Temperature-sensitive Mutants of Bovine Herpesvirus Type 1: Mutants Which Make Unaltered Levels of 'Early' Glycoproteins but Fail to Synthesize a 'Late' Glycoprotein

By RANDY NELSON, ANNE-MARIE ADACHI, HUGH CHISHOLM AND VIKRAM MISRA*

Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada

(Accepted 13 October 1988)

SUMMARY

The major glycoproteins of bovine herpesvirus type 1 showed distinct temporal patterns of expression. The glycoproteins GVP 11 and GVP 6 as well as its cleavage products, GVP 11a and GVP 16, were expressed early in the infectious process, whereas GVP 9 was expressed late. Temperature-sensitive mutants were developed and characterized. Mutants belonging to two complementation groups were unable to synthesize DNA at 40 °C, the non-permissive temperature. In cells infected with these mutants the late glycoprotein GVP 9 was not synthesized at 40 °C, whereas the synthesis of the early glycoproteins GVP 11 and GVP 6 continued at wild-type levels. These studies suggest that the transition from early to late glycoprotein synthesis is linked to viral DNA synthesis.

INTRODUCTION

During infection, herpesviruses control the expression of their genes such that most viral gene products can be categorized as immediate early (α), early (β) or late (γ) depending upon the temporal order of their synthesis and upon whether their expression is dependent on the successful progression of certain physiological events within the cell. Immediate early gene products, which are produced soon after the initiation of infection, are required for the expression of early and late genes. The synthesis of late gene products, many of which are structural polypeptides, requires the prior expression of early genes and the replication of viral DNA (reviewed by Wagner, 1985).

All herpesviruses that have been studied specify a number of glycosylated polypeptides which are embedded in the virus envelope and the cytoplasmic membrane of infected cells. Although these glycoproteins are thought to mediate most, if not all, interactions between virions, infected cells and the immune system there is little concrete information on the role of the individual glycoproteins in the biology of herpesviruses.

The herpesvirus glycoproteins exhibit distinct patterns of expression. In herpes simplex virus (HSV)-infected cells, the glycoproteins gB, gD and gE are synthesized in the early stages of infection (Balachandran et al., 1982; Cohen et al., 1980). The synthesis of gD and gE declines as infection progresses, whereas that of gB continues unabated (Balachandran et al., 1982). In contrast to the early glycoproteins the synthesis of gC does not begin until later in the infectious process, and is dependent upon the initiation of viral DNA replication (Cohen et al., 1980; Frink et al., 1983). It has been suggested that this temporal regulation of glycoprotein synthesis may reflect their different roles in virus replication, morphogenesis and virus–host interactions.

In recent years workers in our laboratory (Misra et al., 1982) and others (Collins et al., 1984; Little-van den Hurk et al., 1984; Little-van den Hurk & Babiuk, 1985, 1986; Marshall et al., 1986; Okazaki et al., 1987) have characterized the glycoproteins specified by the bovine
herpesvirus type 1 (BHV-1). The virus induces at least three, and probably more, distinct glycoproteins. These include GVP 9 (also called gIII or BHV-gC), GVP 11 (also called gIV or BHV-gD) and a disulphide bond-linked complex named GVP 6-11-16 (also called gI or BHV-gB). The synthesis of two BHV-1 glycoproteins, GVP 11 and GVP 6-11-16, begins early in infection and continues in the presence of inhibitors of viral DNA synthesis. Under these conditions the synthesis of the late glycoprotein GVP 9 is greatly reduced (Misra et al., 1981, 1982; Ludwig & Letchworth, 1987).

Previous experiments establishing the early nature of GVP 11 were performed with BHV-1-infected cells that had been treated with inhibitors of viral DNA synthesis. Since these compounds can affect the host cell's metabolism in addition to blocking viral DNA replication, it was desirable to confirm these observations in infected cells arrested in the early phase by means other than the use of metabolic inhibitors. In addition, studies on the functions of individual glycoproteins would be facilitated by the development of mutants that expressed only a subset of the glycoprotein repertoire of BHV-1.

This communication describes the isolation and characterization of a series of temperature-sensitive (ts) mutants of BHV-1. At the non-permissive temperature (NPT) mutants from two separate complementation groups failed to synthesize the late glycoprotein GVP 9. Under these conditions the synthesis of the early glycoproteins was sustained at the level of the wild-type (wt).

