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Construction and Properties of Pseudorabies Virus Recombinants with Altered Control of Immediate-Early Gene Expression

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To investigate how altered control of expression of the essential immediate-early (IE) gene of pseudorabies virus influences virus replication and virulence, we replaced the IE promoter with the tissue-specific promoters of the bovine cytokeratin IV gene (CKIV), the bovine cytokeratin VIb gene (CKVIb), or the inducible promoter of *Drosophila* heat shock gene *HSP70*. We compared expression of the IE gene of the wild-type virus and recombinant viruses in different cell types and at different temperatures and found that IE expression had become cell type or temperature dependent. When a recombinant virus was titrated on nonpermissive cells or was titrated at nonpermissive temperatures in vitro, the plating efficiency was reduced by more than 99%. Mice were inoculated subcutaneously (s.c.), intraperitoneally (i.p.), or intranasally (i.n.) with a dose equal to 100 times the 50% lethal dose of the wild-type virus. After inoculation with temperature-sensitive recombinant N-HSP, two (s.c.), two (i.p.), and four (i.n.) of five mice died. However, at this dose, recombinant N-CKIV, which contains a promoter specific for stratified epithelial tissue of the tongue mucosa, was not lethal when inoculated s.c. or i.p. but killed four mice when inoculated i.n. Recombinant N-CKVIb, which contains a promoter specific for the suprabasal layers of the epidermis, was not lethal after inoculation by any of the three routes. In explant cultures of nasal mucosa of pigs, replication of N-CKIV and N-CKVIb was not markedly reduced in the epithelium. However, in contrast to results obtained with wild-type virus, infection of the stroma was not observed. We conclude that the replicative ability and virulence of pseudorabies virus can be influenced by altering control of expression of the IE gene.

Pseudorabies virus (PRV) is an alphaherpesvirus that causes neuropathological disease (Aujeszky's disease) in swine (31). Although swine are the natural host, PRV can infect many animal species (43) and is able to replicate in nearly any cell type in vitro. Pigs are usually infected by inhalation of the virus in aerosols. The epithelial cells of the nasal mucosa are highly susceptible and are the primary site of infection. From here, the virus disseminates to other parts of the body. Virus can be recovered from tonsil, trachea, lung, liver, kidney, lymphatic system, and placental and fetal tissues. However, the most pronounced clinical signs of Aujeszky's disease arise when PRV infection reaches the central nervous system (20).

To control the disease, pigs are vaccinated with attenuated live PRV vaccines (44). These vaccine strains are attenuated by inactivation of one or more genes that encode nonessential proteins. However, inactivation of viral genes often results in a reduced capacity to replicate. In a study of the pathogenicity of different PRV strains, highly virulent strain NIA-3, intermediately virulent strain 2.4N3A, and nonvirulent strain Bartha were compared and it was shown that the degree of virulence was directly related to the ability of these viruses to replicate in the nasal epithelium (35). Reduced replication lowers the viral antigen supply and may impair the immunogenicity of the vaccine. Pensaert et al. (32) suggested that once the functions of viral genes are more clearly defined, it may be feasible to combine full attenuation with preservation of full infectivity at the primary site of infection. However, no viral gene(s) suitable for achievement of this goal has been identified.

Attenuated PRV strains not only are used to induce a protective immune response against Aujeszky's disease but can also be used as a carrier virus to induce an immune response against other pathogens. Recently, PRV strain 783 was used as a carrier virus. The E1 gene of classical swine fever virus was inserted into the gG (gX) locus of PRV (41). Vaccination of pigs with the chimeric virus resulted in a protective immune response against both viruses, demonstrating the potency of PRV as a viral vector.

Other potential applications of attenuated viruses are delivery of heterologous genes in specific cells to complement genetic defects (13), to kill deranged cells (11), or to interfere with the replication of pathogens (2, 45). The use of herpesviruses like PRV to introduce foreign DNA into cells has a number of attractive advantages. These include the broad host range of PRV and its flexible genome size, which allows the insertion of large DNA fragments. Furthermore, the abilities of PRV to infect postmitotic cells, including neurons, and to establish latency are of particular interest.

The use of PRV as a safe carrier requires the introduction of directed limitations in the replication of PRV. In this study, we examined whether attenuation with preservation of full infectivity in specific tissues, or under specific conditions, can be achieved by altering control of expression of an essential gene of the virus. In certain tissues, or under conditions that allow expression of the essential gene, all viral genes are expressed, thereby preserving full infectivity. In other tissues or under conditions that do not allow expression of the essential gene, viral replication is arrested.

We selected the immediate-early (IE) gene as target gene because it is essential and it is synthesized during the first (IE) phase of PRV replication. The gene encodes a 180-kDa protein (14) that is required for initiation of the second (early) and third (late) phases of PRV replication (15, 24). Although the IE protein induces the expression of several early and late

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viral genes, it represses the transcription of its own gene (23). Furthermore, expression of the IE gene results in rapid degradation of cellular polysomes (4, 5). The promoter region of the IE gene has been well characterized (9, 12, 17, 42, 46). To alter control of expression of the IE gene, we selected the tissue-specific promoters of the bovine cytokeratin IV and VIb genes (7), which both belong to the multigene family of intermediate filaments (28, 39). The cytokeratin IV gene is expressed in the muzzle epidermis, hoof pad, and tongue mucosa and in cell line BMGE+H (6, 7). The cytokeratin VIb gene is expressed in the suprabasal layers of the muzzle epidermis but unfortunately not in cultured cell lines (6, 7, 38). The promoter of *Drosophila* heat shock gene *HSP70* (40) was selected as the inducible promoter. The heat shock protein (HSP) is expressed at high levels in various cells and cultured cell lines under conditions of stress such as heat.

MATERIALS AND METHODS

Cells and virus. PRV strain NIA-3 has been described previously (3). NIA-3 was grown on monolayers of SK6 cells (25). Recombinant strains N-CKIV and N-CKVIb were grown on monolayers of bovine mammary gland epithelial (BMGE+H) cells (38). Recombinant virus N-HSP was grown on monolayers of SK6 cells with the temperature raised from 37 to 42°C for 1 to 9 h after infection. Virus stocks were titrated under conditions which induced IE expression. The physical titer of virus stocks was determined by counting virus particles by using an electron microscope. BALB/c 3T3 cells were obtained from the American Type Culture Collection (CCL 163).

