Proc. Natl. Acad. Sci. USA
Vol. 75, No. 11, pp. 5452–5456, November 1978
Biochemistry

Mammalian cell mutant requiring cholesterol and unsaturated fatty acid for growth

(Chinese hamster ovary cells/induction defect/β-hydroxy-β-methylglutaryl-coenzyme A reductase/unsaturated fatty acid biosynthesis/lanosterol demethylation)

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Communicated by P. Roy Vagelos, September 1, 1978

ABSTRACT **A mutant requiring both cholesterol and oleate for growth has been isolated from mutagenized Chinese hamster ovary cells. By comparison with wild-type cells, sterol and unsaturated fatty acid biosynthetic activities in the mutant cells grown in fetal calf serum medium appear to be nearly intact. However, whole-cell radioactive acetate, mevalonate, dihydrolanosterol, and stearate incorporation studies show that sterol synthesis from acetate, lanosterol demethylation, and fatty acid desaturation are defective in the mutant cells grown in delipidated serum medium. *In vitro* enzyme assays with crude cell extracts demonstrated that β-hydroxy-β-methylglutaryl-coenzyme A reductase is not induced in the mutant. These experiments were substantiated by gas/liquid chromatographic analyses which showed the sterol content and the percentage unsaturated fatty acids in mutant cells to be drastically reduced when the cells are grown in delipidated serum medium. A spontaneous revertant exhibiting prototrophic growth in lipid-free medium has been isolated from 50×10^6 mutant cells. All three defects in this revertant reverted back in parallel, which suggests that these three biosynthetic activities may be controlled by a common regulatory mechanism.**

Specific lipid-requiring mutants isolated from cultured animal cells (1–3) should serve as unique biological tools for studies of regulation of lipid metabolism and for membrane investigations. In this report, we describe the isolation of a Chinese hamster ovary (CHO) cell mutant (designated mutant clone no. 1) defective in the induction of cholesterol biosynthesis. Preliminary biochemical characterization indicates that this mutant is incapable of inducing its β-hydroxy-β-methylglutaryl-coenzyme A (HMG-CoA) reductase activity. This mutant also appears to be defective in lanosterol demethylation activity and in the induction of unsaturated fatty acid biosynthesis. We believe that these three defects are closely associated because a spontaneous revertant has been isolated from the mutant cells in which all three defects are reverted in parallel.

MATERIALS AND METHODS

Lipids were from Sigma. Radioactive chemicals were from New England Nuclear except that [1-^{14}C]acetate was from Amersham/Searle. All other chemicals were of analytical grade.

Cells. CHO cell cultures were grown as monolayers in F-12 medium (linoleic acid-deleted) plus 10% fetal calf serum or 10% delipidated fetal calf serum (De-S) or in Higuchi medium with no protein supplement as described (2, 4). De-S was prepared according to a published procedure (5). Various lipid supplements were added directly to the medium as ≤1% alcoholic solutions.

Mutagenesis and Mutant-Enrichment Procedure. Wild-type CHO cells were grown to confluency in F-12 + 10% serum (medium A) in a 75-cm^2 Corning tissue culture flask and mutagenized with ethyl methanesulfonate (200 μg/ml) in 30 ml of medium A for 7 hr. Afterward, the cells were subcultured in medium A for 6 days. Cells were then trypsinized and plated in 20 75-cm^2 Corning flasks at 0.4×10^6 cells per flask in medium A. After 24 hr, the medium was replaced with 20 ml of F-12 + 0.5% dialyzed serum + 1 mM DL-mevalonate + 0.013% methylcellulose (Fisher, 15 centipoises) (medium B) for 6 or 8 hr. Fresh 1 mM bromodeoxyuridine in saline was added to each flask (0.2 ml/flask). Cells were grown in this medium for 43–47 hr. The medium was then drained off, the cells were washed twice with saline, and the flasks were exposed (bottom-side up) under a long-wavelength UV light source (UVSL-58 Mineralight, Ultraviolet Products, Inc.) at a distance of 5 cm for 15–20 min. Survivors were grown in medium A for 8 days, and the bromodeoxyuridine/light cycle (6) was repeated once more. Survivors were grown and cloned in medium A, and the colonies were tested for lipid auxotrophy by growing them in either 10% serum-supplemented or De-S medium. Potential auxotrophs were recloned before biochemical analyses. Spontaneous revertants were isolated by selecting survivors of 50×10^6 mutant clone no. 1 cells, 2×10^6 cells per 150-cm^2 Corning flask, grown continuously in F-12 + 10% De-S medium with frequent medium changes for 30 days. Revertants were also cloned before biochemical analyses.

