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Madin Darby bovine kidney cell synchronization by lovastatin: application to bovine herpesvirus-1 gene expression

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Summary — The number of investigations involving cell proliferation has increased rapidly in the last years. One of the major difficulties in studying cell-cycle-related events is obtaining highly synchronous cell populations without metabolic imbalance. This study demonstrates that the Madin Darby bovine kidney (MDBK) cells, a commonly used cell line in veterinary research, can be effectively synchronized using lovastatin (Lov), a drug used to treat hypercholesteremia in humans. This was demonstrated by the following results: (i) Lov inhibits cell proliferation in a dose-dependent manner; (ii) Lov synchronizes MDBK cells mainly in the G1 and secondarily in the G2+M cell-cycle phases; (iii) the cytostatic effect of Lov can be specifically inhibited by addition of mevalonate (Mev) (Lov inhibits the synthesis of Mev); (iv) removal of Lov from G1-arrested cultures, followed by addition of Mev, resulted in the synchronous recovery of DNA synthesis; and (v) 5-bromo2'-deoxyuridine incorporation experiments revealed that MDBK cells synchronization by Lov can be followed for at least 3 cycles after removal of Lov and addition of Mev. Furthermore, as an application of investigations based on the availability of synchronized MDBK, we showed that bovine herpesvirus-1 gene expression is independent on the cell cycle.

MDBK cells / cell-cycle synchronization / lovastatin / bovine herpesvirus-1

Résumé — Synchronisation des cellules rénales bovines Madin Darby par la lovastatine : application à l'expression des gènes de l'herpèsvirus bovin-1. *Le nombre de recherches impliquant la prolifération cellulaire a augmenté fortement ces dernières années. La difficulté principale rencontrée dans ce type d'étude est l'obtention de populations cellulaires hautement synchronisées sans déséquilibres métaboliques. Les résultats présentés dans cet article démontrent que les cellules bovines*

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rénales Madin Darby (MDBK), une lignée cellulaire couramment utilisé en recherche vétérinaire, peuvent être efficacement synchronisées par la lovastatine (Lov). Les preuves présentées sont les suivantes : i) la Lov inhibe la prolifération cellulaire suivant une relation dose–effet ; ii) la Lov synchronise les cellules MDBK principalement en phase G1 et accessoirement en phase G2 + M du cycle cellulaire ; iii) l'effet cytostatique de la Lov est neutralisé par l'apport exogène d'acide mévalonique (la Lov inhibe la synthèse endogène de l'acide mévalonique) ; iv) les cellules bloquées en phase G1 par la Lov entrent de manière synchrone en phase S après traitement à l'acide mévalonique ; v) des expériences d'incorporation du 5-bromo-2'- deoxy-uridine démontrent que la synchronisation des cellules MDBK par la Lov se maintient pendant au moins 3 cycles cellulaires. Comme application à l'existence d'une méthode de synchronisation efficace des cellules MDBK, nous avons montré que l'expression des gènes de l'herpèsvirus bovin-1 était indépendante du cycle cellulaire.

cellules MDBK / synchronisation cellulaire / lovastatine / herpèsvirus bovin-1

INTRODUCTION

The number of investigations involving cell proliferation has increased rapidly in recent years. These studies can be classified in 3 groups based on their purposes. The first group includes studies of cellular, biochemical, and molecular mechanisms that regulate cell division in normal cells *versus* tumor cells (Blanchard *et al*, 1985; Rasmussen and Means 1989; Jakobisiak *et al*, 1991). The second group involves studies on the effect of foreign cellular events (for example, viral antigen expression, Pritchard *et al*, 1978; Sladek and Jacobbergger, 1992) on the cell cycle. The last group involves studies on the dependence of various foreign cellular events to the cell cycle, for example, some viral infections (Muller and Hudson, 1977; Kraft and Tischer, 1978; Lenghaus *et al*, 1985; Lewis *et al*, 1992, Lewis and Emerman, 1994).

Keyomarsi *et al* (1991) proposed that in order to develop a procedure effective in synchronizing normal or tumoral cells, the following criteria should be met: (i) both normal and tumoral cells should be arrested in the same specific cell-cycle phase; (ii) the metabolic block should target a specific reaction and must be reversible; (iii) the synchronization must be reversible and non-cytotoxic; (iv) large quantities of synchronous cells must be obtained; (v) the synchronization must be cell-density and medium independent; and (vi) synchrony should be maintainable for more than one cycle, and thus appropriate for studying processes that occur in cycling cells as well as immediately after the initial arrest.

Different procedures have been developed for establishing synchronous cultures. Nevertheless, few of them fulfil the requirements described above. Recently, 2 groups reported that these criteria for synchronization have been met using lovastatin (Lov) (Jakobisiak *et al*, 1991; Keyomarsi *et al*, 1991). Lov was shown to be effective for synchronizing reversibly in the G_1 phase normal and tumoral cells from mouse, hamster (Keyomarsi *et al*, 1991) and human origins (Jakobisiak *et al*, 1991; Keyomarsi *et al*, 1991). This drug is used in human medicine to treat hypercholesteremia and inhibits synthesis of mevalonic acid (Mev) by blocking the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. If a drug blocks a specific reaction, exogenous addition of the product of this reaction abolishes the effect of the drug, and therefore the cytostatic effect of Lov is reversed by addition of exogenous Mev (Keyomarsi *et al*, 1991).

