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Molecular basis of pathogenicity in vaccinia virus

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by

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Abstract

MOLECULAR BASIS OF PATHOGENICITY IN VACCINIA VIRUS

by

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Mutant Z-19 has been isolated from Friend erythroleukemia cells persistently infected with vaccinia virus and characterized. It had 20-21 kb deletion at the left end, including HindIII C and part of HindIII N fragments, and rearrangements at the right end of viral genome. It was able to replicate in different cell lines in vitro, but it did not replicate in the organs of mice. It was completely avirulent when inoculated into mice by intrapeanitoneal (i.p.) injection. However, it was able to protect mice against challenge with wild-type. Since it provides a null virulence background, it is a good model system to study pathogenicity. Furthermore, it was previously reported that in Jurkat cells persistently infected with vaccinia virus, there is stimulation of cytokine synthesis (IL-2, IL-2 receptor-a, and IL-6) and increase in the transactivation of HIV-1LTR transcription.

The aim of this thesis was to study the molecular pathogenicity of vaccinia virus and to investigate virus-host interaction in Jurkat cells persistently infected with vaccinia virus. Three aspects were explored.

(1). To determine if the virulent phenotype can be recovered by introduction of the terminal Hind III C fragment into the attenuated mutant

Z-19. Rescue experiments were performed, recombinant viruses were isolated and characterized. The results showed that recovery of virulence in mice was correlated with the presence and expression of two genes: vaccinia growth factor and vaccinia complement-binding protein, both located at the left terminus. Other "virulence" genes like serpin I and II, located at the right terminus, were present in the mutant Z-19, in recombinant viruses and in the wild-type, and do not seem to contribute to virulence in the IHD-W strain.

(2). To establish a model system to identify which gene(s) is/are responsible for virulence in the IHD-W strain. A shuttle vector was constructed to reintroduce genes into the mutant Z-19. The shuttle vector contained the fusion fragment K' from mutant Z-19, which comprised the end terminal HindIII C and part of the N fragment. The vector also contained the β-galactosidase cassette which included the LacZ gene under the vaccinia promoter P11 for screening purposes. The vaccinia growth factor and vaccinia complement-binding protein genes were inserted back into mutant Z-19. The results showed that these genes were expressed in cells infected with the recombinant viruses and that they became virulent. However, they did not attain the virulence level of the wild-type, suggesting that expression of other genes present in fragment HindIII C may be required.

(3). To study the molecular mechanism by which Jurkat cells persistently infected with vaccinia virus transactivated the LTR of HIV-1 virus, gel mobility shift assays were performed using labeled LTR or synthetic oligonucleotides and nuclear extracts derived from activated or infected Jurkat cells to define which sequences in the LTR were involved. Results indicated that two elements: NFAT-1 and NF-kB, were able to interact with

nuclear extracts derived from Jurkat cells persistently infected with vaccinia virus, suggesting that these elements may play a role in the transactivation of HIV-LTR.

Format of thesis

This thesis is written in accordance with guidelines of the City University of New York which permitted direct incorporation of published research articles as chapters. This thesis has a general introduction, followed by three chapters, chapter II is a published paper as Pi-Yun Chang, Alexander C-K Lai and Beatriz G-T Pogo, Microb. Pathog. 13: 49-59 (1992). Chapter III and IV contain unpublished results. Each chapter has an Abstract, Introduction, Materials and Methods, Results, Discussion and References. A general discussion is provided in Chapter V.

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Chapter I

Introduction

Vaccinia virus is being used as a vector to study gene regulation and function. Based on the success of vaccination to eradicate smallpox, it has been regarded as a good candidate to be used as a live recombinant vaccine in humans and animals. There are several advantages for using vaccinia as a live vaccine, such as: wide host range, potency in the single inoculation, ability to induce humoral and cell-mediated immunity, flexibility of packaging in viral particles, cheap manufacture, ease of administration, and stability without refrigeration (Moss, 1992). It may also be used to carry multiple antigens and to protect against a more than one disease. In 1985, Paoletti et al. (Paoletti et al., 1985; Perkus et al., 1985) constructed a recombinant vaccinia virus vector containing three different antigens (Influenza hemagglutinin, Hepatitis B antigen and herpes glycoprotein D) which was able to induce antibodies against all those antigens in mice. However, the use of vaccinia virus as a live recombinant vaccine is still limited, because the possibility of several adverse reactions, such as generalized vaccinia in skin-damaged individuals, and encephalitis, especially when administrated to immunocompromised individuals. In 1987, Redfield et al. (1987) reported vaccinia dissemination in an HIV positive military recruit who has been vaccinated with multiple vaccines including vaccinia two and an half weeks earlier. Subsequently, the recruit developed AIDS and died. In 1992, it was reported that three AIDS patients who had received fixed vaccinia recombinant infected cells, as part of an experimental protocol, died as a consequence of vaccinia dissemination from incompletely inactivated virus (Picard et al., 1990;

Guillaume et al, 1991). It was later reported from our laboratory that HIV replication was activated in cells persistently infected with vaccinia (Stellrecht et al., 1992a). For vaccinia virus to become a safe vector, it is important to develop strategies to attenuate virulence. Therefore, there is impetus to identify those genes which are responsible for pathogenicity.

Vaccinia virus genome

Vaccinia virus, a prototype of orthopoxviruses, is a DNA virus. The genome consists of double strained DNA with cross-links at both termini (Moss, 1990). Vaccinia virus replicates exclusively in the cytoplasm, unlike other DNA viruses which replicate in the nucleus. Its genome contains 187kb and 10kb terminal repetition at both ends. It codes for more than 200 polypeptides, including enzymes for DNA synthesis, RNA synthesis and structural proteins. The naked DNA is not infectious. Most genes located in the middle region are highly conserved and essential for virus survival in nature. Analysis of spontaneous and artificial deletion mutants, as well as target insertional inactivation mutants, indicated that the genes located at both termini of the viral genome are nonessential for replication in culture cell lines and quite variable (Moss et al., 1981; Panicali et al., 1981; Buller et al., 1985; Paez et al, 1985; Buller et al., 1988a; Lai and Pogo,1989). Deletions, transpositions and duplications occurred frequently at both termini (Moyer et al., 1980; Panicali et al., 1981; Pickup et al., 1984; Kotwal and Moss, 1988). Since the deletion mutants displayed decreased virulence which was also related to host range, it is concluded that these genes play a role in host range and pathogenicity.

Proteins involved in virulence

Proteins which are encoded by genes located at both ends of the viral genome are known to play a proven or possible role in virulence. Their functions are summarized as following:

Table 1

Designation	location	gene product/function
Vaccinia growth factor (VGF)	C 19L	Secreted growth factor, 19Kd protein, may stimulate proliferation of uninfected cells (Buller et al., 1988b)
Vaccinia complement binding protein (VCBP)	C21L	35Kd,complement control binding protein blocks classical complement pathway.(Kotwal and Moss, 1989a; 1990)
ORF N1L	N1L	13.8Kd secreted protein, role in neurovirulence? (Kotwal et al., 1989b)
Human host range (hhr)	C7L	29Kd, prevent early protein synthesis shutoff? (Gillard et al., 1985)
Thymidine kinase	J2R	Nucleotide biosynthesis

(TK)		(Buller et al., 1985)
14kd gene	A27L	Envelope protein, determination of plaque size, role in virus penetration? (Dallo et al., 1987)
SPI-1	B13R	40kd, unknown. (Kotwal and Moss, 1989c)
SPI-2	B24R	38kd serpin, homologous to cowpox virus red pock gene (Kotwal and Moss, 1989c)
IL-1 binding protein	B15R B18R	Related to extracellular domains of the IL-1 and IL-6 receptor, a secreted mouse IL-1β binding protein (Spriggs et al., 1992)
Gamma-Interferon binding protein		Secreted γ-interferon binding protein, role in modulation of cytokines in host immune response against viral infection

(Goebel et al., 1990; Upton et al., 1992)

The location in the genome is indicated in the HindIII map (Fig. 1).

Vaccinia Growth Factor (VGF) :

The VGF has amino acid sequence homology to epidermal growth factor (EGF) and to transforming growth factor α (TGF-α) (Blomquist et al., 1984; Brown et al., 1985; Reisner, 1985). The VGF gene from the WR strain is one of at least three genes lying within the inverted terminal repetitions mapping at position 6.54-7.16kb (Wittek et al., 1980). The gene is transcribed early in the viral cycle and encodes a 19 kd protein. Since VGF can bind to the EGF receptor, it is possible that VGF may be involved in virus entry. VGF also has mitogenic properties for the chorioallantoic membrane of the chicken embryo (Goodpasture et al., 1932) and the tissue culture cells (Buller et al., 1988b). Buller et al. (Buller et al., 1988a) have shown that insertional inactivation of this gene greatly reduced the virulence of the virus. This engineered mutant was both genotypically and phenotypically VGF negative (VGF^-) when examined by Southern blot hybridization and assayed for functional growth factor in cells. The VGF^- mutant virus and wild-type virus (WR strain) had similar plaquing efficiencies in different cell lines and similar production of virus in two lines of actively growing BSC-1 and Swiss 3T3 cells. However, the great difference between VGF^- and WT virus was found in vivo. The VGF^- had an intracerebral LD_{50} in mice more than 1,000 fold greater than that of wild-type, indicating a reduction in its virulence. In addition, smaller amounts of VGF^- progeny virus than of

WT progeny virus were recovered in brain. The VGF$^-$ virus produced less lesions in rabbit skin after intradermal inoculation. These findings indicate that the VGF may be a useful marker for the attenuation of vaccinia.

Vaccinia Complement-binding Protein (VCBP) :

Several viral genes involved in host immune responses have been identified. Kotwal and Moss (Kotwal and Moss,1989a) showed that C21L gene of vaccinia virus, the 21st open reading frame starting from the left end of HindIII C fragment of the genome, encoded a protein, termed vaccinia complement-binding protein (VCBP) with homology to a group of complement control proteins. The complement system is composed of at least 20 plasma glycoproteins, including two receptors (CR1 and CR2) and four inhibitors (factor H, C4b binding protein, membrane cofactor protein and decay-accelerating factor). These proteins consist of tandem arrays of a short consensus repeat of 60 amino acids and can bind to complement components C3 and/or C4. VCBP contains four short consensus repeats that have 38% identity to the first four repeats of C4b binding protein. This protein is secreted into the medium of vaccinia infected cells and can inhibit the classical complement pathway as determined by a hemolysis assay. The VCBP$^-$ mutant, generated by insertional inactivation, grew like wild-type virus in tissue culture and was unable to inhibit the classical complement pathway. In addition, the VCBP$^-$ mutant had little or no effect on antibody dependent complement-enhanced neutralization, and produced fewer skin lesions than that of wild-type virus (Isaacs et al., 1992). Therefore, it has been suggested that VCBP is involved in regulation of the complement cascade to evade the host immune response.

Serine Protease Inhibitors (SPI)

Vaccinia SPI genes, SPI1 and SPI2, are located between 10 and 17 kb from the right end of the genome and encode proteins of 40Kd and 38Kd with amino acid homology to each other. Both are transcribed early during infection. The SPI-2 of vaccinia virus shares 92% amino acid identity with the serpin from cowpox virus which causes red pocks in the chorioallantoic membrane of the chicken embryo. However, vaccinia virus does not generate red pocks. The SPI products may play a role as protease inhibitors of blood coagulation. Alternatively, SPI products in vaccinia virus may interfere with the complement activation. They may act as a protease inhibitor that blocks the processing of viral peptide associated with class I MHC for recognition by cytolytic T cells (CTL). This would be advantageous to the virus since reduced presentation of viral specific peptides would decrease the clearance of infected cells by CTL. These serpin genes are nonessential for virus replication, but it is possible that they play important roles in the pathogenicity of virus infection in vivo. (Kotwal and Moss, 1989c)

Strategies to study pathogenicity

There are two strategies to study the virulence of vaccinia virus. One is using target insertional inactivation of certain genes, however, attenuation may be the result of insertion site interruption, and not due to inactivation of a virulence gene. The second method is to use attenuated deletion mutants, either engineered or spontaneous, to reintroduce the missing gene(s). Some of the spontaneous deletion mutants missing several genes provide a null virulence background to study virulence. Change in phenotype can be attributed to the inserted gene.

In our laboratory, deletion mutants have been isolated from Friend erythroleukemia cells persistently infected with vaccinia virus. One of those mutants, Z-19, has been isolated and characterized (Lai and Pogo, 1989). The mutant had a 20-21 kb deletion at the left end and rearrangements at the right end of viral genome. It was able to replicate in different cell lines in vitro, but it did not replicate in the organs of mice. It was completely avirulent when inoculated into mice by intrapeanitoneal (ip) injection and was able to protect mice against challenge with wild-type virus. Analysis of deleted genes by Southern hybridization showed that the mutant lacked VGF and C4b genes at the left terminus (Chang et al., 1992). Since the Z-19 provides a null virulence background, it can be empolyed as a good model system to evaluate which genes are responsible for pathogenicity.

Effect of virus infection on cellular functions.

Vaccinia virus infection, has a profound and rapid effect on host DNA, RNA, and protein syntheses.

DNA synthesis. Host nuclear DNA synthesis is inhibited 1 to 2 h postinfection with either productive or UV-inactivated vaccinia virus over a MOI range of 40-400 (Hanafusa, 1960; Kit and Dubbs, 1962; Jungwirth and Launer, 1968). Reports indicate that two enzymes associated with the virion: acidic exonuclease and neutral endonuclease, may be involved in this process. After penetration, the core is released in the cytoplasm, and enzymes can be then transferred to the host nuclei to act on the ssDNA, resulting in hydrolysis of nascent host DNA (Pogo and Dales, 1973; 1974).

RNA synthesis. Virus-mediated inhibition of host RNA synthesis or processing occurs after vaccinia virus infection (Becker and Joklik, 1964; Jefferts and Holowczak, 1971; Kit and Dubbs, 1962). The inhibition needs

live virus and requires virus-directed protein synthesis for expression of the inhibition. The mechanism of inhibition occurs at the level of RNA polymerase II activity and mRNA half-life. It has been shown that RNA polymerase II activity decreased after vaccinia virus infection. (Pedley and Cooper, 1984; Morrison and Moyer, 1986). The half-life of the host mRNAs gradually decreased following vaccinia virus infection, especially, the mRNAs for actin and α-tubulin are degraded to levels 50% or less than those of the uninfected cells by 3 hr p.i (normal half-life is longer than 6 hr in uninfected cell) (Oda and Joklik, 1967; Sebring and Salzman, 1967; Rice and Robert, 1983).

Protein synthesis. Vaccinia virus inhibits host protein synthesis quickly and this process depends on the conditions of infection such as MOI and the type of host cell used. Several mechanisms for inhibition of translation have been proposed. (1) Surface tubule element (STE), a structural protein of the virion at the surface, was shown to inhibit host protein synthesis only, not to interfere with DNA and RNA synthesis when added to HEp-2 cells or in vitro to a rabbit reticulocyte protein synthesizing system (Mbuy et al., 1982). (2) 11 kDa basic protein, isolated from virion cores, inhibits in vitro reticulocyte protein synthesis at the binding of stoichiometic ratio, i.e. one molecular 11-kDa protein per ribosome (Ben-Hamida and Beaud, 1978; Person-Fernandez and Beaud, 1986). This protein was shown to block the 40S ribosomal subunit-Met-tRNA f^{Met} initiation complex (Ben-Hamida and Beaud, 1978; Person and Beaud, 1978; Person et al., 1980; Person-Fernandez and Beaud, 1986). (3) Synthesis of small, poly (A) containing RNA molecules in vivo inhibited host protein synthesis (Rosemond-Hornbeak and moss, 1975; Bablanian et al., 1981). Following studies have shown that poly $(A)^+$ RNA made synthetically or by virus

cores, selectively inhibited the translation of mRNA of host origin relative to vaccinia virus (Coppola and Bablanian, 1983; Bablanian et al., 1987). These results definitely indicate that inhibition of host protein synthesis is dependent on viral RNA synthesis.

Vaccinia virus infection results in cytolysis in most infected cell cultures, however, under certain conditions, persistently infected cultures can occur. Inoculation of mouse Friend erythroleukemia cells or human K562 cells with vaccinia virus results in the establishment of persistently infected cell lines. These cell lines retain most of the characteristics of the parental line, such as morphology, growth rate, cloning efficiency, and ability to respond to inducers of erythrodifferentiation, but also showed phenotypic changes, e.g. these cells were less tumorigenic and more spontaneously differentiated (Pogo and Friend, 1982; Pogo et al., 1991). Furthermore, persistently infected lines of human CD4+ T-cells (Jurkat) with vaccinia virus (J_{vac} cells) have been established. It has been shown that the virus influences cellular function, such as increasing expression of IL-2, IL-2 receptor-α and IL-6 (Stellrecht et al., 1992b). Moreover, activation of the HIV-LTR, as shown by CAT assay, was demonstrated (Stellrecht et al., 1992a). The mechanism of HIV-LTR activation in J_{vac} cells is still unclear. However, it is expected that investigation of this model system will provide a new information about host cell responses to permanent vaccinia infection.

Goals of the thesis

The main goal of this thesis was to investigate three different aspects of vaccinia virulence:

1. To determine if the virulence phenotype can be recovered by introduction of the Hind III C fragment into the attenuated mutant Z-19.

Rescue experiments were performed, recombinant viruses were isolated and characterized by Southern blot hybridization and gene expression evaluated by Northern blot hybridization. Their virulence was determined by LD_{50} and per cent of weight loss in mice.

2. To establish a system model to evaluate which gene(s) at the left terminus of the genome were responsible for virulence. For this purpose, an insertion vector was constructed to reintroduce genes into the deletion mutant by homologous recombination. The recombinant viruses containing the test gene were isolated and characterized by Southern blot hybridization and their expression evaluated by Northern blot hybridization. Their virulence was determined by LD_{50} and per cent of weight loss in mice.

3. To study the mechanism(s) by which Jurkat cells persistently infected with vaccinia virus transactivated the LTR of HIV-1 virus DNA mobility shift assays were performed using labeled LTR or synthetic oligonucleotides and nuclear extracts derived from activated or infected Jurkat cells to define which sequences in the LTR were involved.

Results from these studies will provide new insights into the molecular mechanism of pathogenicity of vaccinia virus and its interaction with the host cell. They will be useful in the development of safe vaccinia-based recombinant vaccines.

