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The Replication *in Vitro* of the Gammaherpesvirus Bovine Herpesvirus 4 Is Restricted

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Because several observations have suggested that replication of the gammaherpesvirus bovine herpesvirus 4 (BHV-4) is influenced by the physiological state of the host cell, a study was carried out to determine the relationship between BHV-4 infection and the cell cycle. The temporal expression of BHV-4 late (L) proteins in unsynchronized cell cultures was first investigated by flow cytometry. Interestingly, L protein expression occurred in a limited number of cells infected with a high multiplicity of infection, and a reciprocal correlation between the percentage of positive cells and the cell density at the time of infection was demonstrated. Moreover, the finding that a BHV-4 early–late protein was expressed in nearly all the cells suggested that a blockage in the viral replication cycle occurred in some infected cells at the stage of viral DNA synthesis or L protein expression. Because this blockage could be the consequence of the dependence of one or both of these events on the cell cycle, they were investigated after infection of synchronized cell cultures. The following findings were made. (i) Cell transition through the S phase quantitatively increased the rate of BHV-4 DNA replication. (ii) BHV-4 DNA synthesis could not be detected in cells arrested in G_0. (iii) Synchronization of MDBK cells with Lovastatin before infection increased the percentage of cells expressing L proteins. (iv) In contrast, infection of cells arrested in G_0 led to few positive cells. Taken together these results showed that BHV-4 DNA replication and consequently the expression of L proteins are dependent on the S phase of the cell cycle. This dependence could be of importance for several biological properties of BHV-4 infection *in vitro* and might have implications for the biology of the virus *in vivo.*

INTRODUCTION

Bovine herpesvirus type 4 (BHV-4) is one of the four known bovine herpesviruses. The other three, belonging to the *Alphaherpesvirinae,* are bovine herpesviruses type 1 (BHV-1), type 2 (BHV-2), and type 5 (BHV-5) (Roizman *et al.,* 1992), which cause infectious bovine rhinotracheitis/pustular vulvovaginitis, bovine herpes mammillitis, and bovine encephalitis, respectively (Gibbs and Rweyemamu, 1977a,b). BHV-4 was first isolated by Bartha *et al.* (1966). It has been isolated both from cattle showing various clinical symptoms and from healthy cattle (Thiry *et al.,* 1989). It was initially called bovine cytomegalovirus and was classified as a betaherpesvirus based on its biological characteristics (Storz *et al.,* 1984). However, Bublot *et al.* (1992) have shown, based on molecular data, that BHV-4 belongs to the *Gammaherpesvirinae.*

The expression of herpesvirus proteins is temporally regulated. The proteins are classified into three kinetic classes depending on the order of their synthesis during *in vitro* infection (Honess and Roizman, 1974, 1975). They are expressed chronologically as immediate-early (IE or *alpha*), early (E or *beta*), and late (L or *gamma*) proteins. Immediate-early proteins are expressed directly after release of the viral genome from the capsid into the nucleus (Costanzo *et al.,* 1977). While E protein expression occurs after synthesis of IE proteins, L protein expression depends on the expression of both IE and E proteins and viral DNA synthesis.

In contrast to some simpler viruses, replication of most herpesviruses is generally assumed to be independent of the stage of the host cell in the cell cycle (Cohen *et al.,* 1971; DeMarchi and Kaplan, 1976; Knipe, 1990; Roizman, 1990; Shadan *et al.,* 1994). Nevertheless, such dependence has been described at least in some specific cell lines for the alphaherpesvirus equine herpesvirus 1 (EHV-1) (Lawrence, 1971) and for the betaherpesvirus murine cytomegalovirus (MCMV) (Muller and Hudson, 1977). The possibility that BHV-4 replication could be dependent on the cell cycle was suggested independently by two groups. First, Potgieter and Maré (1974) showed that there were marked differences in the kinetics of BHV-4 plaque formation between confluent cells and actively growing cells. The time at which plaques developed was reduced from 10–12 days to 5–6 days postinfection when confluent cells and freshly seeded

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cells were infected, respectively. Second, Dubuisson *et al.* (1988) observed a second peak in the intracellular viral production curve in freshly seeded cells, but not in confluent cells, despite the multiplicity of infection (m.o.i.) of 7 PFU/cell used.

One of the major difficulties in studying events related to the cell cycle is obtaining highly synchronized cell populations without metabolic imbalance. Recently, it has been demonstrated that Lovastatin (Lov) is effective in reversibly synchronizing cells from different origins in the G_1 phase (Jakobisiak *et al.,* 1991; Keyomarsi *et al.,* 1991; Vanderplasschen *et al.,* 1994). This drug blocks cells in the G_1 phase by inhibiting the synthesis of cellular mevalonic acid (Mev). The cytostatic effect of Lov can be reversed by its removal and the simultaneous addition of Mev.

Because several observations have suggested that BHV-4 replication is influenced by the physiological state of the host cell, we have investigated the relationship between BHV-4 infection and the host cell cycle. This study demonstrates that BHV-4 DNA synthesis and consequently L protein expression are dependent on the S phase of the cell cycle. This dependence could be of importance for several biological properties of BHV-4 infection *in vitro* and might have implications for the biology of the virus *in vivo.*

MATERIALS AND METHODS

Virus strains and cell cultures

Madin Darby bovine kidney (MDBK) (American Type Culture Collection CCL 22) and Georgia bovine kidney (GBK) cell lines and Bovine skin (BSC) and Bovine testicle (BTC) primary cell cultures were used in this study. The cells were cultured in minimum essential medium (MEM) (GIBCO, Gent, Belgium) containing 5% fetal calf serum (FCS) (GIBCO), heat inactivated at 56° for 30 min, and tested for the absence of antibodies against BHV-1 and BHV-4. Cells were used between passage levels 12 and 22 (1:3 split ratio). The BHV-4 V. Test strain isolated from a bull with orchitis (Thiry *et al.,* 1981) and the BHV-1 Cooper strain (kindly provided by Dr. J. T. van Oirschot, Lelystad, The Netherlands) were used in this study. A BHV-4 recombinant strain designated BHV-4 B3 lin (kindly provided by Dr. M. Goltz, Berlin, Germany) was also used. This strain carries the *Escherichia coli* β-galactosidase gene under the control of the human cytomegalovirus IE gene promotor/enhancer (MacGregor and Caskey, 1989). The cassette was integrated into a sequence between two ORFs at the left end of the unique coding part of the BHV-4 strain 66-p-347 genome (Storz, 1968). In MDBK and GBK cells infected with the BHV-4 B3 lin strain β-galactosidase is expressed as an IE antigen (Dr. M. Goltz, personal communication). For infection of cells, virus semipurified from the growth supernatant of infected cells, as described previously, (Vanderplasschen *et al.,* 1993b) was diluted in a medium identical to that used for mock-infected cells.

Monoclonal antibodies

The monoclonal antibodies (Mabs) used in this study were Mab 35 and Mabs 123, 113, and 29, which recognize the early–late (E-L) glycoprotein complex gp6/gp10/gp17 (Dubuisson *et al.,* 1991) and the L glycoproteins gp1 (Dubuisson *et al.,* 1992b), gp8 (Dubuisson *et al.,* 1992a), and gp11/vp24 (Dubuisson *et al.,* 1989), respectively. The E-L classification of the glycoprotein complex gp6/gp10/gp17 is based on the fact that a precursor of two components (gp10/gp17) is already expressed in the early phase, whereas the mature form (gp6/gp10/gp17) is expressed in the L phase (Dubuisson *et al.,* 1991). Mab 35 recognizes both the precursor and the mature forms and was therefore used as a marker of E-L protein expression (Dubuisson *et al.,* 1991).

Mab 1507 raised against BHV-1 gC glycoprotein (expressed as a L protein) was kindly provided by Dr. Letchworth (Marshall *et al.,* 1986; Ludwig and Letchworth, 1987) and anti-β-galactosidase Mab was purchased from Boehringer-Mannheim (Brussels, Belgium).

Fixation and immunological staining of cells for flow cytometry

Adherent cells were harvested with trypsin–EDTA, fixed in cold acetone/phosphate-buffered saline (PBS) (66:34, v/v) (PBS: 3 m*M* KCl, 1.5 m*M* KH_2PO_4, 0.14 *M* NaCl, 6.5 m*M* Na_2HPO_4, pH 7.2) for 20 min at 4°, and then washed with PBS. About 10^6 cells in PBS containing gelatine (PBSG) (0.05 mg/ml) and the appropriate predetermined concentration of Mab were incubated at 37° for 45 min. Cells were washed and further incubated with fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat anti-mouse IgG (H + L chains) (1 μg/10^6 cells per 0.2 ml PBSG) (Becton–Dickinson, Erembodegem, Belgium) at 37° for 30 min. Cells were then washed with PBSG and analyzed by flow cytometry.

