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Correlation of C-Type Virus (WF-1) Production and Heme Synthesis in a Rat Fibroblastic Cell Line (37797)

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We have previously shown that the Friend and Rauscher leukemia viruses can stimulate heme synthesis in murine spleen (1, 2). Mitochondrial δ-aminolevulinic acid (ALA) synthetase, the limiting inducible enzyme of the heme biosynthetic pathway (3), was found to be elevated in this virus-infected tissue relative to normal spleen. In attempts to find a model tissue culture system to further examine this phenomenon, we observed high ALA synthetase activity in a virus-infected Wistar/Furth (W/Fu) rat fibroblastic cell line. This cell line was started from a normal W/Fu rat embryo, but after 2 years the culture started to show a granulomatous cytopathology and C-type particles were detected by electron microscopy (4). Injection of these cells into suckling W/Fu rats caused the development of infiltrating tumors in 90–100% of the animals within 4–5 weeks (4).

Methods. The WF-1 line of transformed rat cells producing C-type virus (4) was originally obtained from Dr. V. Bergs, Miami, FL. The RMTL-8 (5), R-35 (6), and RMTL-9 (Dr. V. Bergs, unpublished results) cell lines originated from mammary tumors in Sprague–Dawley rats. Embryo fibroblasts were obtained by trypsinizing normal W/Fu rat embryos as described by Bergs *et al.* (4). The four virus-containing cell lines exhibit RNA-dependent DNA polymerase activity typical of all RNA oncogenic viruses, whereas the embryo line shows little or no activity (in preparation).

The methods for incubating the cells and for the determination of ALA synthetase activity are described in the legend to Table II. 2-Ketoglutarate-5-^{14}C (Amersham/Searle) was found to be the most efficient substrate for ^{14}C-ALA production with the rat embryo fibroblast system. Succinate-2,3-^{14}C produced similar results, but yielded higher "zero time" values when passed through Dowex 50 columns (7). Addition of partially-purified succinic thiokinase (succinate:CoA ligase (GDP), EC 6.2.1.4) from rat liver to the incubation mixtures did not stimulate ALA synthetase production.

Results and Discussion. The relative activity of the entire heme biosynthetic pathway in the two fibroblastic cultures was studied by incubating the cell lines with glycine-2-^{14}C and isolating labeled hemin. Table I shows

TABLE I. "Heme" Synthesis in W/Fu Embryo and WF-1 Fibroblasts.[a]

Fibroblast culture	MEK-extractable ^{14}C dpm ^{14}C × $10^3/10^6$ cells/4hr
Embryo	4.72
WF-1	17.70

[a]Fibroblast cultures were incubated in Eagles minimal essential media containing 20% fetal calf serum and 2 μCi/ml glycine-2-^{14}C (57 mCi/mmole) for 4 hr. Cells were trypsinized, centrifuged at 1000 *g*, washed twice with Dulbecco's phosphate buffered saline, and suspended in Drabkin's reagent (1:3) overnight at 4°. The "hemin" was extracted into methylethylketone (MEK) as described by Krantz *et al.* (8). The results are the average of 2 experiments. When the MEK extract of the WF-1 cells was subjected to paper chromatography with 2,6-lutidine: water (55:45), only 34% of the total activity migrated with authentic hemin, while 1% of the activity was recovered in the region of glycine. A similar separation was obtained with the embryo cells.

that the transformed tumor cell line produced organic solvent-extractable "hemin" at about 4 times the rate of the normal embryo line.

Since the WF-1 line is not contact-inhibited and grows at a faster rate than the embryo line, it was necessary to compare the biosynthetic activity of both lines when each was actively proliferating. Table II shows the ALA synthetase activity in the embryo and the WF-1 line while proliferating, and at confluency with the former. The proliferating embryo culture showed higher ALA synthetase activity than the confluent culture, indicating a relationship between enzyme activity and growth rate. The WF-1 culture producing C-type virus showed greater than 3 times the enzyme activity of the proliferating embryo line.

TABLE II. ALA Synthetase Activity in Confluent and Proliferating W/Fu Embryo and WF-1 Fibroblasts.[a]

Fibroblast culture	pmoles ALA produced/10^7 cells/hr.
Confluent embryo	38.7
Proliferating embryo	55.2
WF-1	187.5

[a]W/Fu embryo cells were harvested while proliferating and at confluency using trypsin. WF-1 cells, not contact-inhibited, were similarly harvested. Cells were centrifuged and resuspended in 10 m*M* Tris–0.9% NaCl, pH 7.4. Cell counts were performed, and the suspension was quick-frozen in dry ice-ethanol. The mixture was thawed, and aliquots representing 10^7 cells were incubated with 1 μCi 2-ketoglutarate-5-^{14}C (21 mCi/mmole), 75 m*M* Tris-HCl, 100 m*M* glycine, 10 m*M* EDTA, and 0.2 m*M* pyridoxal phosphate, pH 7.2, in a final volume of 2.0 ml for 20 min. The reaction was stopped with 0.5 ml of 25% trichloroacetic acid (TCA), and the ^{14}C-ALA production was determined as described previously (7) with the following modifications. The TCA supernatant solution was adjusted to pH 4.2 with 3 ml of 1 *M* acetate buffer, pH 4.9, and applied to a 2.5 × 1 cm (i.d.) Dowex 50 (AG 50W-X8, sodium form) column. The column was then washed successively with 20 ml 0.1 *M* acetate buffer, pH 4.2, and 20 ml methanol:0.1 *M* acetate buffer, pH 4.2 (4:1, v/v), followed by the removal of the adsorbed ^{14}C-ALA with 4 ml 0.1 *N* NaOH. One-milliliter aliquots were mixed with 10 ml Aquasol (New England Nuclear) and counted in a Packard liquid scintillation counter, Model 3380. Recovery of pure ^{14}C-ALA was 88–95%. The method was occasionally monitored to be certain only ^{14}C-ALA was being eluted from the columns by subjecting the ALA pyrrole derivative of the column-purified ^{14}C-ALA to ascending paper chromatography (7). The ^{14}C-product migrated to the same R_f as authentic ^{14}C-ALA similarly prepared.