Bovine herpesvirus 1 (BHV-1), commonly known as infectious bovine rhinotracheitis virus, belongs to the subfamily *Alphaherpesvirinae*. Herpesvirus genes are expressed in a cascade and are divided into 3 kinetic

classes on the basis of their time of expression during infection *in vitro*. Chronologically, immediate-early (IE), early (E), and late (L) genes are expressed. IE genes are expressed immediately upon infection and do not require prior viral protein synthesis for their expression. Products of IE genes transactivate E and L genes. In addition to IE proteins, viral DNA synthesis is a prerequisite for expression of L genes. Therefore, detection of the late gene product in infected cells indicates that the sequential viral genes expression has been fully completed. The BHV-1 gC gene is expressed as an L gene (Marshall *et al*, 1988).

This study was undertaken in order to develop an effective procedure for synchronizing one of the commonly used cell lines in veterinary research, the Madin Darby bovine kidney (MDBK) cell line. The results presented in this paper demonstrate that Lov can synchronize MDBK cells mainly in the G_1 phase and secondarily in the G_2+M phase of the cell cycle. Moreover, this synchrony is reversible and was followed for at least 3 cycles. Furthermore, as an application based on the availability of synchronized MDBK, we showed that BHV-1 gene expression is independent of the cell cycle.

MATERIALS AND METHODS

Materials

Lov was obtained through the courtesy of Gerda Corbeel (Merck Sharp and Dohme BV, Brussels, Belgium). It was converted from its inactive lactone prodrug form to its active dihydroxy open acid form by first dissolving 20 mg of the lactone in 0.5 ml pure ethanol (heated to 50°C to dissolve the lactone), adding 0.750 ml of 0.1 M NaOH and heating at 50°C for 2 h. The resulting solution was neutralized with 0.1 M HCl to pH 7.2, brought to a volume of 10 ml with culture medium (final concentration 5 mM), and stored in multiple aliquots at −80°C until use.

Virus

The BHV-1 strain used in this study was the Cooper-1 strain (kindly provided by JT van Oirschot, Lelystad, the Netherlands) and was plaque-purified 3 times before use.

Cells and culture conditions

MDBK (ATCC CCL 22) cells were cultured in minimum essential medium (MEM) (GIBCO, Gand, Belgium) containing 5% fetal calf serum (FCS) (GIBCO, Gand, Belgium) as described previously (Vanderplasschen *et al*, 1993). This cell line was maintained free of mycoplasma and of bovine virus diarrhoea virus. To maintain exponential growth, cultures were trypsinized and split at a ratio of 1:6 every 4 d prior to each experiment. Cells were used at passage level 12 to 21.

Synchronization by Lov

MDBK cells (1×10^4) were plated in 24-well cluster dishes (Falcon, Erembodegem, Belgium). Medium was removed 24 h after initial plating and replaced with fresh medium containing Lov with or without Mev at concentrations and for incubation times listed in tables I and II. The cell number (CN) per well, the viability index (VI), and the cell-cycle distribution were then measured. Four measurements from each sample were used to estimate the number of cells. The VI was defined as the percentage of cells excluding trypan blue; about 200 cells were counted for each sample.

To permit the cells arrested in G_1 to enter synchronously the S phase, the medium containing Lov was removed and replaced with fresh medium containing Mev (Sigma, Bornem, Belgium) at a concentration 100 times the Lov concentration use (time 0). Cells were harvested at different times after washing (7, 11, 13, 16, 18, 20, 21 and 23 h) and the cell-cycle distribution was then measured.

Synchronization of cells in G_0 by serum deprivation

The cells were treated as described elsewhere with minor modifications (Keyomarsi *et al*, 1991).

Table I. Effect of Lov and/or Mev on proliferation and viability of MDBK cells.

Lov (μM)	*Mev (μM)*	*Time of incubation (h)*									
		0		*6*		*12*		*24*		*36*	
		CN	*VI*	*CN*	*VI*	*CN*	*VI*	*CN*	*VI*	*CN*	*VI*
0	0	0.54 ± 0.13	99	1.12 ± 0.19	99	1.27 ± 0.15	99	3.60 ± 0.70	99	4.31 ± 0.37	99
0	2 500	0.54 ± 0.13	99	1.20 ± 0.37	99	2.14 ± 0.19	99	3.56 ± 0.62	99	3.64 ± 1.18	95
5	0	0.54 ± 0.13	99	0.90 ± 0.12	99	1.27 ± 0.19	99	1.76 ± 0.37	99	2.14 ± 0.07	91
10	0	0.54 ± 0.13	99	1.43 ± 0.19	99	1.20 ± 0.12	99	1.39 ± 0.31	99	1.46 ± 0.26	85
25	0	0.54 ± 0.13	99	1.20 ± 0.24	99	1.50 ± 0.37	99	1.35 ± 0.47	97	1.27 ± 0.26	83
25	2 500	0.54 ± 0.13	99	1.39 ± 0.58	99	1.42 ± 0.40	99	2.85 ± 0.39	99	4.69 ± 0.68	99
50	0	0.54 ± 0.13	99	0.95 ± 0.19	99	1.31 ± 0.52	99	1.50 ± 0.32	85	1.09 ± 0.26	70

Exponentially growing MDBK cells were incubated at time 0 with Lov (0, 5, 10, 25 and 50 μM) and/or Mev (0 or 2 500 μM). After different time periods (0, 6, 12, 24 and 36 h), the cell number (CN) per well and the viability index (VI) were measured as described in *Materials and methods*. Each reported CN (x 10^4 cells/well) represents the average ± SD for quadruplicate cultures.

Table II. Effect of Lov and/or Mev on the cell-cycle distribution of MDBK cells.

