A somatic cell mutant of the Chinese hamster ovary (CHO)-K1 cell auxotrophic for mevalonic acid has been isolated by means of the bromodeoxyuridine-visible light technique. This mutant can incorporate labeled mevalonate but not labeled acetate into cholesterol and, thus, is apparently defective in mevalonate biosynthesis. The mutant is recessive with respect to the parental cell phenotype. Assessment of the in vitro activities of the enzymes responsible for mevalonate biosynthesis under varying growth conditions indicates that the mutant, MeV-1, is defective in 3-hydroxy-3-methylglutaryl coenzyme A synthase.

The control of cholesterol biosynthesis in cultured mammalian cells by exogenous sterol is an interesting example of metabolic regulation in mammalian systems that is tractable to both biochemical and somatic cell genetic analysis. Both regulatory (1, 2) and auxotrophic mutants (2-4) in cholesterol biosynthesis have been isolated in several laboratories and it appears likely that somatic cell mutants with low density lipoprotein receptor defects analogous to those occurring in patients suffering from familial hypercholesterolemia can be isolated as well (5). Somatic cell auxotrophs in cholesterol biosynthesis can be extremely useful in the further analysis of the regulation of cholesterol biosynthesis, as such mutants would permit the chromosomal localization of genes encoding regulated enzymes (6), tests for cis or trans dominance of regulatory mutations (7), and potential cloning vehicles of genes encoding regulated enzymes (8). In the Chinese hamster ovary cell, one of the more tractable cell lines for somatic cell genetic analysis, it has been demonstrated that the first four enzymes in the cholesterol biosynthesis pathway are regulated by exogenous sterol (9). These enzymes are cytosolic acetoacetate-CoA thiolase, 3-hydroxy-3-methylglutaryl-CoA synthase, 3-hydroxy-3-methylglutaryl-CoA reductase, and mevalonate kinase. Auxotrophic mutants for cholesterol defective in any of these enzymes would thus be extremely useful in the analysis of their regulation. Unfortunately, direct isolation of somatic cell mutants auxotrophic for cholesterol has led almost entirely to mutants which are blocked late in the pathway (3, 4) rather than in any of these first four steps of cholesterol biosynthesis. In this report, we take advantage of the observation that cultured cells exhibit a mevalonate requirement even in the presence of serum cholesterol (10) to develop a bromodeoxyuridine-visible light technique for the direct isolation of mevalonate auxotrophs. Such mutants should be defective in any of the first three steps of cholesterol biosynthesis. The mutant described in this report is shown to be defective in the second step of the pathway, HMG-CoA synthase.

Isolation and Characterization of a Mammalian Cell Mutant Defective in 3-Hydroxy-3-methylglutaryl Coenzyme A Synthase*

(Received for publication, May 26, 1981)

Robin Schnitzer-Polokoff, Charles von Gunten, Judy Logel, Robert Torget, and Michael Sinensky‡

From the Eleanor Roosevelt Institute for Cancer Research, Denver, Colorado 80262

* This work was supported by National Institutes of Health Grants GM 24732, CA 15794, and HD 02080 to Michael Sinensky. This is Contribution No. 360 from the Eleanor Roosevelt Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ An Established Investigator of the American Heart Association.