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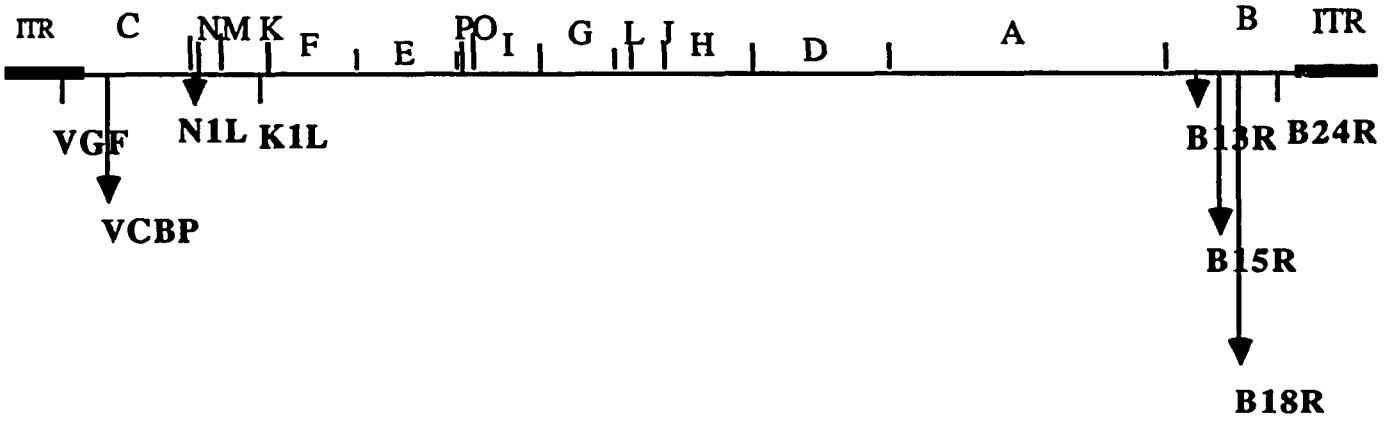
Figure. 1 The HindIII map. The location of the virulent genes indicated in the HindIII map. Taken from Goebel et al., 1990.



Chapter II

Attenuated deletion mutant of vaccinia virus IHD-W recovered virulence by reinsertion of a terminal restriction fragment

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Since vaccinia virus is being considered as a potential immunization vector, it is important to identify genes responsible for pathogenicity. One approach to identify virulence genes is the use of attenuated deletion mutants with a null background to reintroduce regions of the deleted genome material. We have previously described an attenuated deletion mutant of vaccinia virus strain IHD-W (Z-19) that lost 21 kb from the left terminus comprising fragments HindIII C and N, displays rearrangements at the right terminus and is unable to grow in vivo. To establish whether the loss of genes present at the left terminus is the basis for the attenuated phenotype, rescue experiments were performed to reintroduce fragment HindIII C, using growth in vivo as a selection system to isolate recombinant viruses. Several recombinants were isolated and molecularly and biologically characterized. The results indicate that recovery of virulence in mice was correlated with the presence and expression of two genes: vaccinia growth factor (VGF) and C4b binding protein which are located at the left terminus. Serpin I and II genes located at the right terminus were not affected in Z-19 and seem not to play a role in virulence in the IHD-W strain; interestingly, a copy of the serpin I gene was found at the left terminus. Moreover, a gene involved in virulence in the WR strain, the 13.8 kd virokine gene (N1L) was found to be located in fragment HindIII G in the IHD-W strain, and therefore did not contribute to the attenuated phenotype.

Key words: vaccinia virus strain IHD-W, attenuated deletion mutants, virulence genes, rescue experiments.

Vaccinia virus is being considered as a potential immunization vector because of its unique ability to maintain and express large amounts of foreign DNA under its own regulation (1-3). Although vaccinia virus has been successfully used in eradication of smallpox, vaccination with this virus may result in systemic virus infection in immunocompromised individuals (4). Therefore, it is important to identify the genes responsible for pathogenicity in order to develop safer vaccinia virus vectors.

One class of genes, located near the terminal ends of the genome, has been shown to be dispensable for growth in tissue culture (5-9), but necessary for pathogenicity in vivo. This group comprises the genes for vaccinia growth factor (VGF) (5), C4b binding protein (7), 13.8 kd virokine (9) and two serpins (8). Mutants obtained by insertional inactivation of these genes display greatly reduced virulence, suggesting that these genes are involved in pathogenesis.

Another approach to identify virulence genes has been the use of attenuated deletion mutants with a null background to reintroduce regions of the deleted genomic material (10). In our laboratory, deletion mutants of vaccinia virus have been isolated from persistently infected Friend erythroleukemia (FEL) cells (11). One of these mutants, Z-19, was shown to be stable after serial passage in L cells and to have lost 21 kb of its genome at the left terminus, comprising the HindIII C and N fragments and rearrangements at the right terminus (11). This mutant was not host restricted, did not replicate in vivo, was highly attenuated in vivo, and elicited protection against wild-type (wt) virus (11,12). The Z-19 attenuated phenotype has been correlated with the absence of VGF and other genes located at the left terminus, however, the presence of rearrangements and deletions at the right terminus may also have

contributed to the reduction in virulence. To establish whether the loss of the genes present in the left terminus was the basis for the Z-19 attenuated phenotype, rescue experiments were performed to reintroduce fragment HindIII C and to examine the genomic composition and virulence of the recombinant viruses.

Results

Marker rescue with wild-type HindIII C fragment

To evaluate the role of the left terminus in virulence, rescue experiments were performed in which the HindIII C fragment from the wt type virus (IHD-W strain) was transfected into cells infected with Z-19. The recombinants were selected by replication in vivo, since Z-19 was unable to multiply (12) as shown in the Fig 1. Twelve recombinants were isolated in this manner. Three of them, designated A8, A11 and A12 were subjected to several rounds of plaque-purification and amplified in L cells.

Restriction enzyme analysis and southern blot hybridization

Some of the genes located in the left terminus, such as vaccinia virus growth factor (VGF), C4b binding protein and 13.8 kd virokine, have been implicated in pathogenesis (5,7,9). To determine if these genes were present in the recombinants, viral DNA extracted from wild-type, Z-19 and recombinants was restricted and hybridized with labeled plasmid containing the cloned genes. In Fig. 2A, it can be seen that when the plasmid containing VGF gene was used as the probe, it hybridized to one fragment in the wt virus, since only one copy of VGF gene was present in the HindIII C fragment (lane W). As previously shown, Z-19 (lane Z) did not hybridize with the probe (12) while recombinants A11 (lane 2) and A12 (lane 3) displayed a similar pattern of hybridization to that of wt. However, in recombinant A8 (lane 1), VGF is localized in two different

fragments, HindIII C and F (KpnI G). These results indicated that the VGF gene was inserted into the genome of mutant Z-19 by marker rescue.

To examine if the gene of C4b binding protein was present in the recombinants, the blot was probed with plasmid pGK35 which contained the gene of the C4b binding protein. As shown in Fig. 2B, this gene is localized in fragment HindIII C in the wt (lane W) and in A12 (lane 3), but it is absent in Z-19 (lane Z) and is present in fragments HindIII F and G (KpnI D) in A8 (lane 1) and in fragment HindIII D (KpnI B) in A11 (lane 2). These findings indicated that the IHD-W strain contained only one copy of C4b binding protein in the HindIII C fragment of its genome, that Z-19 lacks this gene and that the recombinants have recovered the gene, but in different locations.

The presence of the gene encoding for a 13.8 kd virokine, which is located close to the junction between fragments HindIII C and N (N1L) in WR was also examined (9). Fig. 3 shows the blot that was hybridized with labeled pGK-1 which contained the gene for the 13.8 kd virokine. It can be seen that this gene was present in wt (lane W), Z-19 (lane Z) and all the recombinants (lanes 1,2,3), but in fragment HindIII G. Therefore, the location of this gene in the IHD-W strain is different from that in WR.

Since Z-19 displays rearrangements and small deletions at the right terminus, it was important to establish whether genes located there were also affected. For that purpose two serpin genes localized in fragment HindIII B in the WR strain were studied. In Fig. 4, Southern blot hybridization of the DNA of all the viruses with the plasmid containing the gene for SPI-1 is shown. Fragments HindIII B and C and XhoI E and H were labeled in wt (lane W) and A12 (lane 3), whereas only HindIII B and XhoI E were labeled in Z-19 (lane Z), A8 (lane 1) and A11 (lane 2). On

the other hand, the plasmid containing the SPI-2 gene hybridized to fragments HindIII C and XhoI E in all the viruses (Fig. 5). As expected, a third serpin gene SPI-3 was not affected since it was found to be localized in fragment HindIII K in all the viruses, as in the WR strain (data not shown).

Detection of VGF gene expression by immunoprecipitation

Experiments were also carried out to find out if the recombinants expressed the VGF protein. [^{35}S]-labeled polypeptides isolated from the cytoplasm of wt, mutant or recombinant virus infected L cells were immunoprecipitated with antiserum against epidermal growth factor (EGF) (5,13), and the immune complexes were electrophoresed and detected by radioautography. As shown in Fig. 6, a specific band of MW 24 kd, which corresponds to the membrane associated VGF (13) was detected in the wt (lane W) and recombinant infected cells (lanes 1,2,3), whereas none was observed in Z-19-infected cells (lane Z). These findings confirmed that the mutant lacked the VGF gene and did not express the protein and that the recombinants not only have required the VGF sequences but also expressed them.

Examination of mRNA of C4b by Northern hybridization

Since antibodies against the C4b binding protein were not available, Northern hybridization was used to investigate if this gene was transcribed in the recombinant viruses. mRNA was isolated from the cytoplasmic fraction of infected cells at 2 hr and 10 hr postinfection, electrophoresed in formaldehyde gels and transferred to nytran membrane. The blot was subjected to RNA-DNA hybridization with the plasmid containing the C4b binding protein gene. As shown in Fig. 7, specific transcript was present late in the infectious cycle in cells infected with wt (lane W) and the

recombinants (lanes 1,2,3), but was absent in cells infected with Z-19 (lane Z). These findings indicated that the gene required by the recombinants was also expressed. In addition, Northern hybridization with labeled SPI-1 gene showed that this gene was also transcribed late in infection in all infected cells. However, the level of transcription was less in Z-19 since this gene was only present in fragment HindIII B (data not shown).

Determination of LD50 of the recombinants

To evaluate if the recombinant viruses have recovered virulence, determination of the LD_{50} was performed. Balb/c female mice were inoculated intraperitoneally with serial dilution of wt, mutant or recombinant viruses and the average lethal dose for 50% of the population was measured by the Reed-Meunch method (14). These results are shown in Table 1. Mice inoculated with as much as 10^9 pfu of Z-19 did not die. Whereas some of the mice inoculated with A8 or A11 died. The LD_{50} of recombinant A12 was 3.5 X 10^7 pfu, which when compared to the LD_{50} of the wt 2.1 X 10^7 pfu, indicated that this recombinant recovered virulence. Another sensitive method to evaluate virulence is to measure changes in weight (10). The results shown in Table 1 indicate that the mice inoculated with Z-19 or tissue culture medium did not suffer weight loss, while the mice inoculated with wt and A-12 lost 20-30% of their weights at 10^7 and 10^8 pfu. Interestingly, mice inoculated with A8 and A11 lost weight with 10^8 pfu, but some of them recovered after 7 days.

Discussion

Many genes located in the terminal region of the genome of poxviruses are responsible for viral virulence and are also related to cellular genes involved in the regulation of the host immune system and hemostasis (reviewed in 15). There are at least 23 ORFs in the HindIII C

fragment (16). Some genes have been identified and may be involved in virulence such as VGF, C4b binding protein, one serpin and a host range gene. The latest one is not affected in Z-19 (11).

The results presented here indicated that reintroduction of genes present in the left terminus brings about the recovery of virulence of the attenuated deletion mutant of vaccinia virus, Z-19. They also revealed that the rearrangements and small deletions at the right terminus did not contribute to the attenuated phenotype. In fact, two genes that may be involved in pathogenesis, the two serpin genes were partially altered in Z-19, however, when the HindIII C fragment was reintroduced, virulence was recovered.

From the three recombinants studied, only A12 recovered complete virulence and has the VGF, C4b binding protein and SPI-1 genes reinserted into the HindIII C fragment. Although recombinants A8 and A11 acquired the VGF and C4b protein genes and expressed them, their position in the viral genome is not the same as that of the wild-type. This may have affected their ability to exert their effects in vivo and account for their lack of virulence.

From the results obtained with the gene for the 13.8 kd virokine, it is evident that this gene is localized in fragment HindIII G in the IHD-W strain instead of HindIII N, as reported for the WR strain. Therefore, it was present in wt, Z-19 and recombinant viruses and apparently did not play a major role in pathogenicity of the IHD-W strain.

Finally, it was interesting that the SPI-1 gene was present in fragments HindIII C and B and XhoI E and H in the IHD-W strain. This is unlike the WR and Copenhagen strains in which the gene is localized in fragments HindIII B and C respectively (8,16). Recombinant A12

recovered the SPI-1 gene at the left terminus position and also expressed it. However, the SPI-1 gene may have minor effects on virulence in IHD-W.

The fact that genes related to virulence in the IHD-W strain have been mostly located in fragment HindIII C, makes Z-19 a suitable virus to identify individual virulence genes, since it provides a null background. Reinsertion of genes within the deletion, without selectively inactivating other genes, can be achieved by using an insertion vector containing the flanking sequences of the deletion and a selection system. These experiments are now in progress.

Materials and Methods

Cell and Viruses

L cells were grown as monolayers in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS). Virus infectivity was monitored in L cells according to published procedures (17). The IHD-W strain of vaccinia virus and mutant Z-19 were used when indicated.

Enzyme and chemicals

Restriction enzymes, micrococcus nuclease and nick translation kit were supplied by Bethesda Research Laboratories, Inc., New England Biolabs, Inc., or Boehringer Mannheim Biochemicals, Indianapolis, Ind. and used as specified by the manufacturers.

Animals

Female Balb/c mice between 4 and 6 weeks of age purchased from Charles River Laboratory were inoculated i.p. with different multiplicities of infection and their weight measured daily for two weeks.

Marker Rescue

Confluent monolayers of L cells in plates 15 X 60 mm were infected with 0.5 pfu per cell of mutant Z19. HindIII C fragment was obtained

from wt (IHD-W) DNA digested with HindIII, eluted from agarose gels and phenol extracted. One to two μg were mixed with 20 μg of calf thymus DNA in 1 ml of Hepes-buffered saline and precipitated by addition of $CaCl_2$ to a final concentration of 125 mM (18). The mixture was agitated gently and allowed to precipitate at room temperature for about 45 min. and 0.5 ml added to 6 cm plate monolayers infected for 2 hrs. with Z-19 from which the medium was removed. After 30 min at 37° C, 5 ml of medium containing 8% fetal bovine serum was added to each plate and incubation was continued at 37° C overnight. Then, cells were harvested, sonicated and inoculated into mice by i.p.. After 2 days the animals were sacrificed, the livers were removed, homogenized and plaque assays performed. Twelve plaques were isolated and amplified in L cells. Three recombinants were subjected to several rounds of plaque purification and further characterized.

Screening of recombinants using Southern hybridization

Viral DNA from wt, Z-19 or recombinants was isolated following the procedure of Lai and Chu (19). Briefly, confluent monolayers of L cell plates were infected with the recombinant at m.o.i. of 0.5. After overnight incubation at 37° C, the cells were scrapped off the plates, centrifuged, washed once with PBS, resuspended in 400 μl of a solution containing: 0.5% NP40, 10 mM Tris-HCl buffer pH 7.4 for 30 min at 0° C, vortexed occasionally and transferred to a 1.5 ml Eppendorf tube. To this cell extract, 50 μl of 10 X reaction buffer (20 mM Tris-HCl buffer, pH 7.8, 60 mM KCl and 15 mM NaCl), 1 μl of 1 M $CaCl_2$ and 4 μl of Micrococcus Nuclease (15 units per ml) were added. The reaction was carried out for 2 hr at 37° C and terminated by adding 20 μl of 0.5 M EGTA and 0.5 M EDTA each. Viral DNA was extracted by adding 50 μl

of sarkosyl and proteinase K (1mg/ml) and incubated for 1 hr at 37° C, followed by treatment with phenol and ethanol precipitation. The DNA was resuspended in 0.01 M Tris-HCl buffer pH 7.5 with 0.5 mM EDTA (TE), digested with restriction enzymes, fragments separated by electrophoresis in 0.5% agarose gels and blotted onto nytran membranes. Blots were probed with the following plasmids: pSC16, pGK35, pGk1, SpI-1, SpI-2, SpI-3 which contains the genes for VGF, C4b binding protein, 13.8 kd virokine and the three serpins respectively. The plasmids were gifts from Dr. B. Moss (NIH) and Dr. G.J. Kotwal (Cincinnati, Ohio). They were labelled with [thio-^{35}S] dATP by nick-translation as described by Rigby (20).

^{35}S-Methionine Labelling and preparation of cell extract for immuprecipitation

Confluent monolayers of L2 cells growing on 10 cm plates were infected with 10 pfu per cell and incubated at 37° C for the times indicated. The culture media was then replaced with methionine free medium to which 10 µCi/ml of [^{35}S]-methionine, was added and the cells incubated for another hr. at 37° C. Cells were scraped off the plates, centrifuged at 1,000 rpm, washed once with phosphate buffer saline (PBS), resuspended in a solution containing, 0.02 M Tris-HCl buffer pH 7.8, 0.15 M NaCl and 0.1 mM EDTA, homogenize with a Dounce homogenizer and centrifuged at 800 X g to sediment the nuclei. The supernatant was immunoprecipitated first with preimmune serum and subsequently with antimouse EGF (Collaborative Research) and protein A sepharose CL-4B suspension (Pharmacia) as described by Chang et al. (13). The immunoprecipitated samples were electrophoresed in SDS-15% polyacrylamide gels for 5 hr at 180 V. The gels were fixed for 30 min in

30% methanol-10% glacial acetic acid, enhanced for 30 min in AmplifyTM (Amersham), rinsed twice with water, dried and exposed to Kodak X Omat film at -70° C.

Extraction of cytoplasmic RNA and northern hybridization

Cytoplasmic RNA was extracted as described by Gough (21). Briefly, infected cells were harvested at the times indicated and resuspended in 200 µl of a cold solution containing: 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1.5 mM $MgCl_2$ and 0.65% NP-40. Lysates were subsequently centrifuged to remove nuclei and cytoplasmic RNA was recovered by ethanol precipitation. The RNA was resuspended in TE, precipitated with 5M LiCl and centrifuged at 10,000 rpm for 10 min in a Sorvall centrifuge. The pellets were resuspended in TE and the RNA concentration was determined by absorption at 260 mm in a Gilson spectrophotometer.

Cytoplasmic RNA was resuspended in a buffer containing formamide, formaldehyde and 3-(N-morpholino) propanesulfonic acid (MOPS) and denatured at 70° C for 10 min. Samples were loaded on a 1% (Wt/Vol) agarose gel containing 2.2 M formaldehyde and MOPS. Electrophoresis was conducted for 8 hr at 45 V and the gels were transferred to Nytran filters. The filters were incubated for 2 hr at 42° C in a prehybridization solution containing: 50% formamide, 5 X Denhardt solution, 0.1% sodium dodecylsulfate (SDS), 5 X SSPE and 100 µg/ml fragmented salmon DNA. Subsequently, the probe labeled by nick translation was added and the filters were incubated for 18 hr at 42° C, then washed with 6 X SSPE, 0.1 % SDS, 1 X SSPE and 0.5% SDS at room temperature, dried and exposed to Kodak X-Omat film for 48 hr at -70° C.