Lov (μM)	*Mev (μM)*	*Incubation (h)*	*Cell cycle distribution (%)*		
			G_0/G_1	*S*	G_2/M
0	0	6	37.3 ± 1.12	44.3 ± 1.49	18.4 ± 1.13
25	0	6	36.4 ± 0.78	42.6 ± 2.56	21.0 ± 1.34
0	2 500	6	39.6 ± 1.20	40.7 ± 2.78	19.8 ± 0.50
25	2 500	6	36.4 ± 2.01	44.2 ± 1.21	19.4 ± 0.85
0	0	12	38.3 ± 1.14	44.7 ± 1.28	17.0 ± 1.14
25	0	12	37.5 ± 1.21	40.8 ± 2.06	21.7 ± 1.27
0	2 500	12	39.7 ± 1.56	41.8 ± 1.35	18.5 ± 1.20
25	2 500	12	37.4 ± 2.64	42.5 ± 2.14	20.1 ± 1.49
0	0	24	39.4 ± 1.70	44.8 ± 2.26	15.8 ± 1.32
25	0	24	70.0 ± 1.35	6.60 ± 2.50	23.4 ± 0.87
0	2 500	24	44.4 ± 2.84	40.4 ± 1.63	15.2 ± 1.21
25	2 500	24	41.6 ± 1.99	41.8 ± 1.78	16.6 ± 1.21

Exponentially growing MDBK cells were incubated at time 0 with Lov (0, 25 μM) and/or Mev (0, 2 500 μM). After different time periods (6, 12 or 24 h), the percentage of the cells in the G0/G1, S, and G2+M phases of the cell cycle was determined. Each reported value represents the average ± SD for duplicate cultures.

At 96 h after plating of cells, the medium (MEM/5% FCS) was removed, cells were washed 3 times with medium alone, and then incubated for 72 h with MEM containing 0.2% FCS. Under these conditions, 97.17% of the cells were arrested in G_0 as determined by the percentage of BrdU negative cells after an incubation period of 15 h with BrdU (duration of 1 cell cycle).

Preparation of nuclei for flow cytometry DNA analysis

To analyse the cell-cycle phase distribution, we applied the technique described by Vindeløv *et al* (1983, 1990) using the Cycle Test kit (Becton-Dickinson, Erembodegem, Belgium) following the manufacturer's recommendations. This method involves dissolving the cell membrane lipids with a nonionic detergent, eliminating the cell cytoskeleton and nuclear proteins with trypsin, digesting the cellular RNA with an enzyme, and stabilizing the nuclear chromatin with spermine. The isolated nuclei were then stained with propidium iodide (PI) and analysed by flow cytometry for their relative DNA content.

Quantitative measurement of the number of divided cells (S phase)

MDBK cells (1 x 10^6) were plated in 175 cm^2 flasks (Falcon, Erembodegem, Belgium). Medium was removed 24 h after the initial plating and replaced with fresh medium containing Lov (25 μM) for 24 h. At time 0, the medium was removed and replaced with fresh medium containing 2 500 μM of Mev. The number of cells in the S phase during a determined time period (1 h) was estimated by measuring the incorporation of 5-bromo-2'-deoxyuridine (BrdU) (Gratzner, 1982; Gray 1985). At various times after removal of Lov (every hour from 9 to 53 h), BrdU (Becton-Dickinson, Erembodegem, Belgium) was added to the culture medium (final concentration 10 μM). After an incubation period of 1 h at 37°C, the cells were rinsed, trypsinized and fixed with 70% ethanol at 4°C for at least 30 min. The cells were centrifuged for 10 min at 800 *g*, resuspended in 1 ml of 2 M HCl/0.5% Triton *X*-100 (v/v) and incubated at room temperature for 30 min. Subsequently, the cells were centrifuged and resuspended in 1 ml of 0.1 M $Na_2B_4O_7$ pH 8.5 to neutralize the acid. After spinning the cells down, the pellet was resuspended in 50 μl of 0.5% Tween 20 (v/v)/1.0%

BSA (W/V) in PBS. The anti-BrdU mouse monoclonal antibody (20 µl per 10^6 cells) (Becton-Dickinson, Erembodegem, Belgium) was then added, and incubated for 30 min in the dark at room temperature. The cells were then centrifuged and resuspended in 50 µl of 0.5% Tween 20 (v/v)/1% BSA (w/v) in PBS containing 1 µg of fluorescein isothiocyanate (FITC)-conjugated F(ab')2 goat anti-mouse IgG (H + L chains) (Becton-Dickinson, Erembodegem, Belgium). After incubating at room temperature for 30 min in the dark, the cells were centrifuged and resuspended in 1 ml of PBS containing 5 µg of PI. The cells were then analysed for green (FITC, relative BrdU content) and red (PI, relative DNA content) fluorescences by flow cytometry.

Detection of BHV-1 gC antigen in MDBK cells

The cells were harvested with trypsin-EDTA, and fixed in cold acetone/PBS (66:34, v/v) for 20 min at 4°C, and then washed with PBS. The cells were incubated (45 min at 37°C) in PBS containing gelatine (0.5 mg/ml) (PBSG) (Sigma), and the appropriate predetermined concentration of anti-BHV-1 gC Mab 1507 kindly provided by GI Letchworth (Marshall *et al*, 1988). After being washed, cells were incubated (30 min at 37C°) with fluorescein isothiocyanate (FITC)-conjugated $F(ab)_2$ goat anti-mouse IgG (H + L chains) (Becton- Dickinson, Erembodegem, Belgium) in PBSG. After a final wash step with PBSG, the cells were resuspended in PBSG and analyzed by flow cytometry.

