JOURNAL OF VIROLOGY, June 1996, p. 3807–3814 Vol. 70, No. 6
0022-538X/96/$04.00+0

The Latency-Related Gene of Bovine Herpesvirus 1 Encodes a Product Which Inhibits Cell Cycle Progression

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Received 2 January 1996/Accepted 7 March 1996

Bovine herpesvirus 1 (BHV-1) establishes a latent infection in the sensory ganglionic neurons of cattle. The exclusive viral RNA expressed in a latent infection is the latency-related (LR) RNA, suggesting that it regulates some aspect of a latent infection. During the course of a productive infection, alphaherpesviruses induce certain events which occur during cell cycle progression. Consequently, we hypothesized that a BHV-1 infection might induce events in neurons which occur during cell cycle progression. In agreement with this hypothesis, cyclin A was detected in neurons of trigeminal ganglia when rabbits were infected. Neuronal cell cycle progression or inappropriate expression of cyclin A leads to apoptosis, suggesting that a viral factor inhibits the deleterious effects of cyclin A expression. The BHV-1 LR gene inhibited cell cycle progression and proliferation of human osteosarcoma cells. Antibodies directed against cyclin A or the LR protein coprecipitated the LR protein or cyclin A, respectively, suggesting that the two proteins interact with each other. We conclude that LR gene products inhibit cell cycle progression and hypothesize that this activity enhances the survival of infected neurons.

Bovine herpesvirus 1 (BHV-1) is a significant viral pathogen of cattle which can cause respiratory disease, abortions, or occasionally encephalitis (61). Like other members of the *Alphaherpesvirinae* family, BHV-1 establishes a latent infection in the sensory ganglionic neurons of an infected host (for reviews, see references 49 and 50). Viral DNA persists in a latent state for the lifetime of an infected host but can periodically reactivate and spread to uninfected cattle. In contrast to the 70 to 80 viral genes expressed during a lytic infection of bovine cells, the latency-related (LR) gene (LRG) is the only viral gene which is expressed in latently infected neurons. Transcripts originating from the LRG accumulate in the nuclei of sensory neurons which are latently infected. Recent studies have also demonstrated that a small fraction of LR RNA is poly(A)$^+$ and following splicing is translated into a 41-kDa protein (27). LRG products can inhibit the activity of viral immediate-early genes in transient-transfection assays (3) but the mechanism of action is not known. Although it appears the BHV-1 LRG or the herpes simplex virus type 1 (HSV-1) latency-associated transcripts play a role in reactivation (for reviews, see references 49 and 50) or establishment of latency (51), no mechanism of action for a protein or RNA encoded by these respective genes has been described.

Several independent studies have indicated that HSV-1 or HSV-2 can induce certain events which correlate with cell cycle progression. For example, HSV-1 induces S-phase forms of the transcription factor E2F (23). Viral genes encoding ICP4, ICP27, the single stranded DNA-binding protein necessary for DNA replication (UL29 or ICP8), DNA polymerase (UL30), or helicase (UL5) are necessary for induction of E2F (23). E2F interacts with Rb family members and is critical for progression through the G_1 and S phase of the cell cycle (for reviews, see references 58 and 59). Rb is a tumor suppressor gene product which can inhibit the growth of some tumor cells and regulate cell cycle progression and is important for the survival of neurons during differentiation (7, 28, 33, 36; for reviews, see references 55 and 59). Mutations in the ICP0 (4) or VP16 gene (8) of HSV-1 can be complemented by cellular factors in the G_1 or S phase of the cell cycle. AP-1-dependent transcriptional activity is induced by ICP0 following an infection of cultured cells (29) or in neurons of infected mice (56). In general, transcription factors which bind to AP-1 sites (fos, jun, and ATF-2, for example) play a role in regulating growth control (45). An HSV infection causes unscheduled DNA replication (31) and amplification of integrated simian virus 40 DNA (22) and can rescue nonautonomous parvoviruses, which require S-phase progression for growth (1). Six HSV-1 encoded gene products are involved in the amplification of integrated DNA: UL5, UL8, and UL52 (components of the viral helicase-primase complex which are essential for viral DNA replication) and UL29, UL30, and UL42 (subunits of DNA polymerase) (22). Finally, an HSV-1 infection alters the nuclear localization of Rb (60) and DNA replication complexes or prereplicative site structures (9), suggesting that the ability of Rb to repress cell cycle progression would be altered following an infection. Two recent studies have concluded that UL5, UL8, UL9, UL29, UL30, UL42, and UL52 are necessary for the formation of prereplicative site structures (35, 38). Since alphaherpesviruses establish latent infections in sensory neurons, it would be advantageous for the virus to prevent postmitotic neurons from reentering the cell cycle because the unscheduled proliferation of sensory neurons results in apoptosis (7, 14, 28, 33, 34).

In this study, we demonstrate that an infection of rabbits by BHV-1 induces cyclin A expression in trigeminal ganglia (TG). By immunochemistry, cyclin A was detected in neurons of TG following an acute infection. When latently infected rabbits were injected with dexamethasone to initiate the reactivation of BHV-1, cyclin A RNA expression was consistently detected between 2 and 16 h after the dexamethasone treatment. Since cyclin A RNA expression was only detected at 2 and 4 h after uninfected rabbits were injected with dexamethasone, we con-

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cluded that the reactivation of BHV-1 prolongs cyclin A RNA expression in TG. Further studies demonstrated that LRG products inhibit cell cycle progression and that cyclin A was coprecipitated with the LR protein (LRP) when incubated with an antibody directed against the LRP. Cyclin A is required for S phase entry and progression (5, 15, 47; for a review, see reference 21) and is induced during the early stages of apoptosis (25, 42). Overexpression of cyclin A in the mammary glands of transgenic mice leads to increased apoptosis, adding credence to the concept that cyclin A plays a role in apoptosis (2). Consequently, we hypothesize that the ability of LRG products to inhibit cell cycle progression may enhance neuronal survival during latency.

