Proc. Natl. Acad. Sci. USA
Vol. 78, No. 2, pp. 1077–1080, February 1981
Developmental Biology

Isolation and characterization of mouse mutant embryonal carcinoma cells which fail to differentiate in response to retinoic acid

(teratocarcinomas/*dif*(*RA*)$^-$ mutations/cytoplasmic retinoic acid-binding protein/cytoplasmic retinol-binding protein)

JOEL SCHINDLER, KLAUS I. MATTHAEI, AND MICHAEL I. SHERMAN

Roche Institute of Molecular Biology, Nutley, New Jersey 07110

Communicated by John W. Littlefield, October 13, 1980

ABSTRACT **Murine embryonal carcinoma cells from line PCC4.azalR differentiate readily in response to retinoic acid. By treating PCC4.azalR cells with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, we derived two embryonal carcinoma lines in which the cells failed to differentiate during exposure to retinoic acid. Although these *dif*(*RA*)$^-$ cells maintained the tumorigenic potential of the parental cells, they differentiated poorly in tumor form. Similarly, the tendency of *dif*(*RA*)$^-$ cells to differentiate when aggregated *in vitro* was diminished relative to that of PCC4.azalR cells. The rate of retinoic acid uptake in cells from the two mutant lines did not appear to be reduced compared with the rate in cells from the parental line; however, specific cytoplasmic retinoic acid-binding protein activity was virtually absent in both mutants. These results strengthen the view that differentiation of embryonal carcinoma cells in response to retinoic acid requires formation of retinoic acid–cytoplasmic retinoic acid-binding protein complexes.**

Several different embryonal carcinoma (EC) cell lines have been isolated from teratocarcinomas. Cells from these lines will differentiate to various degrees under conditions that promote differentiation, such as culture at high density, formation of multicellular aggregates, and tumor formation in suitable hosts (1, 2). Strickland and Mahdavi (3) demonstrated that cells that normally differentiate poorly under routine culture conditions can be induced to differentiate at high frequencies upon exposure to retinoic acid (RA). Subsequent studies suggested that promotion of differentiation of EC cell lines by retinoids is a general phenomenon (4, 5). RA can stimulate differentiation even of EC cells that do not differentiate under any other conditions tested (4).

Because retinoids act in a pleiotropic fashion (6) it is conceivable that they promote differentiation of EC cells at any of a number of different levels. However, it has been demonstrated recently that EC cells from line PCC4.azalR have a cytoplasmic binding protein for RA (cRABP) and that there is a correlation between the ability of many naturally occurring and synthetic retinoids to stimulate differentiation and their ability to compete with RA for binding sites on the cRABP (7, 8). Accordingly, it was proposed that the RA–cRABP complex is involved in the differentiation of EC cells (7). To test this hypothesis, we have tried to generate EC lines composed of cells that fail to differentiate in response to RA and then to determine whether these cells form RA–cRABP complexes. We have succeeded in generating two such lines by treating PCC4.azalR EC cells with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). The characterization of these lines is presented below.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Materials. MNNG was purchased from Sigma. Dulbecco's modified Eagle's medium (DME medium) was purchased from GIBCO. Fetal calf serum was obtained from Microbiological Associates (Bethesda, MD). All cultures were carried out in plasticware obtained from Falcon or Corning. *All-trans* RA and retinol were obtained from B. Pawson (Hoffmann–LaRoche). [11,12-^{3}H]RA (30.6 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and [11,12-^{3}H]retinol (8.6 Ci/mmol) were prepared by A. Liebman and C. Perry (Hoffmann–LaRoche). Millipore filters (type HAWP, 0.25 μm) were obtained from Millipore. Hydrofluor and LSC liquid scintillation cocktails were obtained from National Diagnostics (Somerville, NJ) and Yorktown Research (South Hackensack, NJ), respectively.

