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Commitment to Differentiation Induced by Retinoic Acid in P19 Embryonal Carcinoma Cells Is Cell Cycle Dependent

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The rate at which P19 embryonal carcinoma cells in monolayer culture become anchorage dependent during differentiation induced by retinoic acid (RA) was investigated. In both nonsynchronized cultures and cultures synchronized by mitotic selection, the ability to grow in semisolid medium, characteristic of the malignant stem cell, decreased after a lag period of about 12 hr in the continuous presence of RA, prior to an increase in cell generation time. However, striking differences between synchronized and nonsynchronized cultures were observed in their commitment to differentiation following RA removal. After only 2 hr of exposure to RA, synchronized cells continued a program of differentiation in which they became anchorage dependent, while at least 24 hr of exposure was required for exponentially growing cells to become similarly committed. Induction of anchorage dependence by RA was also strikingly cell cycle dependent; 2 or 4 hr of exposure of synchronized cells to RA in G_1 phase, when the intrinsic capacity for soft agar growth is low, was sufficient to commit cells to anchorage dependence, but a similar exposure in S phase was not. Together, these results suggested that interactions between cells in different cell cycle phases in asynchronous cultures influenced commitment since exposure to RA for more than one cycle (13 hr) was required for all cells to become anchorage dependent. Increased plasminogen activator secretion and epidermal growth factor binding, markers of certain differentiated cell types, increased only 3 and 5 days after RA addition, respectively, and were not induced by pulsed exposure to RA of less than 24 hr, even in synchronized cells. © 1987 Academic Press, Inc.

INTRODUCTION

Embryonal carcinoma (EC) cells are the undifferentiated stem cells derived from mouse teratocarcinomas. They are frequently used as a model system to study events involved in early development because of the many properties they have in common with pluripotent embryonic cells (Graham, 1977; Martin, 1980). Most EC cells can be induced to differentiate when cultured in the presence of retinoic acid (RA). The cell types formed following RA exposure depend on the particular EC cell line, on whether the EC cells are cultured in cell aggregates or on solid surfaces, and on the drug concentration (Strickland and Mahdavi, 1978; Hogan *et al.*, 1981; Jones-Villeneuve *et al.*, 1982; Edwards and McBurney, 1983). Differentiation is marked by dramatic changes in cell phenotype, including loss of the capacity for anchorage-independent growth 24–48 hr after induction (Jetten *et al.*, 1979; Strickland *et al.*, 1980). However, the precise temporal relationship between these changes, the extent to which they are reversible, and whether altered proliferation rate is a cause or consequence of differentiation is at present unclear (reviewed in Sherman, 1986).

Several approaches have been used to investigate reversibility and commitment in differentiating EC cells. Ogiso *et al.*, (1982) studied the loss of peanut agglutinin (PNA) binding induced by RA in 311 EC cells; although 4 days of exposure induced an irreversible loss, cells exposed for only 2 days reacquired PNA binding within 5 hr in RA-free medium. Rayner and Graham (1982) have shown that 24 hr of exposure of cloned PC13 EC cells was sufficient to induce the same pattern of changes in growth kinetics as continuous exposure to the inducer; i.e., the cells grew unimpeded for a further 24 hr after RA removal before cell division ceased. Recently, we have analyzed changes in cell cycle kinetics induced by RA in more detail using mitotically synchronized PC13 EC cells and have related these changes to those in cell morphology (Mummery *et al.*, 1984). PC13 EC cells have a restricted differentiation potential but following addition of RA to monolayer cultures an apparently homogeneous cell type PC13 END is formed (Rees *et al.*, 1979). While the flattened phenotype, typical of PC13 END, was acquired in the second cell cycle after induction, cell cycle times only increased from the third cycle. Together these data indicate that (i) a limited exposure to RA is sufficient to induce commitment for differentiation, (ii) the appearance of the differentiated phenotype is related to the cell cycle after induction, and (iii) certain differentiated characteristics are elicited prior to the point at which cell cycle kinetics for treated and untreated cells diverge.

In the present study we have again used mitotically synchronized EC cells but now to characterize in detail

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the differentiation of pluripotent EC cell line, P19 (McBurney and Rogers, 1982; McBurney *et al.*, 1982; Jones-Villeneuve *et al.*, 1982). In particular, we wished to identify early phenotypic alterations associated with commitment to different cell lineages and to establish the relationship with the loss of tumorigenic potential. P19 EC cells form neuroectodermal derivatives or striated muscle when aggregated in the presence of RA and DMSO, respectively, and mesodermal or endodermal derivatives when treated in monolayer with RA. The kinetics of appearance of a number of differentiated characteristics during RA-induced differentiation were compared, and time points at which RA removal was no longer able to affect these kinetics were established. The results demonstrated that only very short exposures to RA were required to commit tumorigenic EC cells to loss of their capacity for growth in semisolid media, while significantly longer exposures were required to commit cells to the expression of certain differentiation markers, namely, plasminogen activator (PA) production and epidermal growth factor (EGF) binding. Furthermore, a striking difference between the response of exponentially growing cultures and synchronized cultures to RA in terms of commitment suggested that sensitivity to RA may be cell cycle dependent. This was confirmed by exposing cells to short (2- or 4-hr) pulses of RA during different cell cycle phases; a 2-hr pulse in G_1 phase was sufficient to abolish growth in soft agar 75 hr later whereas a similar pulse in S phase only marginally reduced growth compared with untreated control cells. The implications of these findings are discussed.

