Defective Elongation of Fatty Acids in a Recessive 25-Hydroxycholesterol-resistant Mutant Cell Line*

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The Chinese hamster ovary recessive mutant, crB, has been selected for its resistance to the cytotoxic effects of 25-hydroxycholesterol in sterol-free media (Sinensky, M., Logel, J., and Torget, R. (1982) J. Cell. Physiol. 113, 314–319). Growth of crB in a chemically defined lipid-poor medium is very slow and is enhanced by a mixture of saturated and unsaturated fatty acids. Incorporation of [3H]acetate into total fatty acids is 4-fold lower in crB compared to that in parental Chinese hamster ovary K1 and in contrast to the wild-type cells, crB cells are unable to synthesize either stearate or oleate. In addition, crB cells cannot elongate exogenous palmitate, while they are capable of desaturating oleate. The mutant cells are also pleiotropically defective in the regulation of mRNA levels for the enzymes of cholesterol biosynthesis. 25-Hydroxycholesterol is a poor regulator of the synthesis and degradation of the rate-limiting enzyme, 3-hydroxy-3-methylglutaryl-coenzyme A reductase in crB in contrast to the wild-type Chinese hamster ovary K1 cells. The defect in the elongation of fatty acids is reversed in revertants of crB selected for their ability to grow in lipid-poor medium. Such revertants exhibit normal regulation of 3-hydroxy-3-methylglutaryl-CoA reductase activity by 25-hydroxycholesterol. Regulation of reductase activity in crB cells can also be restored by supplementing the culture medium with a mixture of fatty acids that restores normal growth rate. The defective regulation of reductase in crB does not appear to be due to nonspecific adverse effects of fatty acid starvation nor is it due to any gross change in the fatty acid composition of cellular phospholipids. These results strongly suggest a direct relationship between the fatty acid auxotrophy of crB and defective regulation of the enzymes of cholesterol biosynthesis.

Somatic cell mutants resistant to the cytotoxic effects of 25-hydroxycholesterol in cholesterol-depleted culture medium have proven to be of interest in elucidating the mechanisms involved in the regulation of cholesterol synthesis in mammalian cells by exogenous sterols (1–4). Mutants resistant to the killing effects of 25-hydroxycholesterol are, when selected under the appropriate conditions, also resistant to the down-regulatory effects of 25-hydroxycholesterol on the enzymes of cholesterol biosynthesis. Analysis of the genotypes of these mutations by somatic cell hybridization techniques have shown that such mutations can be either dominant or recessive in at least two different complementation groups (5).

It has been well documented that 25-hydroxycholesterol can act as a transcriptional regulator of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in mammalian cells (6–8). The mRNA levels of at least two other enzymes of cholesterol biosynthesis, HMG-CoA synthase and farnesyl pyrophosphate synthetase, are also regulated by 25-hydroxycholesterol, presumably also by a transcriptional control mechanism (9–11). We have previously described a dominant somatic cell mutant (CR1) that is pleiotropically defective in transcriptional regulation of enzyme activities of sterol biosynthesis by exogenous sterols (12, 13). However, translational control of HMG-CoA reductase appears to be intact in CR1 (13). The rate of degradation of HMG-CoA reductase is constitutively rapid in this mutant and is not further enhanced by exogenous sterols.

We now report that the recessive somatic cell mutant, crB, has a molecular biological phenotype similar to the one previously described for the dominant mutant, CR1. Surprisingly, in contrast to CR1, crB is auxotrophic for certain fatty acids and we present evidence consistent with the hypothesis that the primary defect conferring the 25-hydroxycholesterol resistance to this mutant appears to lie in the fatty acid elongation pathway.

**MATERIALS AND METHODS**

Cells and Media—crB, a recessive regulatory mutant (5), and CR1, a dominant regulatory mutant (12, 13), were selected from the mutagenized wild-type Chinese hamster ovary (CHO-K1) fibroblasts. Cells were routinely grown in Ham's F-12 medium supplemented with 5% fetal calf serum (F12FC5). Sterol biosynthetic enzymes were derepressed by incubation for 16–24 h in F-12 medium supplemented with 2 or 5% twice-delipidized (14) calf serum (F12DIP5 or F12DIP5E). However, such media did not support well the growth of the primary mutant cells for extended periods. Therefore, for cell growth experiments we utilized F-12 supplemented with 1% Nutridoma-SP (Boehringer Mannheim) and 0.1% dialyzed fetal calf serum (Nutridoma). In certain experiments, the lipid-poor culture media were supplemented either with 1% tissue culture grade bovine albumin (Fraction V, Sigma, catalog no. A8918) or with 0.03% essentially fatty acid-free bovine serum albumin (BSA, Sigma, catalog no. A6003). The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; CHO, Chinese hamster ovary; EGTA, (ethylene glycol bis(oxvethylenenitrilo) tetraacetic acid; HPLC, high performance liquid chromatography; BSA, essentially fatty acid-free bovine serum albumin.

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complexed to various fatty acids. Revertants of crB to fatty acid prototrophy were selected by standard procedures (15) involving overnight mutagenesis with ethyl methanesulfonate (150 µg/ml) followed by incubation in Nutridoma for 10 days. The frequency of spontaneous reversion in crB is less than 1 in 10⁶.