Flow cytometry analysis

Flow cytometric analysis was performed using a Becton-Dickinson fluorescence-activated cell sorter (Facstar Plus), equipped with an argon laser (ILT air cooled with 100 mW excitation lines at 488 nm). The instrument was calibrated daily using chicken erythrocyte nuclei suspensions. The coefficient of variation of the singlet peak in FL2 area at channel 200 was always lower than 2.2%. Debris were excluded from the analysis by the conventional scatter gating method. Cells or nuclei doublets were excluded from the analysis by using the pulse processor boards (Becton-Dickinson, Erembodegem, Belgium). FITC and PI emission signals were collected by using appropriate filters at 530 (band pass 30) and 575 nm (band pass 26), respectively. Ten-thousand events per sample were collected in list mode, stored, and analysed by the Consort 32 system (Becton-Dickinson, Erembodegem, Belgium). The cell-cycle distribution was determined using the Cell Fit program (Becton-Dickinson, Erembodegem, Belgium). One-color analysis data were depicted by histograms in which the relative number of nuclei appeared on the *y* axis and the intensity of fluorescence (in arbitrary units) appeared on the *x* axis. The bivariate BrdU/DNA (green/red) distributions are displayed in contour plots. The lowest contour in each plot is approximately 1% of the maximum in the distribution and the contours increase by factors of 2 (*eg*, 10, 20, 40, 80, *etc*).

Stastistical analysis

Student's *t* test was used to test for significance of the results ($p < 0.01$ level).

RESULTS

Synchronization by Lov

MDBK proliferation was decreased in cultures containing Lov (table I). After an incubation period of 6 and 12 h, the cell numbers were not significantly different between Lov or Mev treated and untreated cultures. After 24 h, the cell number in culture containing 5 µM of Lov was significantly lower than the cell number in control culture. This cytostatic effect was dose-dependent and inhibited by addition of Mev in concentration 100 times the Lov concentration used. At a concentration of 10 and 25 µM, in the absence of any significant cell death, the cells reached a growth plateau by 24 h. The cytotoxic effect of Lov was significant at a concentration of 50 µM after 24 h of incubation. Every dose tested was significantly cytotoxic after 36-h of incubation. Based on

these preliminary results, the cytostatic effect of Lov was further investigated at the concentration of 25 μM and after a maximal exposure period of 24h.

The cell cycle distribution in cultures exposed to 25 μM of Lov for 6 and 12 h was unchanged in comparison with untreated cultures (table II). However, after 24 h incubation, the percentage of cells in the S phase decreased from 44.8% (untreated cells) to 6.6% (treated cells) concomitantly with an increase of cells in G_1 (from 39.4% to 70.0%) and in G_2 plus M (from 15.8% to 23.4%). In this experiment, as well as in the previous one, exogenous Mev (concentration 100 times the Lov concentration used) was capable of blocking the Lov-induced synchrony when present in conjunction with Lov (fig 1 and table II). As measured by incorporation of BrdU, exposure of MDBK cells to 25 μM of Lov for 24 h resulted in over 90.37% inhibition of DNA synthesis (fig 2, time 0).

Removal of Lov from the G_1–arrested cultures followed by addition of 2 500 μM Mev resulted in the synchronous recovery of DNA synthesis (figs 2–4). DNA synthesis resumed after a 9–10 h lag period and reached a peak after 16 h (figs 2, 3). Eighteen hours after removal of Lov, few cells remained in G_1 (3.8%). Twenty-three hours after removal of Lov, the cell-cycle distribution was similar to that observed after 7 h, indicating that nearly all the cells had synchronously accomplished a complete cellular cycle.

Synchronization of MDBK cells by Lov can be followed for multiple cycles (fig 2). During the 53-h period after removal of Lov and addition of Mev, 3 phases of accelerated BrdU incorporation corresponding to 3 successive S phases of the cell cycle were observed. The maximum percentages of cells incorporating BrdU occurred at 16, 30, and 46 h after Lov removal, and were 87.77%, 80.93% and 68.59% respectively.

Expression of BHV-1 gC glycoprotein in MDBK cells infected at different stages of the cell cycle

In order to know whether BHV-1 gene expression is dependent on the cell cycle, the expression of the L gC BHV-1 glycoprotein was analysed after infection (multiplicity of infection (moi) of 10 plaque-forming units (pfu)/cell) of MDBK cells being at different stages of the cell cycle: G_0 arrested cells; Lov synchronized cells 5 h after removal of Lov; and exponentially growing cells. Seventeen hours after infection the cells were harvested and treated for detection of BHV-1 gC by flow cytometry. The percentage of positive cells was similar in the 3 groups and was near the maximum (table III).

DISCUSSION

One of the major difficulties in studying the cell-cycle-related events has been the unavailability of methods to obtain highly synchronous cell populations without metabolic imbalances. Different approaches have been used to investigate events in cycling cultured cells, including synchronization by centrifugal elutriation (Mitchell and Tupper, 1977), mitotic detachment (Teramisa and Tolmach, 1963), and flow cytometry (Dean *et al*, 1982). However, by using these physical methods, only a modest degree of synchrony can be achieved. On the other hand, some chemical methods, such as double thymidine block, isoleucine deprivation, and treatment with hydroxyurea or aphidicolin, induce a high degree of metabolic imbalance and can have severe cytotoxic effects.