MATERIALS AND METHODS

Virus and cells. The growth and maintenance of Cos-7 cells or bovine turbinate (BT) cells were described previously (27). The selection of transfected cells was performed with 600 μg of G418 (Geneticin) per ml where indicated.

The human osteosarcoma cell line U2-OS was obtained from the American Type Culture Collection (Rockville, Md.). U2-OS cells (10^5) were transfected by calcium phosphate precipitation for 6 h with 10 μg of the indicated plasmids and 1.0 μg of pPur. No glycerol shock was performed after transfection. Selection of antibiotic-resistant colonies was performed with 1.5 μg of puromycin per ml.

The human osteosarcoma cell line Saos-2 was obtained from the American Type Culture Collection. Saos-2 cells (10^5) were transfected by calcium phosphate precipitation for 6 h with the designated plasmids. No glycerol shock was performed after transfection. Selection was performed with 1.0 μg of puromycin per ml.

The Cooper strain of BHV-1 was obtained from the National Veterinary Services Laboratory, Animal and Plant Inspection Services, Ames, Iowa.

Infection of rabbits and dexamethasone reactivation. New Zealand White rabbits were tranquilized with acepromazine. BHV-1 (10^7 50% tissue culture infective doses) was inoculated into the right and left conjunctival sacs by instillation. Latent infections in rabbits (45 to 60 days postinfection) were reactivated by single intravenous injections of dexamethasone (2.8 mg/kg of body weight) as described earlier (48). Prior to and after reactivation, ocular swabs were obtained and the presence of infectious virus was assessed by incubation with BT cells. Extensive viral gene expression occurs in TG 4 days after rabbits are infected with BHV-1 and at 16 h after dexamethasone injection (48, 52).

RNA and RT-PCR. RNA was prepared as described previously (27), except that only one phenol extraction was performed. Reverse transcription (RT) reactions were carried out with a highly processive RNase H^- reverse transcriptase (Stratagene, La Jolla, Calif., and Gibco BRL) and were primed with a $T_{(12–18)}$ oligonucleotide. PCR primers for cyclin A were the upstream primer ACCCCCCAGAAGTAGCAGAGTTTGTG and the downstream primer GC CAGCTTTGTCCTGTGACTGTATAGAG. These primers anneal to exon 5 and 6, respectively, in the cyclin A gene. The expected size of amplified products from cyclin A mRNA is 306 bp. Amplification of cyclin A genomic DNA would result in a 765-bp fragment. PCRs were carried out for 30 cycles. Each cycle was 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C (first cycle), increasing the extension step by 2 s per cycle. If more cycles are used it is possible to amplify cyclin A RNA from some TG of uninfected rabbits (52).

Preparation of cellular extracts and immunoprecipitation. The preparation of whole-cell lysates for immunoprecipitation was performed as described previously (27). The P2 antibody is directed against the amino terminus of LR open reading frame 2 (ORF2). A monoclonal antibody directed against cyclin A (sc-239) was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.).

Flow cytometry analysis of transfected U2-OS cells. For flow cytometry analysis, the procedure of Zhu et al. (62) was utilized. Briefly, 2 μg of an expression vector for the B-cell surface antigen CD20 (pCMVCD20) was cotransfected with 20 μg of the designated plasmids. At 48 h posttransfection, cells were washed with phosphate-buffered saline (PBS) and scraped with a rubber policeman. The cells were washed and resuspended in 1 ml of medium containing 20 μl of a fluorescein isothiocyanate-conjugated anti-CD20 antibody (Pharmingen). Cells were incubated with the antibody for 30 min on ice, and this was followed by two washes with PBS containing 1% calf serum. The cells were fixed with 70% ethanol on ice for 1 h. Before flow cytometry analysis, the cells were pelleted, washed once in PBS, and stained with propidium iodide (20 μg/ml) containing RNase (200 μg/ml). Flow cytometry was performed on a Becton-Dickinson FACScan machine. The intensity of propidium iodide staining was analyzed on cell populations that were positive for fluorescein isothiocyanate staining to determine the DNA content. Cell cycle profiles were generated with CellFit cell cycle analysis software.

Plasmids. Plasmids pcDNA3 LRT (LRT) or pcDNA3 LRTΔSph (LRTΔSph) are identical to those previously described (27), except that the respective *Hin*dIII-*Sal*I fragments were inserted into pcDNA3 at *Hin*dIII-*Xho*I sites. pcDNA/LR-1 Stop contains the *Hin*dIII-*Sal*I fragment of the LR promoter and gene with a stop codon linker at the first two *Sph*I sites (27). The stop codon linker contains stop codons in all reading frames (Sigma, St. Louis, Mo.). A diagram of the respective constructs is presented below (see Fig. 4).

Plasmids pCMV-A, pCMVp21 (S. Elledge; Houston, Tex.), or pJ3ΩRb (pRb; P. Robbins; Pittsburgh, Pa.) encode human cyclin A, $p21^{cip1}$, or pRb, respectively. Plasmid pPur (Clontech, Calif.) confers puromycin resistance.

