Isolation of Chinese Hamster Ovary Cell Lines Expressing Human Acyl-Coenzyme A/Cholesterol Acyltransferase Activity

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Abstract. We have previously reported the isolation of Chinese hamster ovary cell mutants deficient in acyl-coenzyme A/cholesterol acyltransferase (ACAT) activity (Cadigan, K. M., J. G. Heider, and T. Y. Chang. 1988. *J. Biol. Chem.* 263:274–282). We now describe a procedure for isolating cells from these mutants that have regained the ability to synthesize cholesterol esters. The protocol uses the fluorescent stain Nile red, which is specific for neutral lipids such as cholesterol ester. After ACAT mutant populations were subjected to chemical mutagenesis or transfected with human fibroblast whole genomic DNA, two revertants and one primary transformant were isolated by virtue of their higher fluorescent intensities using flow cytofluorimetry. Both the revertants and transformant have regained large amounts of intracellular cholesterol ester and ACAT activity. However, heat inactivation experiments revealed that the enzyme activity of the transformant had heat stability properties identical to that of human fibroblasts, while the ACAT activities of the revertants were similar to that of other Chinese hamster ovary cell lines. These results suggest that the molecular lesion in the ACAT mutants resides in the structural gene for the enzyme, and the transformant has corrected this defect by acquiring and stably expressing a human gene encoding the ACAT polypeptide. Secondary transformants were isolated by transfection of ACAT mutant cells with primary transformant genomic DNA. Genomic Southern analysis of the secondary transformants using a probe specific for human DNA revealed several distinct restriction fragments common to all the transformants which most likely comprise part or all of the human ACAT gene. The cell lines described here should facilitate the cloning of the gene encoding the human ACAT enzyme.

Acyl-coenzyme A/cholesterol acyltransferase (ACAT)[1] is an intracellular enzyme that uses cholesterol and fatty acyl-coenzyme A (CoA) to form cholesterol esters (10, 50). The enzyme is localized to the rough endoplasmic reticulum in rat liver (2, 24); is highly regulated in many cell types and tissues; and is believed to play an important role in cholesterol metabolism in various cells and tissues such as the small intestinal mucosa, hepatocytes, and the steroid hormone–producing tissues (10, 50).

Although ACAT has been studied intensively, little is known about its molecular structure. In rat liver, the active site of the enzyme has been localized to the cytoplasmic surface of the microsomal vesicles using a combination of detergent and protease treatments (24, 34), but whether the enzyme spans the entire membrane could not be determined. Recent chemical modification studies have demonstrated that an essential histidyl and sulfhydryl residue(s) may reside at or near the active site of the enzyme. ACAT activities from different rabbit tissues have different sensitivities to the histidyl-modifying reagents, suggesting the existence of different ACAT subtypes (31, 32).

1. *Abbreviations used in this paper*: ACAT, acyl-coenzyme A/cholesterol acyltransferase; CHO, Chinese hamster ovary; CoA, coenzyme A; LDL, low density lipoprotein.

ACAT activity has been solubilized and reconstituted from various cultured cells (4, 17, 29), rat liver (51), and pig liver (16). Although these procedures have allowed enzyme activity to be measured in a defined lipid environment, little progress has been made in purifying the solubilized preparations. Partially purified ACAT fractions that contain up to 100-fold higher enzyme-specific activity than unfractionated pig liver microsomes (16) still contain numerous protein bands when analyzed by gel electrophoresis (unpublished results from this laboratory). The gene(s) encoding this enzyme has not been isolated and no antibodies directed against ACAT have been reported.

Chinese hamster ovary (CHO) cells are a fibroblast-like cell line in which cholesterol ester synthesis is highly regulated by exogenous sources of cholesterol, such as low density lipoprotein (LDL) (8, 17, 33), and by endogenous cholesterol synthesis (7). This laboratory recently reported the isolation of CHO cell mutants almost entirely lacking ACAT activity (5). All of the isolated mutants belonged to the same complementation group and possessed a defect in the ACAT enzyme itself or in a factor needed for production of the enzyme. We now report a procedure for isolating revertants that have regained enzyme activity. The selection uses the fluorescence-activated cell sorter and Nile red, a fluorescent

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stain with high affinity for neutral lipids such as cholesterol esters (22, 23). We have also used this procedure to isolate cells expressing high ACAT activity after transfection of enzyme-deficient mutants with human high molecular weight DNA. A combination of biochemical and DNA hybridization experiments strongly suggests that the transformants have acquired and express a human ACAT structural gene. The results reported here should facilitate the isolation and cloning of the human ACAT gene, which should prove invaluable in efforts to understand the molecular structure and regulation of this enzyme.

Materials and Methods

Reagents

Oleyl-CoA was synthesized as described by Stadtman using oleyl anhydride (49) and [^{3}H]oleyl-CoA was synthesized by another method (3). Quantitation of the oleyl-CoA preparations were made assuming an extinction coefficient at 260 nm of 15.4 mM^{-1} cm^{-1} (57). Purity was judged as 98% by TLC analysis in a solvent system of butanol/acetic acid/water (5:2:3) and by measuring the A_{232}/A_{260} ratio (49). Nile red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) was purified from Nile blue (Sigma Chemical Co., St. Louis, MO) by the method of Thorpe (53) as modified by Greenspan et al. (23). Concentrated stocks were made in ethanol and stored at 4°C protected from light. Compound 58-035 (3-[decyldimethylsilyl]-*N*-[2-(4-methylphenyl)-1-phenylethyl]propanamide) was provided by Dr. John Heider (Sandoz Inc., East Hanover, NJ). Mevinolin was a gift from Alfred Alberts (Merck & Co., Inc., Rahway, NJ). Both of these compounds were added to the culture medium from a concentrated dimethyl sulfoxide stock. pSV2-neo in *Escherichia coli* strain HB101 was a generous gift from Dr. Peter Southern (Research Institute of Scripps Clinic, La Jolla, CA) and pBLUR8 in HB101 was provided by Dr. Joanne Zurlo (Dartmouth Medical School, Hanover, NH) with permission from Dr. Warren Jelinek (New York University Medical Center, New York). The chloroform, methanol, and isopropanol used for the cholesterol analysis were from Mallinckrodt Inc. (Paris, KY) or Fisher Scientific Co. (Pittsburgh, PA) and were nanograde and spectranalyzed grade, respectively. Cholesterol oxidase was generously provided by Dr. Albert Chen (Beckman Instruments, Inc., Fullerton, CA). Horseradish peroxidase (P-6140), cholesterol esterase (C-1892), phosphatidylcholine type XI (P-2772), and all other enzymes and biochemical reagents were from Sigma Chemical Co. Other organic solvents and chemicals were from Fisher Scientific Co. and were of reagent grade quality.