Cell killing (CKA) and infectious center (ICA) assays

MDBK monolayers grown to confluence in 24-well cluster dishes were infected with BHV-4 V. Test strain at a m.o.i. of 10 PFU/cell in MEM containing 2% FCS. The cells were removed with trypsin–EDTA 6 hr postinfection, a time at which no infectious cell-associated progeny virus could be detected (Augsburger and Metzler, 1989). Three hundred infected or mock-infected cells (control) or three hundred cells from a mixture (1/1) of infected and mock-infected cells were sorted into 175-cm^2 flasks (Becton–Dickinson). The sorting was performed in a fluorescence-activated cell sorter (Automatic Cell Deposit Unit—Facstar Plus, Becton–Dickinson). For the cell killing assay, the cells were sorted in flasks containing MEM with 5% FCS taken from a day-old expo-

nentially growing culture. Twelve hours after plating of cells, heparin (176 U/mg) (Sigma, Bornem, Belgium) was added to the growth medium (final concentration of 50 μg/ml) in order to inhibit attachment of extracellular virions (Vanderplasschen *et al.,* 1993b). After 5 days, the colonies were fixed and stained with an aqueous alcoholic solution containing 0.3% crystal violet for quantification by light microscopy. For the infectious center assay, the cells were sorted in flasks containing subconfluent MDBK monolayers (7.8×10^4 cells/cm^2). Twelve hours after plating of cells, the initial medium was replaced with MEM containing 5% FCS and 0.6% carboxymethylcellulose (Sigma). Plaques were counted 9 to 10 days later. The percentage of infected cells for the cell killing assay was calculated as

$$\left(1 - \frac{\text{number of colonies obtained after sorting of 300 infected cells}}{\text{number of colonies obtained after sorting of 300 mock-infected cells}}\right) \times 100.$$

The percentage of infected cells for the infectious center assay was calculated as

$$\frac{\text{number of plaques obtained after sorting of 300 infected cells}}{300} \times 100.$$

Facs-β-galactosidase assay

The β-galactosidase activity in cells infected with the BHV-4 B3 lin was revealed by the procedure described by Nolan *et al.* (1988). Briefly, β-galactosidase activity was detected by the analysis of cells loaded with the fluorogenic substrate fluorescein di-β-D-galactopyranoside (FDG) (Sigma), using a short hypotonic shock. Hydrolytic cleavage of FDG by β-galactosidase frees fluorescein that is locked inside the cells kept on ice.

Synchronization of MDBK by Lovastatin

MDBK cells were synchronized by Lov (Merck Sharpe & Dohme, Brussels, Belgium) as described elsewhere (Vanderplasschen *et al.,* 1994). Briefly 1×10^6 MDBK cells in an exponential growth phase were plated in 175-cm^2 flasks (Becton–Dickinson) containing MEM with 5% FCS. Twenty-four hours following initial plating, medium was removed and replaced with fresh medium containing 25 μM Lov. To allow the cells arrested in G_1 to enter the S phase synchronously, medium containing Lov was removed 24 hr later and replaced with fresh medium containing 2.5 m*M* Mev (Sigma).

Synchronization of MDBK cells in G_0 by serum deprivation

MDBK cells were treated as described by Keyomarsi *et al.* (1991) with some minor modifications. Ninety-six hours after plating, cells were washed three times with medium alone and incubated in MEM containing 0.2% FCS for 72 hr at 37°. During virus infection, cells arrested in G_0 were maintained under conditions of serum starvation.

Preparation of nuclei for analysis of DNA by flow cytometry

The technique described by Vindeløv *et al.* (1983, 1990) using the Cycle Test kit (Becton–Dickinson) was used to determine the cell cycle phase distribution, which was then analyzed by the Cell Fit program (Becton–Dickinson).

Quantitative measurement of the number of dividing cells in the S phase

The number of cells going through the S phase per hour was estimated by measuring the incorporation of 5-bromo-2′-deoxyuridine (BrdU) as previously described by Gratzner (1982).

Flow cytometric 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 line at 488 nm). Debris were excluded from the analysis by the conventional scatter gating method. FITC and propidium iodide emission signals were collected by using appropriate filters at 530 nm (band pass 30 nm) and 575 nm (band pass 26 nm), respectively. In most cases, 10,000 events per sample were collected in a list mode fashion, stored, and analyzed by a Consort 32 system (Becton–Dickinson). The threshold of positivity for fluorescence intensity was arbitrarily set, based on the negative control sample.

Extraction and purification of DNA

DNA from infected and mock-infected cells was extracted and purified as described elsewhere (Muller and Hudson, 1977).

Viral DNA synthesis after infection of MDBK cells at different stages of the cell cycle

The amount of viral DNA in 100 ng of total DNA extracted from infected cells was determined by dot-blot hybridization. To allow comparison, the inoculum used for each infection was calculated such that the m.o.i. used would give the same stoichiometry of viral and cellular DNA at the time of infection. For example, if a pure culture in G_1 is infected with a m.o.i. of *X* PFU/cell, then a pure culture in $G_2 + M$ must be infected with a m.o.i. of 2*X* (i.e., one cell in $G_2 + M$ contains double the amount of DNA in cells in G_1). In our experiments, cell cultures were infected at the ratio of 5 PFU/G_1 cellular DNA con-

tent. The inoculum for each infection was therefore calculated as follows: PFU/culture = number of cells × 5 PFU × (1 × % G_1, G_0/100 + MR × % S/100 + 2 × % G_2 + M/100), in which MR represents the mean relative DNA content of the S phase population. Total DNA was extracted 2, 9, and 14 hr after infection. DNA (100 ng/sample) was denatured by the addition of 1/10 volume of 3 *M* NaOH. After 5 min at room temperature, the solution was neutralized by the addition of 2 *M* ammonium acetate at a volume equal to the total sample volume. The samples were then applied to a nylon membrane (Hybond-N, Amersham, Gent, Belgium) using a 96-well dot-blot manifold (Bio-Rad, Richmond, CA). After air drying, the immobilized samples were fixed to the membrane by UV light irradiation (short wave) for 30 sec. The cloned *Eco*RI K fragment of the BHV-4 V. Test strain (Bublot *et al.,* 1990) and the cloned *Hin*dIII K fragment of the BHV-1 Cooper strain (Mayfield *et al.,* 1983) were used as probes. These cloned fragments were labeled with [α-^{32}P]dCTP by the random primer labeling method using a commercial kit (Boehringer-Mannheim). Conditions for hybridization were similar to those described previously (Vanderplasschen *et al.,* 1993a). Filters were dried and the radioactivity was measured in a liquid scintillation counter.

Statistical analysis

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

RESULTS

Expression of BHV-4 L proteins in infected cells

The ability of MDBK, GBK, BSC, and BTC cells to support BHV-4 or BHV-1 L protein expression was first investigated. Confluent monolayers were inoculated at different m.o.i. with the BHV-4 V. Test strain or the BHV-1 Cooper strain. The percentage of cells expressing L proteins was then determined by flow cytometric analysis.

Preliminary experiments showed that the highest percentage of MDBK cells expressing BHV-4 L proteins was observed at around 44 hr after infection (data not shown). However, only a fraction of the BHV-4-inoculated MDBK cells expressed detectable L proteins irrespective of the high m.o.i. tested (Table 1), e.g., 21% were gp1 positive (Mab 123) 44 hr after infection at a m.o.i. of 10 PFU/cell (Table 1). The results obtained with the other susceptible cells (GBK, BSC, and BTC) were qualitatively similar to those obtained with MDBK cells (Table 1). Moreover, staining with Mabs 29 and 113 raised against other BHV-4 L proteins (gp11/vp24 and gp8, respectively) gave quantitatively similar results to those obtained with Mab 123 (data not shown).

These experiments were independently repeated four times with MDBK cells, with each replication leading to an identical conclusion. However, different percentages ($P < 0.01$) of cells positive for BHV-4 L protein expression were observed between independent experiments, ranging from 18.5 to 34.6% (data not shown). These percentages (*Y*) were observed to correlate with the cell density (*X*) at the time of infection, i.e., the percentage of cells expressing BHV-4 L proteins decreased with increasing cell density ($Y = 105.93 - 15.027 \times \mathrm{Log}(X)$, $R^2 = 0.998$).

After an incubation period of at least 17 hr, the percentage of BHV-1 gC-positive MDBK, GBK, BSC, and BTC cells was nearly maximum and similar for the three m.o.i. tested (Table 1).

Cell killing and infectious center assays

The detection of only a fraction of cells expressing BHV-4 L proteins despite the high m.o.i. of 10 PFU/cell used for infection was rather surprising (Table 1). The cell killing and infectious center assays were chosen to investigate this observation further, as it was thought that either only a fraction of the cells were infected or all the cells were infected but only a fraction of them expressed detectable BHV-4 L proteins under these cell culture conditions.

