JOURNAL OF VIROLOGY, Oct. 1991, p. 5584–5588
0022-538X/91/105584-05$02.00/0

Vol. 65, No. 10

Increased Expression In Vivo and In Vitro of Foreign Genes Directed by A-Type Inclusion Body Hybrid Promoters in Recombinant Vaccinia Viruses

SHIN-ICHI FUNAHASHI,[1] SHIGEYUKI ITAMURA,[2] HISAE IINUMA,[3] KUNIAKI NEROME,[2] MASANOBU SUGIMOTO,[1] AND HISATOSHI SHIDA[4]*

Corporate Research & Development Laboratory, TONEN Corporation, Saitama 354,[1] Department of Virology and Rickettsiology, National Institute of Health, Shinagawa-ku, Tokyo 141,[2] Second Department of Surgery, Teikyo University School of Medicine, Itabashi-ku, Tokyo 173,[3] and Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606,[4] Japan

Received 4 January 1991/Accepted 16 July 1991

We constructed A-type inclusion body (ATI) hybrid promoters, that is, late ATI promoters followed by tandemly repeated early regions of the promoter for the 7.5-kDa protein (the 7.5-kDa promoter). The repetition of the whole early promoter sequence of the 7.5-kDa gene, including the upstream consensus sequence and initiation region, efficiently increased the early expression of the bacterial chloramphenicol acetyltransferase gene in recombinant vaccinia virus. Recombinant vaccinia virus could express influenza virus hemagglutinin via the hybrid promoter more efficiently, induced higher levels of neutralizing antibody and cytotoxic T lymphocytes, and consequently protected mice more efficiently against challenge with influenza virus than did recombinant vaccinia virus containing the widely used 7.5-kDa promoter.

Vaccinia virus (VV)-based live vaccines have been constructed for a variety of viral pathogens whose antigens have been most widely expressed under the control of the VV 7.5-kDa protein promoter (1, 10, 12, 13, 23–25). Many different recombinant VVs (RVVs) have been used to vaccinate animals, conferring protection against challenge with pathogenic viruses (14, 18, 21, 22). In contrast, some RVVs have not been able to elicit a high antibody titer against surface antigens of hepatitis B and human immunodeficiency viruses in chimpanzees, despite effective induction of antibody responses in rabbits (10, 14). These results suggest that larger quantities of antigen may be required to induce a high antibody titer in chimpanzees or in humans immunized with RVV.

Gene expression of VV is regulated temporally before and after DNA replication at the level of transcription by early and late promoters (3). Each promoter has a characteristic structure. The early promoter consists of a 16-bp critical region separated from the transcription initiation region by 11 bp (5, 26). The late promoters comprise an upstream sequence of about 20 bp separated by a spacer region of about 6 bp from a conserved TAAAT transcription initiation region (6, 19). Some late promoters have been thought to direct the synthesis of more foreign products than early promoters, since late promoters control the synthesis of virion structural proteins or large nonvirion constituents (8, 16). One efficient late promoter is that of the A-type inclusion body (ATI) of cowpox virus, which is a large proteinaceous mass apparently consisting of only one protein species. The ATI protein, whose molecular mass is 160,000 Da, is synthesized as the most prominent peptide at a very late stage of cowpox virus infection (8, 16). We and others have cloned and characterized the ATI gene and its promoter, demonstrating that this promoter directs gene expression very efficiently (8, 15, 17).

For effective protection against a variety of pathogens, it would be better to induce both humoral and cellular immunities than to induce only one of the two. Coupar et al. suggested the importance of early expression of the antigens in VV infection to prime cytotoxic T lymphocytes (CTL), in contrast with induction of antibodies by both their early- and late-expressed antigens (4).

This article describes our method of expressing foreign genes efficiently in both early and late VV infection by constructing a hybrid promoter consisting of the ATI promoter and tandemly repeated sets of the early region of the 7.5-kDa promoter (3). The hybrid promoter-directed hemagglutinin (HA) of influenza virus induced higher titers of both humoral and cellular immunities than that directed by the conventional promoter.