Lipid Extraction Procedure. Cells were dissolved in 2 ml of 2 M NaOH as an aqueous slurry and transferred to graduated screw-capped conical extraction tubes (15-ml capacity). An equal volume of 95% ethanol was added and the solution was heated at 80°C for 1 hr. After saponification, each sample was evaporated under N_2 until the volume was reduced to 1 ml or less. The volume was then brought back to 2 ml with H_2O, and the nonsaponifiable lipid fraction was extracted four times with 4 ml of nanograde petroleum ether. The petroleum ether fraction was pooled, concentrated under N_2 to 2 ml, and washed twice with 1 ml of 3% $NaHCO_3$ and once with 1 ml of H_2O. The aqueous fraction after the petroleum ether extraction was acidified with 0.4 ml of 12 M HCl, and the saponifiable lipid fraction was extracted and washed in the same manner as above except that this fraction was washed once with 5 mM HCl instead of 3% $NaHCO_3$. Both fractions were stored in petroleum ether at −20°C before further analyses. For every extraction experiment, [1,2-^{3}H]cholesterol (19,500 cpm; 10 μg per tube) and [1-^{14}C]palmitic acid (55,000 cpm; 10 μg per tube) were used as external standards for recovery purposes.

Thin-Layer Chromatography (TLC). Redi/plates of silica gel G (Analtech, Inc., 20 × 20 cm) were preactivated at 100°C for 1 hr before use. For sterol analyses, a solvent system of methylene chloride/ethyl acetate, 97:3 (vol/vol), was used (2,

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Abbreviations: CHO, Chinese hamster ovary; De-S, delipidated fetal calf serum; HMG-CoA, β-hydroxy-β-methylglutaryl-coenzyme A; GLC, gas/liquid chromatography; TLC, thin-layer chromatography.

Table 1. Whole-cell [^{14}C]acetate incorporation into sterols

Cell clone	^{14}C-Labeled sterol synthesis* fcs medium	De-S medium	Extent of induction†
Wild-type	2833	65,695	23
Mutant no. 1	6397	2,785	0.4
Revertant I	3511	22,748	6.5
Mutant no. 215	2924	9,597	3.3

Cells grown in F-12 + 10% serum (fcs medium) were trypsinized and replated with 30 ml of F-12 + 10% serum medium in 75-cm^2 flasks at 0.4 × 10^6 cells per flask. After 2 days the medium was drained off. The cells were rinsed with 5 ml of saline, fed with 30 ml of F-12 + 10% serum or F-12 + 10% De-S (De-S medium), and grown for 24 hr. The medium was then removed and 2 ml of F-12 + 10% De-S containing sodium [1-^{14}C]acetate (15 μCi/ml; 59.1 mCi/mmol) was added. After the cells were incubated at 37°C for 1 hr, the pulse medium was removed and the cells were washed six times with 5 ml of cold saline. Cells were dissolved in 2 ml of 2 M NaOH and extracted; sterols were analyzed by TLC.

* Sterol synthesis is expressed as cpm incorporated per mg of protein.

† Column 3 ÷ column 2.

7). This TLC system does not distinguish lanosterol from dihydrolanosterol or other C_{30} sterols. For fatty acid methyl ester analyses, TLC plates were impregnated with 10% $AgNO_3$ in methanol, and a solvent system of hexanes/diethyl ether, 90:10 (vol/vol), was used (1, 8). Methyl esters of fatty acids were prepared by using diazomethane (9). For each TLC sample, the entire chromatogram was divided into 16 identical bands, scraped into individual scintillation vials (8-ml capacity), and assayed for radioactivity with a Packard liquid scintillation counter (75% efficiency for ^{14}C and 27% efficiency for ^{3}H).