Isolation of RNA and Northern Blot Analysis—Cells in F12DIPE2 were treated with 0.5 µg 25-hydroxycholesterol for 16 h as described before (16). Total cellular RNA was extracted (16) for Northern blot analysis (13). The blots were probed with either the 3.8-kilobase EcoRI HMG-CoA reductase insert from XD11 (18), the 1.5-kilobase HindIII fragment from the hamster IMSG-CoA synthase clone p50K-312 (19), or the 1.3-kilobase EcoRI fragment from the rat farnesyl pyrophosphate synthetase clone CR39 (11). The human α-tubulin insert (courtesy of Dr. W. Saber, UCLA) was used as an internal standard. The cDNA probes were labeled using random priming to a specific activity of at least 10⁸ cpm/µg (19).

HMG-CoA Reductase—Cells were seeded at 1 x 10⁶/100-mm culture dish in 8 ml of F12PC5 and grown for 24 h (Protocol A). The monolayers were then rinsed twice with FcG media and refed 5 ml of F12DIPE5. Varying amounts of 25-hydroxycholesterol were added in ethanol such that the final concentration of ethanol in the culture medium did not exceed 1% (v/v). After 18 h of incubation, the relative rate of synthesis and the half-life of degradation of HMG-CoA reductase were determined following [3S]methionine labeling as described previously (13). For the measurement of reductase activity, the cells were seeded at 3 x 10⁶/60-mm culture dish in 5 ml of F12PC5 and grown for 24 h (Protocol B). After rinsing twice with saline G, they were refed 2 ml of F12DIPE5. Enzyme activity was assayed in detergent extracts of cells as detailed elsewhere (20). Upon incubation of both CHO-K1 and crB cells in F12DIPE5, there was a rapid induction of HMG-CoA reductase activity during the next 16 h (Fig. 1). The enzyme activity was relatively stable between 16 and 24 h and then declined rapidly. This decrease in reductase activity was not prevented by refeeding of the cells with fresh F12DIPE5 (data not shown). In view of these findings, all measurements of HMG-CoA reductase activity were carried out in cells after 18 h of incubation in F12DIPE5.

25-hydroxycholesterol.

We noticed that while the time course of derepression of reductase activity was very similar in the two cell lines, the in vitro specific activity of reductase in crB was 4-fold lower than that in the wild-type cells (Fig. 1). Treatment of cell extracts with FcG, and Northern Blot Analysis—Cells in F12DIPE2 were grown overnight in F12FC5 and were switched to Nutridoma for 24 h. Total lipids were extracted according to Bligh and Dyer (24). Phospholipids were separated from neutral lipids by silicic acid column chromatography (25) and were saponified in 10% methanolic KOH at 65 °C for 60 min. Extracted fatty acids were methylated with diazomethane and a portion was analyzed by gas chromatography on a 16% DR23-5-PS column (9 mm x 6 feet) using N₂ as the carrier gas at 20 ml/min. Initial column temperature was held at 165 °C for the first 4 min and was then increased at a rate of 8 °C/min to 180 °C. The response of the flame ionization detector was integrated using the System Gold software (Beckman).

RESULTS

Fatty Acid Auxotrophy of crB—In the process of analyzing the nature of the regulatory defect in crB, we attempted to adapt these cells to growth in a chemically defined, lipid-poor culture medium (Nutridoma). We found that CHO K1 cells and the dominant 25-hydroxycholesterol-resistant mutant, CR1 cells, grew in Nutridoma medium at rates comparable to those in F12FC5 (Fig. 3). However, crB cells grew at a much slower rate (1–2 doublings of cell population in 72 h in Nutridoma compared to 6 8 doublings in F12FC6). Essentially similar results were obtained in the lipid-poor

Fig. 1. Time course of derepression of HMG-CoA reductase activity of CHO-K1 and crB cells. Cells were grown as per Protocol B. At indicated time points, cells in F12DIPE5 were lysed in a buffer containing 20 mM imidazole, pH 7.4, 1 mM EGTA, and 0.2% (v/v) Kyro-EOB detergent (Procter and Gamble) for the measurement of HMG-CoA reductase activity (20).

Fig. 2. Immunotitration of IMSG-CoA reductase activity of CHO-K1 and crB cells. Cells were grown as per Protocol B. After 18 h of incubation in F12DIPE5, monolayers were rinsed twice in ice-cold saline and drained well. Cells from each culture dish were solubilized in 300 µl of ice-cold lysis buffer containing 50 mM potassium phosphate buffer, pH 7.0, 100 mM sucrose, 500 mM KCl, 20 mM KCl, 1% (v/v) Triton X-100, 10 mM dithiothreitol, 3 mM phenylmethylsulfonyl fluoride, and 0.3 mM leupeptin (13). Pooled lysates from triplicate dishes were incubated on ice for 30 min and centrifuged at 12,000 x g for 15 min. Aliquots of supernatant fraction (22 µg of protein) were incubated with indicated amounts of anti-reductase IgG at 37 °C for 30 min prior to reductase assay.

[3H]Acetate (10–15 µCi/ml). Cellular lipids were extracted into hexane/isopropyl alcohol (60:40) as described before (22). Lipids were saponified in 1 N KOH in methanol:benzene (80:20), and non-saponifiable lipids were extracted into hexane. The aqueous phase was acidified with HCl and fatty acids were extracted into petroleum ether followed by diethyl ether. Pooled extracts were dried under N₂ and the fatty acids were redissolved in methanol for analysis by gas-phase HPLC essentially as described before (23). Fatty acid synthesis was also measured in vitro in Dounce homogenates of CHO-K1 and crB cells in 1 mM Tris-HCl, pH 7.0, 1 mM EGTA. Aliquots (200–300 µg of protein) were assayed for 30 min at 37 °C in a final volume of 1 ml in 0.1 M potassium phosphate, pH 7, 1 mM dithiothreitol, 50 µM [3H]acetate (3000 cpm/nmol), 50 µM malonyl-CoA, and 2 mM NADPH. The reaction was stopped with 0.1 ml of 0.1 M HCl and fatty acids were extracted into petroleum ether for HPLC analysis.