Acknowledgments

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Table 1 Determination of virulence of vaccinia wild-type, Z-19 and recombinant viruses

Virus	LD_{50} (pfu)	% Weight loss		
		10^6 pfu	10^7 pfu	10^8 pfu
Wt	2.1×10^7	none	20%	30%
Z-19	$> 10^9$	none	none	none
A8	$> 10^8$	none	none	20%
A11	$> 10^8$	none	none	20%
A12	3.5×10^7	none	20%	22%
Control	0	none	none	none

Groups of three Balb/c 4-6 week old female mice were inoculated i.p. with increasing amounts of pfu of the viruses indicated. Control animals received 0.2 ml of MEM. Results from two separate experiments were pooled and the LD_{50} was calculated. The mice were weighed daily and the loss of weight was recorded between 2 and 7 days after inoculation.

Fig. 1 Schematic chart for transfection of HindIII C fragment into Z-19 infected cells

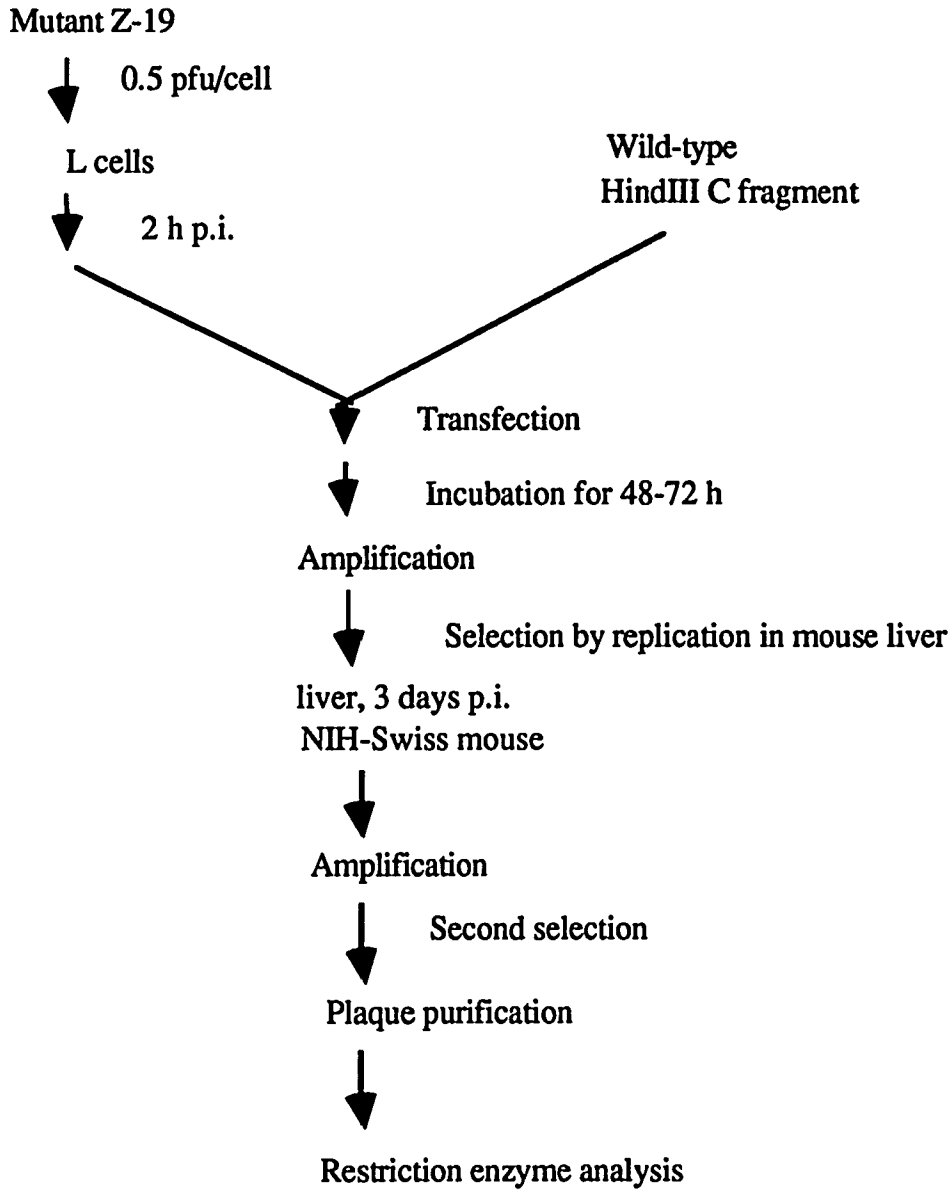


Fig. 2A. Southern hybridization of viral DNA with the vaccinia growth factor gene. Viral DNAs were digested with HindIII and the reaction products separated by electrophoresis in 0.5% agarose gel, blotted to nitrocellulose paper and hybridized with labeled plasmid containing the gene for VGF. M: molecular weight marker, W: wt of IHD-W strain, Z: mutant Z-19, 1: recombinant virus A8, 2: recombinant virus A11. 3: recombinant virus A12. Restriction map from the Copenhagen strain (16).

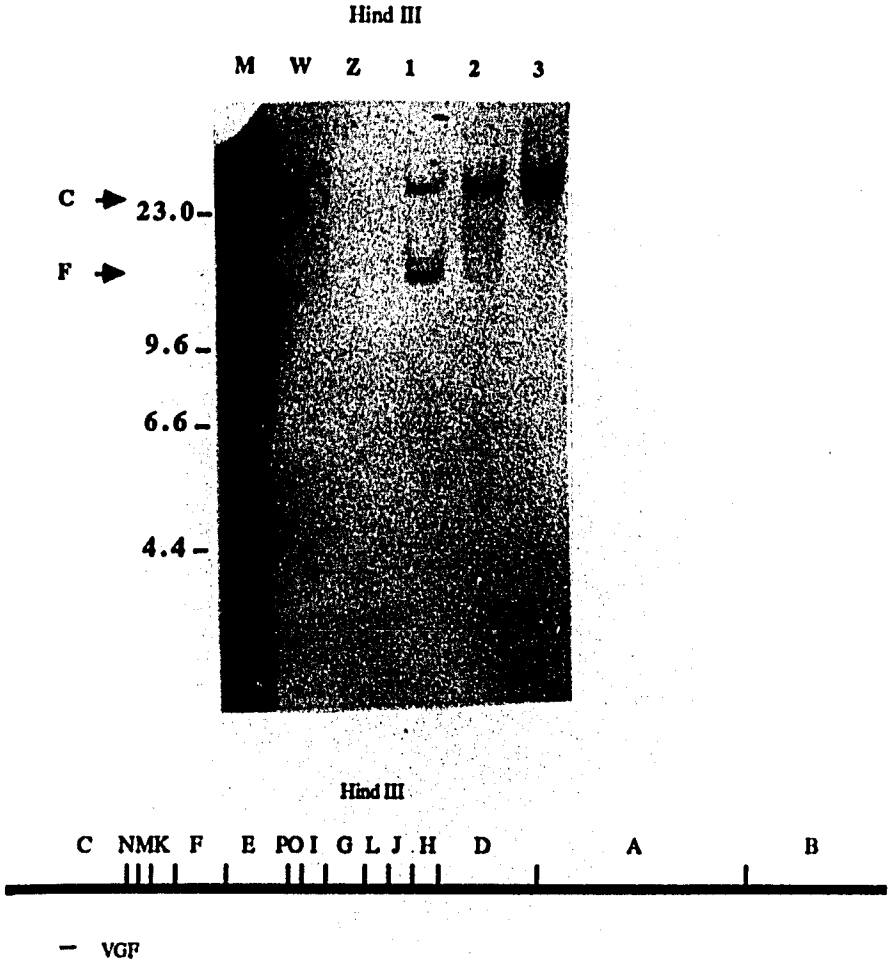


Fig. 2B. Southern hybridization of viral DNA with the C4b binding protein gene. Experimental conditions as in A. M: molecular weight marker, W: wt of IHD-W strain, Z: mutant Z-19, 1: recombinant virus A8, 2: recombinant virus A11. 3: recombinant virus A12.

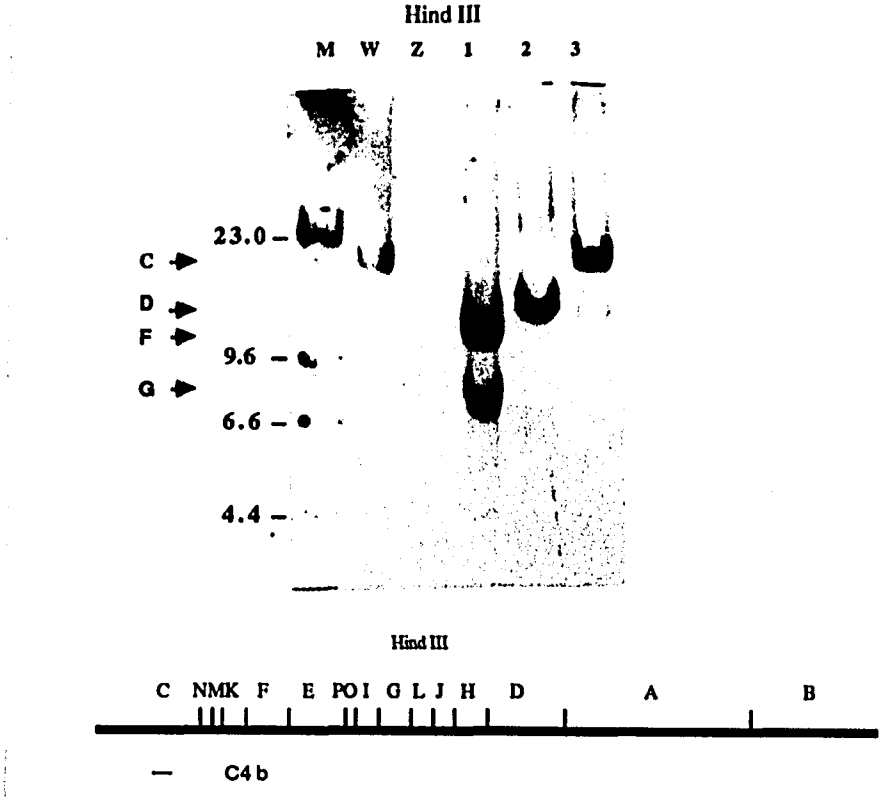


Fig. 3. Southern hybridization of viral DNA with the 13.8 kd protein gene. Viral DNAs digested with HindIII, SalI or KpnI and processed as described in Fig. 2. M: molecular weight marker, W: wt of IHD-W strain, Z: mutant Z-19, 1: recombinant virus A8, 2: recombinant virus A11. 3: recombinant virus A12.

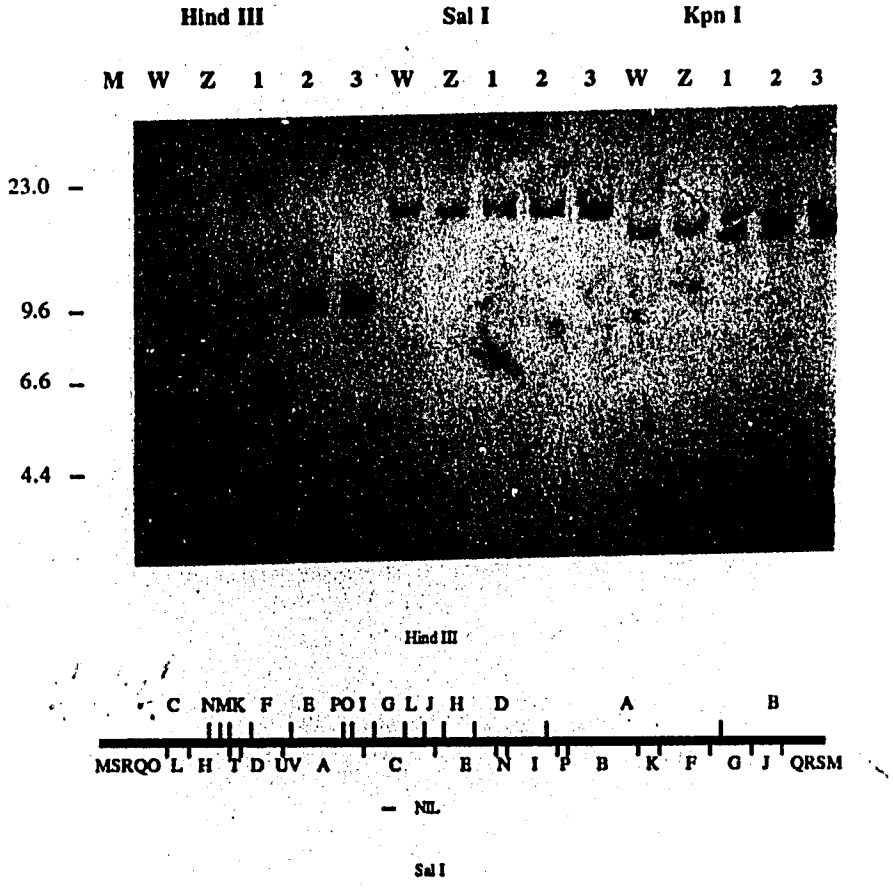


Fig. 4. Southern hybridization of viral DNA with the serpin-1 gene. Viral DNAs were digested with HindIII or XhoI and processed as described in Fig. 2. M: molecular weight marker, W: wt of IHD-W strain, Z: mutant Z-19, 1: recombinant virus A8, 2: recombinant virus A11. 3: recombinant virus A12.

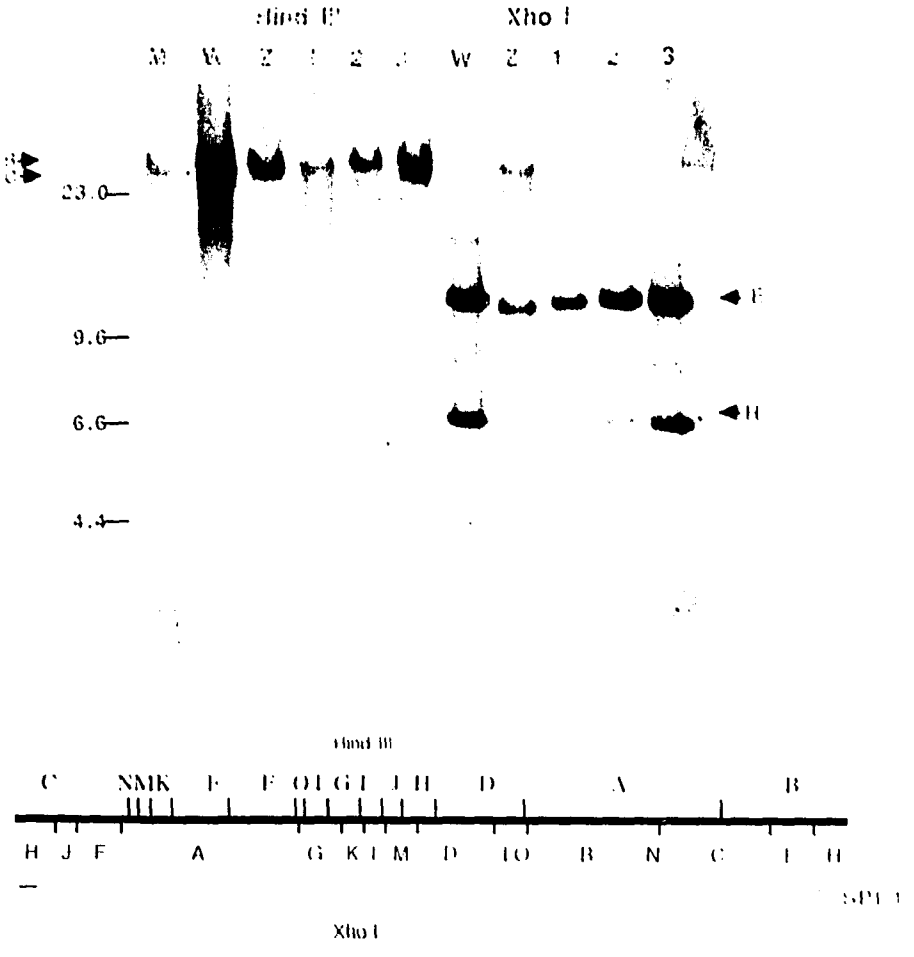


Fig. 5. Southern hybridization analysis of viral DNA with the serpin-2 gene. Viral DNAs were digested with HindIII or XhoI and processed as described in Fig. 2. M: molecular weight marker, W: wt of IHD-W strain, Z: mutant Z-19, 1: recombinant virus A8, 2: recombinant virus A11. 3: recombinant virus A12.

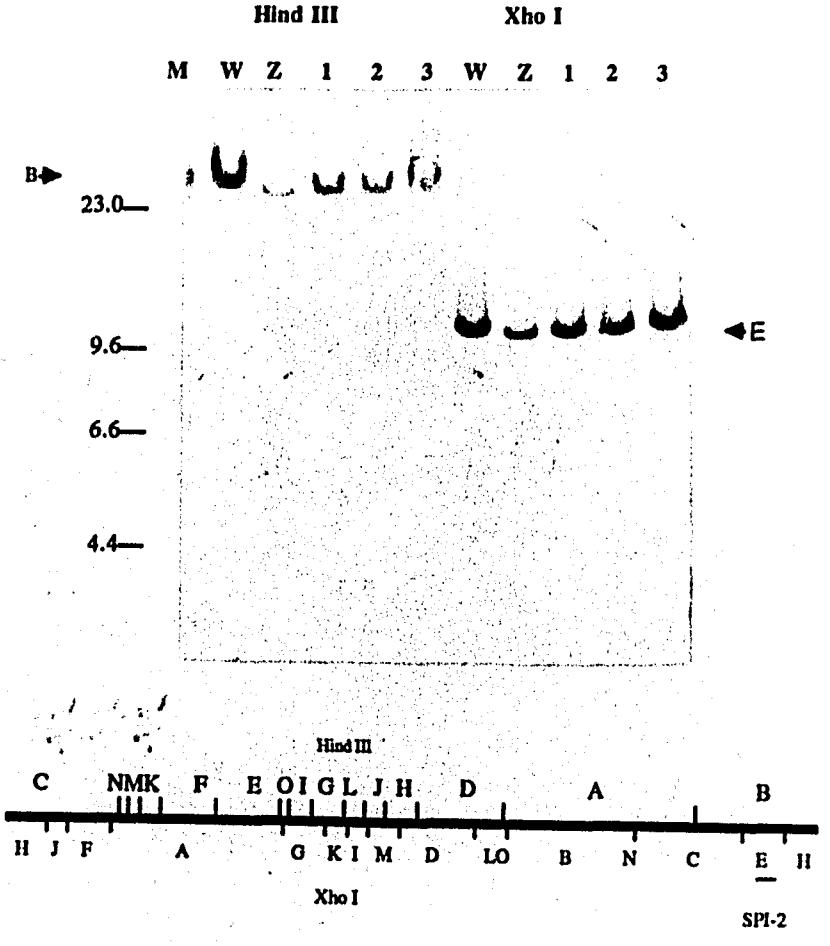


Fig. 6. Immunoprecipation of [^{35}S] methionine-labeled protein extracts from virus-infected cells. L cells monolayer were infected with either wt, mutant Z-19 or recombinant viruses, pulse-labeled with [^{35}S]- methionine at the times indicated, cell extracts prepared and immunoprecipitated with an antibody to epidermal growth factor. M: molecular weight marker. C: mock infection. W: IHD-W strain infected cells. Z: mutant Z-19 infected cells. 1: recombinant virus A8 infected cells. 2: recombinant virus A11 infected cells. 3: recombinant virus A12 infected cells.