Cell Lines. PCC4.azal is an EC cell line (9) that fails to differentiate under normal culture conditions but does give rise to differentiated progeny in tumor form (9) or when grown as aggregates *in vitro* (10). PCC4.azal cells die in the presence of RA at concentrations above 0.1 μM (3, 4). PCC4.azalR cells, derived from PCC4.azal cells (4), appear to be similar to the parental line except that they are tolerant to RA at concentrations as high as 10 μM (4) and they do not differentiate as extensively in tumor form (2). PCC4.azalR cells were mutagenized by exposure to MNNG (3 μg/ml) in DME medium containing fetal calf serum and enriched with extra glucose and glutamine (4) for 18 hr followed by plating at clonal density as described by Boon *et al.* (11). Mutagenized cells were cloned in enriched DME medium with or without 10 μM RA. Two lines, *dif*(*RA*)$^-$1 and *dif*(*RA*)$^-$3, were obtained by cloning and subcloning cells in the continuous presence of RA at 10 μM.

Analyses of Growth. Cells from exponentially growing cultures were dislodged from dishes by a short incubation in calcium- and magnesium-free phosphate-buffered saline (12) followed by flushing with the same buffer, as described (10). Cells were collected by low-speed centrifugation, resuspended in enriched DME medium, and plated in 5 ml vol in 60-mm tissue culture dishes at a density of 10^5 cells per ml of medium. After the indicated periods of incubation at 37°C, cells were collected by trypsinization and counted with the aid of a hemocytometer. Values given are means of triplicate determinations.

Tumor Analyses. Cells were collected and counted as described in the previous section. One million cells were injected subcutaneously into each of five adult male 129/SvS1 mice. Tumors were removed when they reached a diameter of approxi-

Abbreviations: cRABP, cytoplasmic retinoic acid-binding protein; DME medium, Dulbecco's modified Eagle's medium; EC, embryonal carcinoma; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; RA, retinoic acid.

mately 2 cm. After fixation in neutral buffered formalin, the tumors were cut into pieces, blocked in paraffin, sectioned (4 μm), and stained with hematoxylin and eosin; sections representing six or more different levels throughout the tumor were examined.

Formation and Analysis of Aggregates. Aggregates of EC cells were prepared by culture in bacteriological dishes containing enriched DME medium as described (4, 10). After 3 or 10 days of aggregation, with medium changes every other day, aggregates were collected (4, 10) and transferred to tissue culture dishes containing enriched DME medium with or without 10 μM RA. Twenty-four hours later, aggregates were scored for production and secretion of plasminogen activator by the fibrin-agar procedure (13), all as described (4).

[^{3}H]RA Uptake Studies. EC cells (5×10^5) were plated in 60-mm tissue culture dishes containing 5 ml of enriched DME medium. Twenty-four hours later, [^{3}H]RA (10^6 cpm; $\approx$10 nM) was added to each dish. After appropriate periods of incubation, medium was removed and cells were washed rapidly three times with fresh medium, harvested by trypsinization, and collected on a Millipore filter. Filters were washed twice with phosphate-buffered saline, dried, and assayed for radioactivity in toluene-based liquid scintillation fluid. Cell numbers were estimated by counts made on accompanying cultures established and maintained in the same way as those used for uptake analyses except that they were not exposed to [^{3}H]RA.

Retinoid Binding Studies. Retinoid binding analyses were adapted from the procedure described by Jetten and Jetten (7). Cells growing in monolayer cultures were washed with phosphate-buffered saline, dislodged with a rubber policeman, and collected by low-speed centrifugation. Cells were resuspended in 10 mM Tris·HCl, pH 7.6/2 mM $MgCl_2$/7 mM 2-mercaptoethanol. After the cells were disrupted with a glass/Teflon hand homogenizer, cytoplasmic extracts were obtained by centrifugation at 105,000 $\times$ *g* for 60 min and stored frozen at -70°C (multiple freezing and thawing of these extracts has been found not to affect retinoid-binding activities). After the extracts were thawed, 300-μl aliquots ($\approx$1 mg of protein) were incubated for 4 hr at 4°C with *all-trans*-[^{3}H]RA (50 nM; 30.6 Ci/mmol) plus a 200-fold molar excess of unlabeled retinol in the absence or presence of unlabeled RA in 200-fold molar excess. Unbound retinoids were removed by treatment with dextran-coated charcoal (14) or by Sephadex G-25 column chromatography. Samples were then subjected to centrifugation (Beckman SW50.1 rotor, 220,000 $\times$ *g*, 18 hr) in 5–20% sucrose gradients. Fractions were collected, mixed with 8 ml of Hydrofluor, and assayed for radioactivity. Binding of retinol was carried out as described for RA except that aliquots were incubated with 50 nM [^{3}H]retinol (8.6 Ci/mmol) with and without a 200-fold excess of unlabeled retinol.

