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Progesterone increases the incidence of bovine herpesvirus 1 reactivation from latency and stimulates productive infection.

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Abstract

Bovine herpesvirus 1 (BoHV-1), including modified live vaccines, can cause abortions in pregnant cows. Progesterone maintains pregnancy and promotes spermiogenesis and testosterone biosynthesis in males; furthermore, progesterone is a neuro-steroid. Recent published studies demonstrated progesterone stimulated the BoHV-1 immediate early transcription unit 1 (IEt_u1) promoter, and two glucocorticoid receptor response elements within the promoter were required for progesterone mediated transactivation. In this study, we tested whether progesterone induces reactivation from latency in rabbits. As expected, the synthetic corticosteroid dexamethasone consistently induced reactivation from latency in males and females. While progesterone induced reactivation from latency in approximately one-half of male rabbits, virus shedding was sporadic compared to dexamethasone and less efficient in female rabbits. Progesterone significantly increased productive infection in rabbit skin cells, which correlated with stimulating reactivation. These studies suggest progesterone promotes BoHV-1 spread in cattle, in part, by increasing the frequency of reactivation from latency.

Keywords

bovine herpesvirus 1 (BoHV-1); progesterone; reactivation from latency; abortion

INTRODUCTION

Bovine herpesvirus 1 (BoHV-1) is the most frequently diagnosed cause of viral abortion in North American cattle (Chase et al., 2017). Exposure of a susceptible herd to BoHV-1 can result in abortion storms that range from 25% to 60% of cows undergoing abortion. Even commercially available modified live vaccines can cause abortions in pregnant cows. For example, naïve heifers vaccinated with an inactivated BoHV-1 vaccine are less likely to have

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an abnormal estrus cycle and have significantly higher pregnancy rates compared to heifers vaccinated with a modified live (MLV) vaccine (Chase et al., 2017; Miller and Van der Maaten, 1987; O'Toole et al., 2012; O'Toole and Van der Maaten, 2010; Perry et al., 2013). These observations suggest BoHV-1 acute infection and reactivation from latency increase the incidence of reproductive complications in cattle, including abortions. BoHV-1 also causes conjunctivitis and/or upper respiratory tract disease: thus, mucosal surfaces become eroded during acute infection (Hodgson et al., 2005; Jones, 2010), in part because the virus suppresses host immune responses (Jones, 2009, 2019). Consequently, secondary bacterial infections and life-threatening pneumonia can occur (Powell, 2005; Rice et al., 2008). BoHV-1, stress, and other viral pathogens contribute to the poly-microbial disease, bovine respiratory disease complex (BRDC) (Jones and Chowdhury, 2007). A BoHV-1 entry protein encoded by the poliovirus receptor related 1 gene is a BRDC susceptibility gene for Holstein calves (Neibergs et al., 2014) confirming BoHV-1 is an important BRDC cofactor.

Following acute infection of the oral, nasal, or ocular cavity, trigeminal ganglia (TG) are an important site for life-long latency (Jones, 2011, 2013). Increased corticosteroid levels, due to food and water deprivation during shipping of cattle, weaning, and/or dramatic weather changes increase the incidence of BoHV-1 reactivation from latency, (Jones et al., 2013 and Jones, 2014). The synthetic corticosteroid dexamethasone (DEX) mimics the effects of stress, stimulates productive infection (Kook et al., 2015), and consistently induces reactivation from latency (Inman et al., 2002a; Jones, 1998, 2003, Jones et al. 2006; Jones et al., 2000; Rock et al., 1992; Sheffy and Davies, 1972; Shimeld et al., 1990).

Stressful stimuli increase corticosteroids levels, which enter a cell, and bind to GR or mineralocorticoid receptor (MR), reviewed in (Oakley and Cidlowski, 2013). The MR or GR dimer bound to a corticosteroid enters the nucleus and within minutes remodels chromatin and induces transcription. Nuclear GR or MR dimers regulate transcription by binding consensus glucocorticoid response elements (GRE; 5' GGTACANNNTGTTCT-3') (Giguere et al., 1986; Wang et al., 2004). Interestingly, the progesterone receptor (PR) binds and transactivates many GREs (Strahle, 1999), including two GREs located within the BoHV-1 IEtu1 promoter (El-mayet et al., 2019; Kook et al., 2015). PR is expressed in the CNS and sensory neurons (Chan, et al. 2000; Goldsmith et al., 1997; Haywood et al., 1999), and progesterone (P4) is synthesized in the nervous system and is regarded as a neuro-steroid (Schumacher et al., 2012, 2014). P4 levels increase during pregnancy and maintain pregnancy (Lonergan, 2011).

