# RAT ALPHA-FETOPROTEIN: IN VITRO PRODUCTION OF FOUR MOLECULAR VARIANTS BY CLONAL CELL LINES

James B. McMahon, Philip C. Kelleher and Carol J. Smith

Department of Medicine, University of Vermont College of Medicine, Burlington, Vermont 05401 USA

Received May 4,1977

SUMMARY: Mass and six clonal cultures derived from Morris hepatoma 7777 by standard tissue culture techniques synthesize and secrete alpha-fetoprotein in vitro. During serial passage, the alpha-fetoprotein which accumulates in the media of these cultures contains two concanavalin A-affinity molecular variants. Each of the concanavalin A-affinity molecular variants shows two electrophoretic variants. Mass and clonal cell populations of hepatoma cells continue to secrete in vitro four molecular variants of rat alpha-fetoprotein known to occur in vivo. These results demonstrate for the first time that individual hepatoma cells have the potential to synthesize four molecular variants of alpha-fetoprotein and that this phenotypic property is maintained during serial subculture in vitro.

Alpha-fetoprotein synthesis by hepatoma cell lines was first described by Irlin et al. (1). Since this report, the rate of AFP* secretion by hepatoma-derived clonal cell lines (2,3) and the effect of glucocorticoids on AFP secretion rates by hepatoma-derived cell cultures have been studied (4,5).

Four molecular variants of AFP have been identified in fetal and hepatoma-bearing rat serum. Belanger and Dufour (6) demonstrated two electrophoretic variants; the same proportions of the two electrophoretic variants were present in newborn, pregnant and hepatoma-bearing rat serum. Kerckaert et al. (7) found that the mobilities of both electrophoretic variants of rat AFP decreased after neuraminidase treatment. Two concanavalin A-affinity molecular variants of rat AFP were identified by Smith and Kelleher (8). One variant reacts with concanavalin A while the other does not; the two forms can be separated quantitatively by concanavalin A-agarose affinity chromatography (9). The concanavalin A-binding heterogeneity of rat AFP is probably due to differences in mannose content since mannose is the one sugar which is both present as a terminal residue on the oligosaccharides of serum glycoproteins

*Abbreviation: AFP, alpha-fetoprotein.





ISSN 0006-291X

(10) and reactive with concanavalin A (11). Two electrophoretic variants are present in each of the two concanavalin A-affinity variants of rat AFP (6, 12).

Secretion rates of the concanavalin A-affinity molecular variants of rat AFP are under different control mechanism(s) in normal cells and malignant cells (13). The relative amounts of the two concanavalin A-affinity molecular variants of AFP present in newborn rat serum are constant during the first four weeks of life. In contrast, the relative amounts of the two molecular variants present in transplantable hepatoma-bearing rat serum are constant during serial transplantation of an individual tumor line but differ to a varying degree from the relative amounts present in newborn rat serum. Primary hepatomas are associated with widely-differing proportions of the two concanavalin A-affinity molecular variants of AFP. To our knowledge, it has not been demonstrated whether the existence of multiple forms of individual serum glycoproteins (10) is associated with an individual cell's potential to secrete more than one molecular form of individual serum glycoproteins. In the present paper, we report that mass and all six clonal cultures of Morris hepatoma 7777 each secrete four molecular variants of rat AFP.

MATERIALS AND METHODS: Morris hepatoma 7777 was cultured by selective trypsinization of a 1-$cm^3$ piece of tumor (14). The cells were allowed to attach and grow for 72 h in plastic petri dishes before being transferred to 75-$cm^2$ (Corning) plastic flasks. These cultures were designated passage 1, and the standard procedure of inoculation of 1-2 x $10^5$ cells per 75-$cm^2$ flask on the first day, feeding on the third day and trypsinization, dilution (usually 1:20) and transfer on the fifth day defines one passage of these cultures.

Cells were grown in nutrient mixture F12 (Gibco) supplemented with 10% fetal calf serum, 50 µg/ml of aureomycin (Lederle) and 0.2 µg/ml of hydrocortisone hemisuccinate (Sigma). They were incubated at 37°C in a 5% $CO_2$ , 95% air, water-saturated atmosphere. Fibroblast-like cells in the cultures decreased consistently at each passage and could not be detected microscopically by passage 6.

The mass 7777 culture was cloned as follows. Cells in their sixth passage were trypsinized and suspended in culture medium. The cell suspension was adjusted to a concentration of 15 cells/ml, and 0.05ml was planted in each of 24 wells of a Linbro plastic culture dish. Eighteen hours after planting, wells containing only one cell were marked for identification. Populations of cells which grew in six of these marked wells were designated 7777 clones A through F.