The control of cholesterol biosynthesis in cultured mammalian cells by exogenous sterol is an interesting example of metabolic regulation in mammalian systems that is tractable to both biochemical and somatic cell genetic analysis. Both regulatory (1, 2) and auxotrophic mutants (2-4) in cholesterol biosynthesis have been isolated in several laboratories and it appears likely that somatic cell mutants with low density lipoprotein receptor defects analogous to those occurring in patients suffering from familial hypercholesterolemia can be isolated as well (5). Somatic cell auxotrophs in cholesterol biosynthesis can be extremely useful in the further analysis of the regulation of cholesterol biosynthesis, as such mutants would permit the chromosomal localization of genes encoding regulated enzymes (6), tests for cis or trans dominance of regulatory mutations (7), and potential cloning vehicles of genes encoding regulated enzymes (8). In the Chinese hamster ovary cell, one of the more tractable cell lines for somatic cell genetic analysis, it has been demonstrated that the first four enzymes in the cholesterol biosynthesis pathway are regulated by exogenous sterol (9). These enzymes are cytosolic acetoacetate-CoA thiolase, 3-hydroxy-3-methylglutaryl-CoA synthase, 3-hydroxy-3-methylglutaryl-CoA reductase, and mevalonate kinase. Auxotrophic mutants for cholesterol defective in any of these enzymes would thus be extremely useful in the analysis of their regulation. Unfortunately, direct isolation of somatic cell mutants auxotrophic for cholesterol has led almost entirely to mutants which are blocked late in the pathway (3, 4) rather than in any of these first four steps of cholesterol biosynthesis. In this report, we take advantage of the observation that cultured cells exhibit a mevalonate requirement even in the presence of serum cholesterol (10) to develop a bromodeoxyuridine-visible light technique for the direct isolation of mevalonate auxotrophs. Such mutants should be defective in any of the first three steps of cholesterol biosynthesis. The mutant described in this report is shown to be defective in the second step of the pathway, HMG-CoA synthase.

MATERIALS AND METHODS

Chemicals—Mevalonolactone, isopentenyl adenine, dolichol, ubiquinone, cholesterol (recrystallized), CoA, acetyl-CoA, acetoacetyl-CoA, glucose-6-phosphate, NADP, and glucose-6-phosphate dehydrogenase were obtained from Sigma, St. Louis, MO. Radioactively labeled 3-hydroxy-[3,4-14C]-mevalonyl-CoA (54 mCi/mmol), acetyl-[1,2-14C]-CoA (57.7 mCi/mmol), and [4-14C]cholesterol were purchased from New England Nuclear, Boston, MA. Radioactive [3-14C]acetate, sodium salt (38.7 mCi/mmol), and [2-14C]mevalonolactone (22 mCi/mmol) were purchased from Amersham/Searle, Arlington Heights, IL.

Cells and Media—Cells used were the CHO-K1 (pro) cell (11) and a Chinese hamster lung cell defective in purine synthesis. Cells were grown on Ham's F12 medium (12) supplemented with fetal calf serum, dialyzed fetal calf serum, new born calf serum, or serum from which cholesterol had been removed by either organic solvent deplipidization (DIPE) (13) or flotation of lipoproteins (HDF) (14).

Isolation of a Mevalonate Auxotroph—The procedure used was essentially the bromodeoxyuridine-visible light method of Kao and Puck (11). CHO-K1 cells grown in F12 medium + 5% dialyzed fetal calf serum and 0.43 mM mevalonate were mutagenized by 0.4 μl of ethyl methane sulfonate/ml of medium in 150-mm Petri dishes containing 2.5 × 10⁶ cells for 18 h. The medium was then changed to F12 + 5% dialyzed fetal calf serum + 0.43 mM mevalonate and incubated for 3 days with a daily media change. The cells were pooled and 1 × 10⁶ cells were incubated in each of 40 60-mm Petri dishes in F12 + 5% dialyzed fetal calf serum and incubated for 12 h. Bromodeoxyuridine was added at a concentration of 10⁻⁴ M and cells were incubated for 14 h in the dark. Cells were then irradiated with visible light for 1 h. The medium was then changed to F12 + 5% fetal calf serum and 0.43 mM mevalonate and the cells incubated until clones appeared within 3 weeks. Clones were picked and retested for their mevalonate requirement by incubation in F12 + 5% fetal calf serum with and without 0.43 mM mevalonate. Clones that grew in the presence and did not grow in the absence of mevalonate were recloned, restested, and then classified as auxotrophs.