In this study, we tested whether P4 stimulated BoHV-1 reactivation from latency in latently infected rabbits. P4 increased the incidence of reactivation in male rabbits more efficiently than female rabbits. However, P4 was not as effective as DEX with respect to increasing the frequency of virus shedding during reactivation from latency. These studies suggest P4 may increase the incidence of reactivation from latency in cattle and enhance BoHV-1 spread in reproductive tissues in pregnant cows or during estrus.

RESULTS

Measurement of virus shedding during acute infection and reactivation from latency

Studies to test whether P4 can initiate reactivation from latency was performed in rabbits latently infected with BoHV-1. The rabbit is a well-established model to examine BoHV-1 host interactions during the latency-reactivation cycle (Rock et al., 1982; Rock et al., 1987). The rationale for performing this study centers on the following observations: 1) the BoHV-1 genome contains more than 100 GREs (Kook et al., 2015), and PR binds and activate many GREs (Strahle et al., 1999). During acute infection, virus shedding from three male rabbits (R1, R2, and R3) was readily detected in ocular (Figure 1A) and nasal swabs (Figure 1B) until 6 days after acute infection, which was consistent with published reports (Rock et al., 1982). While BoHV-1 clearly replicated in acutely infected rabbits, virus shedding was not as high as calves during acute infection. For example, we consistently detect 1×10^7 and 1×10^8 infectious virus particles in ocular or nasal swabs respectively during the peak of acute infection (Inman et al., 2001) whereas it was approximately 1×10^4 in rabbits. By 10 days after infection, virus was nearly undetectable in ocular and nasal swabs.

To test whether P4 stimulated reactivation from latency, latently infected male rabbits (between 45–60 days after infection), were treated with water-soluble P4 (schematic of experimental protocol is summarized in Figure 2A). As a positive control, latently infected rabbits were given an IV injection of water soluble DEX (2.8 mg/kg of body weight), as this treatment consistently induces reactivation in rabbits latently infected with BoHV-1 (Rock et al., 1992; Rock et al., 1982). Sub-cutaneous injections were also given at 1 and 3 days after the IV injection, which enhance virus shedding in calves during DEX-induced reactivation (Inman et al., 2001). Infectious BoHV-1 was detected in ocular swabs from 5 out of 12 rabbits at 4 days after the P4 treatment, 4 out of 12 at 5 days, and 6 out of 12 at six days after P4 treatment (Figure 2B). Two to four male rabbits were shedding virus until day 9 after the IV P4 injection. Infectious virus was detected in nasal swabs of only 2 male rabbits out of 12 at 4, 5, and 9 days after the initial P4 treatment.

In contrast to P4 treatment, infectious virus was detected in ocular swabs of 5 out of 6 male rabbits 2 days after the IV DEX injection. All 6 male rabbits shed virus from the ocular cavity from 3 to 5 days after DEX treatment. At least 3 out of 6 of the male rabbits shed virus in the nasal cavity from 3–6 days after DEX treatment. Infectious virus was only detected in the ocular cavity of 2 male rabbits at 9 days and not at 10 days after DEX treatment. Three latently infected male rabbits were given a single IV injection of PBS and infectious virus was not detected in ocular or nasal swabs within 10 days after PBS injection indicating that merely handling rabbits and giving the injection did not lead to successful reactivation from latency (data not shown). As expected, infectious virus was not detected in ocular or nasal swabs prior to DEX or P4 injection.

Additional studies were performed in female rabbits, as P4 is an important female sex hormone (Li and O'Malley, 2013). Four female rabbits latently infected with BoHV-1 were treated with P4 to stimulate viral reactivation. Within this group of four rabbits, only one ocular swab (3 days after P4 treatment) contained infectious virus (Figure 3A). From the same experiment, 2 female rabbits treated with DEX shed virus in a similar fashion as in

male rabbits (Figure 3B). These studies indicated there were significantly more days when virus was shed from ocular and nasal swabs of male rabbits compared to female rabbits following treatment with P4 ($p < 0.05$ Students T test).