Labeled RA was stored at -70°C and used over a period of 4 months. Under these conditions, some conversion was observed to a form that comigrated with *cis* RAs on high-pressure liquid chromatography (C. Bugge, personal communication). This conversion did not appear to affect specific binding to the cRABP (ref. 7; unpublished data).

RESULTS

Under our conditions, cloning frequencies for PCC4.azalR cells were greater than 90%. Treatment with MNNG under the regimen used reduced cloning efficiency to approximately 35%. The cloning frequency of PCC4.azalR cells in the presence of 10 μM RA was approximately 25%. After MNNG treatment and subsequent exposure to RA, only 5% of the treated cells formed clones. The majority of the clones that survived the combined

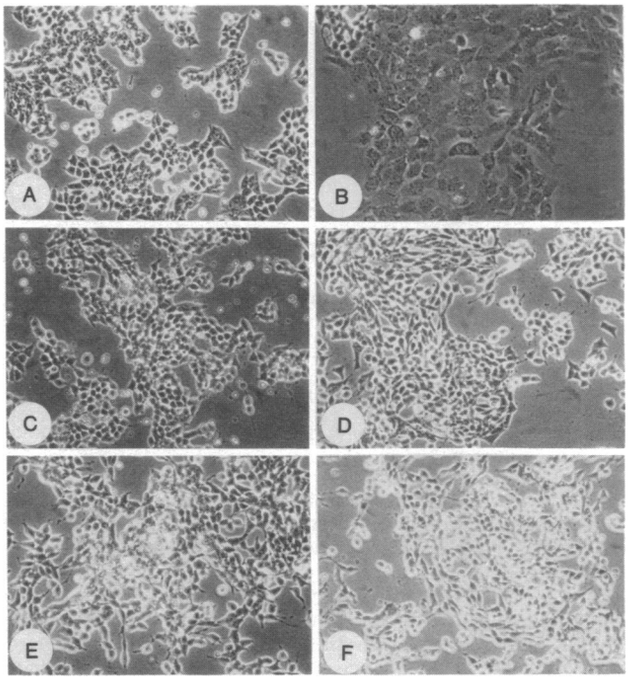

FIG. 1. PCC4.azalR (*A* and *B*), *dif*(*RA*)$^-$1 (*C* and *D*), and *dif*(*RA*)$^-$3 (*E* and *F*) EC cells. (*Left*) Growing exponentially in enriched DME medium. (*Right*) After culture for 6 weeks in the same medium supplemented with 10 μM RA.

treatment had cells with a non-EC, fibroblastic appearance. These cells were similar to those normally observed when PCC4.azalR cells are stimulated to differentiate with RA (2, 4) (Fig. 1*B*). However, a small proportion of cells (estimated frequency, 1:10^5) formed clones containing at least some cells that were EC-like in appearance. Cells from two of these clones were passaged several times in the presence of 10 μM RA. In each case, the majority of the cells had an EC-like appearance. We then subcloned cells from each original clone in the presence of RA and thereby isolated lines that contained cells with a uniform EC appearance (Fig. 1). We have named these clonal cell lines *dif*(*RA*)$^-$1 and *dif*(*RA*)$^-$3. Unlike the parental PCC4.azalR cells, *dif*(*RA*)$^-$1 and *dif*(*RA*)$^-$3 maintained the same appearance in the presence and absence of 10 μM RA, the highest dose of the retinoid that EC cells can tolerate. *dif*(*RA*)$^-$ cells, therefore, fail to differentiate overtly in response to RA.

Cells from the *dif*(*RA*)$^-$ lines were highly tumorigenic when injected into strain 129/SvS1 mice, as are parental PCC4.azalR cells. In all cases, the subcutaneous injection of 10^6 cells led to palpable tumors within 8–12 days. PCC4.azalR cells gave rise to tumors containing primarily EC cells, primitive neuroectoderm-like cells, and glandular epithelium. Tumors from *dif*(*RA*)$^-$1 cells contained a minor primitive neuroectoderm-like component whereas *dif*(*RA*)$^-$3 cells produced apparently pure ECs (unpublished observations).

