

## Cell proliferation and expression of cytokeratin filaments in F9 embryonal carcinoma cells

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### Summary

A double immunofluorescence method was developed for the monitoring of proliferation and differentiation of F9 embryonal carcinoma cells. Cytokeratin filament expression was used as a marker for differentiation, and proliferating cell nuclear antigen (PCNA)/cyclin or bromodeoxyuridine labeling were used as markers for proliferation. F9 cells had a high proliferation rate and were cytokeratin-filament-negative. Upon treatment with retinoic acid and dibutyryl cyclic AMP, cytokeratin-filament-positive cells with differentiated phenotype

appeared. After 3 days, the extent of proliferation of cytokeratin-filament-positive cells was comparable to, but after 5 days significantly lower than, that of cytokeratin-filament-negative cells in the same culture. In differentiating F9 cells, cytokeratin filament expression is associated with, and even slightly precedes, the dramatic decrease in the rate of proliferation.

Key words: cell proliferation, cytokeratin, F9 embryonal carcinoma cells.

### Introduction

Treatment with retinoic acid (RA) and dibutyryl cyclic AMP (dbc-AMP) induces cultured murine F9 embryonal carcinoma (EC) cells to differentiate into endoderm-like (END) cells (Strickland & Mahdavi, 1978; Strickland *et al.* 1980; Hogan *et al.* 1983). This is accompanied by the expression of cytokeratin filaments in a proportion of the cells exhibiting the differentiated morphology (e.g. Lehtonen *et al.* 1983; Tienari *et al.* 1987). Simultaneously with differentiation, a clear reduction in the overall proliferation rate of EC cells can be observed.

The proliferation of F9 cells after RA treatment has been studied at the level of the whole cell population in a differentiating culture (Rosenstraus *et al.* 1982). However, it is obvious that such populations are heterogeneous with respect to both differentiation and rate of proliferation. Thus, the proliferation and differentiation should be studied at the single cell level. Several markers for proliferating cells have been introduced for light microscopic studies (cf. Kurki *et al.* 1988). Such markers might be applicable to the immunofluorescence analysis of the subpopulations of differentiating cells.

In the present study, we apply two methods, labeling of S-phase cells with bromodeoxyuridine (BrdUrd) and

with antibodies to proliferating cell nuclear antigen (PCNA)/cyclin to detect the proliferation at single-cell level. PCNA is an auxiliary protein of DNA polymerase- $\delta$  (Bravo *et al.* 1987; Prelich *et al.* 1987). Cells were double-labeled with antibodies to cytokeratin. Thus, the extent of proliferation could be compared in cytokeratin-filament-negative and -positive F9 cell populations.

### Materials and methods

#### Cell culture and differentiation

The F9 EC cells were cultured as previously described (Lehtonen *et al.* 1983; Tienari *et al.* 1987) on gelatinized plastic dishes with coverslips in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, Scotland). The cells were dissociated with TVP (0.025% trypsin, 1 mM-EDTA, 1% chick serum in phosphate-buffered saline, lacking  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ; Bernstine *et al.* 1973). To induce differentiation, F9 cells were plated on day 1 at a density of  $1.3 \times 10^3$  cells  $\text{cm}^{-2}$ . On day 2,  $5 \times 10^{-8}$  M-RA (Sigma Chemical Co., St Louis, MO) and  $10^{-3}$  M-dbc-AMP (Sigma) were added. The medium was changed on days 4, 5, and 6.

#### Antibodies

Anti-PCNA-positive serum was obtained from a patient with

systemic lupus erythematosus. The antibodies were identified as anti-PCNA by using double immunodiffusion technique and by immunoblotting. The serum was absorbed with a rabbit kidney extract to remove other autoantibodies (Ogata *et al.* 1985).

Mouse monoclonal iododeoxyuridine antibodies (IU-4; Vanderlaan *et al.* 1986) were a gift from Dr M. Vanderlaan (Lawrence Livermore National Laboratory, Livermore, CA).

