMS were cultivated on filter paper, lysed and assayed for DHAP-ATase converting [^{32}P]DHAP with palmitoyl-CoA to acid-insoluble palmitoyl-[^{32}P]DHAP. Assays were carried out at pH 5.5 in the presence of NEM to inhibit any residual activity of microsomal DHAP-ATase (Zoeller and Raetz 1986). Mutants defective in this enzyme were identified by comparing the autoradiogram with the pattern of colonies stained with Coomassie brilliant blue. Two mutants, Z24 and Z65, were isolated from ~25,000 colonies grown from five stocks of EMS-treated CHO cells. Both mutants were severely deficient in DHAP-ATase activity, i.e., <2% of the activity of wild-type cells (Table I). When measured at pH 7.4, considerable DHAP-ATase activity (95–125% of wild-type activity) was detected in the mutants. With the addition of NEM, DHAP-ATase was inhibited in the wild-type cells by 40%, whereas that in the two mutants was all but abolished. Thus, the two mutants were deficient in NEM-insensitive peroxisomal DHAP-ATase, whereas NEM-sensitive DHAP-ATase remained active at pH 7.4. The deficiency in peroxisomal DHAP-ATase was not lethal to the mutants. Cell growth, represented as doubling time, was not effected in the Z65, whereas that of Z24 was reduced to nearly half, presumably because Z24 was almost twice in size, as compared with the wild type and Z65 cells (Table II).

Morphological and Biochemical Analysis of Mutants

Cells were fixed and cytochemically stained with DAB specific for catalase. Electron microscopic analysis demonstrated that the wild-type CHO cell contained numerous ~0.15 μm catalase-containing particles, peroxisomes (Fig. 1, *CHO, P*). No DAB-positive particle was detected in any microscopic section of Z24 and Z65 mutant cells examined (Z24 and Z65). Other intracellular organelles in both mutants appeared to be morphologically normal.

Catalase activity was nearly twice as high in the Z24 cells as in the wild type; the specific activity in the homogenate of Z24 was much the same as that in the wild-type CHO cells (Table II), no doubt due to differences in cell size (see Fig. 7). In the mutant cell Z65, catalase activity was about half that in the wild-type cells. Catalase was probably less in amount in the Z65, as two catalase polypeptides were observed, one of which may be inactive (see below). The subcellular fractionation study revealed that ~80% of the catalase activity of Z24 and Z65 was not sedimentable, whereas 75% of that in the wild-type cells was particle associated (Table II). The residual activity in the supernatant from the wild-type cells presumably reflects the catalase leaked from the broken peroxisomes during homogenization (Lazarow and de Duve, 1973), while that of the particulate fraction in the mutants may represent the unbroken cells. This would suggest that catalase is present, in an active form, in the cytosol of the Z24 and Z65.

In the immunoblot analysis, rabbit antibodies used against rat liver peroxisomal proteins cross-reacted with CHO cell proteins. Anti-catalase antibody recognized the polypeptide of CHO cells, which was slightly smaller than that of rat liver (Fig. 2 *A*). In both mutants, this protein was present mostly in the cytosolic fraction, whereas it was detected in peroxisome-containing particulate fraction from the wild-type cells. Thus, the intracellular localization of catalase activity was confirmed, in both parent and mutant cells. In the Z65, the catalase took the form of a fuzzy band (see below).

In the rat liver, AOx is a heterodimer consisting of 75-kD A, 53-kD B, and 22-kD C polypeptide components (Miyazawa et al., 1987), all of which were detected by immunoblots with anti-rat liver AOx antibody (Fig. 2 *B*, lane *1*). In the parental CHO cells, the antibody reacted with polypeptides comigrating with A and B components of rat liver AOx, respectively; C component was slightly visible (lane *2*). In the mutant Z24 and Z65 cells, only A component was detected (lanes *3* and *4*). The faint band in Z24, with a mobility similar to that of the B component was likely to be nonspecific (see Fig. 3 *A*).

Table II. Characterization of Wild-Type, Mutant, and Hybrid CHO Cells

	Doubling time	Peroxisomal DHAP-ATase	Catalase activity				
			Total		Soluble	Sedimentable	Recovery
	h	*pmol/min per mg*	*mU/mg*	*mU/10^7 cells*	*% of recovered*		*%*
CHO	9.6	285	20	35	25	75	86
Z24	17.0	7	24	77	77	23	88
Z65	9.9	1	12	19	78	22	96
Z24 × Z65	ND	233	19	101	26	74	107

DHAP-ATase was measured at pH 5.5 (see Table I). Cells were homogenized, separated into particulate and soluble fractions, and assayed for catalase as described in Materials and Methods.

