Vol. 82, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 15, 1978 Pages 228-234

REVERSION TO METHIONINE INDEPENDENCE BY MALIGNANT RAT AND SV40-TRANSFORMED HUMAN FIBROBLASTS

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Received March 17, 1978

SUMMARY. Although many lines of malignant and transformed cells are unable to grow in folate- and cobalamin-supplemented medium in which methionine is replaced by homocysteine its immediate metabolic precursor, rare cells from these lines regained the normal ability to grow under these conditions. Six revertant lines, one from Walker-256 rat breast carcinoma cells and five from SV40-transformed human fibroblasts, have been characterized with regard to growth and three measures of methionine biosynthetic capacity: methionine synthetase and methylenetetrahydrofolate reductase activities in cell extracts, and uptake of label from [5-^{14}C]methyltetrahydrofolate by intact cells. When all three measures of methionine biosynthetic capacity were considered, two revertants isolated from SV40-transformed cells had regained the ability to grow like normal cells in homocysteine medium without substantial changes in these measures. Increased methionine biosynthesis thus is not a prerequisite to reversion of the methionine auxotrophy present in the transformed parental lines.

We recently described (1) high levels of endogenous methionine biosynthesis in malignant and SV40-transformed cells that, unlike normal fibroblasts, were unable to grow in folate- and cobalamin-supplemented media in which methionine (Met)* was replaced by homocysteine (Hc), its immediate metabolic precursor. We postulated that the malignant and transformed cells were defective in some step in utilization of the endogenously synthesized methionine rather than in its synthesis. In this report we demonstrate that two SV40-transformed human lines, W18VA2 and SV80, and a malignant rat breast carcinoma, Walker-256 (W-256), can revert spontaneously to methionine independence. The features of the revertants suggest that there are several different mechanisms for reversion and that increased methionine biosynthesis is not a prerequisite to regaining the ability to grow in media in which Hc replaces Met. These

*Abbreviations: Met, methionine; Hc, homocysteine; W-256, Walker-256; H_4PteGlu, tetrahydropteroylglutamic acid.

0006-291X/78/0821-0228$01.00/0

data are consistant with our hypothesis that the original defect in the transformed and malignant cells is not in methionine biosynthesis.

MATERIALS AND METHODS

Cell Lines: The human SV40-transformed lines, W18VA2 and SV80, and rat mammary carcinoma line, W-256, were described previously (1). W18VA2 and W-256 were doubly cloned in non-selective medium. All lines were free of mycoplasma as determined by growth tests.

Media: The following media were used: a) Eagle's minimum essential medium (MEM) with 10% dialyzed fetal calf serum, without methionine and with 0.1 mM L-homocysteine thiolactone, 0.1 mM folic acid 1.5 μM hydroxocobalamin, designated Hc^+Met^- medium. b) MEM with 10% dialyzed fetal calf serum, 0.1 mM methionine, 0.1 mM folic acid and 1.5 μM hydroxocobalamin, designated Met^+ medium. When other media were used, these are specified in the text or tables.

Isolation of revertants: Parental transformed and malignant cells were inoculated in 100 mm dishes with 10 ml of Hc^+Met^- medium and refed for about 1 month. Focal areas of growing cells were isolated with cloning cylinders. These new lines were further cloned and characterized.

Enzyme assays: Methionine synthetase, or 5-methyltetrahydropteroyl-glutamate: L-homocysteine S-methyltransferase (EC 2.1.1.13) was measured as described previously (2) except that the Dowex AG 1-X8 columns used to separate methionine from 5-methyltetrahydrofolate (5-methyl-H_4PteGlu) were equilibrated and eluted with 40 mM phosphate buffer pH 7.4 instead of water. 5,10-Methylene-H_4PteGlu reductase, or methyltetrahydrofolate: NAD^+ oxidoreductase (EC 1.1.1.68), was measured as described previously (3).

Uptake of [5-^{14}C]methyl-H_4PteGlu was measured as described previously (1).

Growth studies were conducted as described earlier (1) except 60 mm petri dishes containing 4 ml of media were used with an initial inoculum of 1.8×10^5 cells per dish. Cells were counted with a Coulter counter.

Protein concentration was measured by the method of Lowry et al. (4).

RESULTS

When parental methionine auxotrophic lines were inoculated in Hc^+Met^- medium, most of the cells eventually detached from the surface despite weekly or twice weekly feedings. However, after 2-4 weeks, focal areas of growing cells appeared. These cells continued to grow in Hc^+Met^- medium and were cloned. The apparent reversion frequency of the W18VA2 line was about $1\text{-}2 \times 10^{-4}$ (data not shown). Revertant lines SV80R2 and W-256R1 were doubly cloned in Met^+ medium. After many generations of growth under nonselective conditions these lines maintained their ability to grow in Hc^+Met^- medium and thus were stable.