MATERIALS AND METHODS

Cell Culture and Differentiation

P19 EC cells were cultured as described previously (Mummery *et al.*, 1985) on gelatinized flasks in a 1:1 mixture of Dulbecco's minimum essential medium (DMEM) and Ham's F12 medium (DF) containing 7.5% fetal calf serum (FCS; Flow Laboratories) and buffered with $NaHCO_3$ (44 m*M*) in a 7.5% CO_2 atmosphere. Cells were subcultured in 0.125% (w/v) trypsin, 50 m*M* EDTA in Ca^{2+},Mg^{2+}-free phosphate-buffered saline. To induce differentiation, EC cells were plated at a density of $1 \times 10^4/cm^2$ in DF + 7.5% FCS and RA was added 24 hr later to a concentration of 1×10^{-6} *M* from a 10^{-2} *M* stock solution, dissolved in DMSO, and stored at −70°C. The duration of RA exposure was varied as required and is indicated in the results of individual experiments.

Cell Synchronization

For synchronization by mitotic shake off, 12×10^6 EC cells were inoculated in a 150-cm^2 gelatinized tissue culture flask in growth medium and incubated for 24 hr at 37°C. This medium was then replaced by 5 ml of growth medium, buffered with Hepes (15 m*M*) and $NaHCO_3$ (15 m*M*), and pregassed in a 2% CO_2 atmosphere to pH 7.4. The flask was then shaken manually, yielding 0.3–0.5 $\times 10^6$ mitotic cells in suspension as large single cells. These cells were replated at a density of $2 \times 10^4/cm^2$; within 20 min, cells attached and more than 95% divided into two, indicating the degree of synchrony. The appearance of cell pairs was designated as time zero. RA was then added for the period required, as indicated under Results. Duration of cell cycle phases was determined from [^{3}H]thymidine incorporation into DNA and film analysis, as previously described for PC13 EC cells (Mummery *et al.*, 1984).

Soft Agar Growth

The percentage of cells able to form colonies in soft agar at particular times during the cell cycle or after induction of differentiation was determined by plating 1×10^4 cells in DF + 20% FCS with 0.375% agar onto a base layer of 0.5% agar in 60-mm petri dishes (Costar), as described by Todaro *et al.* (1981). Colonies larger than 8 cells were scored after 10–12 days by counting 20 random fields corresponding to 2.3% of the total dish area. Data are expressed as the percentage of cells plated able to form colonies.

Plasminogen Activator (PA) Production

Synchronized cells were plated at a density of 2×10^4 cells/cm^2 in 3.5-cm dishes in the presence of RA for the period indicated or in its absence. At $t = 50$ hr, 800 μl of growth medium was added to each dish and incubated for a further 20 hr. At $t = 70$ hr, cells were counted and the PA present in 50 μl of conditioned medium was determined as described previously (Mummery *et al.*, 1985). For exponentially growing cultures, cells were plated for 12 hr before RA addition at $t = 0$. Medium (800 μl) was conditioned for either 8 or 16 hr at various times thereafter and the PA present in 50-μl samples was determined as described above. Data were expressed as equivalent units of urokinase activity per 10^8 cells per hour or per milligram of cell protein per hour, as indicated. Cell protein was determined according to the method of Lowry *et al.* (1951).

^{125}I-EGF Binding

Cells were plated in 3.5-cm-diameter gelatinized petri dishes in the presence or absence of RA at a density such that each contained 0.5–1 $\times 10^6$ cells at the time EGF binding was determined. The binding medium con-

sisted of 1 ml of DMEM, Hepes (25 mM) with 0.1% (w/v) bovine serum albumin containing 1.67×10^{-10} M ^{125}I-EGF (Amersham 250.000 cpm), and varying amounts of unlabeled EGF (0 to 2×10^{8} M), as previously described (Mummery *et al.*, 1985). After labeling, cells were incubated for 2 hr at room temperature (ca. 20°C) aspirated, and the dishes were washed five times with ice-cold PBS. Cells were solubilized in 0.5 M NaOH and radioactivity was counted in a gamma counter. Nonspecific binding, determined by measuring the bound counts in the presence of excess unlabeled EGF (200-fold over ^{125}I-EGF), was less than 10% of the specifically bound counts.

Immunofluorescence

Cells grown on gelatinized plastic coverslips in the presence or absence of RA as required were fixed in ethanol at −20°C for 10 min, washed in PBS, and incubated for 30 min at 37°C with antibody followed by the appropriate FITC-labeled conjugate. The antibodies used were monoclonal anti-SSEA-1 diluted 1:250 (Solter and Knowles, 1978), rabbit anti-mouse laminin diluted 1:20 (BRL, Cambridge, UK), and TROMA-1 diluted 1:10 (Kemler *et al.*, 1981) which recognizes a 55K protein of intermediate filaments on trophoblastoma, trophectodermal, and endoderm cells.

RESULTS

Growth of P19 EC Cells in Semisolid Medium

The ability to grow in semisolid media *in vitro* has been highly correlated with tumor formation *in vivo* (Shin *et al.*, 1975). RA-induced differentiation of EC cells is accompanied by the loss of tumorigenic potential *in vivo* (Rayner and Graham, 1982) and *in vitro* (Rodrigues *et al.*, 1985). In the present study we have used P19 EC cells to investigate the temporal relationships among the reduction of growth in soft agar, the cell cycle, and the appearance of other markers of the fully differentiated progeny.

Cell Cycle Dependence

Initial experiments showed that exponentially growing untreated P19 EC cells rapidly formed colonies in soft agar but that cloning efficiency in medium containing 10% FCS varied considerably between experiments, with 2–30% of the cells forming macroscopic colonies within 10 days (not shown). Similar variability (9–85%) has been observed by others for Nulli-SCCI EC cells (Sherman *et al.*, 1985). Cloning efficiency, however, was significantly increased by increasing the FCS concentration in the soft agar to 20%. Under these conditions, variability between experiments was reduced, with 20–50% of control cells forming colonies. In all subsequent experiments, soft agar growth was routinely determined in 20% FCS.