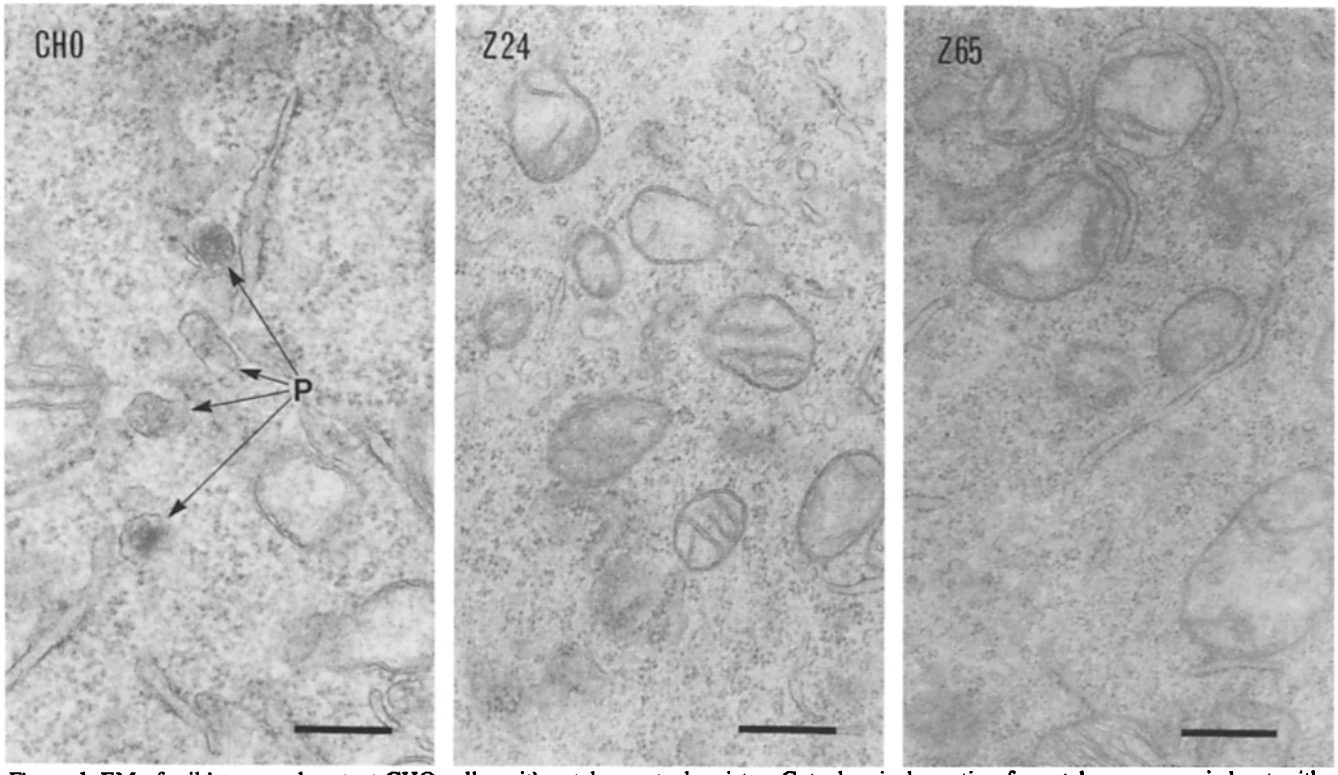

Figure 1. EM of wild-type and mutant CHO cells, with catalase cytochemistry. Cytochemical reaction for catalase was carried out with DAB. Peroxisomes (*P*) were seen only in the wild-type CHO cell. CHO, wild-type CHO cell; Z24 and Z65, CHO cell mutants Z24 and Z65, respectively. ×44,000. Bar, 0.3 μm.

Biosynthesis of Peroxisomal Proteins

To examine the biogenesis of peroxisomal proteins in the wild-type and mutant cells, metabolic labeling of cells was carried out for 48 h with [^{35}S]methionine. Immunoprecipitates of several proteins with specific antibodies against rat liver peroxisomal proteins were analyzed by SDS-PAGE and fluorography (Fig. 3). All the anti-rat liver peroxisomal protein antibodies used recognized the CHO counterparts, as seen in the immunoblot. [^{35}S]catalase was present to the same extent in immunoprecipitates from mutants Z24 and Z65 and parent cells (lanes *7–9, arrowhead*). With a short

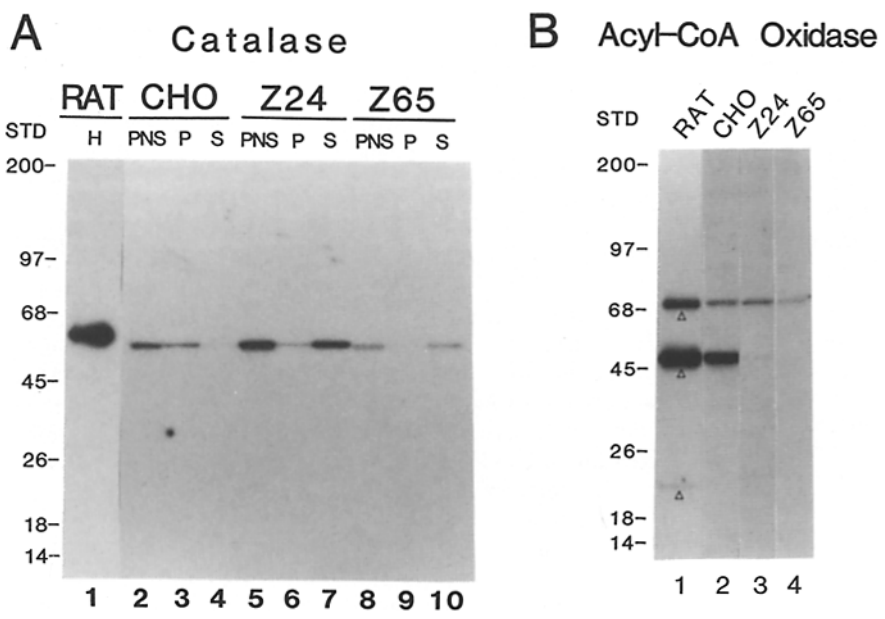

Figure 2. Immunoblot analysis of wild-type and mutant CHO cells. Cells were homogenized and fractionated as described in Materials and Methods. Immunoblot was carried out with rabbit antisera against rat liver catalase (*A*) and rat liver acyl-CoA oxidase (*B*), respectively. Antibodies were detected by ^{125}I-protein A. *A* and *B* are composites of two autoradiographic exposures of a single gel, respectively: lane *1* was exposed for 5 h, the remainder for 18 h. Lanes in *A*: *1*, liver homogenate (15 μg) of a rat treated with a hypolipidemic drug, clofibrate; *2*, postnuclear supernatant fraction (PNS, 100 μg) of wild-type CHO cells; *3* and *4*, particulate and supernatant fractions, respectively, from PNS (100 μg) of parent cells; *5*, PNS (100 μg) of mutant Z24 cells; *6* and *7*, particulate and supernatant fractions, respectively, from PNS of Z24 cells; *8–10*, cell fractions as in lanes *5–7*, but prepared from the mutant Z65. B, lanes: 1, rat liver homogenate (15 μg); *2–4*, PNS (100 μg) of wild-type, Z24, and Z65 cells, respectively. *Open arrowheads*, components *A* (75 kD), *B* (53 kD), and *C* (22 kD) of rat liver AOx. *STD*, standard markers in kilodaltons; *H*, homogenate; *PNS*, postnuclear supernatant fraction; *P*, particulate fraction; *S*, supernatant fraction.

exposure, the [35S]catalase in Z65 was discerned as two bands, one with an authentic size and the other with a slightly smaller mass (not shown).