The following cytokeratin antibodies were used: mouse monoclonal antibodies to oocyte cytokeratin (OCS-1; Lehtonen, 1985), mouse monoclonal antibodies to CK 18 (PKK 3; Virtanen *et al.* 1985), mouse monoclonal antibodies to cytokeratins of PtK 1 cells (LE 65; Lane, 1982; a kind gift from Dr B. Lane, Imperial Cancer Research Fund, Herts, UK), rat monoclonal antibodies to cytokeratin (TROMA 1; Kemler *et al.* 1981; a kind gift from Dr R. Kemler, Max-Planck-Institut, Tübingen, FRG), and rabbit polyclonal antibodies to MDCK cytokeratin (Holthöfer *et al.* 1983). The following FITC-conjugated antisera were used: anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark), anti-rat IgG (Cappel Laboratories), and anti-rabbit IgG (Cappel Laboratories Inc., Cochranville, PA).

#### Double immunofluorescence staining

For PCNA staining, the cells were fixed in 1% paraformaldehyde at room temperature and permeabilized with 0.5% Nonidet P-40. Human PCNA antibodies were followed by FITC-conjugated anti-human IgG (Tago Inc., Burlingame, CA). Thereafter, cytokeratin filaments were labeled by rat monoclonal TROMA 1 antibodies followed by TRITC-conjugated anti-rat IgG (Cappel Laboratories). The level of non-specific binding of human IgG was controlled by using normal human serum instead of anti-PCNA.

Alternatively, S-phase cells were identified by the bromodeoxyuridine-labeling technique. The bromodeoxyuridine-pulsed cells were first fixed in 70% ethanol at  $-20^{\circ}\text{C}$  for 30 min and then in 1% paraformaldehyde for 2 min at room temperature. DNA was denatured by 1 M-NaOH for 15 min at room temperature. Bromodeoxyuridine-containing DNA was stained by mouse monoclonal iododeoxyuridine antibodies followed by FITC-conjugated anti-mouse immunoglobulins (Dakopatts).

#### Flow cytometry

The cells were suspended in ice-cold propidium iodide (PI) solution ( $50\ \mu\text{g ml}^{-1}$ , Sigma) in 0.01 M-Tris-HCl buffer (pH 7.6) containing 0.3% Nonidet P-40 and 0.001 M-EDTA (Titriplex<sup>®</sup>). After 15-min incubation at  $0^{\circ}\text{C}$ , RNase (RNase A, type 1-A; Sigma; final concentration  $1\ \text{mg ml}^{-1}$ ) was added. The cells were then incubated in this solution for 15 min at room temperature and thereafter kept on ice until analyzed. DNA in single cells was quantified by EPICS C flow cytometer (Coulter Electronics, Hialeah, FLA) equipped with a 2 W argon ion laser (Coherent Radiation). The cells were excited at 488 nm, and the fluorescence signal from propidium iodide was collected using a 560 nm dichroic mirror and a 610 nm band pass filter. The percentages of cells in different phases of the cell cycle were analyzed by using Coulter Computer and software.

The duration of the cell cycle was measured by the S-phase entry method by using the Coulter Epics C exciting the cells at 488 nm. A dichroic mirror (560 nm) was used to separate green and red fluorescence. FITC fluorescence was collected between 515–530 nm and PI fluorescence beyond 610 nm.

#### Immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

was performed under reducing conditions by using 8% slab gels. The separated polypeptides were transferred onto nitrocellulose sheets as described earlier (Lehtonen *et al.* 1983; Tienari *et al.* 1987). The sheets were then exposed to TROMA 1 antibodies. The bound antibodies were visualized by using peroxidase-coupled rabbit anti-rat IgG (Dakopatts).

## Results

#### Effect of RA/dbc-AMP treatment on the proliferation of F9 cells

Untreated F9 cells have a cell cycle time of about 12 h as judged by the flow cytometric analysis of sequential samples after a short (10 min) bromodeoxyuridine pulse. Three days after the onset of RA/dbc-AMP treatment, the cell cycle time had increased to 15–16 h. The cell cycle analysis of PI-labeled cells showed that the increase in the cell-cycle time was associated with an increase in the number of cells at the  $G_1$  phase. In one experiment, 21% of untreated F9 cells were in  $G_1$ , whereas the numbers after 3 and 5 days of treatment were 26% and 33%, respectively (Fig. 1).