In the absence of RA, PCC4.azalR, *dif*(*RA*)$^-$1, and *dif*(*RA*)$^-$3 cells had similar doubling times (approximately 8 hr; see Fig. 2). Doubling times were slower in 10 μM RA in all cases: approximate values were 12 hr for *dif*(*RA*)$^-$1 cells, 14 hr for *dif*(*RA*)$^-$3 cells, and 21 hr for PCC4.azalR cells. Aside from having the slowest doubling time in RA-containing medium, the number of PCC4.azalR cells leveled off after 55 hr, consistent with the view that they were giving rise to slower-growing differentiated derivatives.

It has been reported (4) that PCC4.azalR cells in monolayer culture or after 2 days of aggregation did not secrete detectable

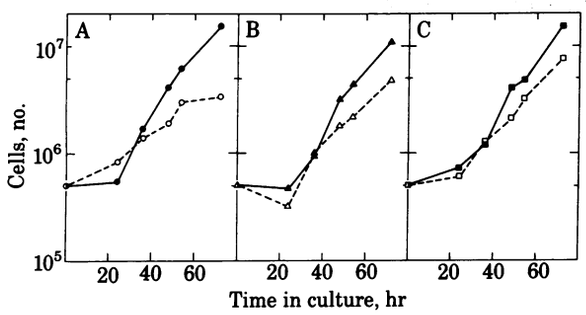

FIG. 2. Growth curves for cells from EC cell lines PCC4.azalR (*A*), *dif*(*RA*)$^-$1 (*B*), and *dif*(*RA*)$^-$3 (*C*) in the absence (solid symbols) or presence (open symbols) of 10 μM RA.

amounts of plasminogen activator. After several months of culture, some of the cells in this line have acquired low levels of plasminogen activator activity even though they have remained EC-like, an observation similar to that made on related PCC4.azal cells by Topp *et al.* (15) and by Lo and Gilula (16). In the current studies, when PCC4.azalR cells were aggregated for 3 days and then transferred to tissue culture dishes for 1 day, approximately 10% of the aggregates secreted detectable levels of plasminogen activator (Table 1). This number was doubled by increasing aggregation time to 10 days. Consistent with previous studies (4), almost all 3-day or 10-day aggregates produced plasminogen activator if they were exposed to 10 μM RA after aggregation. Unlike the parental cells, there was little increase, if any, in the numbers of *dif*(*RA*)$^-$1 or *dif*(*RA*)$^-$3 aggregates secreting plasminogen activator either with increasing times of aggregation or after exposure to RA. The "background" levels of plasminogen activator secretion were substantially lower for *dif*(*RA*)$^-$3 than for the other two lines. We have never observed cells with a non-EC appearance growing out from *dif*(*RA*)$^-$1 or *dif*(*RA*)$^-$3 aggregates in the absence or presence of RA.

To determine whether the lack of response of *dif*(*RA*)$^-$ cells was due to the inability of these cells to take up RA, uptake studies were carried out with [^{3}H]RA. There was a rapid association of [^{3}H]RA with cells during the first 5 min of incubation (Fig. 3). This was followed by a more gradual rate of uptake of [^{3}H]RA from the medium. This pattern is typical of RA association with other cell types (17). If anything, the uptake of [^{3}H]RA from the medium by *dif*(*RA*)$^-$ cells was greater than that by PCC4.azalR cells.

To measure levels of cRABP in PCC4.azalR and *dif*(*RA*)$^-$ cells, we incubated cytoplasmic extracts with [^{3}H]RA with and

Table 1. Plasminogen activator secretion by EC cell aggregates

Cell line	RA	% aggregates secreting plasminogen activator after aggregation for 3 days	10 days
PCC4.azalR	−	9.2	21.5
	+	93.0	98.9
dif(*RA*)$^-$1	−	8.1	6.8
	+	11.0	8.2
dif(*RA*)$^-$3	−	0.6	3.9
	+	1.5	1.2

Aggregates were formed by culture of cells in bacteriological dishes for either 3 or 10 days. Aggregates were then transferred to tissue culture dishes containing medium with or without 10 μM RA. After 24 hr, aggregates were assayed for 6 hr for plasminogen activator secretion. Values are averaged from two independent experiments; at least 100 aggregates were scored for each treatment in each of the experiments.