**METHODS**

**Virus and cells.** The P8-2 strain of BHV-1 was used in these experiments. Virus stocks were prepared from plaque-purified virus and grown as described previously (Misra et al., 1981) in Madin and Darby bovine kidney (MDBK) cells. These cells were obtained from the American Type Culture Collection and were tested at regular intervals and was found to vary by less than 1 °C.

**Choosing the permissive and non-permissive temperatures.** Single-step growth curves were performed for BHV at 30, 32, 34, 36, 38 and 40 °C. Although virus replicated faster at 40 °C than at 32 °C the yield of progeny virus at these temperatures was similar and they were therefore selected as potential permissive temperature (PT) and NPT.

**Mutagenesis and screening.** BHV-1-infected cells were mutagenized by either of two protocols. (i) 5-Bromo-deoxyuridine (B UdR). Actively growing MDBK cells were infected with BHV-1 at an m.o.i. of 10 p.f.u. per cell and then incubated for 48 h at 32 °C in the presence of 7.5 μg of B UdR (Sigma) per ml of tissue culture supernatant. The cells were then frozen and stored at −70 °C until assayed for infectivity. (ii) Nitrosoguanidine (NTG). Cells were infected as for B UdR mutagenesis and incubated at 32 °C in the presence of 20 μg of NTG (Sigma) for 6 h. The infected monolayers were then washed and incubated for an additional 48 h in NTG-free medium before storage at −70 °C.

To screen for ts mutants, MDBK cells in six-well tissue culture plates (Linbro, Flow Laboratories) were infected with the mutagenized stock virus diluted to give about 20 plaques per well. The cells were then overlaid with medium containing 0.5% agarose and incubated at 32 °C. Four days after infection, medium containing 0.01% neutral red was added to the wells. After an additional 24 h at 32 °C the outer margins of the plaques were marked with a fine-tipped felt pen and the plates transferred to 40 °C. After 24 h plaques that had not increased in size at 40 °C were considered to be formed by putative ts mutants and were transferred to fresh MDBK cell cultures for growth. The putative mutants were assayed for infectivity at 32 °C and 40 °C, and isolates that had a 1000-fold or higher titre at the lower temperature were re-assayed at both temperatures to confirm their ts phenotype.

**Recombination analysis.** MDBK cells were infected either with the individual mutants or with a mixture of two at an m.o.i. of 1 p.f.u. per cell. After incubation for 42 h at 32 °C the cells were harvested and assayed for infectivity at 32 °C and 40 °C. Each analysis was performed in triplicate. The recombination frequency was calculated from the equation: recombination frequency = [(A × B)40 − (A40 + B40)]/(A × B)32 × 100, where A × B is the titre of the progeny of a mixed infection assayed at either 32 °C or 40 °C and A and B are the resulting titres of infection by each parent assayed at either temperature.

**Detection of DNA- ts mutants.** Cells were infected with BHV and incubated at either 32 °C or 40 °C. Four h after infection 1.5 μCi of [Me-14C]dT (50 mCi/mmol; Amersham) was added to the culture incubated at 32 °C, and 5 μCi of [Me-3H]dT (20 Ci/mMol; Amersham) was added to the culture at 40 °C. After incubation for an
additional 4 h the two batches of cells were mixed, washed and resuspended in TNE buffer (0.01 M-Tris-HCl pH
8.0, 0.001 M-EDTA and 0.15 M-NaCl). The cells were lysed by adding sodium lauroyl sarcosine (Sigma) to 0.5%
and proteinase K (Sigma) to 0.1 mg/ml. After incubation at 37 °C for 3 h, CsCl was added to a final density of
1.7 g/ml and the solution was centrifuged at 150 000 g for 64 h. Fractions were collected from the bottom Of
the centrifuge tube, the macromolecules were precipitated by cold 5% TCA and 3H and 14C radioactivity associated
with each fraction was determined in a Beckman L-8000 scintillation counter.

Monoclonal antibodies (MAbs) against BHV glycoproteins. Female BALB/c mice were immunized every 2 weeks
for a 6-week period with 50 μg purified BHV-1 (Misra et al., 1982) followed by an intravenous injection 3 days
before fusion. Hybridomas were produced by fusion of spleen cells of antibody-producing mice with cells of the
mouse myeloma line P3-X63-Ag8.653 using 50% polyethylene glycol (Goding, 1983). Antibody-producing cells
were screened for BHV-1-specific antibodies by ELISA using Dynatech Immulon 2 microtitre plates (Fisher
Scientific, Pittsburg, Pa., U.S.A. coated with purified virus (Voller et al., 1976). Hybridoma cells that produced
antibody were expanded and subcloned by limiting dilution. Cell culture fluids from monoclones were used for the
immunoprecipitation of the viral glycoproteins.

