VIROLOGY 185, 768–778 (1991)

Isolation and Characterization of Mutants of Vaccinia Virus with a Modified 94-kDa Inclusion Protein[1]

ALEJANDRO DE CARLOS AND EDUARDO PAEZ[2]

U. E. I. de Virología, Centro de Investigaciones Biológicas (CSIC), Velázquez, 144, 28006 Madrid, Spain

Received May 28, 1991; accepted August 26, 1991

We have characterized one of the most highly expressed genes of vaccinia virus, WR strain, in the wild type and in several spontaneous mutants isolated from persistently infected cells. This gene encodes the 94-kDa inclusion protein, which is the vaccinia virus counterpart of the 160-kDa A-type inclusion (ATI) protein of cowpox virus. The homology index between both genes is greater than 95%. A deletion of two consecutive adenilate residues is responsible for a frameshift mutation and premature translational termination in the vaccinia virus gene. In addition, several point mutations and small deletions occur in the 94K gene. The deduced protein contains 725 amino acids, and 4 of the 10 repeated motifs present in the carboxyl terminus of the cowpox virus 160-kDa protein are conserved. In several mutants independently isolated from untreated and interferon-treated persistently infected cells, the gene encodes a 40-kDa protein. In mutant 87-4, this truncated protein is due to the insertion of a cytidilate residue that produces a frameshift mutation and premature translational termination. The deduced protein contains 366 amino acids and has lost all the repetitions. Transcriptional analysis has shown that the steady-state levels of mRNAs in cells infected with the mutants or wild-type vaccinia virus are similar. However, the accumulation of this protein in cells infected with the mutants is reduced indicating some instability. In addition the mutated protein is not recognized by polyclonal antisera. Existence of tandemly repeated sequences at the carboxyl terminus of this family of inclusion proteins correlates with their antigenicity. These results indicate a high degree of mutability of the ATI gene and products, which apparently has no consequence on replication *in vitro,* but could have relevance to control of the infection by immune responses in animal hosts.

INTRODUCTION

Establishment of persistent infections in cell cultures has proven to be a good method to test the genetic stability of poxviruses (Paez *et al.,* 1985). These virus–cell systems have two characteristics that make them different from the natural infection in animals. First, the virus is under selective pressure to maintain the persistent state in a different cellular environment, and by passage in a new host, one may select virus variants poorly adapted to grow in target animal cells. Second, the virus is not under the immunological pressure from the host and spontaneous mutations in important immunogenic proteins might occur. It has been previously shown that vaccinia virus mutants with reduced infectivity in tissue culture and reduced virulence in mice were obtained from persistent infections in Friend erythroleukemia cells (Paez *et al.,* 1985, 1987). These mutants contain an 8-MDa deletion at the left end of the viral genome (Paez and Esteban, 1988) and modifications in seven structural proteins (Paez *et al.,* 1987). The deletion encodes for a cluster of nonessential genes for virus growth in cell culture, although this region is essential for virulence in animals (Dallo and Esteban, 1987; Paez *et al.,* 1987). On the other hand, five structural proteins, of the seven, altered during virus persistence in different vaccinia virus mutants are immunogenic in animals (Paez *et al.,* 1987). Alteration in size of a 14-kDa protein greatly reduces virus virulence (Dallo and Esteban, 1987; Paez *et al.,* 1987). Other virus genes coding for structural and nonstructural proteins have been shown to influence vaccinia virus virulence. Attenuation of vaccinia virus in animals has resulted from alteration or inactivation of structural genes such as the 14-kDa envelope protein and the hemagglutinin present in both the cellular membrane and the envelopes of the virus (Dallo and Esteban, 1987; Paez *et al.,* 1987; Flexner *et al.,* 1987; Shida *et al.,* 1988) and nonstructural viral genes such as thymidine kinase and growth factor (Buller *et al.,* 1985, 1988).

Among the nonstructural polypeptides of vaccinia virus, the most abundant appears to be the 94-kDa inclusion protein. Patel *et al.* (1986) recently demonstrated that the 94-kDa protein is the LS antigen described by Shedlovsky and Smadel (1942). These authors have shown that the 94-kDa protein is the vaccinia virus counterpart of the cowpox 160-kDa A-type

[1] Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. M76371.

[2] To whom reprint requests should be addressed.

0042-6822/91 $3.00

inclusion (ATI) protein. Other orthopoxviruses produce related proteins with different sizes: raccoonpox (155 kDa), ectromelia (130 kDa), variola (96 kDa), and monkeypox (92 kDa). The gene encoding the 160-kDa ATI protein in cowpox virus has been cloned and sequenced (Funahashi *et al.*, 1988) and its promoter has been used to generate vaccinia virus recombinants that express high levels of foreign genes (Patel *et al.*, 1988).