To construct new promoters that function efficiently during early and late infection, the initiation codon within the sequence TAAATG in the ATI promoter (15, 19) was first changed to TAAATA by site-directed mutagenesis in order to express authentic foreign protein. Next, the tandem repeat of a 36- or 69-bp oligonucleotide fragment making up the early element of the 7.5-kDa promoter was inserted at a *Bam*HI site derived from a multiple-cloning site of a pUC vector and located just downstream of the ATI promoter (Fig. 1). The 69-bp fragment (named the A fragment) contained the sequence of the early region of the 7.5-kDa promoter, which consisted of a consensus critical region, the whole initiation region, and an additional downstream region (nucleotides −35 to +34). The 36-bp fragment (named the B fragment) contained the region of the 7.5-kDa promoter which comprises the sequence from the beginning of the critical region to the initiation site (nucleotides −35 to +1). To quantify the level of expression under control of these promoters, the chloramphenicol acetyltransferase (CAT) gene as a reporter gene was linked to these promoters, and RVVs were constructed by inserting the resultant recombinant plasmids into the HA gene locus of the VV genome by homologous recombination (20, 22).

* Corresponding author.

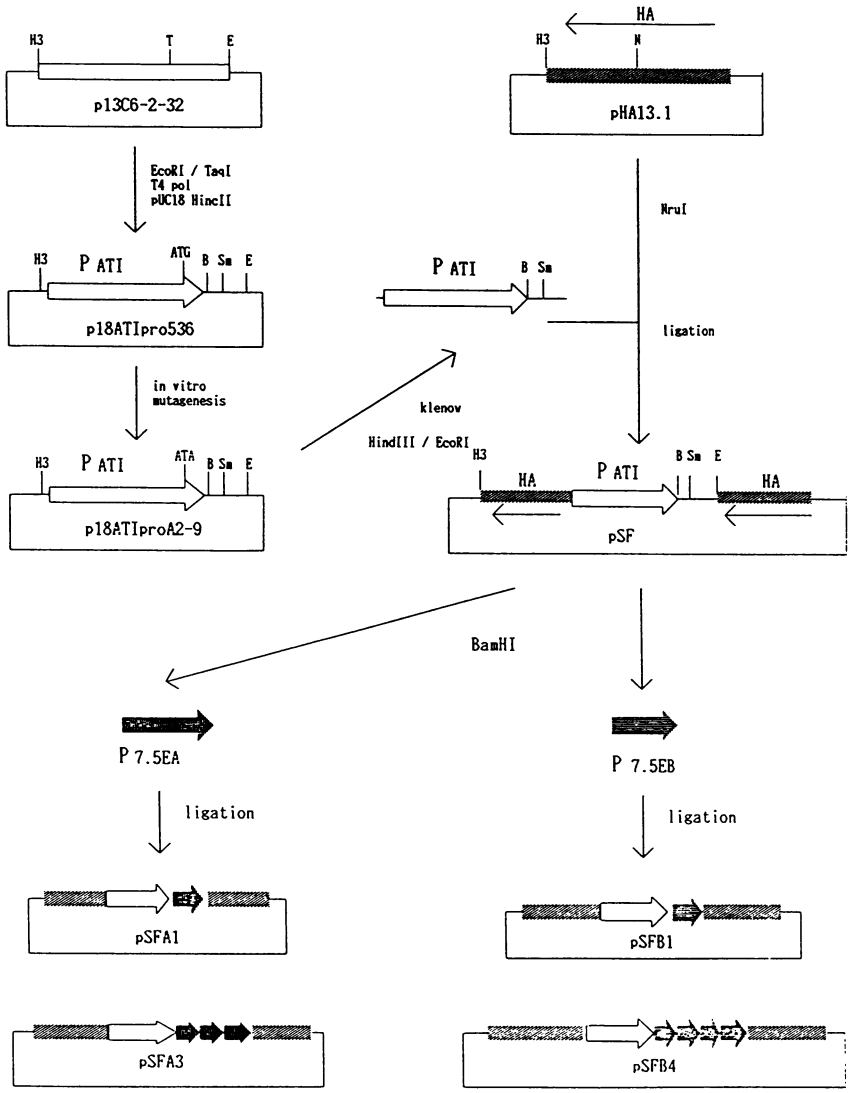

FIG. 1. Construction of VV vectors. p13C6-2-32 was derived from a 3′ deletion mutant of p13C6 (8) used for nucleotide sequencing. p18ATIpro536 and p18ATIproA2-9 contained about 600 bp of the ATI promoter region (from bp −533 to +18) and novel TGA repeats (8). p18ATIpro536 was mutagenized by site-directed mutagenesis (2) to form p18ATIproA2-9. A DNA fragment containing the promoter region (PATI) was excised from p18ATIproA2-9 and inserted into pHA13.1 (20). The vector pSF contained PATI and unique restriction sites flanked with segments of the VV HA gene. Synthetic early promoter elements of 69 (P7.5EA) and 36 (P7.5EB) bp were inserted into the *Bam*HI site of pSF to obtain pSFA1, pSFA3, pSFB1, and pSFB4. Selected restriction sites (B, *Bam*HI; E, *Eco*RI; H3, *Hin*dIII; N, *Nru*I; Sm, *Sma*I; and T, *Taq*I) are indicated.