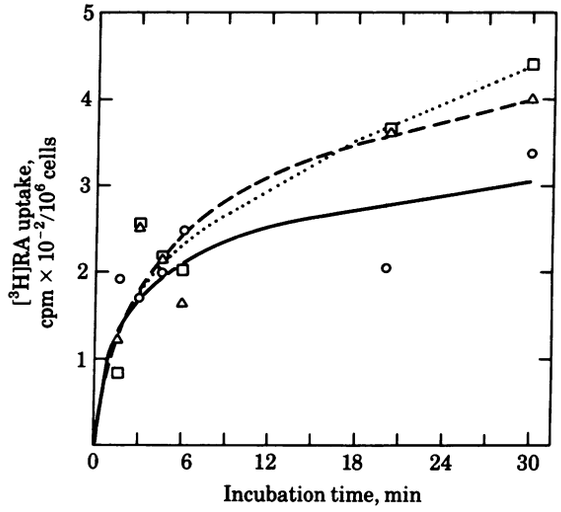

FIG. 3. Uptake of [^{3}H]RA by EC cells. PCC4.azalR (○), *dif*(*RA*)$^-$1 (△), and *dif*(*RA*)$^-$3 (□) EC cells were incubated with [^{3}H]RA for the times indicated and scored for uptake. Each point represents the mean of triplicate determinations in two independent experiments. The sizes of the EC cells in all three lines were approximately the same (cf. Fig. 1 *A*, *C*, and *E*).

without a 200-fold excess of unlabeled RA. After removal of unbound [^{3}H]RA, we centrifuged the extracts on sucrose density gradients. Cytoplasmic extracts of PCC4.azalR cells bound large amounts of [^{3}H]RA in the 2S region of the gradient (Fig. 4*A*). On the other hand, cytoplasmic extracts from *dif*(*RA*)$^-$1 cells (Fig. 4*B*) or *dif*(*RA*)$^-$3 cells (Fig. 4*C*) failed to bind significant amounts of [^{3}H]RA specifically—i.e., the amounts of [^{3}H]RA bound in the absence and presence of the 200-fold excess of unlabeled RA were similar. Identical results were obtained when unbound [^{3}H]RA was removed by Sephadex chromatography instead of adsorption with dextran-coated charcoal. Mixing of *dif*(*RA*)$^-$ cell cytoplasmic extracts with PCC4.azalR extracts did not reduce the ability of the latter to bind [^{3}H]RA specifically. Membrane fraction preparations (18) from PCC4.azalR and from *dif*(*RA*)$^-$ cells failed to bind [^{3}H]RA specifically. Finally, the addition of IgG to the extracts to stabilize cRABP (19) did not increase the amount of [^{3}H]RA bound in the 2S region by any of the cell extracts.

Because several cell types have a specific retinol-binding protein that is different from the cRABP, we tested the same extracts of PCC4.azalR, *dif*(*RA*)$^-$1, and *dif*(*RA*)$^-$3 cells for their ability to bind [^{3}H]retinol. Fig. 4 *D–F* illustrates that all extracts bound [^{3}H]retinol specifically although PCC4.azalR extracts bound 2.4 to 3 times as much ligand as did *dif*(*RA*)$^-$ extracts. A 200-fold excess of unlabeled RA did not diminish the specific binding of [^{3}H]retinol (not shown).

DISCUSSION

We have isolated two mutant cell lines, *dif*(*RA*)$^-$1 and *dif*(*RA*)$^-$3, which retain their EC phenotype even after culture for several weeks in medium containing 10 μM RA. Boon *et al.* (11) first obtained *dif*$^-$ EC cell lines by mutagenizing PCC4.azal cells; tumor analyses revealed that the ability of their mutant cells to differentiate was substantially reduced but not eliminated. Boon *et al.* (11) also described a class of *tum*$^-$ EC mutants that had limited tumorigenic potential. Both of the *dif*(*RA*)$^-$ lines in the present study retain the rapid proliferation rate and capacity for tumorigenesis characteristic of parental PCC4.azalR cells. However, the ability of *dif*(*RA*)$^-$ cells to differentiate in tumor form is severely restricted in one line and possibly elim-