Construction of recombinant viruses. Viruses were constructed by overlap recombination using subgenomic fragments C-179, C-27, C-443, and pN3HB as described by van Zijl et al. (41), with the following modifications. To prevent reconstitution of the wild-type virus, we removed the IE gene promoter, which is located on a 660-bp *Hin*dIII-*Bam*HI fragment on cosmid C-443. DNA of cosmid C-443 was digested with *Hin*dIII, and nucleotides were removed from the 5′-protruding ends with the Erase-a-Base system (Promega).

A 2.9-kb *Xho*I fragment which contains the 660-bp *Hin*dIII-*Bam*HI fragment was cloned in pAT153. The resulting plasmid was named pAT-X. A 730-bp *Hin*dIII-*Bam*HI fragment of pAT-X was exchanged with fragments which contained the promoters of the bovine cytokeratin IV and VIb genes and the promoter of *Drosophila* heat shock gene *HSP70*, resulting in plasmids pAT-IV, pAT-VIb, and pAT-HSP, respectively. The CKIV promoter was isolated as a 600-bp *Hin*dIII-*Bgl*II fragment from plasmid pBLCAT 3 (6). The CKVIb promoter was isolated as a 4,600-bp *Hin*dIII-*Bam*HI fragment from plasmid pBLCAT (plasmids were kindly provided by W. Franke), and the subcloned 468-bp *Alu*I fragment containing the *HSP70* promoter (40) was isolated as a *Hin*dIII-*Bam*HI fragment from plasmid pBN247 (kindly provided by P. Krimpenvoort).

For transient expression of the IE gene in eukaryotic cells, we inserted *Bam*HI fragment 8 of PRV, which contains the IE coding information, into the *Bam*HI site of expression vector pEVhis14 (29), resulting in plasmid pEV-IE.

Viruses were constructed in BMGE+H cells by cotransfecting 330 ng each of cosmids C-179 and C-27; 330 ng of modified cosmid C-443; 110 ng of pN3HB; 1 μg of either pAT-X, pAT-IV, pAT-VIb, or pAT-HSP; and 1 μg of pEV-IE. Virus plaques were isolated, plaque purified, and characterized by Southern blot analysis by using ^{32}P-labeled, nick-translated NIA-3 DNA as the probe. All DNA manipulations were performed as described by Sambrook et al. (37).

IE expression. Expression of the IE gene by NIA-3, N-CKIV, and N-CKVIb was measured in quadruplicate in BMGE+H and SK6 cells. Monolayers of both cell types were grown in microtiter culture plates and infected at a multiplicity of infection of 10. For N-CKVIb, the number of physical particles used for infection was equal to that of the NIA-3 virus.

Expression of the IE gene by N-HSP was determined in quadruplicate on SK6 cells. Monolayers of SK6 cells were infected at a multiplicity of infection of 10. The cells were incubated at 37°C or incubated at 42°C after an adsorption period of 30 min at 37°C. The NIA-3 virus served as a control.

At different times after infection, monolayers were washed with phosphate-buffered saline, dried, and stored at −20°C. Expression of the IE gene was determined by using an immune peroxidase monolayer assay with a monospecific, polyvalent rabbit anti-IE serum (described below) and the soluble chromogen 5-aminosalicylic acid as the substrate. A_{450} was measured after 2 h with a Titertek Multiscan. The highest value obtained after infection with NIA-3 under each condition was arbitrarily set to 100.

Monospecific, polyvalent anti-IE serum. A 1.3-kb *Bam*HI-*Pst*I fragment containing the C-terminal part of the IE gene was isolated from *Kpn*I fragment E of PRV and cloned into the polylinker of prokaryotic expression vector pUEX (8). The fusion protein was expressed in *Escherichia coli* DH5α. Rabbits were inoculated with 50 μg of the fusion protein, once in complete Freund's adjuvant and four times in incomplete Freund's adjuvant. Seroconversion was examined by using an immune peroxidase monolayer assay of SK6 cells infected with NIA-3.

Plating efficiencies. The plating efficiencies of N-CKIV and N-CKVIb were examined by counting plaques that developed after plating of the viruses on monolayers of BMGE+H, BALB/c 3T3, and SK6 cells. The plating efficiency of N-HSP was examined under inducing and noninducing conditions. Under noninducing conditions, the virus was absorbed for 1 h at 37°C, whereafter cells were incubated at 37°C until plaques developed. Under inducing conditions, the virus was absorbed for 1 h at 37°C, whereafter the temperature was shifted to 42°C for 1 h. Cells were then incubated for 23 h at 37°C. This incubation schedule was repeated for 3 days, after which plaques had developed.

To examine whether the time span between infection and induction of IE expression influenced the plating efficiency, a time-delayed induction experiment was performed. After virus adsorption for 1 h at 37°C, monolayers were not induced (kept at 37°C) or were induced for 1 h at 42°C after a delay of 1, 2, 3, 4, 5, 6, or 24 h. The number of plaques that developed was determined after 3 days.

Inoculation of mice. The virulence of the recombinant viruses was examined by inoculating 4- to 6-week-old BALB/c mice intraperitoneally (i.p.), intranasally (i.n.), or subcutaneously (s.c.) in the neck. The virus dose administered was 10^4 PFU for NIA-3, N-HSP, and N-CKIV. For N-CKVIb, the same number of physical particles was administered as for NIA-3. Mice were observed every 6 h for clinical signs of disease. Three weeks after inoculation, blood samples of surviving mice were examined for seroconversion by using a commercial enzyme-linked immunosorbent assay (Bommeli, Aujeszky-kit; Hoechst). Subsequently, mice were challenged i.p. with 10^4 PFU of NIA-3.

Nasal mucosa explants. Pieces of nasal mucosa were isolated from the turbinates and nasal septa of specific-pathogen-free pigs as described by Pol et al. (34). The explants, with a total surface area of 2.5 cm^2, were placed in dry 35-mm-diameter macroplates (Costar) and infected with 10^6 PFU of virus in a volume of 1 ml. Explants infected with N-HSP were incubated at 37 or at 42°C after an adsorption period of 1 h at 37°C. Explants were collected at 24 and 48 h after infection, fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin wax. Slices 5 μm thick were processed for immunological staining, and infected areas were identified by using rabbit anti-PRV serum.