Previous studies on cell cycle regulation during RA-induced differentiation of PC13 EC cells have shown that using synchronized cells prevents any complications arising from interactions between cells in different cell cycle phases. It does require, however, that intrinsic variations of particular parameters within one cell cycle also be characterized so that their values are known at the time of addition of the inducer. For the soft agar growth of P19 EC cells, the variations during the first cell cycle after synchronization by mitotic selection are shown in Fig. 1. Cells in G_1 phase form colonies less efficiently than cells in S phase. Although a number of factors inducing soft agar growth of nontransformed cells have been identified (van Zoelen *et al.*, 1986), the biochemical and genetic basis for the anchorage-independent phenotype of tumor cells remains largely unknown; furthermore, 100% colony formation of tumor cells is never observed. The reason for the cell cycle variation in soft agar growth shown by P19 EC cells is therefore unclear although the data in Fig. 1 demonstrate that asynchrony is not the reason why a large proportion of exponentially growing cultures fail to form colonies.

Effects of RA: Kinetics of Reduced Colony Formation in Exponentially Growing and Synchronized Cells

In order to establish when P19 cells lost colony-forming ability after RA addition, exponentially growing cells

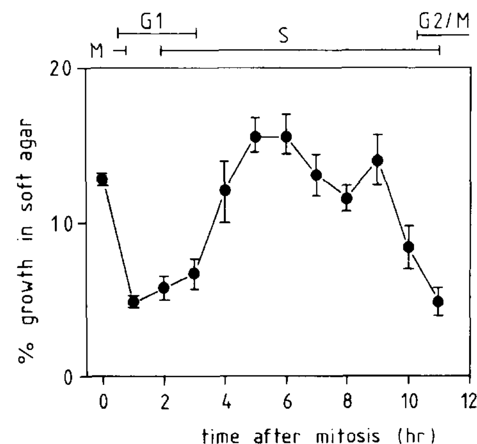

FIG. 1. Soft agar growth during the cell cycle of P19 EC cells. Cells were synchronized by mitotic selection and plated in gelatinized petri dishes at time zero in DF + 7.5% FCS. At the time indicated, cells were trypsinized, resuspended, and plated in soft agar in DF + 20% FCS. Cell cycle phases based on [^{3}H]TdR incorporation and film analysis (not shown) are indicated by horizontal bars. Data given are the means (±SEM) of three separate experiments with each point determined in fourfold.

were treated in monolayer for various lengths of time introduced into soft agar in the absence of RA, and colony formation was scored 10 days later. Figure 2 shows that 10 hr elapsed before anchorage-independent growth decreased and that at least 24 hr of exposure was required before colony formation became insignificant. A similar time course has been observed by others in exponentially growing F9 cells treated with RA (Rodrigues *et al.*, 1985). A lag period also occurred before a reduction in growth rate was observed in exponentially growing PC13 EC cells treated with RA (Mummery *et al.*, 1984). Using synchronized cells to follow the effects of RA on cell cycle progression more precisely, the lag period was shown to consist of two complete cell cycles (24–26 hr) in both PC13 and P19 EC cells (Mummery *et al.*, 1984, 1987). To investigate whether the loss of anchorage-independent growth showed a similar cell cycle-dependent lag period, P19 EC cells were synchronized in mitosis, treated for various periods with RA, and then introduced into soft agar in the absence of RA. The results showed that exposure for one complete cell cycle (13 hr; Mummery *et al.*, 1987) was required before colony formation significantly decreased but that after exposure for two cell cycles, no colonies formed (Fig. 2). A lag period of only one complete cell cycle is therefore observed before RA reduces growth in soft agar. The data in Fig. 2 are expressed relative to control values since soft agar growth itself is cell cycle dependent (Fig. 1). The apparent decrease following exposure of synchronized cells for 0–2 hr with RA is probably not significant since the intrinsically low capacity for soft agar growth in G_1 phase leads to a relatively large error in this data point.

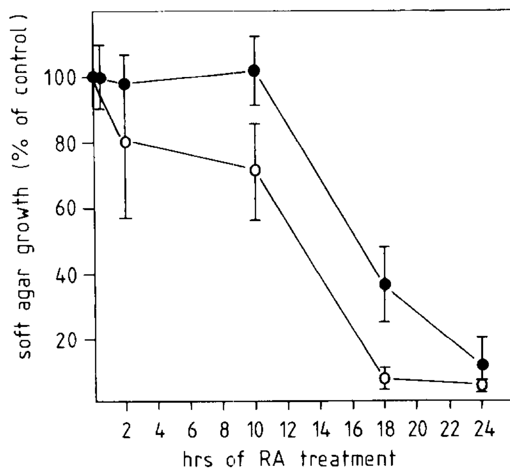

FIG. 2. Effect of retinoic acid on soft agar growth of P19 EC cells. Exponentially growing (● — ●) or synchronized (○ — ○) P19 EC cells in monolayer at a density of 2×10^4 cells/cm^2 were exposed to RA (10^{-6} *M*) in DF medium + 7.5% FCS; at the time indicated cells were trypsinized, resuspended, and immediately plated in soft agar containing DF medium + 20% FCS. Colonies were counted 10 days later and expressed as a percentage of control cultures taken at the same time point.