Cell Culture

A primary culture of human fibroblasts was obtained from the foreskin of a healthy newborn. The tissue was dissociated using bacterial collagenase and trypsin as described by Dayer et al. (15) and the culture was used between the seventh and fifteenth passage. Human fibroblasts and CHO cell lines were grown as monolayers; human fibroblasts in MEM (Gibco Laboratories, Grand Island, NY) supplemented with 2 mM glutamine; and CHO cells in F-12 medium minus linoleic acid. Both media were supplemented with antibiotics as previously described (4, 5) and 10% FCS (Sigma Chemical Co.). When delipidated FCS was used, it was prepared according to a published procedure (6) as modified by Chin and Chang (14). Human LDL (d = 1.019-1.063 g/ml) was prepared from human plasma by sequential flotation in the presence of protease inhibitors as previously described (5).

Mutagenesis and DNA Transfections

The ACAT mutant cell line, AC29, was mutagenized with 125 μg/ml *N*-nitroso-*N*-ethylurea as previously described (5). Cotransfections of AC29 with the plasmid pSV2-neo, which confers resistance to the cytotoxic drug G418 (48), and human high molecular weight DNA were carried out as follows. High molecular weight DNA was prepared from cultured cells according to a published procedure (56). AC29 cells were transfected by the calcium phosphate coprecipitation technique of Graham and van der Eb (21) as modified by Wigler et al. (55) except that the precipitate was left on the monolayers for 12 h. During the course of this work, it was found that the frequency of G418-resistant colonies obtained by transfection could be increased significantly by the addition of 100 μM chloroquine to the medium (Hasen, M., and T. Y. Chang, manuscript in preparation). This modification was used in the isolation of the secondary transformants. 48 h after the initial plating, the transfected cells were grown in medium containing 500 μg/ml G418 (Gibco Laboratories). Medium was replaced every other day for 1 wk, and then the cells were allowed to grow for 1 wk more without a change of medium. The resistant colonies were then pooled and maintained at 100 μg/ml G418 while the brightest Nile red-stained cells were isolated.

Isolation of Nile Red-positive Cells

All solutions used in preparing the cells for the cytofluorograph and fluorescence microscopy were in sterile Hank's balanced salt solution containing no phenol red. Confluent monolayers were washed three times and incubated with 0.003% trypsin for 15-20 min at 37°C. Cells were transferred to a sterile tube containing 1/10 vol of 0.075% soybean trypsin inhibitor (Sigma Chemical Co. T-9253) and then 2 vol of 150 ng/ml Nile red (final ethanol concentration, 0.2%) was added. The cells were gently mixed and allowed to sit for 15 min at room temperature protected from light. The stained cells were analyzed for green fluorescence (excitation wavelength 488 nm; emission wavelength 515-530 nm) using the Ortho Diagnostic Systems, Inc. (Raritan, NJ) cytofluorograph system 50H with the brightest 0.1% or 0.2% cells sorted into plates containing medium without sodium bicarbonate (to keep the pH from becoming too alkaline). The media containing the sorted cells were diluted two- to threefold with media containing bicarbonate and placed in a 5% CO_2 incubator at 37°C. The next day the medium was replaced with bicarbonate-containing medium. After 10 d colonies were visible and could be prepared for another round of cell sorting or examined under a low power phase microscopy for the presence of intracellular lipid droplets.

Fluorescence Microscopy

Cells were grown on glass cover slips. To prepare for viewing, the cover slips were rinsed five times and then stained for 6 min at room temperature with 100 ng/ml Nile red. After staining, the coverslips were rinsed, mounted onto slide chambers, and kept moist with the salt solution. Immediately thereafter, the cells were viewed using a Carl Zeiss, Inc. (Thornwood, NY) universal microscope and a 67× achromat oil-immersion phase-contrast objective (Carl Zeiss Inc.) with differential-interference contrast or epifluorescence (excitation 485 ± 10 nm; emission 520-560 nm) optics. Paired micrographs were taken with TMAX-400 film (Eastman Kodak Co., Rochester, NY) and developed according to instructions given by the manufacturer.

Sterol Analysis

Cells were grown in 25-cm^2 flasks and harvested in 1 ml 0.2 M NaOH as described previously (5). The NaOH sample was neutralized by adding HCl and phosphate buffer (8) and then Folch extracted (19). After drying under N_2, the samples were resuspended in 1 ml isopropanol and aliquots (30 or 60 μl) were taken for cholesterol determination using a fluorometric procedure (25) either with or without cholesterol esterase preincubation. Control experiments using radiolabeled cholesterol revealed recoveries of between 90 and 100% after extraction. The procedure for preparing the samples described above differs from the one previously used by this laboratory (5). In the previous procedure, aliquots were taken from the NaOH sample, extracted, dried, and resuspended in a small volume of isopropanol. The effect of the two different protocols upon the values obtained is discussed in the Results section.

ACAT Assays

For the [^{3}H]oleate pulse, all F-12 media were supplemented with 1.5 mM $CaCl_2$ because F-12 medium is low in calcium (0.3 mM) and the binding of LDL to its receptor is calcium dependent (20). The monolayers were pulsed with a [^{3}H]oleate/BSA solution and analyzed for incorporation of radiolabel into cholesterol oleate as previously described (5, 8) except that the blank was determined and subtracted from the reported values by pulsing AC29 cells grown in the presence of 58-035, a specific inhibitor of ACAT (43). The blank value was between 1.2 and 2.0 pmol/min per mg. For the in vitro ACAT assays, cell homogenates were prepared by the hypotonic-shock and scraping method (12) and used immediately. The microsomal assay has been described in detail previously (5, 8, 17). The reconstituted ACAT assay was performed as described in Cadigan and Chang (4). During this study it was found that solubilized cell extracts diluted into cholesterol-

phosphatidylcholine vesicles prepared with different lots of Sigma Chemical Co. type XI phosphatidylcholine gave distinct but reproducible enzyme-specific activities. Using the phosphatidylcholine lot 67F-8410, ACAT-specific activities were approximately half that of the values obtained with the lot previously used (46F-8430; these results can be found by comparing activities reported in Figs. 5 and 6). TLC analysis revealed no detectable contaminants in the two lots. This phenomenon is currently being pursued in this laboratory and will not be discussed further in this report. In all three assays described above, control experiments using [^{14}C]cholesterol oleate as a standard revealed recoveries of 70–77% after extraction and TLC. All protein determinations were made using the Peterson modification (40) of the method of Lowry et al. (36); no TCA precipitation was performed on the NaOH cell extracts in the sterol analysis and the [^{3}H]oleate pulse.