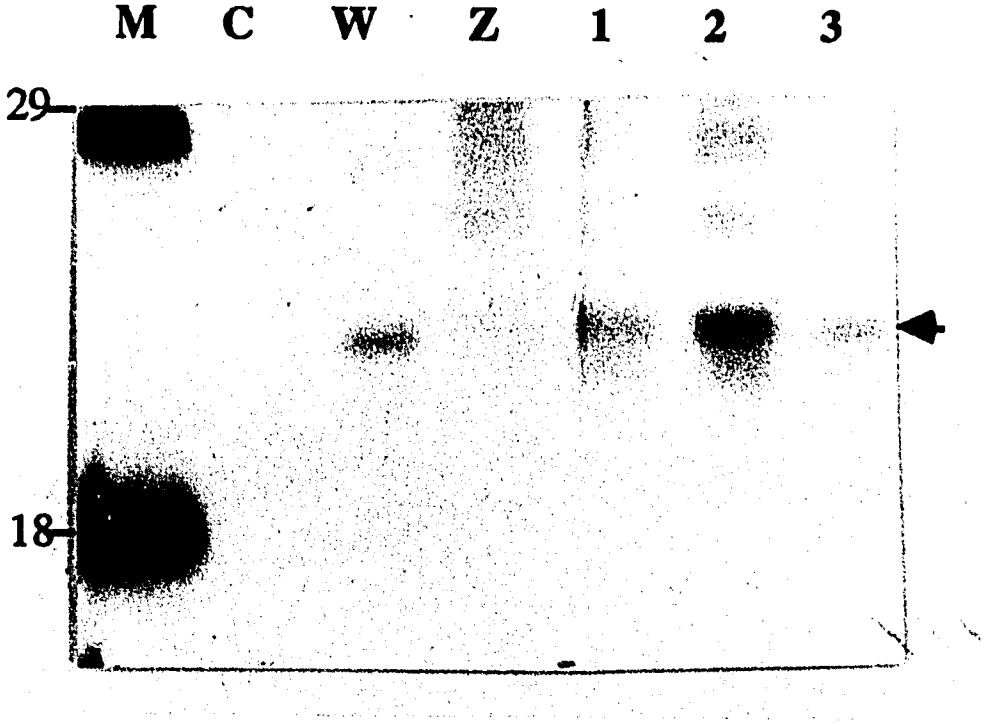
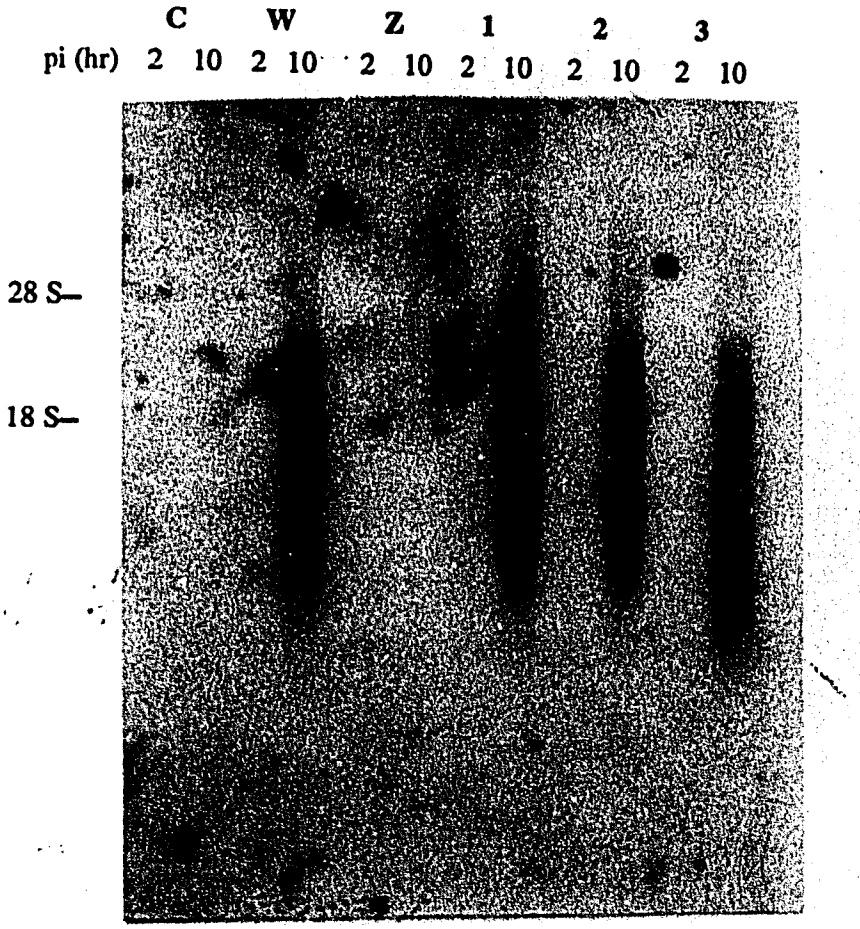


Fig. 7. Northern (RNA) blot analysis of cytoplasmic RNA. Cytoplasmic RNA was isolated from L cells monolayers infected with 10 pfu per cell of either wt, mutant Z-19 or recombinant viruses at the times indicated. The RNA was size fractionated by electrophoresis on formaldehyde-agarose gel and transferred to nytran membrane. The blot was hybridized with labeled plasmid containing the gene of C4b binding protein as described in material and methods. C: mock infection. W: IHD-W strain infected cells. Z: mutant Z-19 infected cells . 1: recombinant virus A8 infected cells. 2: recombinant virus A11 infected cells. 3: recombinant virus A12 infected cell.



Chapter III

Reintroduction of gene(s) into attenuated deletion mutant of vaccinia virus

Pi-Yun Chang and Beatriz G-T Pogo

Abstract

To evaluate individual genes responsible for virulence of vaccinia virus, a shuttle vector containing a viral fusion fragment from a deletion mutant Z-19 was constructed. The gene in question was reinserted into the mutant Z-19 by homologous recombination. The vector also included a β-galactosidase cassette which contained the LacZ under the control of the vaccinia late promoter P11 to screen recombinants. The vaccinia growth factor and the vaccinia complement-binding protein were inserted into this shuttle vector and transferred by recombination to mutant Z-19. The presence of the inserted gene was examined by Southern hybridization and its expression by Northern blot hybridization. The virulence of the recombinants was investigated by inoculation into mice. Results indicate that the recombinants contain the inserted gene and expressed it. Although virulence was recovered, it did not reach the level of the wild-type, either when inoculated alone or in combination.

Introduction

Vaccinia virus is being used as a vector to study gene regulation and function. Based on the success of vaccination to eradicate smallpox, it has been regarded as a good candidate to be used as a live recombinant vaccine in humans and animals. There are several advantages for using vaccinia as a live vaccine, such as: wide host range, potency in the single inoculation, ability to induce humoral and cell-mediated immunity, flexibility of packaging in viral particles, cheap manufacture, ease of administration, and stability without refrigeration (Moss, 1992). It may also be used to carry multiple antigens and to protect against a more than one disease. In 1985, Paoletti et al. (Paoletti et al., 1985; Perkus et al., 1985) constructed a recombinant vaccinia virus vector containing three different antigens (Influenza hemagglutinin, Hepatitis B antigen and herpes glycoprotein D) which was able to induce antibodies against all those antigens in mice. However, the use of vaccinia virus as live recombinant vaccine is still limited, because of the possibility of several adverse reactions, such as generalized vaccinia in skin-damaged individuals, and encephalitis, especially when administrated to immunocompromised individuals. In 1987, Redfield (Redfield et al., 1987) reported vaccinia dissemination in an HIV positive military recruit who had been vaccinated with multiple vaccines including vaccinia two and an half weeks earlier. Subsequently, the recruit developed AIDS and died. Moreover, in 1992, AIDS patients within a study group in Paris received fixed vaccinia recombinant infected cells died due to vaccinia dissemination derived from inactivated virus (Picard et al., 1990; Guillaume et al., 1991). Therefore, it is important to study pathogenicity to design safe vaccinia-based recombinant vaccines.

Characterization of spontaneous deletion mutants (Drillen et al., 1981; Panicali et al., 1981; Moss et al., 1981; Perkus et al., 1986; Dallo and Esteban, 1987; Lai and Pogo, 1989a), or target insertional inactivated mutants (Buller et al., 1985; Buller et al., 1988; Kotwal et al., 1989; Kotwal et al., 1990) indicates that genes mostly located at both ends of the viral genome are variable, and not necessary for virus replication in tissue culture. These genes are related to host range, and virulence, as well as the immune defense response.

There are two ways to identify whether a gene is involved in pathogenicity. One approach is insertional inactivation of target gene or systematic removal of selected regions of DNA to produce mutants (Buller et al., 1985; Buller et al.,1988; Kotwal et al., 1989; Kotwal et al., 1990). Insertional inactivation mutants have shown alterations in virulence, but sometimes the site selected for insertion may influence the virulence by the insertion itself, such as is the case of the thymidine kinase gene (Buller et al., 1985). To identify a virulence gene, another approach is to reintroduce the gene in question into an attenuated deletion mutant. One advantage of this method is that deletion mutant provides a null background. Therefore, if the recombinant virus shows an alteration of virulence it will be the consequence of the insert. Bloom et al. (1991) have used such a system for systemic reintroduction of overlapping segments of deleted DNA into an attenuated deletion mutant of the rabbitpox virus (RPV) which has restricted host range and a deletion at the left end of the genome. It was found that two regions at the left end of the rabbitpox virus genome were involved in virulence.

In our laboratory, previous studies showed that mutant Z-19 had a large deletion at the left terminus of the viral genome (Lai and Pogo, 1989a), lacking vaccinia growth factor (VGF) and vaccinia complement-binding protein (VCBP) genes and was avirulent in vivo (Lai and Pogo, 1989b, Chang et al., 1992). It provides a good model system for reinsertion of deleted genes to evaluate the virulence. We have reintroduced the deleted fragment HindIII C into mutant Z-19, to evaluate which genes were responsible for the attenuated phenotype. The results showed that one of the recombinants recovered virulence (Chang et al., 1992). We also found that two genes, VGF and VCBP, involved in virulence, were present and expressed in this recombinant, in addition to other 21 genes located in fragment HindIII C. To identify which gene(s) are responsible for pathogenicity, we report the construction of a shuttle vector containing fusing fragment K' from Z-19 and a β–galactosidase cassette (pK'). The test gene was inserted into pK' and this chimeric plasmid used to introduce test gene(s) into mutant Z-19 by homologous recombination. The resulting recombinant viruses were analyzed for gene expression and virulence in mice. The results suggested that when, either gene VGF or VCBP, was reintroduced into mutant Z-19, the resulting recombinant viruses showed increased virulence, suggesting that the alteration of the phenotype was provided by the insert. However, neither of them alone, or in combination, were capable of restoring complete virulence.

Material and methods

Cells and viruses

L cells were grown as monolayer in minimum essential medium (MEM) supplemented with 5% fetal calf serum (Gibco). The IHD-W strain of vaccinia virus, mutant Z-19, and recombinant viruses containing VGF or VCBP were propagated in L cells and purified by sucrose gradients (Joklik, 1962). Virus infectivity was monitored in L cells, as described previously (Dales, 1962).

Enzyme and chemicals.

Restriction enzymes, micrococcus nuclease and nick translation kit were supplied by Bethesda Research Laboratories, Inc., New England Biolabs, Inc., or Böehringer Mannheim Biochemicals, and used as specified by the manufacturers.

Restriction endonuclease and DNA hybridization analyses of virus DNA.

DNA was isolated as described previously and digested with restriction endonucleases (Chang et al., 1992). The restriction fragments were resolved by electrophoresis in a 0.5% agarose gel, stained with ethidium bromide, and photographed by UV transillumination. DNA fragments were transferred to nytran membrane by the procedure of Southern (Southern, 1975). DNA was nick-translated (Rigby et al., 1977) and hybridized to the immobilized DNA as described (Chang et al., 1992).

Animals.

Female Balb/c mice between 4-6 weeks of age purchased from Charles River Laboratory were inoculated by the intraperitoneal (ip) route with 10-fold dilutions: 10^6, 10^7 and 10^8 pfu (four or five mice per

dilution) of each vaccinia recombinant. The LD_{50} was determined in mice by calculating the 50% end point by the Reed-Muench method (Reed and Meunch, 1938) and all mice were weighed between 0 and 7 days.

Construction of chimeric plasmids.

1) **Construction of plasmid including K' fragment.**

The fragment K' was isolated from mutant Z-19 DNA digested with HindIII, and then ligated into HindIII-cleaved pUC19, and the competent Escherichia coli $DH5\alpha$ cells were transformed.. β-Gal negative colonies were isolated by using the X-Gal-IPTG screening system. The plasmid was designated pK'.

2) **Construction of plasmid containing VGF gene.**

The fragment containing VGF gene was isolated from pSC16 (kindly supplied by Dr. Moss) which was digested with HindIII and HincII. This fragment was repaired by using the Klenow fragment of the DNA polymerase, ligated to an EcoRI-linker and then ligated into EcoRI-cleaved pK' and used to transform competent cells $DH5\alpha$. Colonies were isolated by using hybridization with labeled VGF. This plasmid designated pK'V contained the VGF gene flanked by the K' fragment.

3) **Construction of plasmid containing VCBP gene.**

A 1.1 kilobase-pair HincII restriction endonuclease fragment containing the complete nucleotide sequence of the VCBP gene was isolated from pGK35 (kindly supplied by Dr. Moss). This fragment was ligated to an EcoRI-linker and then into EcoRI-cleaved pK' and used to transform

competent cells DH5α. The white colonies were picked and the DNA was extracted and separated by agarose gel electrophoresis and blotted onto nytran membrane. The blot was hybridized with labeled VCBP gene. The resulting plasmid, termed pK'C, contained a complete VCBP sequence flanked by K' fragment. This plasmid was sequentially digested with KpnI and dephosphorylated with calf intestinal phosphatase and ligated to a modified XbaI-SmaI fragment from pSC10 (kindly supplied by Dr. Moss), which contained the vaccinia virus late promoter P11 coupled to Ecoli lacZ gene (β-galactosidase cassette). The competent DH5α cells was transformed and the blue colonies (β-Gal$^+$) were isolated. The DNA from blue colonies was digested with KpnI restriction endonuclease and hybridized with labeled vaccinia virus promoter p11. The resulting plasmid was termed pK'CZ.

4) **Transfection of plasmids and isolation of recombinant viruses.**

Modified procedures described by Faith et al. (1986) were followed. Briefly, A 60 X mm dish of confluent L cells was infected with mutant virus Z-19 at a multiplicity of infection of 0.5 pfu/cell in unsupplemented MEM. The viruses were allowed to adsorb for one hour at 37°C and 5% CO_2. The inoculated solution was aspirated and MEM with 5% fetal calf serum was added to the monolayer of infected L cells. During incubation, 20 μg of BglI-digested plasmid pK'V or pK'CZ and 1 μg of mutant Z-19 viral DNA were mixed with 0.5 ml of 1 X hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered saline, then 62 μl of 1 M $CaCl_2$ was added. The mixture was allowed to precipitate at room temperature for one hour. After two hours postinfection, the medium was removed from

the monolayer, and the cells were washed once with unsupplemented MEM, then 0.5 ml mixture of calcium phosphate-precipitated DNA was added and incubated for 30 min at room temperature. Thereafter, the fresh medium was added to the monolayer, which was incubated for another 3.5 hour at 37°C. The medium was removed, fresh medium was added, and the monolayer was incubated for 24 hours: The transfected cells were harvested by scraping the dish with a rubber policeman and centrifuged at 8000 rpm at 4°C for 30 min. The pellet was resuspended in 1 ml of 1 x unsupplemented MEM and sonicated to release the virus. The recombinant virus contained VGF gene was isolated by serial dilution and dot blot hybridization or by in vivo replication (Lai and Pogo, 1989b). The recombinant virus containing VCBP gene (blue plaques) was screened by X-Gal. The recombinants were purified by plaque assay and amplified in the L cells for analysis of virulence.

Extraction of cytoplasmic RNA and Northern blot hybridization.

The procedures used were previously described (Chang et al., 1992).

Results

Mutant Z-19 has a large deletion at the left terminus of the viral genome, including VGF and VCBP (Lai and Pogo 1989b; Chang et al., 1992). To investigate which gene is responsible for pathogenicity in vaccinia virus, rescue experiments were performed. Since vaccinia virus DNA is not infectious, the test gene is reintroduced into mutant viral genome by homologous recombination. For this purpose, first, a chimeric

plasmid containing the test gene flanked by viral DNA was constructed. Second, introduction of the chimeric plasmid by transfection into mutant virus Z-19 infected cells has to be performed. Previous experiments showed that mutant Z-19 generated a new fragment, K', which included part of the terminal repetition and part of HindIII N fragment (Lai and Pogo, 1989a). The K' fragment was cloned into pUC19, and the plasmid was designated pK' (Fig.1a). It was used as a shuttle vector for reinsertion of test genes into mutant Z-19 by homologous recombination.

Construction of recombinant vaccinia virus containing VGF.

To determine the role of VGF gene in the pathogenicity of vaccinia virus, we have constructed a recombinant virus containing the VGF gene, vV. The plasmid, pK'V, containing the entire VGF gene flanked by fragment K', was used for transfection (Fig. 1b). The strategy used to reintroduce the VGF gene into mutant Z-19 is shown in Fig. 2A. The chimeric plasmid pK'V lacked a selection system, recombinant viruses were selected by in vivo replication since mutant Z-19 cannot replicate in the liver of mouse as previously demonstrated (Lai and Pogo,1989b). The recombinant isolated from mouse liver was then characterized.

Southern hybridization was carried out to examine if the VGF gene was reinserted into the mutant Z-19 genome. The results showed that there was a band in the recombinant virus (Fig. 3a, lane 3), compared to parental virus Z-19 (Fig. 3a, lane 2), indicating that the VGF gene was reintroduced into Z-19. However, after a second round of virus amplification, the inserted gene VGF was missing in the recombinant genome, as indicated by the results of hybridization shown in Fig. 3b, lane

3. Another method for selection of recombinant virus, vV, was carried out by serial dilutions of the virus mixture and screening by dot blot hybridization, then plaque purification and amplification. The results of hybridization showed that VGF gene was present in the recombinant virus, vV (Fig. 3b, lane 5). After several rounds of amplification, the VGF gene was still present in the recombinant genome, as shown by hybridization (Fig. 3c, lane 3).

Construction of recombinant vaccinia virus containing VCBP gene.

To evaluate the role of the VCBP gene in the virulence of vaccinia virus, construction of a recombinant virus, vC, was performed, as shown in Fig. 2B. The plasmid, pK'CZ, contained the entire sequence of VCBP gene and LacZ under the control of vaccinia virus promoter, P11, flanked by fragment K' (Fig. 1c). This plasmid, pK'CZ, was used as a shuttle vector for transfection of the VCBP gene into mutant Z-19. The resulting recombinant viruses were selected by X-gal screening as blue plaques.

