distal CAAT box at the −117 position of the $^{A}\gamma$ globin gene) (*6*) with MEL cells. Six hybrids that contained chromosome 11 were analyzed. Three hybrids contained the wild-type chromosome and expressed only adult human globin (Fig. 2D). The other three hybrids contained only the HPFH chromosome (Fig. 3C) and expressed γ globin (Fig. 2, B and D), providing evidence that nondeletion globin developmental mutations are activated in the environment of erythroleukemia cells (*7*).

The activation of both deletion and promoter HPFH-mutated genes, but not wild-type fetal globin genes, in these hybrids suggests that the HPFH lesions affect developmental expression by promoting interactions between the γ genes and the adult erythroid environment. A detailed structural and functional analysis of mutant chromatin may lend insight into how this is brought about. It is difficult to obtain sufficient numbers of pure nucleated cells needed for a study of this nature. In addition, cells with developmental mutations like HPFH are usually heterozygous; the presence of normal genes in the homologous chromosome complicates structural analysis. However, hybrids hemizygous for a mutant chromosome can be easily obtained (either directly or through cell subcloning) in large numbers. We performed DNA methylation analysis on a sequence brought into the vicinity of the γ globin genes by the HPFH deletions. This region has an erythroid-specific methylation pattern (*8, 9*) and acts as an enhancer in transient gene transfer assays (*9*). Juxtaposition of this region with the γ globin genes causes the activation of the γ globin genes in cells with HPFH deletion mutations (*1, 9*). When Bam HI–digested human DNA is hybridized with a probe specific for this region, a fragment of approximately 2.5 kb is seen. When the Hpa II restriction site located within this region is unmethylated, Hpa II digestion truncates this 2.5-kb Bam HI fragment to 2 kb (Fig. 4, lanes 1 and 2). In lymphoid cells, a 2.5-kb fragment is obtained after Bam HI–Hpa II digestion, which indicates that this site is methylated (Fig. 4, lanes 4 and 7). In wild-type lymphoid × MEL hybrids, however, this site was unmethylated in that a 2-kb band was present (Fig. 4, lane 9). In both deletion HPFH × MEL hybrids and promoter mutation HPFH × MEL hybrids this position was also undermethylated (Fig. 4, lanes 5, 6, and 8). Thus, in these cells the juxtaposed enhancer region, normally located 100 kb downstream of the globin cluster, acquired an erythroid-specific methylation pattern.

These observations, like those from nuclease sensitivity studies (*10*), imply that the structure adopted by the human globin cluster within the MEL cell is representative of that seen in normal human erythroid cells. Hybrids of this sort should therefore prove useful in functional analyses of globin gene developmental mutants. Indeed, by fusing lymphocytes from mutant individuals with suitably differentiated, established cell lines, one may be able to activate and therefore study other human developmental mutations.

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11. Supported by NIH grant DK30852.

6 June 1988; accepted 19 September 1988

Protection of Cattle Against Rinderpest with Vaccinia Virus Recombinants Expressing the HA or F Gene

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Rinderpest is a highly contagious ruminant viral disease manifested by a rapid course and greater than 90% mortality. Infectious vaccinia virus recombinants were constructed that express either the hemagglutinin or the fusion gene of rinderpest virus. All cattle vaccinated with either recombinant or with the combined recombinants produced neutralizing antibodies against rinderpest virus and were protected against the disease when challenged with more than 1000 times the lethal dose of the virus.

RINDERPEST, OR CATTLE PLAGUE, IS a devastating animal disease with serious ecological, social, and economic consequences. It is an acute, febrile, and contagious viral disease, primarily of cattle and buffalo, that is characterized by inflammation, hemorrhage, necrosis, and erosion of the gastrointestinal tract. The mortality rate approaches 100%; until recently rinderpest accounted for a loss of over 2 million animals per year (*1*).

The Plowright tissue culture vaccine (PTCV) provides protective immunity for the practical life of the animal and is widely used for vaccination against rinderpest in preference to the caprinized and lapinized vaccines (*2*). Because of economic problems in production of PTCV and logistical problems in delivery to the field, rinderpest is again rampant in Africa and Asia (*3*). Thus, the most effective rinderpest vaccine must possess more practical characteristics such as heat stability and ease of production, transport, and administration in the field, which are our reasons for using vaccinia virus recombinant vaccines.