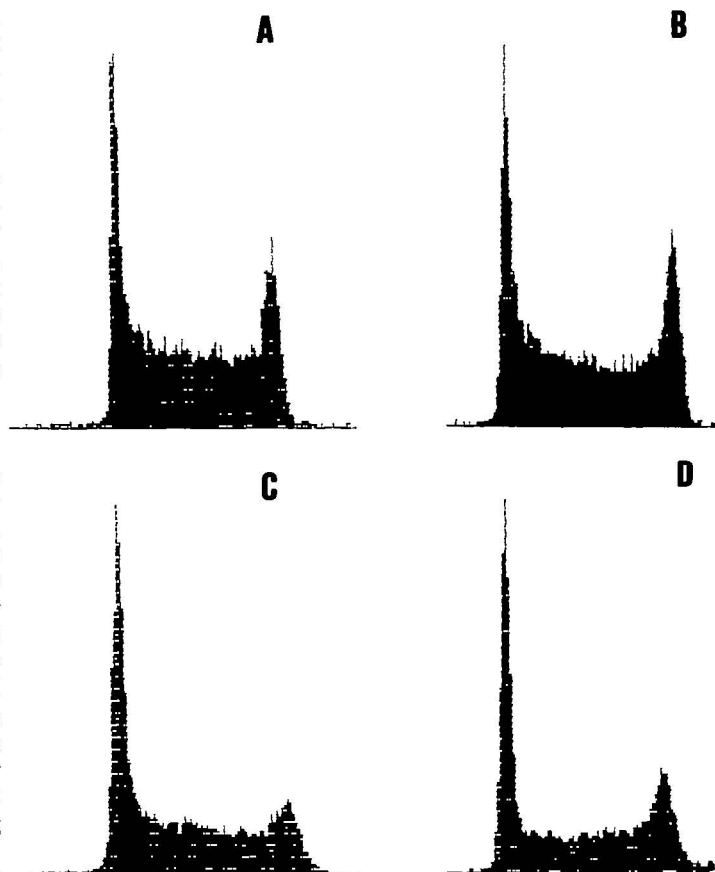
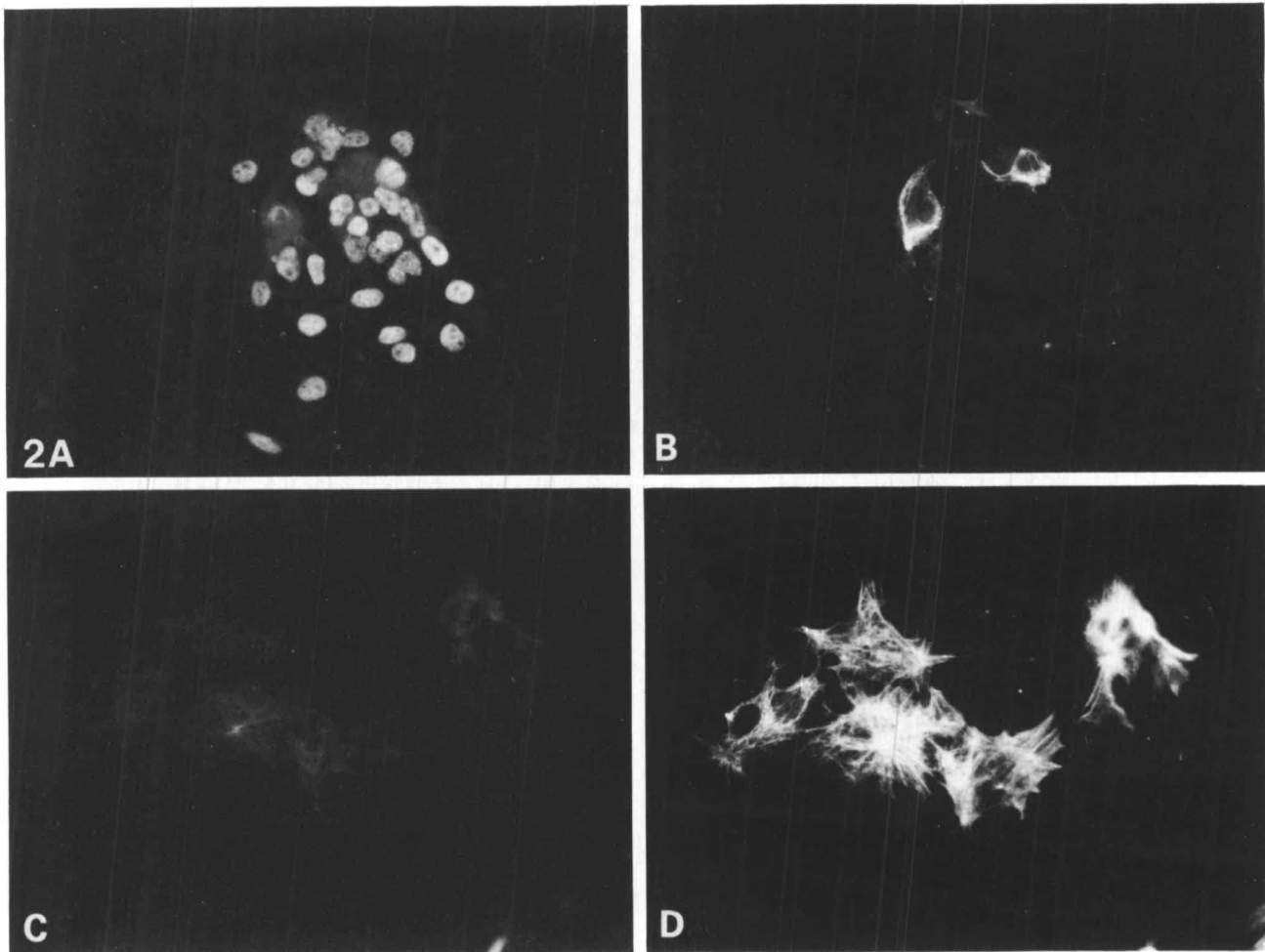