Metabolism of Radiolabeled Fatty Acids by Cells—Cells were grown for 24 h in F12PC5 and were then incubated for 24 h in F12DIPE5. Total cellular lipid was analyzed with either [3H]palmitate or with [1-14C]palmitate (1 µCi/dish) complexed to fatty acid-free BSA. The final concentration of BSA was 0.17%. Fatty acids were isolated and analyzed as described above.

Acyl-CoA:Cholesterol Acyltransferase—The activity of cellular acyl-CoA:cholesterol acyltransferase was assayed indirectly by following the esterification of endogenous cholesterol by exogenous [3H]oleate (22).

Composition of Phospholipid Fatty Acids of Cultured Cells—Cells were grown overnight in F12FC5 and were switched to Nutridoma for 24 h. Total lipids were extracted according to Bligh and Dyer (24). Phospholipids were separated from neutral lipids by silicic acid column chromatography (25) and were saponified in 10% methanolic KOH at 65 °C for 60 min. Extracted fatty acids were methylated with diazomethane and a portion was analyzed by gas chromatography on a 16% DR23-5-PS column (9 mm x 6 feet) using N₂ as the carrier gas at 20 ml/min. Initial column temperature was held at 165 °C for the first 4 min and was then increased at a rate of 8 °C/min to 180 °C. The response of the flame ionization detector was integrated using the System Gold software (Beckman).
Fatty Acids and Regulation of Sterol Biosynthesis

Auxotrophy in CHO-K1 and crB—To determine the site of the biosynthetic lesion of fatty acid synthesis, we incubated CHO-K1 and crB cells with radiolabeled acetate and examined the lipid products. Both cell lines incorporated similar amounts of radiolabeled non-saponifiable lipids (mostly sterols) and fatty acids (10 μM each) ranging in chain length from 14 to 20 carbons and in unsaturation from 0 to 4 double bonds were complexed to a small amount (0.03%) of fatty acid-free BSA for presentation to the cells either singly or in several combinations. Maximal growth of crB cells could be restored by supplementation of lipid-poor culture medium with a mixture of a saturated and an unsaturated fatty acid, particularly stearate (18:0) and oleate (18:1) (Fig. 3). These fatty acids supplements stimulated growth somewhat when added singly (data not shown), but complete restoration of growth was reproducibly achieved only with the combination shown. Fatty acid-free BSA did not enhance the growth of crB cells even at 1% level (data not shown).

Nature of the Biosynthetic Defect Leading to Fatty Acid Auxotrophy in crB—To determine the site of the biosynthetic lesion of fatty acid synthesis, we incubated CHO-K1 and crB cells with radiolabeled acetate and examined the lipid products. Both cell lines incorporated similar amounts of radiolabeled into total cellular lipids. However, the distribution of the label between non-saponifiable lipids (mostly sterols) and fatty acids was quite different in the two cell types (Table I).

Incorporation of [3H]acetate into total fatty acids in crB was 4-fold lower than that in CHO-K1 while that into non-saponifiable lipids was 3-fold greater. This difference in the distribution of radiolabel persisted (data not shown) over a wide range of exogenous acetate concentrations (1 μM to 4 mM), suggesting that the acetate pool sizes in the two cell lines were not very different (26, 27). Analysis of the radio-labeled non-saponifiable lipids of CHO-K1 and crB by thin layer and high performance liquid chromatography (20) revealed that >95% of the radiolabel migrated as C27-sterols, with cholesterol accounting for 50 and 85% of the total in CHO-K1 and crB, respectively. As expected, treatment of CHO-K1 cells with 26-hydroxycholesterol caused a 5-fold inhibition of the rate of sterol synthesis (Table I). A similar treatment of crB cells resulted in a much smaller degree of inhibition such that the rate of synthesis of sterols even in oxysterol-treated cells was greater than that in untreated CHO-K1 cells (Table I).

A comparison of the fatty acids formed from exogenous acetate in CHO-K1 cells and crB cells (Fig. 4A) indicated that, whereas in wild type cells substantial amounts of stearate (18:0) and oleate (18:1) are formed, in crB cells there did not appear to be any elongation of palmitic acid (16:0). Instead, a significant accumulation of radioactivity in myristate (14:0) could be observed. Examination of the specific activities of acetyl-CoA carboxylase and fatty acid synthetase by direct enzyme assay in cell-free homogenates indicated that these enzyme activities were present at near normal levels in crB (data not shown). When the products of the in vitro fatty acid synthetase reaction were analyzed (Fig. 4B), the myristate accumulation seen in intact crB cells was not observed. The absence of fatty acids of chain length longer than 16 in assays with CHO-K1 homogenates is explained by the short incubation period employed (30 min).