Detection of cyclin A in TG by immunoperoxidase. TG of rabbits were fixed overnight at 4°C in 10% buffered formalin, and thin sections were prepared. Paraffin was removed from the thin sections, and the sections were rehydrated and subsequently treated with 0.3% H_2O_2 in methanol for 20 min to suppress endogenous peroxidase activity. Slides were washed in PBS for 20 min and then blocked with 0.015% nonimmune horse serum in PBS for 30 min. A monoclonal cyclin A antibody, 6 μg/μl, (sc-239; Santa Cruz Biotechnology) was suspended in PBS containing 0.015% normal serum and incubated with the respective sections for 30 min. After being washed with PBS for 10 min, slides were incubated with a biotin-conjugated secondary antibody for 30 min. The slides were then washed in PBS for 10 min and incubated with Avidin-Biotin Enzyme reagent (Santa Cruz Biotechnology), following the instructions of the manufacturer. After the last PBS wash, slides were developed by incubation with the substrate for 15 to 20 min and the color reaction was stopped by washing the slides in tap water for 10 min. Sections were counterstained with 0.5% methyl green for approximately 5 min. All incubations were performed at room temperature (20°C).

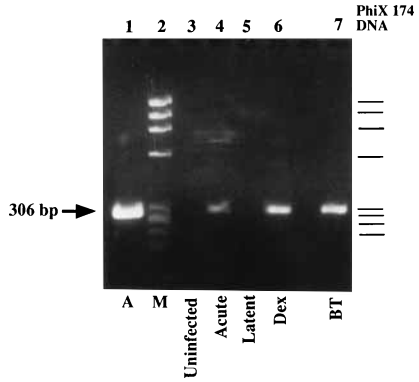

FIG. 1. BHV-1 infection induces cyclin A expression in TG. Detection of cyclin A RNA in TG of uninfected rabbits (lane 3), rabbits 4 days postinfection (lane 4), latently infected rabbits (90 days postinfection) (lane 5), or rabbits 16 h after dexamethasone-induced reactivation (lane 6). The positive control, 100 ng of pCMV-A, is shown in lane 1. The molecular weight marker was ϕX174 DNA digested with *Hae*III (lane 2). The lines to the right of the gel represent the size standards (1,353, 1,078, 872, 603, 310, 281, 272, and 234 bp, from top to bottom). Lane 7, BT cells. The arrow indicates amplified products from cyclin A RNA (or cDNA in lanes 1).

RESULTS

Induction of cyclin A expression in TG of rabbits infected with BHV-1. Several independent studies suggested that alphaherpesviruses induce certain events which occur during S phase progression (1, 4, 8, 9, 22, 23, 31). Consequently, we hypothesized that similar events may occur when BHV-1 colonizes TG or when reactivation from a latent infection occurs. To determine if events which correlate with S-phase progression occur when sensory neurons are infected by BHV-1, the expression of cyclin A RNA in the TG of rabbits following an ocular infection was examined. Cyclin A was chosen as a marker for S phase because it is expressed only in S and G_2 and it is required for S phase progression (5, 15, 43, 47; for a review, see reference 21). Furthermore, terminally differentiated cells, including neurons, do not express cyclin A (5, 14, 15) unless they reenter S phase (5). Cyclin A RNA was detected in rabbit TG 4 days after an ocular infection with BHV-1 (Fig. 1, lane 4) or following dexamethasone-induced reactivation of latent virus (lane 6). In contrast, cyclin A RNA was not detected in the TG of uninfected (lane 3) or latently infected (lane 5) rabbits. As expected, amplification of cyclin A RNA was detected in proliferating BT cells (lane 7) or in the skin of uninfected rabbits (data not shown).

FIG. 2. Localization of cyclin A in neurons of TG from an uninfected rabbit and a rabbit infected with BHV-1. Sections were prepared from TG with immunoperoxidase and the cyclin A antibody as described in Materials and Methods. (A) TG from a rabbit infected with BHV-1 for 4 days (note the positive nuclear signals in the neurons). Arrowheads point to neurons expressing cyclin A in their nuclei. Small arrows point to neurons which are not expressing detectable cyclin A. (B) TG from an uninfected rabbit. Note that even after prolonged development times there are no positive-staining neurons. Magnification for both panels, ×400.

To determine what cell type in TG was expressing cyclin A protein, thin sections of rabbit TG were prepared and incubated with a monoclonal antibody directed against cyclin A. Cyclin A protein was detected in neurons of sections prepared from the TG of rabbits infected with BHV-1 for 4 days (Fig. 2A). These results also demonstrated that cyclin A was primarily localized to the nucleus. It was also evident that most neurons of infected rabbits did not express cyclin A. The lack of cyclin A staining in the sections of TG prepared from uninfected rabbits (Fig. 2B) correlated well with the RT-PCR results shown in Fig. 1.

The kinetics of cyclin A induction during dexamethasone-induced reactivation was examined by RT-PCR. Dexamethasone will reproducibly induce the reactivation of BHV-1 in nearly all latently infected rabbits (48), making it a good model to study the early events that occur during reactivation. Two hours after latently infected rabbits were injected with dexamethasone, the 306-bp cyclin A RT-PCR product was detected (Fig. 3, lane 9). Cyclin A RNA was also detected at 4, 8, and 16 h after dexamethasone-induced reactivation (Fig. 3, lanes 10 to 12). When uninfected rabbits were injected with dexamethasone, cyclin A was detected at 2 and 4 h after treatment (Fig. 3, lanes 4 and 5) but not at 8 or 16 h (lanes 6 and 7). The small band (approximately 100 bp) present in lanes 4, 8, 9, 10, 11, and 12 was occasionally seen in samples lacking template and thus may be a result of primer dimer formation. The kinetics of cyclin A RNA expression in latently infected rabbits or uninfected rabbits was the same when the experiment was repeated with another rabbit for each time point (52).