Southern Analysis

Whole genomic DNA samples were digested with restriction enzymes (15 U/μg DNA) for 36 h at 37°C. The digested samples were run on an 0.8% agarose gel and transferred to nylon filters (ICN Laboratories, Inc., Plainview, NY) by the method of Southern (47) as modified by Reed and Mann (41). Filters were prehybridized in a solution containing 25 mM KPO_4, pH 7.4, 5× SSC, 5× Denhart's solution, 100 μg/ml sonicated and denatured salmon sperm DNA, 50% formamide, and 1% SDS for 12 h at 42°C and then incubated with an identical solution containing 10% dextran sulfate and the denatured ^{32}P-probe. The 300-bp Bam HI fragment of the plasmid pBLUR8, which contains a human repetitive element of the Alu family (44), was used as the probe. The plasmid was digested with Bam HI and the Alu-containing fragment was excised from a low melting agarose gel and radiolabeled by the oligolabeling method of Feinberg and Vogelstein (18). After incubation in the hybridization buffer for 48 h at 42°C, filters were washed two times in 2× SSC/0.1% SDS supplemented with 0.05× bovine lacto transfer technique optimizer (27), followed by a 0.1× SSC/0.1% SDS wash, both at room temperature, before a final wash in 0.1× SSC/0.1% SDS at 55°C for 60 min. The filters were air dried and exposed to Kodak X-OMAT AR film with a Dupont Co. (Wilmington, DE) Lightning Plus intensifying screen for 3–5 d before developing.

Results

Isolation of ACAT Revertants and Transformant

Nile red is a highly fluorescent compound which preferentially partitions into hydrophobic environments such as intracellular neutral lipid droplets (22, 23). The Nile red fluorescent patterns of the ACAT mutant, AC29, and its parental cell line, 25-RA, were compared. 25-RA was isolated from wild-type CHO cells by its resistance to the cytotoxic effects of 25-hydroxycholesterol (11). Unlike wild-type cells, the uptake of LDL and the rate of endogenous cholesterol synthesis in 25-RA cells are partially resistant to suppression by exogenous sterols (5, 11). This results in an elevated rate of cholesterol ester synthesis leading to a large accumulation of intracellular cholesterol ester (5, 9). In contrast, the mutant cell line AC29 contains <1% of the ACAT activity of 25-RA and has greatly reduced intracellular cholesterol ester (5).

As shown in Fig. 1, there is a clear difference in the appearance of 25-RA and AC29 cells when viewed with differential-interference contrast (Fig. 1, *a* and *c*) and epifluorescence (Fig. 1, *b* and *d*) optics. 25-RA contained numerous birefringent perinuclear particles (Fig. 1 *a*) which were brightly stained with Nile red (Fig. 1 *b*). These particles were not found in AC29 cells, or in 25-RA cells grown in the presence of the specific ACAT inhibitor, 58-035 (data not shown). The light, diffuse fluorescence seen in AC29 cells (Fig. 1 *d*) is seen in all CHO cells examined thus far that lack cholesterol ester. The filters used for the fluorescent micrographs in Fig. 1 allowed only green fluorescence to be seen (520–560 nm).

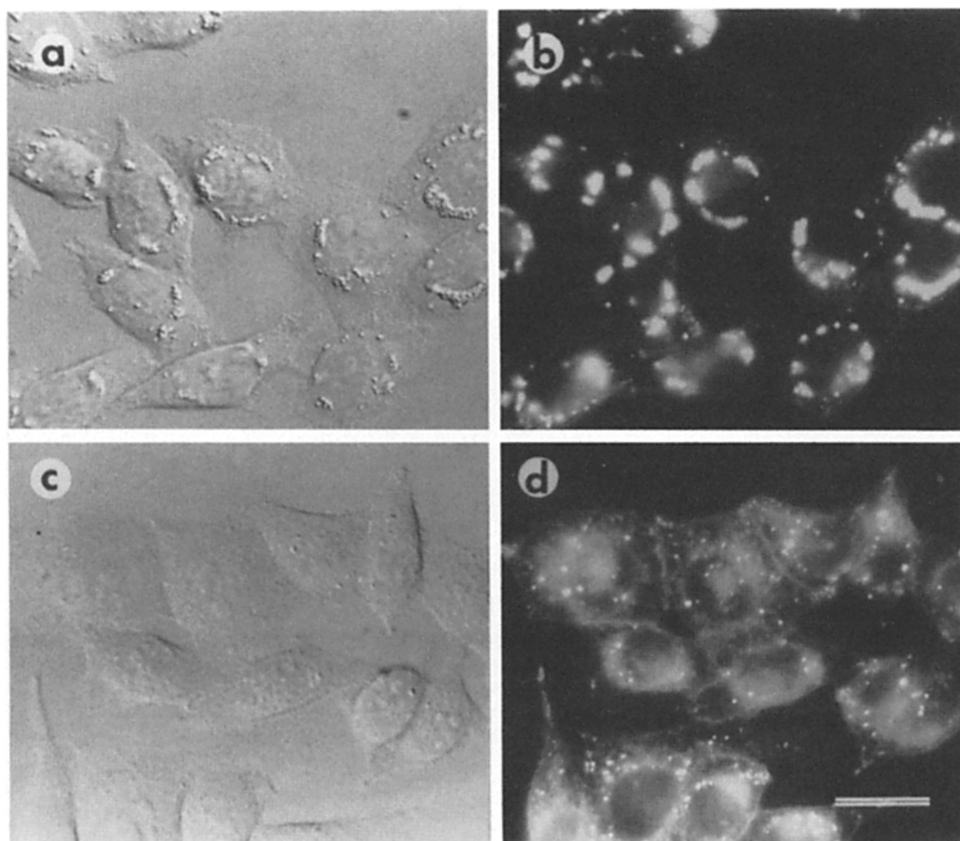

Figure 1. Nile red–stained 25-RA and AC29 cells viewed with differential-interference contrast or fluorescence microscopy. 25-RA (*a* and *b*) and AC29 (*c* and *d*) were plated at a density of 6.5 × 10^4 cells per 8-cm^2 well containing a glass coverslip and F-12 medium plus 10% FCS and grown for 62 h with a medium change at 48 h. Coverslips were prepared and viewed with differential-interference contrast (*a* and *c*) or fluorescence (*b* and *d*) microscopy as described in Materials and Methods. Bar, 20 μm.