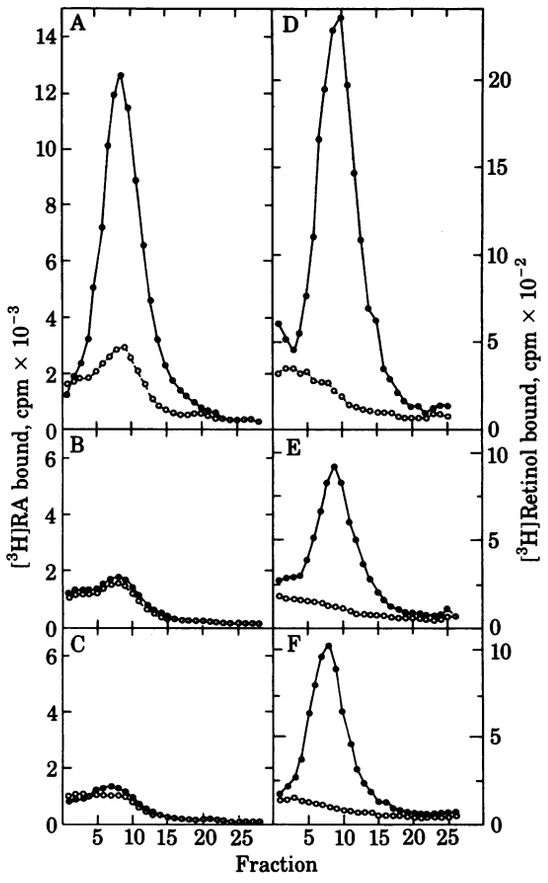

FIG. 4. Binding of [^{3}H]RA (*Left*) and [^{3}H]retinol (*Right*) by cytoplasmic extracts of EC cells. Cytoplasmic extracts from PCC4.aza1R (*A* and *D*), *dif*(*RA*)$^-$1 (*B* and *E*), and *dif*(*RA*)$^-$3 (*C* and *F*) cells were exposed to [^{3}H]RA without (●) or with (○) a 200-fold molar excess of unlabeled RA or to [^{3}H]retinol without (●) or with (○) a 200-fold molar excess of unlabeled retinol. After incubation and removal of unbound ^{3}H label, extracts were subjected to sucrose density gradient centrifugation and analyzed. The top of the gradients is at the left. The peak binding activity has been found to comigrate with a 2S myoglobin marker (ref 7; unpublished data). Data are expressed in terms of cpm bound per mg of cytoplasmic protein in the original incubation mixture.

inated in the other. Similarly, the effectiveness of aggregate formation in stimulating differentiation, especially when combined with exposure to RA, appears to be markedly diminished in *dif*(*RA*)$^-$ cells as measured by plasminogen activator production.

Uptake studies with [^{3}H]RA do not point to altered permeability as the immediate cause of failure of *dif*(*RA*)$^-$ cells to differentiate in response to RA. However, although similar to RA uptake studies performed by others (17), the studies carried out do not discriminate between membrane-associated and cytoplasmic RA. It is conceivable that RA uptake into the cytoplasm requires the presence of cRABP. In any event, comparisons of RA uptake into the cytoplasm of *dif*(*RA*)$^-$ and of PCC4.aza1R cells will be difficult because the absence of cRABP activity in the former cells could influence the rates of exit of RA from, as well as its entry into, the cytoplasmic compartment.

RA at 10 μM slows the proliferation rate of both normal and mutant EC cells. This supports the view of Lotan *et al.* (20) that modulation of growth rate by RA is not mediated through cRABP. On the other hand, the lack of detectable levels of cRABP activity in *dif*(*RA*)$^-$ cells provides strong support for the proposal (7) that the complexing of RA to cRABP is an essential step in the sequence of events leading to the RA-induced stimulation of differentiation of EC cells. It is notable that cytoplasmic retinol-binding protein activity was not similarly eliminated in *dif*(*RA*)$^-$ cells; this is not unexpected because retinol fails to stimulate differentiation of EC cells (4, 7, 8). Although levels of cytoplasmic retinol-binding protein or the affinity of the binding protein for retinol were reduced in *dif*(*RA*)$^-$ EC cells relative to levels in PCC4.aza1R cells, the values obtained were nevertheless within the range found for other EC cell lines which are capable of differentiating in response to RA and to other stimuli (unpublished data).