To test if the VCBP gene was reinserted into the mutant Z-19, Southern hybridization was carried out with labeled VCBP gene. DNA from the recombinant was hybridized with p^{32}-labeled VCBP gene. The results indicated that VCBP gene was inserted into the mutant Z-19 (Fig. 4, lane 5). In addition, the P^{32}-labeled-P11 was used to examined if p11 was inserted into recombinant virus. The P11 promoter included in the β-galactosidase cassette was located between the HindIII E and F fragments. Two bands were labeled: one in the HindIII F fragment and the other in the same band that the VCBP gene was located (Fig. 5). When labeled lacZ

gene was used as a probe, the results showed that the LacZ was inserted into the recombinant virus and did not appear in the wild-type and mutant Z-19 (Fig. 6). The hybridization indicated that VCBP gene and β-galactosidase cassette were inserted into the mutant Z-19 and the resulting recombinant virus was designated vC.

Expression of the recombinant viruses.

To investigate expression of the inserted genes, Northern blot hybridization with the labeled inserted gene as a probe was performed. The cytoplasmic RNA was isolated and fractioned by eletrophoresis in 1% formaldehyde gels and transferred to a nitrocellulose membrane. The blots were subjected to RNA-DNA hybridization with labeled VGF gene or VCBP gene obtained from plasmids pSC16 or pGK35. RNAs isolated from cells infected with wild-type virus (lane 1, 4), mutant Z-19 (lane 2, 5), and vV recombinant virus (lane 3, 6) are shown in Fig. 7A. The VGF transcripts are present at early and late times in the infection cycle of the recombinant virus and the wild-type, but not in Z-19 infected cells. RNA isolated from recombinant virus, vC, infected cells is shown in Fig. 7B. The transcripts are expressed at late time in the infection cycle of recombinant virus, vC, (lane 3) and of wild-type virus (lane 1). The characteristic of the late transcript is the heterogeneous size of the RNA. As expected, cells infected with Z-19 do not contain VCBP transcripts.

Virulence of vaccinia recombinant viruses.

To evaluate the virulence of the recombinant viruses, recombinants vV and vC were inoculated i.p. in mice at three dilutions (10^8, 10^7, and 10^6 pfu). Intraperitoneal injection was used because it caused less damage

to mice than intracranial injection. The LD_{50} of wild-type was 6 X 10^6 pfu, indicating that wild-type was virulent, compared with mutant Z-19 whose LD_{50} was > 10^9 pfu . The LD_{50} of recombinants containing vC or vV was 4.2 X 10^7 pfu and >10^8 pfu, respectively, suggesting that both recombinant viruses have an increase in virulence.

Another parameter, per cent of weight loss, was used to evaluate the virulence. Mice were inoculated with wild-type or recombinants at three dilutions (10^8, 10^7 and 10^6 pfu) and weighed daily for one week. The results showed in table 1. Of five mice inoculated with 10^8 pfu of the recombinant, vC, four died and the one left lost 17 % of weight during 3 and 4 days after inoculation, but recovered after seven days. Five mice were inoculated with the recombinant virus, vV, two died, but three survived and lost 15%-25% of weight during third and sixth day after inoculation, and recovered after eight days. The surviving mice also displayed serious symptoms such as arched back and ruffled appearance and inactivity during 2 to 5 days postinoculation. Neither symptoms nor deaths occurred in the mice inoculated with 10^7 pfu of either recombinant vC or vV, in contrast to mice inoculated with 10^7 pfu of wild-type, all of which died.

Since neither recombinant containing VGF or VCBP gene completely restored virulence, a combination of the two different recombinants, vV and vC, was inoculated. The same amount of each of recombinant was mixed for inoculation, and double amount of wild-type was used. With 10^8 pfu, of six mice inoculated with the combined recombinants only two died. Although symptoms occurred in the other four mice, they recovered after 5 days. However, neither symptoms nor

deaths occurred in the mice inoculated with 10^7 pfu of both combined recombinants. In contrast, mice inoculated with wild-type virus died between 2-4 days and only one mouse recovered after 5 days postinoculation with 10^7 pfu.

Discussion

In this communication, we used the attenuated mutant Z-19 to study molecular pathogenicity of vaccinia virus. For that purpose, the shuttle vector, pK'Z, was constructed. This vector contained the viral fragment K' from mutant Z-19 and the β-galatosidase cassette under control by vaccinia late promoter P11. This vector is useful to reintroduce any gene into Z-19, which is a highly attenuated deletion mutant.

Two recombinants containing the VGF or the VCBP were constructed using this vector through homologous recombination. Northern blot hybridization showed that the recombinants vV transcribed VGF at early and late times in the infection cycle, and that recombinant vC transcribed VCBP late in the infection cycle. Analysis of LD_{50} and per cent of weight loss showed that both recombinants displayed an increase in virulence.

Analysis of the LD_{50} of recombinants indicated that recombinant vC (4.2×10^7 pfu) was more virulent than recombinant vV ($> 10^8$ pfu). It was reported that VCBP could block the classical complement pathway and bind to C4b and was synthesized through late infection cycle (Kotwal and Moss, 1988; Kotwal et al., 1990). Therefore, VCBP may play an important role in the inhibition of host defense mechanism. Recovery of this gene function partially reestablished virulence in Z-19. Although this

recombinant lacks VGF it is still able to induce death in mice contrary to previous assumptions (Buller et al., 1988; Lai and Pogo, 1989b) that VGF is necessary for replication in vivo and virulence. Interestingly, when two recombinants were inoculated at a high pfu dose, only two out of six mice died. By contrast, when five mice were inoculated with one recombinant vC, four mice died. This may be due to intrinsic variation of the biological system or to interference between the recombinants in vivo resulting in a less virulent infection.

Taken together, results indicated that more than VGF and CBP are necessary to reestablish virulence and that other genes present in fragment HindIII C may be necessary. Our results are in agreement with those reported by Bloom et al. (1991) who reintroduced overlapping segments of the deleted region into an avirulent mutant of rabbitpox virus. It was found that one recombinant virus containing only one complete open reading frame (LD_{50} $>10^7$ pfu) was less virulent than other containing several open reading frames (LD_{50} 3.8×10^5). This data and ours data support the notion that more than one gene is necessary for pathogenicity.

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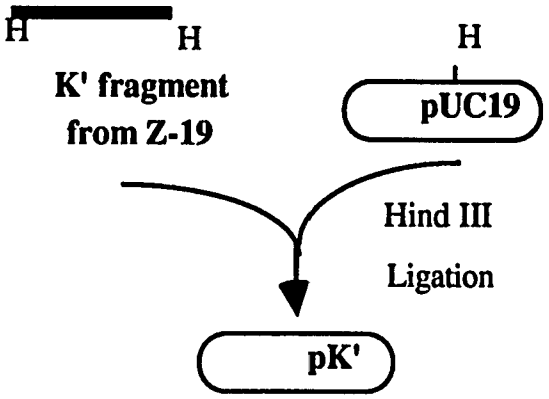
Table 1. Results of animal experiments

Virus	Inoculation PFU	No. of mice	Dead	% weight loss	No. of mice Recovered
WT	10^6	4	0	0%	4
	10^7	4	4	0%	0
	2×10^7	5	5	0%	0
	10^8	4	3	24%	1
	2×10^8	6	6	0%	0
Mutant Z-19	10^6	6	0	0%	6
	10^7	6	0	0%	6
	10^8	6	0	0%	6
vC	10^6	4	0	3%	4
	10^7	4	0	3.5%	4
	10^8	5	4	17%	1
vV	10^6	4	0	3%	4
	10^7	4	0	4.5%	4
	10^8	5	2	15-25%	3
vC+vV	2×10^7	5	1	5%	4
	2×10^8	6	2	20%	4

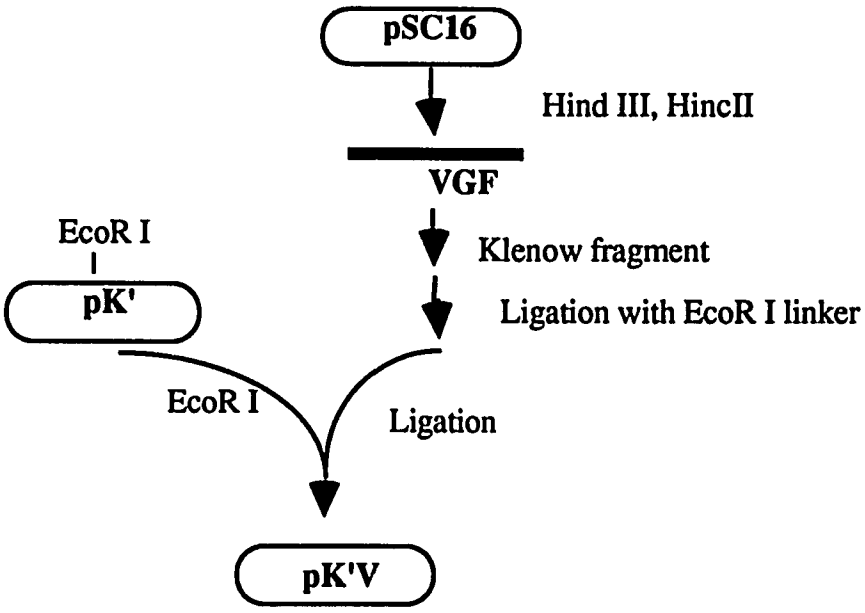
Groups of 4-6 Balb/c 4-6 week-old female mice were inoculated i.p. with increasing amounts of pfu of the viruses as indicated. The mice were weighed daily. Recovery occurred between 2-8 days after inoculation. Five control animals received 0.2 ml of MEM, none showed any effects.

Figure1. Construction of chimeric plasmids

a) Construction of plasmid pK'



b) Construction of plasmid pK'V
containing VGF gene



(c) Construction of plasmid pK'CZ containing VCBP gene

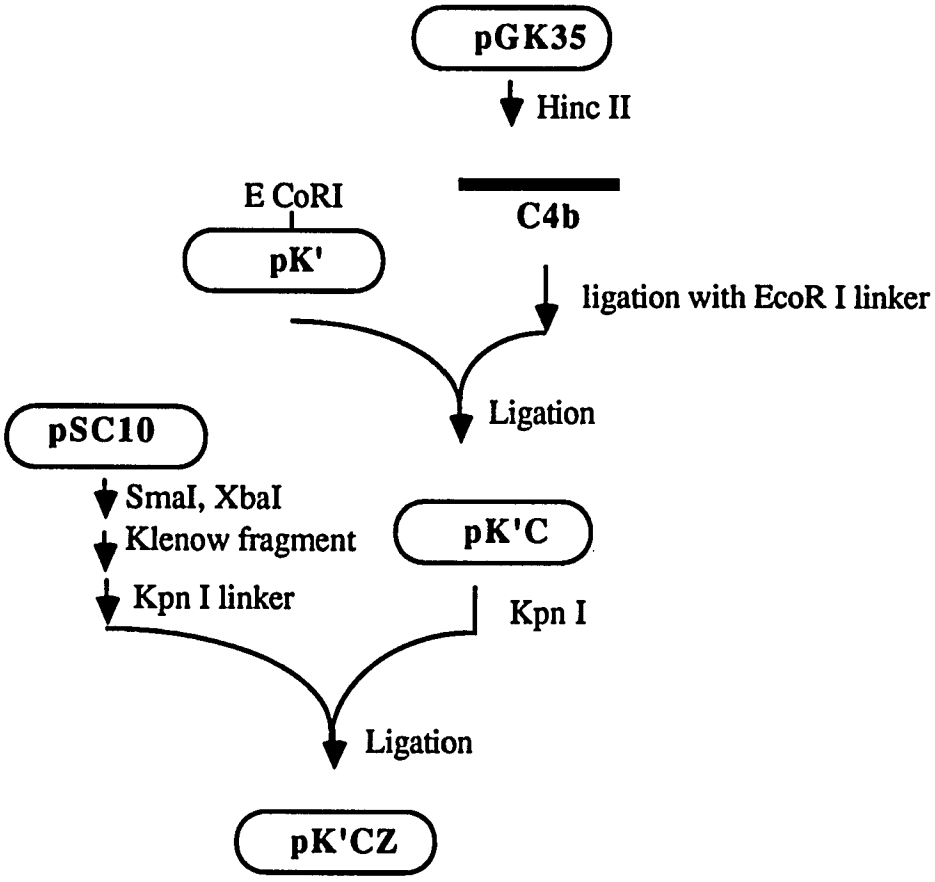
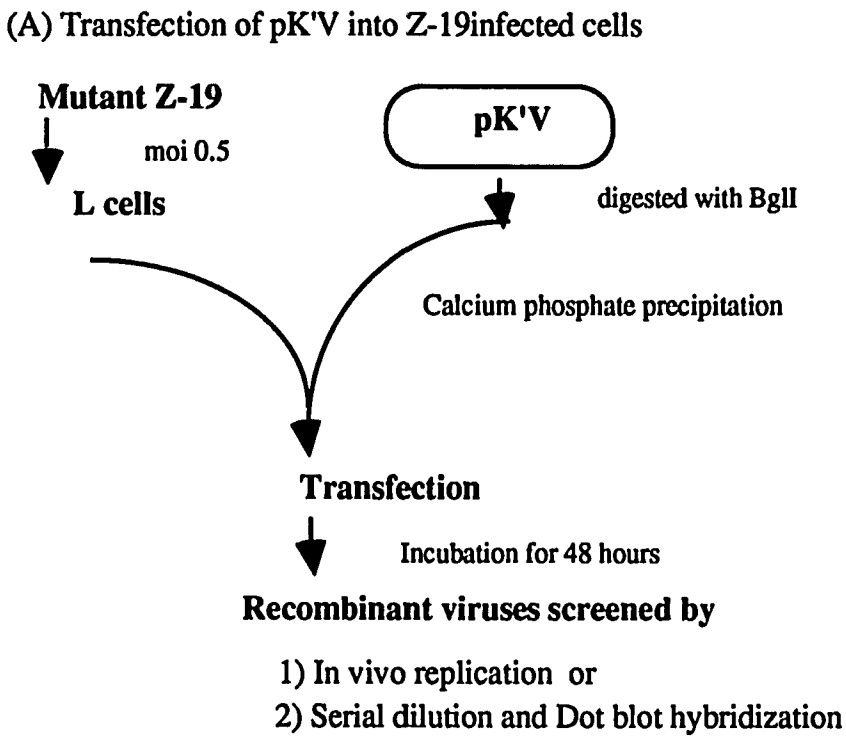


Figure 2. Schematic chart for transfection.



(B) Schematic chart for transfection of pK'CZ into Z-19 infected cells.

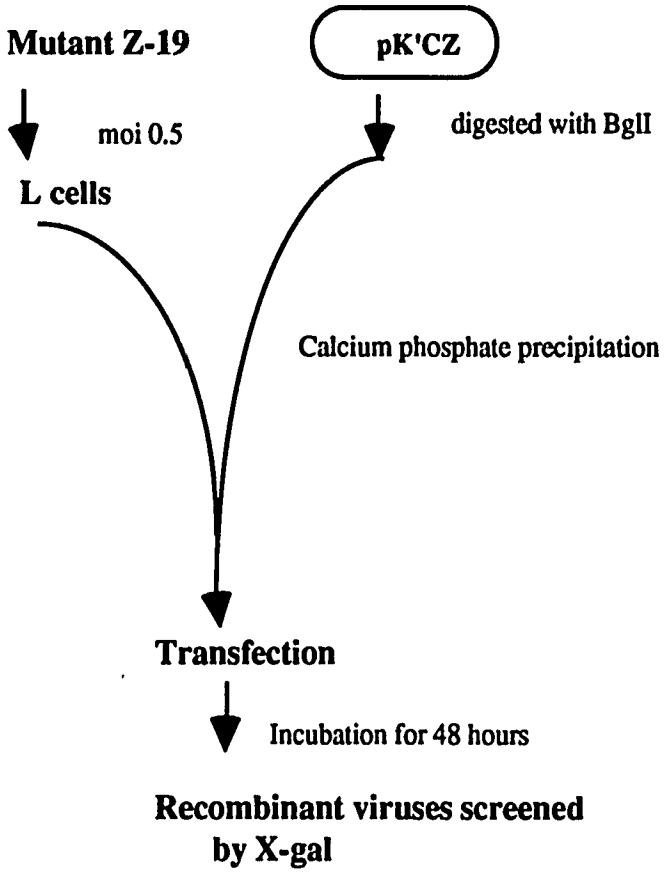


Figure 3: Southern hybridization of viral DNA with the vaccinia growth factor gene. Viral DNAs were digested with HindIII, the restriction fragments were separated by electrophoresis in 0.5% agarose gel and blotted to nytran membrane and hybridized with labeled 1.3 kb HindIII-HincII fragment from plasmid pSC16, containing the gene for VGF. (a) Recombinant viruses isolated from mice liver. Lane1, wild-type strain IHD-W ; lane 2, mutant Z-19; lane 3, recombinant. (b) Lane 1, wild-type; lane 2, mutant Z-19, lane 3, recombinant isolated from mouse liver; lane 5-11, recombinants isolated from serial dilution and dot blot hybridization. (c) Recombinant isolated from serial dilution and dot blot hybridization. Lane 1, wild-type; lane 2, mutant Z-19; lane 3, recombinant.

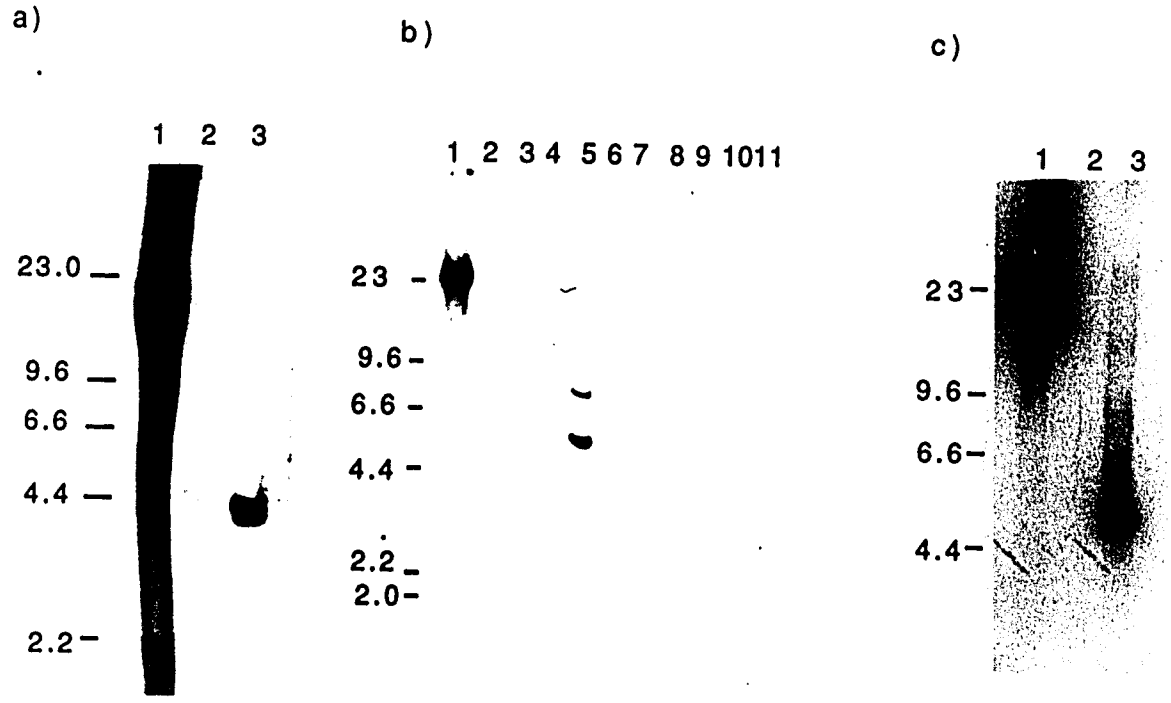


Figure 4: Southern hybridization of viral DNA from recombinant viruses containing the VCBP gene. The viral DNA was digested with HindIII and processed as described in Fig. 3. Hybridization with labeled HincII fragment containing entire gene of VCBP. Lane 1, wild-type IHD-W strain; Lane 2, mutant Z-19; Lane 3-6, recombinant viruses.