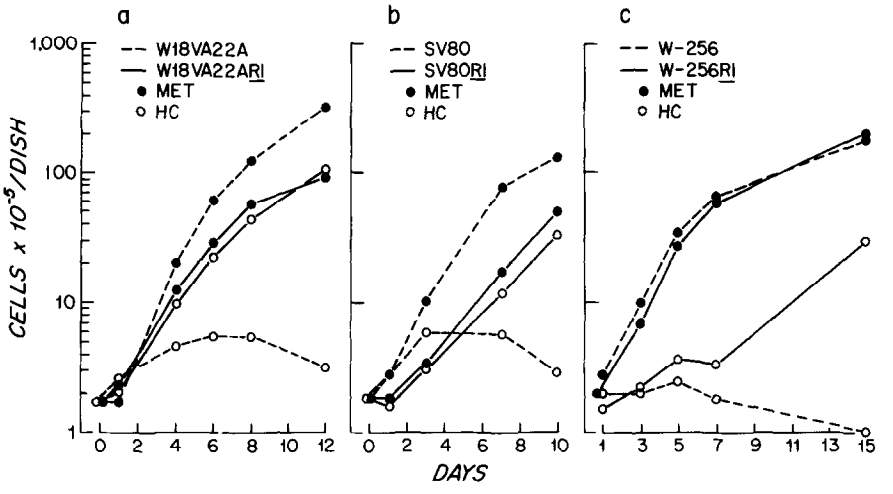

Figure 1: Growth of parental malignant and transformed methionine auxotrophs and their methionine independent revertants in Hc^+Met^- (O) and Met^+ medium (●). a, W18VA22A and W18VA22AR1; b, SV80 and SV80R1; c, W-256 and W-256R1.

Fig. 1 compares the growth of a representative revertant of each parental line with its respective parent in Hc^+Met^- medium and Met^+ medium. Revertants W18VA22AR1 (Fig. 1a) and SV80R1 (Fig. 1b) grew as well in Hc^+Met^- as in Met^+ medium in striking contrast to the parental lines which grew only transiently in Hc^+Met^- medium. The revertants grew almost as well as their parents in Met^+ medium. The revertant rat line W-256R1, although growing in Hc^+Met^- medium unlike its parent, grew much faster in Met^+ medium (Fig. 1c).

Table 1 compares the activity of methionine synthetase in extracts of the revertants and their respective parental lines. Although five of the six revertants contained somewhat greater levels of methionine synthetase activity under one or more of the conditions tested, revertant SV80R1 contained parental levels of the enzyme under all of these conditions indicating that increased methionine synthetase activity is not essential for reversion. In extracts of revertants grown in Hc^+Met^- medium supplemented with cobalamin, conditions that might be expected to derepress the methionine synthetase activity (2), none of the five revertant lines tested showed increased activity.

Table 1

Growth media	Assay conditions cyano-cobalamin	Methionine synthetase activity in cell extracts (nmol of methionine formed per mg of protein)								
		W18VA22A*	W18VA22AR1*	SV80	SV80R1*	SV80R2*	SV802L*	SV802LR1*	W-256*	W-256R1*
MEM + OH-Cbl	+	6.8	14.9	8.2 7.4	8.3 7.3	13 14.7	9.6	27	4.2	7.6
	-	1.9	4.7	1.9	2.5	2.1	1.7	9.2	1.5	2.2
MEM	+	0.5 1.9	2	1.2	1.1	1.9	2.1 1.4	1.1	0.65 0.74	0.72
	-	0	0.02	0.03	0.09	0.04	0.08	0.1	0.04 0.1	0.17
MEM, -Met, +Hc, +OH-Cbl	+	#	8.4	#	8.8	9.9	#	15.9	#	5.1

*Doubly cloned line

#The inability of cells to grow under these conditions precluded activity measurements.

Further evidence that increased methionine biosynthesis is not a prerequisite for reversion was provided by studies of *in vivo* methionine synthesis measured by the uptake of label from [5-^{14}C]methyl-H_4PteGlu as described previously (1). Fig. 2a-c show three revertants that do not take up more label than their parents. However, Fig. 2d and 2e show revertants with intermediate increments while the rat cell revertant W-256R1 (Fig. 2f) took up substantially more label than its parent.

Table 2 shows the activities in cell extracts of 5,10-methylene-H_4PteGlu reductase, the enzyme catalyzing the synthesis of 5-methyl-H_4PteGlu, the methyl donor in methionine biosynthesis. Although revertant W18VA22AR1 contained about 5 fold greater activity than its parent, other revertants showed small increments and the activities in SV80R2 and its parent were equal indicating that increased reductase activity is also not essential for reversion.

DISCUSSION

In a previous report (1) we described malignant and SV40-transformed cells that were unable to grow in media in which Met was replaced by Hc despite high levels of endogenous methionine biosynthesis. The malignant and transformed