RESULTS

Construction of viruses with heterologous IE promoters. To alter control of expression of the essential IE gene, we exchanged the promoter of this gene with two tissue-specific promoters (CKIV and CKVIb) or an inducible promoter (*HSP70*) (outlined in Fig. 1). The viruses were constructed by overlap recombination with subgenomic PRV fragments C-179, C-27, C-443, and pN3HB as described by van Zijl et al. (41). The promoter of the IE gene is located on subgenomic fragment C-443. Immediately upstream of the IE promoter are located two *Hin*dIII sites separated by 73 bp (17). To remove the promoter of the IE gene, we digested cosmid C-443 with *Hin*dIII and sequentially removed nucleotides from the 5′ protruding ends by using the Erase-a-Base system (Promega). When approximately 600 bp were removed, overlap recombinations became unsuccessful even after addition to the recombination mixture of the 2.9-kb *Xho*I fragment (which overlaps the deleted region) of wild-type strain NIA-3. Ihara and Ben-Porat (22) demonstrated that the mutation of a temperature-sensitive IE mutant of PRV could be corrected by marker rescue only when performed at the permissive temperature. This suggested that expression of the biologically active IE protein is required for recombination. We therefore constructed plasmid pEV-IE (see Materials and Methods), which was used to express the IE gene transiently. The recombinant viruses, designated N-CKIV, N-CKVIb, and N-HSP, were generated by cotransfection of pEV-IE with transfection mixtures that contained C-179, C-27, C-443 (treated as described above), pN3HB, and the *Xho*I fragments of plasmid pAT-IV, pAT-VIb, or pAT-HSP. N-CKIV grew well on BMGE+H cells, and the plaque size did not markedly differ from that of the wild-type virus. In contrast, N-CKVIb was difficult to amplify and only after repeated collection of plaques and reinfection of BMGE+H cells was a virus stock obtained. To amplify N-HSP, several temperature regimens were tested. Virus adsorption and growth at 42°C reduced the plating

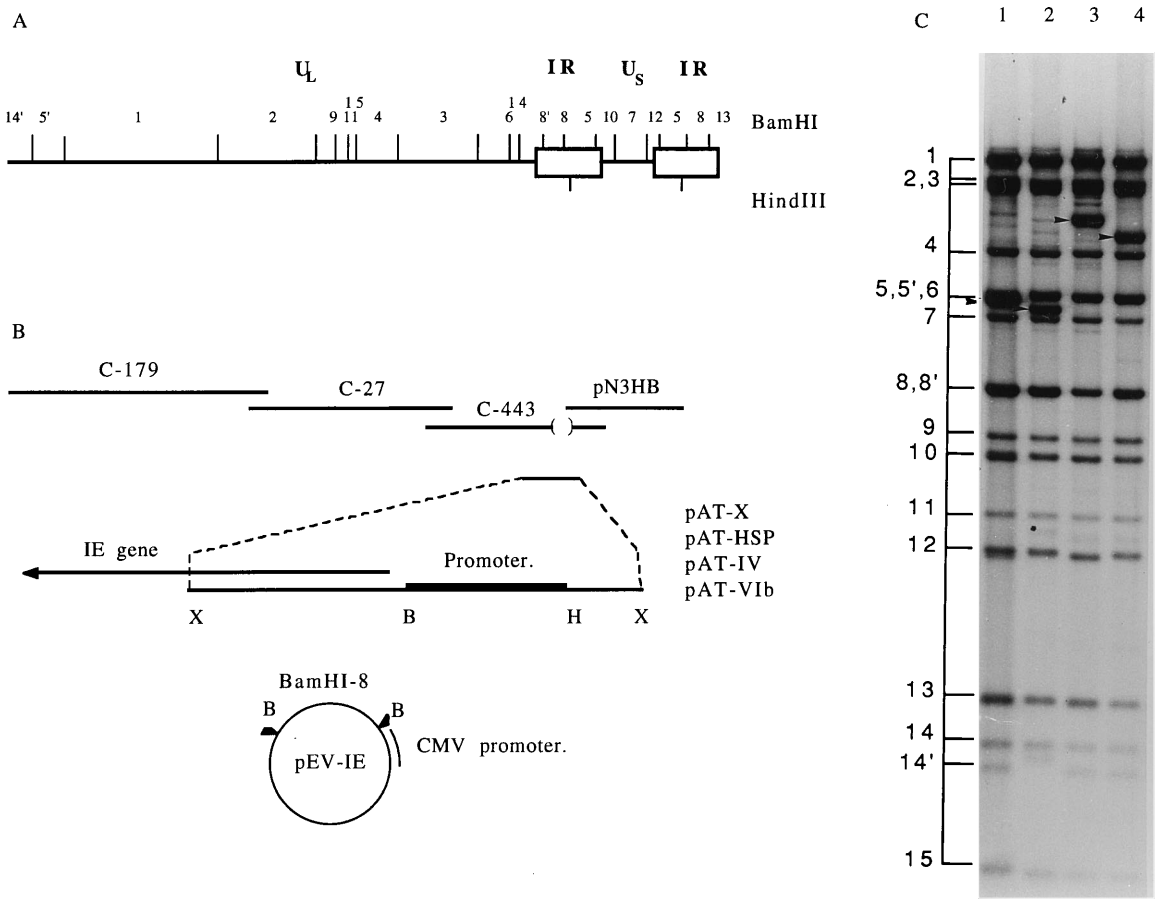


FIG. 1. Construction of recombinant viruses N-HSP, N-CKIV, and N-CKVIb. (A) *Bam*HI map of the genome of NIA-3. U_L, unique long region; U_S, unique short region; IR, inverted repeat. (B) PRV subgenomic fragments used to reconstruct mutant viruses by means of overlap recombination as described by van Zijl et al. (41). The IE promoter was removed from cosmid C-443 by digestion with *Hin*dIII and subsequent removal of approximately 600 bp (Erase-a-Base system [Promega]). Replacement of the IE promoter was performed in the 2.9-kb *Xho*I fragment. pAT-X, subcloned *Xho*I fragment of PRV; pAT-HSP, pAT-CKIV, and pAT-CKVIb, pAT plasmids with the *Xho*I fragment containing the HSP, CKIV, and CKVIb promoters, respectively; -()-, erased sequence. Plasmid pEV-IE was used for transient expression of the IE protein (see text). CMV, cytomegalovirus. (C) Southern blot analysis of recombinant viruses. Viral DNA was digested with *Bam*HI and probed with ^{32}P-labeled, nick-translated NIA-3 DNA. Lanes 1 to 4 contained DNAs of NIA-3, N-HSP, N-CKIV, and N-CKVIb, respectively. *Bam*HI fragment 5 contains the heterologous promoters that control expression of the IE gene. Because of the difference in size between the inserted fragments, *Bam*HI fragment 5 has a different size in each of the recombinant viruses (arrowheads; also, see text).