Fig. 1. DNA histograms of F9 cells before (A) and after 2 days (B), 3 days (C), and 5 days (D) of RA/dbc-AMP treatment. The X-axis indicates the DNA content and the Y-axis the relative number of cells. After RA/dbc-AMP treatment, there is a relative increase in the number of  $G_1$ -phase cells and decrease in the number of S-phase cells.



**Fig. 2.** Double immunofluorescence staining of F9 cells with anti-PCNA antibodies (A,C) and with anticytokeratin antibodies (B,D). (A,B) RA/dbc-AMP treatment for 2 days. The figures show an island of rapidly proliferating cells with a few cytokeratin-filament-positive cells. (C,D) RA/dbc-treatment for 5 days. The figures show a field with nonproliferating cytokeratin-filament-positive cells.  $\times 400$ .

#### *Cytokeratin filament expression after RA/dbc-AMP treatment*

Cytokeratin filament expression did not occur during the first 24 h of RA/dbc-AMP treatment as judged by indirect immunofluorescence staining with the five cytokeratin antibodies used in this study. After 2 days of treatment, 0–3% of the cells expressed cytokeratin filaments. During the following days the number of cytokeratin-filament-positive cells increased steadily. After 5 days of RA/dbc-AMP treatment, 5–40% of the cells, the percentage depending on the antibody used, displayed cytokeratin filaments. The TROMA-1 antibody proved to be the most sensitive probe, and the results described in this paper were obtained by using this antibody. In a representative experiment, cytokeratin filaments were found in 10% and 23% of the cells after 3 and 5 days of treatment, respectively (Table 2, Fig. 2). After 3 days, the majority of the cytokeratin-filament-positive cells were fusiform, whereas after 5 days, the positive cells were large and flat.

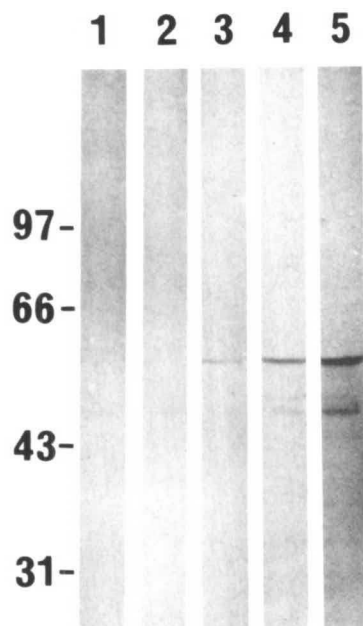
Immunoblotting with TROMA 1 antibodies revealed two polypeptide bands, representing the  $M_r$  46 000 and

$M_r$  52 000 cytokeratins, in F9 cells treated with RA/dbc-AMP for 5 days (Fig. 3). The  $M_r$  52 000 band but not the  $M_r$  46 000 band was visible as early as after a 2-day RA/dbc-AMP treatment. No polypeptide bands definitely reacting with the TROMA 1 antibodies were found in cultures not treated with RA/dbc-AMP (Fig. 3).