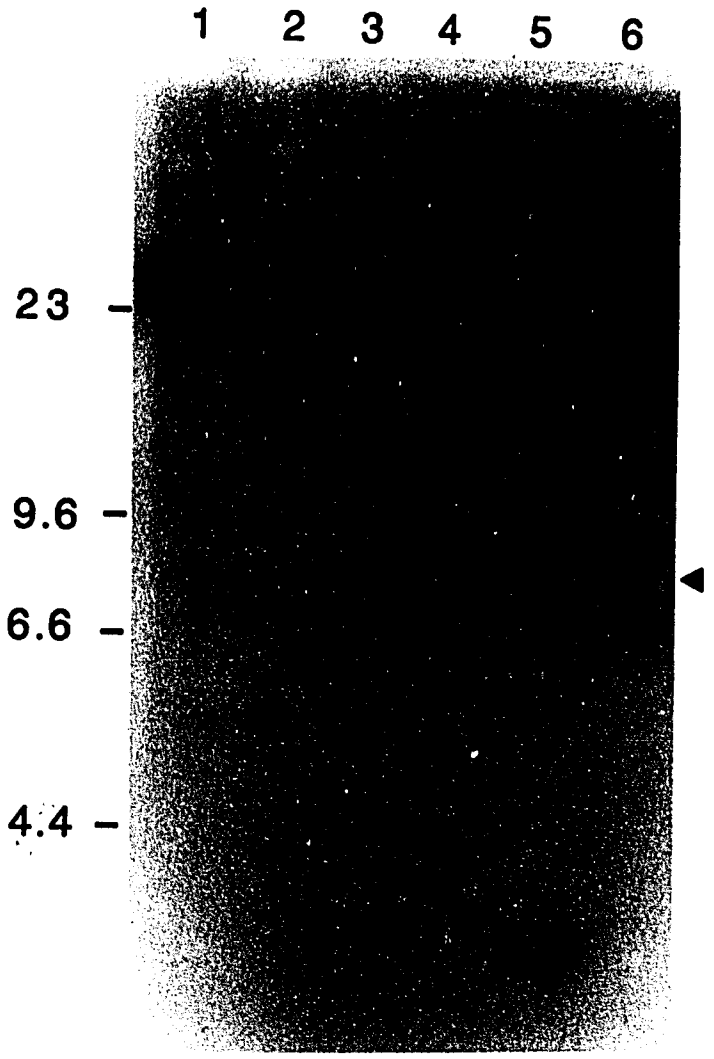


Figure 5: Southern hybridization of viral DNA from recombinant viruses containing VCBP gene with labeled vaccinia late promoter, P11, from plasmid pSC10. Method and material as described in Fig. 3. Lanes 1, wild-type IHD-W strain; lane 2, mutant Z-19; lanes 3-7, recombinant viruses.

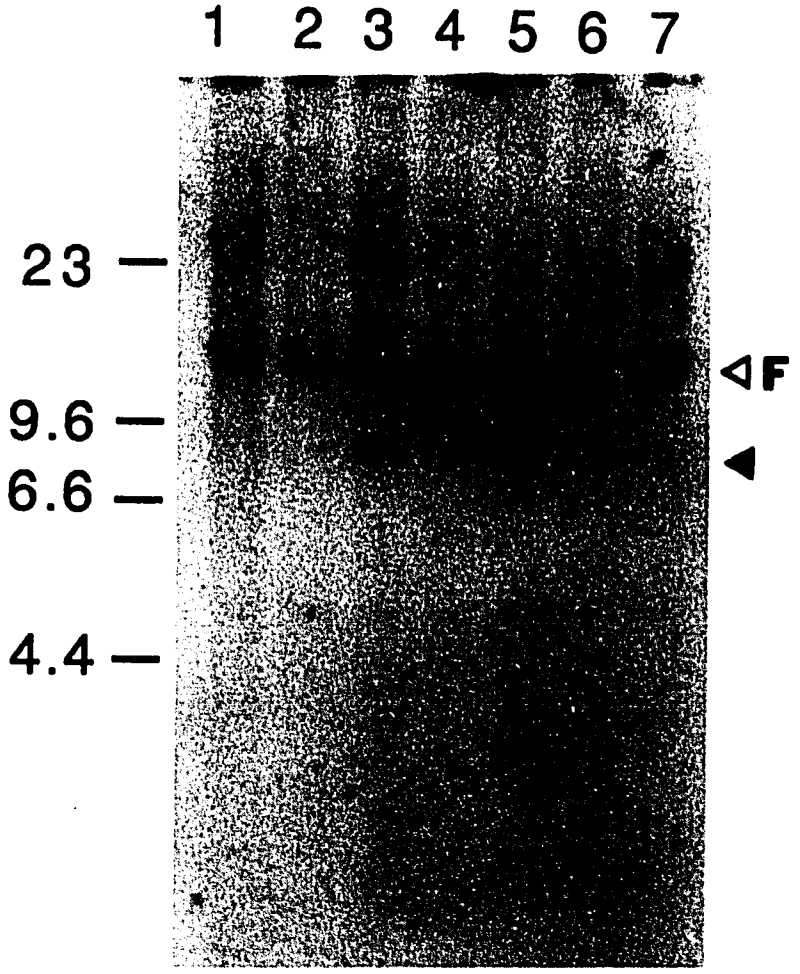


Figure 6: Southern hybridization of viral DNA from recombinant vC with labeled Lac Z gene from pSC10. Method and material as described in Fig. 3. Lanes as described in Fig. 5.

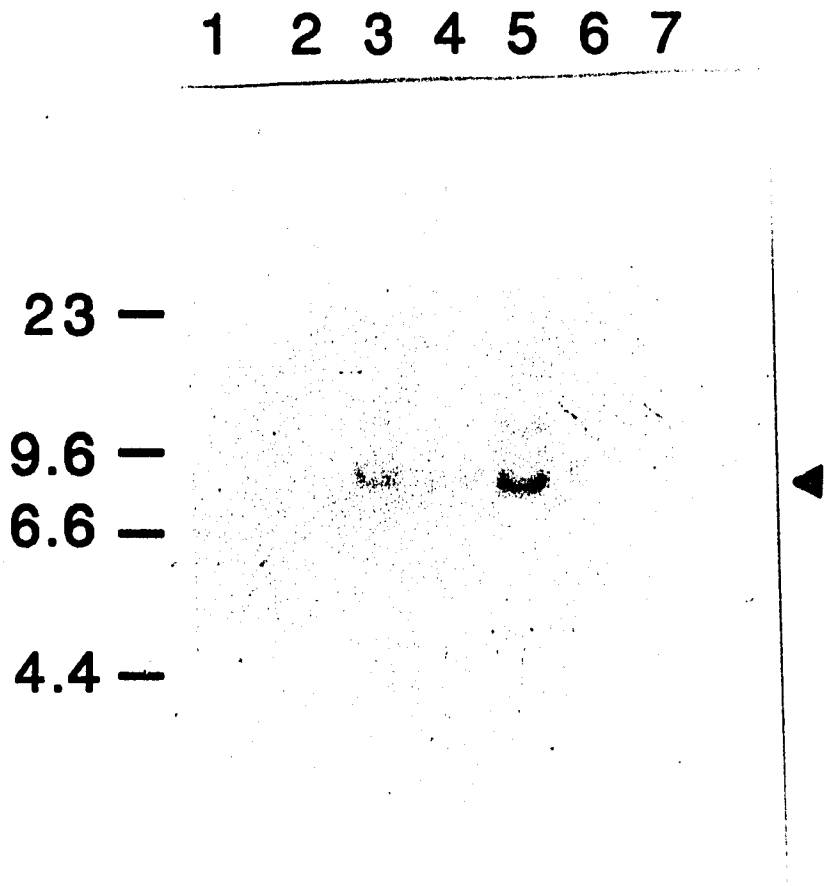
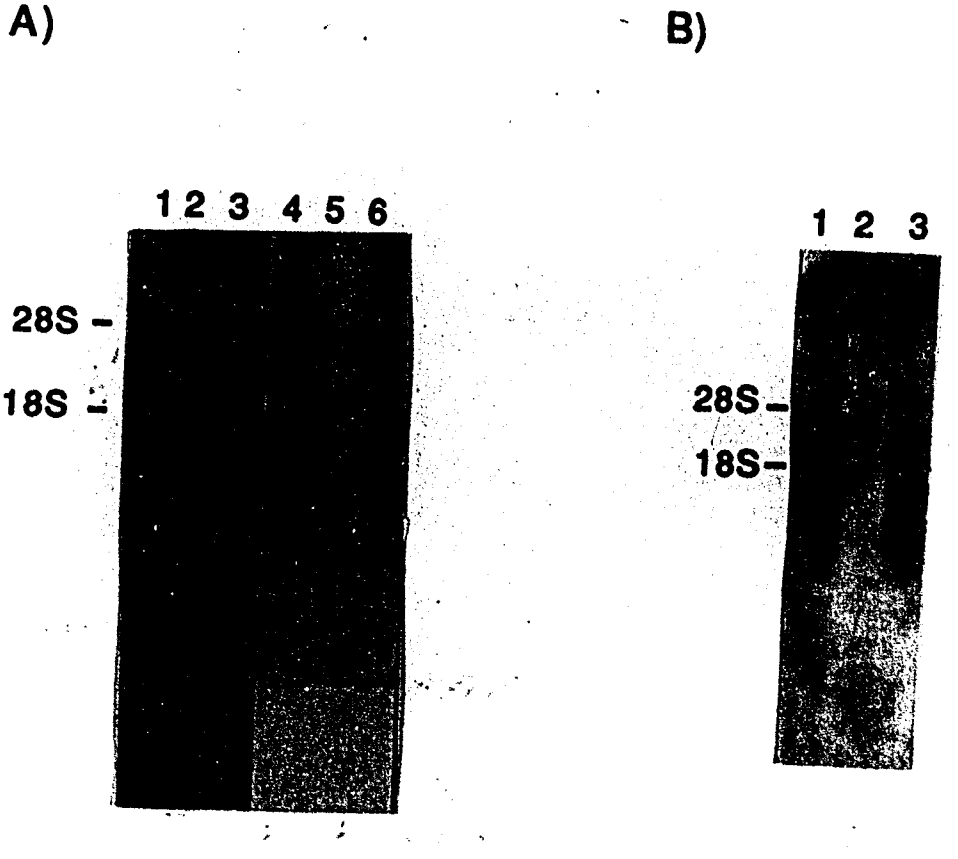


Figure 7: Northern (RNA) blot analysis of cytoplasmic RNA. Cytoplasmic RNA was isolated from L cells monolayers infected with 10 pfu per cell of either wild-type, mutant Z-19, recombinant viruses vV or vC, at the times indicated. RNAs were size fractionated by electrophoresis on formaldehyde-agarose gel and transfer to nitrocellulose filter. The blot was hybridized with labeled fragment containing VGF or VCBP gene. (A) Cytoplasmic RNA from cells infected with wild-type, mutant Z-19 or recombinant vV. Lanes 1-3 : postinfection 2 hr, lanes 4-6: postinfection 10 hr. Lane 1 and 4 : wild-type infected cells; lane 2 and 5: mutant Z-19 infected cells; lane 3 and 6: recombinant vV infected cells.

(B) Cytoplasmic RNA from cells infected with wild-type, mutant Z-19 or recombinant vC at 10 hr postinfection. lane 1: wild-type infected cells; lane 2: mutant Z-19 infected cells; lane 3: recombinant vC infected cells.



Chapter IV

Identification of elements in HIV LTR that interact with nuclear extracts from Jurkat cells persistently infected with vaccinia virus

Pi-Yun Chang, Kathleen Stellrecht, Stella Melana and Beatriz G-T Pogo

Abstract

Previous reports showed transactivation of HIV-1LTR in Jurkat cells persistently infected with vaccinia virus. In this communication, gel mobility shift assays were used to characterize the elements in HIV-1LTR which might be responsible for the mechanism of transactivation. The results indicated that two elements, NF-κB and NFAT-1, were able to interact with nuclear extracts derived from Jurkat cells persistently infected with vaccinia virus, suggesting that they may play a role in the transactivation of HIV-1LTR.

Introduction

HIV-1 replication can be shown in a small population of T-cells. The virus can persist for a long time without inducing clinical symptoms, remaining at a low-level of transcription during the latent period. However, activation of $CD4^+$ cells by antigen, mitogens or superinfection with other viruses interacting with HIV-1 via viral and/or cellular trans-acting factors, may terminate HIV-1 latency resulting in a productive HIV-1 infection. Several DNA viruses, including the papovavirus (Gendelman et al., 1986), adenovirus (Kliewer et al., 1989; Laspia et al., 1989; Rice et al., 1987), herpes simplex virus type 1 and 2 (Gendelman et al., 1986; Mosca et al., 1987; Ostrove et al., 1987; Rando et al., 1987; Gimble et al., 1988; Albrechtet et al., 1989; Laurence, 1990; Margolis et al., 1992), cytomegalovirus (CMV) (Davis et al., 1987; Mosca et al., 1987; Kowalik et al., 1993), Epstein-Barr virus (Kenney et al., 1988), human herpesvirus 6 (Ensoli et al., 1989), and hepatitis B virus (Seto et al., 1988; Twu and Robinson., 1989) , encode gene products that augment HIV-1 transcription in various host cells.

The function and structure of HIV-1LTR has been extensively investigated. The HIV-1LTR contains a TATA box, three tandem repeats of Sp-1, two direct repeats of NF-κB, one major site and one minor site of NFAT-1 and one Ap-1 site. The regulatory elements such as Sp-1 and TATA are able to bind cellular proteins that are constitutively expressed in most cells (Dynan et al., 1983, Garcia et al., 1989, Jones at al., 1986). These sequences are necessary for basal-level HIV-1 gene expression. Other elements such as NF-κB and NFAT-1 interact with factors synthesized only in activated T cells (Durand et al., 1988, Kawakami et al., 1988, Nabel et al., 1987, Shaw et al., 1988). The mechanism that triggers

a change from low-level transcription or latent infection to productive viral replication is still poorly understood. Immune activation of infected T-cells can increase HIV-1 replication probably via effects on regulatory elements on the HIV-1 LTR interacting with cellular or viral proteins induced by mitogens or viruses.

Previous works from this laboratory indicate that in T lymphocytes persistently infected with vaccinia virus (J_{vac} cells), there is an increase in the HIV-1LTR expression (Stellrecht et al., 1992a). In this communication, gel shift assays were used to study the interaction HIV-1LTR with factors present in the J_{vac} cells. The results suggest that proteins induced by persistent infection with vaccinia virus interacted with two elements in the HIV-1 LTR and may be responsible for the increased HIV-1 transcription.

Method and materials

Cells and virus

Jurkat cells, a $T4^+$ human T-leukemia cell, was persistently infected with vaccinia viruses and cultured in the RPMI 1640 (Gibco) contained 10 % fetal calf serum (Gibco), as previously described (Stellrecht et al., 1992a). Cells were stimulated with 2% Bacto-phytohaemagglutinin (PHA) from Difco, and/or 50 ng of phorbol-12 myristate13-acetate (PMA) per ml for 24 h.

Preparation of nuclear extract

Nuclear extracts were prepared from J_{vac}, PHA/PMA stimulated and control cells, according to the procedure of Böhnlein et al., 1988, and stored in aliquots at -70°C. The amount of protein in the extract was determined by the Bio-Rad protein assay (Bio-Rad).

DNA preparations

DNA from HIV-1LTR plasmid pUR III (Rosen et al., 1985) was restricted and fragments were isolated as follows. The 700 bp of XboI-HindIII fragment containing HIV-1LTR was obtained by digestion of the plasmid pUR III (Rosen et al, 1985) with HaeIII and electrophoresed in 1 % agarose gel followed by eletroelution and ethanol precipitation. This fragment was digested with Hae III, and several fragments were isolated after electrophoresis in 5% native polyacrylamide gel by soaking the minced gel slice in elution buffer containing 500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS. After a 6-8 hr incubation at 37°C, the eluate was spun down and the supernatant was extracted once with phenol and chloroform and the DNA was precipitated by adding 2 volumes of ethanol. The fragments involving 300 bp, 160 bp, 120 bp, 80 bp, and 60 bp, were used as competitors or for gel binding assay after 5'-end labeling.

Gel binding assay

Binding reactions were carried out in a solution containing 25 mM Hepes pH 7.5, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$ and 10% glycerol for 30 min at room temperature. Poly d(IC) was added as a nonspecific carrier plus 0.5 ng of end-labeled DNA (specific activity 10^7 cpm per µg) and 5-7 µg of nuclear extracts. Following binding, the mixture was eletrophoresed through a native 5% polyacrylamide gel (acrylamide: bisacrylamide ratio 30:1) containing 45 mM Tris-HCl, pH 8, 45 mM boric acid, and 1 mM EDTA. The gel was preelectophoresed for 1 hr at 11 v/cm in 0.5 x TBE buffer (22.5 mM Tris-HCl, pH 8, 22.5 mM boric acid, and 1 mM EDTA). Eletrophoresis was carried out at the same voltage for 2 hr in the cold room. The gel was dried and autoradiographed with a screen at -70°C. For competition experiments the conditions were as described

above, except that specific and nonspecific competitor DNAs were added to the mixture prior to addition of the nuclear extract. Synthetic oligonucleotides were also used as labeled probes and competitors in the binding assay. The following oligonucleotides were used:

NF-κB: gatccAGGGACTTTCC

Sp-1: gaattCGAGGCGTGGCCTGGGCGGGACTGGGGAGTGCG

NFAT-1: gaattCTTGTTACACCCTGTGAGCCTGCATGGGATGGATG

Ap-1: gatccATGAGTCAT

Mutated NF-κB: ACTCACTTTCC (as described by Nabel and Baltimore, 1987). They were synthesized in the Core Facilities of the Brookdale Center for Molecular Biology.