PCR analysis of virus in swabs during DEX and progesterone treatment

PCR was performed using viral specific primers to detect the BoHV-1 thymidine kinase (TK) gene from swabs of 5 latently infected male rabbits in which reactivation was induced with P4 and 2 treated with DEX (Figure 4). Consistent with the results in Figure 2, viral DNA was not detected on day 0 (latency) after P4 or DEX treatment of all animals confirming they were not shedding virus. With respect to P4 induced reactivation, sporadic detection of viral DNA was detected in ocular swabs and less frequently in nasal swabs: in fact, these results were identical to virus isolation from the same rabbits (Figure 2). These studies also confirmed virus shedding began on day 2 after DEX treatment in ocular swabs and was consistently detected until day 10. Finally, virus shedding from the nasal cavity after DEX treatment did not begin until after ocular shedding. In summary, P4 induced reactivation from latency in latently infected rabbits: however, P4 induced reactivation was less efficient compared to DEX.

Virus specific neutralizing antibodies increase after reactivation from latency

Prior to treating latently infected male rabbits with DEX or P4 to induce reactivation, rabbits were bled and serum prepared. To measure the effect of reactivation on virus neutralizing antibodies, male rabbits were bled and serum prepared at 6 and 12 days after the initial DEX or P4 treatment. During DEX induced reactivation, the 5 male rabbits examined contained significantly higher levels of serum neutralizing antibodies at 6 and 12 days after DEX treatment (Figure 5A), which was consistent with previous studies in calves (Inman et al., 2002). The 8 male rabbits treated with P4 contained significantly higher levels of serum neutralizing antibodies at 12 days after the first P4 injection when compared to day 0 control serum. Relative to calf infection studies (Inman et al., 2002a), the levels of viral specific antibodies were lower in rabbits, which correlates with reduced viral replication during acute infection (Inman et al., 2001).

P4 and PR stimulate productive infection

A BoHV-1 recombinant virus that contains the Lac Z gene inserted downstream of the gC promoter in place of the gC ORF (BoHV-1 gCblue virus) was used for this study. The gCblue virus grows to similar titers as wt BoHV-1. β -Gal expression directly correlates with viral replication because the gC promoter is a late promoter and its expression is low prior to viral DNA replication. Twenty-four hours after transfection of rabbit skin cells was used to count β -Gal⁺ cells to minimize the number of virus positive cells that resulted from virus spread (data not shown). Rabbit skin cells were transfected with BoHV-1 gCblue DNA instead of infecting cells because VP16 and other regulatory proteins in the virion, bICP4 for example (Barber et al., 2017), diminish the stimulatory effects of DEX and cellular or viral genes on productive infection (El-mayet et al., 2018; El-Mayet et al., 2017; El-mayet, 2019; Geiser et al., 2002; Geiser et al., 2003). As previously reported, BoHV-1 DNA is not very infectious (Figure 6A and B), in part because IETu1 promoter activity is dependent on the tegument protein, VP16 (Misra et al., 1994; Misra et al., 1995; Wirth et al., 1992). However,

cotransfection with a plasmid that expresses the human P4 receptor (equivalent amount of the two receptors were included in the transfection) stimulated viral replication, as judged by a significant increase in the number of β -Gal+ cells (Figure 6). Addition of P4 further stimulated viral replication. Addition of the PR and GR antagonist (RU486) (Bardon et al., 1985) reduced the effects of P4. Similar results were observed at 48 hours after transfection; however, it was clear β -Gal+ cells fused as a result of cell-cell spread of BoHV-1 making it difficult to quantify the results (data not shown). Collectively, these studies revealed P4 and PR significantly stimulated BoHV-1 productive infection in rabbit skin cells.