Gas/Liquid Chromatography (GLC). Into each sample, 3 μg of 5-cholesten-3β-ol methyl ether and 10 μg of heptadecanoic acid were added as internal standards for quantitation. A mixture containing a fixed ratio of cholesterol, lanosterol, 25-hydroxycholesterol, cholesterol methyl ether, palmitic acid, palmitoleic acid, stearic acid, and oleic acid (10 μg of total sterols and 10 μg of total fatty acids) was used as an external standard to check for selective lipid extraction. Sterols were analyzed on a 3% OV-17 column (Supelco) at 270°C; fatty acid methyl esters were analyzed on a 10% DEGS (Supelco) column at 180°C. A Varian 2100 gas/liquid chromatograph equipped with CDS-111 automatic integrator was used. Proteins were determined by a microbiuret method (10). Other specific procedures are described in figure legends and tables.

Table 2. Whole-cell [^{3}H]mevalonate and [^{3}H]dihydrolanosterol incorporation into sterols by cells grown in De-S medium

Fraction	Incorporation, cpm/mg protein Wild-type	Mutant no. 1
^{3}H-Labeled lipids*		
Total nonsaponifiable lipids	13,439	7,857
Lanosterol	3,338	5,150
Cholesterol	7,968	1,038
^{3}H-Labeled sterols†		
Total sterols	14,009	15,861
Dihydrolanosterol	10,553	15,030
Cholesterol	2,549	156

Cells were grown as described in Table 1.

* After 36 hr in F-12 + 10% De-S, medium was removed and 3 ml of F-12 + 10% De-S containing 1 mM [5-^{3}H]mevalonic acid (20 mCi/mmol) was added. Cells were incubated at 37°C for 1 hr. The pulse was stopped and the cells were harvested as described in Table 1. Lipids were extracted and analyzed by TLC.

† After 27 hr in F-12 + 10% De-S, medium was removed and 16 ml of F-12 + 10% De-S containing [2-^{3}H]dihydrolanosterol (16 μg/ml; 23.1 μCi/mg) was added. Cells were incubated for 24 hr. The incorporation was stopped and the cells were harvested essentially as described in Table 1 except that the saline washes were preceded by two rinses with F-12 + 10% serum. Sterols were extracted and analyzed by TLC. The [^{3}H]dihydrolanosterol used was 99% pure as determined by GLC analysis on an OV-17 column.

Table 3. Whole-cell [^{14}C]acetate incorporation into fatty acids

Cell clone	% desaturation* fcs medium	De-S medium	Extent of induction†
Wild-type	16	22	1.4
Mutant no. 1	15	8.3	0.55
Revertant I	15	16	1.1
Mutant no. 215	18	25	1.4

Experiments were done as described in Table 1. Fatty acids were extracted, methylated, and analyzed by TLC.

* Percent desaturation is expressed as radioactivity in unsaturated fatty acids divided by radioactivity in total fatty acids.

† Column 3 ÷ column 2.

RESULTS

Growth Properties of Mutant Clone No. 1 and Revertant I Cells. As shown in Fig. 1*A*, wild-type CHO cells proliferate in De-S medium. Their growth is enhanced slightly by medium supplemented with oleic acid (1) but is unaffected by cholesterol. The revertant I clone, which was isolated as a spontaneous revertant of mutant clone no. 1 cells, also grew healthily in De-S medium but multiplied at a slightly slower rate. In De-S medium without lipid supplement, mutant clone no. 1 cells died after dividing only once; however, they grew as healthily as wild-type cells when the medium was supplemented with both cholesterol and oleic acid. As shown in Fig. 1*B*, both lipids are required because addition of either cholesterol or oleic acid to the medium alone gave only minimal growth support to the mutant. Stearate, a saturated fatty acid, had no effect on the growth of the mutant cells. These experiments indicate that mutant clone no. 1 is a specific auxotroph for both cholesterol and unsaturated fatty acid.