The causative agent of the disease, the rinderpest virus (RPV), is enveloped and has a single-stranded RNA genome with a negative polarity. The virus is in the family Paramyxoviridae and is a member of the morbillivirus group, as are measles virus of humans, distemper virus of dogs, and pestedes-petits-ruminants virus of goats and sheep. In paramyxoviruses, the hemaggluti-

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nin (HA) and fusion (F) surface proteins provide protective immunity (*4*).

In preparation for the development of the recombinant vaccine, we propagated the highly virulent Kabete "O" strain of RPV in primary bovine kidney cells, characterized eight viral proteins, made cDNA copies of the HA and F mRNAs, and determined the complete nucleotide sequence of both the HA and F genes (*5–7*). Standard procedures were used to construct vaccinia virus recombinants expressing the HA gene of RPV (*8*). The gene was excised from plasmid pRVH6 by digestion with Eco RI (*6*). It was then cloned into the Sma I site of the vaccinia virus vector pSC11, generating pvRVH. Homologous recombinations were performed with pvRVH and the Wyeth or the WR strain of vaccinia virus in CV-1 cells. Vaccinia virus recombinants containing the HA gene (vRVH) were selected by their thymidine kinase (TK) phenotype and by their ability to express β-galactosidase (*8*).

The F gene lacks a convenient restriction site within 100 nucleotides upstream from its initiation codon (*7*). A new Eco RI site, engineered 4 bp upstream from the ATG, facilitated the cloning of an Eco RI fragment of the entire coding region in the Sma I site of pSC11 to generate pvRVF (Fig. 1). Otherwise, the manipulations used to make the recombinant expressing the F gene (vRVF) were identical to those used for vRVH. The expression of authentic HA protein by vRVH was demonstrated by specific immunoprecipitation. However, no immunoprecipitable F protein could be demonstrated in cells infected with vRVF. This was anticipated, as we failed to immunoprecipitate the F protein efficiently from lysates of RPV-infected cells treated with rabbit or bovine hyperimmune serum directed against RPV (*5*).

Immune response studies in cattle were

Table 1. SN titers of cattle and response to challenge with RPV. Cows were vaccinated intradermally with 10^8 PFU of vaccinia virus recombinants on days 0 and 28. As a positive control, two cows were vaccinated with PTCV and challenged 42 days after the primary vaccination with 10^3 $TCID_{50}$ of RPV. SN titers are expressed as the reciprocal of the dilution of serum that gave complete protection against cytopathic effect of 550 $TCID_{50}$ of RPV.

Cow number and vaccine	Prechallenge titers on day							Postchallenge titer on day
	0	7	14	21	28	35	42	63
1. None*	0	0	0	0	0	0	0	Dead
2. None*	0	0	0	0	0	0	0	Dead
3. PTCV	0	256	374	1024	1024	1024	512	1024
4. PTCV	0	4	374	512	256	256	256	512
5. vRVH	0	32	374	384	384	1024	1536	3072
6. vRVH	0	64	512	1024	394	1536	2048	512
7. vRVH + vRVF	0	16	256	394	512	1536	2048	1024
8. vRVH + vRVF	0	16	256	128	256	768	768	768
9. vRVF	0	0	8	16	16	64	64	256
10. vRVF	0	3	8	8	8	48	128	64

*Cows 1 and 2 (unvaccinated controls) died on days 5 and 6 after challenge, respectively.

