Proteins Specified by the Short Unique Region of the Genome of Pseudorabies Virus Play a Role in the Release of Virions from Certain Cells

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Two pseudorabies virus vaccine strains (Bartha and Norden) that have a similar deletion in the short unique (U_S) region of the genome have been identified previously (B. Lomniczi, M. L. Blankenship, and T. Ben-Porat, *J. Virol.* 49:970-979, 1984). These strains do not code for the glycoprotein gI, a glycoprotein that has been mapped on the wild type virus genome by T. C. Mettenleiter, N. Lukacs, and H. J. Rziha (*J. Virol.* 53:52-57, 1985) to the sequences deleted from the vaccine strain. Restoration of these deleted sequences to the Bartha strain genome restores to the virus the ability to specify the gI glycoprotein. The Bartha vaccine strain grows as well as wild-type virus in pig kidney and in rabbit kidney (RK) cells, but is not released efficiently from and forms small plaques in RK cells. The rescued Bartha 43/25a strain (which has an intact U_S) is released considerably more efficiently than the Bartha vaccine strain, but less efficiently than wild-type virus from RK cells; it also forms larger plaques on RK cells than does the parental Bartha vaccine strain. The Norden vaccine strain, which has a deletion in the U_S , is released better from RK cells than is the Bartha strain, but not as well as is wild-type virus. We conclude that whereas the sequences in the U_S that are deleted from the Bartha and Norden strain genomes specify functions that play a role in the release of virions from some cell types, at least one other function (which is defective in the Bartha strain but not in the Norden strain) also affects release of virus from these cells. Since restoration to the Bartha strain of an intact U_S restores to the virus both the ability to grow in chicken brains (B. Lomniczi, S. Watanabe, T. Ben-Porat, and A. S. Kaplan, *J. Virol.* 52:198-205, 1984) and to be released from RK cells, the possibility that the lack of virulence of the Bartha vaccine strain may be related to its limited release from some target cells is discussed.

Attenuated, avirulent strains of pseudorabies virus (PrV) that grow well in vitro but do not cause disease in swine (their natural hosts) have been isolated (5). Little is known, however, about the molecular basis for the lack of expression of virulence (or neurovirulence) of PrV or, for that matter, of any member of the herpesvirus family. It has been established that the expression of the virus thymidine kinase gene is required for neurovirulence of herpes simplex virus and PrV (4, 13, 14, 18, 19; for review, see reference 10). Furthermore, a few reports have appeared describing attempts to localize the genes involved in the neurovirulence of herpes simplex virus (3, 20, 21) and of PrV (2, 12, 13), but the functions involved in virulence, as well as the detailed interactions of the avirulent mutants with their hosts, remain to be elucidated.

We showed previously that virulence of PrV, not surprisingly, is determined multigenically; i.e., more than one gene required for the expression of virulence may be defective in a particular vaccine strain (13). A region in the short unique sequence (U_S) which had been deleted from the genomes of two avirulent strains and which plays a role in virulence was identified (2, 12, 13). The expression of the genes located in this region does not appear to be essential for virus growth in vitro. Several mRNAs originate from this region (13); recently, Mettenleiter et al. (15) mapped a glycoprotein (gI) to this region of the genome.

Marker rescue involving cotransfection of the PrV Bartha strain genome with a restriction fragment of wild-type DNA which spans the sequences of the genome that are deleted from the U_S region of the Bartha strain resulted in the

isolation of a population of virions (Bartha 43/25a) to which an intact U_S had been restored (13). These virions, though avirulent, have the ability to replicate in the brains of chickens after intracerebral inoculation, in contrast to the parental Bartha strain, which does not have this ability.