DISCUSSION

The finding that P4 induced reactivation from latency less efficiently than DEX was surprising because GR and PR bind and activate the same sequences in promoters (Strahle et al., 1999). Furthermore, DEX and GR stimulated productive infection in rabbit skin cells (El-Mayet et al., 2017) 3 fold less relative to P4 and PR. Hence, the ability of P4 to stimulate productive infection more effectively than DEX did not directly correlate with the efficiency of reactivation from latency in TG neurons of rabbits. The ability of Krüppel like transcription factor 15 (KLF15) to form a feed forward loop with GR to cooperatively stimulate the IETu1 promoter may be a key difference for DEX mediated reactivation versus P4 induced reactivation (El-Mayet et al., 2017) because PR+KLF15 stimulated IETu1 promoter activity at least 3-times less efficiently (El-mayet et al., 2019). It is also possible DEX stimulates expression of different viral and/or cellular genes in TG of latently infected rabbits relative to P4, including cellular transcription factors important for stimulating viral gene expression during reactivation. GR is expressed in approximately 1/2 of all TG neurons in rats (DeLeon et al., 1994); however, it is not clear what percentage of TG neurons express PR. Attempts to perform immunohistochemistry to evaluate the number of TG neurons that express PR were unsuccessful because the commercially available antibodies we tested did not work well in formalin fixed paraffin embedded TG thin sections (data not shown). If fewer TG neurons express PR or lower levels of PR were expressed in TG neurons relative to GR, one would expect P4 to stimulate reactivation less efficiently.

These studies also revealed P4 induced reactivation more efficiently in male rabbits and virus shedding occurred less frequently in the nasal cavity. This surprising result can partially be explained by the fact that females generally mount a stronger immune response to viral infections compared to males, in part due to a more robust humoral and cellular immune response, reviewed in (Ghosh et al., 2018). While P4 is primarily produced in ovaries, males produce P4 in testicles during production of testosterone from testis and smaller amounts in adrenal glands. Finally, it is conceivable that a metabolite of P4 stimulated reactivation more efficiently in male rabbits versus females because males can convert P4 into testosterone and other hormones.

While the rabbit is an accepted model to study molecular events during BoHV-1 reactivation from latency following DEX treatment (Rock et al., 1992), it is not clear how the P4 results obtained in rabbits can be translated into viral replication and spread in cattle. We suggest stress-induced reactivation may enhance virus spread in reproductive tissue that expresses high levels of P4 and PR because BoHV-1 targets the ovary and corpus luteum during estrus

and early in gestation in BoHV-1 sero-negative heifers (Chase et al., 2017). BoHV-1 present in semen can also be sexually transmitted (van Oirschot, 1995) suggesting female reproductive tissues expressing P4 are potential sites for enhanced viral replication and shedding. Studies focused on understanding the effects of P4 on acute infection and reactivation from latency need to be performed in calves.

MATERIALS AND METHODS

Cells and Virus

Rabbit skin cells, and bovine kidney cells (MDBK) were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), penicillin (10 U/ml), and streptomycin (100 µg/ml).

A BoHV-1 mutant containing the β-Gal gene in place of the viral gC gene was obtained from Dr. S. Chowdury (LSU School of Veterinary Medicine: BoHV-1 gCblue virus) and stocks of this virus grown in MDBK cells. The gCblue virus grows to similar titers as the wt parental virus and expresses the Lac Z gene. Procedures for preparing viral genomic DNA were described previously (Inman et al., 2001).

Infection of rabbits and reactivation from latency

Male and female New Zealand White rabbits (5 months old; approximately 7 lbs) were lightly anesthetized with Xylazine and inoculated in the right and left conjunctival sacs with 1×10^7 PFU of BoHV-1 (Cooper strain). An IV injection of water-soluble progesterone (Sigma; P7556, 2.8 mg/kg of body weight) or water-soluble DEX (Sigma; D2915, 2.8 mg/kg of body weight) was given to latently infected rabbits on 45 days after infection (latency). Two additional sub-cutaneous P4 or DEX injections (0.7 mg/Kg) were given at 1 and 3 days after the initial IV injection.

Sample collection and viral isolation

Swabs from the ocular or nasal cavities were collected daily for 10 days post-reactivation and paced in 4 ml viral transport medium [MEM containing 2% FCS, penicillin (10 U/ml), streptomycin (100 µg/ml) and amphotericin B (10 µg/ml)]. Samples were then vortexed and centrifuged at 5,000 rpm for 5 min at 4°C. To avoid cross-contamination of samples, swabs were processed separately using sterile and clean labware for each specimen. One-half of the supernatant of each swab was inoculated on to MDBK cells in 6-well tissue culture dishes and cytopathic effects monitored for 3–5 days by microscopy and plaque assays. Specimens were considered negative for BoHV-1 if no cytopathic effects appeared after three successive blind passages on MDBK cells.