In order to confirm the results of the whole cell experiments and rule out the possibility that the normal and WF-1 lines may have different mitochondrial pool sizes, it was necessary to examine the specific enzyme activity in the mitochondria. Table III shows that there is 4 times the specific activity of ALA synthetase in mitochondria from WF-1 cells compared to that found in mitochondria from normal embryo fibroblasts. This finding suggests that, in addition to growth, the presence of virus or transformation may be inducing enzyme activity.

Four transformed cell lines exhibiting various rates of virus production have been described (5, 6, 10). Table IV shows the correspondence of virus concentration with ALA synthetase activity in the normal embryo cell line and in the 4 lines producing virus. The virus content of the cell lines could be more precisely graded by immunofluorescence than by electron microscopy as shown in Table IV. The lowest activities of ALA synthetase were observed in the embryo and RMTL-9 lines, and these were essentially the same, while the R-35 and RMTL-8 lines showed greater amounts of virus than the former

TABLE III. ALA Synthetase Activity in Mitochondria Isolated from W/Fu Embryo and WF-1 Fibroblasts.[a]

Source of mitochondria	pmoles ALA produced/mg protein/hr
Embryo fibroblasts	104
WF-1	423

[a]Approximately 10^8 cells were trypsinized, centrifuged, and suspended in 0.25 *M* sucrose, 0.01 *M* EDTA, pH 7.0. Cells were disrupted twice by sonication for 20 sec. Mitochondria were isolated from the cell lines by differential centrifugation (9), and this pellet was suspended in 0.25 *M* sucrose, 0.01 *M* EDTA, 0.02 *M* $MgCl_2$, 0.05 *M* phosphate buffer, pH 6.9. Aliquots were incubated with 2-ketoglutarate-5-^{14}C (17.5 mCi/mmole), and ALA synthetase activity was determined as described in the legend to Table II. The results are the average of 2 experiments.

TABLE IV. ALA Synthetase Activity as a Function of Virus Concentration in 5 Rat Cell Lines.[a]

Cell line	Relative numbers of virus particles/cell		pmoles ALA produced/10^7 cells/hr
	Immunofluorescence	Electron microscopy	
Embryo	0	0	74.8[b]
RMTL-9	1	0–1	96.3
R-35	2	2	218
RMTL-8	3	2	202
WF-1	4	3	1230

[a]ALA synthetase activity was determined as described in Table II. Electron microscopy and immunofluorescence assays were performed as described previously (10). The specific activity of the 2-ketoglutarate-5-^{14}C was 14.3 or 17.5 mCi/mmole.

[b]Data from 1 experiment. The remaining data are the average of 3 experiments.

lines, but the differences in the rate of virus production could not be delineated by electron microscopy. Similarly, ALA synthetase activity in these two lines was about the same and 2–3 times greater than that found in the embryo and RMTL-9 lines. By all criteria examined, the WF-1 line showed the greatest relative amount of virus and the highest enzyme activity. There was a good qualitative correspondence between the quantity of virus particles assayed by electron microscopy and the amount of enzyme activity in the five cell lines.

Since there is a correlation between the quantity of virus particles and enzyme activity, attempts were made to determine if the virus contained any enzyme activity. WF-1 viral pellets were obtained by centrifuging tissue culture supernatant fluid at 100,000*g* following a run at 10,000*g* to remove cell debris. When the pellets were incubated in the standard incubation mixture containing succinate-2,3-^{14}C and added succinic thiokinase to provide a succinyl-CoA generating system, no enzyme activity could be detected. As shown in Table III, the increased enzyme activity in the WF-1 line can be accounted for in the mitochondrial fraction. Therefore virus production is apparently altering the cellular synthesis of porphyrins by some unknown mechanism.

ALA synthetase activity does not appear to be related to transformation of the cell. Although the RMTL-8, RMTL-9, R-35, and WF-1 fibroblastic lines obtained from 2 different strains of rats are all transformed cells, the ALA synthetase activity varied as much as 30-fold (Experiment 3) between the lowest and highest virus-producer. In addition, the transformed line, RMTL-9, producing relatively low quantities of virus, showed similar enzyme activity to that of the normal embryo line. The requirement of a virus-transformed cell for porphyrin-containing enzymes or cytochromes has received little attention. Richert and Hare (11) have recently suggested that mitochondria may provide functions necessary for virus production and transformation in the Rous sarcoma virus-chick embryo fibroblast system. ALA synthetase induction and resultant heme biosynthesis has recently been shown to be associated with increased mitochondrial protein synthesis and cytochrome formation (12). Studies are now in progress to determine which enzymes or porphyrin-containing compounds are altered to accommodate virus production.

Summary. Some characteristics of heme biosynthesis were investigated in normal Wistar/Furth rat embryonic fibroblasts and spontaneously transformed cells producing rat C-type virus (WF-1). The activity of the control enzyme of the heme biosynthetic pathway, δ-aminolevulinic acid synthetase, was increased in mitochondria of the WF-1 line to 4 times the level of the normal embryo line, suggesting a relationship between transformation and heme biosynthetic activity. However, a direct correlation to ALA synthetase activity was observed when five rat fibroblastic cell lines were graded according to in-

creasing levels of virus production suggesting that heme biosynthetic activity is directly related to the rate of virus production.

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