In summary, these results demonstrated that (i) during acute infection, BHV-1 induces cyclin A RNA in TG and cyclin A protein expression in neurons of TG (Fig. 1 and 2), (ii) dexamethasone induced the transient expression of cyclin A RNA in the TG of uninfected rabbits (Fig. 3, lanes 3 to 7), and (iii) prolonged expression of cyclin A RNA occurred in TG when latently infected rabbits were treated with dexamethasone (Fig. 3, lanes 8 to 12).

Transient transfection of U2-OS human osteosarcoma cells with LRG inhibits cell cycle progression. The LRG is the only viral gene expressed during latency, suggesting that it regulates some aspect of a latent infection. We hypothesized that LRG might neutralize the deleterious effects of cyclin A expression. To understand the effect that LRG products have on mammalian cells, BT or Cos-7 cells were transfected with a plasmid containing the LRG (LRT [Fig. 4]). Numerous cells were resistant to antibiotic selection, but these "flat" cells did not proliferate to the extent that stable cell lines were obtained. When BT or Cos-7 cells which were transfected with LRT were trypsinized, they attached to plastic and did not proliferate but the cells were viable for at least 1 month (52). In summary, studies with BT or Cos-7 cells suggested that LRG products inhibited cell cycle progression and/or proliferation (52).

Since the studies with BT or Cos-7 cells suggested that LRG products inhibited proliferation or cell cycle progression, further studies were conducted to confirm this hypothesis. Two human osteosarcoma cell lines, U2-OS and Saos-2, have been widely used for assessing the effect that regulatory genes have on the cell cycle (6, 10, 12, 16–18, 24, 30, 36, 37, 40, 41, 44,

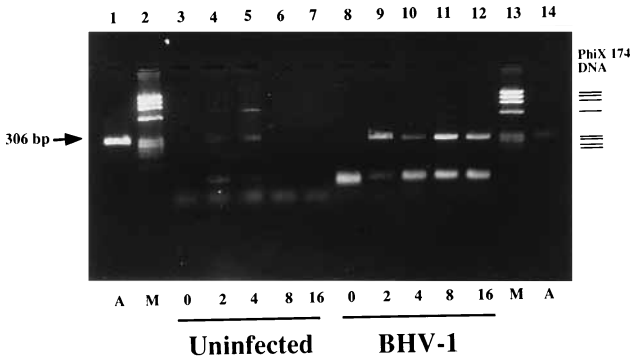

FIG. 3. Kinetics of cyclin A expression in TG following dexamethasone-induced reactivation. Uninfected rabbits were injected with dexamethasone and euthanized at different times after treatment. RNA was prepared from the TG of one rabbit at 2, 4, 8, or 16 h (lanes 4 to 7, respectively) after treatment. Latently infected rabbits were injected with dexamethasone and RNA prepared from the TG of one rabbit at 2, 4, 8, or 16 h (lanes 9 to 12, respectively) after treatment. Prior to dexamethasone injection, RNA was prepared from the TG of an uninfected rabbit (lane 3) or a latently infected rabbit (lane 8). cDNA was synthesized from the respective RNA samples as described in Materials and Methods. The positive control, 100 ng of pCMV-A, is shown in lanes 1 and 14. The molecular weight marker was ϕX174 DNA digested with *Hae*III (lanes 2 and 13). The lines to the right of the gel represent the size standards (1,353, 1,078, 872, 603, 310, 281, 272, and 234 bp, from top to bottom). The arrow indicates amplified products from cyclin A cDNA.

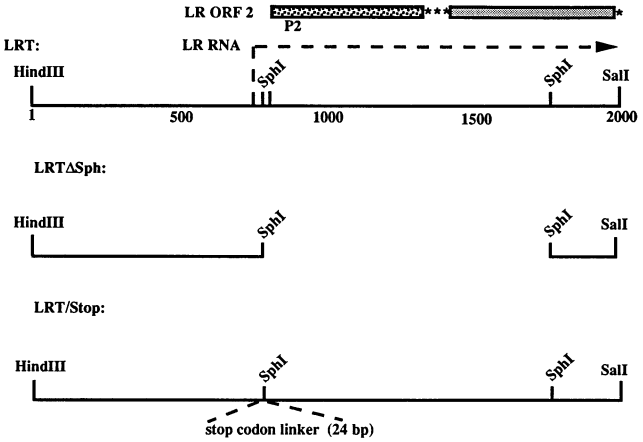

FIG. 4. Schematic diagram of BHV-1 LRG and plasmids used. The positions of the LR RNA initiation site, LR ORF2, and the peptide sequence to generate the P2 antibody were described previously (25). The stars after LR ORF2 (the bar with large black dots) represent three in-frame stop codons, and the bar with small black dots represents an ORF without an initiating methionine that is in frame with LR ORF2 (31).

53–55, 62). Transfection of U2-OS cells with plasmids encoding cell cycle inhibitors reduces colony numbers after antibiotic selection and represses cell cycle progression (17, 18, 36, 62). In six experiments, LRT reduced the number of U2-OS colonies at least fourfold relative to cells transfected with LRTΔSph or pcDNA3 (Fig. 5). Furthermore, colonies of LRT-transfected cells were consistently smaller than colonies obtained from U2-OS cells transfected with LRTΔSph or pcDNA3.