In this report we have tested the alterations in nonstructural viral proteins of vaccinia virus mutants. We have characterized mutants with a modified 94-kDa inclusion protein. This protein is a major immunogen during vaccinia virus infection in mice. We suggest that a relationship exists between a set of tandem repeats in the amino acid sequence of these proteins with regard to immunogenicity.

MATERIAL AND METHODS

Cells and virus

BSC-40 monkey kidney cells were grown at 37° in Dulbecco's modified Eagle (DME) medium supplemented with 10% newborn calf serum (NCS) and antibiotics. Wild-type vaccinia virus, WR strain, and vaccinia virus mutants obtained from untreated and interferon (IFN)-treated, persistently infected Friend erythroleukemia cells as previously described (Paez *et al.*, 1987) were used. The viruses were purified as described by Joklik (1962) and virus titration was done in BSC-40 cells.

Analysis of proteins

Cells were infected with vaccinia virus at 10 PFU/cell and after 1 hr of adsorption in DME, the virus inoculum was removed and cells were supplemented with DME containing 2% NCS. Proteins were labeled for 1 hr with 10 mCi/ml of L-[^{35}S]methionine (1200 Ci/mmol, Amersham) in DME lacking methionine at various times after infection. Samples were analyzed by one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) on linear 15% gels. Labeled proteins were detected by autoradiography on XAR-5 film of dried gels, and unlabeled proteins were visualized by staining with Coomassie brilliant blue. Molecular weight markers (Bio-Rad Laboratories) included phosphorylase B (97 kDa), bovine seroalbumin (66 kDa), ovoalbumin (45 kDa), carbonic anhidrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lisozyme (14 kDa).

Polyclonal antibodies

A rabbit polyclonal antiserum against the components of the ATI bodies of cowpox virus, CPRC1 strain (Patel *et al.*, 1986), was kindly provided by David Pickup (Duke University Medical Center, Durham, NC). This antiserum recognizes a 160-kDa protein which is the major component of the ATIs of cowpox virus and cross-react with a 94-kDa inclusion protein of vaccinia virus. Polyclonal antibodies against live vaccinia virus were generated after 2 weeks of immunization in mice inoculated intraperitoneally with 10^6 PFU/mouse of purified vaccinia virus.

Immunoblot analysis

Protein from purified virions (20 to 40 μg) or cell extracts (60 μg) was subjected to electrophoresis on linear SDS–PAGE. The proteins were transferred to nitrocellulose paper (BA-85, Schleicher and Schuell Inc., Keene, NH) in Towbin buffer [25 m*M* Tris–HCl, pH 8.3, 192 m*M* glycine, 20% methanol (v/v)] with a semidry blotting apparatus for 30 min at 400 mA. Immunoblots were developed for alkaline phosphatase staining or with ^{125}I-Protein A as described previously (Rodriguez *et al.*, 1987).

Immunoprecipitation analysis

BSC-40 cells growing in 60-mm petri dishes were infected with vaccinia virus at 10 PFU/cell. Cells were pulse-labeled for 4 hr with 70 μCi of L-[^{35}S]methionine at 20 hr postinfection. Labeled cells were dissolved in 250 μl of dissociation buffer [phosphate-buffered saline (PBS) with 0.5% NP-40, 0.1% SDS, 0.75% 2-mercaptoethanol, and 1 m*M* PMSF]. Cellular debris were removed by centrifugation at 10K in a Sorvall SS-34 rotor for 60 min, and the supernatants were incubated with 10 μl of rabbit preimmune serum for 15 min on ice. After addition of 50 μl of Protein A–Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden), the samples were further incubated for 15 min on ice and spun at 16,000 *g* for 5 min in an Eppendorf microfuge, and the supernatants were incubated overnight at 4° with 10 μl of rabbit anti-160-kDa immune serum. Protein A–Sepharose (50 μl) was added to the mixture and incubated for 30 min at 4°, the mixture was centrifuged, and the pellet was washed four times each with PBS with 0.5% NP-40. After the final wash, 30 μl of 2× sample buffer (62.5 m*M* Tris–hydrochloride, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) was added and the mixture was boiled for 5 min and centrifuged, and the supernatants were subjected to SDS–PAGE and autoradiography.

DNA extraction

DNA from either vaccinia virus (WR strain) or mutants was extracted from infected cells. Briefly, BSC-40

monolayers were infected at an input m.o.i. of 0.01–0.5 PFU per cell and further incubated for 48–72 hr at 37° depending upon WR or mutants infectivity. The infected cells (ca. 10^8 cells) were harvested by low-speed centrifugation and the resulting pellets were processed exactly as described by Esposito *et al.* (1981). After digestion with the appropriate restriction endonuclease, individual fragments were purified from agarose gels by the silica matrix binding method using the GENECLEAN kit (Bio 101, La Jolla, CA) according to the manufacturers instructions.