Percentages of MDBK cells (3.4×10^5 cell/cm^2) infected with the BHV-4 V. Test strain (m.o.i. 10 PFU/cell) as determined by the cell killing and infectious center assays were 98.8 and 96.4%, respectively (Table 2). The results obtained with the mixture (1/1) of infected and mock-infected cells assured us that there was no secondary infection of the cells during and/or after sorting (Table 2).

Although only a fraction of confluent infected cells expressed detectable BHV-4 L proteins, nearly all of the cells were shown to be infected. This observation implies a probable arrest or delay of the viral replication cycle in some cells of the monolayer, either during or at a stage before the L protein expression (this includes stages of attachment, entry, decapsidation, IE and E protein expression, DNA replication, or L protein expression).

In order to determine if this arrest or delay occurred at an early stage of the viral replication cycle (attachment, entry, or decapsidation), the expression kinetics of β-galactosidase (expressed as an IE antigen) in MDBK cells (4.2×10^5 cell/cm^2) infected with the BHV-4 B3 lin strain was investigated by a Facs-β-galactosidase assay (Fig. 1). The results showed that β-galactosidase expression in infected cells occurred synchronously in virtually all of the cells of the monolayer. Seven hours after infection 98.5% of the cells expressed β-galactosidase (Fig. 1). The control, which contained an equal proportion of infected and mock-infected cells, gave 54.5% positive cells, indicating that there was no contamination of negative cells with leaked fluorescein generated by β-galactosidase-positive cells (Fig. 1F).

Since results of L protein and β-galactosidase expres-

TABLE 1

Percentages of L Proteins Expressing Cells after Infection at Different m.o.i. with BHV-4 or BHV-1[a,b]

	m.o.i.	MDBK	GBK	BSC	BTC
Detection of BHV-4 gp1 (Mab 123)	0	0.29 ± 0.13	0.22 ± 0.08	0.37 ± 0.11	1.43 ± 0.29
	0.1	1.31 ± 0.35	8.66 ± 0.92	ND[c]	ND
	1	8.01 ± 0.61	16.4 ± 0.19	18.01 ± 0.91	14.50 ± 0.70
	10	21.45 ± 0.81	24.57 ± 0.49	31.14 ± 1.13	28.17 ± 1.01
Detection of BHV-1 gC (Mab 1507)	0	1.71 ± 0.32	0.79 ± 0.31	1.24 ± 0.17	1.89 ± 0.03
	0.1	99.18 ± 0.17	98.49 ± 0.41	ND	ND
	1	99.39 ± 0.07	98.67 ± 0.16	99.75 ± 0.07	99.40 ± 0.78
	10	99.15 ± 0.11	99.81 ± 0.04	ND	99.08 ± 0.19

[a] Each reported value represents the average ± SD for triplicate cultures.

[b] Confluent monolayers of MDBK, GBK, BSC, and BTC cells in six-well cluster dishes were inoculated with the BHV-4 V. Test strain or with the BHV-1 Cooper strain. After an incubation period (44 and 17 hr for BHV-4- and BHV-1-infected cells, respectively), the cells were harvested and treated for immunological detection of BHV-4 gp1 (Mab 123) or BHV-1 gC (Mab 1507), followed by flow cytometric analysis.

[c] Not done.

sion were obtained with two different strains of BHV-4 (V. Test and B3 lin strains, respectively), analysis of L protein expression for the BHV-4 B3 lin strain was also carried out. The highest percentage of cells expressing BHV-4 L gp1 protein was observed at around 42 hr after infection. At this time, 91 and 30% of cells were positive for β-galactosidase (immunological staining) and gp1 expression, respectively (Fig. 2).

In order to characterize further the rate-limiting step in the replication cycle of BHV-4, monolayers of MDBK cells (6×10^4 cell/cm^2) infected with BHV-4 (V. Test strain, m.o.i. 10 PFU/cell) were harvested and stained with Mabs 35 and 123. Of the infected cells 93% were positive for gp6/gp10/gp17 in contrast to 31% of the gp1-positive cells (Fig. 3). The distribution of cells positive for gp6/gp10/gp17 was in two populations, in which 34% of the cells presented a bright fluorescence and 58% had a dim fluorescence (Fig. 3D). The results suggest that E proteins (at least for the E-L gp6/gp10/gp17 protein complex) were expressed in nearly all of the cells, whereas the expression of L proteins was only in a fraction of the infected cells. It therefore seems that the viral replication cycle was blocked at a stage after E protein expression. This includes stages of DNA replication or L protein expression. Because this blockage could be the consequence of the dependence of one or both of these events on the cell cycle, they were further investigated in synchronized cells infected at different stages of the cell cycle. The Lov and serum deprivation methods were used for cell synchronization. The same stocks of synchronized MDBK cells were used to study concurrently: (i) the synchronization of mock-infected cells (cell cycle distribution and BrdU incorporation), (ii) viral DNA replication, and (iii) expression of L and E-L proteins after infection at different stages of the cell cycle.

Synchronization of MDBK cells by Lovastatin and by serum deprivation

The synchronization of mock-infected cells by Lov is illustrated in Figs. 4A and 6. It was noted that (i) cells treated with Lov for 24 hr (25 μM) were reversibly blocked primarily in G_1 (65%) and secondarily in G_2 + M (28%) cell cycle phases (Fig. 4A, time 0; Fig. 6A); (ii) removal

TABLE 2

Determination of the Percentage of Infected Cells by a Cell Killing Assay and an Infectious Center Assay[a]

	CKA (colonies)	ICA (plaques)	Percentage of infected cells: CKA	Percentage of infected cells: ICA
Mock-infected cells	269.3 ± 12.9	0	0	0
BHV-4-infected cells[b]	3.3 ± 3.2	289.3 ± 5.0	98.8	96.4
Mixture (1/1) of infected and mock-infected cells[b]	139.3 ± 12.7	153.7 ± 13.2	48.3	51.2

[a] Each reported value represents the average ± SD for triplicate cultures.

[b] MDBK cells were infected at a m.o.i. of 10 PFU/cell with the BHV-4 V. Test strain. After an incubation period of 6 hr, the percentage of infected cells was determined by CKA and ICA as described under Materials and Methods.