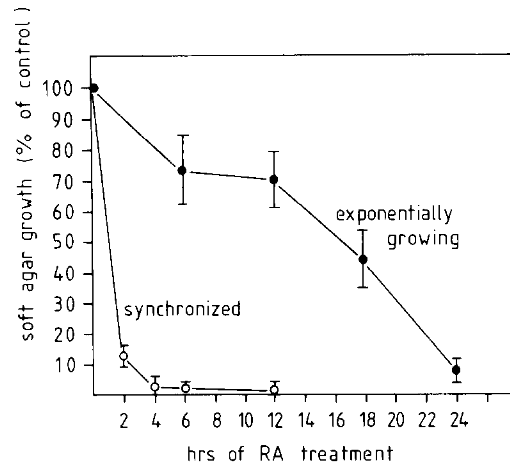

FIG. 3. Commitment of P19 EC cells to differentiation: soft agar growth. Exponentially growing (● — ●) or synchronized (○ — ○) EC cells were plated and allowed to attach, and RA (10^{-6} *M*) was added at $t = 0$. Cell density at $t = 0$ was 2×10^4 cells/cm^2 in both cases. RA was then removed at the time indicated and the cultures were incubated further in its absence in DF + 7.5% FCS before plating in soft agar at $t = 80$ hr in DF + 20% FCS. Data are expressed relative to controls taken at the same time point.

Effects of RA: Commitment and Reversibility in Exponentially Growing and Synchronized cells

The preceding experiments demonstrated that reduced colony formation in soft agar in P19 EC cells treated in monolayer with RA occurs after a lag period of at least one cell cycle. The question of when the cells become irreversibly committed to lose the capacity for anchorage-independent growth then arose. P19 EC cells were therefore treated in monolayer for different periods with RA returned to RA-free medium until a fixed time point, and then colony formation in soft agar was tested as above.

In exponentially growing P19 EC cells, short exposures (6–12 hr) to RA were sufficient to induce a 30% reduction in the fraction of the cell population still able to grow in soft agar 75 hr later, although at least 24 hr was necessary for all cells to lose their colony-forming ability irreversibly (Fig. 3). In themselves, these short exposures did not affect colony-forming ability when cells were plated in soft agar immediately after RA removal (Fig. 2). By contrast much shorter exposures were sufficient to induce commitment for anchorage-dependent growth in synchronized P19 EC cells (Fig. 3). Addition of RA to mitotic cells for 2 hr or more at the beginning of the first cell cycle induced complete loss of colony-forming potential 75 hr later; cells had divided at least three

times during this period. The results have several implications. First, the ability of RA to induce anchorage-dependent growth may be cell cycle dependent. Second, progression of differentiation appears to require substrate attachment, since synchronized cells exposed to RA for up to 10 hr and then immediately plated in agar (Fig. 2) still form colonies while those attached to a substrate for a certain period after RA removal lose this capacity entirely even though their exposure to RA may have been much shorter (Fig. 3). Third, reduced soft agar growth is observable in the second cell cycle after induction; it therefore preceded the increase in cell cycle duration induced by RA by one cycle (Mummery *et al.*, 1987; see also Mummery *et al.*, 1984), suggesting that these events are uncoupled and that reduced colony formation is not simply the result of a general decrease in growth rate.

RA-Induced Commitment for Anchorage-Dependent Growth Is Cell Cycle Dependent

The apparent cell cycle dependence of RA-induced commitment for anchorage-dependent growth suggested by the previous experiments was investigated further by exposing synchronized EC cells in monolayer to pulses of RA during different cell cycle phases and assaying for their capacity to grow in soft agar 75 hr later. The results of these experiments are shown in Fig. 4. In control cultures, synchronized at $t = 0$ but not treated with RA, 50 ± 5% (mean ± SEM; $n = 6$) of the cells formed colonies when plated in soft agar 75 hr later. Parallel

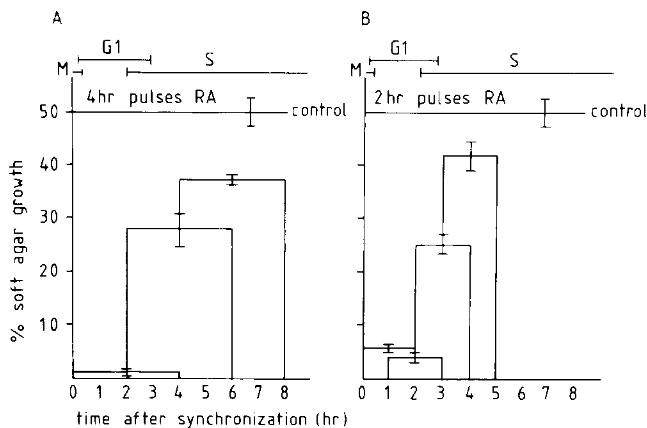

FIG. 4. Effect of pulsed RA treatment on the commitment of synchronized P19 EC cells to differentiation: soft agar growth. Cells were synchronized at $t = 0$, and RA (10^{-6} *M*) was added for 2- or 4-hr pulses at the times indicated. After removal of RA, cultures were incubated further in DF + 7.5% FCS before plating in soft agar at $t = 75$ hr in DF + 20% FCS. Data shown are the percentages of cells plated which had formed macroscopic colonies after 10 days. Each point shows the mean of four separate dishes (±SEM). Control cultures were synchronized at $t = 0$ but were further untreated until $t = 75$ hr.

cultures treated with RA for the first 4 hr after replating ($t = 0$–4 hr) but further incubated in RA-free medium had completely lost their ability to form colonies 75 hr later. Their morphology at the time of replating in agar is shown in Fig. 5. By contrast, a similar 4-hr period of exposure to RA between $t = 4$ and 8 hr induced only a small decrease in soft agar growth of cells 75 hr later with almost 40% of the cells still forming colonies (Fig. 4). Thus the reduction induced by RA exposure immediately after synchronization was not due simply to inadequate removal of RA. Like PC13 EC cells (Mummery *et al.*, 1984), P19 EC cells are synchronized in mitosis primarily as large single cells, form sister pairs when replated at $t = 0$, and then proceed through G_1 phase. [^{3}H]TdR incorporation shows that the G_1/S-phase transition occurs between 2 and 3 hr after mitosis and most cells are in S phase at $t = 4$ hr (data not shown, but indicated schematically in Figs. 1 and 4). Exposure of cells to RA for a 4-hr period including G_1 phase clearly had a greater effect than a similar period in S phase. The effect became even more striking when cells were exposed to pulses of 2 hr duration, as shown in Fig. 4B.