We report here that the rescued Bartha 43/25a (to which an intact U_S has been restored) synthesizes the gI glycoprotein, whereas the Bartha and Norden vaccine strains (from which part of the U_S has been deleted) do not. These results are expected because this protein was mapped by Mettenleiter et al. (15) to the genome sequences deleted from the Bartha and Norden strains. Furthermore, the Bartha 43/25a virions form larger plaques on rabbit kidney (RK) cells than do the parental Bartha virions; in contrast to the Bartha vaccine strain, which is inefficiently released from RK cells, the rescued Bartha 43/25a is released relatively efficiently from these cells. We conclude from these results that the gene products specified by the region of the genome spanning 0.855 to 0.882 map units play a role in the ability of the virus to be released from certain types of cells, as well as in the ability of the virions to replicate in chicken brains, as reported previously (13).

MATERIALS AND METHODS

Virus strains. PrV (Ka) is a strain which has been carried in our laboratory for 25 years; its origin is uncertain (8). The Norden and the Bartha strains were obtained from P. S. Paul; the origins of these strains have been described previously (17). The Bartha 43/25a strain is a Bartha strain to which an intact U_S has been restored by marker rescue (13).

Media and solutions. The following abbreviations for media and solutions are used: ES, Earle saline; EDS, Eagle

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FIG. 1. PAGE of glycoproteins of various PrV strains. PK cultures were infected and labeled with [3 H]glucosamine. Extracellular virions were purified as described in Materials and Methods.

synthetic medium plus 3% dialyzed bovine serum; EDS-FU, EDS plus 5-fluorouracil (20 μ g/ml) and thymidine (5 μ g/ml); PBS, 0.136 M NaCl–2.6 mM KCl–8 mM Na_2HPO_4 –1 mM KH_2PO_4 –20 mM MgCl_2 –1.8 mM CaCl_2 (pH 7.0); TBS, the same as PBS, but the phosphate is replaced by 0.01 M Tris hydrochloride; TBSA, the same as TBS, plus 1% crystalline bovine albumin; NT buffer, 150 mM NaCl–50 mM Tris–0.05% Triton X-100.

Radiochemicals. [3 H]thymidine (specific activity, 45 Ci/mmol) was purchased from Schwarz/Mann, and [3 H]glucosamine (specific activity, 32 Ci/mol) was purchased from New England Nuclear Corp.

Treatment of cells to inhibit cellular DNA synthesis. In the experiments dealing with the synthesis of virus DNA, the cultures were pretreated with EDS-FU for 24 h before the experiment. This treatment completely inhibits cellular DNA synthesis in RK cells without affecting virus DNA synthesis or the production of infectious virus (7).

Labeling and purification of virions. A continuous line of pig kidney (PK) or primary RK cells were infected (multiplicity, 5 PFU/cell) and incubated for 48 h in EDS containing either [3 H]thymidine or [3 H]glucosamine (50 μ Ci/ml). To obtain intracellular virus, the cells were sedimented by centrifugation at $1,000 \times g$ for 5 min, suspended in TBSA, and sonicated, and the virions were purified as described previously (1). The extracellular virus was purified from the supernatant fluid obtained after sedimentation of the cells.

Preparation of cell extracts for immunoprecipitation. Infected cells labeled with [3 H]glucosamine (50 μ Ci/ml) were scraped into the medium 48 h after infection and centrifuged at $1,000 \times g$ for 5 min. The cells in the pellet were suspended in PBS. The virus in the supernatant fluid was collected by

centrifugation at 13,500 rpm for 1 h in an SW50.1 rotor, suspended in PBS, mixed with the cell suspension, and sonicated. Triton X-100 (final concentration, 1%) was added, and the samples were incubated for 20 min at 37°C and centrifuged at $20,000 \times g$ for 1 h. The supernatants (containing at least 90% of all of the [3 H]glucosamine-labeled macromolecules in the samples) were used for immunoprecipitation.

Immunoprecipitation with monoclonal antibodies. Immunoprecipitation with monoclonal antibodies was performed essentially as described by Kessler (9). In brief, the samples in PBS containing 0.2% Triton X-100 were incubated with the appropriate monoclonal antibody for 90 min on ice. An excess of freshly washed, Formalin-treated *Staphylococcus aureus* Cowan strain was added to the samples, which were kept on ice for 30 min. The samples were centrifuged for 1 min in an Eppendorf centrifuge. The pellets were washed six times with NT buffer and resuspended in 6 M urea–2% sodium dodecyl sulfate, boiled for 3 min, and centrifuged for 1 min in an Eppendorf centrifuge, and the supernatant was collected and analyzed by polyacrylamide gel electrophoresis (PAGE).