Incorporation of Labeled Acetate and Mevalonate into Saponifiable Lipids—2.5 × 10⁶ cells were incubated into 100-mm plates and incubated overnight in F12 + 5% fetal calf serum + 0.43 mM mevalonate. The medium was then changed to F12 + 5% fetal calf serum for 4 h and 50 μCi of [14C]acetate or 5 μCi of [14C]mevalonolactone was added. After incubation for 22 h, the medium was changed to F12 + 5% fetal calf serum + 0.43 mM mevalonate and cells were incubated for an additional 24 h. Cells were harvested by scraping into 1.0 ml of 0.05 M Tris, pH 7.4, and lipids were extracted, saponified, and chromatographed.

Lipid Analysis—Cellular lipids were extracted by the method of Bligh and Dyer (15). Saponification was performed at 100 °C in 2.0
ml of 5% ethanolic KOH (16). After extraction 3 times with petroleum ether, lipids were separated by silica gel thin layer chromatography in petroleum ether/diethyl ether/acetic acid either 80:20:1 or 90:10:1 (1). The identity of the labeled reaction product of the HMG-CoA synthase assay was determined by silica gel thin layer chromatography in butanol/acetic acid/water (5:2:3, v/v) using authentic [14C]hydroxymethylglutarate as a standard (17). Chromatographed lipids were visualized with iodine vapor and then autoradiographed using Kodak X-omat film and 'H-Enhance (New England Nuclear).

Preparation of Microsomal and Cytosolic Fractions—For the assay of acetoacetoy-CoA thiolase, cells were scraped into 0.2 ml of 0.25 m sucrose, 0.1 mM EDTA, and 2.0 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hapes), pH 7.2, and broken by 3-5 s sonic bursts. The homogenate was centrifuged at 8000 g for 15 min and the resulting supernatant fraction was spun at 100,000 g for 1 h. The supernatant from this spin (cytosolic fraction) was used directly.

For the assay of HMG-CoA synthase, cells were scraped into 0.2 ml of PBS, pH 7.2, broken by sonication, and the cytosol prepared as described above. This fraction was then dialyzed for 24 h at 4 °C against 20 mM potassium phosphate buffer, pH 7.2, containing 0.1 mM EDTA and 0.5 mM dithiothreitol, and stored at -70 °C until used. The microsomal pellet obtained from the 100,000 g spin in both cytosolic preparations was frozen at -70 °C and used for the assay of HMG-CoA reductase.

Enzyme Assays—HMG-CoA reductase was assayed as previously described (18). Cytosolic acetoacetoy-CoA thiolase was assayed by the spectrophotometric procedure of Clinkenbeard et al. (19) in a final volume of 0.5 ml of 118 mM Tris-chloride, pH 8.2, and 0.118 mM EDTA containing 12 mM acetoacetoy-CoA, 90 μM CoA, and 2 to 16 μg of cytosolic protein. Cytosolic HMG-CoA reductase was assayed by a modified procedure of Balasubramanian et al. (17). The standard assay mixture contained in a final volume of 0.2 ml of 0.25 M Tris, pH 8.0, 80 μM acetoacetyl CoA, 400 μM [1-14C]acetyl-CoA (2 pCi/μmol), and 20 to 100 μg of cytosolic protein.