A cotransfection assay using a B-cell surface marker, CD20, was developed by E. Harlow and colleagues (30, 62) to examine the effect of transfected genes on the cell cycle in a transient-transfection assay. After transfection, cells that contained transfected DNA were identified by staining with an anti-CD20 monoclonal antibody and the DNA content of the transfected cells determined by propidium iodide staining. When U2-OS cells were cotransfected with a plasmid expressing CD20 (pCMVCD20) and LRT, ~80 to ~86% of the $CD20^+$ cells were in the G_1 phase of the cell cycle, compared with ~31 to ~40% of the $CD20^+$ cells cotransfected with pCMVCD20 and pcDNA3 or LRTΔSph (Table 1). As expected, the cell cycle distribution of mock-transfected cells was similar to that of cells transfected with pCMVCD20 and pcDNA3 or LRTΔSph. Taken together, these studies demonstrated that LRG products inhibited S phase progression of U2-OS cells.

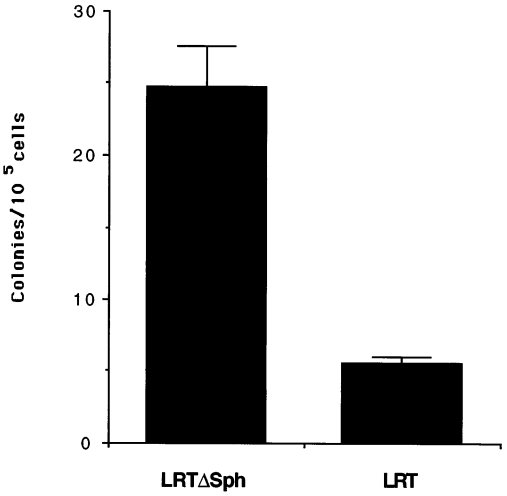

FIG. 5. BHV-1 LRG inhibits colony formation in U2-OS cells. U2-OS cells (10^5) were transfected by calcium phosphate precipitation as described in Materials and Methods with 10 μg of the indicated plasmids and 1 μg of pPur. Colony formation of U2-OS cells was performed as described previously (18, 24). The values are the means of three independent studies, and the bars indicate standard errors of the means.

TABLE 1. Flow cytometry analysis of transfected U2-OS cells

Type of transfection[a]	% Cells in phase:					
	Expt 1			Expt 2		
	G_1	S	G_2 or M	G_1	S	G_2 or M
LRT	85.7	8.9	5.4	79.8	11.3	8.9
pcDNA3	38.1	41.5	20.4	31.5	42.7	25.8
LRTΔSphI	39.4	42.9	17.7	32.7	45.8	21.5
Mock	37.8	39.4	22.8	33.2	43.6	23.2

[a] U2-OS cells were cotransfected with pCMVCD20 and the plasmids indicated or were mock transfected.

LRG inhibits cell cycle progression of Saos-2 cells which express pRb and cyclin A. Transfection of a wild type retinoblastoma (pRb) gene into Saos-2 cells (pRb−/−) induces a flat cell morphology because cells are arrested in G_0 (6, 10, 12, 16, 18, 24, 37, 40, 41, 44, 53–55) (Fig. 6 and 7). When pRb is cotransfected with a plasmid expressing cyclin A (pCMV-A), the number of flat cells decreases because cell cycle progression occurs and consequently the cells are killed during antibiotic selection (24). Furthermore, integration of the respective plasmids is a rare event. Thus, the frequency of flat cells being induced by a gene directly correlates with the ability of the gene's product to inhibit cell cycle progression in Saos-2 cells. Since several cell cycle inhibitors require the expression of wild-type pRb to elicit their effects (for examples, see references 18, 37, and 41), Saos-2 cells are a useful model to study cell cycle progression. Cotransfection of Saos-2 cells with pRb, cyclin A, and LRT increased the frequency of flat cells compared with that for cells transfected with pRb, cyclin A, and pcDNA3 (Fig. 6A and 7). However, transfection of LRT alone or with cyclin A did not result in efficient flat-cell formation (Fig. 6A and 7). The frequency of flat-cell formation induced by LRG products was less efficient than that induced by p21 (pCMVp21), a known cell cycle inhibitor (Fig. 6B). In the absence of cyclin A, p21 but not LRT enhanced flat-cell formation of Rb. In summary, these studies demonstrated that LRG products inhibited cell cycle progression of Saos-2 cells when cotransfected with pRb and cyclin A.