Results

Binding of the HIV LTR fragment to nuclear extracts.

The 700 bp XboI-HindIII fragment of the HIV-1LTR from plasmid pURIII was subdivided into 300 bp (LTR300), 160 bp (LTR160), 120 bp (LTR120), 80 bp (LTR80), and 60 bp (LTR60) segments by digestion with Hae III. As shown in Fig. 1, these segments contain different sites for interaction with nuclear factors from J_{vac} cells. The eletrophoretic mobility shift assay (EMSA) was used to investigate protein factors that interact with the LTR of HIV. When end labeled fragments (LTR160, LTR120, LTR80, and LTR60) were used in such an assay, DNA-protein complexes migrating more slowly than the free DNA segments were observed with nuclear extracts derived from the J_{vac} cells (Fig. 2, lanes 1, 3, 5, and 7, respectively).

Characterization of binding site in the subfragments LTR160 and LTR60 in the HIV LTR.

To define whether the new bands found in the EMSA represented specific complexes, binding reactions in the presence of varying amounts of nonradioactive competitor fragments as well as synthetic oligonucleotides were carried out. From the structure of HIV-1LTR, HaeIII-digested fragment LTR160 contained Sp-1 site (Fig. 1). Therefore, the synthetic oligonucleotide including the Sp-1 sequence was used as competitor. The formation of DNA-protein complex was not inhibited by addition of the synthetic oligonucleotide (Sp-1) nor by another oligonucleotide with Ap-1 sequences, indicating that this complex was not specific for Sp-1 or Ap-1 responsible element(Fig. 3, lanes 2 and 3, respectively). In addition, the complex formed with fragment LTR60, which includes an Ap-1 sequence, was not inhibited by addition of the oligonucleotide Ap-1. The results showed that this complex was not competed with either unlabeled oligonucleotide Ap-1 or Sp-1, suggesting that the band was not specific for either of these responsible elements (Fig. 4, lanes 2 and 3, respectively). Taken together, these results indicate that new bands produced by the nuclear extract from J_{vac} cells interacting with end labeled fragments LTR160 and LTR60 were not specific for Sp-1 or Ap-1 responsible element.

Characterization of NFAT-1 sequence in the subfragment LTR120.

To examine proteins reacting with other sites, the subfragment LTR120 was used. LTR120 contained the NFAT-1 sequence which comprises -216 to -254 in the HIV-1 LTR (Shaw et al., 1988). To identify protein-DNA complexes specific for this sequence, competitors such as the total HindIII-XhoI fragment (LTR), subfragment LTR120, and synthetic oligonucleotide NFAT-1 (-216 to -254) were used. Competition analysis

showed that there was a decrease in the amount of complex in the presence of either LTR or subfragment LTR120 (Fig. 5a, lanes 2-4). Furthermore, when the synthetic oligonucleotide including the NAFT-1 site (-216 to -254) was used as a competitor, the formation of the complex was inhibited with 100 ng of unlabeled oligonucleotide (Fig 5b, lanes 2 and 3). In contrast, formation of the complex was not inhibited by the oligonucleotides containing Sp-1 and NF-κB sequences, but part of complex (upper complex) was inhibited by Ap-1 sequence (Fig. 5b, lanes 4-6). The results indicated that DNA-protein complexes contained at least two complexes that specifically bound to NFAT-1 sequence. The upper complex was inhibited by addition of oligonucleotide Ap-1 (Fig. 5b, lane 4).

Characterization of NF-κB site in the subfragment LTR80.

To define the complex with fragment LTR80 more precisely, fragment LTR80 and a synthetic oligonucleotide containing the NF-κB sequences were used in the binding reaction. Fragment LTR80 contains two copies of the NF-κB site which is homologous to IL-2R-α promoter (Nabel and Baltimore, 1987). Binding reactions were carried out using end labeled fragment LTR80 and nuclear extracts derived from J_{vac} cells in the presence of increasing amounts of the nonspecific competitor poly d(IC) (Fig. 6, lanes 1, 8 and 9). Fragment LTR80 formed a major band with the nuclear extract even in the presence of 3 μg of poly d(IC) (Fig. 6, lane 9). The complex generated with LTR80 was specifically inhibited by incubation with 100 ng of unlabeled LTR fragment and LTR80, respectively (Fig. 6, lanes 2 and 3, 4), and also competed by incubation with 40 ng of unlabeled oligonucleotide containing one copy of NF-κB sequence (Fig. 6, lane 5, 6). These results indicated that protein(s) are

present in the nuclear extract that can recognize the NF-κB site in the HIV-1LTR. To confirm that the nuclear extract specifically bind to the NF-κB site, the end labeled synthetic oligonucleotide NF-κB site was used as a probe. The formation of complex was inhibited in the presence of increasing amounts of unlabeled oligonucleotide NF-κB(Fig. 7, lanes 7-11). Similar results were observed when the nuclear extract from Jurkat cells stimulated by PHA/PMA was used (Fig. 7, lanes 2-6). In addition, the mutated NF-κB site was used in which the conserved binding site GGG in NF-κB sequence was replaced by CTC (Nabel and Baltimore, 1987). The competition reaction in the presence of mutated NF-κB showed that formation of the complex was not inhibited by the mutated NF-κB (Fig. 8, lanes 6 and 9) and same results were also shown in the nuclear extract from Jurkat cells activated by PHA/PMA (Fig 8, lanes 3). Taken together, these results indicate that the nuclear extract prepared from J_{vac} cells can generate a specific complex with NF-κB site. Nuclear extracts prepared from different culture conditions including unstimulated control cells (Fig. 9, lanes1, 2), PHA/PMA stimulated cells (Fig. 9, lanes 3,4), and J_{vac} cells showed different degrees of NF-κB binding (Fig. 9, lanes 5, 6). Some activity was seen occasionally in control cells as shown in Fig. 7, lane 1.

Discussion

We have previously reported that HIV-1LTR was transactivated in J_{vac} cells, as shown by CAT assays (Stellrecht et al.,1992a). To understand the molecular mechanism of transactivation of HIV-1LTR by vaccinia virus, we examined the interaction of proteins induced by vaccinia virus with HIV-1LTR using an EMSA. It was found that nuclear extracts react with two sites in the HIV-1LTR. Within the HaeIII digested fragment LTR80, nuclear extract from virus infected Jurkat cells could specifically

bind to a NF-κB site since the formation of the complex was inhibited by competition with the fragment itself and by the oligonucleotide containing the NF-κB site. The other site is in the fragment LTR120, where NFAT-1 is located. The nuclear extract bound to this site was specific competed by the fragment itself and by the oligonucleotide containing NFAT-1 site. In addition, similar results were obtained when nuclear extracts from Jurkat cells stimulated by PHA/PMA or persistently infected with the mutant Z-19 which has a large deletion at the left terminus of the viral genome (Lai and Pogo, 1989) were used.

Protein NF-κB binds to a 10 bp site in the κ light chain enhancer, called κB (Sen and Baltimore, 1986). The κB element is found in the regulation region of interleukin-2 receptor-α (IL-2R-α) gene, which is expressed during activation of T cells (Böhnlein et al., 1988). NF-κB is also induced by T cell mitogens that mimic physiologic T cell activation (Sen and Baltimore, 1986; Tong-Starksen et al., 1989) and by the tax protein of HTLV (Siekevitz et al., 1987). Cytomegalovirus (CMV) and hepatitis B virus also induce trans-activators including NF-κB activity (Cherrington and Mocarski, 1989; Siddiqui, 1989). Moreover, herpes simplex virus type 1 has been shown to activate HIV transcription, and to induce two NF-κB-specific proteins of 55 and 85 kd in the nucleus (Vlach and Pitha, 1992). The results presented here indicate that nuclear extracts from J_{vac} cells formed a specific complex with the NF-κB site of the HIV-1LTR, which is not present in unstimulated Jurkat cells.

Increase in NF-κB like proteins in J_{vac} cells is possibly related to the mechanism of T cell activation. Stimulation of certain cytokines that use the NF-κB motif has been found in the J_{vac} cells (Stellrecht et al., 1992b). Alternatively, vaccinia virus protein kinase (Lin et al, 1992) could

phosphorylate the inhibitor IκB in the cytoplasm, resulting in the release of NF-κB.

Another possibility is that some vaccinia virus product may interact with the p50 subunit of NF-κB. NF-κB is a heterodimer that contains two subunits: p50 and p65. The p50 family (p50 and p50B) is generated from precursor p105 by proteolytic cleavage and binds to NF-κB sites as a homodimer or as a heterodimer with p65, c-Rel or Rel-B (Schmid et al., 1991; Neri et al., 1991; Bours et al., 1992; Mercurio et al., 1992). The p50 homodimer does not transactivate and can act as a competitive inhibitor of transactivation (Schmitz and Baeuerle, 1991; Franzoso et al., 1992). An oncoprotein, Bcl-3, which is structurally related to the ankyrin domain of the p50 precursor, can form a complex with the κB DNA-binding p50 homodimers, changing such inhibitory dimers into potent transactivating complexes (Bours et al., 1993). Analysis of the vaccinia virus genome indicates that it encodes for proteins which contain ankyrin repeats (Howard et al., 1991). It is possible that in J_{vac} cells, some proteins containing ankyrin repeats will bind to p50B dimer and directly transactivate HIV-1LTR. Experiments are in progress to discriminate between these possibilities.

NFAT-1 (nuclear factor of activated T cells) is a transcription factor found in the activated T cells which is involved in initiation of IL-2 gene expression and also interacts with sequences -252 to -216 in the HIV-1 LTR (Shaw et al. , 1988). It has been shown that NFAT-1 was constitutively expressed in a small population of cells in the dermis of mice transgenic with the oligomerized NFAT-1 and SV40 T antigen gene, some of which developed skin damage (Verweij et al., 1990). Interestingly, results from EMSA showed that nuclear extracts from J_{vac} cells complexed with

NFAT-1 sequences in the HIV-1LTR, suggesting that these proteins may play a role in the dermal activation of HIV-1LTR. The EMSA results showed that two complexes were formed with the NFAT-1 oligonucleotide, one of which was also inhibited by AP-1, suggesting that participation of other nuclear factors in the complexes.

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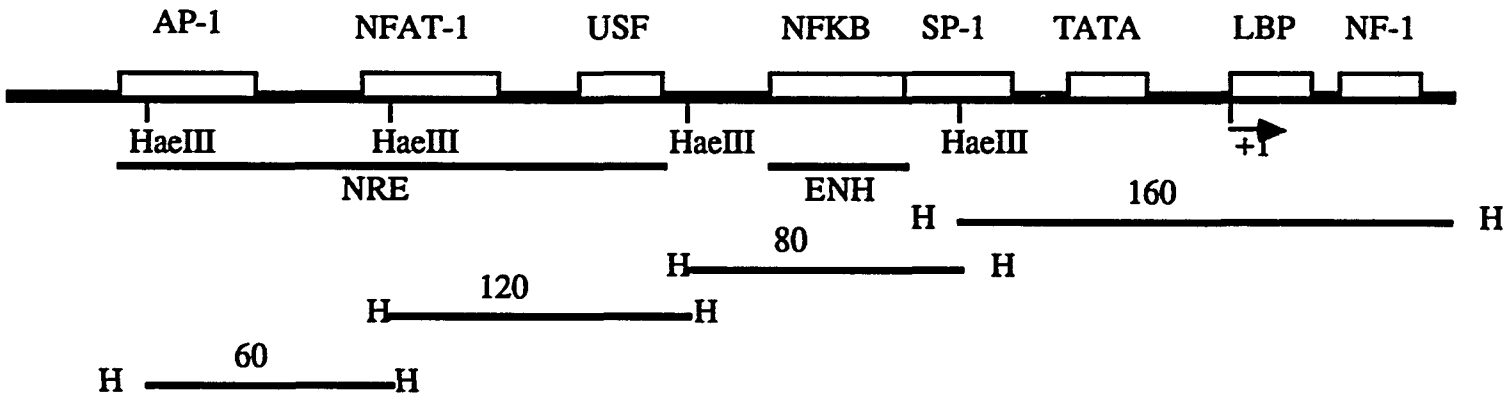
Figure 1. The map for HIV-1 LTR is illustrated in the figure, indicating the position of regulatory elements. Taken from Lu. et al., 1989..



H: Hae III

ENH: enhancer

NRE: negative response element

Figure 2. Electrophoretic mobility shift assay of HIV-1 LTR fragments (LTR160, LTR120, LTR80, and LTR60). End-labeled LTR160, LTR120, LTR80, or LTR60 (0.5 ng) were incubated with 5-6 μg of a nuclear extract prepared from J_{vac} cells in the presence of 1 μg of poly d(IC), followed by electrophoresis as described in materials and methods. Lane 1: labeled LTR160 ; lane 3: labeled LTR120; lane 5: labeled LTR80; lane 7: labeled LTR60. lanes 2, 4, 6, 8: free DNA fragment.

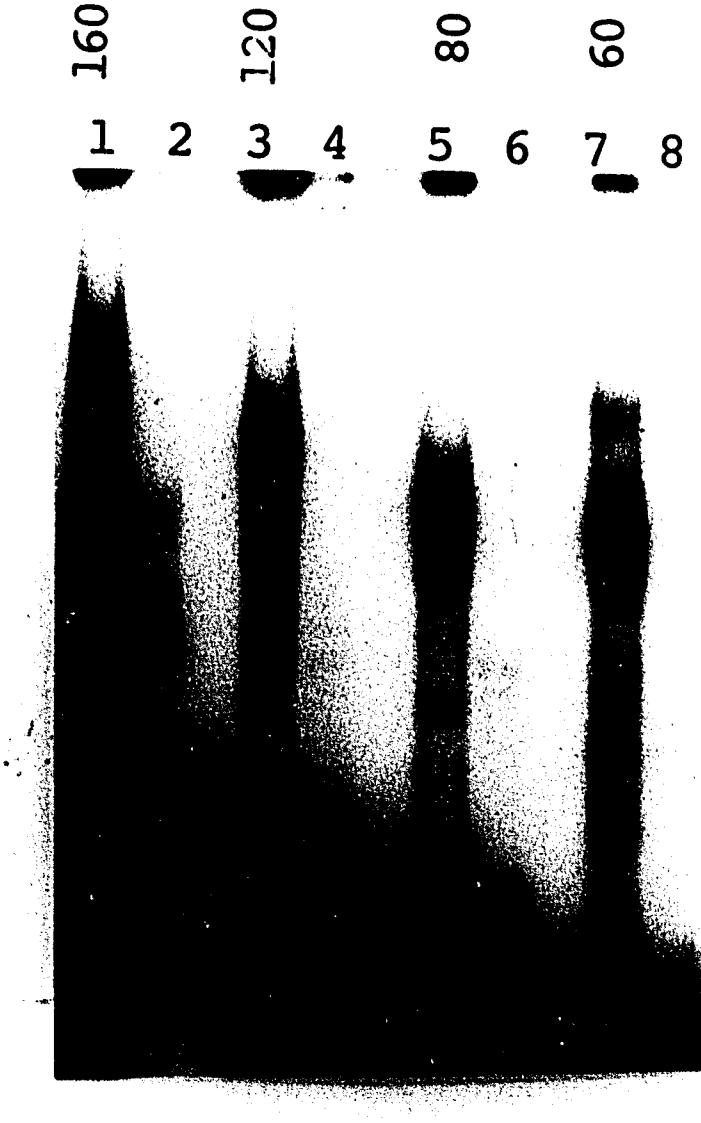


Figure 3. Competition analysis of subfragment of LTR160. Binding reaction was carried out in a final 15 µl volume with 6 µg of a nuclear extract derived from J_{vac} cells with 0.5 ng of end labeled LTR160 and 1 µg poly d(IC). Lane 1: no competitor DNA added; lanes 2 and 3: binding reaction in the presence of 100 ng of the sequences indicated, added prior to addition of the nuclear extract; lane 4: free DNA fragment.

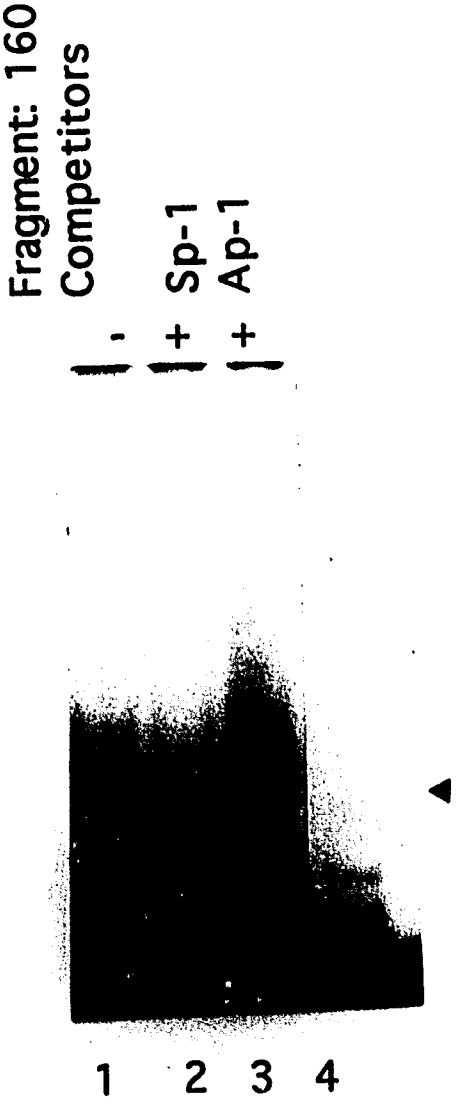


Figure 4. Competition analysis of subfragment LTR60. Binding reaction was carried out in a final 15 µl volume with 6 µg of a nuclear extract derived from J_{vac} cells with 0.5 ng of end labeled LTR60 and 1 µg poly d(IC). Lane 1: no competitor DNA added; lanes 2 and 3: binding reaction in the presence of 100 ng of different sequences as indicated, added prior to addition of the nuclear extract; lane 4: free DNA fragment.

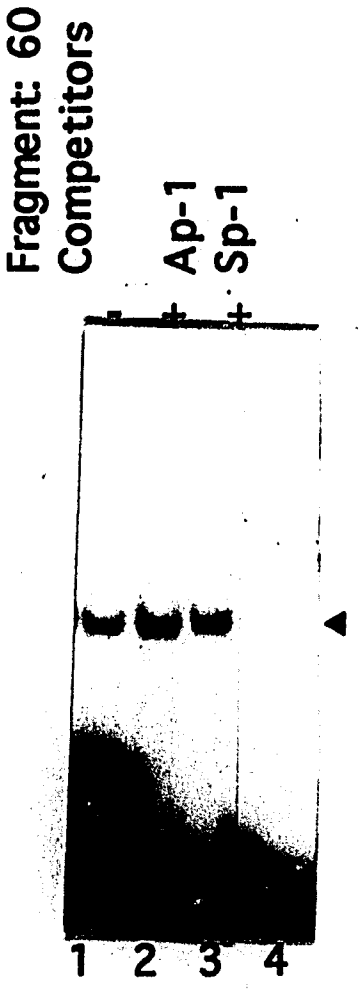


Figure 5. Electrophoretic mobility shift assay of LTR120.