RESULTS

Nutritional Characterization of a Mevalonate Auxotroph—Using the bromodeoxyuridine-visible light technique, a somatic cell mutant (Mev-1) of the CHO-K1 cell was isolated that exhibits mevalonate auxotrophy in medium containing serum (Fig. 1). No growth of the mutant was observed at less than 10 μg/ml of mevalonate supplement to the culture medium. Concentrations of mevalonate greater than 20 μg/ml in the culture medium produced the expected 4-fold increase in cell number during a 48-h incubation period (11). Starvation of Mev-1 for mevalonate for 60 h produced a drastic reduction in cell viability as measured by relative plating efficiency (Fig. 2). To determine whether the cholesterol requirement of the Mev-1 mutant could be met by mevalonate as would be expected for a mutant blocked in one of the first three steps of cholesterol biosynthesis, the capacity of mevalonate to support the growth of the Mev-1 mutant in cholesterol-poor medium was examined. The results (Table I) indicated that at concentrations comparable to that of the mixture of cholesterol and mevalonate supplements in which growth was originally selected, neither mevalonate nor cholesterol alone could support growth of the mutant. In contrast, high concentrations of mevalonate (13.4 mM) promoted Mev-1 growth, although at a slower rate than that observed in media containing both cholesterol and mevalonate. The requirement for mevalonate supplements at such high concentrations to meet a requirement for cholesterol biosynthesis has previously been reported (2).

The failure of a 10 μg/ml supplement of cholesterol to permit growth of Mev-1 suggested that some mevalonate metabolite other than cholesterol might be required for cell survival. Siperstein and co-workers (10, 20) have demonstrated a regulatory role of mevalonate in S phase DNA replication which appears to be mediated by isopentenyl adenine and not other mevalonate derivatives. Accordingly,
the ability of dolichol, ubiquinone, and isopentenyl adenine to replace the mevalonate requirement of MeV-1 in medium containing serum was examined. While wild type cell growth was unaffected, the growth of MeV-1 measured over a 65-h period was not sustained by any of these three mevalonate metabolites added either separately or in combination over a broad range of concentrations (data not shown).

**Conversion of Cholesterol Precursors to Cholesterol in CHO-K1 and MeV-1**—The ability of mevalonate alone to support the growth of the MeV-1 mutant implies that this cell is blocked in mevalonate biosynthesis. If this is the case, it should be possible to demonstrate comparable conversion of labeled mevalonate to cholesterol in MeV-1 and CHO-K1. However, conversion of acetate to cholesterol under the same labeling conditions should be far less efficient in MeV-1 than in CHO-K1. The results of such an experiment (Fig. 3) indicate that Mev-1 is indeed blocked in the conversion of acetate to cholesterol relative to the wild type cell, but that conversion of mevalonate to cholesterol is comparable in the two cell types. It should be noted that efficient conversion of mevalonate to cholesterol in Mev-1 could only be observed when cells were subsequently incubated with mevalonate supplements capable of supporting growth. These observations suggested that Mev-1 was defective in de novo cholesterol biosynthesis and that the defect was either at the level of, or prior to, HMG-CoA reductase.

**Activities of Acetoacetyl-CoA Thiolase, HMG-CoA Synthase, and HMG-CoA Reductase in CHO-K1 and Mev-1**—To determine which early steps of cholesterol biosynthesis were defective in Mev-1, the activities of the first three enzymes in the cholesterol biosynthetic pathway were investigated. Since the activities of these enzymes increase when cells are grown in the absence of sterol (9), cells were incubated in delipidated medium without mevalonate for 24 h prior to harvest to maximize activity. No difference in cytosolic acetoacetyl-CoA thiolase activity was observed between CHO-K1 and MeV-1, while microsomal HMG-CoA reductase activity was actually stimulated 4-fold in the mutant (Table II). In contrast, a striking reduction of cytosolic HMG-CoA synthase activity (45-fold) was apparent in Mev-1. To determine whether the decrease in HMG-CoA synthase activity was a consequence of reduced cellular metabolism due to incubation in nonpermissive growth medium, HMG-CoA synthase activity was measured and compared in cells incubated with and without added mevalonate. The addition of mevalonate produced a 2-fold reduction in synthase activity in CHO-K1 (Table III). However, no effect of added mevalonate on synthase activity was detectable in the mutant. These results suggested that the failure of Mev-1 to incorporate [3-14C]acetate into cholesterol in vivo was due to a defect in HMG-CoA synthase activity.