The lack of formation of stearate and oleate observed in intact crB (Fig. 4A) is most consistent with a defect in fatty acid elongation reactions. To test this possibility, we examined the incorporation of labeled palmitic acid into products in CHO-K1 cells and crB. The results (Fig. 5A) demonstrate that exogenously added palmitate could not be converted to stearate in crB although this process could clearly be observed in the wild-type CHO-K1 cell. The data also indicate that the source of myristate in crB cells is not β-oxidation of palmitate. These observations are consistent with a defect in the chain elongation reaction that adds a two-carbon unit onto palmitic acid. Also, in both crB and CHO-K1 cells exogenous stearic acid was converted to oleic acid (Fig. 5B). The extent of desaturation of cell-associated [14C]stearate ranged from 47 to 52% in CHO-K1 cells and 33 to 41% in crB cells. While this experiment was not designed to yield quantitative results, these data do demonstrate that crB cells are capable of fatty acid desaturation at a level not very different from that in

**Table I**

| Lipid class               | CHO-K1 Control | CHO-K1 +25-Hydroxycholesterol | crB Control | crB +25-Hydroxycholesterol |
|---------------------------|----------------|-------------------------------|-------------|---------------------------|
| Non-saponifiable lipids   | 12.04 ± 1.58   | 2.326 ± 251                   | 39.507 ± 1.457 | 22.700 ± 210             |
| Fatty acids               | 24.266 ± 1.329 | 21.078 ± 826                  | 5.751 ± 2.133 | 5.470 ± 142              |

FIG. 3. Growth of Chinese hamster ovary cells in Nutridoma. Cells were seeded at 5 × 10⁴/60-mm culture dish in F12FC5 and incubated for 4 h. The culture medium was then changed to Nutridoma containing either no addition (A), 1% tissue culture grade albumin (B), 0.03% fatty acid-free BSA (C), or 10 μM each of stearate and oleate complexed to 0.03% BSA (D). Cells were harvested after 5 days by trypsinization and counted. The results are presented as a percentage ± S.E. of the cell number for cells maintained throughout in F12FC5 (1.75 ± 106, 1.32 ± 106, and 1.94 ± 106 cells/dish for CHO-K1, crB, and CHO-K1, respectively).
previously been described (28). The relationship between fatty acid auxotrophy and defective regulation of cholesterogenic enzymes by 25-hydroxycholesterol—These observations immediately raised the question of whether the defective regulation of cholesterol synthesis by exogenous 25-hydroxycholesterol in crB arises from the same genetic defect responsible for the fatty acid auxotrophy. In order to examine this question, we first attempted to isolate revertants of crB to fatty acid prototrophy by selection for growth in lipid-poor medium after mutagenesis. Isolation of such mutants was achieved by standard methods (15) and the ability of the putative revertants to synthesize long chain fatty acids was examined. The results with one such revertant, crB R7, clearly demonstrate synthesis of stearate and oleate (Fig. 6) similar to that observed with the wild-type cells (Fig. 4A). The putative revertants were then analyzed for the regulatory response of HMG-CoA reductase activity to 25-hydroxycholesterol (0.25 PM) in wild type CHO-K1 cells. The results (Fig. 7A) indicate that in the two revertants examined, regulation of HMG-CoA reductase activity by 25-hydroxycholesterol was essentially re

![Radioactivity (cpm)](image)

**Retention time (min)**

**Fig. 4. Biosynthesis of fatty acids by CHO-K1 (upper panels) and crB (lower panels).** Intact cells in F12DIPE5 (A) or Dounce homogenates of cells (B) were incubated with [3H]acetate or [3H]acetil-CoA, respectively, and the labeled fatty acids were isolated as described under “Materials and Methods.” Individual fatty acids were resolved by reverse phase HPLC as described in Fig. 4. The putative revertants were then analyzed for the regulatory response of HMG-CoA reductase activity to 25-hydroxycholesterol (0.25 PM) in wild type CHO-K1 cells. The results (Fig. 7A) indicate that in the two revertants examined, regulation of HMG-CoA reductase activity by 25-hydroxycholesterol was essentially restored to the same levels as that found in parenta CHO-K1 cells. This finding clearly establishes a genetic link between fatty acid elongation and sterol-mediated regulation of isoprenoid biosynthesis.

The capacity of exogenous fatty acid supplements to restore regulation of HMG-CoA reductase by 25-hydroxycholesterol in the crB mutant was also tested (Fig. 7B). With a mixture of fatty acids (oleate and stearate) that enhanced the growth of crB in lipid-poor medium, regulation of HMG-CoA reductase activity by 25-hydroxycholesterol was essentially restored. The restoration of sterol-mediated regulation by fatty acid supplementation of crB culture medium could be shown to occur at the level of reductase synthesis (Table IV). When added singly, oleate restored the regulation partially and stearate was ineffective (Fig. 7B). Single supplements of myristate, palmitate, linoleate, or arachidonate were also incapable of restoring oxysterol regulation of HMG-CoA reductase (data not shown). We also tested the possibility that the peculiar accumulation of myristate in crB cells (Fig. 4A) might somehow confer resistance to oxysterols by examining the ability of exogenous myristate supplements (5–100 μM) to interfere with the regulation of HMG-CoA reductase activity by 25-hydroxycholesterol (0.25 μM) in wild type CHO-K1 cells. No such interference with the regulation of reductase activity by 25-hydroxycholesterol could be observed (data not shown).

The relationship between fatty acid chain elongation and sterol-mediated regulation of HMG-CoA reductase could be alternatively demonstrated in the wild-type CHO-K1 cells treated with hexadecynoic acid, an inhibitor of fatty acid chain elongation (29). CHO-K1 cells were adapted to grow in Nutridoma so that their fatty acid requirements were completely dependent on de novo synthesis. Treatment of these wild-type cells. Desaturation mutants of CHO-K1 cells have previously been described (28).