Recently, 2 groups reported that Lov was highly effective in reversibly synchronizing in the G_1 phase, normal and tumoral cells of mouse, hamster and human origins, without modification of the general RNA, pro-

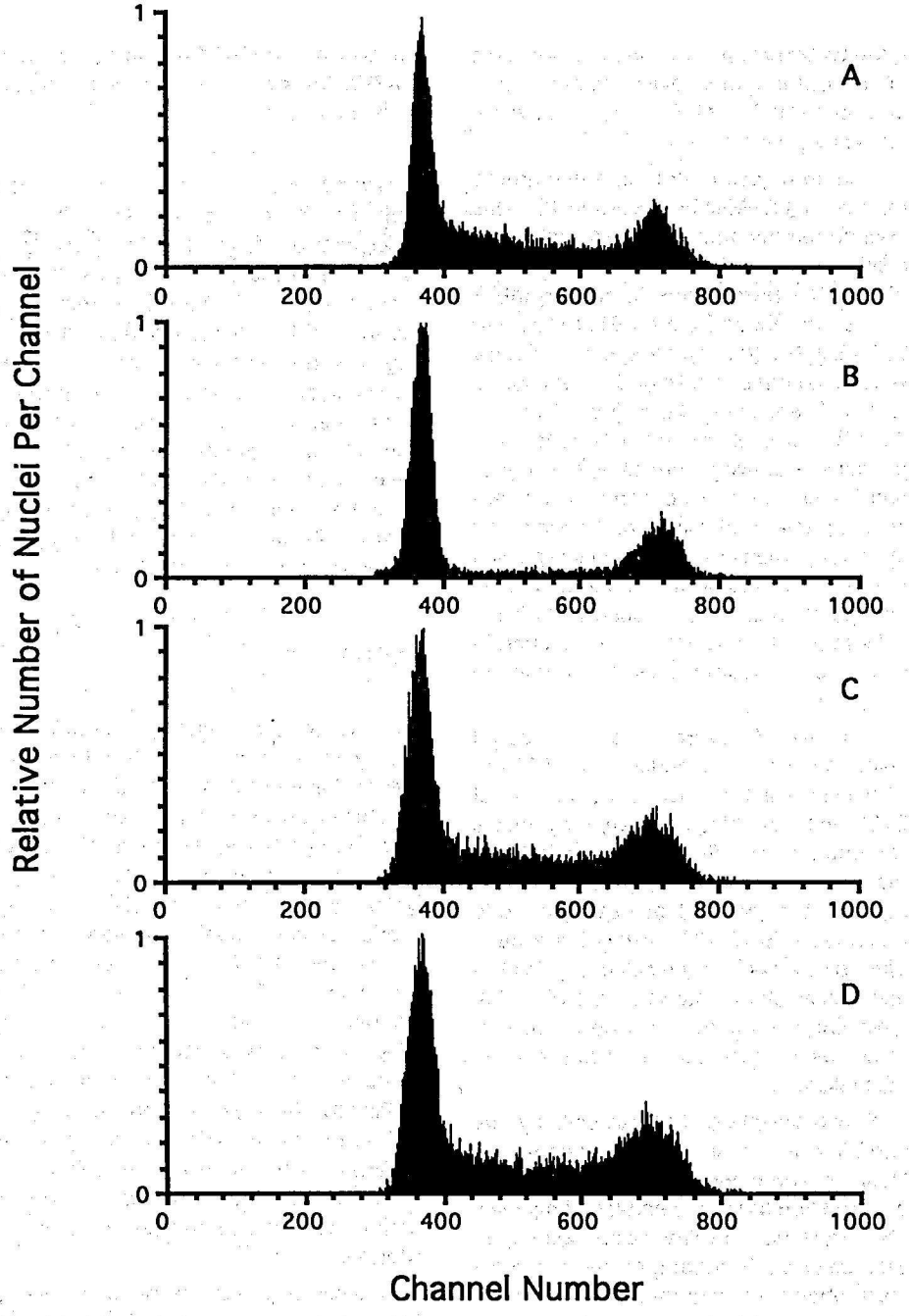

Fig 1. Effect of Lov (0.25 μM) and/or Mev (0, 2 500 μM) on the cell-cycle distribution of MDBK cells. Exponentially growing MDBK cells were incubated with Lov and/or Mev. After an incubation period of 24 h, the cell-cycle distribution was analysed by flow cytometry. From A to D, the concentrations of Lov and Mev (Lov/Mev) were respectively 0/0, 25/0, 0/2 500, and 25/2 500 μM.

tein and DNA metabolisms (Jakobisiak *et al*, 1991; Keyomarsi *et al*, 1991).

This study demonstrates that MDBK cells can also be effectively synchronized using Lov. This was suggested by the following results: (i) Lov inhibits cell proliferation in a dose-dependent manner; (ii) Lov synchronizes MDBK cells in G_1 and secondarily in G_2+M; (iii) the cytostatic effect of Lov can be specifically inhibited by addition of Mev; (iv) removal of Lov from the G_1-arrested cultures, followed by addition of Mev resulted in the synchronous recovery of DNA synthesis; and (v) BrdU incorporation experiments revealed that MDBK cell synchronization by Lov can be followed for at least 3 cycles after removal of Lov and addition of Mev.

Our results show that Lov arrested MDBK cells mainly in the G_1 phase and secondarily in the G_2 + M phase of the cell cycle. The maximal cytostatic effect without any significant cytotoxic effect was obtained after an incubation period of 24 h with a concentration of 25 μM Lov. Under these conditions, 70.0% of the Lov-treated cells were in the G_1 phase in contrast with 39.4% of untreated cells. Because few cells traversed the S phase in the presence of the drug, the rate of entrance into G_2 was decreased, and thus fewer cells were dividing (G_2+M). The presence of a relatively large number of cells in G_2+M phases in Lov-treated cultures (23.4% in contrast with 15.8% in untreated cells) indicated that cell progression through G_2+M was also delayed in the presence of Lov.