Labelling of infected cells and immunoprecipitation. Details of immunoprecipitation have been published earlier
(Misra et al., 1982).

RESULTS

MAbs against BHV glycoproteins

The use of MAbs allows the separation and estimation of individual proteins from the mixture
of virus and host proteins present in infected cells. We therefore prepared a set of MAbs against
BHV-1 antigens, pertinent details of which are outlined in Table 1.

Temporal control of glycoprotein synthesis

To confirm previous results indicating that BHV-1 glycoproteins exhibited different temporal
patterns of synthesis (Misra et al., 1981, 1982; Ludwig & Letchworth, 1987), BHV-infected cells
were labelled with [35S]methionine for 2 h at various times post-infection (p.i.). The cells were
then solubilized and the three major glycoproteins, GVP 9, GVP 6-11-16 and GVP 11, were
precipitated with MAbs under conditions that ensured quantitative precipitation of each
glycoprotein. The immunoprecipitates were analysed by PAGE and the relative amounts of the
glycoproteins synthesized during each labelling period were estimated (Fig. 1). Both GVP 11
and GVP 6-11-16 could be detected as early as 2 to 4 h p.i. The synthesis of GVP 11 continued to
increase throughout the experiment, whereas the rate of synthesis of GVP 6-11-16 declined 8 h
p.i. In contrast, GVP 9 could not be detected until 6 to 8 h p.i.

Ts mutants

BHV-1 stocks were mutagenized with BUdR or NTG and screened for ts mutants. Seven
independently isolated mutants were selected for further study (Table 2). Six of the mutants
reverted to the wt phenotype with a frequency of less than 0.001, while one, TSG, reverted with a
frequency of 0.004. With the exception of TSB and TSG, which could not recombine with each
other to yield wt virus, the remaining five mutants belonged to distinct recombination groups.

Identification of DNA-· ts mutants

Since the transition from early to late gene expression appears to be linked to viral DNA
replication we identified mutants which were unable to synthesize virus DNA at the NPT. To
Fig. 1. Changes in the rate of synthesis of BHV glycoproteins GVP 11 (●), GVP 11a (▲), GVP 16 (▼) (GVP 11a and GVP 16 are the cleavage products of GVP 6) and GVP 9 (■) during infection. Cultures of MDBK cells were infected with BHV-1 at an m.o.i. of 10 p.f.u./cell. At 2 h intervals [35S]methionine was added to the cultures. After incubation for an additional 2 h the cells were harvested and the individual glycoproteins precipitated from samples of the solubilized cells were analysed by PAGE and autoradiography. The conditions of immunoprecipitation ensured quantitative precipitation of the target protein, and each lane contained immunoprecipitates from the same number of cells. The relative amount of radioactivity precipitated in each sample was estimated by scanning the autoradiogram in a Helena Quickscan Junior densitometer. For each glycoprotein the absorbance scale on the densitometer was calibrated by setting the absorbance of the most dense band to 100%. Analysis of autoradiograms exposed for twice the time period did not alter the results of the analysis.

Table 2. Recombination between ts mutants of BHV-1*

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Mitogen</th>
<th>Reversion frequency of mutant in self-crosses</th>
<th>Recombination frequencies† from cross between ts mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TSA</td>
<td>TSB</td>
</tr>
<tr>
<td>TSA</td>
<td>BUdR</td>
<td>&lt;0.00016</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(0.15)</td>
</tr>
<tr>
<td>TSB</td>
<td>BUdR</td>
<td>&lt;0.00014</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2.46)</td>
</tr>
<tr>
<td>TSC</td>
<td>NTG</td>
<td>&lt;0.0001</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(3.9)</td>
</tr>
<tr>
<td>TSD</td>
<td>BUdR</td>
<td>&lt;0.00019</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(3.9)</td>
</tr>
<tr>
<td>TSE</td>
<td>NTG</td>
<td>&lt;0.00065</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1.69)</td>
</tr>
<tr>
<td>TSF</td>
<td>NTG</td>
<td>&lt;0.00016</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1.69)</td>
</tr>
<tr>
<td>TSG</td>
<td>NTG</td>
<td>0.004</td>
<td>-</td>
</tr>
</tbody>
</table>

* MDBK cells were infected with the individual mutants or with mixtures of the mutants at an m.o.i. of 1 p.f.u./cell. After incubation for 3 h at 32 °C the infected cells were harvested and assayed for p.f.u. at 32 °C and 40 °C. Each analysis was performed in triplicate.