In order to eliminate the possibility that the susceptibility of cells to RA immediately after synchronization was not related to cell cycle phases but was an artefact introduced by mitotic shake off, we also exposed cells to RA for 2-hr pulses at the end of the first cell cycle and the beginning of the second cell cycle following synchronization. These results (Table 1) again showed that a 2-hr pulse during the first G_1 phase was sufficient to eliminate soft agar growth 75 hr later while a 2-hr pulse in S phase had only a marginal effect. More importantly however, the results showed that a 2-hr pulse from 12–14 hr (not shown) or 13–15 hr (Table 1), coincident with the second G_1 phase after synchronization, was again sufficient to eliminate soft agar growth 75 hr later.

Other Differentiation Markers: Plasminogen Activator Production and EGF Binding

RA has been shown to induce plasminogen activator production and the expression of EGF receptors in a variety of EC cell lines (Strickland and Mahdavi, 1978; Ress *et al.*, 1979; Rodrigues *et al.*, 1985) which are thought to be markers of certain differentiated cell lineages. We have followed the kinetics with which these markers become detectable during RA-induced differentiation of P19 EC cells to establish the temporal relationship with the loss of malignant phenotype. Figure 6 shows that in the continuous presence of RA, the increase in plasminogen activator occurred only after a lag period of at least 16 hr in exponentially growing cells, while 3–5 days elapsed before EGF receptor expression became detectable (Fig. 6).

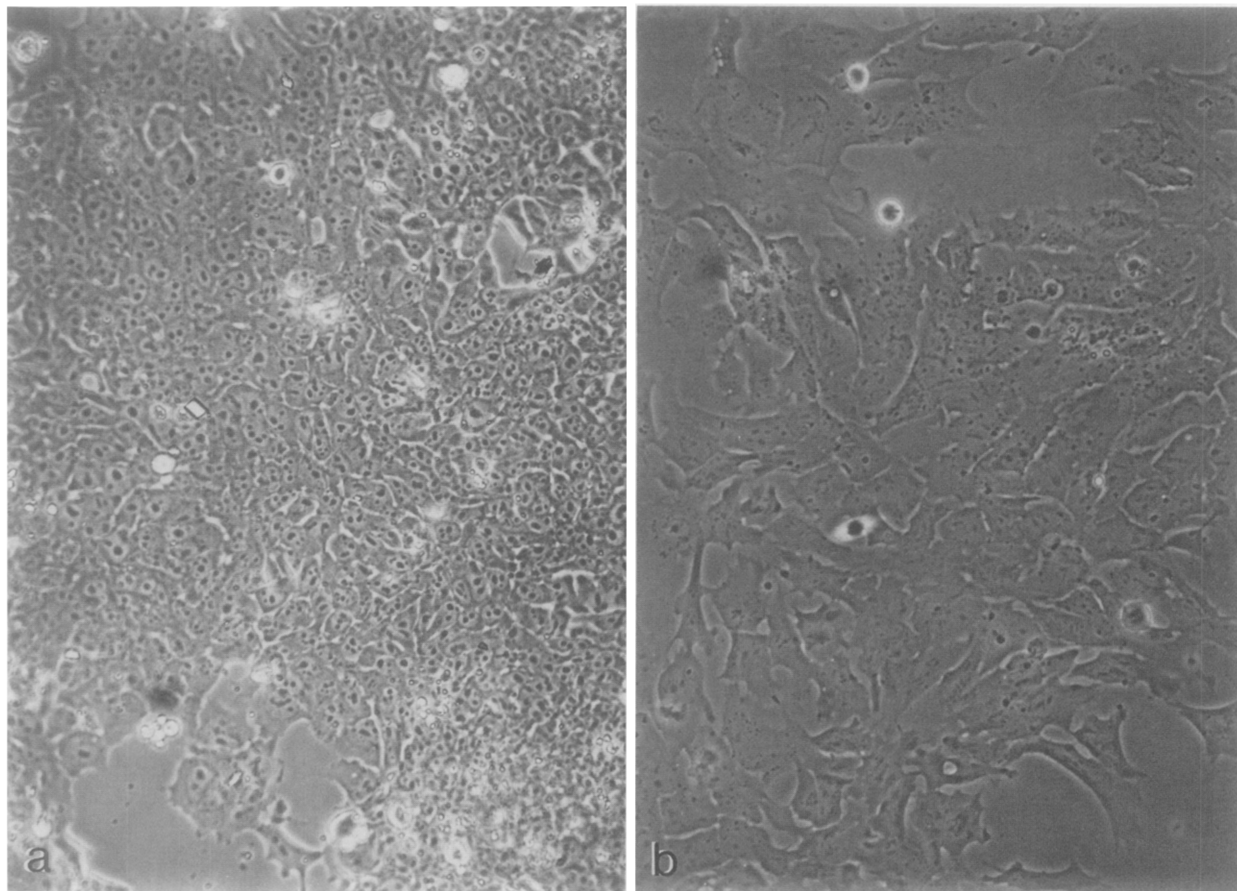

FIG. 5. Effect of pulsed RA treatment on the commitment of synchronized P19 EC cells to differentiation: morphology. Cells were synchronized at t = 0 and replated, and RA was added for 4 hr as required. Photomicrographs were made at t = 75 hr just prior to plating in soft agar as in Fig. 6. (a) Control. (b) RA exposure from t = 0 to t = 4 hr.