If a drug blocks a specific reaction, exogenous addition of the product of this reaction should abolish the effect of the drug. Because cytostatic and cytotoxic effects of Lov were inhibited by exogenous addition of Mev, we could confirm that the effect of Lov is due to its capacity to inhibit Mev synthesis. The role of Mev in cell-cycle regulation is not yet clearly understood. Nevertheless, it seems to be required for the post-translational modification of a number

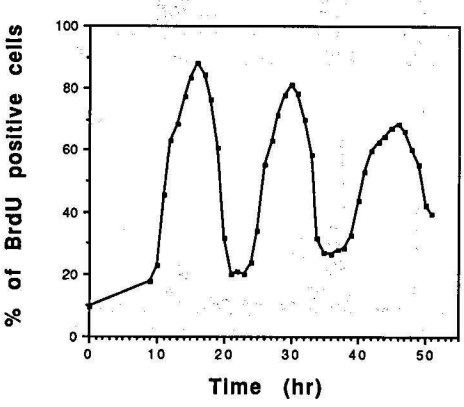

Fig 2. Synchronization of MDBK cells can be followed for 3 cycles. Exponentially growing MDBK cells were incubated with culture medium containing 25 μM of Lov for 24 h. At time 0, cells were extensively rinsed and cultured in medium containing 2 500 μM of Mev. At the indicated time after removal of Lov, the percentage of proliferating cells was estimated by measuring the percentage of cells incorporating BrdU as described in *Materials and methods.*

Table III. BHV-1 gC expression in MDBK cells infected at different stages of the cell cycle.

	% of BHV-1 gC positive cells	
	Infected	*Mock infected*
G_0 arrested	99.27 ± 0.56	0.79 ± 0.23
Synchronized by Lov	98.51 ± 0.13	0.29 ± 0.09
Exponentially growing	97.74 ± 0.89	1.16 ± 0.78

G_0 arrested cells, Lov synchronized cells 5 h after removal of Lov, and exponentially growing cells were infected with BHV-1 at the moi of 10 pfu/cell. Seventeen hours after infection the cells were harvested and treated as described in *Materials and methods* to detect

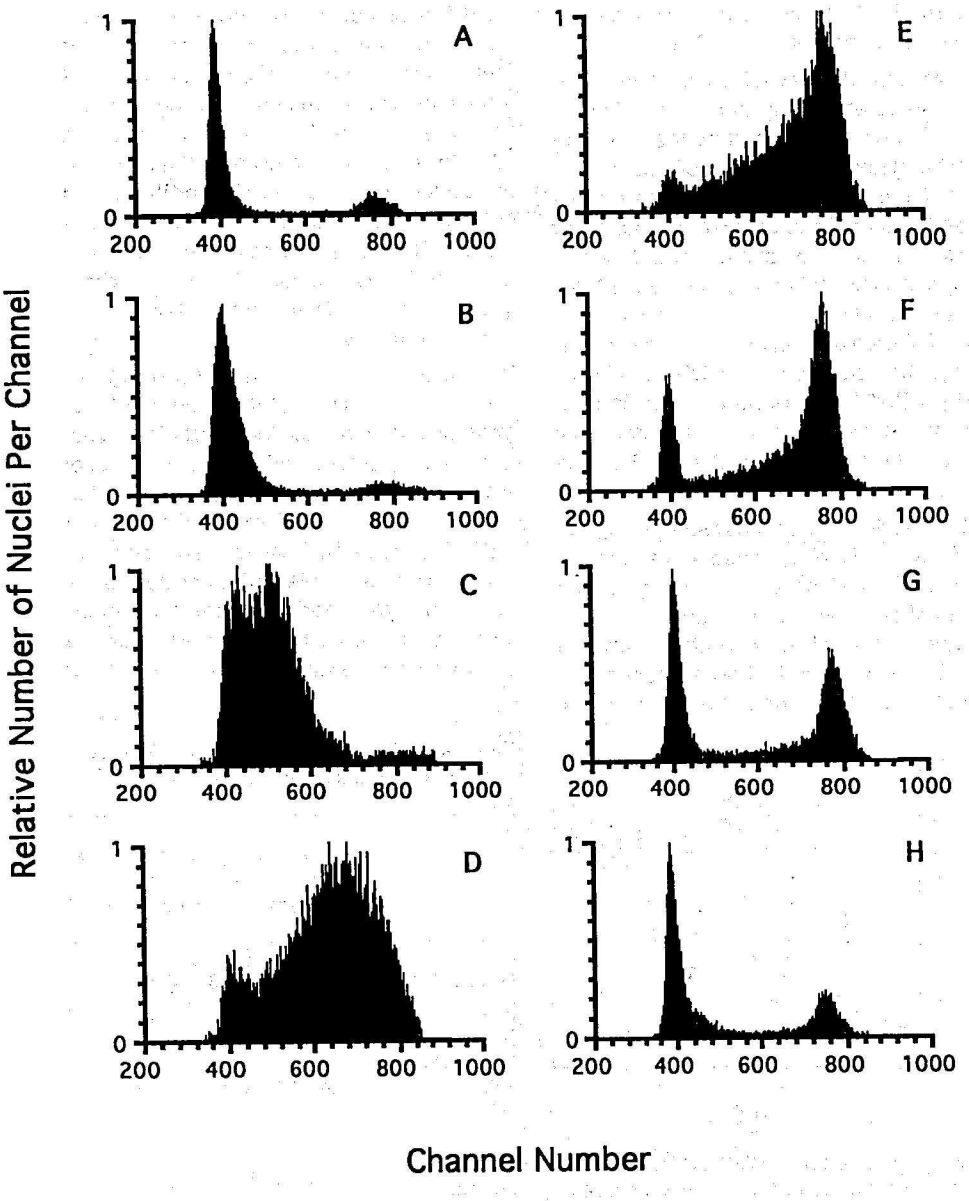

Fig 3. The cell-cycle distribution of MDBK cells progressing through the cell cycle following release from a G_1 phase arrest. Exponentially growing MDBK cells were incubated with culture medium containing 25 μM of Lov for 24 h. At time 0, cells were extensively rinsed and cultured in medium containing 2 500 μM of Mev. The cell-cycle distribution was analysed by flow cytometry at various times after removal of Lov; from A to H (respectively) 7, 11, 13, 16, 18, 20, 21 and 23 h.