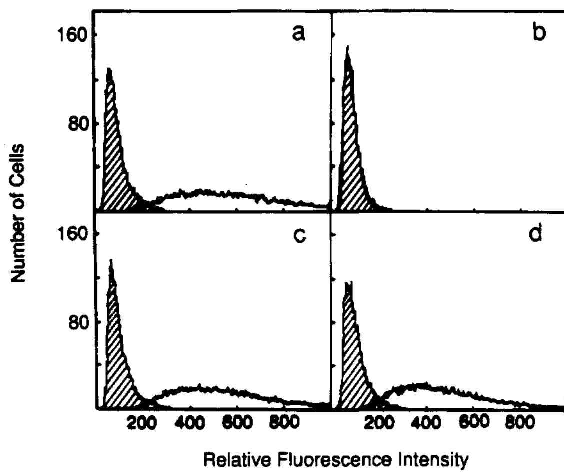

Figure 2. Flow cytometric analysis of Nile red–stained 25-RA (*a*); AC29 (*b*); and a revertant, 29CS1 (*c*), and transformant, 29T1 (*d*), of AC29. 2×10^5 cells were plated in 8-cm² wells and grown as described in Fig. 1. 29T1 cells were grown in the presence of 100 μg/ml G418. Cells were resuspended in buffer containing 100 ng/ml Nile red and analyzed by flow cytometry as described in Materials and Methods. Each histogram corresponds to 10^4 cells and the hatched histograms represent cells that had been plated and grown in medium containing 200 ng/ml 58-035.

When a different filter set is used (one that allows light >520 nm to be seen) the perinuclear droplets in 25-RA cells appeared yellow-gold and the diffuse signal in AC29 cells appeared orange-red. Greenspan and Fowler (22) have demonstrated that at low concentrations (100 ng/ml), Nile red emitted maximal fluorescence at 576 nm (yellow-gold) when it partitioned into hydrophobic environments, such as neutral lipid droplets. When Nile red interacted with phospholipids, maximal emission of fluorescence occurred at 628 nm (red). Apparently all the Nile red taken up by 25-RA cells is associated with the lipid droplets, so no diffuse staining due to other more polar lipids is seen.

It was previously shown that after Nile red staining, cholesterol ester–loaded, mouse peritoneal macrophages could be distinguished from unloaded macrophages by the cytofluorograph (23). As shown in Fig. 2, this was also true in CHO cells. A sevenfold difference was found in relative mean fluorescent intensities between Nile red–stained AC29 and 25-RA cells when analyzed for green fluorescence (mean ± SD for AC29 cells, 83.5 ± 34.4; and 25-RA cells, 578.8 ± 229.1). When grown in the presence of 58-035, the profile of AC29 cells was unaffected, but that of 25-RA was dramatically altered to become almost identical to AC29 (Fig. 2, *a* and *b*; *hatched curves*).

AC29 cells were mutagenized with *N*-nitroso-*N*-ethylurea and grown for 5–6 d to allow time for an altered phenotype to be expressed. The mutagenized cells were then stained with Nile red and sterilely sorted with the cytofluorograph as described in Materials and Methods. After two rounds of sorting, two putative revertants were isolated independently, 29CS1 and 29CS3. After Nile red staining, these cell lines had an identical fluorescent profile compared to 25-RA as analyzed by the cytofluorograph (Fig. 2 *c* and data not shown), and appeared similar to 25-RA when viewed with differential-interference or phase contrast and epifluorescence optics (data not shown).

It was found that the cell survival after sorting was extremely low (2–10%). Changes in the flow rate of the sorting (500–2,000 cells/s) or the time between staining and sorting (15–120 min) did not affect the survival rate (data not shown). To determine the cause of cell death, the experiment described in Table I was performed. Unstained or Nile red–stained cells were plated directly into dishes (200 cells/dish), or 200 of the 0.2% brightest cells were sorted via cytofluorography into dishes. For unsorted cells, the results indicated that Nile red had no effect on cell plating efficiency, which ranged from 25 to 41% for all cell types examined. There was a small reduction in cell survival in all cell types when unstained cells were passed through the flow cytometer (11–19%). When Nile red–stained cells were sorted, only the cell types with high Nile red fluorescence had survival rates comparable to unstained cells. The cells with low Nile red fluorescence (AC29 and AC29-G418^r, which are AC29 cells transfected with the plasmid pSV2-neo as described below) had a two- to fivefold lower survival rate than the other cell types. For our particular purpose, this was an unforeseen advantage, since AC29 cells were being selected against by their lower fluorescence and by a lower survival rate.

Approximately 7×10^6 mutagenized AC29 cells were sorted and ~300 cells (from six separate groups) survived. From these survivors, two independent revertants were isolated after an additional round of sorting. At the setting used for the first sort, 0.1% of all AC29 cells and ~40% of all 25-RA cells would be sorted. Since 29CS1 and 29CS3 cells have almost identical fluorescent profiles to 25-RA (Fig. 2 *c* and data not shown), there was an $\sim 4 \times 10^2$-fold enrichment of the revertants after the sort. In addition, there was probably a twofold difference in cell survival between the revertants and AC29 (Table I). Thus the frequency of isolation of revertants in this selection was $\sim 2/3 \times 10^2 \times 1/4 \times 10^2 \times 1/2 = 1/1.2 \times 10^5$.

Table I. Effect of the Fluorescent Stain Nile Red and the Cytofluorograph on Cell Survival

	Cell survival			
	Unsorted		Sorted	
Cell type	Unstained	Nile red	Unstained	Nile red
	%	%	%	%
25-RA	29.6 ± 3.3	30.6 ± 3.7	16.3 ± 2.0	23.6 ± 3.8
AC29	25.5 ± 2.2	28.6 ± 3.7	16.1 ± 2.2	7.1 ± 0.7
29CS1	28.3 ± 1.0	29.0 ± 2.5	19.3 ± 1.0	13.8 ± 3.3
AC29-G418^r	32.0 ± 0.5	32.1 ± 3.0	10.8 ± 3.0	3.3 ± 0.3
29T1	33.0 ± 4.7	41.0 ± 3.0	18.3 ± 2.7	16.6 ± 0.7

Monolayers were grown to confluency in F-12 medium plus 10% FCS (AC29-G418^r and 29T1 were grown in medium containing 100 μg/ml G418) and cells were resuspended in buffer with or without 100 ng/ml Nile red as described in Materials and Methods. Each sample was then subjected to the cytofluorograph and 200 of the 0.2% brightest cells were sorted into 100-mm dishes as described in Materials and Methods. In addition, the cell concentration of each unsorted sample was quantitated using a hemocytometer and 200 cells were plated directly into 100-mm dishes. After 10 d, the cell survival was determined by counting the number of colonies in each dish. The values are given as the mean of three dishes ± SD.