efficiency of NIA-3. In addition, prolonged incubation of the monolayers at 42°C caused cell damage. We therefore used an adsorption period of 60 min at 37°C followed by amplification of strain N-HSP by an alternating-temperature regimen of 8 h at 42°C and 16 h at 37°C. For titration of N-HSP, an incubation period of 1 h at 42°C after adsorption appeared to be sufficient to induce virus replication.

All mutant viruses were plaque purified three times. Viral DNA was isolated as previously described (16) and analyzed by Southern blotting. Figure 1C shows the restriction patterns of viral DNAs digested with *Bam*HI and hybridized with ^{32}P-labeled, nick-translated NIA-3 DNA. The restriction pattern of wild-type NIA-3 is shown in Fig. 1C, lane 1. In lane 2, the restriction pattern of N-HSP is shown. Because the inserted HSP promoter (450 bp) is smaller than the original IE promoter region (730 bp), *Bam*HI fragment 5 is reduced in size. Lane 3 contains DNA of N-CKIV. Because of the insertion of the *Hin*dIII-*Bgl*II fragment (600 bp), which contains the CKIV promoter, the *Bam*HI site between *Bam*HI fragments 5 and 8 is lost, yielding a fusion fragment migrating between *Bam*HI fragments 3 and 4. Lane 4 contains DNA of N-CKVIb. The insertion of the 4.6-kb CKVIb promoter causes modified *Bam*HI fragment 5 to migrate between *Bam*HI fragments 3 and 4.

Comparison of IE expression. After infection of BMGE+H and SK6 cells with wild-type strain NIA-3 and recombinant strains N-CKIV and N-CKVIb, expression of the IE gene was measured by using an IE-specific immune peroxidase monolayer assay and a soluble chromogen (Fig. 2A to D). As expected, expression of the IE gene was detected quickly after infection of both cell types with NIA-3. At 2 h after infection, the increase in the IE protein started to decline, and at 3 h after infection, the amount of the IE protein reached a constant level. Compared with that in NIA-3-infected cells, expression of the IE gene in BMGE+H cells infected with N-CKIV (Fig. 2A) began slowly and at 3 h after infection started to increase at the same rate as that in NIA-3-infected cells. In contrast, when SK6 cells were infected with this virus, expression of the IE gene remained very low (Fig. 2B). This indicated that IE expression of N-CKIV was cell type dependent.

Expression of the IE gene in N-CKVIb-infected BMGE+H or SK6 cells was low compared with that in NIA-3-infected