Table 4. Whole-cell [^{14}C]stearate incorporation experiment with cells grown in various media

Cell clone	Growth medium	Incorporated radioactivity* × 10^{-3}	% desaturation†
Wild-type	I	55.2	27
	II	45.7	32
	III	49.6	37
	IV	51.6	37
Mutant no. 1	I	53.9	22
	II	35.4	13
	III	41.2	21
	IV	53.3	18

Cells were grown and switched to various media as described in Fig. 4. A 1-hr pulse was performed with 3 ml of F-12 + 10% De-S containing 18 μM [18-^{14}C]stearate (4.7 mCi/mmol) and 0.57 mg of bovine serum albumin per ml. The stearate/albumin complex used was prepared as described (1, 13). After the pulse, cells were washed twice with cold F-12 + 10% serum, twice with cold 2% fatty acid-free bovine serum albumin in saline, and five times with cold saline and then harvested as described in Table 1. The fatty acids were extracted and analyzed by TLC. Refer to Fig. 4 for composition of growth media I–IV.

* Values are expressed as cpm incorporated per mg of protein.

† Expressed as 100 × (radioactivity in unsaturated fatty acids divided by radioactivity in total fatty acids).

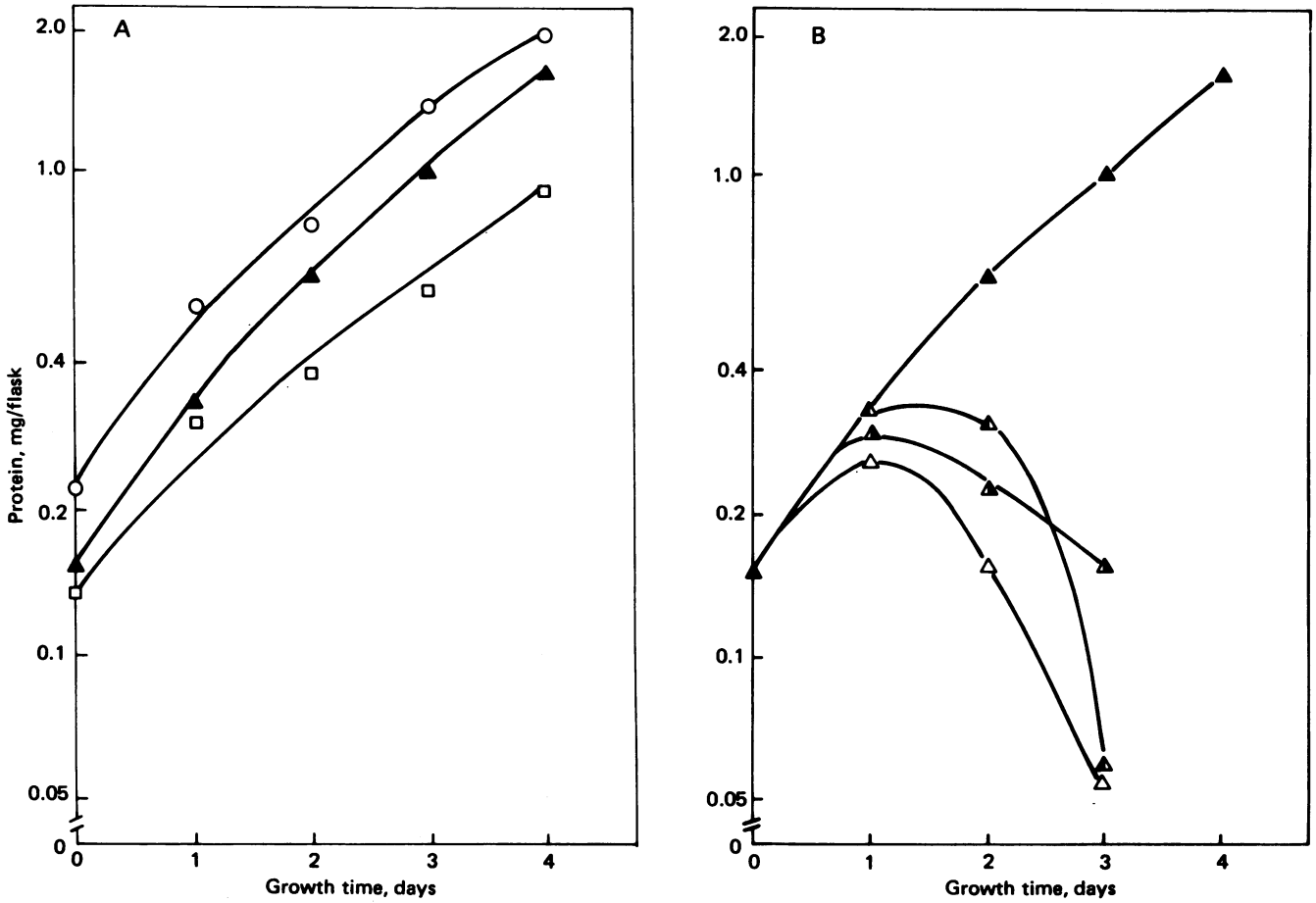

FIG. 1. Growth curves of various cell clones in F-12 + 10% De-S with or without lipid supplements. Cells grown in F-12 + 10% fetal calf serum were trypsinized and replated at 0.1 × 10^6 cells per 25-cm^2 flask in 10 ml of the same medium. After 2 days, the medium was drained off, the cells were rinsed with 5 ml of saline, and the medium was replaced with 10 ml of F-12 + 10% De-S with or without lipid supplements. Medium was renewed every day. Cell growth was followed in duplicate flasks by measurement of cellular protein content. (*A*) ○, Wild-type cells, no supplement; ▲, mutant no. 1 cells, supplemented with oleate (5.7 μg/ml) and cholesterol (5 μg/ml); □, revertant I cells, no supplement. (*B*) Mutant no. 1 cells with or without lipid supplements. △, No supplement or with stearate (5.7 μg/ml); ▲, with cholesterol (5 μg/ml); ▲, with oleate (5.7 μg/ml); ▲, with oleate (5.7 μg/ml) and cholesterol (5 μg/ml).