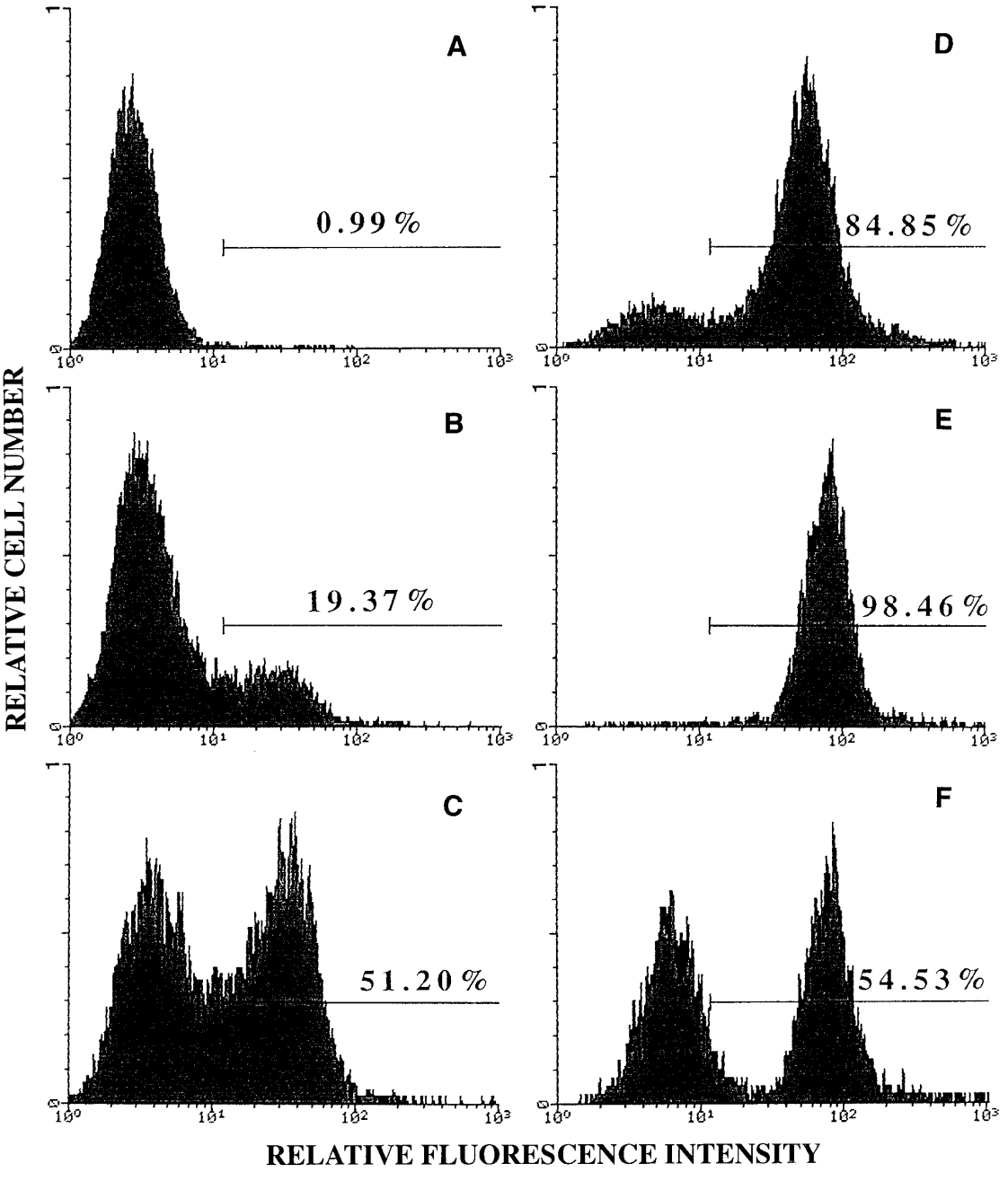

FIG. 1. Expression of β-galactosidase in MDBK cells infected with the BHV-4 B3 lin strain. Monolayers of MDBK cells were infected with the BHV-4 B3 lin strain at a m.o.i. of 10 PFU/cell. After different time periods (1, 3, 5, and 7 hr), the cells were harvested and treated for quantification of β-galactosidase-expressing cells by a Facs-β-galactosidase assay. (A) Mock-infected cells; (B to E) cells harvested 1, 3, 5, and 7 hr after infection, respectively; and (F) a mixture (1/1) of mock-infected and infected (7 hr postinfection) cells. The percentage of positive cells is indicated in each panel.

of Lov from the G_1-arrested cultures, followed by addition of Mev (2.5 m*M*), resulted in the synchronous recovery of DNA synthesis after a 5- to 10-hr lag period and reached a peak after 15 hr. At this time 85% of the cells were proliferating as shown by the BrdU incorporation experiment (cells were pulse labeled during 1 hr) (Figs. 4A and 6C); (iii) 20 hr after Lov removal and Mev addition, 72% of the cells were in G_2 + M phases, although 11% of the cells remained in S phase (Fig. 4A); and (iv) synchronization could be followed to at least a second cell cycle. Thirty hours after removal of Lov, a second peak of BrdU-incorporating cells was observed (68% of the cells) (data not shown).

Following the G_0 synchronization procedure, 94% of the cells were arrested in G_0, as determined by the percentage of BrdU-negative cells after an incubation period of 15 hr (a duration of one cell cycle) (Vanderplasschen *et al.,* 1993a). When G_0-synchronized cells were maintained under condi-

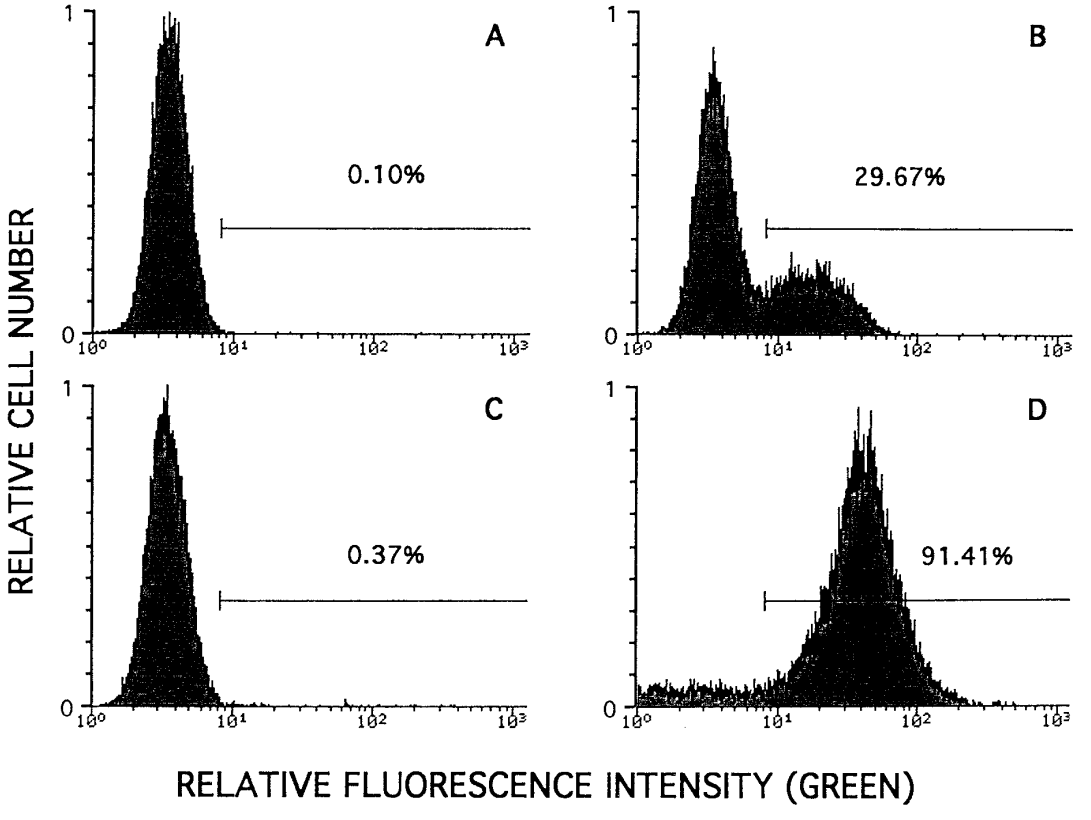

FIG. 2. Quantification of BHV-4 gp1 (A and B)- and β-galactosidase (C and D)-expressing cells 42 hr after infection with the BHV-4 B3 lin strain (m.o.i. 10 PFU/cell). A and C represent mock-infected cells; B and D represent infected cells. The percentage of positive cells is indicated in each panel.

tions of serum starvation for an additional period of 44 hr, the amount of cells arrested in G_0 increased to 97%.

BHV-4 DNA replication in Lov-synchronized, G_0-arrested, and randomly growing MDBK cells

The rate of the BHV-4 DNA synthesis was compared in synchronized MDBK cells infected at different stages of the cell cycle by a Southern dot blot assay. Preliminary experiments with MDBK-synchronized and unsynchronized cell cultures showed that at 2 hr postinfection BHV-4 and BHV-1 DNA replication could not be demonstrated by the dot blot assay used (data not shown). Therefore, the amount of viral DNA detected at this time of infection represents the amount of virus taken up.

The results in Fig. 4B show that the uptake of BHV-4 was similar among cells infected at different stages of the cell cycle. However, the stage of the cells in the cell cycle during the infection period had an effect on the rate of BHV-4 DNA synthesis. Experiments performed with Lov-synchronized MDBK cells revealed that the amount of BHV-4 DNA synthesized increased in a quantitative manner during cell transition through the S phase (Fig. 4B). Data from DNA extracted 9 and 14 hr after infection led to similar conclusions. Infection of the cells from 5 hr before, up to 5 hr after Lov removal, progressively increased the amount of viral DNA synthesized. The highest amount of BHV-4 DNA was synthesized when MDBK cells were infected 5 hr after Lov removal (Fig. 4B). This time corresponds to G_1/S boundary–early S phase (Fig. 4A). At the end of the 9- and 14-hr infection periods, most of the mock-infected cells were in mid S and G_2 + M phases, respectively (Fig. 4A). Late infection of the cells 5 hr post-Lov removal, up to 15 hr, progressively lowered the amount of BHV-4 DNA synthesized. Little or no BHV-4 DNA synthesis could be demonstrated 9 and 14 hr after infection of cells arrested in the G_0 phase (Fig. 4B). Nevertheless, the percentages of G_0-infected cells as determined by the cell killing and infectious center assays were 97.4 and 96.1%, respectively.

The results presented in Fig. 4C indicate that both viral uptake and the rate of BHV-1 DNA synthesis are not influenced by the phase at which the cell is in the cell cycle. Similar amounts of viral DNA were found in DNA samples extracted from cells infected at different stages of the cell cycle.