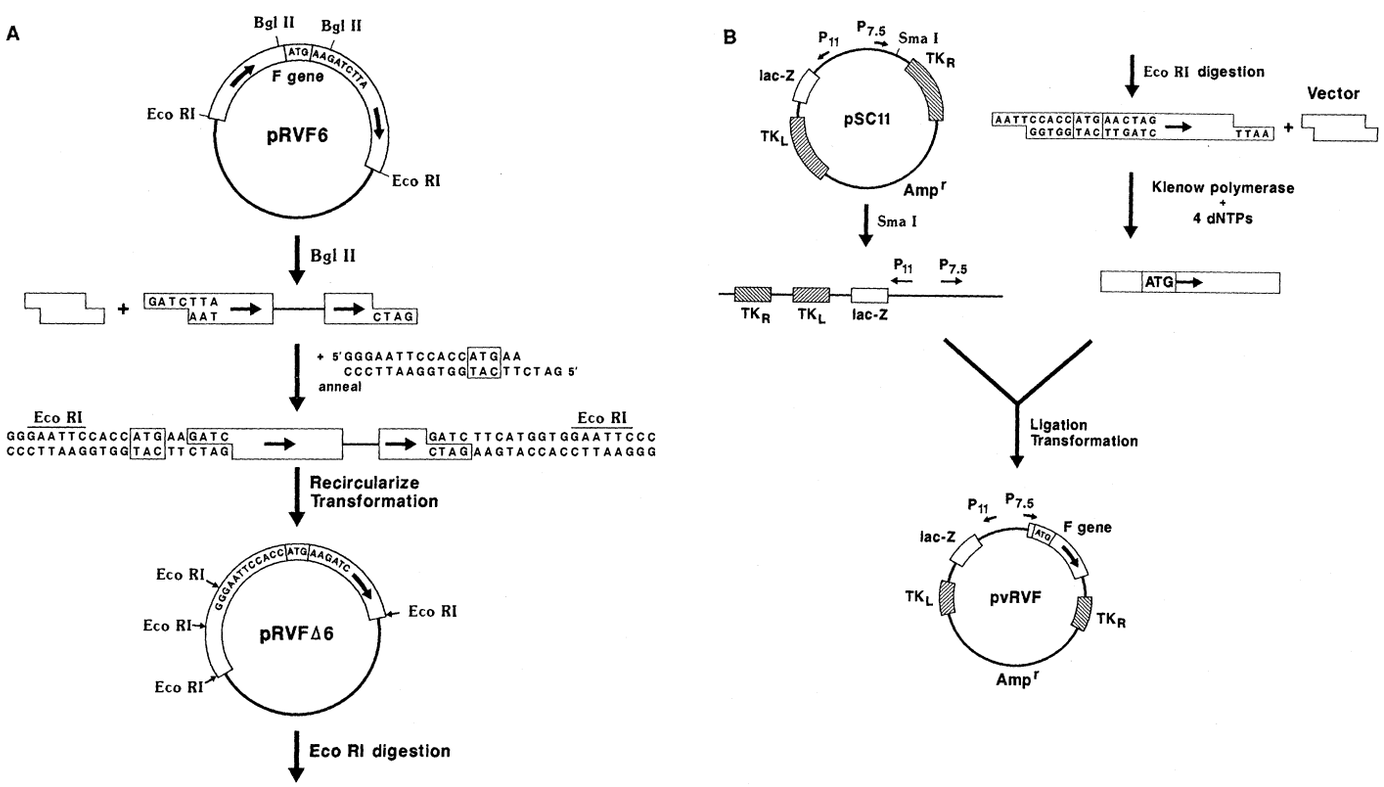

Fig. 1. Strategy for the construction of infectious vaccinia virus recombinants expressing the F protein of RPV. (**A**) To create an Eco RI restriction site and a Kozak consensus sequence immediately upstream from the second ATG, we replaced the small Bgl II fragment in pRVF6 with a 17-bp adapter that had a four-nucleotide overhang. The adapter contained an Eco RI site (GAATTC) followed by a Kozak consensus sequence (CCACC) and an initiation codon (ATG). The adapter also repaired that part of the original coding sequence removed with the Bgl II fragment to generate pRVFΔ6. pRVF6 was first digested with Bgl II restriction enzyme, and 100 times excess of adapter was added to the digest before ligation. To prevent the formation of concatamers, only the 21-base oligonucleotide was phosphorylated at the 5′ end. The ligation mixture containing the linearized pRVFΔ6 was treated with T_4 DNA kinase before recircularization of the plasmid. (**B**) The coding region of the F gene of RPV was excised by digestion with Eco RI from pRVFΔ6, the overhangs were filled with the Klenow polymerase, and cloned by blunt-end ligation in the Sma I site of pSC11 to generate pvRVF (*7*).

Table 2. SN titers of cattle after a single vaccination and challenge with RPV. Cows were vaccinated intradermally with 10^8 PFU of vaccinia virus recombinants on day 0. As a positive control, two cows were vaccinated with PTCV; for vaccinia virus control, two cows were vaccinated with v50 (*12*); two unvaccinated cows, as controls, were also included. All were challenged 35 days after vaccination with 10^3 $TCID_{50}$ of RPV. Titers are expressed as the reciprocal of the dilution of serum that gave complete protection against cytopathic effect of 125 $TCID_{50}$ of RPV.