Table II. Cholesterol and Cholesterol Ester Analysis of 25-RA, AC29, and a Revertant (29CS1) and Transformant (29T1) of AC29

Cell type	Cholesterol	Cholesterol ester
	nmol/mg protein	
25-RA	81.9 ± 7.7	234.0 ± 29.6
AC29	105.5 ± 21.6	3.8 ± 1.2
29CS1	72.1 ± 12.6	146.3 ± 13.7
29T1	77.8 ± 16.7	131.6 ± 15.0
25-RA (58-035)	86.8 ± 13.0	12.5 ± 2.9
AC29 (58-035)	81.6 ± 11.4	2.7 ± 5.1

5×10^5 cells were plated in 25-cm^2 flasks and grown in F-12 medium plus 10% FCS for 66 h with a medium change at 48 h and 64 h. 29T1 was grown in the presence of 100 μg/ml G418 throughout the experiment. The cells were harvested and analyzed for sterol content as described in Materials and Methods. Where indicated, 25-RA and AC29 were grown in medium containing 200 ng/ml 58-035. Duplicate aliquots were taken from duplicate dishes and the results are shown as the mean value ± SD.

Next, we transfected AC29 cells with exogenous DNA to determine if we could isolate transformants that had regained the ability to synthesize cholesterol ester. AC29 cells were cotransfected with high molecular weight DNA from human fibroblasts and pSV2-neo, a plasmid containing the gene conferring G418 resistance. The transfected cells were isolated by their acquired resistance to the toxic neomycin analogue, G418 (see reference 48). In several experiments, transfection frequencies ranging from 5×10^{-4} to 1.7×10^{-4} were obtained. It is known that cells transfected with selectable genes by the calcium phosphate precipitation technique also take up large amounts of the carrier DNA used (39), in our case human fibroblast genomic DNA. Of the 1.2×10^4 G418-resistant colonies obtained, one putative transformant, 29T1, was isolated (Fig. 2 *d*). The transformant had numerous Nile red–positive perinuclear particles similar to 25-RA and the revertants, which were not present when the cells were grown in the presence of the ACAT-specific inhibitor, 58-035 (data not shown).

Cholesterol Ester Metabolism in the Revertants and Transformant

As shown in Table II, analysis of the cholesterol ester mass of 25-RA, AC29, a revertant (29CS1), and the transformant (29T1) was consistent with the Nile red data, shown in Fig. 2. The majority of the cholesterol in 25-RA, 29CS1, and 29T1 cells was in the esterified form, the revertant and transformant always observed to have less cholesterol ester (~55–65%) than 25-RA cells. AC29 and cells grown in the ACAT inhibitor 58-035 had very little cholesterol ester. The values shown in Table II are almost twice as high as the values reported previously from this laboratory, although the results are qualitatively very similar (5). We believe this is due to differences in the preparation of the samples before the cholesterol analysis was performed (see Materials and Methods for details). The earlier method of preparing the samples, in which aliquots were taken from a NaOH-dissolved cell extract, has been found to be nonlinear in the amount of cholesterol detected with increasing volume of the aliquots (data not shown) which lead to an underestimation of the absolute values. The new method, in which the entire sample is extracted and then resuspended in isopropanol before aliquoting for analysis, was found to give a linear response (data not shown) and was used in all subsequent experiments.

The fact that the revertants and transformant had a large decrease in Nile red fluorescence and cholesterol ester when grown in 58-035 suggested that ACAT is active in these cells. To confirm this and to examine whether cholesterol ester synthesis is regulated normally in the isolated cell lines, the rate of cholesterol ester synthesis in response to LDL present in the growth medium was examined by [^{3}H]oleate pulse (Fig. 3). LDL is known to activate ACAT in CHO cells (8, 17, 33). The rates of cholesterol ester synthesis in 25-RA, 29CS1, 29CS3, and 29T1 cells were all activated at least 50-fold by LDL while AC29 showed no response. In this experiment mevinolin, a competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase (1), was added to the cells shortly before and during the incubation with LDL to inhibit endogenous cholesterol biosynthesis, which also activates ACAT and thus partially masked the activation by LDL in 25-RA and its derived cell lines (data not shown). A small amount of mevalonate was added with the mevinolin, to permit the synthesis of nonsteroidal isoprenoids (45).

Table III shows the in vitro ACAT activities of the above mentioned CHO cells plus that of human fibroblasts. As seen

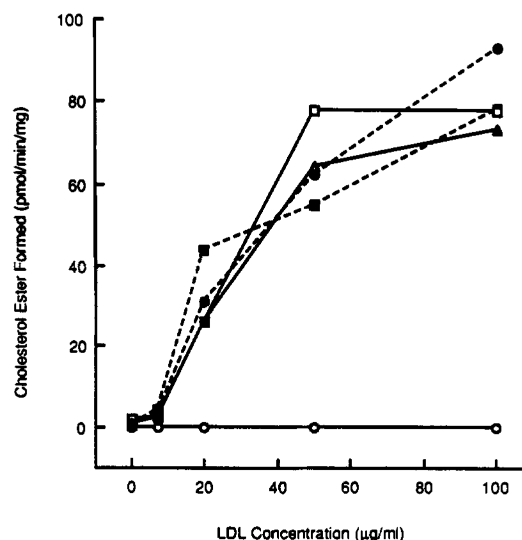

Figure 3. Activation of cholesterol ester synthesis by LDL in 25-RA (●; *dashed line*); AC29 (○; *solid line*); and two revertants, 29CS1 (□; *solid line*) and 29CS3 (■; *dashed line*), and a transformant, 29T1 (△; *solid line*), of AC29. 1.2×10^5 cells were plated in 25-cm^2 flasks and grown for 2 d in F-12 medium containing 10% FCS supplemented with $CaCl_2$ as described in Materials and Methods. After 2 d, the monolayers were washed three times with PBS and switched to F-12 medium supplemented with 10% delipidated FCS + 35 μM oleic acid and grown for an additional 36 h with a medium change 24 h after the switch. Then cells were changed to delipidated FCS medium containing 10 μM mevinolin and 230 μM mevalonate and grown for 6 h before a fresh medium containing increasing amounts of LDL was added. 29T1 cells were grown in the presence of 100 μg/ml G418 throughout this experiment. After 6 h of additional growth, the cells were pulsed with [^{3}H]oleate and analyzed for cholesterol-[^{3}H]oleate formed as described in Materials and Methods. Values are the average of duplicate flasks and ranged within 10% of the mean.