Cloning protocols

The appropriate viral restriction fragments from WR and mutants were ligated to either pUC13 or pBluescriptIISK (Stratagene, La Jolla, CA) linearized recipients to create chimeric plasmids. After transforming *Escherichia coli* TG2 competent cells, plasmids were amplified and further purified from isopycnic cesium chloride gradients using standard procedures (Sambrook *et al.,* 1989).

Isolation of RNA from infected cells

Monolayers of BSC-40 cells were infected with WR and mutants at 10–20 PFU/cell. After 1 hr of adsorption the virus inoculum was removed and replaced by fresh DMEM supplemented with 2% NCS. Total cytoplasmic RNA was extracted at 22 hr postinfection by lysing the cells in guanidium isothiocyanate/sarkosyl and pelleting through CsCl by overnight centrifugation (Chirgwin *et al.,* 1979). Unless otherwise indicated, all viral RNAs were extracted from cells in this late stage of infection.

Northern blot analysis

Virus RNA samples were resolved by electrophoresis through denaturing agarose gels containing formaldehyde and further transferred to nitrocellulose membranes (BA-85, Schleicher and Schuell) following standard procedures (Sambrook *et al.,* 1989). Hybridization was carried out overnight at 42° in 50% deionized formamide, 5× SSC, 1× Denhardt's, 20 m*M* sodium phosphate buffer, pH 6.5, 0.1% SDS, 100 mg/ml single-stranded salmon sperm DNA, and 5% dextran sulphate. Hybridization probe was obtained by ^{32}P-nick-translating an appropriate double-stranded DNA fragment containing almost the complete 94K gene sequence.

Nuclease S1 analysis

The extent of complementarity between the transcripts of the 94K gene from WR and mutants and viral DNA was determined by high resolution nuclease S1 protection procedures (Berk and Sharp, 1977). The viral DNA fragments used to detect the 5′ ends of the 94K gene transcripts were ^{32}P-labeled at their 5′ ends using polynucleotide kinase (Boehringer) in the presence of 50 μCi of [γ-^{32}P]ATP (Amersham, 3000 Ci/mmol). The denatured probes were hybridized with 10 μg of viral RNA in 30 ml of a buffer containing 0.4 *M* NaCl, 40 m*M* piperazine-*N*-*N*′-bis[2-ethane sulfonic acid], pH 6.4, 0.1 m*M* EDTA, and 80% deionized formamide at 37° for 16 hr. Residual single-stranded nucleic acids were digested with nuclease S1 (Boehringer) in 300 ml of S1 buffer (0.25 *M* NaCl, 30 m*M* NaOAc, pH 4.5, 1 m*M* $ZnSO_4$) at 30° for 60 min. The ethanol-precipitated products were then electrophoresed on 6% polyacrylamide sequencing gels using an unrelated M13 sequence ladder as size marker. The resulting protections were visualized after autoradiography of dried gels for the required period.

94K gene sequencing

Supercoiled DNA recombinant plasmids containing a 94K gene copy from WR or mutant 87-4 were purified by centrifugation to equilibrium in CsCl–ethidium bromide gradients following standard procedures (Sambrook *et al.,* 1989). A set of oligonucleotide primers was designed from cowpox virus ATI coding sequence (Funahashi *et al.,* 1986) with an average of 200 nt. Sequencing was performed by the dideoxy chain termination method, using the Sequenase kit (USB, Cleveland, OH) and [^{35}S]dATP (Amersham, 1000 Ci/mmol) on double-stranded templates denatured by the method of Hattori and Sakaki (1986). The resulting reactions were resolved on 6% polyacrylamide Tris–borate–EDTA (TBE) buffer gradient sequencing gels.

RESULTS

Analysis of polypeptides synthesized in cells infected with vaccinia virus mutants

Vaccinia virus DNA encodes for more than 200 proteins (Carrasco and Bravo, 1986). Alterations of structural and nonstructural proteins might be detected as long as they are not lethal for virus replication. We have isolated a collection of vaccinia virus mutants from untreated and IFN-treated Friend erythroleukemia (FEL) cells which have remained persistently infected with vaccinia virus after more than 2 years of continuous passage. For this study, we selected vaccinia virus mutants from early passages (48) or late passages (87 and 101) of persistently infected cells in the presence (clones 48-42 and 87-4) or absence of IFN (clones 48-7