Cells from the mass and clonal cultures were planted in 150-$cm^2$ flasks (Corning) and allowed to grow until near confluency (4-5.5 x $10^6$ cells/flask) The medium was then replaced with 50 ml of nutrient mixture F12 without fetal calf serum and the cells were incubated for an additional 48 h. This procedure minimized the effects of fetal calf serum proteins on the chromatographic

TABLE 1. AFP PRODUCTION AND CONCANAVALIN A-AFFINITY AFP MOLECULAR VARIANT RATIOS OF 7777 CULTURES IN VITRO

| Cell line | No. passages assayed | μg AFP/$10^6$ cells/ 48 h | $\frac{\text{Non-reactive AFP}}{\text{Total AFP}} \times 100$ |
|---|---|---|---|
| 7777 mass | 7 | 33 - 85* | 12 - 33* |
| 7777 clone A | 7 | 40 - 68 | 13 - 34 |
| clone B | 8 | 28 - 52 | 14 - 43 |
| clone C | 6 | 17 - 42 | 17 - 52 |
| clone D | 6 | 30 - 72 | 27 - 44 |
| clone E | 7 | 30 - 43 | 32 - 48 |
| clone F | 6 | 20 - 62 | 20 - 45 |
| 7777 passage 11 | --- | 64 ± 14** | 26 ± 5** |
| 7777 passage 13 | --- | 54 ± 14** | 28 ± 6** |

*Range of values obtained.

**Mean ± 2 S.D. obtained when 6 replicate plantings from one passage were assayed.

and electrophoretic analyses. The cells secreted AFP into the medium at the same rate during this time period irrespective of the presence or absence of the serum supplement. Following the 48 h incubation, the cell-free media were concentrated 30 to 50 times by Amicon ultrafiltration using XM-50 or PM-30 membranes.

Concentrations of AFP in media and column eluates were determined by quantitative radial immunodiffusion (15). AFP standard was purified as described previously (9). The variation of the quantitative immunodiffusion assay in our laboratory is 2%. Unused fetal calf serum-supplemented media concentrated 30 to 50 times showed no immunological reaction with the antisera.

Concanavalin A-affinity molecular variants were separated quantitatively by concanavalin A-agarose affinity chromatography (9). Ratios of the concanavalin A-affinity AFP molecular variants, expressed as the percentage of the total AFP not reactive with concanavalin A, were determined on fresh samples of media using 10-ml columns of concanavalin A-agarose. The non-reactive fraction was collected in a single tube and, if necessary, concentrated by ultrafiltration. The amount of concanavalin A non-reactive AFP and the amount of AFP applied to the column were measured and the ratio calculated.

Two-dimensional single electroimmunodiffusion (16) was carried out as follows. Duplicate 10 μl samples of sera, media or column eluate fractions containing 0.5-1.5 mg/ml AFP were electrophoresed in gel electrophoresis apparatus GE-4 on gradient acrylamide gel slabs PAA 4/30 (Pharmacia) for 20 min at a constant voltage of 70V, followed by 15 h at a constant voltage of 125V in a 0.09M Tris-0.08M borate-0.003M $Na_2EDTA$ buffer, pH 8.7, at $4^oC$. The slabs were sectioned vertically into 0.75-cm wide sections. One section of each duplicate run was fixed in 10% trichloroacetic acid, stained with 0.05% Coomassie Brilliant Blue, and destained with 7% acetic acid. The other gel section was embedded in 0.75% agarose containing 1% (v/v) specific anti-rat AFP antiserum on an 8 x 10-cm glass plate and electrophoresed for 1.5 h at a constant voltage