Three polypeptides with apparent masses of 75, 53, and 22 kD were detected by anti-AOx antibody in the wild-type cells, whereas only the 75-kD component was found in a small quantity, in both Z24 and Z65 mutants (Fig. 3, lanes *10–12*). These three proteins found in CHO cells had a mobility on SDS-PAGE that was indistinguishable from the A, B, and C components of rat liver AOx, respectively, as observed in the immunoblot. Anti-3-ketoacyl-CoA thiolase antibody immunoprecipitated a 41-kD [35S]polypeptide in the parent cells, whereas only the 44-kD protein was detected in Z24 and Z65 (lanes *1–3*, *solid* and *open arrowheads*). These two proteins comigrated with the thiolase precursor and mature protein of rat liver, respectively (not shown).

Rabbit antiserum against 70 IMP of rat liver peroxisomes recognized a [35S]polypeptide of 70 kD in both wild-type and mutant cells (Fig. 3, lanes *4–6*). A [35S]protein with a mass of ~79 kD was equally present in all types of cells, by immunoprecipitation with anti-rat liver bifunctional protein, hydratase-dehydrogenase (lanes *13–15*); it comigrated on SDS-PAGE with rat liver bifunctional protein (not shown).

The [35S]polypeptides of CHO cells, immunoprecipitated by anti-peroxisomal proteins of rat liver, were all displaced during immunoprecipitation by purified rat liver proteins, thereby indicating that the CHO cell proteins were specifically immunoprecipitated (not shown). We interpreted these events to mean that the immunoprecipitated proteins of CHO cells were the counterparts corresponding to those of rat liver peroxisomes.

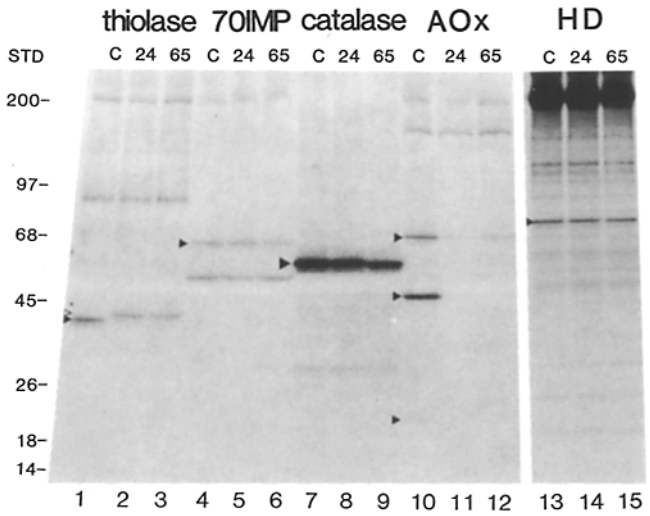

Figure 3. Biosynthesis of peroxisomal proteins in wild-type and mutant CHO cells. Wild-type and mutant CHO cells were labeled for 48 h with [35S]methionine. The same amount of radioactivity (3.7 × 10^6 dpm) of cell lysates was subjected to immunoprecipitation with respective specific rabbit anti-rat liver peroxisomal protein antibody. Immunoprecipitates were analyzed by SDS-PAGE (7–15% gel) and fluorography. *STD*, standard marker proteins in kilodaltons; *70 IMP*, 70-kD integral membrane protein; *AOx*, acyl-CoA oxidase; *HD*, bifunctional hydratase-dehydrogenase. Solid arrowheads indicate the positions of respective protein; three arrowheads in AOx indicate those of components *A*, *B*, and *C*, respectively. *Open arrowhead*, migration of a larger precursor of 3-ketoacyl-CoA thiolase. *C*, wild-type CHO cells; *24*, mutant cell Z24; *65*, mutant cell Z65. Exposure, 18 d except for HD, 22 d.