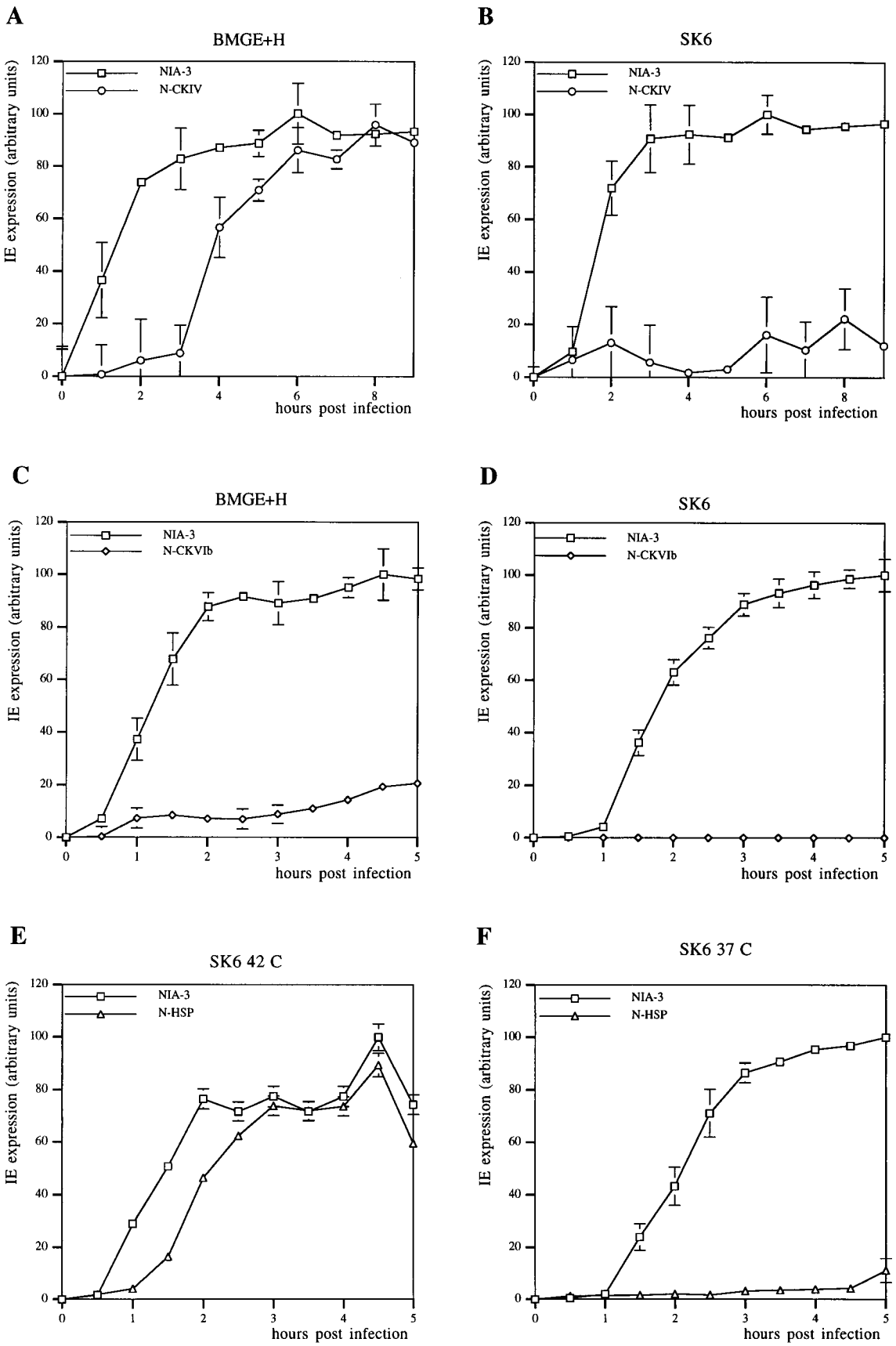

A
BMGE+H
NIA-3
N-CKIV
IE expression (arbitrary units)
hours post infection
B
SK6
NIA-3
N-CKIV
IE expression (arbitrary units)
hours post infection
C
BMGE+H
NIA-3
N-CKVIb
IE expression (arbitrary units)
hours post infection
D
SK6
NIA-3
N-CKVIb
IE expression (arbitrary units)
hours post infection
E
SK6 42 C
NIA-3
N-HSP
IE expression (arbitrary units)
hours post infection
F
SK6 37 C
NIA-3
N-HSP
IE expression (arbitrary units)
hours post infection

FIG. 2. Analysis of expression of the IE gene. Monolayers were infected in quadruplicate at a multiplicity of infection of 10 (CKVIb with the same number of physical particles as NIA-3), and expression of the IE gene was measured at the indicated times postinfection by using an immune peroxidase monolayer assay with the soluble chromogen 5-aminosalicylic acid as the substrate (error bars indicate standard deviations). Comparison of IE expression of the NIA-3 and N-CKIV viruses in BMGE+H (A) and SK6 (B) cells. Comparison of IE expression of NIA-3 and N-CKVIb in BMGE+H (C) and SK6 (D) cells. Comparison of IE expression of NIA-3 and N-HSP in SK6 cells at 42°C (E) and 37°C (F).

cells (Fig. 2C and D), indicating that expression of the IE gene of this virus was not induced in these cell types.

IE expression in SK6 cells infected with NIA-3 and N-HSP was compared after incubating these cells at 37 and 42°C (Fig. 2E and F). At 42°C, the amount of IE protein increased quickly in both NIA-3- and N-HSP-infected cells although expression in N-HSP-infected cells started 30 min later. Expression of the IE gene in NIA-3-infected cells was almost equal at 37 and 42°C, indicating that temperature hardly affected IE expression in NIA-3-infected cells. In contrast, at 37°C, expression of the IE gene in N-HSP-infected cells was hardly detectable. We therefore conclude that expression of the IE gene by N-HSP is temperature dependent.

Plating efficiencies of viruses with induced and noninduced IE expression. Knowing that IE expression of the mutant viruses was dependent on proper induction, we examined the effect of IE expression on the initiation of virus replication. Mutant virus N-CKIV was titrated on BMGE+H, SK6, and BALB/c 3T3 cells. BALB/c 3T3 cells were used as an additional control because it has been demonstrated that the promoter of the bovine cytokeratin IV gene is not stimulated in this cell line (6). Compared with the titer of N-CKIV on BMGE+H cells, the titer on 3T3 and SK6 cells was reduced by more than 99% (Table 1). In contrast, NIA-3 virus titers were similar on BMGE+H and SK6 cells but were 10-fold lower on 3T3 cells. These findings indicated that initiation of replication of N-CKIV had become cell type dependent. When N-CKVIb was titrated on BMGE+H, SK6, and BALB/c 3T3 cells, relatively low titers (4.1, 4.5, and 1.7, respectively) were obtained compared with the number of physical particles (8.7) present in the inoculum. The virus titer of N-HSP at 37°C was reduced by 99% compared with the virus titer obtained after titration at 42°C. NIA-3 virus titers were similar at both temperatures. This finding indicated that the initiation of replication of N-HSP had become temperature dependent.

To examine the influence of delayed induction of IE expression on the plating efficiency of N-HSP, a time-delayed induction experiment was performed with SK6 and BALB/c 3T3 cells. When the delay time was increased, the number of viruses that initiated replication decreased, irrespective of the cell line used (Fig. 3; only plating efficiencies with SK6 cells are shown). After approximately 2.5 h, 50% of the viruses could no longer be activated. At 24 h after infection, induction no longer resulted in an increase over the background level.

Virulence in mice. To examine the effect of the altered control of IE expression on virulence, we infected BALB/c mice with N-HSP, N-CKIV, and N-CKVIb (Table 2). Virulent strain NIA-3 was used as a control. In each experimental group, five mice were inoculated either s.c., i.p., or i.n. with 10^4 PFU. The virus dose of the recombinant strains was equal to approximately 100 times the 50% lethal dose of wild-type strain NIA-3 (5a). As expected, the NIA-3 virus killed all of the mice independently of the route of inoculation. However, the route of inoculation influenced the mean time to death (MTD) and mice died earlier after i.n. and s.c. inoculations (56 and 60 h, respectively) than after i.p. inoculation (71 h).

The N-HSP virus showed reduced virulence compared with NIA-3 and killed only two of five mice when inoculated s.c. or i.p. and four of five mice when inoculated i.n. The MTD after inoculation with N-HSP was also considerably longer than after inoculation with the NIA-3 virus, especially when the virus was inoculated i.n. (96 versus 56 h) or i.p. (138 versus 71 h).

N-CKIV was lethal only when inoculated i.n. and killed four of five mice with an MTD of 108 h. This result indicated that the virulence of N-CKIV was strongly reduced. Nevertheless, mice that died showed clinical signs of disease, such as extensive scratching, which is characteristic for Aujeszky's

TABLE 1. Titers of PRV IE mutant viruses in different cell types or at different temperatures

Virus	No. of pp[a]	Virus titer (log_{10} PFU)				
		BMGE+H	SK6	3T3	37°C	42°C
NIA-3	9.6	7.8	7.7	6.8	7.7	7.7
N-CKIV	9.9	7.5	4.8	4.7	ND[b]	ND
N-CKVIb	8.7	4.1	4.5	1.7	ND	ND
N-HSP[c]	9.3	ND	ND	ND	6.0	8.3

[a] pp, physical particles expressed as log_{10}.
[b] ND, not determined.
[c] N-HSP plating efficiency data have been published previously (18) and were reproduced to complete the data.