BHV-4 L and E-L protein expression in Lov-synchronized, G_0-arrested, and randomly growing MDBK cells

The percentages of MDBK cells expressing the BHV-4 L gp1 and E-L gp6/gp10/gp17 glycoproteins were com-

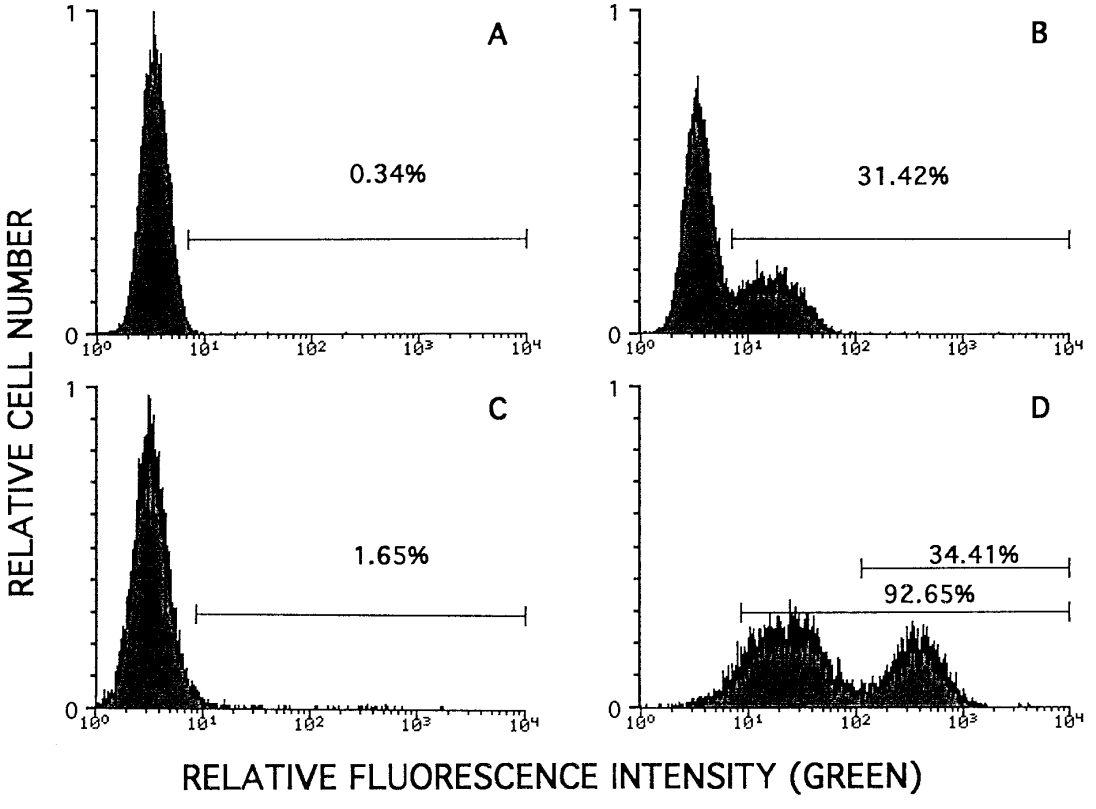

FIG. 3. Quantification of BHV-4 gp1 (A and B)- and gp6/gp10/gp17 (C and D)-expressing cells 44 hr after infection with the BHV-4 V. Test strain (m.o.i. 10 PFU/cell). A and C represent mock-infected cells; B and D represent infected cells. The percentage of positive cells is indicated in each panel.

pared after infection at different stages of the cell cycle. The percentage of BHV-4 gp1-positive cells increased when MDBK cells were synchronized by Lov before infection. Sixty-seven and twenty-nine percent of positive cells were observed after infection of Lov-synchronized and unsynchronized cell cultures, respectively (Fig. 5). In contrast, only 3% of gp1-positive cells was observed after infection of G_0-arrested cells (Fig. 5).

In spite of the infection of cells at different stages of the cell cycle, the percentage of gp6/gp10/gp17-positive cells did not differ significantly and remained at an elevated level (Fig. 5). A mean percentage of 93% of positive cells was observed. However, it is clear that the relative distribution of positive cells between the dim and bright populations was influenced by the cell synchronization. The percentage of bright gp6/gp10/gp17-positive cells evolved similar to that of gp1-positive cells (Fig. 5).

Results from synchronized BHV-1-infected cells (Lov-synchronized and G_0-arrested cells) harvested 17 hr postinfection showed similar percentages of gC-positive cells, and a mean of 98.6% of positive cells was obtained (data not shown).

DISCUSSION

This study shows that the success of BHV-4 infection *in vitro* is restricted by the dependence of its DNA replication on the S phase of the cell cycle. This was suggested by the following results. (i) BHV-4 L proteins are expressed by a limited number of MDBK-infected cells (Table 1), although nearly all the cells expressed a BHV-4 E-L protein (Fig. 3). (ii) Cell transition through the S phase quantitatively increased the rate of BHV-4 DNA replication (Fig. 4B). (iii) Synchronization of MDBK cells with Lov before infection increased the percentage of cells positive for BHV-4 L protein expression (Fig. 5B). (iv) BHV-4 DNA synthesis and L protein expression could barely be detected in cells arrested in G_0 (Figs. 4B and 5D).

Only a fraction of BHV-4-infected cells expressed detectable L proteins. This observation was not limited to one specific cell type or to one BHV-4 strain (Table 1 and Fig. 2), suggesting that the phenomenon is not artifactual to a specific cell type and is a general property of BHV-4.

The replication cycle of BHV-4 is blocked at a late stage and not earlier. Although only a fraction of infected cells expressed detectable BHV-4 L proteins (Table 1), nearly of all the cells were shown to be infected by the cell killing and infectious center assays (Table 2). The inability of some of the infected cells to express BHV-4 L proteins is likely due to the arrest of the viral replication cycle in these cells, either during or at a stage before L protein expression. The detection of β-galactosidase

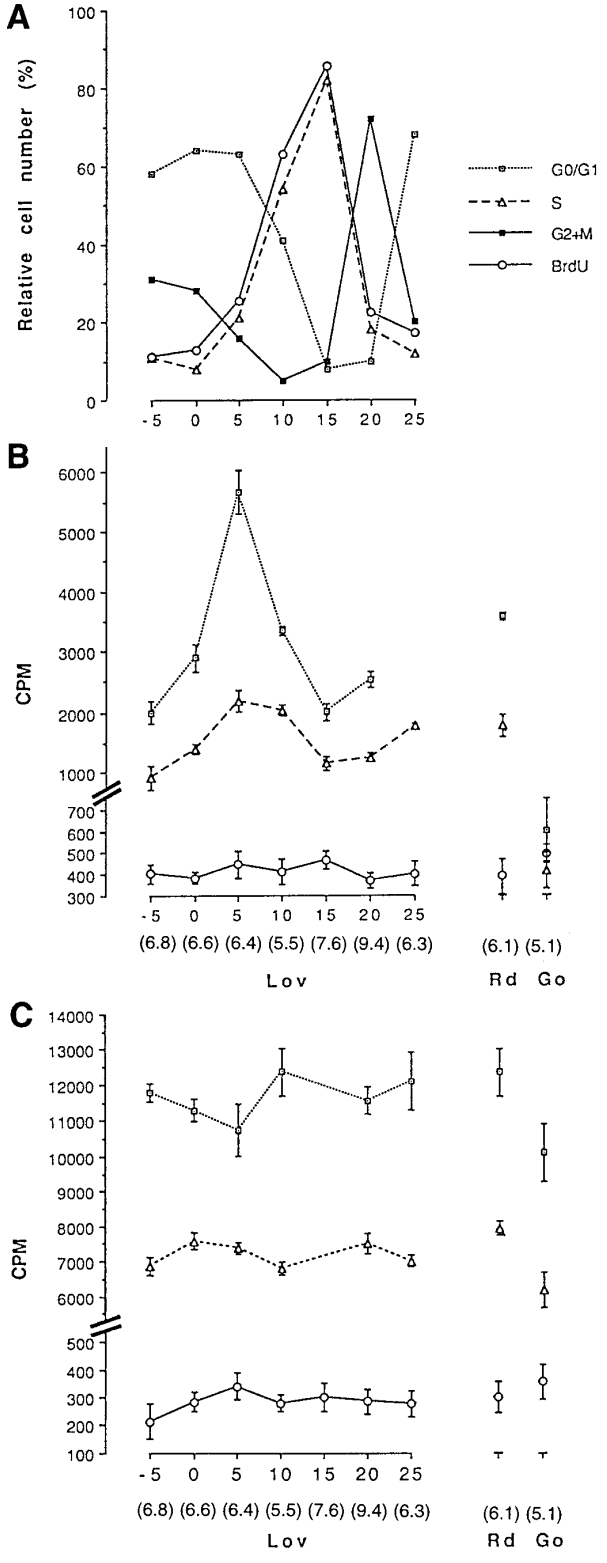

FIG. 4. BHV-1 and BHV-4 DNA replication in MDBK randomly growing cells (Rd) or in MDBK-synchronized cultures infected at different stages of the cell cycle. MDBK cells were synchronized by Lov (Lov) or by serum deprivation (G_0) as described under Materials and Methods. The cell cycle distribution and the fraction of BrdU-incorporating cells were determined for each time of infection of Lov-synchronized cells (5 hr before removal of Lov, at the time of Lov removal, and 5, 10, 15, 20, and 25 hr after removal of Lov) (A). Cells were infected with BHV-4 V. Test strain (B) or BHV-1 Cooper strain (C) for 1 hr. After infection, cells were washed and incubated with medium identical to that of mock-infected cells. The m.o.i. used to infect the cell cultures with a ratio of 5 PFU/G_1 DNA content are in brackets. The relative amount of BHV-4 (B) or BHV-1 (C) DNA in 100 ng of total DNA extracted 2 (○), 9 (△), and 14 (□) hr after infection was determined by dot-blot hybridization. Each value represents the average ± SD for triplicate measures corrected for background (113 ± 17 and 104 ± 12 cpm were detected in BHV-4 and BHV-1 mock-infected cells, respectively) (B and C).