Association of LRP with cyclin A. A protein encoded by the LRG (LRP) was recently identified and partially characterized (27). A domain in LRP has 48% similarity (27% identity) with $p21^{cip}$, also referred to as Waf1 or Cip1 (11, 20). Cip1 is a protein that binds cyclin-dependent kinases that are complexed with cyclins and inhibits cell cycle progression (11, 20). Previous studies demonstrated that the *Hin*dIII-*Sal*I fragment containing the LRG expressed a 41-kDa protein after Cos-7 cells were transfected (27) (Fig. 8C). LRP was immunoprecipitated by the monoclonal antibody directed against cyclin A in Cos-7 cells transfected with LRT (Fig. 8A). Cyclin A was also immunoprecipitated by the P2 antibody when Cos-7 cells were transfected with LRT (Fig. 8B). The P2 antibody did not cross-react with cyclin A, because when Cos-7 cells were transfected with plasmids which do not express LRP (LRTΔSph or LRT/

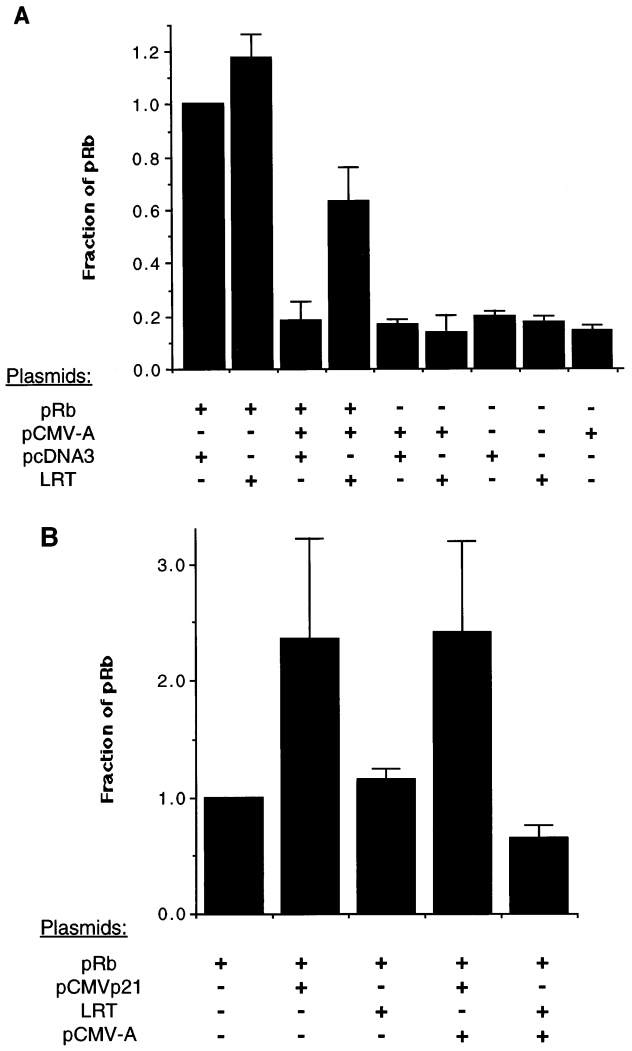

FIG. 6. BHV-1 LRG induces flat-cell formation in Saos-2 cells expressing Rb and cyclin A. (A) Saos-2 cells (10^5) were transfected by calcium phosphate precipitation as described in Materials and Methods with 2 μg of pJ3ΩRb (or empty vector), 4 μg of pCMV-A (or empty vector), and 8 μg of LRT or pcDNA3, plus 1.2 μg of pPur. Flat-cell analysis was performed as described previously (10, 20, 37). The number of flat cells produced by transfection of pRB alone in each experiment was assigned the value of 1. The number of flat cells produced by the other combinations of plasmids was expressed as a fraction of the number of colonies in cells transfected with pRb alone. (B) Comparison of LRT with a plasmid expressing p21 (pCMVp21). Flat-cell analysis was performed as described above. The values are the means of three independent studies, and the bars indicate standard errors of the means.

Stop) cyclin A was not immunoprecipitated by the P2 antibody. As expected, the monoclonal antibody directed against cyclin A recognized a protein migrating near 60 kDa, the known molecular mass of cyclin A (Fig. 8D). The smaller protein migrating as a 56-kDa band may be a proteolytic breakdown product of cyclin A or a modified form of cyclin A. These results suggested that cyclin A was associated with the LR protein in transfected Cos-7 cells.

DISCUSSION

In this study, we presented evidence that LRG products inhibit proliferation and cell cycle progression of human osteosarcoma cells, BT cells, or Cos-7 cells. This activity could be important, because the infection of TG results in the expression of a cellular factor, cyclin A, which promotes cell cycle progression or apoptosis.

The finding that cyclin A RNA was induced in TG following an acute infection implied that BHV-1 induced cell cycle progression in neurons. Since prolonged expression of cyclin A was detected in latently infected rabbits following dexamethasone-induced reactivation, it appears that similar events occur during reactivation. In support of these observations, we have recently demonstrated that certain G_1 and S phase cyclin-dependent kinase-cyclin complexes are induced following an infection of permissive cells with BHV-1, HSV-1, or HSV-2 (26, 52). Recent studies have demonstrated that cyclin A induces cell cycle progression (47) and that cell cycle progression leads to apoptosis in postmitotic neurons (14, 34). Expression of cyclin A can also precede apoptosis (2, 25, 42), implying that certain signals leading to proliferation also play a role in apoptosis. Postmitotic sympathetic neurons are susceptible to apoptosis if intracellular levels of dATP are increased, even in the presence of nerve growth factor (57). During an acute infection or reactivation, it is likely that intracellular levels of dATP in sensory neurons increase because viral nucleotide-metabolizing enzymes, such as ribonucleotide reductase or thymidine kinase, are expressed. We currently do not know whether cyclin A induction in sensory neurons reflects cell cycle progression or is indicative of apoptosis. Regardless of which mechanism is involved, unscheduled cyclin A expression would have deleterious effects on sensory neurons.