**Characterization of Regulatory Defect in crB Cells—**Recent work from our laboratory indicated that the pleiotropic loss of regulation of cholesterogenic enzymes in a dominant 25-hydroxycholesterol-resistant mutant (CR1) corresponded to a defect in the regulation of mRNA levels for HMG-CoA synthase and HMG-CoA reductase by 25-hydroxycholesterol (13). We, therefore, compared the effects of 25-hydroxycholesterol on the mRNAs encoding HMG-CoA reductase, HMG-CoA synthase and farnesyl pyrophosphate synthetase in the recessive mutant crB and the parental CHO-K1 cell line. The results (Table II) indicate that like CR1, crB is also pleiotropically defective in oxysterol-mediated regulation of mRNA levels for enzymes of sterol biosynthesis. We also examined the effect of 25-hydroxycholesterol on the rates of synthesis and degradation of HMG-CoA reductase in crB and CHO-K1 cells. The results (Table III) demonstrate that in contrast to the findings in wild-type cells, 25-hydroxycholesterol is a poor regulator of the synthesis and degradation of this enzyme in crB cells. These observations, including the constitutionally rapid rate of HMG-CoA reductase degradation in crB are similar to the previously reported findings with CR1 (13).
Materials and Methods. Isolated fatty acids were resolved by HPLC as described under "Materials and Methods." The data, normalized to α-tubulin mRNA levels in each case, are expressed as the mean ± S.E. of three determinations except where indicated. The numbers in parentheses refer to the percent mRNA remaining after treatment with 25-hydroxycholesterol, taking the corresponding control values to be 100%.

| Enzyme                        | CHO-K1            | crB               |
|-------------------------------|-------------------|-------------------|
| HMG-CoA reductase Control     | 0.63 ± 0.03 (100) | 1.25 ± 0.14 (100) |
| 1A-Hydroxycholesterol         | 0.01 ± 0.00 (49)  | 0.87 ± 0.09 (70)  |
| HMG-CoA synthase Control      | 0.24 ± 0.03 (100) | 0.31 ± 0.04 (100) |
| +25-Hydroxycholesterol        | 0.07 ± 0.01 (29)  | 0.22 ± 0.00 (71)  |
| Farnesyl pyrophosphate synthetase Control | 0.57 ± 0.03 (100) | 0.40 ± 0.08 (100) |
| +25-Hydroxycholesterol        | 0.09 ± 0.02 (16)  | 0.20 ± 0.09 (30)  |

* Average of two determinations.

**TABLE III**

Regulation of the rates of synthesis and degradation of HMG-CoA reductase in CHO-K1 and crB cells by 25-hydroxycholesterol

Cells were grown and treated as described in Table II. The relative rates of synthesis and the rates of degradation of HMG-CoA reductase polypeptide were determined following [35S]methionine labeling as described under "Materials and Methods." The rate of synthesis is expressed as a ratio (~10^3) ± S.E. of 35S counts/min in reductase to those in total proteins determined as trichloroacetic acid-precipitable material and is the average of triplicate cultures. The numbers in the parentheses show normalized results taking the control values to be 100.

| Treatment                     | Relative rate of synthesis | Half-life (h) |
|-------------------------------|-----------------------------|---------------|
|                               | CHO-K1 | crB       | CHO-K1 | crB |
| Control                       | 3.63 ± 0.08 (100)           | 4.5 ± 0.9     | 2.2 ± 0.2 |
| +25-Hydroxycholesterol        | 0.50 ± 0.01 (14)            | 2.81 ± 0.45   | 1.4 ± 0.1  |

**FIG. 6. Biosynthesis of fatty acids in a revertant of crB to fatty acid prototrophy.** The revertant crB R7, capable of growth in Nutridoma, was labeled with [14C]acetate as described under "Materials and Methods." Isolated fatty acids were resolved by HPLC as described in Fig. 4.

Cells with hexadecynoate caused a substantial abolition of the synthesis of fatty acids longer than palmitate (Fig. 8), resulting in a phenotype quite similar to that of crB. As shown in Table V, hexadecynoate treatment of CHO-K1 resulted in resistance to regulation of HMG-CoA reductase by 25-hydroxycholesterol. Similar treatment of crB did not produce any additional resistance. Thus, defective elongation of fatty acids, produced either through mutation or through the action of 25-hydroxycholesterol, was labeled with [3H]acetate as described under "Materials and Methods." The rate of synthesis is expressed as a ratio (~10^3) ± S.E. of 35S counts/min in reductase to those in total proteins determined as trichloroacetic acid-precipitable material. The numbers in parentheses refer to the percent mRNA remaining after treatment with 25-hydroxycholesterol, taking the corresponding control values to be 100%.

**TABLE IV**

Effect of fatty acid supplementation on the regulation of synthesis of HMG-CoA reductase of crB by 25-hydroxycholesterol

Cells were grown and treated as described in Table II except that the culture medium for one set of crB dishes was supplemented with fatty acids (B). Cells grown according to Protocol B, were treated with indicated concentrations of 25-hydroxycholesterol in F12DIPE5 + 10 μM of fatty acids in 0.03% BSA for 10 h before the measurement of reductase activity. The results are the average ± S.E. of data from triplicate cultures at each point. A, control (100%) enzyme activities (nanomoles of mevalonate formed per min/mg protein) were 0.94, 0.16, 0.94, and 0.53 for CHO-K1 (A), crB (B), crB R2 (C), and crB R7 (D), respectively. B, control (100%) enzyme specific activities (nanomoles/min/mg protein) were 0.16, 0.19, 0.30, and 0.44 for crB cells supplemented with either none (O), 10 μM stearate (Q), 10 μM oleate (Ω), or 10 μM each of stearate and oleate (⊕), respectively.

| Treatment                      | CHO-K1            | crB               |
|-------------------------------|-------------------|-------------------|
|                               | 2.90 ± 0.31 (29)  | 2.79 ± 0.54 (29)  |
| +25-Hydroxycholesterol        | 0.56 ± 0.13 (10)  | 2.16 ± 0.04 (10)  |