Cow number and vaccine	Prechallenge titers on day						Postchallenge titers on day	
	0	7	14	21	28	35	42	63
1. None*						0	Dead	Dead
2. None*						0	Dead	Dead
3. v50	0	0	0	0	0	0	Dead	Dead
4. v50	0	0	0	0	0	0	Dead	Dead
5. PTCV	0	16	768	768	1536	384	384	512
6. PTCV	0	6	1024	384	768	—	512	768
7. vRVH	0	16	256	512	384	256	384	1024
8. vRVH	0	24	64	256	768	64	128	128
9. vRVH	0	16	192	128	256	64	96	128
10. vRVH	0	32	128	64	96	48	256	1024
11. vRVH	0	24	128	384	384	96	128	384
12. vRVH + vRVF	0	16	192	256	512	256	512	256
13. vRVH + vRVF	0	16	96	64	256	64	96	96
14. vRVH + vRVF	0	6	48	24	128	32	256	768
15. vRVH + vRVF	0	6	192	256	512	128	96	128
16. vRVH + vRVF	0	4	256	192	256	256	256	256
17. vRVF	0	3	0	8	8	16	16	1024
18. vRVF	0	4	8	8	6	8	8	16
19. vRVF	0	4	8	8	8	8	256	> 4096
20. vRVF	0	0	6	12	6	12	32	64
21. vRVF	0	0	6	2	2	4	48	1536

*Control cows 1 to 4 died on day 6 after challenge.

conducted in the high-containment facility at the Plum Island Animal Disease Laboratory according to proper institutional guidelines (*9*). All animals used were seronegative to RPV before being vaccinated. Two separate studies were conducted; in both studies serum samples were taken weekly, and humoral responses of cattle were assessed by a serum neutralization (SN) assay (*10*).

In the first study, two animals were vaccinated with vRVH or vRVF, or both recombinants combined (vRVH + vRVF), or with PTCV (Table 1). The PTCV-inoculated animals were used as a positive control, and two animals were left unvaccinated for a negative control. We vaccinated the animals that received the vaccinia virus recombinants by intradermal inoculation and scarification with 10^8 plaque-forming units (PFU) of recombinant vaccine at two sites on both sides of the neck (*11*). One milliliter of PTCV at 10^6 times the median tissue culture infectious dose ($TCID_{50}$) was administered subcutaneously. Four weeks after the primary vaccination, each animal that had received a recombinant vaccine was revaccinated with the same preparation, dosage, and method of administration. Animals were not revaccinated with PTCV.

In the second study (Table 2), only a single vaccination was administered. Five animals were used per group for vRVH, vRVF, or both recombinants combined (vRVH + RVF). Two animals were vaccinated with PTCV for a positive control, and two animals were left unvaccinated for a negative control. For a vaccinia virus control, two animals were vaccinated with v50, a vaccinia virus recombinant expressing the G gene of vesicular stomatitis virus (*12*).

Pock lesions developed within 4 days in all animals vaccinated with the recombinants but were limited to the site of inoculation and were healed completely by 2 weeks. All animals vaccinated with the recombinants or PTCV produced serum neutralizing antibodies to RPV. As expected, control animals had no detectable antibody titer (Tables 1 and 2).

To establish a reproducible challenge system, we determined the minimum dose and best route of administration. Intravenous and subcutaneous routes were evaluated in 19 animals. As little as 1 $TCID_{50}$ administered subcutaneously in the prescapular lymph node region induced clinical rinderpest with a 100% mortality rate. To evaluate protection, all animals were challenged with a heavy dose (10^3 $TCID_{50}$) of RPV subcutaneously in the prescapular lymph node region. In the first study animals were challenged on day 42, and in the second study on day 35, after primary immunization.