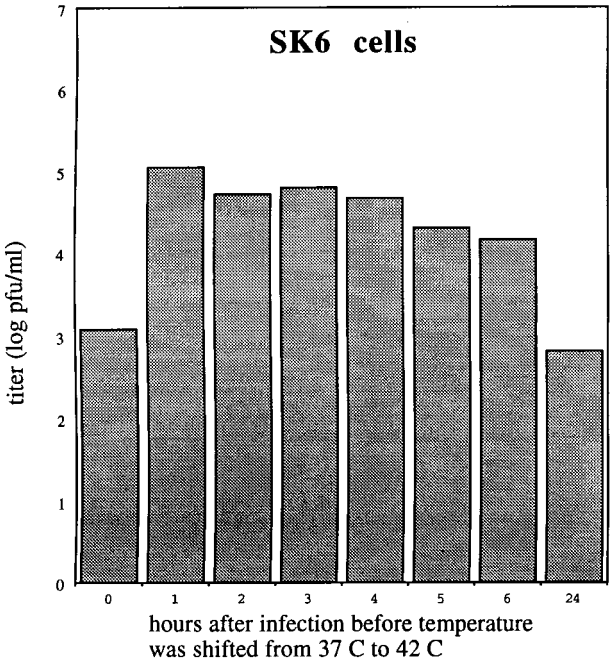


FIG. 3. Influence of delayed induction of IE expression on plating efficiency. The N-HSP virus was plated on monolayers of SK6 cells. One monolayer was incubated at 37°C throughout the experiment (lane 0), while the other monolayers were induced for 1 h at 42°C at 1, 2, 3, 4, 5, 6, and 24 h after infection.

TABLE 2. Virulence of NIA-3, N-HSP, N-CKIV, and N-CKVIb in mice after s.c., i.p., or i.n. application

Virus	s.c.		i.p.		i.n.	
	M, MTD[a]	MTD[b]	M, MTD[a]	MTD[b]	M, MTD[a]	MTD[b]
NIA-3	5, 60 ± 6		5, 71 ± 6		5, 56 ± 3	
N-HSP	2, 78 ± 0	68 ± 3	2, 138 ± 0	72 ± 6	4, 96 ± 12	90 ± 0
N-CKIV	0	72 ± 10	0	92 ± 52	4, 108 ± 7	66 ± 0
N-CKVIb	0	74 ± 9	0	66 ± 0	0	73 ± 3

[a] M, mortality in a group of five mice. All mice received 10^4 PFU of a virus. MTD (± the standard deviation) in hours after inoculation is given.

[b] MTD (± the standard deviation) in hours after i.p. challenge with virulent strain NIA-3 is given. The MTD of control mice was 61.2 ± 2.7 h after challenge.

disease, suggesting that the i.n.-inoculated virus even affected and replicated in neural tissue. The MTD of mice that died after i.n. inoculation with N-CKIV was twice as long (108 versus 56 h) as that of mice after inoculation with NIA-3.

N-CKVIb did not kill any mice, regardless of the route of inoculation, indicating that the virulence of N-CKVIb was even more reduced than that of N-CKIV. Mice that survived infection with N-HSP, N-CKIV, and N-CKVIb showed abnormal behavior, such as repeated tail biting and extensive digging. These symptoms were observed for over 3 weeks until the mice were challenged.

Three weeks after infection, blood samples of all surviving mice were collected and examined for seroconversion. Only three mice, two of which were inoculated i.p. with N-HSP and one of which was inoculated i.n. with N-CKVIb, showed an antibody response against PRV. Because inoculation of mice with 10^4 PFU of NIA-3 is lethal, it was not possible to obtain a positive control serum. Although this control was lacking, we considered a serum response of more than two times the background as positive. All surviving mice were challenged i.p. with 10^4 PFU of NIA-3. Unexposed control mice died with an MTD of 61.2 h. None of the mice that survived inoculation with recombinant virus were protected against the challenge with NIA-3. However, mice that had previously been inoculated s.c. and i.p. with N-HSP or N-CKIV and mice that had been inoculated s.c., i.p., or i.n. with N-CKVIb survived significantly longer ($P < 0.05$; Mann-Whitney test) than the control mice did. This observation suggested that 10^4 PFU of N-HSP, N-CKIV, or N-CKVIb induced a weak immune response in mice.

Infection of porcine nasal epithelium. To examine the effect of the replaced IE promoters on virus replication in cytokeratin-expressing tissues, we infected explants of porcine nasal epithelium with N-CKIV, N-CKVIb, N-HSP, and NIA-3 as described by Pol et al. (34). The course of infection was examined by using immunohistochemical staining of paraffin-embedded sections (Fig. 4).