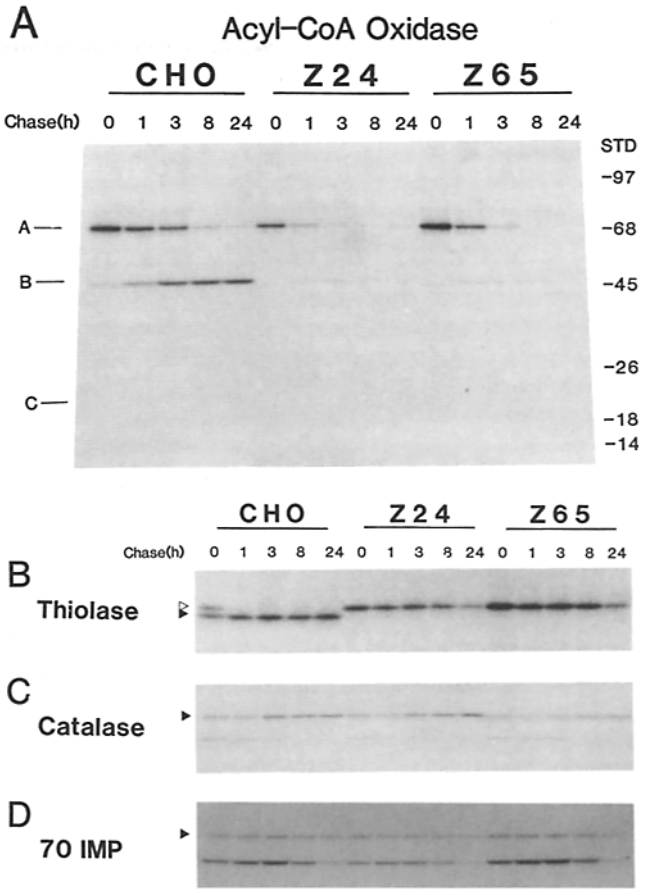

Figure 4. Kinetics of synthesis of peroxisomal proteins in wild-type and mutant cells. All types of cells were pulse-labeled with [35S]methionine for 1 h and chased for 1, 3, 8, and 24 h as described in Materials and Methods. (*A*) biosynthesis of acyl-CoA oxidase. *CHO*, wild-type CHO cell; *Z24*, mutant cell Z24; *Z65*, mutant cell Z65. *A*, *B*, and *C* on the left indicate the positions of the components *A*, *B*, and *C* of AOx, respectively. *STD*, standard mass markers. *B–D*, synthesis of thiolase, catalase and *70 IMP*, respectively. *Open* and *solid arrowheads* in *B*, positions of a larger precursor and mature thiolase, respectively. *Solid arrowheads* in *C* and *D* indicate the positions of labeled proteins. Exposure: *A*, 11 d; *B*, 41 d; *C*, 4 d; *D*, 11 d.

We next examined the kinetics of labeling of peroxisomal proteins in pulse/chase radiolabeling experiments. Cells were pulse-labeled with [35S]methionine for 1 h in a methionine-free medium, and the radioactivity was chased for 1, 3, 8, and 24 h in normal Ham's F12 medium (Fig. 4, *A–D*). In the wild-type cells, immunoprecipitation with anti-AOx antibody revealed a [35S]protein, identified as the 75-kD A form, as described above, then disappeared with an apparent half-life of 2–3 h (*CHO*). The [35S]polypeptide, found to be the B component of AOx, was barely seen at 0 h but increased in amount with time. The [35S]protein comigrating with the AOx-C component was little evident after only an 8-h chase, presumably because of the low content of methionine, as noted for rat liver AOx (Miyazawa et al., 1987). On the other hand, in both mutants, only the A component polypeptide was seen at 1 h labeling (chase, 0 h) of cells and dis-

appeared with an apparent half-life of <1 h. Neither B nor C polypeptide was apparent (panel Z24 and Z65). The faint band in Z24 and Z65 with a mass similar to that of AOx-B was apparently nonspecific, as it was not displaced by purified AOx during the immunoprecipitation (not shown). These results show that AOx is synthesized as component A and proteolytically converted to B and C subunits in the wild-type cells, whereas the AOx A component is synthesized in the mutants as in the wild-type cells but is not processed to B and C, and then rapidly degrades. This finding of conversion of A to B and C components is in good agreement with reports on the biosynthesis of rat liver AOx, in vivo and in vitro (Furuta et al., 1982; Miura et al., 1984; Miyazawa et al., 1989).

Immunoprecipitation with anti-3-ketoacyl-CoA thiolase antibody yielded 44- and 41-kD [^{35}S]polypeptides in the wild-type cells (Fig. 4 *B*, *CHO*, *open* and *solid arrowheads*). The larger polypeptide disappeared within 1 h in the chase, with a concomitant increase in the 41-kD protein. In the mutants, the 44-kD [^{35}S]polypeptide was synthesized and remained in the same form. The radioactivity of the band gradually decreased during the 24-h chase (panels Z24 and Z65). These findings are interpreted to mean that 3-ketoacyl-CoA thiolase was synthesized as a larger precursor of 44 kD, in both wild-type and mutant cells, then processed to 41-kD mature form in the wild-type but remained as the 44-kD precursor in the mutants.

[^{35}S]catalase was immunochemically detected in all types of cells (Fig. 4 *C*). The radioactivity of the polypeptide increased with the time of chase, although the significance of this was not clear. One possible explanation is reutilization by the cells of [^{35}S]methionine that was depleted in the medium. Catalase was observed in Z65 as a fuzzy band.

The anti-70 IMP antibody immunoprecipitated [^{35}S]protein, identified as 70 IMP (see above), from both parent and mutant cells (Fig. 4 *D*). The 70 IMP was stable for at least up to 24 h during the chase, in the mutants and the wild-type cell.

Cell-free Synthesis of Peroxisomal Proteins

RNA from the wild-type and mutant cells was translated in vitro with [^{35}S]methionine as the label; several peroxisomal proteins were respectively immunoprecipitated from the translation products by antibodies against peroxisomal proteins of the rat liver. [^{35}S]Catalase was detected in all types of cells, which migrated in SDS-PAGE more rapidly than did the 60-kD rat liver catalase (Fig. 5 *A*). Two polypeptide bands were seen in the mutant Z65; the larger band comigrated with authentic catalase and the other migrated slightly faster (*solid* and *open arrowheads*). These results are consistent with those obtained from the metabolic labeling of cells (Figs. 3 and 4) as well as the immunoblotting (Fig. 2 *A*), albeit the two polypeptides not being clearly discerned. Accordingly, the lower protein is unlikely to be a degradation product from the authentic polypeptide. One allele of catalase gene may be mutated by treatment with EMS.