DNA extraction and PCR

The remainder of each swab was processed for DNA extraction. Supernatants (2 mL) were treated with 100 µl lysis buffer (10mM Tris-HCl pH 7.4, 25mM EDTA pH 8, 100mM NaCl, 0.5% SDS and proteinase K (20 mg/mL) and digested overnight at 42 °C. Total DNA was extracted twice from samples with phenol: chloroform: isoamyl alcohol (25:24:1). 3M sodium acetate and 2 µl glycogen (20 mg/ml) were added to samples (10% of the total

volume) and then DNA precipitated with 2.5 volumes of 100% cold ethanol, washed with 70% ethanol, dried in a vacuum microfuge, and suspended in 30 µl nuclease free water. DNA concentrations were measured by absorbance at 260 nm (Nanodrop 2000, Thermo Scientific) and 50 ng DNA was used for each PCR assay. PCRs were performed using primers that amplify the thymidine kinase (TK) gene: forward primer is 5'-GCCGCCGTACTGGACATGCG-3' and reverse primer is 5'-GCCGAGTCCCCGTAAGGCGAT-3'. These primers yield a product that migrates as a 200 bp fragment. After initial denaturation of 95°C for 5 min, each cycle consisted of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec (28 cycles total). To ensure complete elongation of amplified products, reactions were incubated at 72°C for an additional 5 min. PCR products were subjected to electrophoresis through 1.5% agarose gels stained with ethidium bromide and visualized under ultraviolet light.

Plaque Reduction Neutralization Test

BoHV-1 neutralizing antibodies levels were measured by 50% plaque reduction neutralization test. Rabbit sera was inactivated at 56° C for 30 min before assays were performed. Test sera, positive control sera and negative control sera were two-fold serially diluted with MEM. 100 µl of the diluted virus stock, which contained approximately 100 plaque forming unit of BoHV-1 was added to each serum dilution. Virus titer was confirmed by back titration in MDBK cells. Tubes were mixed by gentle vortexing and then incubating in a water bath at 37° C for 1 h. A 0.2 ml of virus-serum mixture was inoculated to each well of 12-well plates containing confluent MDBK cells. Dishes were incubated at 37° C for 90 min in a 5% CO₂ incubator to allow virus adsorption. Then, 1 ml of 1.2% agarose-containing overlay medium was added to each well, and dishes were incubated in a 5% CO₂ incubator at 37° C for 3–5 days. Cells were fixed with 10% formaldehyde solution in PBS and stained with crystal violet solution in distilled water. Plaques were then counted. Neutralization titer was defined as the highest serum dilution which reduced the numbers of plaques by 50%.

Plasmids

The human progesterone receptor A and B isoforms in the pSG5 expression vector were obtained from Dr. Pierre Chambon (University of Strasbourg, Strasbourg, France). Plasmids were prepared from bacterial cultures by alkaline lysis and 2 rounds of cesium chloride centrifugation.

Quantification of β-Gal positive cells

Rabbit skin cells grown in 60 mm dishes were cotransfected with 1.5 µg of the gCblue viral genome and the designated amounts of plasmid expressing PR (A and B isoforms) using Lipofectamine 3000 (catalog no. L3000075; Invitrogen). At 48 h after transfection, cells were fixed with a solution containing 2% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline [PBS] and then stained with a solution containing 1% Blueo-Gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 0.5 M MgCl₂ in PBS. The number of β-galactosidase (β-Gal)-positive cells was determined as described previously (Geiser et al., 2002; Inman et al., 2001a; Inman et al., 2001b; Inman et al., 2002b). In brief, the number of β-Gal positive cells in cultures expressing the blank vector was set at 1 for

each experiment. To calculate fold change of β -Gal positive cells, the number of blue cells in cultures transfected with the plasmids of interest were divided by the number of blue cells in cultures transfected with the blank vector. The effect P4, RU486 and over-expression of PR had on productive infection is expressed as fold induction relative to the control. This representation of the data minimized the differences in cell density, Lipofectamine 3000 lot variation, and transfection efficiency.

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RESEARCH HIGHLIGHTS

- Progesterone stimulates bovine herpes virus 1 (BoHV-1) reactivation from latency.
- Progesterone induced reactivation was more efficient in male rabbits.
- Progesterone and the progesterone receptor stimulated productive infection in rabbit skin cells.

Figure 1. Virus shedding during acute infection of rabbits with BoHV-1.