In the first study, up to a fivefold rise in titer (anamnestic response) was observed in the group vaccinated with vRVH, eightfold with vRVF, and fourfold with vRVH + vRVF after the second vaccination (Table 1). This shows that anamnestic responses can be mounted in the presence of antibody to vaccinia virus (*12*). The animals vaccinated with the recombinants or PTCV were completely protected from rinderpest, exhibiting no detectable illness and a normal temperature of 38°C. The two unvaccinated control cows had high fever (42°C) by day 2 after challenge and died on days 5 and 6. The cows also had lesions typical of severe rinderpest, characterized by sloughing and erosion of the epithelial lining of the gastrointestinal tract and bloody diarrhea. After 2 weeks of daily monitoring we terminated the experiment, as there was no clinical disease in the vaccinated animals.

In the second single-vaccination study, all cattle vaccinated with the recombinants or PTCV produced serum-neutralizing antibody to RPV by day 14 (Table 2). On the day of challenge, the group vaccinated with vRVH + vRVF and vRVH alone had 15-fold and 10-fold higher titers, respectively, than the group vaccinated with vRVF alone. All animals in the control group had high fever (107° to 108°F) by day 2 and died from massive bloody diarrhea by day 6 of challenge. Regardless of the level of SN titer, all animals vaccinated with the recombinants or PTCV were completely protected and, with a single exception, had no fever or any other clinical signs of rinderpest. One animal vaccinated with vRVF developed a temperature of 40°C 2 days after challenge. However, the animal had no fever after 3 days and showed no other signs of rinderpest. Two weeks after challenge, we terminated the experiment because no clinical signs of rinderpest could be detected.

None of the animals in the PTCV or vRVH + vRVF groups showed any increase in SN titers after challenge, indicating a lack of replication of challenge virus (Table 2). However, in the group vaccinated with vRVF alone, three animals had a rise in SN titer, with the animal that developed transient fever (40°C) having a titer greater than 4096 SN units. Similarly, two animals vaccinated with vRVH had a fourfold increase in SN titers after challenge, although neither had fever or other clinical signs of rinderpest. No control animal survived beyond 6 days after challenge, whereas naturally infected animals usually die 6 to 12 days after exposure (*1*).

We have developed vaccinia virus recombinant vaccines that provide complete protection against rinderpest. The use of vaccinia virus as a vector that can express heterologous viral antigens allows its use as a live virus vaccine for rinderpest, combining the safety advantages of a subunit vaccine with the antigen amplification and native presentation of an attenuated live virus vaccine

(*11*). Previous experience with the production, storage, and use of vaccinia virus for mass smallpox vaccination suggests that the recombinant vaccine for rinderpest may overcome logistical and economic disadvantages of PTCV. For example, cattle can be vaccinated with the recombinant vaccine at any age, even in the presence of antibody to RPV (*13*), since antibody to vaccinia virus is not normally found in cattle. Additionally, pock lesions serve as markers of effective vaccination.

Because safety considerations must be addressed before introducing live recombinant viruses into the environment, we constructed the recombinant rinderpest vaccine with the attenuated strain (Wyeth) used worldwide in successful smallpox eradication. Insertional inactivation of the TK gene further attenuates this virus (*14*). Lymphokine genes such as interferon-γ or interleukin-2 may also be useful in enhancing the immune response and further attenuating vaccinia virus recombinant vaccines (*15*); expression of interleukin-2 has been shown to prevent disseminated vaccinia virus infection in immunodeficient mice (*16*).

Rinderpest is a potential candidate for eradication with the vaccinia virus recombinant vaccine. There is only one serotype of RPV, although there are different strains manifesting different degrees of pathogenicity in the field. A vaccine against one strain will immunize against all strains, including peste-des-petits-ruminants virus of sheep and goats. Because of the close antigenic relation among the morbilliviruses, the recombinant vaccine for rinderpest may also protect against distemper in dogs and measles in humans (*1*).

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17. We thank B. Moss of NIH for providing vaccinia virus plasmid vectors, and J. Shine and B. Cordell of California Biotechnology for guidance and assistance. We dedicate this manuscript to J. Callis for his many years of service as director of the Plum Island Animal Disease Center and to D. DeTray and the late A. Fetene for their services in the JP/15 rinderpest eradication program. This work was supported by Cooperative Agreement DAN-4178-A-00-6040-00 from USAID of the State Department. We acknowledge the extensive assistance of K. A. Jones and C. Mullenax of USAID, and B. Osburn of the University of California, Davis.

9 June 1988; accepted 12 September 1988