Table III. Microsomal and Reconstituted ACAT Activity of 25-RA, AC29, Human Fibroblasts, Revertants (29CS1 and 29CS3), and a Transformant (29T1) of AC29

Cell type	ACAT specific activity	
	Microsomal	Reconstituted
	pmol/min/mg	
25-RA	90.8	184.8
AC29	0.6	0.0
29CS1	68.8	91.1
29CS3	50.9	88.2
Human fibroblasts	6.3	30.1
29T1	69.5	67.8

For CHO cell lines, 3 × 10^6 cells were plated in 150-cm^2 flasks containing F-12 medium plus 10% FCS and grown for 66–70 h with a medium change at 48 h and 2 h before harvest. 29T1 was grown in the presence of 100 μg/ml G418 at all times. 7 × 10^5 human fibroblasts were plated in 150-cm^2 flasks containing MEM plus 10% FCS and grown for 7 d, with medium changes on day 4 and 6, and 2 h before harvest. Cells were harvested and assays were performed as described in Materials and Methods. The values are the means from duplicate assays and ranged within 10% of the mean.

previously, after reconstitution into cholesterol/phosphatidylcholine vesicles, the enzyme activity was elevated compared to the activity of the enzyme in the native microsomal membrane (4, 8, 17). In three separate experiments (Table III, Fig. 5, and data not shown), the reconstituted activities of 29CS1 and 29CS3 ranged from 39 to 51% and 42 to 48% of 25-RA–reconstituted ACAT activity, respectively. The ACAT activity of the putative transformant was tenfold higher than the activity found in human fibroblasts in the microsomal assay and approximately twice as high in the reconstitution assay.

Heat Stability of ACAT

As outlined in the previous section, the revertant and transformant cell lines isolated from AC29 have similar characteristics. Even though the frequency of obtaining the transformant was an order of magnitude higher than was found for the revertants isolated from mutagenized AC29 cells, the possibility existed that 29T1 was a G418-resistant clone which had reverted to an ACAT-positive phenotype during or after transfection.

While characterizing the ACAT activity of human fibroblasts, it was found that it was more stable at elevated temperatures than the enzyme activity of CHO cells. If 29T1 is an AC29 clone which has acquired the human ACAT gene, then its enzyme activity should have the heat stability characteristics similar to that found in human fibroblasts. This turned out to be the case. Fig. 4 shows the heat inactivation curves of microsomal enzyme activity at 45°C for the relevant cell lines. The inactivation curves, which do not follow simple first-order kinetics, revealed a distinct difference between the various cell types. The curves for the human fibroblasts and the transformant 29T1 were very similar, and demonstrated an enzyme activity substantially more heat stable than those of the other CHO cell lines. The curve for revertant 29CS1 was almost identical to the one for 25-RA, while the curve for the other revertant 29CS3 was much more heat labile.

The different heat inactivation curves between the cell lines examined in Fig. 4 could arise in part from differences in the cellular membrane environment where the ACAT enzyme resides. To rule out this possibility, heat inactivation experiments using ACAT activities reconstituted into liposomes were performed. In this procedure the ACAT enzyme is solubilized from its native membrane by detergent and inserted into cholesterol-phospholipid vesicles of defined concentration (4). As shown in Fig. 5, all of the reconstituted enzyme activities were less stable than the microsomal activities, but the difference between the heat stability of the human and 25-RA enzyme was even more striking ($t_{1/2}$ for the human fibroblast enzyme was 5.5 min; for the 25-RA enzyme it was 1.3 min). The enzyme inactivation profile of 29T1 was almost identical to the one for human fibroblasts, while those for 25-RA, 29CS1, and 29CS3 were very similar.

When cells are transfected by the calcium phosphate coprecipitation technique used in this report, up to 2,000 kb of exogenous DNA are stably integrated into each transformant (39). Therefore, it is probable that many human genes have been taken up and expressed in the primary transformant, 29T1. To remove superfluous human DNA not involved in transforming AC29 to an ACAT-positive cell line, we isolated secondary transformants. This was accomplished by transfecting AC29 with pSV2-neo and whole genomic DNA isolated from 29T1 cells. Three secondary transformants, 29T2-4, 29T2-8, and 29T2-10, were isolated from 3.4 × 10^4 G418-resistant colonies. These cell lines had the

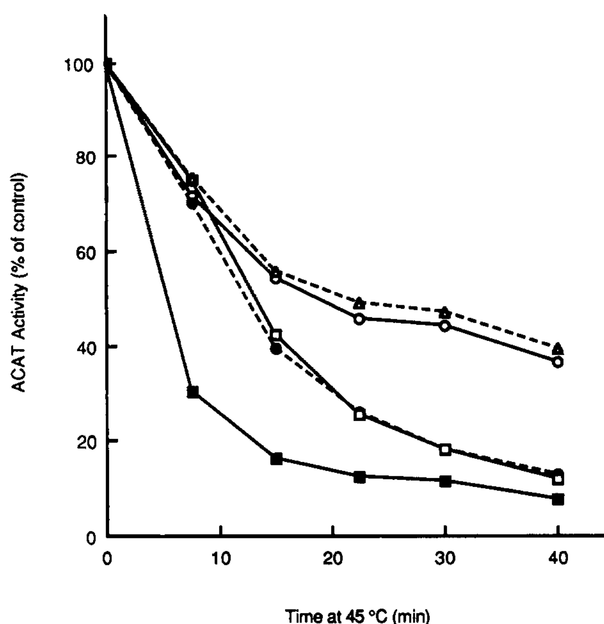

Figure 4. Heat inactivation of microsomal ACAT activity from cell extracts of 25-RA (●; *dashed line*); human fibroblasts (○; *solid line*); and two revertants, 29CS1 (□; *solid line*) and 29CS3 (■; *solid line*), and a transformant, 29T1 (△; *dashed line*), of AC29. CHO cells and human fibroblasts were grown and harvested as described in Table III. Cell extracts at 4°C were preincubated in a 20°C water bath for 3 min before incubation in a 45°C water bath for the indicated times. Samples were then placed on ice until assayed for enzyme activity as described in Materials and Methods. The control values for 25-RA, 29CS1, 29CS3, human fibroblasts, and 29T1 were 71.3, 51.0, 36.9, 5.9, and 68.5 pmoles/min per mg, respectively. Duplicates ranged within 10% of the mean.