**FIG. 7. Restoration of regulation of HMG-CoA reductase activity by 25-hydroxycholesterol.** Regulation of reductase activity by 25-hydroxycholesterol was studied in revertants of crB (A) as well as in crB cells supplemented with fatty acids (B). Cells grown according to Protocol B, were treated with indicated concentrations of 25-hydroxycholesterol in F12DIPE5 + 10 μM of fatty acids in 0.03% BSA for 18 h before the measurement of reductase activity. The results are the average ± S.E. of data from triplicate cultures at each point. A, control (100%) enzyme activities (nanomoles of mevalonate formed per min/mg protein) were 0.94, 0.16, 0.94, and 0.53 for CHO-K1 (A), crB (B), crB R2 (C), and crB R7 (D), respectively. B, control (100%) enzyme specific activities (nanomoles/min/mg protein) were 0.16, 0.19, 0.30, and 0.44 for crB cells supplemented with either none (O), 10 μM stearate (Q), 10 μM oleate (Ω), or 10 μM each of stearate and oleate (⊕), respectively.

**TABLE V**

Regulation of the rates of synthesis and degradation of HMG-CoA reductase in CHO-K1 and crB cells by 25-hydroxycholesterol

Treatments with hexadecynoate caused a substantial abolition of the synthesis of fatty acids longer than palmitate (Fig. 8), resulting in a phenotype quite similar to that of crB. As shown in Table V, hexadecynoate treatment of CHO-K1 resulted in resistance to regulation of HMG-CoA reductase by 25-hydroxycholesterol. Similar treatment of crB did not produce any additional resistance. Thus, defective elongation of fatty acids, produced either through mutation or through the action of 25-hydroxycholesterol, was labeled with [3H]acetate as described under "Materials and Methods." The rate of synthesis is expressed as a ratio (~10^3) ± S.E. of 35S counts/min in reductase to those in total proteins determined as trichloroacetic acid-precipitable material. The numbers in parentheses refer to the percent mRNA remaining after treatment with 25-hydroxycholesterol, taking the corresponding control values to be 100%.

| Treatment                      | CHO-K1            | crB               |
|-------------------------------|-------------------|-------------------|
|                               | 0.63 ± 0.03 (100) | 1.25 ± 0.14 (100) |
| +25-Hydroxycholesterol        | 0.01 ± 0.00 (49)  | 0.87 ± 0.09 (70)  |
| Farnesyl pyrophosphate synthetase Control | 0.24 ± 0.03 (100) | 0.31 ± 0.04 (100) |
| +25-Hydroxycholesterol        | 0.07 ± 0.01 (29)  | 0.22 ± 0.00 (71)  |
|                               | 0.57 ± 0.03 (100) | 0.40 ± 0.08 (100) |
| +25-Hydroxycholesterol        | 0.09 ± 0.02 (16)  | 0.20 ± 0.09 (30)  |

* Average of two determinations.
of a specific inhibitor, appears to cause a loss of regulation of reductase by sterols.

Specificity of the Regulatory Defect in crB—Since the experiments on the regulation of HMG-CoA reductase in crB were conducted on cells incubated for 18-24 h in lipid-poor media, we considered the possibility that growth arrest resulting from fatty acid starvation may have played a part in the observed loss of regulation. However, as shown in Fig. 9, fatty acid depletion had no adverse effect on the rate of total protein synthesis in crB cells for up to 24 h. It may be pointed out that in this experiment as well as in other experiments of short duration (<24 h) investigating the regulation of HMG-CoA reductase activity, the initial cell density was 6-fold higher than that used in cell growth experiments (Fig. 3). The rate of total protein synthesis in crB under these culture conditions did decline upon longer incubation in F12DIPE5 (data not shown). It should be noted that the derepressed rates of synthesis of HMG-CoA reductase protein are also very similar in crB and CHO-K1 (Tables III and IV). In addition, treatment of crB with mevalonate produced a dose-dependent suppression of reductase activity which was only slightly smaller than that seen in CHO-K1 cells (Fig. 10). This finding suggests that the reductase activity in crB can indeed be down regulated in the presence of suitable regulators. The small difference in the magnitude of inhibition of reductase activity by mevalonate between the two cell lines can be explained as due to the loss of the sterol component of inhibition in crB. We have previously reported (20) that specific blockage of the pathway of sterol synthesis in CHO-K1 cells results in a similar minor loss of inhibition of reductase activity by mevalonate. Thus, these results indicate that the component of regulation mediated by non-sterol products of mevalonate remains intact in crB cells starved for fatty acids. We have observed that incubation of crB cells in F12DIPE5 beyond 18-20 h resulted in progressive loss of DNA synthesis as measured by the incorporation of [3H]thymidine (Fig. 11). The extent of inhibition at 20 h was 25-30% compared to controls supplemented with fatty acids. However, in other CHO cell lines in which we caused a similar degree of inhibition of DNA synthesis such as in CHO-K1 cells treated with 0.2 μM aphidicolin (31) or in the inositol auxotroph Ino-A3 cells3 starved for myo-inositol, regulation of reductase activity by 25-hydroxycholesterol was not compromised (Table VI).