Table 1 shows a comparison of the CAT synthesized in cells infected with RVVs which have various promoters. To quantify the amount of early or both early and late CAT produced, RVV-infected cells were cultured in the presence or absence of cytosine arabinoside (araC), an inhibitor of DNA replication. The ATI promoter directed the synthesis of severalfold more CAT than the 7.5-kDa promoter directed, confirming a previous report (17). The hybrid promoter (PATI-P7.5A1) containing one A fragment showed activity similar to the early activity of the 7.5-kDa promoter at early times of infection, whereas the hybrid promoter (PATI-P7.5B1) containing the B fragment had one-third to one-half the activity of the 7.5-kDa promoter. In contrast, the A fragment(s) had a marginal inhibitory effect on the ATI promoter activity, but the B fragment did not inhibit it at all. Marked enhancement of the early promoter activity was observed when the several sets of A and B fragments were placed in tandem. Thus these results indicated that the combinations of the ATI promoter and tandemly repeated A or B fragments were very strong promoters at both early and late times of infection.

To examine whether the antigen synthesized under the direction of the ATI hybrid promoter efficiently induced an immune response, we constructed two RVVs, vR-7 and vSFB4infl, in which the 7.5-kDa promoter and ATI hybrid (PATI-P7.5B4) promoter, respectively, express the HA gene of influenza virus A/SW/Cambridge/39 (H1N1) (Fig. 1). At first, expression in vitro was measured by immunoprecipitation of influenza virus HA protein labeled metabolically in RVV-infected cells (Fig. 2). In the lysates of cells infected

TABLE 1. Expression of the CAT gene from various promoters in RVVs

Promoter	AraC	CAT activity (%)[a] in expt:		
		1	2	3
P7.5	−		27.9	19.2
	+		4.2	2.7
PATI	−	71.4	87.1	72.7
	+	0.3	0.4	0.2
PATI-P7.5B1	−	67.3	83.3	74.0
	+	1.1	1.4	1.2
PATI-P7.5B4	−	79.7	87.7	66.8
	+	9.3	11.2	7.6
PATI-P7.5A1	−	54.0	69.8	57.5
	+	2.2	4.7	3.3
PATI-P7.5A3	−	72.4	78.8	61.1
	+	10.4	16.3	8.8

[a] The bacterial CAT gene was inserted into the *Sma*I site of each of a series of pSF plasmid vectors and pVR2 (12), and RVVs were obtained by inserting the recombinant plasmid by homologous recombination into the HA gene locus. CV-1 cells were infected with RVV at 10 PFU per cell and cultured in the presence or absence of 40 μg of araC per ml. At 24 h postinfection, the cells were harvested and the CAT activity in the cell extract was assayed (3, 9). The CAT activity is represented as percent conversion of the chloramphenicol to acetylated forms.