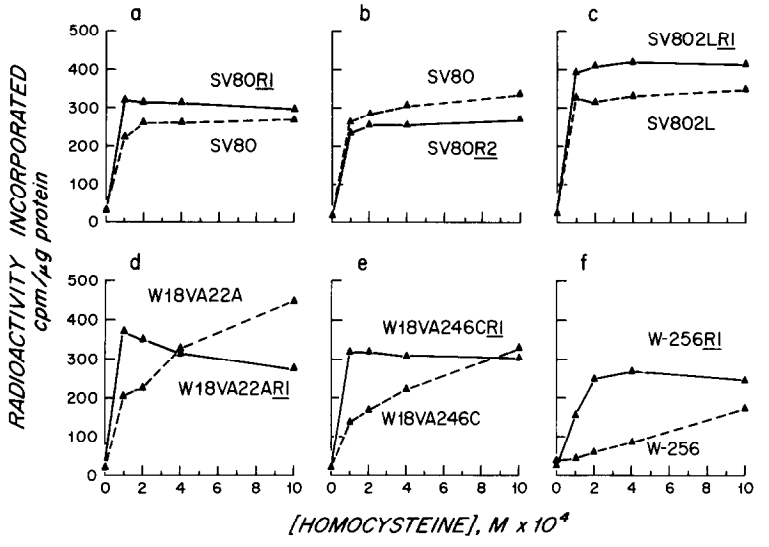

Figure 2: Effect of homocysteine on the cellular uptake of radioactive label from [5-^{14}C]methyl-H_4PteGlu in methionine auxotrophic parents (--) and methionine independent revertants (—). The medium in which the cells were incubated contained homocysteine at the concentration indicated on the abscissa but lacked methionine and folic acid. After 26 hours, intracellular radioactivity was measured as described in Materials and Methods. Each point is the average of two determinations.

Table 2

Methylene-H_4PteGlu Reductase Activity in Cell Extracts

(nmol of formaldehyde per mg of protein)

Growth Media	W18VA22A*	W18VA22ARI*	SV80	SV80RI*	SV80R2*	SV802L*	SV802LRI*	W-256*	W-256RI*
MEM + OH-Cbl	3.15 7.0	23.3 29.8	7.4 8.4	14.9 20.3	5.5 8.7	11.7 14.1	19.7	17.6	26.7
MEM, -Met, +Hc, +OH-Cbl	#	19.5	#	14.0	4.0	#	23.6	#	16.5

*Doubly cloned line

#The inability of cells to grow under these conditions precluded activity measurements.

cells, however, appeared to use exogenous methionine normally. We postulated that these cells were defective in some step in the utilization of endogenously

Table 3

Ratio of Revertant to Parent Methionine Biosynthetic Capacity

	Methionine Synthesis		5-Methyl-H_4PteGlu Synthesis
Line	Revertant/Parent Methionine synthetase in extracts	Revertant/Parent Incorporation of label from [5-^{14}C]methyl-H_4PteGlu in 0.1 mM homocysteine	Revertant/Parent 5,10-methylene-H_4PteGlu reductase
SV80R1	1	1.5	2.2
SV80R2	1.8	0.9	0.9
W18VA22AR1	2.2	1.8	5.2
W18VA246CR1	3.2	2.5	1.2
W-256R1	1.8	11.5	1.5
SV802LR1	2.8	1.2	1.5

synthesized methionine rather than in its biosynthesis. This hypothesis is now further supported by the studies of revertants described here. The revertant/parent comparisons are shown in Table 3. Of particular importance are SV80R1 and SV80R2 which have regained methionine independence without increases in any of the three measures of methionine biosynthesis. Thus these together with our previous results (1) indicate that rates of methionine synthesis do not distinguish normal, methionine-independent, diploid cells from methionine-dependent transformed cells or from these two methionine independent revertants.

Other revertants, however, had increased levels of one or more of the activities measured. The about three fold greater level of methionine synthetase activity in extracts of SV802LR1 and W18VA246CR1 grown and assayed in high levels of cobalamin may be the basis of reversion in these lines, possibly correcting the parental defect by compensation. The uptake of label from [5-^{14}C]methyl-H_4PteGlu, a measure of *in vivo* methionine synthesis (1), by revertant W-256R1 was about 11 fold greater than its parent in the presence of 0.1 mM homocysteine. This large increment may reflect the basis for reversion

of W-256R1. Methylene-H_4PteGlu reductase was thought to be deficient and possibly rate limiting in a methionine auxotrophic SV40-transformed baby hamster kidney cell line studied by Jacobsen et. al (5). The 5 fold greater level of this reductase in W18VA22AR1 may be the basis of reversion in this line.

Our initial attempts have thus yielded revertants that are heterogenous. These revertants have demonstrated that increments in three measures of methionine biosynthesis alone or in combination are not necessary for reversion. Since revertant lines SV80R1 and SV80R2 appear unaltered in regard to methionine biosynthesis, they may have regained methionine independence by correcting the defect that originally produced the methionine auxotrophy. Although not yet identified such a defect could involve specific deficiencies in protein synthesis due to altered charging of transfer RNA or, via S-adenosylmethionine synthesized from methionine, specific abnormalities in transmethylation or polyamine synthesis. Further studies will be necessary to explore these possibilities.

ACKNOWLEDGEMENTS

RMH was supported by fellowships from The Medical Foundation, Inc., Boston, and from N.I.H. (1 F32 CA05850), SJJ by an Institutional National Research Service Award (1 T32 07092) and this investigation by grants CA16838 and HD06356 from the National Institutes of Health. We thank Helen H. Wong for excellent technical assistance, and Martha K. Conant and Leslie Jalbert for aid in preparation of this manuscript.

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