† Recombination frequency defined in Methods.
‡ Numbers in parentheses are standard deviations.

avoid effects due to differences in the amount of infecting virus we compared DNA synthesis in cells maintained at either temperature but infected under identical conditions with the same mutant or wt virus. Infected cells maintained at the NPT were labelled with [3H]dT, while parallel cultures of infected cells were incubated at the PT in the presence of [14C]dT. The cells from each paired set were then mixed and the DNA was analysed on isopycnic CsCl gradients, in which cellular DNA can be separated from the more dense virus DNA. At either temperature, in BHV-1 strain P8-2-infected cells (Fig. 2a), only viral DNA was labelled with...
Regulation of BHV-1 glycoprotein expression

Fig. 2. DNA synthesis in cells infected with wt and ts BHV-1. Infected cells (a, P 8-2 strain; b, TSA mutant; c, TSB mutant; d, TSC mutant) maintained at 32 °C were labelled with [14C]dT while parallel cultures incubated at 40 °C were labelled with [3H]dT. The cells from matched sets were mixed after harvesting and analysed on CsCl gradients. The 3H and 14C content of the individual fractions was estimated and are represented here as a percentage of the radioactivity in the most radioactive fraction of the gradient. The results have been corrected for 'spillover' between the 3H and 14C channels of the scintillation counter. Arrowheads indicate the position of virus DNA and arrows show the position of host cell DNA. NPT (○) and PT (●).

Radioactive thymidine. The mutants exhibited one of three patterns of DNA synthesis (Fig. 2b, c and d). The mutant TSA was not affected at the NPT (Fig. 2b). The mutants, TSB (Fig. 2c), TSE and TSG synthesized virus DNA at the NPT but, unlike the wt virus, were unable to turn off host DNA synthesis. Radioactive host DNA synthesized in these cells at the NPT banded in CsCl at a density that was less than that of virus DNA. The mutants TSC (Fig. 2d) and TSF did not synthesize viral DNA at the NPT.

Glycoprotein synthesis by DNA- mutants

To determine whether at the NPT the DNA- mutants TSC and TSF were arrested in the early phase of gene expression, glycoprotein synthesis in cells infected with the wt or mutant viruses was examined (Fig. 3). There were no differences in the amounts of the three glycoproteins synthesized, at either 32 °C or 40 °C by the wt virus (Fig. 3a). In contrast, the two mutants, when grown at the NPT, either synthesized greatly reduced levels of GVP 9 or failed to synthesize detectable levels of this glycoprotein (Fig. 3b and c). The synthesis of the glycoproteins GVP 6-11-16 and GVP 11 was not affected at the NPT.

DISCUSSION

Among virus-induced glycoproteins, those expressed on the surface of the host cell early in the infectious process would be of particular interest for the following reasons. (i) During virus replication after a primary infection, early glycoproteins would be the first virus gene products recognized by the afferent arm of the host's immune system. (ii) During subsequent infections or episodes of reactivation, early glycoproteins would again be the first virus targets available to the efferent arm of the immune system. (iii) Early glycoproteins may be the only targets
Fig. 3. Glycoprotein synthesis at 32 °C and 40 °C in cells infected with wt (a) and DNA−ts mutants of BHV-1 (b, TSF; c, TSC). Infected cells maintained at 32 °C and 40 °C were labelled with [35S]methionine. At 36 h p.i. the cells were harvested and the individual glycoproteins were precipitated and analysed by PAGE. The conditions of immunoprecipitation ensured quantitative precipitation of the target protein, and each lane contained immunoprecipitates from the same number of cells. Precipitates in odd-numbered lanes were from cells grown at the PT (32 °C) while those in even-numbered lanes were from cells grown at the NPT (40 °C). Whole cell lysates (lanes 1 and 2) were immunoprecipitated with MAbs against GVP 9 (lanes 3 and 4), GVP 11 (lanes 5 and 6) or GVP 6-11a-16 (lanes 7 and 8).
expressed in the absence of virus DNA synthesis in latently infected cells. (iv) If early glycoproteins had a modulating effect on the host animal's immune response, such interactions would affect the host's response to virus antigens expressed subsequently, and might influence the outcome of infection.