Immunoprecipitation with anti-AOx antibody yielded a single [^{35}S]polypeptide possessing electrophoretic mobility indistinguishable from that of rat liver AOx A component

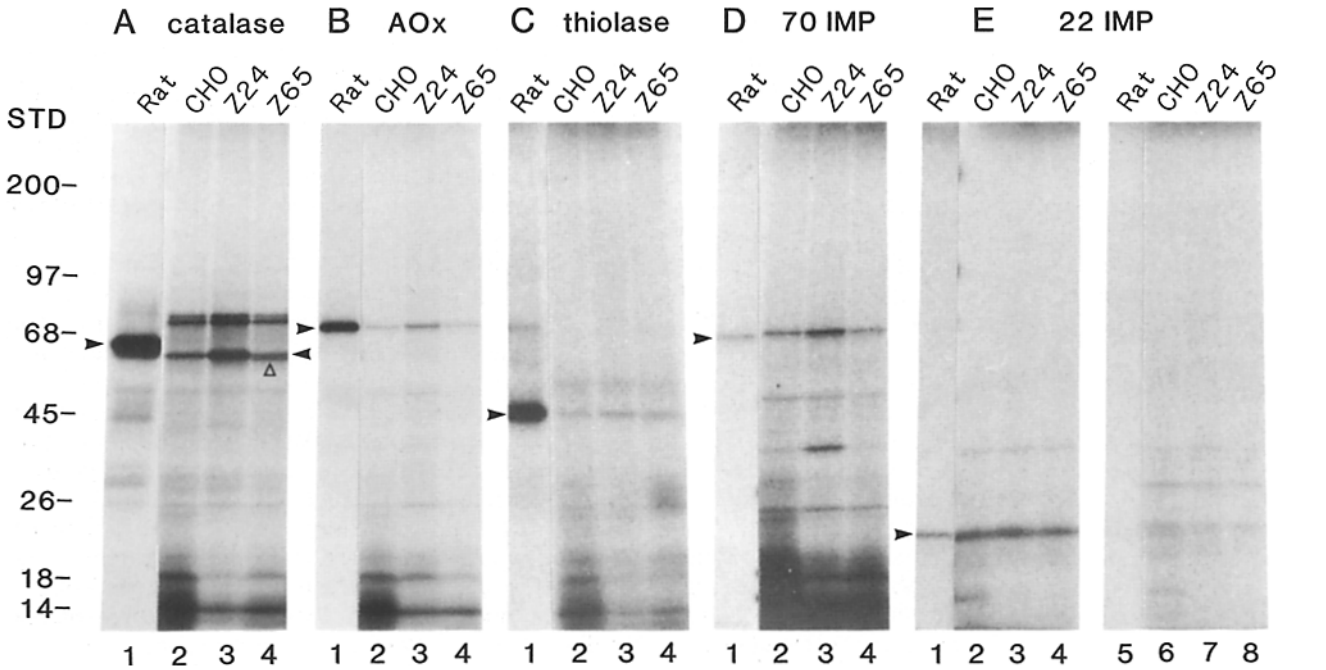

Figure 5. Cell-free translation of RNA from wild-type and mutant cells. RNA translation and immunoprecipitation of peroxisomal proteins were carried out as described in Materials and Methods. The same amount of radioactivity (2.6×10^6 dpm) was subjected to immunoprecipitation, except that 20% of the input radioactivity was used in lanes related to rat liver. (*A*) catalase; (*B*) acyl-CoA oxidase; (*C*) 3-ketoacyl-CoA thiolase; (*D*) 70 IMP; (*E*) 22 IMP. Lanes: *1*, normal rat liver; *2*, wild-type CHO cells; *3*, mutant cell Z24; *4*, mutant cell Z65. [^{35}S]22 IMP was immunoprecipitated in the presence of unlabeled authentic 22 IMP of rat liver peroxisomes (*E*, lanes *5–7*). Exposure: *A* and *B*, 11 d; *C* and *D*, 54 d; *E*, 20 d. *Arrowhead*, the position of respective protein. *Upward open arrowhead* in *A*, a catalase band with higher mobility as compared with the authentic monomer.

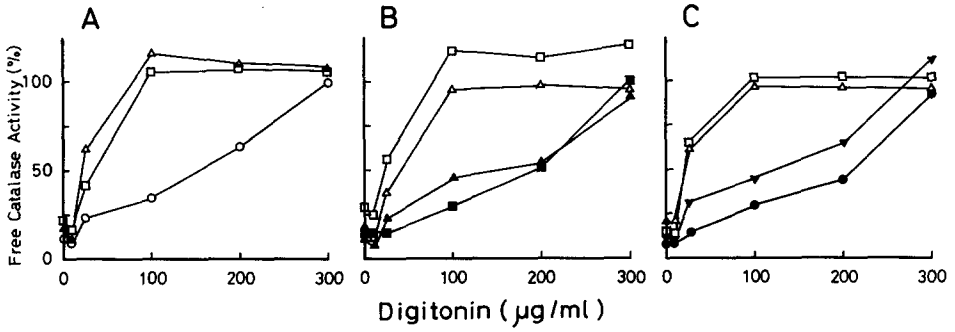

Figure 6. Latency of catalase in wild-type, mutant, and hybrid cells. Cells were treated with digitonin at the concentration indicated and assayed for catalase activity in an isotonic medium. Relative free catalase activity is expressed as a percentage of total activity measured in the presence of 1% Triton X-100. (*A*) wild-type (CHO, ○) and two mutants (Z24, △; Z65, □). (*B*) variants of the mutants, Z24TG^{r}Oua^r (△) and Z65TG^{r}Oua^r (□); hybrid cells, wild-type x Z24TG^{r}Oua^r (▲) and wild-type x Z65TG^{r}Oua^r (■). (*C*) heterologous hybrids, Z24 x Z65-TG^{r}Oua^r (▼) and Z65 x Z24TG^{r}Oua^r (●); homologous hybrids, Z24 x Z24TG^{r}Oua^r (△) and Z65 x Z65TG^{r}Oua^r (□).