Rates of Sterol Biosynthesis in Whole Cells. Table 1 compares the rates of incorporation of [^{14}C]acetate into sterols in four different cell types grown either in 10% serum or in 10% De-S medium. Although the rate of sterol biosynthesis in wild-type cells was greatly enhanced (23-fold increase) by deprivation of lipids in the growth medium, such induction did not occur in mutant clone no. 1 cells. Revertant I cells exhibited subnormal but clearly measurable induction (6.5-fold increase). Mutant clone no. 215 cells, characterized previously (2) as being defective at the stage of lanosterol demethylation, also exhibited measurable induction (3.3-fold increase).

Table 5. Lipid composition of cells grown in F-12 + 10% De-S for 3 days

Cell clone	Sterol content*	% unsaturated fatty acids†
Wild-type	18	72
Mutant no. 1	6.9	31
Revertant I	20	70

Cells were grown as described in Fig. 1. Cellular lipids were analyzed by GLC.

* Expressed as μg of sterol per mg of protein; cholesterol constituted over 98% of the total sterols in each case.

† Percentage unsaturated fatty acids is expressed as 100 × (amount of palmitoleic acid and oleic acid divided by amount of total fatty acids). Palmitic acid, palmitoleic acid, stearic acid, and oleic acid constituted over 90% of the total cellular fatty acids in these cell clones.

The composition of the ^{14}C-labeled sterols synthesized from [^{14}C]acetate in these four cell types is shown in Fig. 2. In serum medium, in contrast to results with mutant clone no. 215 cells, the lanosterol demethylation activity of mutant clone no. 1 cells appeared to be normal by comparison with that of the wild-type and revertant I cells. In De-S medium, the lanosterol demethylation activity of mutant clone no. 1 cells was defective compared with that of wild-type and revertant I cells.