TABLE 2. Neutralizing-antibody response against influenza virus and VV induced by RVVs[a]

Expt	Immunizing virus	Immunization dose (PFU/mouse)	Immunization duration (wk)	Antibody titer[b] against:	
				Influenza virus	VV
1	vR-7	5×10^3	2	160	145
	vSFB4infl	5×10^3	2	3,000	220
2	vR-7	5×10^5	2	70	400
	vSFB4infl	5×10^5	2	400	1,000
3	vR-7	5×10^5	6	1,150	7,800
	vSFB4infl	5×10^5	6	15,000	13,000

[a] Groups of six or seven BALB/c (*H-2*^{d}) mice were inoculated intravenously with 5×10^3 or 5×10^5 PFU of RVV vR-7 or vSFB4infl. Two or six weeks later, sera were collected. The sera were examined for neutralizing-antibody titers against influenza virus A/SW/Cambridge/39 (H1N1) after receptor-destroying enzyme treatment and heat inactivation (56°C for 30 min).
[b] The neutralizing-antibody titer is expressed as the reciprocal of the serum dilution causing 50% reduction of plaque formation on MDCK cells. Assays of neutralizing antibody to VV were carried out as described previously (7).

with vR-7 and vSFB4infl, polypeptides ranging from 75 to 80 kDa in size were specifically immunoprecipitated with antiserum against the H1 subtype of influenza virus but were not immunoprecipitated with normal serum. HA protein was expressed at higher levels in vSFB4infl than in vR-7, either in the absence or presence of araC, reflecting the results of the CAT assay (Table 1). Titration of hemagglutination activity confirmed this result (data not shown).

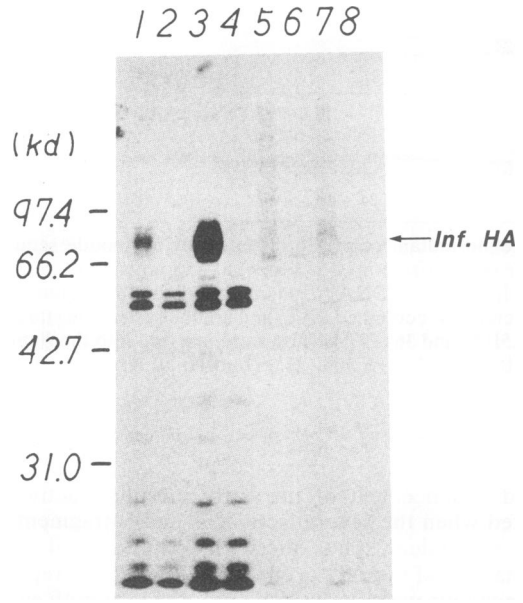

FIG. 2. Immunoprecipitation of influenza virus HA protein expressed by RVVs. Monolayers of CV-1 cells were infected with vR-7 (lanes 1, 2, 5, and 6) or vSFB4infl (lanes 3, 4, 7, and 8) at 5 PFU per cell and labeled with [^{35}S]methionine (100 μCi/ml) at 4 h postinfection for 24 h in the absence (lanes 1 to 4) or presence (lanes 5 to 8) of araC (40 μg/ml). Influenza virus HA protein was immunoprecipitated with anti-influenza virus A/SW/Cambridge/39 (H1N1) (odd-numbered lanes) or nonimmune rabbit serum (even-numbered lanes) as described previously (12). Immunoprecipitates were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and detected by fluorography.