(Fig. 5 *B*). Catalase and AOx were both present to a greater extent in the mutant Z24 than in the wild-type and mutant Z65. The 3-ketoacyl-CoA thiolase translation product was detected in each type and had the same molecular mass as that of the rat liver 44-kD thiolase precursor (Fig. 5 *C*). These three enzyme proteins, i.e., AOx, catalase, and thiolase, were much less abundant in the CHO cells than in rat liver (compare lanes *1* with *2–4*).

We then examined the biosynthesis of two peroxisomal integral membrane proteins, 70 IMP and 22 IMP. Immunoprecipitation of in vitro translation products with anti-rat liver 70 IMP antiserum gave a [^{35}S]polypeptide band in all three types of cells, practically comigrating with rat liver 70 IMP (Fig. 5 *D*). Among the cell-free products, [^{35}S]polypeptide with the same mobility as rat liver 22 IMP was similarly immunoprecipitated by anti-rat liver 22 IMP antiserum (Fig. 5 *E*, lanes *1–4*). ^{35}S-labeled 22 IMP of the parent and mutant cells was displaced during immunoprecipitation by isolated cold 22 IMP of rat liver, thereby indicating that the polypeptide band was the 22 IMP translation product of CHO cells (lanes *5–8*).

Complementation Analysis of Mutant Cell Lines

To analyze genetics of the two mutant cell lines, we carried out a complementation test by means of cell fusion with polyethylene glycol. After a combination of fusions among the wild-type and mutant cells, the hybridized cells were selected by growing in the HAT plus ouabain medium. The efficiency of cell fusion was ~1%.

To observe the intracellular localization of catalase, we examined the latency of catalase by means of digitonin titration (Fig. 6). Total catalase activity (as 100%) of each type of cell was measured in the presence of 1% Triton X-100; 10–20% activity was detected in the absence or at 10 μg/ml of digitonin for both wild-type and mutant cells (Fig. 6 *A*). At the concentration of 25 μg/ml of digitonin, ~25% of catalase was noted for the parent cells, whereas ~50% was detected for the mutants. Full catalase activity was obtained at 100 μg/ml for both mutants, but only 35% catalase was observed in the case of wild-type cells. Treatment with 200 and 300 μg/ml of digitonin released 65 and 100% activity, respectively, for the wild-type CHO cells. At the concentration >100 μg/ml of digitonin, 100–115% of catalase was constantly detected for the mutants. These results indicate that the catalase of the mutants is completely released at 100 μg/ml of digitonin, whereas nearly 65% of the catalase remains latent in the wild-type CHO cells. This striking difference in the latency of catalase seems useful to distinguish cell type.

The variants of mutants Z24 and Z65 resistant to thioguanine (TG^r) and ouabain (Oua^r) showed the same profile of catalase latency as seen in the parent cells (Fig. 6 *B*). When the mutant cells Z24 and Z65 were fused, respectively, with the wild-type cell, both hybrids showed, upon treatment with digitonin, nearly the same profile of catalase activity as seen in the wild-type CHO cells, thereby indicating that these mutants are recessive to the wild-type cells (Fig. 6 *B*).

Catalase activity of the hybrid between the mutant Z24 and Z65 was detected at a rate of 30–40% at 100 μg/ml of digitonin and full activity was released at 300 μg/ml, thereby indicating the catalase latency of Z24 × Z65 hybrid (Z24 × Z65TG^{r}Oua^r and Z65 × Z24TG^{r}Oua^r) was close to that of the wild-type cells. This was confirmed in the subcellular fractionation study (Table II). The latency of catalase was restored after cell hybridization between Z24 and Z65. Thus, the mutants Z24 and Z65 apparently belong to different complementation groups. Cell fusion between homologous types of cells, i.e., Z24 × Z24TG^{r}Oua^r and Z65 × Z65TG^{r}Oua^r showed that the catalase latency was the same as observed in the respective cell mutant, before the fusion, hence the hybridization procedure itself did not alter the property of the cells (Fig. 6 *C*).

Morphological analyses of the wild-type, mutant, and hybrid cells were performed by means of immunocytochemistry with anti-catalase antibody and horseradish peroxidase-labeled second antibody (Fig. 7). Numerous particles immunoreactive with anti-catalase antibody were found in the wild-type cells, but no particle was seen in either mutant, Z24 or Z65. There the cytosol was stained with DAB. In the hybrid of Z24 with Z65TG^{r}Oua^r, catalase-positive particles were as numerous as in the wild-type cells (Fig. 7, Z24 × Z65). The results are consistent with findings in the measurements of catalase latency described above.

DHAP-ATase activity of the hybrid of Z24 with Z65TG^r-Oua^r was restored to nearly that seen in the wild-type (Table II).

To investigate the biosynthesis of peroxisomal proteins in the hybrid of Z24 with Z65TG^{r}Oua^r, radio-labeling experiments were carried out (Fig. 8). Unlike observations of the mutants, [^{35}S]polypeptides corresponding to A, B, and little C components of AOx as well as the 41-kD mature thiolase were detected by immunoprecipitation after 48 h of continuous labeling of the fused cells (Fig. 8, *continuous label*).