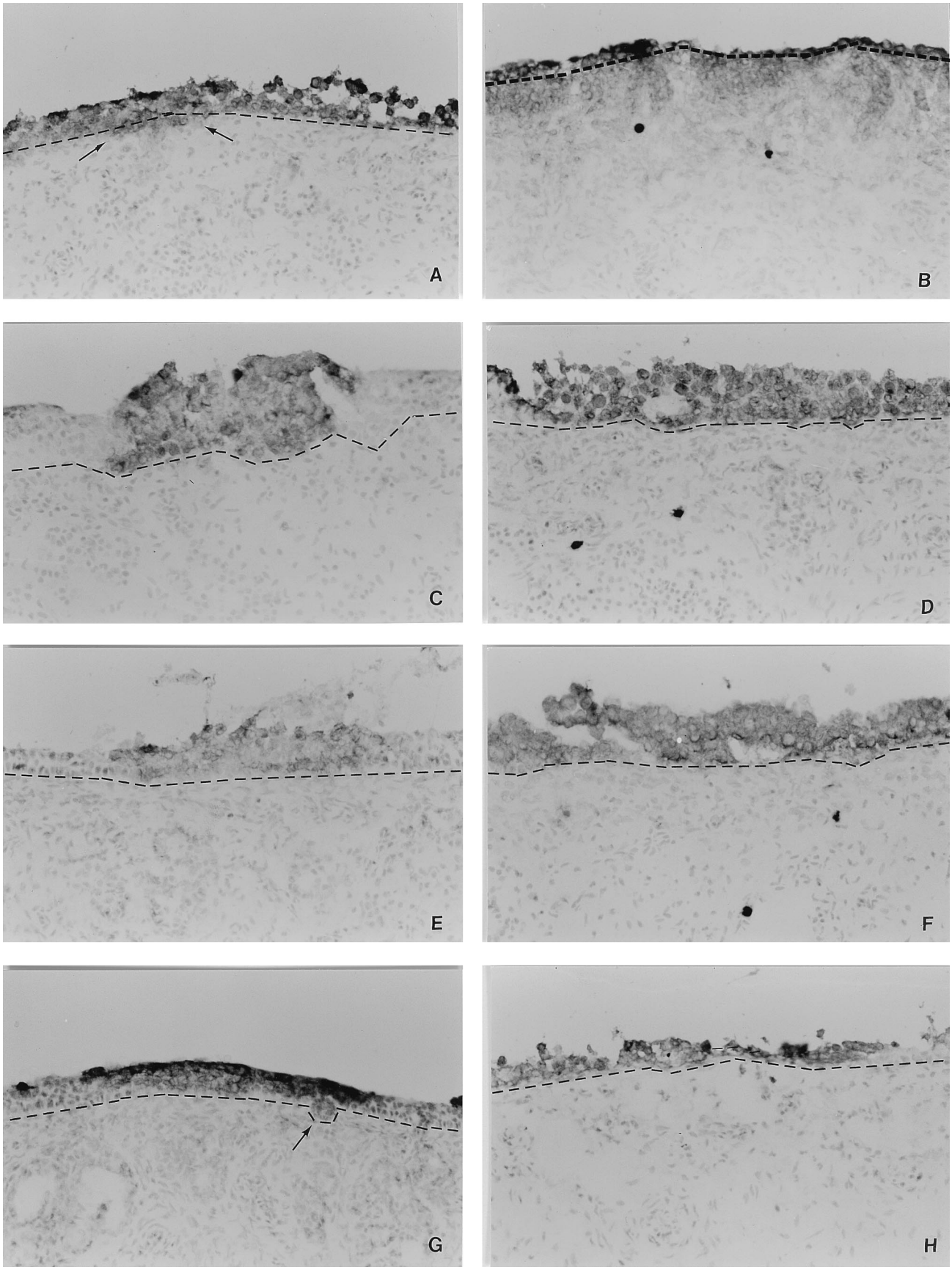
At 24 h after inoculation with NIA-3, viral antigen was detected in most of the epithelial surface and small spots of viral antigen were observed in the stroma (Fig. 4A). At 48 after inoculation, large areas of the epithelium had become infected and NIA-3 had clearly invaded the stroma (Fig. 4B). At 24 h after inoculation, N-CKIV (Fig. 4C) and N-CKVIb (Fig. 4E) were detected in the epithelial surface. At 48 and 72 h after inoculation, these viruses (Fig. 4D and F) had spread through the epithelium to the same extent as NIA-3 (72-h data not shown). Therefore, no significant reduction in virus replication in the nasal epithelium of pigs was observed after replacement of the IE gene promoter by the CKIV or CKVIb promoter. However, in contrast to NIA-3 (Fig. 4A and B), N-CKIV and N-CKVIb did not invade the stroma, suggesting that viral replication was restricted to epithelial cells (Fig. 4C to F). N-HSP infected the epithelium equally well at 37 and at 42°C (only N-HSP 42°C data are shown) (Fig. 4G and H). Because infection with viruses induces synthesis of HSP70 (21, 33), we assume that N-HSP supports its own induction independently of the temperature. The stroma was not invaded by N-HSP at both temperatures at 48 h after infection, indicating that replication of N-HSP was less efficient than that of wild-type PRV.

DISCUSSION

In this study, we examined whether replication of PRV can be regulated by altering control of expression of the essential IE gene. We replaced the promoter of the IE gene with a tissue-specific or inducible promoter. Without expression of the multifunctional IE gene, transition from the IE phase to the early and late phases of PRV replication does not occur, and therefore viral replication is arrested (15, 22). Expression of the IE gene furthermore results in inhibition of cellular protein and DNA synthesis (23) and causes quick degradation of cellular polysomes (4, 5). The promoter-enhancer which controls expression of the IE gene contains several conserved regulatory elements, like transcription factor Sp1 binding sites, inverted TAATGARATTC motifs, CCAAT boxes which bind cellular factors, GCGGAA motifs which are important for induction by binding factor IEF_{ga} (9, 42), and a repeated sequence which is recognized by the IE protein itself and which probably autoregulates IE transcription (46). Replacement of the IE promoter-enhancer region with the heterologous promoters of the bovine cytokeratin IV or VIb gene or *Drosophila* heat shock gene *HSP70* resulted in a viable virus, which demonstrates that the conserved regulatory elements are not essential for lytic replication of PRV. Without knowing the optimal condition for growth, N-CKIV could be amplified to a titer of $10^{7.5}$ in the BMGE+H cell line, whereas N-HSP could be amplified to a titer of 10^8 under induced conditions (42°C) in SK6 cells. The parental NIA-3 virus could be amplified to a titer of 10^9 PFU in SK6 cells. We therefore conclude that replacement of the promoter of the IE gene with the CKIV or *HSP70* promoter only slightly reduces the reproductive capacity of NIA-3 in vitro.