The monoclonal antibodies against gI were the generous gift of H.-J. Rziha. The characteristics of monoclonal antibody against the gII and gIII glycoproteins have been described previously (6); the two monoclonal antibodies against gIII (M4 and M7) used in this study react with different epitopes of the gIII glycoprotein (M. Gurish, personal communication).

PAGE. Urea and sodium dodecyl sulfate (final concentrations, 1 M and 2%, respectively) were added to the protein samples, which were reduced by incubation with dithioerythritol (final concentration, 0.01 M) at 37°C for 30 min. The proteins were then electrophoresed on acrylamide gels as described previously (11).

RESULTS

Restoration of an intact U_S to the Bartha strain genome restores its ability to specify the gI glycoprotein. Virus glycoproteins are of particular interest in studies of pathogenesis because virus-host cell receptor interactions are probably determined by glycoproteins and may therefore play a role in determining the host range specificity of the virus. With the help of monoclonal antibodies, we previously identified five different glycoproteins (gI through gV) of PrV, one of which is processed into three antigenically related polypeptides of different sizes (6). To determine whether differences could be detected in the glycoprotein component of wild-type and vaccine virus strains, we analyzed the glycoproteins of these virions by PAGE. Figure 1 shows the autoradiographic image of [3 H]glucosamine-labeled, purified wild-type PrV [PrV (Ka)], and of two vaccine strains, Bartha and Norden, which have deletions in the U_S region of their genomes (0.855 to 0.882 map units). The glycoproteins of Bartha 43/25a (a Bartha strain to which an intact U_S has been restored by marker rescue [13]) are also shown. The main difference observed between the PAGE patterns of the glycoproteins of the four strains is that the 98K (gIII) glycoprotein appears to be underrepresented in both the Bartha and the Bartha 43/25a strains. The reason for the underrepresentation of the gIII glycoprotein is not clear; gIII maps in the U_L of the genome (A. Robbins, personal communication), and the defects in this region of the genome in the Bartha strain have not yet been identified. On the other hand, Mettenleiter et al. (15) have mapped a 130K glycoprotein, designated gI or gA (6, 15), to the region of the

genome which has been deleted from the Bartha and Norden strains. These virions should therefore not specify this glycoprotein. The absence of this glycoprotein in the virion of the Bartha and Norden vaccine strains cannot, however, be detected by PAGE, probably because the gI (130K) glycoprotein is obscured by the presence of the 125K component of the gII glycoprotein (6).

That the gI glycoprotein is indeed not synthesized by cells infected with PrV strains from which 0.855 to 0.882 map units has been deleted is shown in Table 1 and Fig. 2, which summarize the results of a representative immunoprecipitation experiment. Whereas cells infected with the Bartha and Norden vaccine strains (which have a deletion in the U_S) did not synthesize the gI (130K) glycoprotein, cells infected with Bartha 43/25a (to which an intact U_S has been restored) did synthesize this glycoprotein. Thus, as expected, after restoration of the region of the genome deleted from the Bartha strain, the ability to specify the gI glycoprotein is restored.

Figure 2 and Table 1 also corroborate the finding illustrated in Fig. 1 which shows that both the parental Bartha vaccine strain and the Bartha 43/25a strain (which has an intact U_S) underproduce the gIII (98K) glycoprotein, as determined by immunoprecipitation with two different monoclonal antibodies against this glycoprotein. As mentioned above, the reason for the underproduction of the gIII glycoprotein by Bartha-infected cells remains to be ascertained.