Synchronized cells treated with RA for a period incorporating the G_1 phase of the cell cycle were shown to be committed to developing both anchorage dependence for growth and a flattened morphology of cells during the 75 hr following RA removal. The extent to which these cells had retained EC-specific markers or had acquired those of differentiated cells derived by continuous RA exposure was investigated. Cells were therefore synchronized, treated for various periods with RA, and then PA production and EGF binding were determined at the time maximum levels were normally reached (see Figs. 5a and 5b); i.e., PA levels were determined in medium conditioned between 50 and 70 hr after initial synchronization and EGF binding was determined on Day 7. Undifferentiated EC cells were not evident in the cultures at the time the experiments were carried out. Figure 7A shows that minimally 24 hr of exposure to RA was necessary to induce PA production. Similarly,

TABLE 1
EFFECT OF PULSED TREATMENT ON COMMITMENT OF SYNCHRONIZED P19 EC CELLS TO DIFFERENTIATION: COMPARISON OF EFFECTS IN FIRST AND SECOND CELL CYCLE

	Controls	G_1 phase	S phase	S/G_2 phase	Second G_1 phase
RA pulse (time after synchronization, hr)	None	0–2	5–7	10–12	13–15
Soft agar growth (% of control)	100	1.7 ± 1.1	89.4 ± 14.4	53.9 ± 15.5	1.7 ± 1.7

Note. RA (10^{-6} *M*) was present for periods of 2 hr as indicated (cells synchronized in mitosis at t = 0 hr); the cells were then allowed to grow in the absence of RA until introduction into soft agar at t = 75 hr.

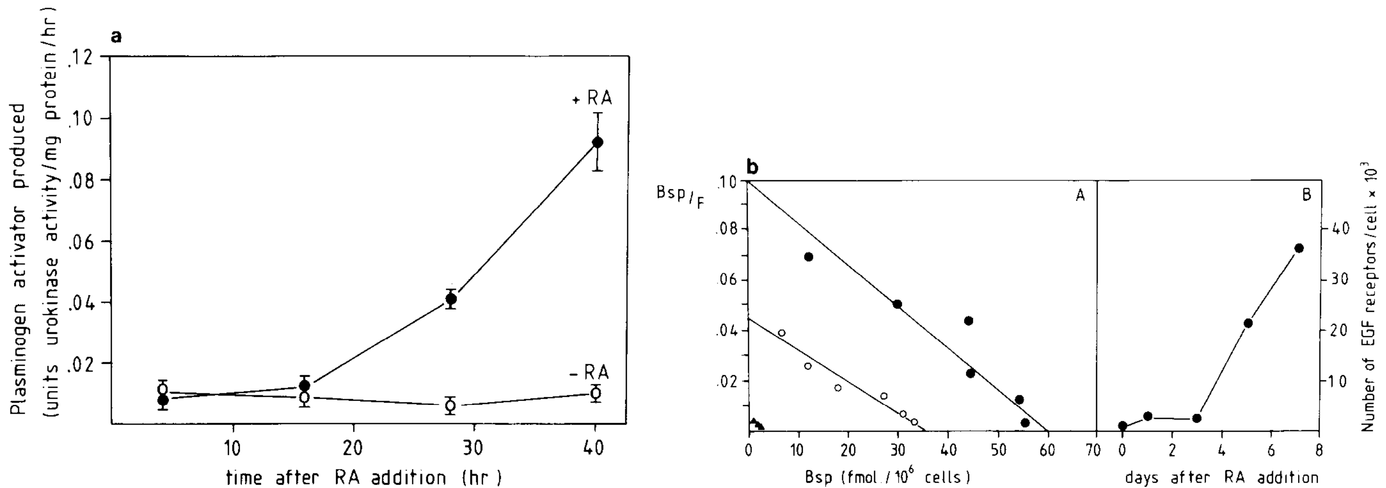

FIG. 6. Effect of RA on differentiation markers in exponentially growing P19 EC cells. (a) Plasminogen activator production. Cells were plated in gelatinized petri dishes and RA (10^{-6} M) was added 12 hr later at time zero. Fresh medium (800 μl) was conditioned for 8 or 16 hr at various times and levels of plasminogen activator were determined as described under Materials and Methods. Data are expressed relative to human urokinase as standard per milligram of cell protein at the end of the conditioning period. Production rates (mean ± SEM; n = 3) are indicated in the middle of the conditioning period. (b) EGF binding. Cells were plated as in a, and EGF binding was determined as described under Materials and Methods. (A) Scatchard analysis of binding data 3 (▲ — ▲), 5 (○ — ○), and 7 (● — ●) days after RA addition. (B) number of EGF receptors per cell determined as the intercept of Scatchard curves shown in A.

short exposure to RA in G_1 phase did not induce detectable increases in EGF receptor expression (Fig. 7B). Again, at least 24 hr was required before levels of expression comparable to those in cells exposed continuously to RA were found, i.e., more than 40,000 receptors/cell (Mummery *et al.*, 1985, 1986, and Figure 6).

Anti-SSEA-1, TROMA-1, and Laminin Markers

SSEA-1 determinants are characteristically expressed by EC cells, while laminin, an extracellular matrix protein, and Endo A, a cytoskeletal protein recognized by TROMA-1 antibodies, are expressed by differentiated endodermlike cells. Using indirect immunofluorescence and a monoclonal antibody against SSEA-1 (Solter and Knowles, 1978), we showed that P19 EC cells expressed SSEA-1 determinants as expected (Figs 8a and 8b), but the majority of synchronized cells exposed to RA for 4 hr at the beginning of the first cycle no longer expressed SSEA-1 75 hr later (Figs. 8c and 8d). Very few cells with EC morphology were present; these had retained anti-SSEA-1 binding. No specific labeling with the anti-laminin antibody was detected although the cytoskeleton of approximately 10% of the cells was specifically labeled by TROMA-1 (Table 2). Virtually all cells continuously exposed to RA for this period bound anti-laminin antibodies and TROMA-1. Results using indirect immunofluorescence were confirmed in a more sensitive ELISA assay (data not shown). Thus, although morphological changes were induced by pulsed RA exposure, they were not the result of changes in the secretion of laminin or on the intermediate filaments of the cytoskeleton recognized by TROMA-1.