**Characterization of Cytosolic HMG-CoA Synthase Activities in CHO-K1 and MeV-1**—Mev-1 has less than 10% as much cytosolic HMG-CoA synthase activity as CHO-K1 (Tables II, III, and V). The identity of this activity in both cell types was confirmed by the isolation of a 117-kDa protein that showed identical migration to lanosterol on gel-permeation chromatography. The labeled material in the band immediately above cholesterol co-chromatographs with fatty acid. Some un-}

![Fig. 3. Incorporation of labeled acetate or mevalonate into saponified lipids in CHO-K1 and Mev-1.](http://www.jbc.org/)

| Table II |
|---|

**A comparison of cholesterogetic enzyme activities in CHO-K1 and Mev-1**

| Cell type | Cytosolic acetoacetyl-CoA thiolase | Cytosolic HMG-CoA synthase | Microsomal HMG-CoA reductase |
|---|---|---|---|
| CHO-K1 | 147.6 ± 17.6 | 2.68 ± 0.74 | 42 ± 6.0 |
| Mev-1 | 194.3 ± 29 | 0.06 ± 0.02 | 158 ± 20 |

| Table III |

**The effect of added mevalonate on the activity of HMG-CoA synthase in CHO-K1 and Mev-1**

| Cell type | Specific activity of HMG-CoA synthase |
|---|---|
| CHO-K1 | 2.68 ± 0.74 |
| Mev-1 | 0.06 ± 0.02 |
25-hydroxycholesterol (Table V). In contrast, MeV-1 synthase activity was unaffected by sterol supplementation. The activity of HMG-CoA synthase in CHO-K1 and Mev-1 in the absence and presence of 25-hydroxycholesterol was stimulated 10-fold by incubating cells in delipidized mevalonate-supplemented medium and this stimulation was prevented by the addition of 0.43 mM mevalonate and incubated for 24 h. The medium was then changed to F12 + 2% DIPE with and without 0.43 mM mevalonate and cells incubated an additional 24 h. Cells were harvested and enzyme activity assayed as described under "Materials and Methods." Results are expressed as the mean of 2 separate determinations. Table V

| Incubation condition                  | Specific activity of HMG-CoA synthase (nmol/min/mg) |
|---------------------------------------|-----------------------------------------------------|
| CHO-K1                                | 0.27 ± 0.07                                         |
| MeV-1                                 | 0.03 ± 0.02                                         |
| CHO-K1 and MeV-1                      | 0.10 ± 0.03                                         |

The data presented in this report describe the isolation and characterization of a mutant of the CHO-K1 cell line that is recessive for the mevalonate requirement of Mev-1. Mev-1 cells were fused with a Chinese hamster fibroblast auxotrophic for adenine and thymidine by means of irradiated Sendai virus. Ten thousand treated cells were inoculated into F12 + 5% fetal calf serum deficient in hypoxanthine thymidine and mevalonate. The cells were incubated for 6 days and then fixed and stained.