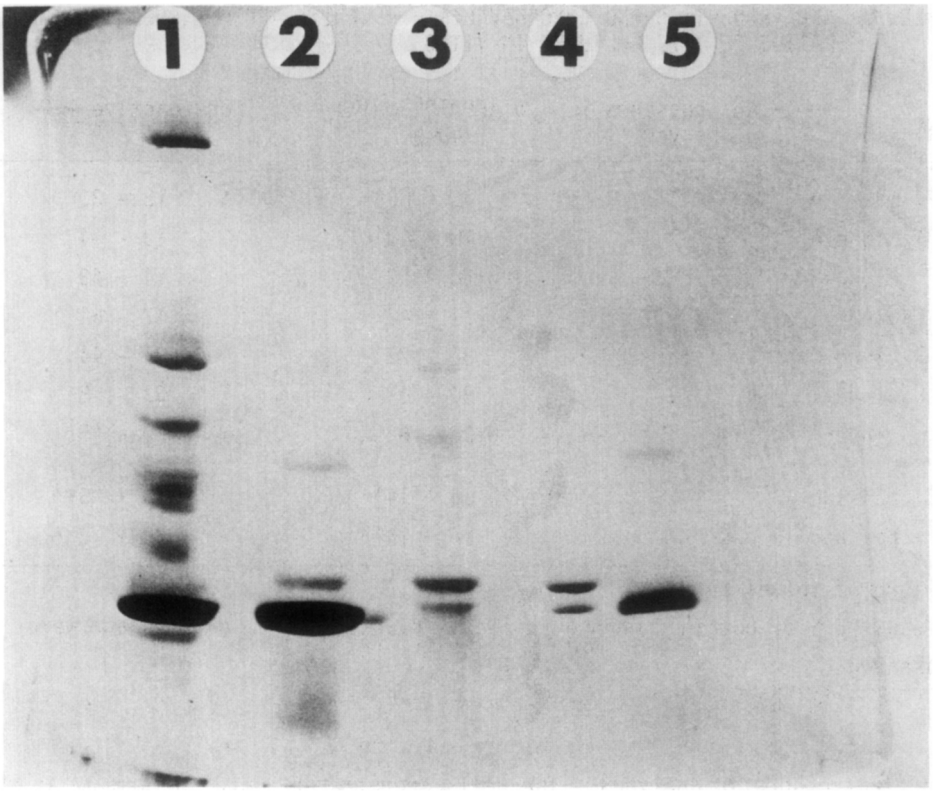


FIGURE 1. Gradient acrylamide gel electrophoresis. Samples: 1) whole serum from Buffalo rat bearing Morris hepatoma 7777; 2) partially-purified concanavalin A non-reactive AFP fraction from 7777 serum; 3) partially-purified concanavalin A-reactive AFP from 7777 serum; 4) pure concanavalin A-reactive AFP; and 5) rat albumin.

of 200V in the second dimension. The embedded acrylamide sections were removed; the agarose plates were washed to remove unprecipitated protein, dried in air, stained with 0.05% Coomassie Brilliant Blue and destained with $H_2O$: isopropanol:acetic acid (2.6:1:0.4, v/v/v).

RESULTS AND DISCUSSION: Table 1 lists the amount of AFP secreted and the concanavalin A-affinity molecular variant ratios obtained from the mass and clonal cell lines derived from Morris hepatoma 7777. Both the amounts of AFP secreted and the molecular variant ratios found varied from cell line to cell line and, within each line, from passage to passage. When the replicate flasks were assayed, however, the amount of AFP secreted and the variant ratios were more uniform. No trend in either the rate of AFP secretion or the proportions of

concanavalin A-affinity molecular variants present in the culture media was evidenced during serial passage. Differences in culture conditions and/or phenotypic drift during growth in vitro may account for the variation in the total amount of AFP and concanavalin A-affinity molecular variant ratios obtained at each passage.

Addition of the individual concanavalin A-affinity molecular variants to a newborn rat liver-derived hepatocyte cell line which itself does not secrete AFP did not result in a change in reactivity of the AFP with concanavalin A; that is, there was no in vitro interconversion of the two concanavalin A-affinity molecular variants of AFP. Recovery of added AFP from these incubations was 100%.

Figure 1 shows the electrophoretic pattern of pure and partially-purified AFP from serum of a rat bearing Morris hepatoma 7777. This gradient acrylamide gel offers excellent separation of electrophoretic variants of AFP. The electrophoretically-faster variant cannot be distinguished in gels #1 and #2 because it co-migrates with rat serum albumin. Electrophoretic analysis of AFP produced in vitro by the 7777 mass and clonal cell lines gave the same type of pattern but the presence of residual fetal calf serum proteins rendered visual detection of the electrophoretic molecular variants difficult. This problem was circumvented by electrophoresing sections of the gradient acrylamide gel perpendicularly to the direction of initial electrophoresis into agarose containing AFP-specific antiserum. Figure 2 shows the results from the two-dimensional electrophoresis of concanavalin A-reactive AFP and non-reactive AFP isolated from media of 7777 Clone D passage 5. Both electrophoretic variants were found in the concanavalin A-reactive and non-reactive AFP fractions from the media of the 7777 mass culture and each of the six clonal cell lines. Although this two-dimensional single electroimmunodiffusion procedure is semi-quantitative, the peak heights of the two electrophoretic variants were similar in all cases, indicating that the amounts of each electrophoretic variant present were approximately equal.