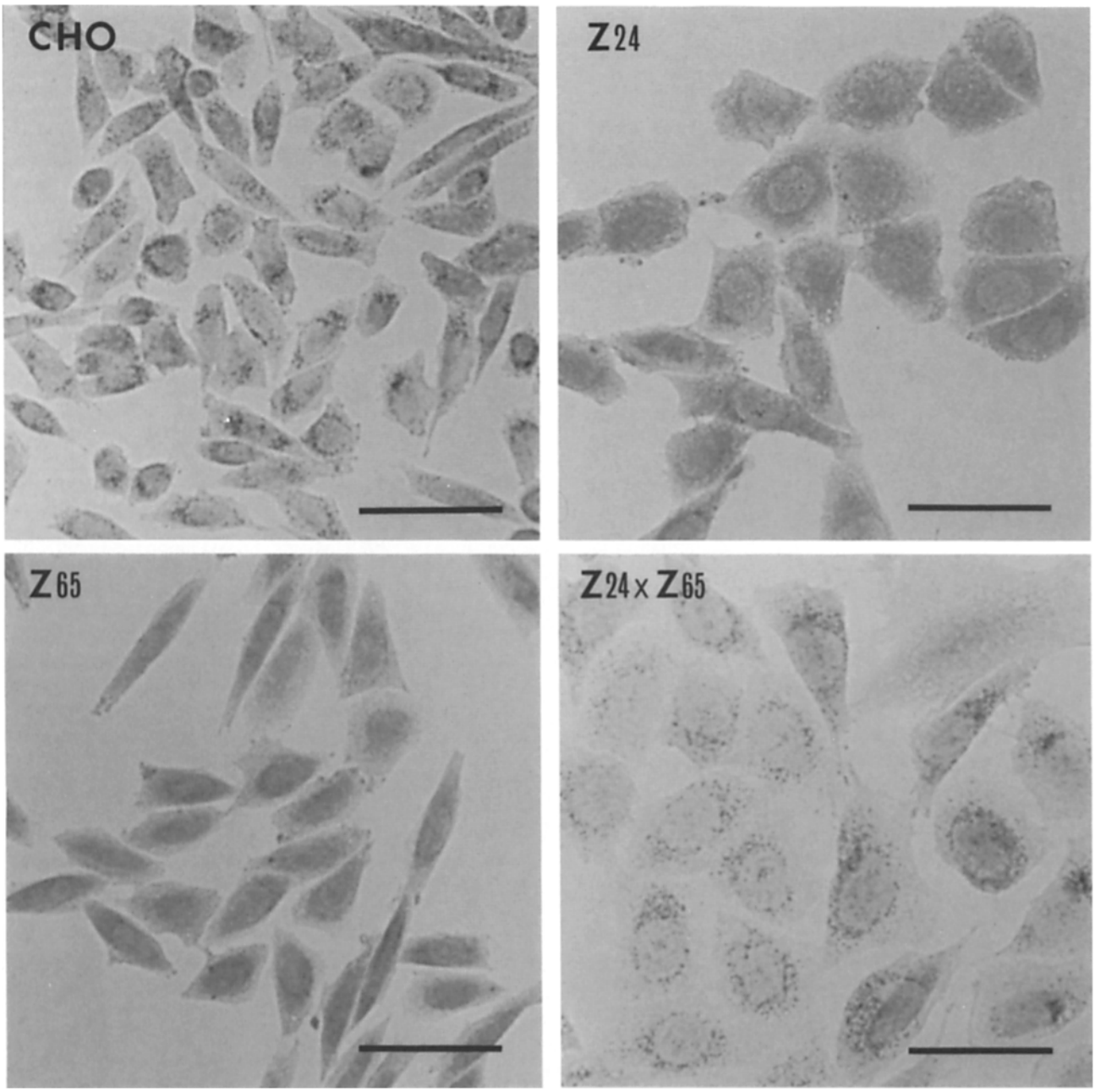

Figure 7. Immunocytochemical analysis of wild-type, mutant, and hybrid cells. Wild-type (CHO), mutants (Z24 and Z65), and hybrid (Z24 x Z65TG^{r}Oua^r) cells were respectively stained for catalase by the immunocytochemical procedure described in Materials and Methods, and examined under an Olympus BH-2 microscope. ×450. Bar, 50 μm.

Pulse–chase radio-labeling experiments revealed AOx initially in the 75-kD A form; B and C components increased with time during the chase for 24 h, with a concomitant decrease in the A component (Fig. 8, *pulse–chase*). Immunoprecipitation with anti-thiolase antibody at 1 h-labeling (chase, 0 h) yielded two [^{35}S]polypeptides corresponding to a larger precursor and to a mature polypeptide, respectively. The thiolase precursor disappeared with an apparent half-life within 1 h; only the 41-kD mature form was evident after a 3-h chase. Catalase and 70 IMP were also synthesized and remained as observed in the wild-type CHO cells (not shown). Therefore, the hybrid of two mutants apparently synthesizes and processes peroxisomal proteins, as so do the wild-type cells.

All these observations taken together show that the genetic lesions in these two mutants differ and that the mutations are recessive.

Discussion

Genetic disorders in which peroxisomes are deficient and/or their functions are impaired have been investigated (Goldfischer and Reddy, 1984; Moser, 1987; Schutgens et al., 1986; Zellweger et al., 1988). The biogenesis of peroxi-

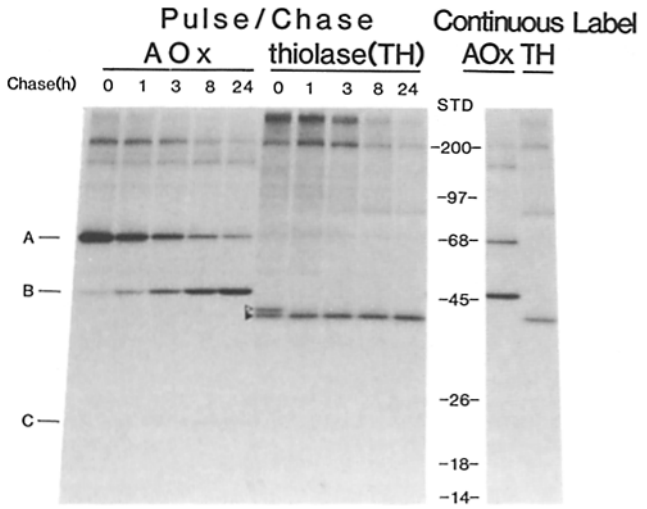

Figure 8. Biosynthesis of peroxisomal proteins in hybrid cells. Hybrid cells (Z24 x Z65TG^{r}Oua^r) selected in HAT plus ouabain medium after fusion were labeled with [^{35}S]methionine and analyzed as described in Figs. 3 and 4. *STD*, molecular mass standards; *TH*, thiolase. *A*, *B*, and *C* on the left indicate the components *A*, *B*, and *C* of AOx, respectively. *Open* and *solid arrowheads* are as in Fig. 4. Exposure, 7 d.

somes appears to be severely impaired in Zellweger syndrome, a prototype and the most severe disorder among the peroxisomal diseases (Wanders et al., 1988).