Another possible explanation for the defective regulation of crB cells by exogenous 25-hydroxycholesterol is that the fatty acid starvation may have produced a structural change in the cell membrane leading to a defect in 25-hydroxycholesterol uptake. We examined this possibility by measuring the response of cellular acyl-CoAcholesterol acyltransferase activity to exogenous 25-hydroxycholesterol in crB cells incubated in F12DIPE5 for 24 h. The results in Fig. 12 demonstrate a stimulation of acyl-CoAcholesterol acyltransferase activity of crB cells by 25-hydroxycholesterol similar to that seen in CHO-K1 cells. This finding also indicates that the activity of acyl-CoA ligase in crB cells is normal. Comparison of the fatty acids of CHO-K1 and crB phospholipids by gas chromatography after a 24-h incubation of both cell types in lipid-poor medium also did not reveal any obvious differences in the pattern of fatty acids between the two cells (Fig. 13). The ratio of palmitate (16:0) to stearate (18:0) plus oleate (18:1) in total phospholipids was thus unchanged by this incubation.

**DISCUSSION**

We have previously reported that crB is one of two complementation groups of recessive 25-hydroxycholesterol-resistant mutants that have been isolated to date (5). In both dominant and recessive resistant mutants isolated thus far, resistance to the cytotoxic effects of 25-hydroxycholesterol appears to correlate with resistance to regulation by exogenous sterols of the enzymes of sterol synthesis, particularly of HMG-CoA reductase. Significant regulation of reductase activity by 25-hydroxycholesterol has been observed in many human cell lines (32) as well as in the yeast Saccharomyces cerevisiae (33). We have previously reported (20) that specific blockage of the pathway of sterol synthesis in CHO-K1 cells results in a similar minor loss of inhibition of reductase activity by mevalonate. Thus, these results indicate that the component of regulation mediated by non-sterol products of mevalonate remains intact in crB cells starved for fatty acids. We have observed that incubation of crB cells in F12DIPE5 beyond 18-20 h resulted in progressive loss of DNA synthesis as measured by the incorporation of [3H]thymidine (Fig. 11). The extent of inhibition at 20 h was 25-30% compared to controls supplemented with fatty acids. However, in other CHO cell lines in which we caused a similar degree of inhibition of DNA synthesis such as in CHO-K1 cells treated with 0.2 μM aphidicolin (31) or in the inositol auxotroph Ino-A3 cells3 starved for myo-inositol, regulation of reductase activity by 25-hydroxycholesterol was not compromised (Table VI).

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**TABLE V**

| Treatment               | CHO-K1       | crB          |
|-------------------------|--------------|--------------|
|                         | -25-Hydroxy- | +25-Hydroxy- | -25-Hydroxy- | +25-Hydroxy- |
|                         | cholesterol | cholesterol | cholesterol | cholesterol |
| Control                 | 0.40 ± 0.01  | 0.03 ± 0.001 | 0.20 ± 0.01  | 0.11 ± 0.001 |
| +Hexadecynoate          | 1.49 ± 0.06  | 0.74 ± 0.07  | 0.31 ± 0.03  | 0.17 ± 0.02  |

*Ino-A3 cells were kindly provided by Dr. David Patterson of the Eleanor Roosevelt Institute for Cancer Research, Denver, CO.*

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*Fig. 8. Effect of hexadecynoic acid on the fatty acid species synthesized by CHO-K1 cells. CHO-K1 cells, continuously grown in Nutridoma, were seeded in the same medium at 3 x 10^5/60-mm culture dish. The next day, they were refed fresh Nutridoma medium with (A) or without (B) 20 μM hexadecynoate complexed to 0.06% BSA. After 1 h, [3H]acetate (15 μCi/ml) was added and the cells were incubated for 16 h. Fatty acids were isolated and analyzed by reverse phase HPLC as described in Fig. 4.*
Therefore, it is not surprising that as we have previously indicated time points were pulsed with \([3H]\)thymidine and at 30 min. Incorporation of radiolabel into DNA was determined as trichloroacetic acid-insoluble counts as described previously (30).

![Fig. 9. Effect of fatty acid starvation on total protein synthesis in crB cells. Cells were grown as per Protocol B and were switched to methionine-free F12DIPES containing either no addition (experimental) or a mixture of 10 \(\mu\)M each of palmitate, stearate, oleate, and linoleate complexed to 0.03% BSA (control). Cells were pulsed with \([35S]\)methionine (2 \(\mu\)Ci/ml) for 2 h at indicated time intervals. Protein synthesis was measured as the incorporation of \[^{35}S\] label into trichloroacetic acid-precipitable proteins and is expressed as a percent of the value for control cultures at each time point.](image)

![Fig. 10. Down-regulation of HMG-CoA reductase activity of crB cells by mevalonate. Cells grown as per Protocol B, were incubated with the indicated concentrations of mevalonate in F12DIPES for 18 h before the determination of HMG-CoA reductase activity in detergent extracts. Control (100%) activities (nanomoles/min/mg protein) were 0.91 and 0.19 for CHO-K1 and crB, respectively.](image)

![Fig. 11. Effect of fatty acid starvation on the rate of DNA synthesis in crB cells. CHO-K1 and crB cells grown according to Protocol B were switched to F12DIPES containing either no addition (experimental) or a mixture of 10 \(\mu\)M each of palmitate, stearate, oleate, and linoleate complexed to 0.03% BSA (control) cells. Cells were pulsed with \([3H]\)thymidine (1 \(\mu\)Ci/ml) for 30 min. Incorporation of radiolabel into DNA was determined as trichloroacetic acid-insoluble counts as described previously (30).](image)

![Fig. 12. Stimulation of acyl-CoA:cholesterol acyltransferase activity of CHO-K1 and crB cells by 25-hydroxycholesterol. Cells were seeded at 1 \(\times\) 10\(^6\)/60-mm culture dish in F12FC5 on day 0. They were refed F12DIPES on day 3 and F12DIPES containing the indicated amounts of 25-hydroxycholesterol on day 4. After 2 h, \([3H]\)oleate (100 \(\mu\)M, 5 \(\mu\)Ci/ml) complexed to BSA (0.5%) was added and the cells were incubated for an additional 2 h. Labeled cholesteryl esters were isolated as described (22).](image)