Our data suggest that the lack of cRABP activity in *dif*(*RA*)$^-$ cells not only interferes with their ability to respond to RA exposure but also hampers their capacity for differentiation during tumor formation or aggregation *in vitro*. These observations give the impression that cRABP is universally involved in the differentiation of EC cells. However, studies with other EC lines might not support this view. For example, whereas cells from lines such as PCC4.aza1R and F9 differentiate extensively *in vitro* only when exposed to RA (2–4), cells from other lines differentiate readily in response to aggregation or continuous culture at high density in the absence of exogenous RA (2, 4). The latter cells might have a greater propensity for differentiation because they differ from the former cells by being capable of efficiently converting retinol in the serum to RA, by being able to produce some other ligand for cRABP, or by having much higher levels of cRABP. On the other hand, alternative pathways not involving retinoids or cRABP might be operative in cells that differentiate readily in the absence of exogenous RA.

We thank Dr. Peter McCue for assistance with tumor analyses and Drs. McCue, A. Shatkin, and S. Shapiro for comments on the manuscript.

1. Graham, C. (1977) in *Concepts in Mammalian Embryogenesis*, ed. Sherman, M. I. (MIT, Cambridge, MA), pp. 315–394.
2. Sherman, M. I., Matthaei, K. I. & Schindler, J. (1981) *Ann. N.Y. Acad. Sci.* **359**, 192–199.
3. Strickland, S. & Mahdavi, V. (1978) *Cell* **15**, 393–403.
4. Jetten, A. M., Jetten, M. E. R. & Sherman, M. I. (1979) *Exp. Cell Res.* **124**, 381–391.
5. Adamson, E. D., Gaunt, S. J. & Graham, C. F. (1979) *Cell* **17**, 469–476.
6. Lotan, R. (1980) *Biochim. Biophys. Acta* **605**, 33–91.
7. Jetten, A. M. & Jetten, M. E. R. (1979) *Nature (London)* **278**, 180–182.
8. Trown, P. W., Palleroni, A. V., Bohoslawec, O., Richelo, B. N., Halpern, J. M., Gizzi, N., Geiger, R., Lewinski, C., Machlin, L. J., Jetten, A. & Jetten, M. E. R. (1980) *Cancer Res.* **40**, 212–220.
9. Jakob, H., Boon, T., Gaillard, J., Nicolas, J. F. & Jacob, F. (1973) *Ann. Microbiol. Inst. Pasteur* **124B**, 269–282.
10. Sherman, M. I. (1975) in *Teratomas and Differentiation*, eds. Sherman, M. I. & Solter, D. (Academic, New York), pp. 189–205.
11. Boon, T., Kellermann, O., Mathy, E. & Gaillard, J. A. (1975) in *Teratomas and Differentiation*, eds. Sherman, M. I. & Solter, D. (Academic, New York), pp. 161–166.
12. Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167–182.
13. Beers, W. H., Strickland, S. & Reich, E. (1975) *Cell* **6**, 387–394.
14. Marcal, J. M., Chew, N. J., Salomon, D. S. & Sherman, M. I. (1975) *Endocrinology* **96**, 1270–1279.
15. Topp, W. D., Hall, J. D., Marsden, M., Teresky, A. K., Rifkin, D., Levine, A. J. & Pollack, R. (1976) *Cancer Res.* **36**, 4217–4223.
16. Lo, C. W. & Gilula, N. B. (1980) *Dev. Biol.* **75**, 78–92.
17. Lotan, R., Neumann, G. & Lotan, D. (1981) *Ann. N.Y. Acad. Sci.* **359**, 150–170.
18. Sani, B. P. (1979) *Biochem. Biophys. Res. Commun.* **91**, 502–507.
19. Ong, D. E. & Chytil, F. (1975) *J. Biol. Chem.* **250**, 6113–6117.
20. Lotan, R., Ong, D. E. & Chytil, F. (1980) *J. Natl. Cancer Inst.* **64**, 1259–1262.