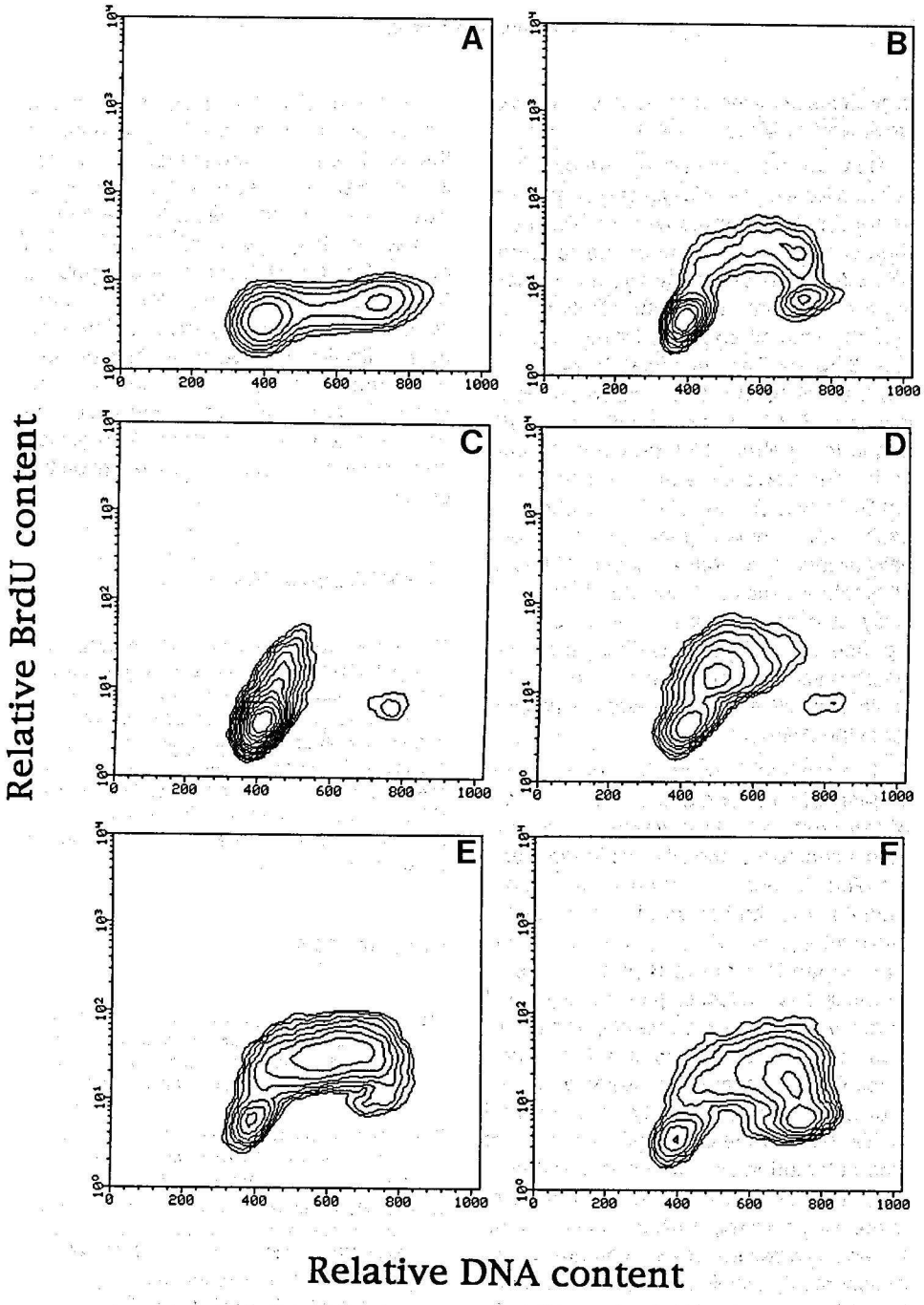

Fig 4. Quantitative measurement of the number of divided cells (S phase). MDBK cells were pulsed with BrdU (1 h at 37°C) followed by staining of DNA (PI) and BrdU (FITC) as described in *Materials and methods*. A. Exponentially growing MDBK cells, which were not pulsed with BrdU (control). B. Exponentially growing MDBK cells, which were pulsed with BrdU 24 h after plating. C–F. Lov synchronized MDBK cells, which were pulsed 9, 13, 16 and 18 h respectively after removal of Lov and addition of Mev.

of proteins involved in cell-cycle regulation (reviewed by Maltese, 1990).