activity in 98.5% of cells infected with the BHV-4 B3 lin strain (Fig. 2E) gave the indication that the arrest did not occur at an earlier stage (attachment, entry, or decapsidation) as reported for herpesvirus saimiri (Randall *et al.,* 1985). Unfortunately, a thorough characterization of the rate-limiting step in the replication cycle of BHV-4 could not be determined due to the nonavailability of specific Mab probes for IE and E proteins. However, since 93% of the BHV-4-infected cells were positive for expression of the E-L gp6/gp10/gp17 (Mab 35) in contrast to 31% of the L gp1-positive cells (Fig. 3), it seems very probable that the viral replication cycle was arrested at a stage after E protein expression. Glycoprotein gp6/gp10/gp17-positive cells were distributed into two populations (Figs. 3 and 5). The percentage of bright fluorescent cells was similar to the percentage of gp1-positive cells (Figs. 3 and 5). Nevertheless, these results were not sufficient to clearly and unambiguously show that the dim and bright fluorescences of gp6/gp10/gp17 corresponded to expression of E (p(gp10/gp17)) and L (gp6/gp10/gp17) proteins, respectively.

BHV-4 DNA replication and consequently L protein expression are dependent on the cell cycle. The observations in this study suggested that the replication cycle of BHV-4 was blocked at the stage of DNA replication and/or L protein expression in some cells of the monolayer. The results obtained with Lov-synchronized cells revealed that cell transition through the S phase increased quantitatively the amount of BHV-4 DNA synthesized (Fig. 4B). This observation could not be accounted for by the quantitative differences in the rate of viral uptake according to the stage of the cells in the cell cycle. Indeed, similar amounts of viral DNA were detected in samples extracted 2 hr postinfection (before viral DNA replication could be detected) (Fig. 4B). Moreover, the experiment with the BHV-4 B3 lin strain presented in this study showed that attachment, entry, and decapsidation occurred in nearly all of the cells of a nonsynchronized confluent MDBK monolayer (Fig. 1). The observed increase in the rate of BHV-4 DNA synthesis associated with the S phase does not seem to be due to the adjustment of the m.o.i. (used to infect the cells at the ratio of 5 PFU/G_1 cellular DNA content), as the highest BHV-4 DNA synthesis rate was observed after infection with one of the lowest m.o.i. used (Fig. 4B). The observed results could not be artifactual consequences of the cell

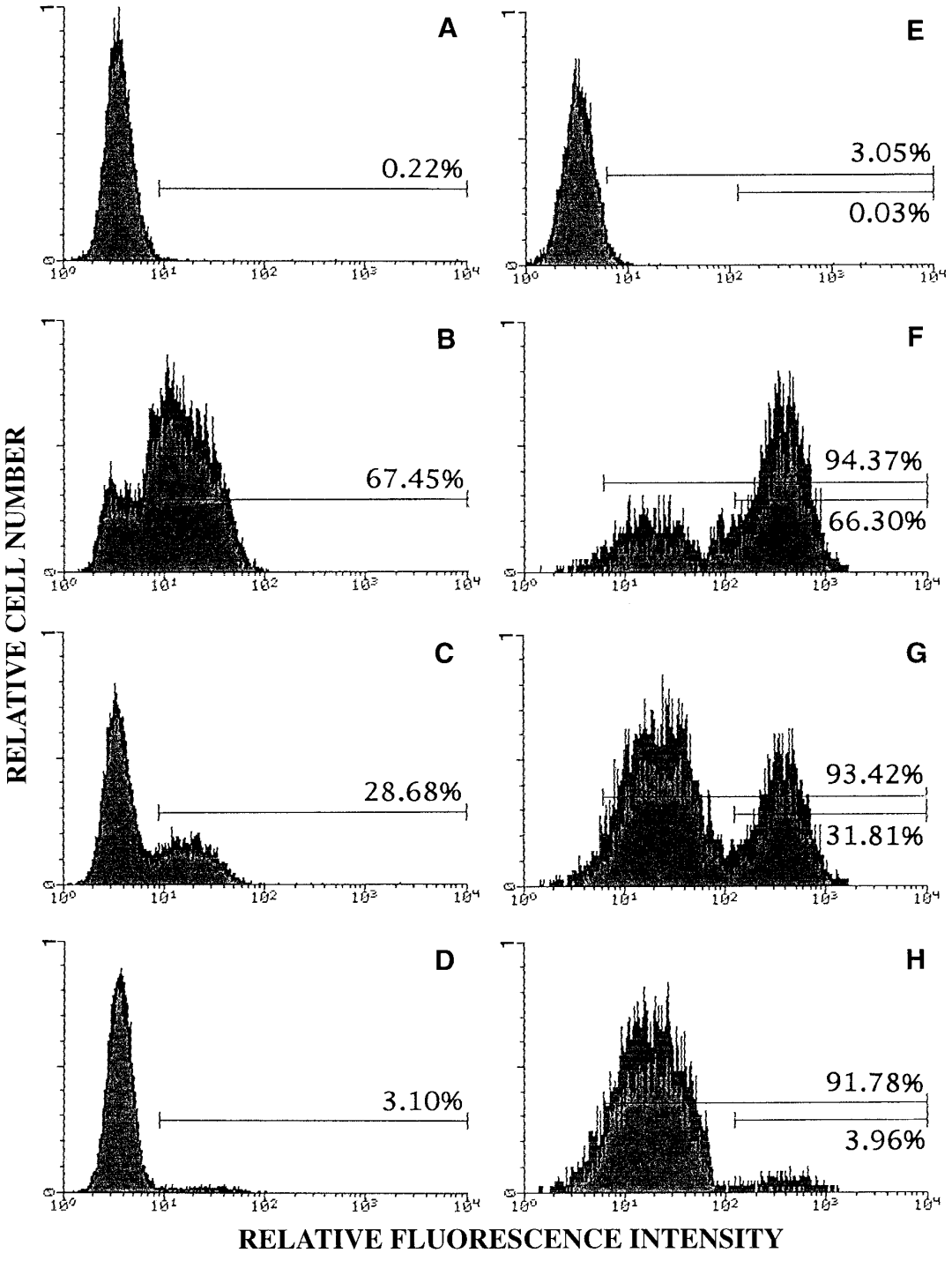

FIG. 5. Quantification of BHV-4 gp1 (A to D)- and gp6/gp10/gp17 (E to H)-expressing MDBK cells 44 hr after infection of randomly growing cells (C and G) and synchronized cell cultures. MDBK cells were synchronized by Lov (B and F) or by serum deprivation (D and H) as described under Materials and Methods. Cells were infected with the BHV-4 V. Test strain (m.o.i. 10 PFU/cell). Lov-synchronized cell cultures were infected at the time of Lov removal and Mev addition. A and E represent mock-infected cells. The percentage of positive cells is indicated in each panel.

synchronization, as similar amounts of BHV-1 DNA were detected in Lov-synchronized cells infected at different stages of the cell cycle (Fig. 4C). Cell synchronization by Lov prior to BHV-4 infection increased the percentage of cells expressing L proteins (as compared with the results obtained with nonsynchronized confluent monolayers) (Fig. 5). A maximum of 67% positive cells was observed when MDBK cells were infected at the time of Lov removal. The inability to obtain nearly 100% of positive cells could be explained by either (i) the inability of complete Lov synchronization of the cell cycle, which is clearly shown in Fig. 4A, or (ii) the use of adherent cell staining