Data in Table 1 and Fig. 2 indicate the following: (*i*) mutant clone no. 1 is defective in the induction of sterol biosynthesis and is defective in lanosterol demethylation activity in De-S medium, (*ii*) because mutant clone no. 215 was shown to be able to induce sterol biosynthesis, the induction defect in sterol biosynthesis in mutant clone no. 1 cannot simply be attributed to its defective lanosterol demethylation. [^{3}H]Mevalonate pulse and [^{3}H]dihydrolanosterol incorporation experiments were next carried out in wild-type and mutant clone no. 1 cells grown in De-S medium (Table 2). The results confirmed the acetate pulse data by showing lanosterol demethylation activity in the mutant cells grown in De-S medium to be defective.

HMG-CoA Reductase Assay *In Vitro*. As shown in Fig. 3, when cells were switched from serum medium to De-S medium for 24 hr, the HMG-CoA reductase activity in wild-type cells was enhanced about 12-fold but such induction did not occur

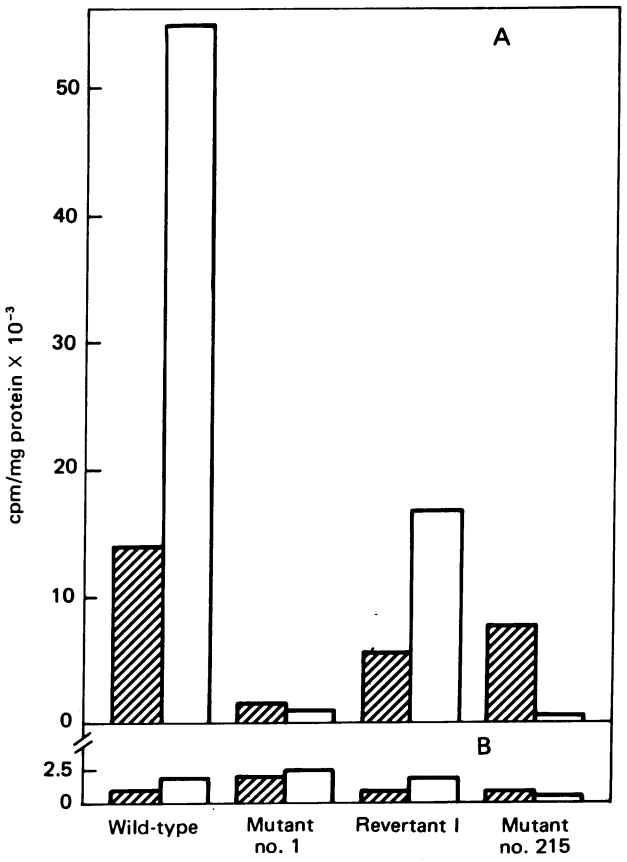

FIG. 2. Incorporation of ^{14}C into sterols after [^{14}C]acetate pulse. Cells were grown and treated as described in Table 1. ^{14}C-Labeled sterols were analyzed by TLC. Hatched area, lanosterol; open area, cholesterol. Experiments were done with cells grown in De-S (*A*) or in serum (*B*) medium.