Comparison of the growth characteristics of the Bartha and Bartha 43/25a strains. The attenuated Bartha and Norden strains grow well in PK cells; the virus titer obtained in these cells is approximately the same as that of wild-type PrV (Ka) virus. On the other hand, only low virus yields were obtained when the Bartha strain was similarly grown in RK cells (Table 2). Indeed, cultures of RK cells infected with this virus strain at a relatively low multiplicity of infection (0.1 PFU/cell) did not show extensive virus-induced cytopathic effects until approximately 1 week after infection, whereas cultures similarly infected with wild-type virus degenerated within 24 h after infection (data not shown).

In contrast to the parental Bartha strain, Bartha 43/25a grew relatively well in RK cells (Table 2). Thus, the lack of ability of RK cells infected with the Bartha strain to yield high titers of infectious virus can be attributed to the absence of functions specified by the region in the U_S that had been deleted from this strain. The Norden strain, however, which

FIG. 2. PAGE of glycoproteins immunoprecipitated by monoclonal antibodies. The immunoprecipitates were obtained as described in footnote *a* of Table 1. Shown are extracts from cells infected with PrV(Ka) (lane 1), Bartha (lane 2), Bartha 43/25a (lane 3), and Norden (lane 4). The lanes marked gII were immunoprecipitated with M5. This monoclonal antibody precipitates the gII complex (125K, 74K, 57K) from virions (6). Another glycoprotein (50K) coprecipitates from extracts of infected cells. The lanes marked gIII were immunoprecipitated with M7. The lanes marked gI were immunoprecipitated with monoclonal antibody 1/14.

lacks similar sequences in the U_S, yielded infectious virus with titers that were only slightly lower than those yielded by wild-type virus. The possible reason for this observation is discussed below.

Synthesis of virus DNA in RK cells infected with the Bartha strain. To determine the basis for the low titer of infectious virus produced by RK cultures infected with the Bartha strain, we first determined whether these cells synthesize virus DNA. Table 3 shows that the amount of virus DNA synthesized by either RK or PK cells infected with all four strains used in this study was approximately the same. Furthermore, in contrast to cell DNA, of which more than 95% was rendered acid soluble by digestion with DNase (data not shown), a relatively large proportion of the virus DNA synthesized by cells infected with all four PrV strains became insensitive to the action of DNase by 48 h after infection. These results show that, despite the low titer of infectious virus produced by cultures of Bartha-infected RK

TABLE 1. Immunoprecipitation of glycoproteins from extracts of cells infected with different strains of PrV^a

Cells infected with:	Immunoprecipitation by monoclonal antibodies ^b (cpm)					
	gI		gII		gIII	
	1/14	3/6	M5	M3	M7	M4
PrV (Ka)	8,900	4,950	18,350	13,850	47,413	14,370
Bartha	795	520	13,747	15,370	2,722	1,365
Bartha 43/25a	5,620	3,970	12,534	12,130	3,707	1,430
Norden	807	730	21,727	14,160	23,709	8,750

^a PK cells were infected (multiplicity of infection, 10 PFU/cell) with the indicated strains of PrV. After 1 h of adsorption, the cells were washed and incubated at 37°C for 48 h in EDS containing [³H]glucosamine (50 µCi/ml). Extracts were prepared and immunoprecipitated as described in Materials and Methods.

^b Background (i.e., cell extracts immunoprecipitated with a monoclonal antibody against an unrelated cellular protein) was approximately 500 cpm and was not subtracted.

TABLE 2. Production of infectious virus by cells infected with different strains of PrV^a

Infecting virus	PFU/ml	
	PK cells	RK cells
PrV (Ka)	8 × 10 ⁸	8 × 10 ⁸
Bartha	9 × 10 ⁸	1 × 10 ⁶
43/25a	7 × 10 ⁸	1 × 10 ⁸
Norden	8 × 10 ⁸	2 × 10 ⁸

^a PK or RK cultures were infected with the appropriate virus strain at multiplicity of infection of 0.1 PFU/cell. After 1 h of adsorption, the cells were washed extensively to remove unadsorbed virus and further incubated for 48 h. The cells were scraped into the culture fluid and sonicated, cellular debris was removed by centrifugation (1,500 × g for 10 min), and the virus yield was determined by plaque assay on RK cells.