DISCUSSION

The present study has shown that P19 EC cells respond differentially to RA when they are at different stages of the cell cycle. By using cells synchronized by mitotic

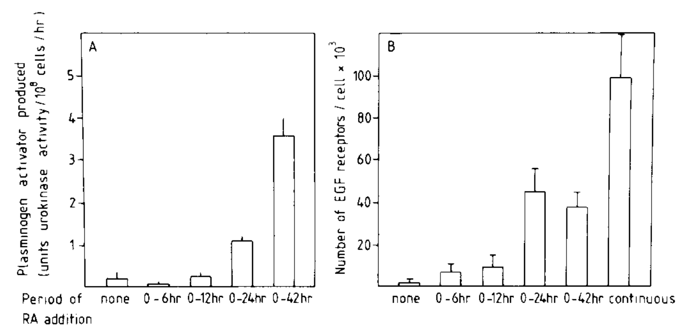

FIG. 7. Commitment to differentiation. (A) Plasminogen activator production induced in synchronized P19 EC cells following pulsed exposure to RA. Cells were synchronized in mitosis and replated in the presence of RA at t = 0. At t = 6, 12, 24, and 42 hr, RA was removed by changing the medium. From t = 50 hr to t = 70 hr, 800 μl of medium was conditioned and the amount of plasminogen activator produced in that period was determined, as described under Materials and Methods. (B) EGF receptor expression induced in synchronized P19 EC cells following pulsed exposure to RA as for PA production in A. EGF binding was determined on Day 7 after initial synchronization, as described under Materials and Methods. The number of EGF receptors per cell was determined from a Scatchard analysis of the binding data, as in Figure 6b.

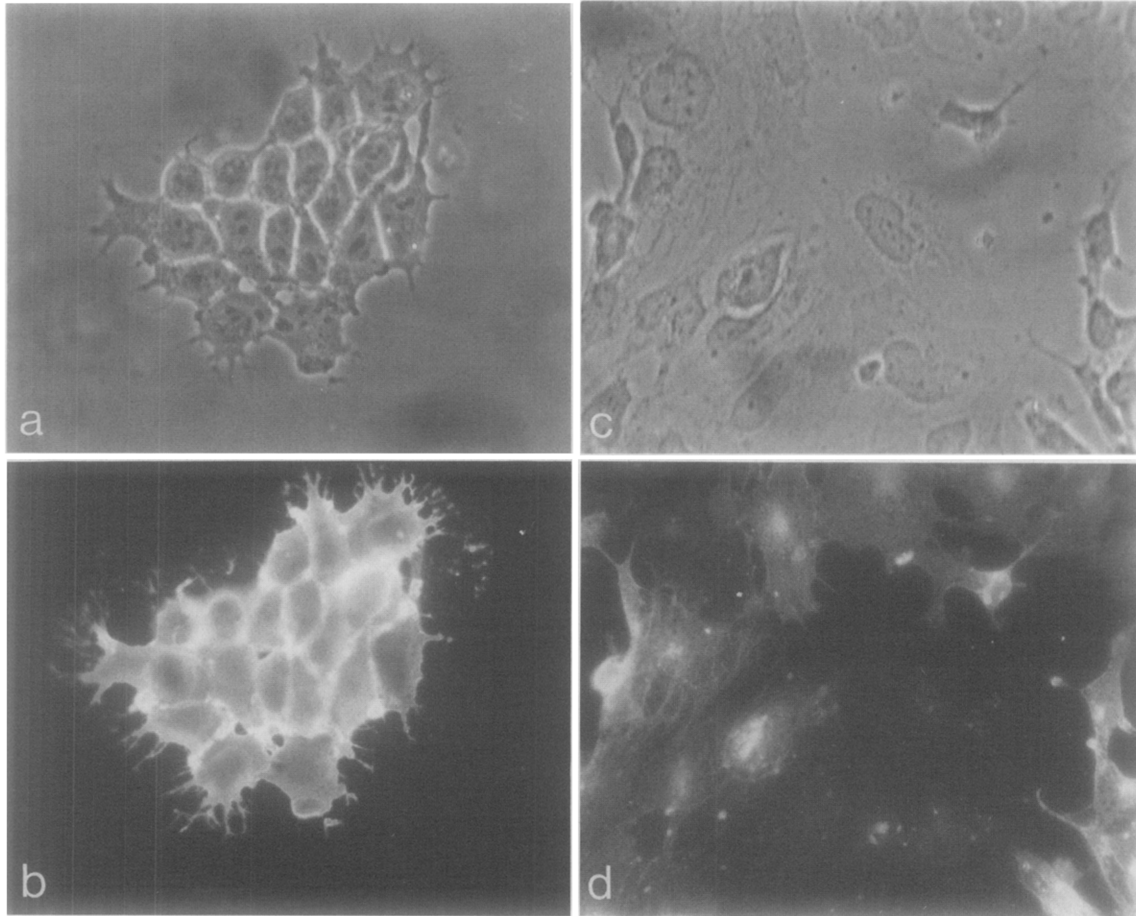

FIG. 8. Effect of pulsed RA treatment on SSEA-1 expression. Cells were synchronized at $t = 0$ and replated, and RA was added for 4 hr as required. Expression of SSEA-1 was determined at $t = 75$ hr by immunofluorescence as described under Materials and Methods. (a, b) without RA; (c, d) with RA for $t = 0$–4 hr. (a, c) phase contrast; (b, d) fluorescence.