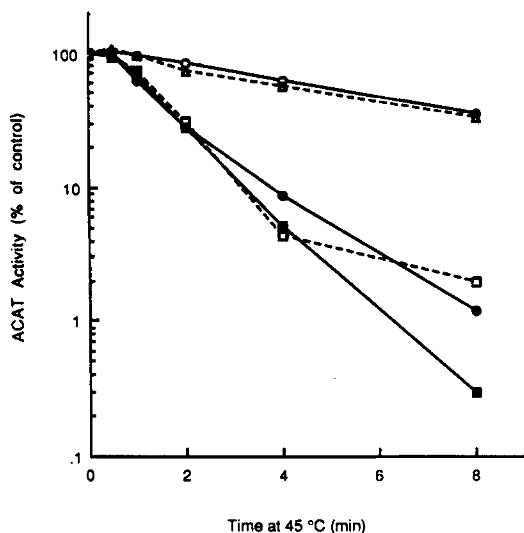

Figure 5. Heat inactivation of reconstituted ACAT activity from 25-RA (●; *solid line*); human fibroblasts (○; *solid line*); and two revertants, 29CS1 (□; *dashed line*) and 29CS3 (■; *solid line*), and a transformant, 29T1 (△; *dashed line*), of AC29. CHO cells and human fibroblasts were grown and harvested as described in Table III. Cell extracts were solubilized and diluted into cholesterol-phosphatidylcholine vesicles according to Cadigan and Chang (4). The reconstituted enzyme preparations were treated as described in Fig. 4 and assayed for activity. The control values for 25-RA, 29CS1, 29CS3, human fibroblasts, and 29T1 were 260.0, 101.2, 109.2, 52.0, and 93.9 pmoles/min per mg. Duplicates ranged within 10% of the mean.

perinuclear particles characteristic of 25-RA and the revertants and transformant isolated earlier as well as increased Nile red fluorescence and high ACAT activity. Fig. 6 demonstrated that the reconstituted enzyme activities of the secondary transformants had a rate of inactivation at elevated temperatures similar to the ones seen in the primary transformant and human fibroblasts, and distinct from the one in 25-RA cells.

Southern Analysis of Transformants Using a Labeled Human Repetitive Element

Alu repeats have been reported to be present in as many as 6–9 × 10^5 copies per haploid human genome (26, 42) and are found, on average, every few kilobases throughout the genome (52). To directly demonstrate that the isolated primary and secondary transformants have indeed stably integrated human DNA into their genomes, Southern analysis using a human repetitive element of the Alu family as the probe (44) was performed on restriction enzyme–digested, whole genomic DNA from the primary and secondary transformants. The results of one such experiment are shown in Fig. 7. The radiolabeled probe hybridized strongly to human DNA but not at all to AC29 DNA (Fig. 7, lanes *1* and *2*). Note that there is 2,000 times more AC29 DNA blotted onto the nylon filter than human DNA. The primary transformant, 29T1, was found to have a large amount of human sequences integrated into its genome. The secondary transformants, however, contained a relatively small amount of human sequences (see Fig. 7, lanes *4*–*9*). These results are similar to the ones previously reported by other laboratories (28, 35, 37, 46). As indicated in Fig. 7, there were discrete restriction fragments that the probe hybridized to, which are common to all three secondary transformants in the Hind III/Eco RI double digest (Fig. 7, lanes *7*–*9*). Common bands of 23, 9.7, 6.8, and 2.2 kbp were also found in the Hind III digest (Fig. 7, lanes *4*–*6*). In addition, there were also bands common to all three transformants in Eco RI digests (data not shown). It is highly likely that these common fragments contain at least part of the gene which confers human ACAT activity to AC29 cells. The sum of the common bands in each digest added up to 26–42 kbp. The functional gene could be smaller than the sum of the common fragments, or larger due to significant portions of the gene that do not contain an Alu repeat. Further experiments will be needed to more exactly define the size of the functional human ACAT gene.

Discussion

This report describes a new selection procedure for the isolation of CHO cells that have regained their ability to synthesize cholesterol ester from a population of AC29 mutant cells which are deficient in ACAT activity. The selection procedure uses cytofluorography and Nile red, a fluorescent dye that partitions preferentially into neutral lipid droplets (22, 23). As shown in Fig. 2, the majority of the fluorescent signal is due to cholesterol ester synthesized intracellularly, since there is an approximate sevenfold reduction in fluorescence intensity when the cells were grown in the presence of the ACAT inhibitor 58-035. Although there is a small discrepancy between the flow cytometric data in Fig. 2 and the

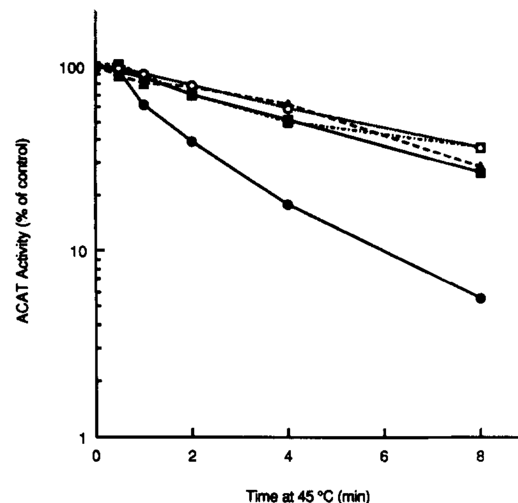

Figure 6. Heat inactivation of reconstituted ACAT activity from 25-RA (●; *solid line*); a primary transformant, 29T1 (△; *dashed line*); and three secondary transformants, 29T2-4 (□; *dashed/dotted line*), 29T2-8 (■; *solid line*), and 29T2-10 (○; *dotted line*), of AC29. CHO cells were grown and harvested as described in Table III. Extracts were reconstituted according to the procedure of Cadigan and Chang (4). The samples were treated as described in Fig. 4 and assayed for enzyme activity. The control activities for 25-RA, 29T1, 29T2-4, 29T2-8, and 29T2-10 were 137.2, 41.7, 39.6, 45.7, and 62.6 pmoles/min per mg. Duplicates ranged within 7% of the mean.