TABLE 3. Synthesis of virus DNA by cells infected with various strains of PrV^a

Infecting virus	PK cells		RK cells	
	Total (cpm × 10 ³)	DNase resistant (%)	Total (cpm × 10 ³)	DNase resistant (%)
PrV (Ka)	520	42	190	53
Bartha	460	46	150	56
Bartha 43/25a	370	52	205	47
Norden	690	38	220	43
Mock infected	<1	ND ^b	<1	ND

^a RK cultures were pretreated with ELS-FU for 24 h to inhibit cellular DNA synthesis. They were then infected (multiplicity of infection, 5 PFU/cell) and incubated in EDS containing [³H]thymidine (50 µCi/ml). At 48 h after infection, the cells were scraped into the medium and sonicated. Portions were taken to determine the amount of [³H]thymidine incorporated into acid-precipitable material before and after treatment with DNase (10 µg/ml). Part of the samples was also treated with sodium dodecyl sulfate (1%) mixed with [¹⁴C]thymidine-labeled cellular DNA and centrifuged in a CsCl density gradient, and the amount of [³H]thymidine incorporated into virus and cell DNA was determined. In all cases all the [³H]thymidine label was found associated with virus DNA; none was found in cell DNA.

^b ND, Not determined.

cells, the infective process proceeds normally in these cells up to and including encapsidation of the virus DNA.

Bartha strain is not released efficiently from RK cells. Since virus DNA synthesis and encapsidation occur normally in RK cells infected with the Bartha strain, the low yield of infectious virus produced by these cells (Table 2) could be due to (i) a lack of formation of infectious virions or (ii) a lack of release of virions from the cells (at the low multiplicity of infection used to propagate the virus, recycling may not have occurred).

To distinguish between these possibilities, we determined whether release of virions occurred in RK cells infected with the Bartha strain. Table 4 shows that the total number of virions produced by either RK cells or by PK cells infected with any one of the four different virus strains was approximately the same. However, the Bartha strain was not released as efficiently from the RK cells as were the other virus strains.

Bartha 43/25a was released from the RK cells much more efficiently than was the parental Bartha strain. These results show that the genes in the U_S which were deleted from the genome of the Bartha strain (and restored to Bartha 43/25a), although not required for the processes leading to the replication of the virus, play a role in the release of the virions from certain cell types; they appear to be required for release of the virus from RK cells but not PK cells.

Interestingly, the Norden strain, which has a deletion in the U_S similar to that of the Bartha strain (12), was released relatively efficiently from RK cells (Table 4). This observation indicates that at least two functions may affect the release of virus from RK cells, but that the expression of just one function still allows partial release of the virus. Indeed, both the Norden and Bartha 43/25a, although released more efficiently than the Bartha strain, were not released as well as PrV (Ka). Thus, the Bartha strain appears to be defective in at least two functions necessary for release of the virus from RK cells; restoration of the function normally present in the U_S allows partial release of the virus. Because the Norden strain is also partially released from RK cells, the other region(s) of the genome involved in release must be functional.

Plaque sizes of virus strains on RK cells. Since the Bartha

strain is not released efficiently from RK cells, whereas the Bartha 43/25a strain is, one may expect differences in the plaque sizes that these strains produce in RK cells. Figure 3 shows that the Bartha strain produces small plaques (<1 mm) and that PrV (Ka) produces much larger plaques (>3 mm). Bartha 43/25a produces plaques that are intermediate in size. The plaques produced by Bartha 43/25a are of about the same size (2 to 2.5 mm) as those of the Norden strain. Thus, the strains that are released relatively well from RK cells (Table 4) are also those that form larger plaques on these cells (Fig. 3).

DISCUSSION

As part of a study designed to identify the genes responsible for the virulence of PrV, we have examined several aspects of the biology and molecular biology of avirulent vaccine strains. We reported previously that the genomes of two vaccine strains (Norden and Bartha) have deletions in the U_S between 0.855 and 0.882 map units. This deletion appears to be a characteristic of these attenuated strains (12) and has not been found in any wild-type strains isolated in the United States and in Europe (>100) that we have analyzed (unpublished results).