### Table VI

| Cells and treatment | Specific activity of HMG-CoA reductase | Relative rate of DNA synthesis (nmol/min/mg protein ± S.E.) |
|---------------------|---------------------------------------|--------------------------------------------------------|
| crB                 |                                       |                                                        |
| Control             | 0.43 ± 0.02                           | 0.04 ± 0.006                                          |
| -Stearate, oleate   | 0.22 ± 0.01                           | 0.11 ± 0.003                                          |
| CHO-K1              | 0.88 ± 0.02                           | 0.07 ± 0.01                                          |
| +Aphidicolin        | 0.91 ± 0.03                           | 0.06 ± 0.01                                          |
| Ino-AB              | 0.21 ± 0.01                           | 0.02 ± 0.004                                          |
| Control             | 0.43 ± 0.02                           | 0.04 ± 0.006                                          |
| +Stearate, oleate   | 0.22 ± 0.01                           | 0.11 ± 0.003                                          |
| CHO-K1              | 0.88 ± 0.02                           | 0.07 ± 0.01                                          |
| +Aphidicolin        | 0.91 ± 0.03                           | 0.06 ± 0.01                                          |
| Ino-AB              | 0.21 ± 0.01                           | 0.02 ± 0.004                                          |
response. The link between long chain fatty acids and sterol regulation of cholesterogetic enzymes is further demonstrated by the fact that resistance to regulation by 25-hydroxycholesterol can be generated in the wild-type cells by treatment with hexadecynoate, a specific inhibitor of fatty acid elongation.

Some tentative conclusions regarding the mechanisms by which fatty acids may be regulating HMG-CoA reductase can also be drawn from the data reported in this manuscript. We have determined that under the conditions in which regulation of HMG-CoA reductase activity by 25-hydroxycholesterol is compromised there is no gross change in the fatty acid composition of cellular phospholipids. This result implies that the regulatory response is produced by fatty acids in a labile pool. We have also noted that the regulatory response to 25-hydroxycholesterol in crB is restored to near normal by fatty acids corresponding to those which are normal components of bulk lipids of cells. Some known signaling mechanisms that might involve fatty acids such as post-translational modification by acylation with myristate or palmitate, or through oxidation of arachidonic acid do not, therefore, seem to be responsible for the regulation observed in this case. Neither myristate, palmitate, linoleate nor arachidonate when given alone were able to restore regulation of HMG-CoA reductase activity in crB. There is some prior evidence of the effects of fatty acids on hepatic cholesterol biosynthesis (35-41). The fatty acid composition of dietary lipids is reported to affect the rate of cholesterol biosynthesis measured either as $[^{14}C]$-acetate incorporation or as the activity of HMG-CoA reductase in crB. There is some prior evidence of the effects of fatty acids on hepatic cholesterol biosynthesis (35-41). The fatty acid composition of dietary lipids is reported to affect the rate of cholesterol biosynthesis measured either as $[^{14}C]$-acetate incorporation or as the activity of HMG-CoA reductase (35-37). Dietary stearate is found to stimulate cholesterogetic in rat liver more than other fatty acids tested (36). Stimulation of hepatic HMG-CoA reductase activity by dietary fatty acid is reported to be proportional to the chain length and inversely related to the degree of unsaturation. In perfused rat liver, however, oleate caused the most pronounced rise of reductase (39, 40). Treatment with oleic acid is also reported to enhance HMG-CoA reductase activity of rat hepatocytes in culture by 2-3-fold (41, 42). Other fatty acids such as palmitate, stearate, linoleate and linolenate were much less effective than oleate. Arachidonate treatment of hepatocytes actually led to a small (33%) decrease in reductase activity (42). Oral administration of olive oil is reported to enhance the hepatic activities of several other enzymes of cholesterol biosynthesis (43).

We have noted that the specific activity of HMG-CoA reductase in crB incubated in lipid-poor medium was 4-5-fold lower than that observed in CHO-K1 cells, and was stimulated by fatty acids. The lower in vitro activity of reductase is inconsistent with the observations of higher mRNA levels for HMG-CoA reductase in crB than in CHO-K1, an equivalent rate of synthesis of the enzyme, and comparable amounts of immunotitratable mass (see Tables II-IV and Fig. 2). In addition, the conversion of $[^{3}H]$acetate into non-saponifiable lipids was 2.4-fold higher in crB compared to that in CHO-K1 over a wide range of exogenous acetate concentrations (Table I). These data are consistent with the conclusion that while the amount of HMG-CoA reductase protein in crB cells is comparable to that in the wild-type cells, the enzyme from the mutant cells may be rapidly inactivated upon cell lysis. It should, however, be pointed out that despite the lower specific activity of HMG-CoA reductase in crB lysates, the magnitude of the changes in the measured activity upon treatment of cells with 25-hydroxycholesterol is consistent with the observed in the levels of reductase mRNA and the rate of enzyme synthesis. Thus, measurement of reductase activities has proven to be a reliable preliminary measure of the resistance of crB cells to 25-hydroxycholesterol.

The crB mutant provides a system in which to examine several questions related to the enzymology of fatty acid chain elongation. The chain length specificity of fatty acid elongating enzymes and their subcellular distribution have been difficult to analyze by biochemical procedures alone. The crB mutation should allow us to answer the question of whether or not there are fatty acid elongating enzymes with different chain length specificities and whether the functional site of fatty acid elongation, at least in CHO cells, is mitochondrial or microsomal.

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