(a) Competition experiment to show that the complex on LTR120 is sequence-specific. Binding reaction was carried out in a final 15 µl volume with 6 µg of a nuclear extract derived from J_{vac} cells with 0.5 ng of end labeled LTR120 and 1 µg poly d(IC). Lane1: no competitor DNA added; lanes 2, 3 and 4: binding reaction in the presence of 100 ng of LTR fragment or 10 ng or 100 ng of fragment LTR120 added prior to the nuclear extract.

(b) Competition experiment to show that the complex is specific for the NFAT-1 sequence. Binding reaction was carried out in a final 15 µl volume with 6 µg of a nuclear extract derived from J_{vac} cells with 0.5 ng of end labeled synthetic oligonucleotide NFAT-1 and 1 µg poly d(IC). Lane 1: no competitor DNA added; lanes 2 and 3: 10 ng or 100 ng of unlabeled oligonucleotide NFAT-1 were added before the nuclear extract; lane 4: 100 ng Ap-1 was added; lane 5: 100 ng Sp-1 was added; lane 6: 40 ng NF-κB was added; lane 7: pUC-19 added; lane 8: labeled probe.

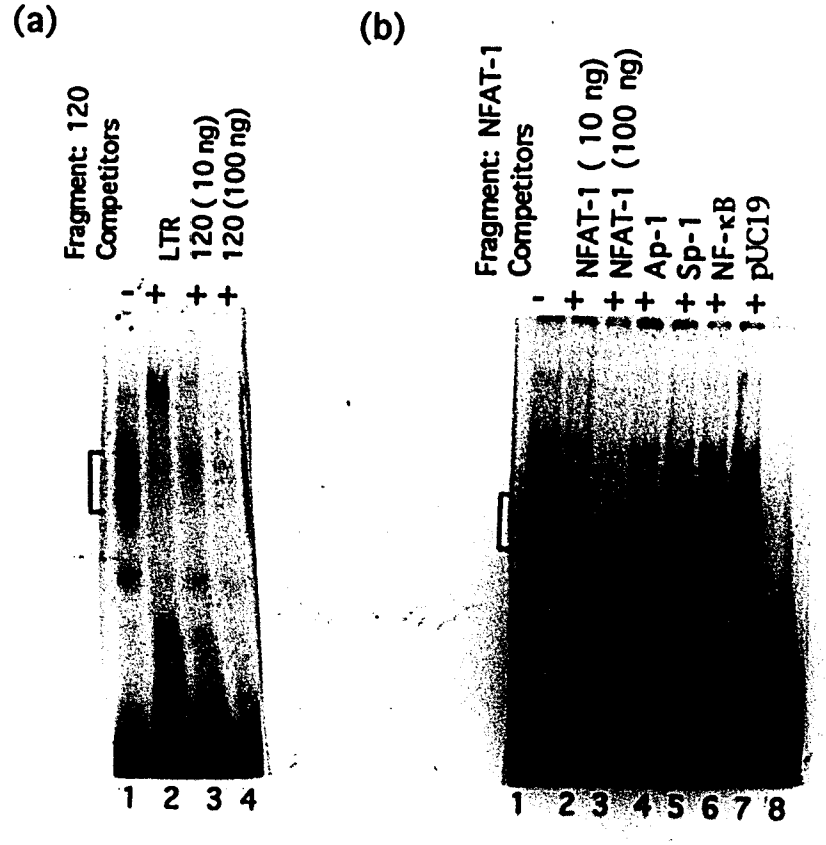


Figure 6. EMSA of subfragment LTR80.

Competition experiment to show that the complex formed with LTR80 is sequence-specific. Binding reaction was carried out in a final 15 µl volume with 6 µg of a nuclear extract derived from J_{vac} cells with 0.5 ng of end labeled LTR80 and 1 µg poly d(IC). Lanes 1, 8, and 9 : binding reaction in the presence of 1 µg, 2 µg and 3 µg of poly d(IC). Lanes 2 to 4: 100 ng of LTR and 10 ng or 100 ng of fragment LTR80 were added; lanes 5 and 6: 10 ng or 40 ng of synthetic oligonucleotide NF-kB was added; lane 7: labeled fragment LTR80 probe.

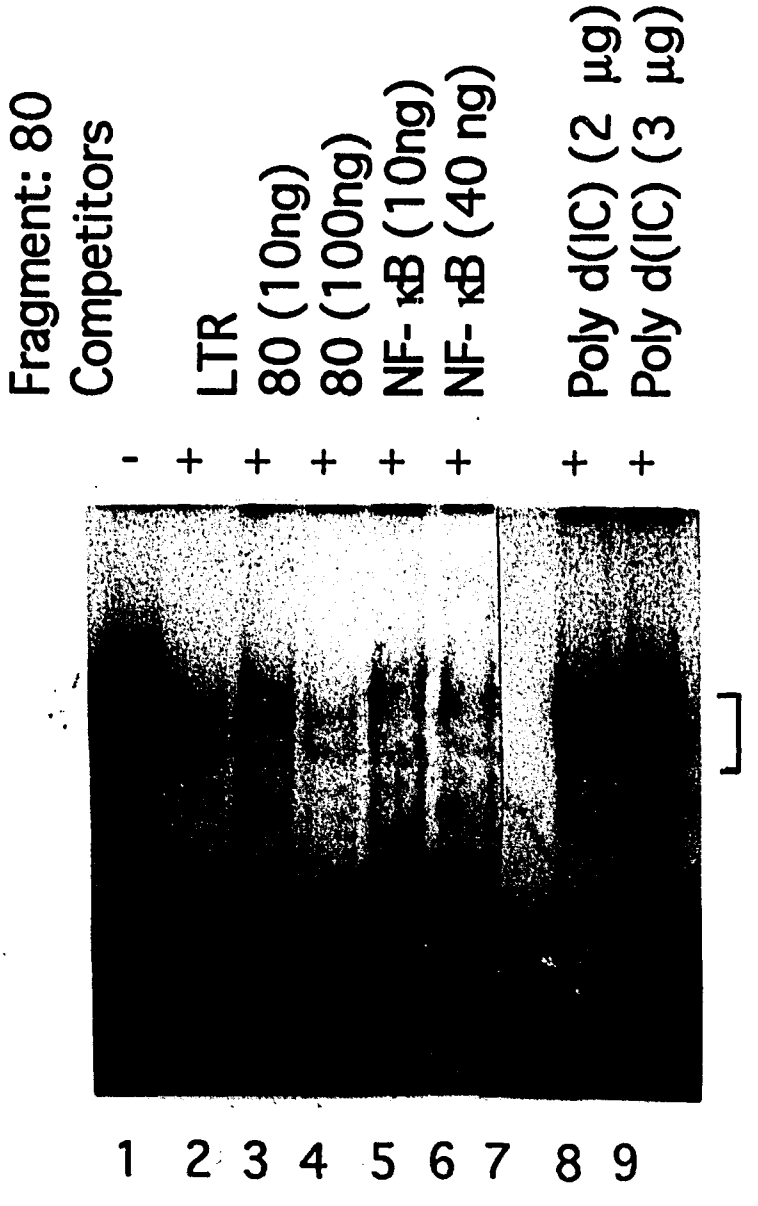


Figure 7. Competition experiment to show that complexes were formed with the NF-κB sequence. Binding reaction was performed in the presence of different amounts of oligonucleotide NF-κB with labeled probe NF-κB. Nuclear extracts from Lane 1:unstimulated Jurkat cells; lanes 2 to 6: from Jurkat cells stimulated by PHA/PMA; lanes 7 to 11: from J_{vac} cells, lane 12: labeled probe NF-κB.

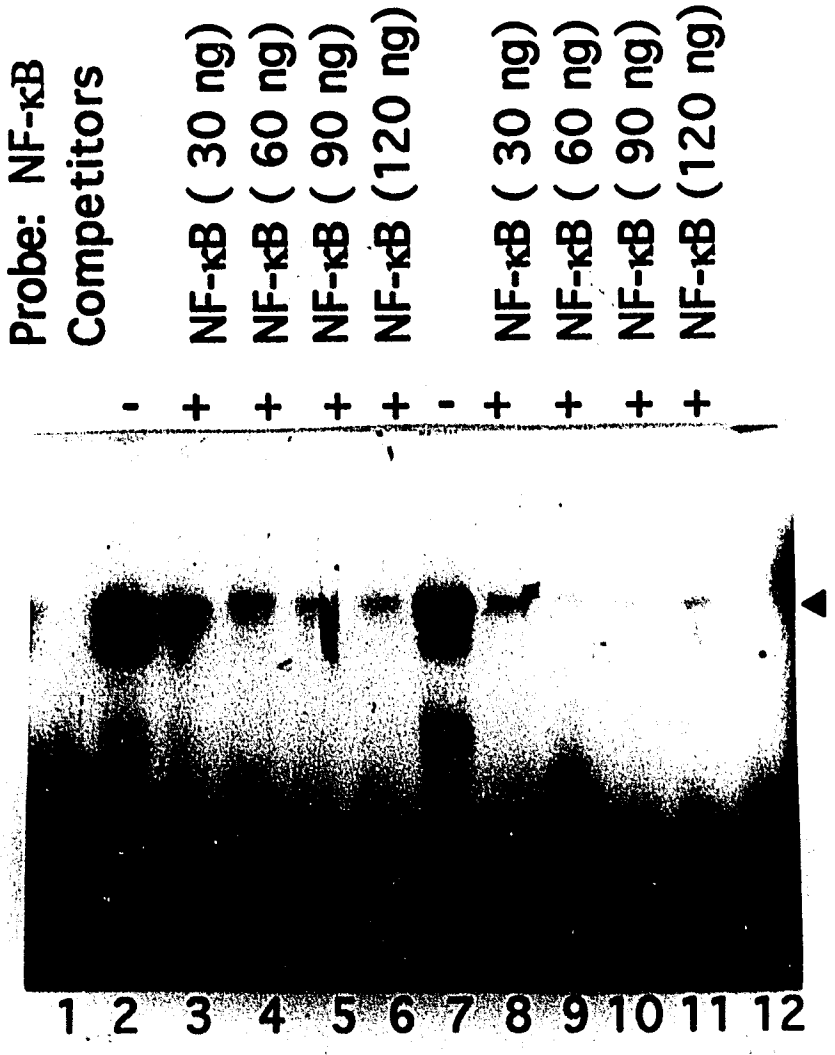


Figure 8. Competition experiment with mutated NF-κB and NF-κB using labeled probe NF-κB. Lanes 1 to 3: Jurkat cells stimulated by PHA/PMA; lanes 4 to 6 and 7 to 9: Jurkat cells infected with different pfu of vaccinia virus; lanes 2, 5, and 8: competitor NF-κB; lanes 3, 6, and 9: competitor mutated NF-κB.

Figure 9. Binding reactions of NF-κB with nuclear extracts isolated from unstimulated and stimulated Jurkat cells. Lanes 1 and 2, unstimulated Jurkat cells; lanes 3 and 4, Jurkat cells stimulated by PHA/PMA; lanes 5 and 6, J_{vac} cells; lane 7, probe: labeled NF-κB.

Chapter V

General Discussion

Taken together the results described here provided new insights into the molecular mechanism of pathogenicity of vaccinia virus and its interaction with the host cell.

Results from chapter II indicated that reintroduction of genes present in the 23 kb sequences of left terminus brings about the recovery of virulence of the attenuated deletion mutant of vaccinia virus, Z-19. It was found that genes, VGF and VCBP, were present as one copy in the HindIII C fragment in the IHD-W strain, in contrast to two copies of VGF in the HindIII C and B fragments in the WR strain. Therefore, these genes may be involved in pathogenesis in the IHD-W strain. VGF is related to epidermal growth factor and binds to epidermal growth factor receptor stimulating proliferation of uninfected cells in vivo and in vitro (Buller et al., 1988a). In addition, the VCBP encoded by vaccinia virus is structurally related to the superfamily of complement control proteins, binds to C4b and can block the classical complement pathway (Kotwal and Moss, 1988; Kotwal et al., 1990).

Other virulence genes, like serpin protease inhibitors (SPI), (SPI-1, SPI-2, and SPI-3) have been found in the vaccinia virus (Kotwal and Moss, 1989a; Smith et al., 1989). The function of SPI-1 was not clear, its amino acid sequence was related to human plasminogen activator inhibitor-2 (PAI-2) (Kruithof, 1988; Smith et al., 1989). Deletion of SPI-1 increased its antibody response to a foreign antigen expressed from recombinant

viruses (Zhou et al., 1990) and may block antigen presentation to cytotoxic T cells (Townsend et al., 1988). We have found that the SPI-1 gene is present in two copies located in fragments HindIII C and B and XhoI E and H in the IHD-W strain (chapter II). This is unlike the WR and Copenhagen strains in which the gene is localized in fragments HindIII B and C, respectively (Kotwal and Moss, 1989a; Goebel et al., 1990). However, the SPI-1 gene may have minor effects on virulence in IHD-W strain, since it is present in fragment B of the mutant Z-19 which is attenuated.

Insertional inactivation of the 13.8 kd virokine gene reduces neurovirulence for both normal and nude mice injected intracranially (Kotwal et al., 1989b). We have found that this gene is localized in fragment HindIII G in the IHD-W strain instead of HindIII N, as reported for the WR strain. It was present in Z-19 and apparently does not play a major role in pathogenicity of the IHD-W strain.

Several laboratories have constructed attenuated mutants by using molecular biological methods to inactivation of specific genes (Buller et al., 1988b; Buller et al., 1985; Flexner et al., 1987; Kotwal et al., 1989b; Morita et al., 1987; Lee et al., 1992; Tartaglia et al., 1992). These alterations attempt to abolish functions that are exclusively related to viral virulence, host defense response and to understand vaccinia virus pathogenicity and attenuation. In fact, our results (chapter II) showed that genes related to virulence in the IHD-W strain are mostly located in fragment HindIII C, making Z-19 a good model system to study virulence.

Insertional inactivation of target gene has been applied to study virulence. When a nonessential gene such as TK is inactivated by insertion of another gene, the virus became less virulent (Buller et al., 1985).

Therefore, insertional inactivation is not a good procedure to identify virulence genes. Another approach to study virulence is reinsertion of a test gene (or overlapping region) into a deletion attenuated mutant. Our results (chapter III) showed that deletion mutants in which the VGF or VCBP genes were introduced, displayed an increase in virulence, suggesting that the alteration of the phenotype was provided by the insert. This model provides an useful tool to study virulence. Bloom et al. (1991) have used similar methodology to find two nonessentional regions of the rabbitpox virus genome involved in virulence. Our results also indicated that more than VGF and VCBP expression is necessary to reestablish virulence and that other genes located in fragment HindIII C may be required. Studies with engineered deletion mutants of the Copenhagen strain (Tartaglia et al., 1992) also indicate that virulence is likely to be multifactorial and that one gene is not completely responsible for virulence.

Thus far, genes located at both ends of the viral genome are not essential for viral replication in vitro, and are related to regulate the metabolic machinery of the host cell and to block the host defense mechanism. Using the shuttle vector described here (chapter III), it will be possible to identify a number of new nonessential genes in vaccinia virus strain IHD-W and gain more information about the pathogenicity of this strain.

When the vaccinia based-vaccines were administrated to immunocompromised individuals, adverse reactions occurred. To avoid these severe complications, vaccinia vectors with reduced dissemination properties have been developed. The ALVAC is a poxvirus known to replicate only in avian species, it has been used as a recombinant fowlpox

virus vaccine and induces protective immunity in nonavian species (Taylor, 1988). A similar vector, NYVAC, has been constructed from Copenhagen strain, it is restricted to replicate in cells derived from human and other species, but still retains the ability to induce immune response to foreign antigens (Tartaglia et al., 1992). Similar findings were observed with the mutant Z-19 which in spite of the fact that it can not replicate in vivo still provides protection against challenge with wild-type in mice (Lai and Pogo, 1989). This is an interesting approach to develop new safer vaccines.

The mechanism of transactivation of HIV-1LTR in J_{vac} cells was not understood. Our results (chapter IV) indicated that two elements, NF-κB and NFAT-1, were able to interact with nuclear extracts from J_{vac} cells. It is known that the NF-κB is an abundant protein that binds to κB element in the κ light chain enhancer. It is also found in the non-B cells such as activated T-cells and is induced by T cell mitogens (Sen and Baltimore, 1986; Tong-Starksen et al., 1989) and by the tax protein of HTLV (Siekevitz et al., 1987). Some DNA viruses like CMV and hepatitis B virus also induce trans-activators including NF-κB activity (Cherrington and Mocarski, 1989; Siddiqui et al., 1989). Moreover, herpes simplex virus type 1 has been shown to activate HIV transcription by increasing the NF-κB-like proteins (Vlach and Pitha, 1992). Our results suggested that nuclear extracts from J_{vac} cells may play a role in the transactivation of HIV-1LTR via NF-κB site.

Several mechanisms may be involved: (1) Increase in NF-κB-like proteins in J_{vac} cells, (2) Phosphorylation of the inhibitor IκB in the cytoplasm by a vaccinia protein kinase releasing the active NF-κB forms, that can translocate to the nuclei. Both mechanisms will result in increased

level of active NF-κB and possibly mimic T cell activation. (3) Some vaccinia virus product may interact directly with the p50 subunit of NF-κB. NF-κB is a heterodimer that contained two subunits: p50 and p65. The p50 family (p50 and p50B) is generated from precursor p105 by proteolytic cleavage and binds to NF-κB sites as a homodimer or as a heterodimer with p65, c-Rel or RelB (Schmid et al., 1991; Neri et al., 1991; Bours et al., 1992; Mercurio et al., 1992). The p50 homodimer does not transactivate and can act as a competitive inhibitor of transactivation (Schmitz and Baeuerle, 1991; Franzoso et al., 1992). An oncoprotein, Bcl-3, which is structurally related to the ankyrin domain of the p50 precursor, can form a complex with the κB DNA-binding p50 homodimers, changing such inhibitory dimers into active transactivating complexes (Bours et al., 1993). Analysis of vaccinia virus genome indicates that it encodes for proteins which contain ankyrin repeats (Howard et al., 1991). It is possible that in J_{vac} cells , some proteins containing ankyrin repeats will bind to p50B dimer and directly transactivate HIV-1LTR. Experiments are in progress to discriminate between these possibility.

Another transcription factor, NFAT-1 (nuclear factor of activated T cells), is involved in initiation of IL-2 gene expression and also interacts with sequences -252 to -216 in the HIV-1LTR (Shaw et al. , 1988). Our results suggested that NAFT-1 may play a role in the transactivation and also complexes with other transcription factors as indicated by Ap-1 competition. It seems that persistent infection with vaccinia virus may induce viral proteins or change some cellular proteins that indirectly or directly interact with elements in the HIV-1LTR, which in turn, may activate HIV-1LTR transcription.

In summary, we have shown that mutant Z-19 provided an useful system to study pathogenicity and it could also be a good candidate for development of safer vaccinia-based recombinant vaccines. The study of mechanism of transactivation of HIV-1LTR in cells persistently infected with vaccinia virus provided new insights into the interactions between virus and host during long term conditions.

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