Fig. 5. Recessive nature of the mevalonate requirement of Mev-1. Mev-1 cells were fused with a Chinese hamster fibroblast auxotrophic for adenine and thymidine by means of irradiated Sendai virus. Ten thousand treated cells were inoculated into F12 + 5% fetal calf serum deficient in hypoxanthine thymidine and mevalonate. The cells were incubated for 6 days and then fixed and stained.
phenotypically a mevalonate auxotroph. The mutant is shown to have an absolute requirement for mevalonate for growth and viability even with fetal calf serum or cholesterol in the culture medium. Our experiments indicate that Mev-1 is incapable of converting acetate to cholesterol, apparently because of a defect in the enzyme HMG-CoA synthase. The data are consistent with this defect being due to an alteration in the structural gene for this enzyme, rather than an effect due to faulty regulation or the presence of a cytosolic inhibitor in Mev-1. Although these studies confirm those of other workers in suggesting that mammalian cells have a requirement for mevalonate itself or for some known or unknown metabolite of mevalonate. Our inability to support the growth of Mev-1 with dolichol, ubiquinone, or isopentenyl adenine supplements does not rule out the possibility that one of these products may in fact be required for growth in the absence of endogenous mevalonate synthesis since we have not yet confirmed cellular uptake of these compounds. The observation that cholesterol in the growth medium can reduce the mevalonate requirement of Mev-1 argues against the possibility of a defect in the enzyme HMG-CoA synthase. The elevation of HMG-CoA reductase activity in Mev-1 is worthy of note. It has previously been observed that such observations may be due to an inhibition of mevalonate biosynthesis by these compounds. It is anticipated that with depressed levels of HMG-CoA synthase and elevated levels of HMG-CoA reductase activity, Mev-1 will serve as a useful tool in gene-mapping studies, further experiments designed to elucidate the regulation of cholesterol biosynthesis, and the preparation of cDNA probes for both enzymes.

REFERENCES

1. Sinensky, M. (1977) Biochem. Biophys. Res. Commun. 78, 863-867
2. Sinensky, M., Armagast, S., Mueller, G., and Torget, R. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 6621-6623
3. Chang, T. Y., Telakowski, C., Vanden Heuvel, W., Alberts, A. W., and Vagelos, P. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 77, 832-836
4. Saito, Y., Chou, S. M., and Silbert, D. F. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3730-3734
5. Goldstein, J. L., Brown, M. S., Krieger, M., Anderson, R. G., and Mintz, B. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 2843-2847
6. Ruddle, F. H., and Creagan, R. P. (1975) Annu. Rev. Genet. 9, 497-486
7. Hartman, P. E., and Suskind, J. R. (1965) Gene Action, pp. 104-105, Prentice-Hall, New York
8. Perucchini, M., Hanahan, D., Lipsich, L., and Wigler, M. (1980) Nature 285, 207-211
9. Chang, T. Y., and Limanek, J. S. (1980) J. Biol. Chem. 255, 7787-7795
10. Quesney-Huneeus, V., Wiley, M. H., and Siperstein, M. D. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 5066-5060
11. Kao, F. T., and Puck, T. T. (1969) J. Cell Physiol. 74, 245-258
12. Ham, R. G. (1965) Proc. Natl. Acad. Sci. U. S. A. 53, 286-289
13. Cham, B. E., and Knowles, B. R. (1976) J. Lipid Res. 17, 176-181
14. Sinensky, M. (1979) FEBS Lett. 106, 129-131
15. Bligh, E. G., and Dyer, W. H. (1959) Can. J. Biochem. Physiol. 37, 911-917
16. Ditmer, J. C., and Wells, M. A. (1969) Methods Enzymol. 14, 493
17. Balasubramanian, S., Goldstein, J. L., and Brown, M. S. (1977) Biochemistry 74, 1421-1425
18. Sinensky, M., Duwe, G., and Pinkerton, F. (1979) J. Biol. Chem. 254, 4482-4486
19. Clinkenbeard, K. D., Sugiyama, T., Moss, J., Reed, W. D., and Lane, M. D. (1973) J. Biol. Chem. 248, 2275-2284
20. Quesney-Huneeus, V., Wiley, M. H., and Siperstein, M. D. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5842-5846
21. Brown, M. S., Faust, J. R., Goldstein, J. L., Kaneko, I., and Endo, A. (1978) J. Biol. Chem. 253, 1121-1128
Isolation and characterization of a mammalian cell mutant defective in 3-hydroxy-3-methylglutaryl coenzyme A synthase.

R Schnitzer-Polokoff, C von Gunten, J Logel, R Torget and M Sinensky

J. Biol. Chem. 1982, 257:472-476.

Access the most updated version of this article at http://www.jbc.org/content/257/1/472

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/257/1/472.full.html#ref-list-1