Preservation of the infectious properties of the recombinants was demonstrated in cell culture under conditions that induced expression of the IE gene, as well as in tissue explants of nasal mucosa of porcine origin. In BMGE+H and SK6 cells,

FIG. 4. Photomicrograph (magnification, ×150) of porcine nasal mucosa explants infected with different PRV strains at 24 and 48 h after inoculation. Viral antigens were stained black (immunoperoxidase method). The basal lamina is indicated by a dashed line. (A) NIA-3 infection at 24 h after inoculation. Viral antigens are present in epithelial cells. In one focus, fibroblasts under the basal lamina are infected (arrows). (B) NIA-3 infection at 48 h after inoculation. The number of infected fibroblasts has markedly increased. (C) N-CKIV infection at 24 h after inoculation. Viral antigens are confined to epithelial cells. (D) N-CKIV infection at 48 h after inoculation. The infection has spread in the epithelial cells, but viral antigens are still confined to epithelial cells. (E) N-CKVIb infection at 24 h after inoculation. Viral antigens are confined to epithelial cells. (F) N-CKVIb infection at 48 h after inoculation. The infection has spread in the epithelial cells, but viral antigens are still confined to epithelial cells. (G) N-HSP infection at 24 h after inoculation. Viral antigens are confined to epithelial cells. The arrow indicates glandular epithelial cells. (H) N-HSP infection at 48 h after inoculation. The infection has spread in the epithelial cells, but viral antigens are still confined to epithelial cells.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we observed that the amount of IE protein expressed after NIA-3 infection increased quickly and reached 80% of the maximum amount measured within 3 h. For unknown reasons, in BMGE+H cells infected with N-CKIV, there was a delay of 3 h before IE expression started. However, at 8 h after infection the same amount of IE protein was detected in BMGE+H cells infected with NIA-3 as in BMGE+H cells infected with N-CKIV. The delayed IE expression of N-CKIV might be explained by the stimulatory effect of the IE protein on other promoters (1). In BMGE+H cells, this might result in initially weak induction of the IE gene by the CKIV promoter, which induction is strengthened when enough IE protein has accumulated to stimulate the CKIV promoter. The amount of IE protein expressed by N-HSP-infected SK6 cells after incubation at 42°C equalled that expressed by NIA-3-infected SK6 cells. However, expression was delayed for 30 min. This delay was most likely caused by the 30-min period of adsorption at 37°C which preceded the incubation at 42°C.

Analysis of the plating efficiencies of NIA-3, N-CKIV, and N-HSP revealed that the capacity to initiate infection was dependent on induction of the heterologous promoter that controlled expression of the IE gene. Under conditions of induced IE expression, the plating efficiencies of N-CKIV and N-HSP was similar to that of the wild-type virus. Unfortunately, it was impossible to study the plating efficiency of N-CKVIb since cultured cells that express the CKVIb gene, and thus should stimulate IE expression by N-CKVIb, are not available (7).

Explants of nasal tissues of pigs have been successfully used to demonstrate differences in replication among a virulent PRV strain, a mildly virulent PRV strain, and a nonvirulent PRV strain (34). By using these explants, we showed that in epithelial tissue, N-CKIV and N-CKVIb replicated as efficiently as NIA-3. The same was true for N-HSP and NIA-3 when the explants were incubated at 42°C. The observation that N-CKVIb replicated efficiently in epithelial cells was surprising because in tissue culture cells, N-CKVIb yielded low titers and expressed small amounts of IE protein. Furthermore, N-CKVIb was not lethal for mice. Altogether, the above findings indicate that N-CKIV, N-CKVIb, and N-HSP have preserved the ability to replicate under conditions that stimulate IE expression.

A vast reduction in the replication ability of the recombinants under conditions that did not induce expression of the IE gene was demonstrated in cell culture and in mice. Analysis of the level of IE expression showed that under conditions that did not induce IE expression, the amount of IE protein in monolayers infected with N-CKIV, N-CKVIb, and N-HSP was reduced to less than 20% of the amount measured under induced conditions. Furthermore, titration of the recombinants under conditions that did not induce IE expression resulted in a titer reduction of 99% compared with the titers obtained under conditions that induced IE expression. In addition, an increase in the delay of induction of N-HSP resulted in an increase in the number of abortive infections. The latter finding is in agreement with that of Ihara and Ben-Porat (22), who showed that infections became abortive when the temperature of cells infected with temperature-sensitive mutant tsG_1 was shifted to the permissive temperature at 5 h after infection. These results indicate that the virus is vulnerable in the time between penetration and the onset of replication. After infection of cells, viruses are surrounded by a hostile environment. At the cellular level, antiviral defense is mediated by interferons which induce the synthesis of inhibitors of viral replication, such as the double-stranded RNA-activated inhibitor (19, 27). To counteract host cell defense, viruses produce proteins or RNAs which interfere with the defense mechanisms of the cell. Whether expression of the multifunctional IE protein which causes inhibition of cellular protein synthesis (23) and quick degradation of cellular polysomes (4, 5) is a PRV counter measure against the defense mechanisms of the host cell is unclear. Nevertheless, our results indicate that the potential to initiate a productive infection is strongly reduced under conditions that do not allow IE expression.

Mice are extremely sensitive to PRV; the 50% lethal dose of the NIA-3 virus after s.c. or i.p. inoculation is 120 or 70 PFU, respectively (5a). The conventionally attenuated vaccine strain Bartha is lethal for mice, and even thymidine kinase-negative viruses are still lethal for mice when administered intracerebrally (50% lethal dose, 500 PFU [26]). We found that N-HSP, N-CKIV, and N-CKVIb had reduced virulence for mice. N-HSP was lethal for some, but not all, mice when inoculated s.c., i.p., or i.n. at a dose of 10^4 PFU. At this dose, N-CKIV was lethal only after i.n. inoculation whereas N-CKVIb was not lethal at all. We furthermore found that N-HSP, N-CKIV, and N-CKVIb were able to induce neurological signs of disease without killing mice. This indicates that these viruses affected and most likely replicated in neural tissue without destroying or reaching vital neural centers. However, this was not investigated. Recently, Card et al. (10) and Rinaman et al. (36) described differences between neuronal infections with virulent PRV strain Becker and attenuated strains of PRV. A correlation was found between the lytic effect of virus strains on neurons and the overt symptoms displayed by infected rats. In addition, postmortem examination of mice infected with PRV mutants that lacked envelope protein gD (gp50), gI (gp63), or both revealed that the mutant viruses had infected brain tissue much more extensively than the wild-type virus (30). These findings indicate that infection of the brain by attenuated PRV strains may be accompanied by moderate signs of disease.

We conclude that PRV can be attenuated and that tissue tropism or growth can be influenced by altering control of expression of the essential IE gene. The choice of the inserted promoter determines the limitations of viral replication. Replacement of the promoter of essential genes may therefore contribute to the design of viral vectors, especially when delivery of genetic information at specific locations or under specific conditions is required.

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