Recently, Mettenleiter et al. (15) mapped the gI PrV glycoprotein to the region of the genome deleted from the Norden and Bartha strains. Our results show that, not unexpectedly, cells infected with these strains do not synthesize this glycoprotein. A similar result has also been reported by Mettenleiter et al. (16). Furthermore, restoration of the sequences deleted from these strains restores to the virus the ability to synthesize the gI glycoprotein.

The Bartha and Norden vaccine strains grow well in several cell types in vitro. Thus, in PK cells the vaccine virus strains grow well, the virus can recycle in these cells, and virus yields similar to those of wild-type virus are produced. On the other hand, only a low level of infectious Bartha virus is produced by RK cells infected at a low multiplicity (Table 2). This is probably related to the limited release of the virus from these cells and its consequent slow recycling. Indeed, RK cells infected with either the Bartha or the PrV (Ka) strain at high multiplicities (20 PFU/cell) produce similar numbers of infectious virions (as assayed after sonication of these cells at 48 h postinfection [data not shown]). We conclude that whereas the Bartha vaccine strain is synthesized normally by RK cells, it is not released efficiently from these cells.

The slow release of the Bartha strain from RK cells is

TABLE 4. Release of different strains of PrV from infected RK and PK cells^a

Cells	Virus	PrV (Ka)	Bartha	Bartha 43/25a	Norden
RK	Extracellular	60 (72.5)	4 (4.5)	29 (36.4)	24 (33.4)
RK	Intracellular	23	85	51	48
PK	Extracellular	85 (42.8)	50 (48.5)	37 (39.9)	17 (25.8)
PK	Intracellular	113	53	56	49

^a Cells were pretreated with ELS-FU to inhibit cellular DNA synthesis. They were then infected (multiplicity of infection, 5 PFU/cell) and incubated in EDS containing [³H]thymidine (50 µCi/ml). At 48 h after infection, the extracellular virus in the medium and the intracellular virus obtained after sonication of the cells were collected separately, and the virions were banded in sucrose gradients as described in Materials and Methods. The amount of radioactivity associated with the virus peaks was determined. The numbers in parentheses indicated the percentage of total virions (extracellular plus intracellular) that were released. Data are given as cpm × 10³.

FIG. 3. Size of plaques formed by the various virus strains on RK cells. Panels: A, Bartha; B, Bartha 43/25a; C, PrV(Ka); D, Norden.

overcome partially after marker rescue with sequences that span the region of the U_S that have been deleted from the genome of this strain. Thus, Bartha 43/25a is released much more efficiently than is the Bartha strain, but not as well as is wild type PrV (Ka) virus. The Norden strain, which also lacks the same sequences in the U_S as the Bartha strain, is released more efficiently from RK cells than the latter, but not as efficiently as is PrV (Ka). Furthermore, the size of the plaques formed by the Bartha 43/25a and Norden strains in RK cells, although larger than the plaques formed by the Bartha strain, are not as large as those formed by the PrV (Ka) strain (Fig. 3). These observations indicate that both of these strains may express only some of the functions which normally play a role in the release of the virus from RK cells. We conclude that release of virus from RK cells is determined by at least two different functions, one of which is specified by the sequences in the U_S that are deleted from the two vaccine strains.

Both the Bartha 43/25a strain and the Norden strain replicate much better in the brains of chickens than does the

parental Bartha strain (13; unpublished results). A correlation between the ability of the virus to be amplified in RK cultures and its ability to grow in chicken brains appears to exist. We also previously observed a good correlation between the ability of different PrV strains to grow in chicken brains and their virulence for piglets. An intriguing possibility is that, in some cases (for example, in the case of the Bartha vaccine strain), a virus strain is avirulent, at least in part, because although it is synthesized at a normal rate by the target cells, it is not released from these cells. Infection of neighboring cells in the target tissue would therefore be reduced in animals infected with this virus strain. As a consequence, extensive replication and tissue damage would not occur before immunological controls are activated, thereby allowing the survival of the infected animals.

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