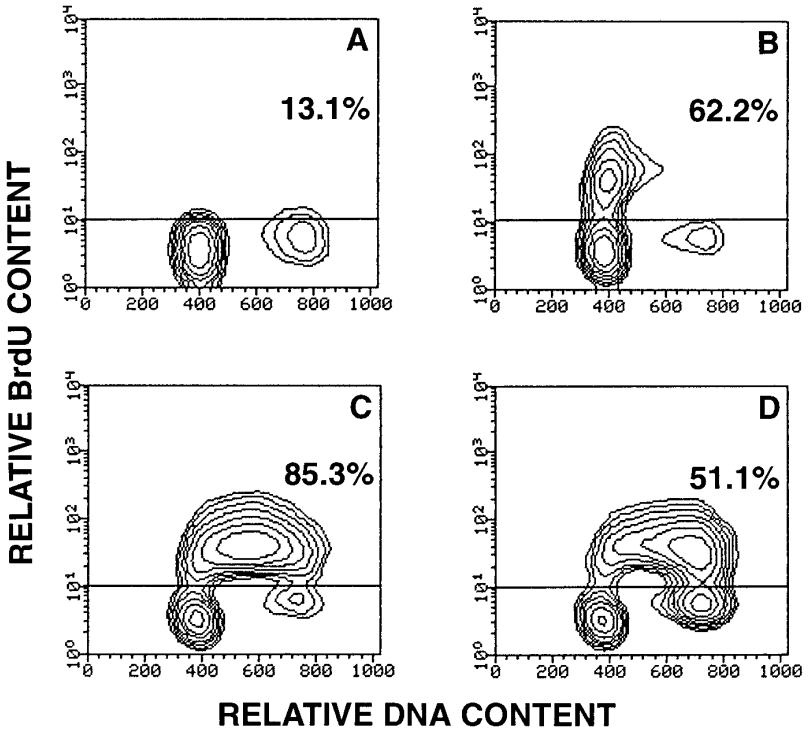

FIG. 6. A quantitative measurement of the number of dividing cells (S phase). Lovastatin-synchronized MDBK cells were pulsed with BrdU (1 hr at 37°) at the time of Lov removal and Mev addition (A) and 10 (B), 15 (C), and 17 (D) hr later. The percentage of positive cells is indicated in each panel.

for detection of viral antigen to avoid nonspecific attachment of Mab on fixed dead cells, leading to the exclusion of cells, which had been killed by the virus, and which certainly contained L proteins.

Synthesis of BHV-4 DNA could not be demonstrated in cells arrested in G_0. This observation further supports the dependence of BHV-4 DNA replication on the S phase. Since viral DNA replication is a prerequisite for L protein expression, it is not surprising that BHV-4 L protein expression did not occur in cells arrested in G_0. These observations do not seem to be an artifact of the G_0 synchronization, as it was proved that G_0-arrested cells are capable of supporting BHV-1 DNA replication (Fig. 4C) and L protein expression. The inability of BHV-4 L protein expression to occur in G_0-arrested cells can explain why the percentage of cells expressing BHV-4 L proteins decreased with increasing cell density at the time of infection. Indeed, normal cells are sensitive to contact inhibition, which induces cell growth arrest in the G_0 phase. Therefore, the fraction of G_0-arrested cells in a monolayer increases with the cell density.

In order to achieve their DNA replication irrespective of the status of the cell cycle, herpesviruses encode a set of proteins which is involved directly in DNA replication and nucleotide biosynthesis (Hammerschmidt and Sugden, 1990; Hammerschmidt and Mankertz, 1991; Shadan *et al.,* 1994). Nevertheless, the dependence of herpesvirus DNA replication on the cell cycle has been described for the alphaherpesvirus EHV-1 (Lawrence, 1971) and the betaherpesvirus MCMV (Muller and Hudson, 1977). The present study has demonstrated that the DNA replication of the gammaherpesvirus BHV-4 is dependent on the S phase of the cell cycle and therefore extends to include a member of the *Gammaherpesvirinae* in the group of herpesviruses known to be dependent on the S phase for their DNA replication. The mechanism by which EHV-1 and MCMV DNA replication are dependent on the S phase of the cell cycle is at present unknown. In this communication, we did not determine the S phase factor(s) required for BHV-4 DNA replication. Any event(s) displaying a transient appearance in the cell cycle limited to the S phase would be a candidate (Wintersberger, 1991). The cellular factor(s) whose presence is critical for replication of certain herpesviruses is currently unknown. It is also not determined whether these factors are identical, linked, or different in alpha-, beta-, and gammaherpesviruses. These points require further work and are important for our understanding of herpesvirus evolution.

The S phase dependence in BHV-4 replication established in this study could be of importance for some biological properties of the infection *in vitro.* First, the slow replicative cycle of BHV-4 could be due to its slow rate of thymidine kinase induction in infected cells as suggested by Kit *et al.* (1986). However, another explanation could be the dependence of BHV-4 DNA replication

on the S phase. Second, most herpesviruses rapidly induce the shutoff of host cell protein expression and DNA synthesis after infection (Fenwick *et al.,* 1979). This feature is, of course, incompatible with S phase dependence of viral replication. Interestingly, BHV-4 does not induce a host cell shutoff (Augsburger and Metzler, 1989), probably to allow the host cell to go through the S phase.

The S phase dependence of BHV-4 replication may also have implications for the biology of the virus *in vivo.* First, it can be suggested that lytic BHV-4 infection is limited to dividing cells. Second, it can be postulated that the physiological state (resting or dividing) of the host cell can determine the induction of lytic or latent infection as it was suggested for cytomegaloviruses (CMV) (Stinski, 1983, 1990). CMV infection of some cells could lead to productive or nonproductive infection, depending on the physiology of the cell at the time of infection. Moreover, changes in the physiology of the infected cell can induce a transition from a nonproductive to a productive infection. Stinski (1990) hypothesized that nonproductive infection may favor latency, while further changes in the host cell physiology can induce the transition from latency to lytic infection. A similar model could be postulated for BHV-4, knowing that the physiology of the host cell determines its capacity to support a nonproductive (resting cell) or productive infection (dividing cell). BHV-4 was demonstrated to be latent in bovine peripheral mononuclear blood cells (PBMC) (Osorio and Reed, 1983). Taking into account that more than 98% of PBMC *in vivo* are resting cells, BHV-4 infection of these cells could lead to nonproductive infection, which in turn may favor latency. Further activation and division of these latently infected cells could induce and allow virus reactivation. Interestingly, BHV-4 infection of unactivated bovine PBMC cultures *in vitro* can lead to a nonproductive infection, while some cells in activated cultures can support virus replication (A. Vanderplasschen, G. Czaplicky, E. Thiry, and P.-P. Pastoret, unpublished data).

This study demonstrates that BHV-4 DNA synthesis is dependent on the S phase of the cell cycle. This feature should be considered in further work on BHV-4 replication. Furthermore, due to this dependence, the role of the host cell physiology (resting or dividing) in the establishment of latency and reactivation should be investigated.

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REFERENCES

Augsburger, H. R., and Metzler, A. E. (1989). In vitro growth characteristics of bovine herpesvirus 4 (BHV-4) as revealed by indirect immunofluorescence assay with monoclonal antibodies and polyvalent antisera. *Arch. Virol.* 104, 309–321.

Bartha, A., Juhasz, M., and Liebermann, H. (1966). Isolation of a bovine herpesvirus from calves with respiratory disease and keratoconjunctivitis. *Acta Vet. Hung.* 16, 357–358.

Bublot, M., Van Bressem, M.-F., Thiry, E., Dubuisson, J., and Pastoret, P.-P. (1990). Bovine herpesvirus 4 genome: Cloning, mapping and strain variation analysis. *J. Gen. Virol.* 71, 133–142.

Bublot, M., Lomonte, P., Lequarre, A.-S., Albrecht, J.-C., Nicholas, J., Fleckenstein, B., Pastoret, P.-P., and Thiry, E. (1992). Genetic relationships between bovine herpesvirus 4 and the gammaherpesviruses Epstein–Barr virus and herpesvirus saimiri. *Virology* 190, 654–665.

Cohen, G. H., Vaughan, R. K., and Lawrence, W. C. (1971). Deoxyribonucleic acid synthesis in synchronized mammalian KB cells infected with herpes simplex virus. *J. Virol.* 7, 783–791.

Costanzo, F., Campadelli-Fiume, G., Foà-Tomasi, L., and Cassai, E. (1977). Evidence that herpes simplex virus DNA is transcribed by cellular RNA polymerase B. *J. Virol.* 21, 996–1001.

DeMarchi, J. M., and Kaplan, A. S. (1976). Replication of human cytomegalovirus DNA: Lack of dependence on cell DNA synthesis. *J. Virol.* 18, 1063–1070.

Dubuisson, J., Thiry, E., Thalasso, F., Bublot, M., and Pastoret, P.-P. (1988). Biological and biochemical comparison of bovid herpesvirus-4 strains. *Vet. Microbiol.* 16, 339–349.