shake off and exposing them to pulses of RA at different times thereafter, we have demonstrated that cells in the G_1 phase of the cell cycle are exquisitely sensitive to the induction of differentiation as defined by the loss of colony-forming ability in soft agar. The conclusion from the present experiment is similar to that derived by Griep and De Luca (1986) in more indirect experiments using partially synchronized F9 EC cells. In these experiments, 80% synchrony in early S phase was induced by growing cells at low serum and high thymidine concentrations. Addition of RA for a few hours immediately after release of the S phase block induced significantly greater numbers of differentiated cells 36 hr later than RA addition for a similar period 13 hr after release. The effect of using thymidine for cell synchronization in these studies may however have been significant since 17–24% of colonies contained differentiated cells even without RA treatment. Although this increased to 85% after early RA treatment, it may have been greater if the degree of synchrony had been as high as that achieved by mitotic shake off. Cell cycle specificity in the inducation of myeloid differentiation by RA has also been observed in HL60 cells and has been correlated with enhanced uptake of the inducer during the sensitive phases (Yen and Albright, 1984).

Loss of anchorage-independent growth is one of the

TABLE 2
EXPRESSION OF SSEA-1 ANTIGEN AND LAMININ SECRETION BY SYNCHRONIZED P19 EC CELLS EXPOSED TO RA DETERMINED BY INDIRECT IMMUNOFLUORESCENCE

Period of RA exposure	SSEA-1	Laminin	TROMA-1
None	+	−	−
0–4 hr	−	−	±
Continuously	−	+	+

Note. RA was present as indicated; labeling was determined 75 hr after initial synchronization.

earliest effects of RA on the EC cell phenotype (Jones-Villeneuve *et al.*, 1983; Edwards *et al.*, 1983; Rodrigues *et al.*, 1985); in synchronized P19 cells, this could be clearly shown to occur in the second cycle after induction. Similarly, the flattened morphology typical of differentiated cells was first evident in the second cycle after induction by RA, and, like anchorage dependence, was specifically induced in synchronized cells by G_1-phase exposure to RA. Cells therefore became anchorage dependent before the cell cycle duration increased and also before EGF receptor expression and PA production were detectable.

EGF receptor expression and PA production appeared to be relatively late markers of differentiation in cells continuously exposed to RA and were only expressed at significant levels in synchronized cells treated for at least 24 hr with RA. This may indicate that the anchorage-dependent cells derived by G_1-phase exposure to RA represent intermediate states of differentiation. Alternatively, and in view of previous observations that different doses of RA induce the development of different cell types in P19 cells (Edwards and McBurney, 1983), the length of exposure to RA may play a role in determining which cell types eventually develop. If this interpretation is correct, then the requirement of a long exposure to retinoic acid for the appearance of PA and EGF receptor is a consequence of RA action upon cells committed to differentiate but not yet committed to the particular developmental lineage for which PA and EGF receptors are markers.

G_1-phase exposure was clearly sufficient to commit synchronized cells to anchorage dependence, but comparison of Figs. 3 and 4 suggested that more complex cellular interactions were involved in the commitment of nonsynchronized cells. During a 13-hr period, it would be expected that all cells in exponentially growing cultures would have been exposed to RA during G_1 phase and, therefore, that all cells would be committed. Figure 3 showed that this was not the case; after 13 hr of exposure, only a fraction of the population was committed while 24 hr was required for commitment to be complete. Communication via diffusible factors or direct cell contact between cells in different cell cycle phases may inhibit the ability of the G_1-phase cells to respond to the inducer and become committed to anchorage dependence and might be the reason for this discrepancy. Cell interaction is indeed known to affect P19 differentiation in DMSO, although not in RA (Campione-Piccardo *et al.*, 1985). Alternatively, RA may be metabolized more rapidly in unsynchronized cultures and, therefore, reduced more quickly to ineffective concentrations. Further experimentation is required to distinguish these possibilities.

The reason for the the cell cycle specificity in the induction of initial events leading to the differentiation of P19 cells is unclear at present. Changes in gene expression are known to be induced following binding or interaction of RA with intracellular retinoid binding proteins (Linder *et al.*, 1981). Specific RA-binding proteins (RABP) have been detected in various cell types (reviewed by Schroder *et al.*, 1983), and cell cycle-dependent variations in cytoplasmic levels of cellular retinoic acid-binding protein (CRABP) may well be involved in the cell cycle dependence of RA induction. Certain differentiation-defective EC cell lines derived by mutagenesis (McCue *et al.*, 1983) indeed contain little or no CRABP and metabolize retinoic acid poorly (Gubler and Sherman, 1985). On the other hand, in several tumor cell lines, anchorage-independent growth is unaffected by RA, despite the presence of CRABP (Mukherjee *et al.*, 1983). Another possible mechanism may involve the alteration of specific cell surface membrane glycoconjugates (De Luca *et al.*, 1979; Lotan, 1980). Again in certain transformed cell lines, the ability of RA to induce changes in cell surface glycoproteins has been correlated with its ability to affect soft agar growth. In P19 EC cells we have demonstrated that soft agar growth is cell cycle dependent; cells are most sensitive to induction by RA in the cell cycle phase where soft agar growth is intrinsically low. Cell surface glycoproteins may change in a cell cycle-dependent manner and the ability of RA to affect these changes may be cell cycle dependent. Synchronization of EC cells by mitotic shake off as in the present study has clearly provided a simple means of unraveling some of the complex events of early differentiation, including identification of the stage of differentiation at which pluripotency is lost. As such, this method has significant potential as a system for studying the relationship between growth regulation and differentiation.

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