#### *Cell proliferation and cytokeratin filament expression*

In control F9 cell cultures, 64% of the cells in the whole population were in S phase as determined by bromodeoxyuridine labeling and indirect immunofluorescence microscopy. After 3 days of RA/dbc-AMP treatment, 57% of the cells were in S phase and after 5 and 7 days, 53% and 41%, respectively. At each time point, the numbers of S-phase cells were smaller in the cytokeratin-filament-positive cell population than in the filament-negative population (Table 1).

The numbers of cells expressing proliferating cell nuclear antigen (PCNA) did not change significantly in the whole cell population during the first 5 days of RA/dbc-AMP treatment (Table 2). There were less



**Fig. 3.** Electrophoretically separated polypeptides of F9 cell cultures transferred onto nitrocellulose sheets and incubated with TROMA 1 cyokeratin antibodies. Lane 1, untreated F9 cells; lanes 2-5, F9 cells treated with RA/dbc-AMP for 1, 2, 3, and 5 days, respectively. In lanes 3-5, the antibodies reveal a polypeptide band of  $M_r$  52 000. In lane 5 and, less distinctly, in lane 4, an additional band of  $M_r$  46 000 reacts with the antibodies.

**Table 1.** Expression of cyokeratin filaments in S-phase cells in RA/dbc-AMP-treated F9 cell population

Days of treatment	% S-phase	% S-phase/CK
3	57	47
5	63	31
7	41	31
Control	64	nd

% S-phase: proportion of S-phase cells in the whole population.

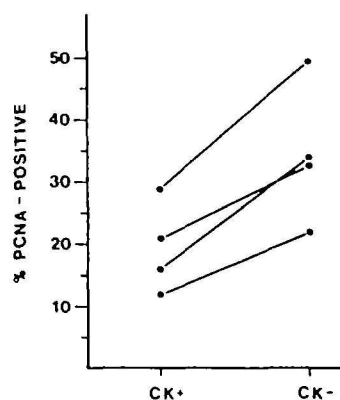
% S-phase/CK: proportion of S-phase cells in the cyokeratin-filament-positive population.  
nd: not determined.

**Table 2.** Expression of PCNA/cyclin and cyokeratin filaments in RA/dbc-AMP-treated F9 cells

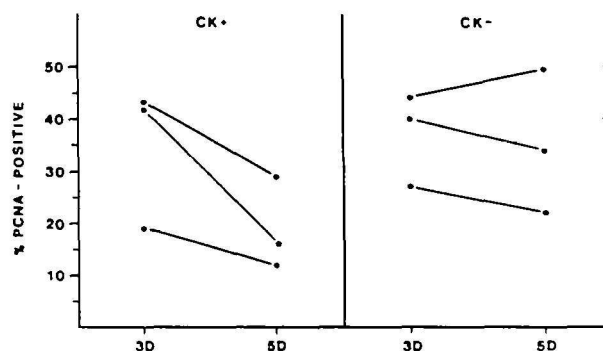
Days of treatment	% PCNA	% CK	% PCNA/CK
1	49	0	nd
2	47	2	nd
3	43	10	44
5	49	23	29
Control	49	0	nd

% PCNA, % CK: proportion of positive cells in the whole population.

% PCNA/CK: proportion of PCNA-positive cells in the cyokeratin-filament-positive population.  
nd: not determined.



**Fig. 4.** The percentage of proliferating cell nuclear antigen (PCNA)-positive F9 cells after 5 days of RA/dbc-AMP treatment. Cyokeratin filament (CK)-positive and -negative cell populations were analyzed separately. The solid lines indicate the results from the same culture.



**Fig. 5.** The percentage of proliferating cell nuclear antigen (PCNA)-positive F9 cells in cyokeratin filament-positive (CK+) and -negative (CK-) F9 cell populations after 3 and 5 days of RA/dbc-AMP treatment. The solid lines indicate the results from the same culture.

PCNA-positive cells among the cyokeratin-filament-positive cells, especially after 5 days (Table 2). The difference in the PCNA expression in F9 cells after 2 and 5 days of RA/dbc-AMP treatment is demonstrated in Fig. 2. In repeated experiments, the number of PCNA-positive cells varied, but in the cyokeratin-filament-positive cell population, the number of PCNA-positive cells was always smaller than in the cyokeratin-filament-negative population (Fig. 4). The decrease in the number of PCNA-positive cells occurring between days 3 and 5 was clearly seen in the cyokeratin-filament-positive cell population; this change was less pronounced among the cyokeratin-filament-negative cells (Fig. 5).