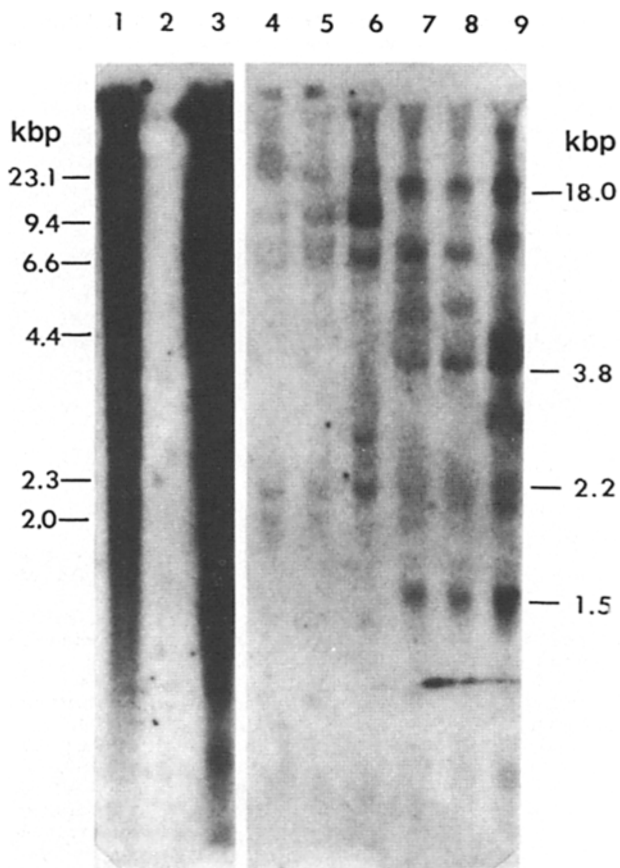

Figure 7. Southern analysis of enzyme-restricted, whole-genomic DNA from AC29, human fibroblasts, and the transformants probed with a radiolabeled human repetitive element. Genomic DNA of human fibroblasts (lane *1*), AC29 (lane *2*), 29T1 (lane *3*), 29T2-4 (lanes *4* and *7*), 29T2-8 (lanes *5* and *8*), and 29T2-10 (lanes *6* and *9*) were digested with Hind III (lanes *1–6*) or Hind III and Eco RI (lanes *7–9*), run on a 0.8% agarose gel, transferred to a nylon filter, and probed with radiolabeled Alu repetitive element as described in Materials and Methods. 20 μg of genomic DNA was used for each sample except for human fibroblasts (10 ng) and 29T1 (10 μg). A Hind III digest of lambda DNA served as molecular weight markers and are indicated on the left in kilobase pairs and the Eco RI/Hind III restriction fragments common to all the secondary transformants are indicated on the right in kilobase pairs.

chemical determination of cholesterol ester content in Table II (29CS1 and 29T1 have ~60% of the cellular cholesterol ester as 25-RA cells, while their Nile red fluorescent profiles are almost the same), Nile red can be viewed as an indirect assay for cholesterol ester in the CHO cell lines examined.

The survival of Nile red–stained AC29 cells after sorting through the cytofluorograph was very low. The data in Table I suggested that the Nile red staining plus cell sorting preferentially killed cholesterol ester–deficient cells, while cholesterol ester–rich cells are relatively unaffected. Although the reason for this specific killing is not known for certain, it may be that after excitation by the laser beam in the cytofluorograph, the Nile red compound becomes cytotoxic. In cells with large amounts of cholesterol ester, the toxic compound is trapped in the lipid droplets, thereby enabling the cells to survive. Since this is the first report using Nile red for sorting actively growing cells in the cytofluorograph, further experiments will be needed to determine if the unexpected cytotoxicity is observed when other cell types are examined. This phenomenon was beneficial in the selections described in this report, since cells with high amounts of cholesterol ester were enriched. However, it may prove difficult to use Nile red in selections where cells are selected for lower Nile red fluorescence.

The two revertants, 29CS1 and 29CS3, isolated from mutagenized populations of AC29 cells, have highly regulated cholesterol ester synthesis in intact cells and relatively high ACAT activity in vitro (Fig. 3 and Table III). The reconstituted enzyme activities of the revertants are between 39 and 51% of that found in 25-RA cell extracts (Table III, Fig. 5, and data not shown). This reconstitution assay is entirely dependent on the exogenous cholesterol present in the vesicles, which was at saturating amounts in our experiments, thus eliminating any differences in ACAT activities due to differences in the cholesterol composition of the microsomal membranes (4). Therefore, the revertants appear to have approximately half the enzyme content of 25-RA cells. The frequency of obtaining the revertants from mutagenized AC29 cells was calculated to be 8×10^{-6}, a value consistent with other reports of reversion of single genes (9, 13, 30, 38, 54). The low frequency of isolation of the ACAT mutants suggested that more than one gene needed to be inactivated to produce the ACAT mutant phenotype (5). We now propose, based on the biochemical data and the frequency of isolation of the revertants, that there are two active genes in the parental cell line 25-RA and that one of the two genes has been reactivated in the revertants.

A transformant was isolated after AC29 cells were transfected with whole genomic DNA obtained from human fibroblasts. This transformant, termed 29T1, appears to be very similar to the revertants with respect to cholesterol ester metabolism (Fig. 3 and Tables II and III). However, the heat inactivation curves shown in Figs. 4 and 5 clearly demonstrated that the ACAT activity of 29T1 has heat stability properties identical to that of the human fibroblast ACAT activity and distinct from the other CHO cells. AC29 could contain a defect in a structural gene encoding ACAT or in a gene needed for enzyme production. The heat inactivation data strongly favors the first possibility. If AC29 is a production mutant and transfection with human DNA corrected the mutation, the transformant's enzyme activity should have biochemical properties similar to that found in CHO cells, not human fibroblasts.

Three secondary transformants were isolated independently by transfecting AC29 with genomic DNA from 29T1 and were shown to possess human-like enzyme activity (Fig. 6). The finding that all three secondary transformants had heat stability properties similar to the primary transformant suggested that they have acquired the heat-stable (i.e., human) gene from the 29T1 genomic DNA used in the transfection. With the transfection protocol used, a very small amount of human DNA should remain in the secondary transformants (28, 35, 37, 46). This was confirmed by Southern analysis using a human repetitive element as the probe. Restriction fragments common to all three secondary transformants allowed a tentative approximation of the size of the structural gene for the human ACAT enzyme. We cannot rule out the possibility of more than one structural gene for the enzyme, but at present there is no genetic or biochemical evidence to support this possibility.

It is interesting to note that the transformants, which most

likely contain one human ACAT gene, have much higher enzyme activity than human fibroblasts (Table III), which may have two active genes. The microsomal activity of 29T1 is ten times that of human fibroblasts, while the reconstituted activity is only twice as high. This suggests that the enzyme has more cholesterol available to it in the CHO cell microsomal membrane than in the fibroblasts, not surprising since 29T1 is a 25-RA-derived cell line and has an elevated rate of cholesterol biosynthesis and increased number of LDL receptors compared to wild-type CHO cells (data not shown). The remaining twofold difference in enzyme activity could be explained by an increased level of expression, or a CHO cell–specific posttranslational modification of the human ACAT enzyme in the transformants.

In conclusion, the results reported in this paper strongly suggest that the molecular lesion in the ACAT-deficient mutants isolated previously in this laboratory (5) resides in the structural gene for the enzyme, which either prevents expression or leads to the production of an inactive enzyme. There are normally two active ACAT genes in CHO cells, but the reversion or transfection of one ACAT gene is sufficient to restore an ACAT-positive phenotype. The secondary transformants isolated possess one human gene encoding the ACAT enzyme and all three contain common restriction enzyme fragments which hybridize to a cloned human-specific repetitive element. These common fragments probably form part or all of the human ACAT gene. It should now be possible to isolate the gene from a secondary transformant genomic library, as has been accomplished for other human genes (28, 35, 46). The isolation of a functional ACAT gene will be the first step towards the preparation of the molecular tools necessary for probing the molecular structure and regulation of this enzyme.

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