BALB/c (*H-2*^{d}) mice were inoculated intravenously with various quantities of these RVVs. Two to six weeks later, they were bled, and the levels of neutralizing antibodies against influenza virus and VV were measured. As shown in Table 2, vSFB4infl induced a 6- to 20-fold-higher level of anti-influenza virus antibody than vR-7, whereas anti-VV antibodies were induced similarly. The ability of these RVVs to prime influenza virus-specific CTL was also examined. Six weeks after inoculation with RVVs, splenocytes were obtained and stimulated in vitro with influenza virus-infected cells, after which CTL activity was measured by using various ratios of effector cells to target cells. As shown in Fig. 3, vSFB4infl primed mice for an influenza virus H1-specific CTL response more efficiently than vR-7.

To demonstrate that such immunities augmented by vSFB4infl work for the protection of animals against the infection of influenza virus, challenge experiments were performed. Three weeks after immunization, influenza virus was injected as a challenge, and the amounts of influenza virus in lungs were measured by titration 4 days after challenge. Whereas all of the mice immunized by higher doses of both of the RVVs were protected, immunization with the lowest dose of vR-7 failed to protect four of five mice, in contrast to complete protection by vSFB4infl (Table 3). Similar amounts (450 or 370 U) of anti-VV neutralizing antibody were present in the serum obtained from mice immunized by 50 PFU of vR-7 or vSFB4infl, respectively (data not shown), indicating that similar amounts of the RVVs were really inoculated.

This article describes the construction of a strong VV promoter by combining the ATI promoter with several copies of the entire early region of the 7.5-kDa promoter. These promoters directed the synthesis of several-fold-greater amounts of foreign proteins at both early and late times of VV infection than the widely used 7.5-kDa promoter (3). These data suggest that the repetition of the whole early region of the 7.5-kDa promoter, including the critical, tran-

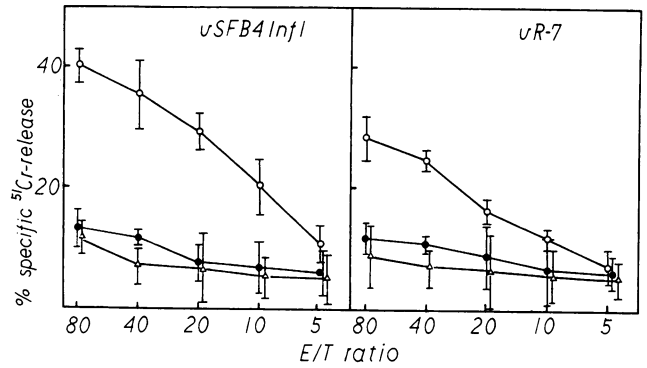

FIG. 3. Ability of RVVs to prime influenza virus-specific CTL response. BALB/c (*H-2*^{d}) mice were immunized intravenously with 5×10^5 PFU of vR-7 or vSFB4infl. Six weeks later, splenocytes were stimulated in vitro with autologous splenocytes infected with influenza virus A/SW/Cambridge/39 (H1N1) as described previously (11). Effector cells stimulated in vitro were assayed for specific cytotoxicity against influenza virus A/SW/Cambridge/39 (H1N1) (○)-, influenza virus A/Niigata/102/81 (H3N2) (●)-, and mock (△)-infected mastocytoma P815 (*H-2*^{d}) cells as target cells at various effector cell-to-target cell (E/T) ratios. Cytotoxic activity was measured by a ^{51}Cr release assay (11) and was expressed as the percentage of specific ^{51}Cr release from target cells, which was calculated by the formula [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. Spontaneous release from labeled target cells in medium alone ranged between 26 and 30% of the maximum release. Each point indicates the mean of triplicate measurements, and standard deviations are shown by bars.

scription initiation, and downstream sequences, is more effective in increasing gene expression than the repetition of only the critical region, which has been shown to enhance β-galactosidase gene expression twofold (5). The increased expression of the foreign antigen induced humoral and cellular immune responses in vivo more efficiently, which led to better protection of animals. Thus, the construction of a better VV promoter is a good way to improve VV-based live vaccines.