Dubuisson, J., Boulanger, D., Bublot, M., Thiry, E., and Pastoret, P.-P. (1989). Proteins specified by bovine herpesvirus type 4: Structural proteins of the virion and identification of two major glycoproteins by using monoclonal antibodies. *J. Gen. Virol.* 70, 1743–1753.

Dubuisson, J., Pastoret, P.-P., and Thiry, E. (1991). Temporal control of bovine herpesvirus type 4 glycoprotein synthesis. *J. Gen. Virol.* 72, 1429–1434.

Dubuisson, J., Koromyslov, I., Pastoret, P.-P., and Thiry, E. (1992a). Proteins of bovine herpesvirus type 4 released into the culture medium of productively infected cells: Identification of a 135K glycoprotein involved in viral attachment. *J. Gen. Virol.* 73, 189–194.

Dubuisson, J., Pastoret, P.-P., and Thiry, E. (1992b). Identification and characterization of glycoprotein gp1 of bovine herpesvirus type 4. *J. Gen. Virol.* 73, 1293–1296.

Fenwick, M., Morse, L. S., and Roizman, B. (1979). Anatomy of herpes simplex virus DNA. XI. Apparent clustering of functions affecting rapid inhibition of host DNA and protein synthesis. *J. Virol.* 29, 825–827.

Gibbs, E. P. J., and Rweyemamu, M. M. (1977a). Bovine herpesviruses. Part I. Bovine herpesvirus 1. *Vet. Bull.* 47, 317–342.

Gibbs, E. P. J., and Rweyemamu, M. M. (1977b). Bovine herpesviruses. Part II. Bovine herpesvirus 2 and 3. *Vet. Bull.* 47, 411–425.

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

Hammerschmidt, W., and Sugden, B. (1990). DNA replication of herpesviruses during the lytic phase of their life-cycles. *Mol. Biol. Med.* 7, 45–57.

Hammerschmidt, W., and Mankertz, J. (1991). Herpesviral DNA replication: Between the known and unknown. *Semin. Virol.* 2, 257–269.

Honess, R. W., and Roizman, B. (1974). Regulation of herpesvirus macromolecular synthesis I. Cascade regulation of the synthesis of three groups of viral proteins. *J. Virol.* 14, 8–19.

Honess, R. W., and Roizman, B. (1975). Regulation of herpesvirus macromolecular synthesis: Sequential transition of polypeptide synthesis requires functional viral polypeptides. *Proc. Natl. Acad. Sci. USA* 72, 1276–1280.

Jakobisiak, M., Bruno, S., Skierski, J. S., and Darzynkiewicz, Z. (1991).

Cell cycle-specific effects of lovastatin. *Proc. Natl. Acad. Sci. USA* 88, 3628–3632.

Keyomarsi, K., Sandoval, L., Band, V., and Pardee, A. B. (1991). Synchronization of tumor and normal cells from G_1 to multiple cell cycles by lovastatin. *Cancer Res.* 51, 3602–3609.

Kit, S., Kit, M., Ichimura, H., Crandell, R., and McConnell, S. (1986). Induction of thymidine kinase activity by viruses with group B DNA genomes: Bovine cytomegalovirus (bovine herpesvirus 4). *Virus Res.* 4, 197–212.

Knipe, D. M. (1990). Virus–host–cell interactions. *In* "Virology" (Fields *et al.,* Eds.), 2nd ed., Vol. 1, pp. 293–316. Raven Press, New York.

Lawrence, W. C. (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.

Ludwig, G. V., and Letchworth, G. J., III (1987). Temporal control of bovine herpesvirus 1 glycoprotein synthesis. *J. Virol.* 61, 3292–3294.

MacGregor, G. R., and Caskey, C. T. (1989). Construction of plasmids that express *E. coli* β-galactosidase in mammalian cells. *Nucleic Acids Res.* 17, 2365.

Marshall, R. L., Rodriguez, L. L., and Letchworth, G. J., III (1986). Characterization of envelope proteins of infectious bovine rhinotracheitis virus (bovine herpesvirus 1) by biochemical and immunological methods. *J. Virol.* 57, 745–753.

Mayfield, J. E., Good, P. J., Van Oort, H. J., Campbell, A. R., and Reed, D. E. (1983). Cloning and cleavage site mapping of DNA from bovine herpesvirus 1 (Cooper strain). *J. Virol.* 47, 259–264.

Muller, M. T., and Hudson, J. B. (1977). Cell cycle dependency of murine cytomegalovirus replication in synchronized 3T3 cells. *J. Virol.* 22, 267–272.

Nolan, G. P., Fiering, S., Nicolas, J.-F., and Herzenberg, L. A. (1988). Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β-D-galactosidase activity after transduction of *Escherichia coli lacZ. Proc. Natl. Acad. Sci. USA* 85, 2603–2607.

Osorio, F. A., and Reed, D. E. (1983). Experimental inoculation of cattle with bovine herpesvirus-4: Evidence for a lymphoid-associated persistent infection. *Am. J. Vet. Res.* 44, 975–980.

Potgieter, L. N. D., and Maré, C. J. (1974). Assay and antigenic interrelationships of the recently isolated bovine herpesviruses, DN 599, FTC, and V11. *Arch. Gesamte Virusforsch.* 46, 238–247.

Randall, R. E., Newman, C., and Honess, R. W. (1985). Asynchronous expression of the immediate-early protein of herpesvirus saimiri in populations of productively infected cells. *J. Gen. Virol.* 66, 2199–2213.

Roizman, B. (1990). Multiplication of viruses. An overview. *In* "Virology" (Fields *et al.,* Eds.), 2nd ed., Vol. 1, pp. 87–94. Raven Press, New York.

Roizman, B., Desrosiers, R. C., Fleckenstein, B., Lopez, C., Minson, A. C., and Studdert, M. J. (1992). The family *Herpesviridae:* An update. *Arch. Virol.* 123, 425–449.

Shadan, F. F., Cowsert, L. M., and Villarreal, L. P. (1994). *n*-butyrate, a cell cycle blocker, inhibits the replication of polyomaviruses and papillomaviruses but not that of adenoviruses and herpesviruses. *J. Virol.* 68, 4785–4796.

Stinski, M. F. (1983). Molecular biology of cytomegaloviruses. *In* "The Herpesviruses" (B. Roizman, Ed.), Vol. 2, pp. 67–113. Plenum, New York/London.

Stinski, M. F. (1990). Cytomegalovirus and its replication. *In* "Virology" (Fields *et al.,* Eds.), 2nd ed., Vol. 2, pp. 1959–1980. Raven Press, New York.

Storz, J. (1968). Comments on malignant catarrhal fever. *J. Am. Vet. Med. Assoc.* 152, 804–806.

Storz, J., Ehlers, B., Todd, W. J., and Ludwig, H. (1984). Bovine cytomegaloviruses: Identification and differential properties. *J. Gen. Virol.* 65, 697–706.

Thiry, E., Pastoret, P.-P., Dessy-Doizé, C., Hanzen, C., Calberg-Bacq, C. M., Dagenais, L., Vindevogel, H., and Ectors, F. (1981). Réactivation d'un herpèsvirus en culture de cellules testiculaires prélevées chez un taureau atteint d'orchite et d'azoospermie. *Ann. Med. Vet.* 125, 207–214.

Thiry, E., Bublot, M., Dubuisson, J., and Pastoret, P.-P. (1989). Bovine herpesvirus 4 (BHV-4) infections of cattle. *In* "Herpesvirus Diseases of Cattle, Horses, and Pigs" (G. Wittmann, Ed.), pp. 96–115. Kluwer Academic, Norwell, MA.

Vanderplasschen, A., Bublot, M., Pastoret, P.-P., and Thiry, E. (1993a). Restriction maps of the DNA of cervid herpesvirus 1 and cervid herpesvirus 2, two viruses related to bovine herpesvirus 1. *Arch. Virol.* 128, 379–388.

Vanderplasschen, A., Bublot, M., Dubuisson, J., Pastoret, P.-P., and Thiry, E. (1993b). Attachment of the gammaherpesvirus Bovine herpesvirus 4 is mediated by the interaction of gp8 glycoprotein with heparinlike moieties on the cell surface. *Virology* 196, 232–240.

Vanderplasschen, A., Hanon, E., Benarafa, C., Greimers, R., Op De Beeck, A., Loncar, M., and Pastoret, P.-P. (1994). Madin Darby bovine kidney cell synchronization by lovastatin: Application to bovine herpesvirus-1 gene expression. *Vet. Res.* 25, 555–567.

Vindeløv, L. L., Christensen, I. J., and Nissen, N. I. (1983). A detergent–trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* 3, 323–327.

Vindeløv, L. L., and Christensen, I. J. (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.

Wintersberger, E. (1991). Biochemical events controlling initiation and propagation of the S phase of the cell cycle. *Rev. Physiol. Biochem. Pharmacol.* 118, 49–95.