The growth of U2-OS, BT, or Cos-7 cells was consistently arrested when these cells were transfected with plasmid constructs containing LRG. Efficient flat-cell formation in Saos-2 cells was achieved only when Saos-2 cells were cotransfected with pRb and cyclin A. Since there are several examples of cell cycle inhibitory genes which require pRb for activity (18, 37, 41), it is possible that LRG products require functional pRb to inhibit growth and cell cycle progression. Although it could be argued that LRG products are toxic to cells, several lines of evidence suggest that this is not the case. (i) Transfected Cos-7, BT, or Saos-2 cells were viable in culture for long periods of time but did not proliferate. (ii) After transfected cells were

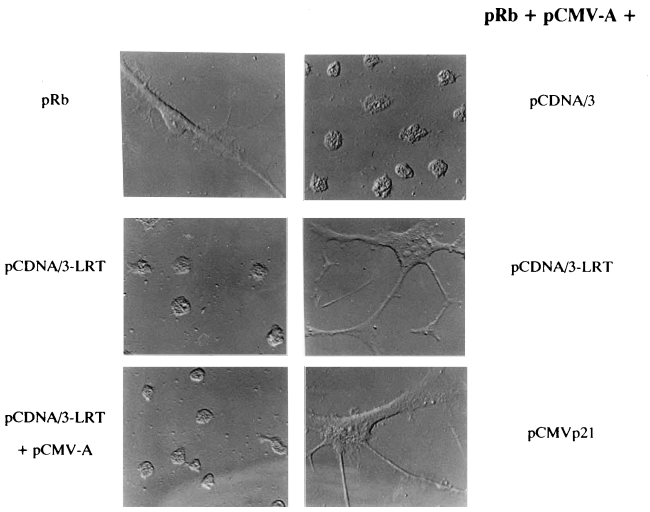

FIG. 7. Morphology of Saos-2 cells after puromycin selection. Saos-2 cells were transfected with the designated plasmids as described in Materials and Methods and the legend to Fig. 7. Left column, cells transfected with the designated plasmids; right column, cells transfected with pRb + pCMV-A and with the designated plasmids.

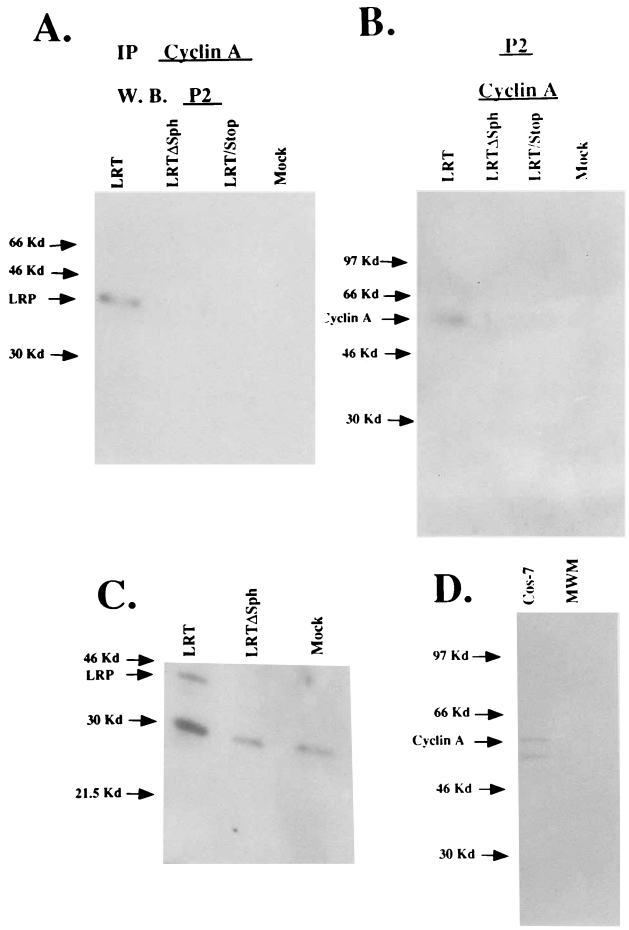

FIG. 8. The BHV-1 LRG encodes a protein which interacts with cyclin A. Lysates from transfected cells (100 μg of protein) were prepared and then immunoprecipitated (IP) with the monoclonal antibody directed against cyclin A (A) or with the P2 antibody (27) (B). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed, Western blotting (immunoblotting) (W.B.) was performed with the P2 antibody (A) or a monoclonal antibody directed against cyclin A (B) as described previously (27). (C) Immunoprecipitation was performed with P2, and the Western blot was probed with P2. (D) The whole-cell lysate (50 μg of protein) from Cos-7 cells was subjected to SDS-PAGE, and the resulting Western blot was probed with the monoclonal antibody directed against cyclin A. Arrows indicate the positions of cyclin A (60 kDa) or LRP (41 kDa). Lanes: LRT, Cos-7 cells transfected with LRT; LRTΔSph, Cos-7 cells transfected with LRTΔSph; LRT/Stop, Cos-7 cells transfected with LRT/Stop; Mock, mock-transfected Cos-7 cells; MWM, molecular weight markers.