Removal of Lov from the growth-arrested cultures followed by addition of Mev resulted in the synchronous recovery of DNA synthesis. Sixteen hours after the release, 87.77% of the cells did proliferate indicating a high degree of synchronization. Lov synchronization could be followed for at least 3 cycles. The periodic DNA synthesis occurred at 16, 30, 46 h. Based on these data the MDBK cell-cycle duration was estimated to be about 15 h (mean of 14 and 16 h). The percentage of cells incorporating BrdU was progressively reduced with each cycle, suggesting that the cells gradually escape synchrony over time. This loss of synchrony may be explained in 2 ways, firstly, by large variations in the rate of cycle traverse by individual cells during the post-synchronization period, and secondly, by contact inhibition of cells present in the center of the clone.

The replication cycle of herpesviruses is generally independent of the cell cycle of the host cell, but it was nevertheless demonstrated that the DNA replication (and therefore, L gene expression) of murin cytomegalovirus (Muller and Hudson, 1977) and equine herpesvirus 1 (Lawrence, 1971) was dependent of the S phase. L gene expression is the last step in the sequential herpesvirus genes expression, and therefore detection of L product in a cell indicates that viral genes expression and DNA replication has been totally accomplished in this cell. The late BHV-1 gC gene product was detected in cells infected at different stages of the cell cycle. The percentage of expressing cells was similar in the 3 groups tested (G_0 arrested, Lov synchronized, and exponentially growing cells) and was near to 100%. Taken together these results indicated that BHV-1 gene expression and DNA replication are independent of the cell cycle, at least in the model used in this study.

To summarize, this paper describes a procedure that is effective in synchronizing the MDBK cell line, a commonly used cell line in veterinary research. This procedure was cell-density and medium independent and allowed large quantities of synchronous cells to be obtained. Due to the metabolic block of 3-hydroxy-3-methylglutarylcoenzyme A reductase, the synchronization could be specifically reversed by addition of Mev. Furthermore, cell synchronization could be followed for at least 3 cycles. Moreover, we demonstrated that BHV-1 gene expression and DNA replication are independent on the cell cycle.

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REFERENCES

Blanchard JM, Piechaczyk M, Dani C *et al* (1985) c-*myc* gene is transcribed at high rate in Go-arrested fibroblasts and is post-transcriptionally regulated in response to growth factors. *Nature (Lond)* 3, 443-445

Dean PN, Gray JW, Dolbeare PA (1982) The analysis and interpretation of DNA distributions measured by flow cytometry. *Cytometry* 3, 188-195

Gratzner HG (1982) Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication. *Science* 218, 474-475

Gray JW (1985) Monoclonal antibodies against bromodeoxyuridine. *Cytometry* 6, 501-673

Jakobisiak M, Bruno S, Skierski JS, Darzynkiewicz Z (1991) Cell cycle-specific effects of lovastatin. *Proc Natl Acad Sci USA* 88, 3628-3632

Keyomarsi K, Sandoval L, Band V, Pardee AB (1991) Synchronization of tumor and normal cells from G1

to multiple cell cycles by lovastatin. *Cancer Res* 51, 3602-3609

Kraft V, Tischer I (1978) Cell-cycle dependent multiplication of avian adenoviruses in chicken embryo fibroblasts. *Arch Virol* 57, 243-254

Lawrence WC (1971) Evidence for a relationship between equine abortion (herpes) virus deoxyribonucleic acid synthesis and the S phase of the KB cell mitotic cycle. *J Virol* 7, 736-748

Lenghaus C, Mun TK, Studdert MJ (1985) Feline panleukopenia virus replicates in cells in which cellular DNA synthesis is blocked. *J Virol* 53, 345-349

Lewis P, Hensel M, Emerman M (1992) Human immunodeficiency virus infection of cells arrested in the cell cycle. *EMBO J* 11, 3053-3058

Lewis PF, Emerman M (1994) Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J Virol* 68, 510-516

Maltese WA (1990) Posttranslational modification of proteins by isoprenoids in mammalian cells. *FASEB J* 4, 3319-3328

Marshall RL, Israel BA, Letchworth GJ (1988) Monoclonal antibody analysis of bovine herpesvirus-1 glycoprotein antigenic areas relevant to natural infection. *Virology* 165, 338-347

Mitchell BF, Tupper JT (1977) Synchronization of mouse 3T3 and SV40 3T3 cells by way of centrifugal elutriation. *Exp Cell Res* 106, 351-355

Muller MT, Hudson JB (1977) Cell-cycle dependency of murin cytomegalovirus replication in synchronized 3T3 cells. *J Virol* 22, 267-272

Pritchard C, Bates RC, Stout ER (1978) Levels of cellular DNA polymerases in synchronized bovine parvovirus-infected cells. *J Virol* 27, 258-261

Rasmussen CD, Means AR (1989) Calmodulin is required for cell-cycle progression during G_1 and mitosis. *EMBO J* 8, 73-82

Sladek TL, Jacobbergger JW (1992) Simian virus 40 large T-antigen expression decreases the G1 and increases the G2+M cell-cycle phase durations in exponentially growing cells. *J Virol* 66, 1059-1065

Terasima T, Tolmach LJ (1963) Growth and nucleic acid synthesis in synchronously dividing populations of HeLa cells. *Exp Cell Res* 30, 344-362

Vanderplasschen A, Bublot M, Dubuisson J, Pastoret PP, Thiry E (1993) Attachment of the gamma-herpesvirus bovin herpesvirus 4 is mediated by the interaction of gp8 glycoprotein with heparin-like moieties on the cell surface. *Virology* 196, 232-240

Vindeløv LL, Christensen IJ, Nissen NI (1983) A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* 5, 323-327

Vindeløv LL, Christensen IJ (1990) A review of techniques and results obtained in one laboratory by an integrated system of methods designated for routine clinical flow cytometric DNA analysis. *Cytometry* 11, 753-770