TABLE 3. Protection from challenge infection with influenza viruses in mice immunized with RVVs[a]

Virus used for vaccination	Dose (PFU)	No. of mice shedding virus (n = 5)	Mean virus titer [$\log_{10}$(PFU/ml)][b]	Protection %[c]
vSFB4infl	5×10^5	0	<2.0 ± 0	100
	5×10^3	0	<2.0 ± 0	100
	5×10	0	<2.0 ± 0	100
vR-7	5×10^5	0	<2.0 ± 0	100
	5×10^3	0	<2.0 ± 0	100
	5×10	4	4.5 ± 1.6	20
WR (HA$^-$)	5×10^5	5	5.5 ± 0.1	0
	5×10^3	5	5.5 ± 0.1	0
	5×10	5	5.4 ± 0.4	0

[a] Groups of five BALB/c mice each were vaccinated intravenously with RVVs at the indicated doses. Three weeks later, the mice were challenged intranasally with influenza virus A/SW/Cambridge/39 (H1N1). Protection was evaluated by assaying virus titers in lungs 4 days postchallenge.

[b] Values represent the mean ± standard deviation of all virus titers obtained within a group, including those from protected mice, for which the maximum possible titer of 2.0 was assigned.

[c] Mice were considered protected from infection if no virus [$\log_{10}$(PFU/ml) < 2.0] was detected in their lungs.

We thank Y. Hirosaki and A. Endo for their assistance.

REFERENCES

1. **Cantin, E. M., R. Eberle, J. L. Baldick, B. Moss, D. E. Willey, A. L. Notkins, and H. Openshaw.** 1987. Expression of herpes simplex virus 1 glycoprotein B by a recombinant vaccinia virus and protection of mice against lethal herpes simplex virus 1 infection. Proc. Natl. Acad. Sci. USA **84**:5908–5912.
2. **Carter, P., H. Bedoulle, and G. Winter.** 1985. Improved oligonucleotide site-directed mutagenesis using M13 vectors. Nucleic Acids Res. **13**:4431–4443.
3. **Cochran, M. A., C. Puckett, and B. Moss.** 1985. In vitro mutagenesis of the promoter region for a vaccinia virus gene: evidence for tandem early and late regulatory signals. J. Virol. **54**:30–37.
4. **Coupar, B. E. H., M. E. Andrew, G. W. Both, and D. B. Boyle.** 1986. Temporal regulation of hemagglutinin expression in vaccinia virus recombinants and effects on the immune response. Eur. J. Immunol. **16**:1479–1487.
5. **Davison, A. J., and B. Moss.** 1989. Structure of vaccinia virus early promoters. J. Mol. Biol. **210**:749–769.
6. **Davison, A. J., and B. Moss.** 1989. Structure of vaccinia virus late promoters. J. Mol. Biol. **210**:771–784.
7. **Finney, D. J.** 1964. Statistical method in biological assay, 2nd ed. Charles Griffin & Co., Ltd., London.
8. **Funahashi, S., T. Sato, and H. Shida.** 1988. Cloning and characterization of the gene encoding the major protein of the A-type inclusion body of cowpox virus. J. Gen. Virol. **69**:35–47.
9. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
10. **Hu, S.-L., P. N. Fultz, H. M. McClure, J. W. Eichberg, and G. Todoro.** 1987. Effect of immunization with a vaccinia-HIV env recombinant on HIV infection of chimpanzees. Nature (London) **328**:721–723.
11. **Itamura, S., H. Iinuma, H. Shida, Y. Morikawa, K. Nerome, and A. Oya.** 1990. Characterization of antibody and cytotoxic T lymphocyte responses to human influenza virus H3 haemagglutinin expressed from the haemagglutinin locus of vaccinia virus. J. Gen. Virol. **71**:2859–2865.
12. **Itamura, S., Y. Morikawa, H. Shida, K. Nerome, and A. Oya.** 1990. Biological and immunological characterization of influenza virus haemagglutinin expressed from the haemagglutinin locus of vaccinia virus. J. Gen. Virol. **71**:1293–1301.
13. **Moss, B., and C. Flexner.** 1987. Vaccinia virus expression vectors. Annu. Rev. Immunol. **5**:305–324.
14. **Moss, B., G. L. Smith, J. L. Gerin, and R. H. Purcell.** 1984. Live recombinant vaccinia virus protects chimpanzees against hepatitis B. Nature (London) **311**:67–69.
15. **Patel, D. D., and D. J. Pickup.** 1987. Messenger RNAs of a strongly-expressed late gene of cowpox virus contain 5′-terminal poly(A) sequences. EMBO J. **6**:3787–3794.
16. **Patel, D. D., D. J. Pickup, and W. K. Joklik.** 1986. Isolation of cowpox virus A-type inclusions and characterization of their major protein component. Virology **149**:174–189.
17. **Patel, D. D., C. A. Ray, R. P. Drucker, and D. J. Pickup.** 1988. A poxvirus derived vector that directs high levels of expression cloned genes in mammalian cells. Proc. Natl. Acad. Sci. USA **85**:9431–9435.
18. **Piccini, A., and E. Paoletti.** 1988. Vaccinia: virus, vector, vaccine. Adv. Virus Res. **34**:43–64.
19. **Rosel, J. L., P. L. Earl, J. P. Weir, and B. Moss.** 1986. Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes deduced by structural and functional analysis of the *Hin*dIII H genome fragment. J. Virol. **60**:436–449.
20. **Shida, H.** 1986. Nucleotide sequence of the vaccinia virus hemagglutinin gene. Virology **150**:451–462.
21. **Shida, H., Y. Hinuma, M. Hatanaka, M. Morita, M. Kidokoro, K. Suzuki, T. Maruyama, F. Takahashi-Nishimaki, M. Sugi-**