## Discussion

It is not completely clear how retinoic acid (RA) induces differentiation and how it alters the proliferative behavior of cultured cells. RA is dislocated into the

nucleus, and it binds to an inducible receptor protein that seems to be a *trans*-acting enhancer factor (Sherman *et al.* 1985; Petkovich *et al.* 1987). Proliferation and differentiation may be regulated by the same factor(s). Alternatively, differentiation may be a direct result of changes in cell proliferation, especially if there are modifications in the proportions of the different phases of the cell cycle. The susceptibility of the undifferentiated cells to RA is cell-cycle-dependent (Griep & Deluca, 1986; Mummery *et al.* 1987*a,b*). Furthermore, there is a close temporal relationship between the onset of differentiation and changes in the proliferation of EC cells (Rayner & Graham, 1982).

The cell cycle of undifferentiated F9 cells mainly consists of an S phase with a short G<sub>2</sub>/M phase (Rosenstrauss *et al.* 1982; Sennerstam & Strömberg, 1984). The duration of G<sub>2</sub>/M does not change during RA-induced differentiation (Rosenstrauss *et al.* 1982). Thus, the alterations in the proliferation rate are due to changes in the G<sub>1</sub> and S phases. The proliferation markers used in this study, the IU-4 antibody to iododeoxyuridine (Vanderlaan *et al.* 1986) and the antibody to proliferating cell nuclear antigen (PCNA)/cyclin (Celis & Celis, 1985; Kurki *et al.* 1986, 1988) principally detect S-phase cells. The advantage of the PCNA antibodies in detecting proliferating cells is that PCNA is an *in vivo* marker that is not dependent on active DNA synthesis (Kurki *et al.* 1986) and that it can be readily detected simultaneously with other antigens (Kurki *et al.* 1987).

In general, the proliferation of differentiated cells is mainly regulated during the G<sub>1</sub> phase that usually also determines the length of the cell cycle (Tsuda *et al.* 1986; Clegg & Hauschka, 1987; Clegg *et al.* 1987). Thus, it is possible that alterations in the cell cycle are required for differentiation. The G<sub>1</sub> phase of undifferentiated F9 cells is extremely short or even nonexistent (Rosenstrauss *et al.* 1982; Sennerstam & Strömberg, 1984; Griep & Deluca, 1986); the same is true for early embryo cells (Gamow & Prescott, 1970; Streffer *et al.* 1980). Upon RA treatment of F9 cells, G<sub>1</sub> is established and the relative contribution of the S phase to the length of the cell cycle is diminished. Correspondingly, we observed an increase of G<sub>1</sub>-phase cells and decrease of S-phase cells during RA/dbc-AMP-induced differentiation. In these conditions, the number of S-phase cells correlates to the proliferation rate.

At the single cell level, this trend should be more marked among the differentiated cells. Our results show that this indeed is the case. It should be noted that the most remarkable changes in proliferation took place between the third and fifth days after the onset of RA/dbc-AMP treatment. At that time the cells not only increased their cytokeratin filament expression but also underwent dramatic morphological changes. Thus, the appearance of filamentous cytokeratin is a rather late event in the differentiation process of F9 cells.

In the present study, employing cytokeratin as a marker for differentiation, a clear correlation was observed between cell proliferation and cytokeratin expression. It has been suggested in a number of studies

that there is an association between growth control and cytoskeleton (e.g. Connell & Rheinwald, 1983; Ben-Ze'ev, 1986). In this connection, it should be noted that expression of high levels of cytokeratin is well compatible with high growth rate (Kim *et al.* 1987; Ben-Ze'ev *et al.* 1988; Stoler *et al.* 1988) and, in fact, the differentiated F9 cells still have a relatively high average rate of proliferation.

The present analysis of the cytokeratin expression was based on light microscopy. The same staining technique is also applicable to flow cytometry which is far more sensitive and offers the possibility of detailed analysis of the cell cycle. Our preliminary results by flow cytometry suggest that cytokeratin, in an extractable form, is expressed in a considerable proportion of differentiating F9 cells as early as after 2 days of RA/dbc-AMP treatment. Thus, this kind of a dual parameter analysis is a useful tool in the study of the relationship between cell proliferation and differentiation.

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