Male New Zealand White rabbits (5 months old) were infected with BoHV-1 as described in the materials and methods. At the designated times after infection, ocular and nasal swabs were collected and plaque assays performed to measure virus titers in MDBK cells. The BoHV-1 plaque assay titers in plaque-forming units (PFU)/mL reached the maximum titer at 2-day post-infection in both ocular and nasal swabs then steadily decreased to become undetectable at 10-day post-infection. R1, R2, or R3 refers to rabbit#1, rabbit #2, or rabbit #3 respectively. The mean of the titers of three rabbits are shown.

Figure 2. Induction of reactivation by P4 and DEX in male rabbits latently infected with BoHV-1.

Male New Zealand White rabbits (5 months old) were infected with BoHV-1 as described in the materials and methods. At 45 days after infection, rabbits were given a single IV injection of DEX or P4 as described in the materials and methods (Panel A). At day 1 and 3 after treatment, P4 (Panel B) and DEX (Panel C) were administered via subcutaneous injection as described in the materials and methods. Ocular and nasal swabs were collected and virus shedding monitored by incubating with MDBK cells. Two independent experiments were performed using 6 latently infected rabbits treated with P4 and 3 latently infected rabbits treated with DEX. A total of 12 latently infected rabbits were treated with P4 and 6 with DEX for these studies.

Figure 3. Induction of reactivation by P4 and DEX in female rabbits latently infected with BoHV-1.

Female New Zealand rabbits (5 months old) were infected with BoHV-1 as described in the materials and methods. At 45 days after infection, rabbits were given a single IV injection of P4 (Panel A) or DEX (Panel B) as described in the materials and methods. At day 1 and 3 after treatment, P4 or DEX was administered via subcutaneous injection as described in the materials and methods. Ocular and nasal swabs were collected and virus shedding monitored by incubating with MDBK cells. Four latently infected rabbits were treated with P4 and 2 with DEX for these studies.

Figure 4. Detection of viral DNA in ocular and nasal swabs during reactivation from latency. DNA from ocular or nasal swabs was prepared as described in material and methods from latently infected male rabbits treated with P4 (R6-R0) or DEX (R3 or R4). PCR was performed using primers specific for the BoHV-1 thymidine kinase (TK) gene. These primers yield a specific product of 200 base pairs. Zero-day swabs were included as negative controls. PCR products were subjected to electrophoresis through 1.5% agarose gels stained with ethidium bromide and visualized under ultraviolet light. Molecular weight markers (100-bp ladder) were used to estimate the size of the PCR products: bottom band is 100 bp.

Figure 5. Comparison of BoHV-1 specific neutralizing antibodies during latency to those 10 days after reactivation from latency.

Blood samples from latently infected male rabbits were collected from the ear vein at 6 and 12 days after the initial DEX or P4 injection to induce reactivation. Sera were prepared and stored at -20°C until examined for the development of BoHV-1 neutralizing antibody titers by virus plaque reduction neutralization assay. Standard testing was performed with 100 pfu of BoHV-1 and two-fold serial dilutions of heat-treated rabbit sera. The data are depicted as the mean of 5 DEX treated male rabbits (Panel A) or 8 P4 treated male rabbits (Panel B). An asterisk denotes a significant difference ($P < 0.05$) compared to day 0 (students T test).

Figure 6. P4 and PR cooperate to stimulate productive infection.

Rabbit skin cells (Panel B) were used for these studies. Twenty-four hours prior to transfection 2% “stripped” FBS was added to media. Stripped FBS was used for these studies because normal serum contains steroid hormones, including P4. Cells incubated with stripped FBS for 24 hours contain little or no nuclear GR. Cells were then transfected with 1.5 ug BHV-1 gC-Blue and where indicated a plasmid that expresses human PRA and PRB protein (0.5 ug DNA of each PR isoform). To maintain the same amount of DNA in each sample, empty vector was included. Designated cultures were then treated with water soluble P4 (10 uM; Sigma). At 24 hours after transfection, the number of β -Gal+ cells were counted. Representative results after β -Gal staining are shown (Panel A: arrows denote β -Gal+ cells). The value for the control (gC-Blue virus treated with PBS after transfection) was set at 1. The results from P4 treated cultures were compared to the control and are an average of three independent studies (Panel B). An asterisk denotes a significant difference between rabbit skin cells transfected with BoHV-1 DNA and PR then treated with P4 ($P < 0.05$) when compared to all other combinations tested in these studies, using the students T test.