moto, R. Kitamura, T. Miyazawa, and M. Hayami. 1988. Effects and virulences of recombinant vaccinia viruses derived from attenuated strains that express the human T-cell leukemia virus type I envelope gene. J. Virol. **62**:4474–4480.

22. **Shida, H., T. Tochikura, T. Sato, T. Konno, K. Hirayoshi, Y. Ito, M. Hatanaka, Y. Hinuma, M. Sugimoto, F. Takahashi-Nishimaki, T. Maruyama, K. Miki, K. Suzuki, M. Morita, H. Sashiyama, N. Yoshimura, and M. Hayami.** 1987. Effect of the recombinant vaccinia viruses that express HTLV-I envelope gene HTLV-I infection. EMBO J. **6**:3379–3384.
23. **Smith, G. L., K. Mackett, and B. Moss.** 1983. Infectious vaccinia virus recombinants that express hepatitis B virus surface antigen. Nature (London) **302**:490–495.
24. **Stott, E. J., L. A. Ball, K. K. Young, J. Furze, and G. W. Wertz.** 1986. Human respiratory syncytial virus glycoprotein G expressed from a recombinant vaccinia virus vector protects mice against live-virus challenge. J. Virol. **60**:607–613.
25. **Tsukiyama, K., Y. Yoshikawa, H. Kamata, K. Imaoka, K. Asano, S. Funahashi, T. Maruyama, H. Shida, M. Sugimoto, and K. Yamanouchi.** 1989. Development of heat stable recombinant rinderpest vaccinia. Arch. Virol. **107**:225–235.
26. **Weir, J. P., and B. Moss.** 1987. Determination of the promoter of an early vaccinia virus gene encoding thymidine kinase. Virology **150**:451–462.