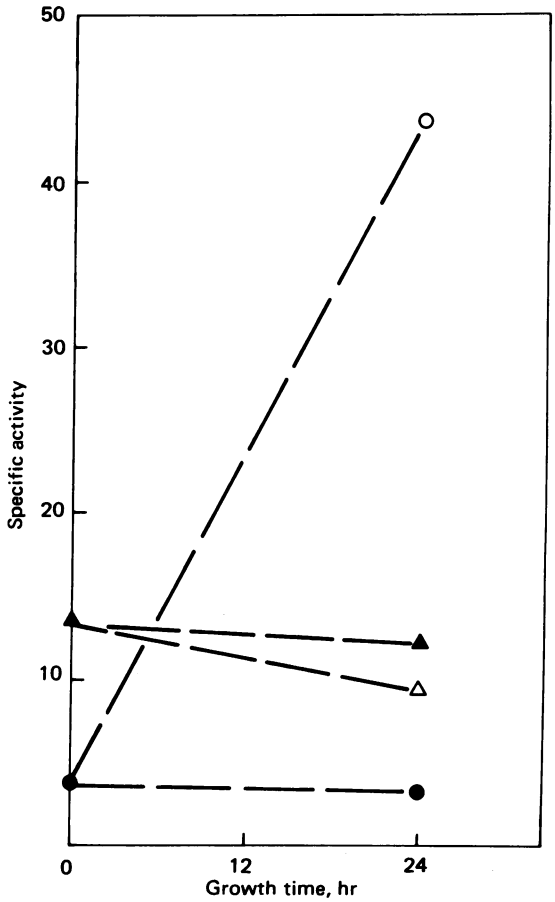

FIG. 3. HMG-CoA reductase activities (nmol of mevalonate formed per mg of whole cell protein per hr) in wild-type (●, ○) and mutant clone no. 1 (▲, △) cells grown in F-12 + 10% serum (●, ▲) or in F-12 + 10% De-S (○, △). Cells grown in F-12 + 10% serum were trypsinized and replated at 2.0×10^6 cells per 75-cm^2 flask in 25 ml of the same medium. After 30 hr, the cells were rinsed with 5 ml of saline and the medium was replaced with 25 ml of F-12 + 10% serum or F-12 + 10% De-S. For reductase assay, cells were rinsed five times with cold saline (4°C) and twice with cold hypotonic buffer [1 mM $MgCl_2$/1 mM Tris·HCl, pH 7.6 (buffer J)]. The cells were then scraped into 0.2 ml of buffer J, transferred to a glass Dounce homogenizer, and allowed to swell at 4°C for 3 min. After a few strokes with a tight-fitting pestle, complete cell breakage was checked under a phase-contrast microscope. Small aliquots of this homogenate were taken for protein measurement, and another 120 μl was mixed with 30 μl of hypertonic buffer (250 mM KH_2PO_4, pH 7.4/12.6 mM $MgCl_2$/150 mM EDTA/385 mM KCl/50 mM dithiothreitol) and used for HMG-CoA reductase assay. The assay used was a modification of the micro-method described by Shapiro *et al.* (11). Quantitation of mevalonate formed was done by TLC as described (12).

in mutant clone no. 1 cells, confirming the whole-cell radioactive precursor pulse data.

Rates of Unsaturated Fatty Acid Biosynthesis. Table 3 compares the percentage desaturation of ^{14}C-labeled fatty acids derived from incorporated [^{14}C]acetate [reflecting whole-cell fatty acid desaturation activity (1)] in four different cell types grown either in serum or De-S medium. The relative rate of desaturation of ^{14}C-labeled fatty acids in mutant clone no. 1 cells grown in serum medium was nearly the same as that in the wild-type cells and revertant I cells (15–16%). However, in De-S medium, in contrast to wild-type cells and revertant I cells, mutant clone no. 1 cells showed less desaturation activity. As also shown in this table, although mutant no. 215 cells had slightly higher fatty acid desaturation activity than the wild-type cells, induction was normal (1.4-fold). This indicates that decreased cellular cholesterol content does not cause the defect in fatty acid desaturation; therefore, the defect in sterol biosynthesis and the defect in unsaturated fatty acid biosynthesis as seen in mutant clone no. 1 cells are concomitant rather than sequential events.

Effects of Lipid Supplements on Lipid Biosynthetic Activities. In order to rule out the possibility that any of the observed defects described above in mutant clone no. 1 cells is due to nonspecific arrest of cell growth, whole-cell [^{14}C]stearate and [^{14}C]acetate pulse experiments were carried out in wild-type and mutant clone no. 1 cells grown in various growth media. The results are shown in Table 4 and Fig. 4. The amounts of cholesterol (5 μg/ml) and oleic acid (5.7 μg/ml) used in these experiments did not suppress the induction of sterol and unsaturated fatty acid biosyntheses in wild-type cells; in fact, the oleic acid supplement with or without cholesterol supplement potentiated these inductions [presumably, by promoting cell growth as described (1)]. The same lipid supplements, which variously support the growth of mutant clone no. 1 cells (Fig. 1), did not correct the induction defects in these cells.