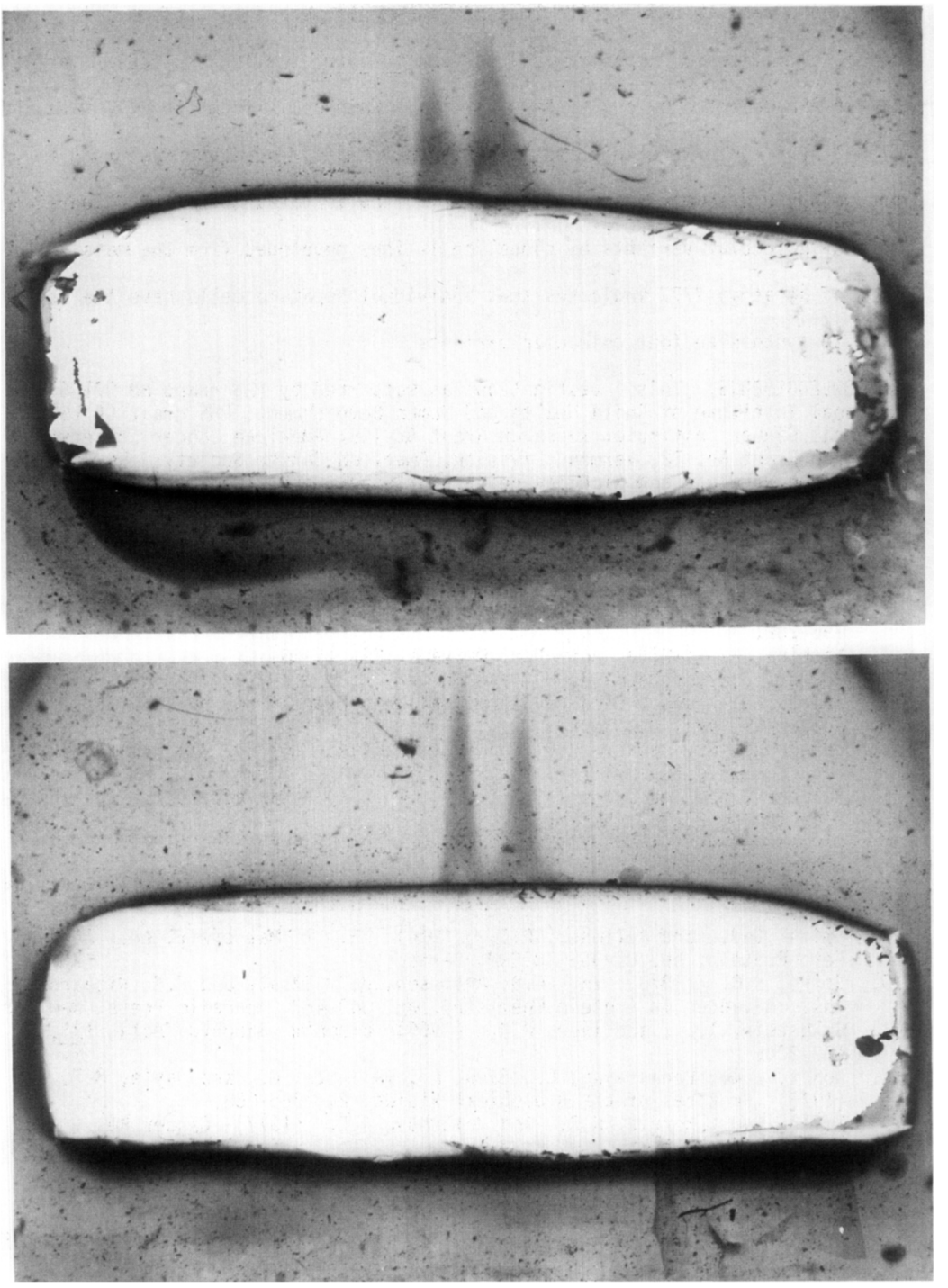

FIGURE 2. Two-dimensional single electroimmunodiffusion. First direction - gradient acrylamide gel electrophoresis; anode at right. Second direction - agarose gel electrophoresis; anode at top. Concanavalin A non-reactive fraction (upper) and concanavalin A-reactive fraction (lower) from medium of 7777 clone D passage 5. The gradient acrylamide gel section was removed (white rectangular area of photo) prior to staining of immunoprecipitates in the agar gel.

These results demonstrate that the mass culture and each of the six clonal cell lines of hepatoma 7777 secrete *in vitro* the four molecular variants of rat AFP detected by our systems *in vivo*. When cells from the 7777 mass and clonal cell lines were injected into syngeneic rats, they gave rise to AFP-producing tumors which secreted the four molecular variants of AFP. Secretion of four molecular variants by clonal cell lines developed from the mass culture of hepatoma 7777 indicates that individual hepatoma cells have the potential to synthesize four molecular variants.

ACKNOWLEDGEMENTS: This investigation was supported by PHS grant HD 07136, National Institute of Child Health and Human Development; PHS grant CA 15222, National Cancer Institute; Research Grant BC-158, American Cancer Society; Research Grant No. 12, Vermont Division, American Cancer Society. We wish to thank John G. Wieja and Nicholas Heintz for expert assistance.

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