Viral glycoprotein expression in BHV-1-infected cells is temporally regulated such that the glycoproteins GVP 6-11-16 and GVP 11 are synthesized in the early stages of infection, whereas GVP 9 is expressed late in infection (Misra et al., 1981, 1982; Ludwig & Letchworth, 1987). Other herpesviruses also exhibit distinct patterns of glycoprotein expression. In HSV-1- and HSV-2-infected cells the glycoproteins gB, gD and gE are synthesized early, whereas gC is expressed late (Balachandran et al., 1982; Cohen et al., 1980; Frink et al., 1983). The sequence for the BHV-1 gene for the glycoprotein GVP 6-11-16 (Misra et al., 1988) has considerable homology with the HSV gene for gB (Bzik et al., 1984). In addition, the properties of GVP 11 and GVP 9 suggest that they are analogous to HSV gD and gC (Babiuk et al., 1987).

It therefore appears that the distinct patterns of expression of the homologous glycoproteins are conserved among distantly related herpesviruses. This lends credence to the possibility that the regulation of glycoprotein expression in herpesvirus-infected cells may reflect the distinct roles these glycoproteins may play in the replication, morphogenesis and social interactions of the virus.

The appearance of viral glycoproteins on the surface of infected cells before the virus has had an opportunity to replicate presents a conundrum. We have shown that at least one of the BHV-1-induced glycoproteins, GVP 11, can act as a target for antibody–complement-mediated cytolysis (Misra et al., 1982). If indeed these proteins can act as targets in vivo, it would not be advantageous to the virus to cause the infected cell to become susceptible to cytolysis before virus replication had begun. Such a protein would be selected against, unless of course it provided a function that was vital enough to balance this disadvantage. It is also possible that early glycoproteins do not act as significant immunological targets in vivo. Recently, Rosenthal et al. (1987) have shown that of the major HSV glycoproteins only gC, a late protein, can be recognized by cytotoxic T cells. If the action of cytotoxic T cells is the most important mechanism for the destruction of infected cells, expression of early glycoproteins may not present a significant risk to the virus.

To develop a system which would allow us to examine the role of early glycoproteins and the immunological responses to them, we attempted to isolate ts mutants which at the NPT would express only the two early glycoproteins. Since the initiation of virus DNA replication is often considered a prerequisite for the expression of late genes, we examined DNA− mutants for the absence of the late glycoprotein. At the NPT both DNA− mutants failed to express the late glycoprotein but synthesized the two early glycoproteins normally.

Although the exact mechanism for the switch of gene expression from the early to the late phase is unclear, viral DNA replication is often considered a distinct step that separates the two phases. Although this connection may be fortuitous, the data presented here argue for a more direct link. The two mutants have a recombination frequency of 18% and probably belong to distinct complementation groups. Given the complex nature of viral DNA replication, while it is not surprising for DNA− mutants to belong to two, or even several, complementation groups, it is unlikely that both mutants would have the same effect on the switching on of a late gene unless DNA replication was a necessary prerequisite for late expression.

The HSV glycoprotein gD, which is synthesized at early times after infection, is considered to be a βf protein rather than an early or β protein (Johnson & Spear, 1984). This is because although it is synthesized only at early times, the synthesis of its mRNA continues through the infectious process. In addition, although the synthesis of gD continues in the absence of viral DNA replication it does so at reduced levels. When DNA replication was inhibited with cytosine arabinoside BHV-1-infected cells also expressed reduced levels of the early glycoproteins GVP 6-11-16 and GVP 11, suggesting that like gD these may also be βf proteins. However, at the NPT the mutants TSC and TSF continued to synthesize normal levels of GVP 6-11-16 and GVP 11. It is possible that the cessation of DNA replication and consequently the continued expression of early proteins and absence of late proteins may be more complete in
these cells than those treated with inhibitors. The mutants should, therefore, allow us to study selectively the interactions of early glycoproteins expressed on cells in a 'natural' context.

This study was supported by a grant to V. Misra from the Medical Research Council of Canada.

REFERENCES