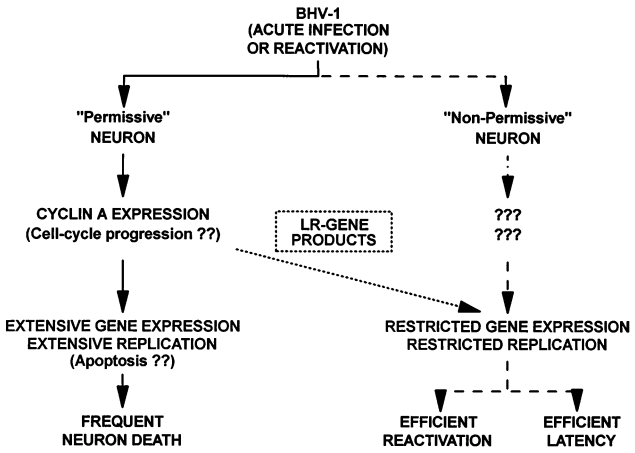

FIG. 9. A hypothetical model of the role the LRG plays in latency.

trypsinized, they attached and assumed a flat-cell phenotype. (iii) A block in cell cycle progression was observed only in Saos-2 cells which were cotransfected with LRT, pRb, and cyclin A and was not observed when they were transfected with LRT alone. (iv) Saos-2 cells transfected with LRT, pRb, and cyclin A had enhanced survival compared with Saos-2 cells cotransfected with pRb and cyclin A.

The association of LRP with cyclin A may be important for inhibition of cell cycle progression. Recent studies have demonstrated that the first 90 amino acids of Cip1 can bind cyclin-dependent kinase-cyclin complexes (6, 39). The amino termini of Cip1 and LRP have 48% similarity and 27% identity, suggesting that the interaction between LRP and cyclin A has functional significance. To prove that LRP is a Cip1-like homolog, it will be necessary to demonstrate that cyclin A is bound to LRP after biochemical purification and that LRP alters cyclin-dependent kinase activity bound to cyclin A. Since proliferation of mammalian cells can also be inhibited by untranslated regions of RNA (46) or RNA molecules which do not encode protein (19), it is conceivable that LR transcripts repress cell cycle progression. Earlier studies suggested that HSV-1 latency-associated transcripts (13) or LR transcripts (3) are functional RNAs which regulate some aspect of a latent infection. Experiments designed to understand the mechanism by which LRG products inhibit cell cycle progression are currently in progress.

What is the biological significance of the findings presented in this study? We hypothesize that during a primary infection, BHV-1 induces the expression of cyclin A in a subset of sensory neurons, thus facilitating viral gene expression and replication. In these neurons, LRG products would neutralize the deleterious effects of cyclin A and perhaps other factors, viral or cellular, to ensure neuronal survival and promote latency. In nonpermissive neurons, extensive viral gene expression and replication would not occur and LRG products would not be required. An earlier study (48) demonstrated that only 20% of neurons latently infected with BHV-1 actually reactivate following dexamethasone injection, suggesting that approximately 80% of latently infected neurons survive unsuccessful reactivation events. Since cyclin A RNA expression was detected during reactivation, LRG products may prevent unsuccessful reactivation events from inducing cell cycle progression or apoptosis in sensory neurons and/or allowing reactivation to be completed by preventing premature death of sensory neurons (a summary of this hypothetical model is shown in Fig. 9). In order for BHV-1 to persist in cattle, it would be advantageous for a high percentage of latently infected sensory neurons to survive multiple reactivation events. With these stringent constraints placed on BHV-1, LRG products may be more important than anticipated.

ACKNOWLEDGMENTS

This research was supported by grants from the USDA (9402117, 9203562, and 9502236).

We thank Todd Holt for preparing extracts and providing assistance with the CD20 assays. We thank M. Dickman, F. Osorio, R. Thompson, and D. Rock for critically reading the manuscript and/or providing insightful discussion. Finally, we thank S. Elledge, L. Zhu, E. Harlow, and P. Robbins for plasmids which were invaluable for these studies.

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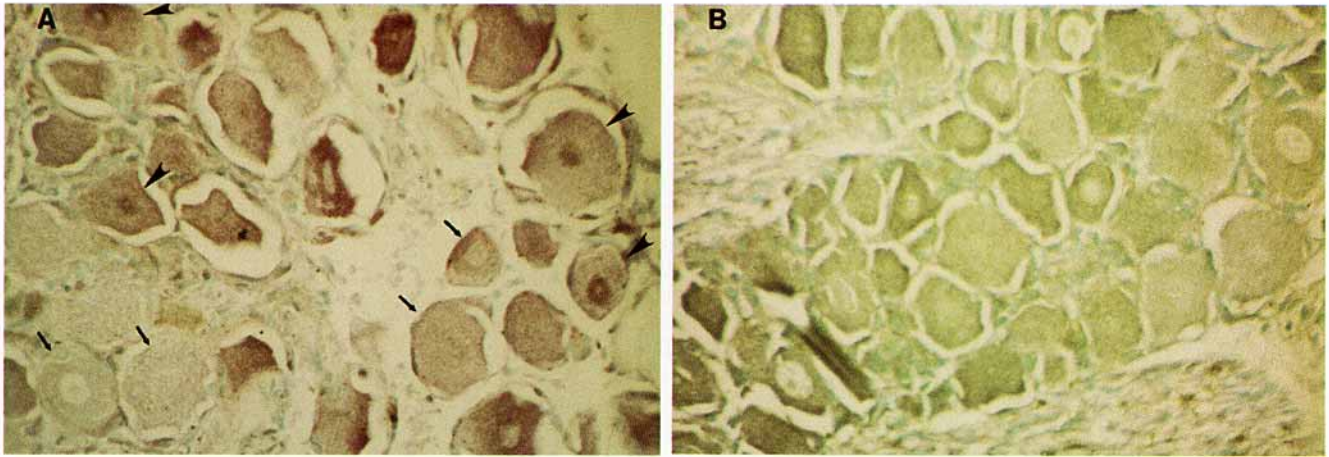
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