Cellular Lipid Composition of Cells Grown in De-S Medium. On the third day of the growth experiment described in Fig. 1, cells were harvested for sterol analyses and fatty acid analyses by GLC. The sterol content in the mutant cells was significantly lower than that in the wild-type cells and revertant I cells; also, the percentage of unsaturated fatty acid in the mutant cells was significantly lower than that in the wild-type cells and revertant I cells (Table 5).

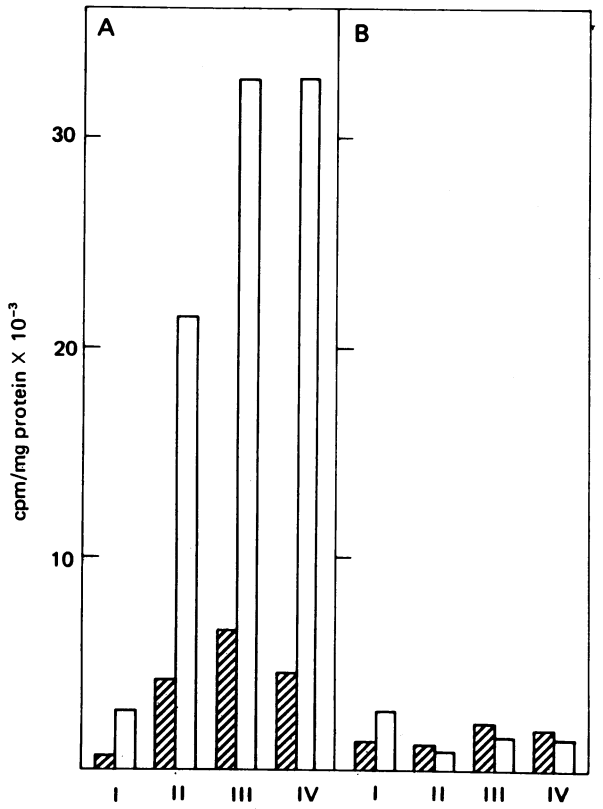

FIG. 4. Incorporation of [^{14}C]acetate into sterols in wild-type (*A*) and mutant clone no. 1 (*B*) cells grown with various lipid supplements. Cells were trypsinized and replated with 15 ml of F-12 + 10% serum in 75-cm^2 flasks at 0.12 × 10^6 cells per flask. On the third day the medium was drained off, the cells were rinsed with 5 ml of saline, and the medium was replaced with 20 ml of one of the following: I, F-12 + 10% serum; II, F-12 + 10% De-S; III, F-12 + 10% De-S + oleate at 5.7 μg/ml; IV, F-12 + 10% De-S + oleate at 5.7 μg/ml + cholesterol at 5.0 μg/ml. The cells were grown for 24 hr and then treated with [^{14}C]acetate as described in Table 1. Hatched area, lanosterol; open area, cholesterol.

DISCUSSION

All of the evidence presented in this paper is consistent with the idea that mutant clone no. 1 cells are defective in the induction of cholesterol biosynthesis and in the induction of unsaturated fatty acid biosynthesis. A specific enzyme assay *in vitro* indicated that the induction of HMG-CoA reductase activity is defective in the mutant cells. When cells are grown in De-S, lanosterol demethylation activity (14) also appears to be defective in this mutant (Table 2). This has yet to be confirmed by specific enzyme assays. The phenotypic expression of this mutant has been stable for at least 1 year without recloning. The successful isolation of a spontaneous revertant (revertant clone I) in which all three of these defects had reverted in parallel suggests that the expression of these three lipid biosynthetic activities may actually be tightly controlled by a common regulatory mechanism. This regulatory mechanism can be either at the transcriptional/translational level or at the level of protein–protein interaction. Recent biochemical evidence by Spence and Gaylor (14) suggests that a coordinated control may exist for HMG-CoA reductase and 4-methylsterol oxidase. The availability of mutant clone no. 1 cells and other mutant clones should prove useful in delineating such a control process at the molecular level.

Critical comments made by the referees for this manuscript are appreciated. These studies were supported in part by National Institutes of Health Grant HL 21246 and Contract NO1-CN-55199.

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