We isolated CHO cell mutants defective in peroxisomal DHAP-ATase activity, the enzyme responsible for the synthesis of ether-glycerolipids such as plasmalogens. The two mutants we obtained both possessed only 2% or less of the wild-type DHAP-ATase activity, consistent with the data of Zoeller and Raetz (1986) who isolated several CHO cell mutants. The frequency of mutation by EMS treatment described here, one mutant out of 10^4 cells, is consistent with the generally accepted concept (Thompson and Baker, 1973).

We characterized morphological and biochemical properties of the mutants. Peroxisomes were never detected in either of the mutants. Catalase is synthesized and is active as in the wild-type cells, but is localized in the cytosol. Several peroxisomal proteins, including β-oxidation enzymes, are likewise synthesized as in the wild-type cells. AOx is synthesized as a 75-kD A polypeptide component but is not converted to B and C components and is rapidly degraded, thereby implying that the conversion depends on the presence of peroxisomes. This may be explained by the finding by several groups, including ours, that the proteolytic cleavage of A form to B and C occurs inside the peroxisomes (Miura et al., 1984; Miyazawa et al., 1989).

Thiolase is synthesized as a larger precursor and is processed to its mature form in the wild-type cells, as was noted for the rat liver (Miura et al., 1984; Fujiki et al., 1985), whereas it remains as a larger precursor in both mutants. This suggests that processing of thiolase occurs with translocation to the peroxisomes, as is the case with rat liver thiolase (Miura et al., 1984; Miura, S., and Y. Fujiki, manuscript in preparation). It is noteworthy that the proteolytic processing of thiolase proceeds much more rapidly than the conversion of component A of AOx to B and C (Fig. 5). The bifunctional protein was equally present in all types of cells (Fig. 4). This enzyme, however, has been reported to be greatly reduced or absent in human peroxisome deficiency disorders, such as Zellweger syndrome (Wanders et al., 1988). It may be stable in the CHO mutants.

Peroxisomal integral membrane proteins are also synthesized, one of which, 70 IMP is as stable in the mutant cells as in the wild-type, but the subcellular localization in the mutants is unknown. 22 IMP was detected when total RNA was translated (Fig. 5), however, we did not observe 22 IMP in the radio-cell labeling experiment. Whether 22 IMP is degraded or stable in the mutant cells was not determined.

Together, the mutants appear to de defective in the assembly of peroxisomes.

Complementation analyses revealed that the mutation(s) in mutants Z24 and Z65 are recessive to parental cells. The mutants belong to different complementation groups, because cell fusion between two mutants resulted in a hybrid where the morphological and biochemical properties of peroxisomes are restored. As seen in the wild-type cells, catalase is particle bound; DHAP-ATase activity is fully active; the biosynthesis and processing of enzymes are normal. This implies that the genetic lesion(s) in the two mutants reside most likely on the different genes needed for assembly of the peroxisomes. It was noted by Allen et al. (1987. *J. Cell Biol.* 105:157*a*.) that all of the CHO mutants isolated by Zoeller and Raetz (1986) appear to belong to the same complementation group.

It is generally agreed that peroxisomal proteins are synthesized on free polyribosomes in the cytosol and posttranslationally transported to preexisting peroxisomes, whereupon new peroxisomes are formed by growth and division (Lazarow and Fujiki, 1985; Borst, 1986). Several possibilities can be considered for the impairment of peroxisome assembly in the mutants we have described. First, peroxisomal constituent proteins are synthesized but cannot be transported, because there is no membrane vesicle formed to be targeted. Second, cytosolic protein factors necessary for import are defective. Peroxisomal membrane polypeptides are synthesized and remain, but the localization is not clear. Third, membrane proteins may locate on other endomembranes, hence peroxisomal membrane vesicles do not form. Another possibility is that membrane proteins are indeed localized in peroxisomal membrane vesicles but they are defective in translocation of newly synthesized peroxisomal content proteins. Santos et al. (1988) suggested that such peroxisomal ghost vesicles seemed to be present in fibroblasts from a patient with Zellweger syndrome. These peroxisomal membrane vesicles would not be recognizable by conventional catalase-cytochemistry. Finally, it is also possible that a combination of these factors may be involved.

Plasmalogens may possibly be essential for the formation of peroxisomal membrane vesicles, however, we found that supplementation of plasmalogens to the CHO cell mutants, Z24 and Z65 did not lead to formation of peroxisomes (data not shown), a finding consistent with the results of Zoeller et al. (1988).

All the features of the mutants described above are similar to those noted in biopsy samples and fibroblasts from Zellweger patients (Moser, 1987; Wanders et al., 1988). Among human autosomal recessive peroxisome diseases such as Zellweger syndrome and neonatal adrenoleukodystrophy, five complementation groups have thus far been character-

ized by means of somatic cell fusion followed by the measurement of DHAP-ATase and particle-bound catalase activities, and the number is on the increase (Brul et al., 1988). Thus, it is of interest to determine to which complementation group the mutants Z24 and Z65 belong. The CHO cell mutants are a pertinent model which can be used to study molecular bases and primary defects of these human peroxisomal diseases and related syndromes that are characteristic in dysfunctions of peroxisomes. These mutant cell lines will also facilitate identification and characterization of gene(s) and gene product(s) essential for assembly of fully functional peroxisomes.

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