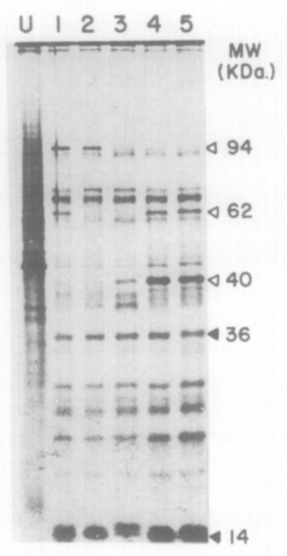

FIG. 1. Alterations of viral-induced proteins in cells infected with different mutants of vaccinia virus. BSC-40 cells were infected with 10 PFU per cell of different variants and labeled for 2 hr with 10 μCi of [^{35}S]methionine/ml at 24 hr postinfection. Proteins were solubilized and electrophoresed in a 15% linear SDS–PAGE. U, uninfected cells. Lane 1, cells infected with wild-type virus; lanes 2–5, cells infected with mutants isolated from untreated (clones 48-7 and 101-14) or IFN-treated (clones 48-42 and 87-4), persistently infected cells, respectively. The low synthesis of the 40-kDa polypeptide in lane 3 is most likely due to a lower m.o.i. of mutant 101-14 compared with that of other mutants.

and 101-14). Each mutant is characteristic of the viral population at the cell passage number, as previously shown (Paez *et al.*, 1987). We examined whether protein modifications occur in mutants by one- and two-dimensional gel electrophoresis of ^{35}S-pulse-labeled polypeptides from vaccinia virus-infected cells. Early and late viral-induced proteins synthesized in the presence or absence of 40 μg/ml of cytosin arabinoside were analyzed. No major changes have been observed in early viral-induced proteins (data not shown). However, several modifications of late viral-induced proteins occurred in cells infected with vaccinia mutants (Fig. 1). By one-dimensional SDS–PAGE, we found an alteration in the size of a 94-kDa late protein in extracts of cells infected with several of the mutants (clones 101-14, 48-42, and 87-4) with the appearance of a new 40-kDa late protein in the extracts of cells infected with the same mutants. We also found another late protein of 62 kDa that was apparently not synthesized by some of the mutants, which had been isolated from untreated, persistently infected cultures (clones 48-7 and 101-14). These results were confirmed and extended by two-dimensional gel electrophoresis analysis (data not shown). Because the pattern of the 94-kDa protein appeared to change in size in several mutants and because this protein is highly expressed during virus infection, we decided to focus our studies on the nature of this protein.

It has been previously shown that the vaccinia virus counterpart of the cowpox virus 160-kDa ATI protein is a 94-kDa nonstructural protein highly expressed at late times after infection, which can be visualized after Coomassie blue staining of a SDS–PAGE of proteins from infected cells (Patel *et al.*, 1986). This protein was apparently not synthesized in cells infected with several vaccinia virus mutants (data not shown). We have found a good correlation between the presence of the 94-kDa protein in extracts from cells infected with vaccinia virus mutants and the results described in Fig. 1. However, we could not detect a 40-kDa protein accumulating to levels similar to those of the 94-kDa protein in cells infected with these mutants, indicating some instability of the mutated protein.

Identification of the 94-kDa protein as the vaccinia virus inclusion protein

To confirm that the 94-kDa protein which is modified in our vaccinia virus mutants is the component of the cytoplasmic inclusion bodies, we used a rabbit polyclonal antiserum prepared against the 160-kDa protein of cowpox virus that cross-reacts with the vaccinia virus 94-kDa protein homolog. This type of study was carried out by immunoblot analysis (Fig. 2A). By this method we identified the vaccinia virus 94-kDa protein in extracts from cells infected with wild-type virus (lane 1) and mutant 48-7 (lane 2). This antiserum did not recognized either a 94-kDa polypeptide or a 40-kDa polypeptide that appeared in cells infected with mutants 101-14, 48-42, and 87-4 (lanes 3–5).

We tested whether this protein could be recognized by a polyclonal antiserum raised against live vaccinia virus. Figure 2B shows a Western blot of extracts from cells infected with different vaccinia mutants and reacted with a polyclonal vaccinia virus antiserum obtained from BALB/c mice after 2 weeks of immunization with 10^6 PFU/mouse of purified wild-type vaccinia virus. A highly immunoreactive virus protein of 94 kDa was recognized by this antiserum in extracts of cells infected with wild-type vaccinia virus (lane 1) and mutant 48-7 (lane 2), but no reactivity occurred in extracts of cells infected with mutants 101-14 (lane 3), 48-42 (lane 4), and 87-4 (lane 5) or extracts of purified vaccinia virions (lane WR). This antiserum did not recognize a highly expressed 40-kDa late protein specific for these mutants, confirming the results described above with a monospecific ATI protein antiserum. These re-

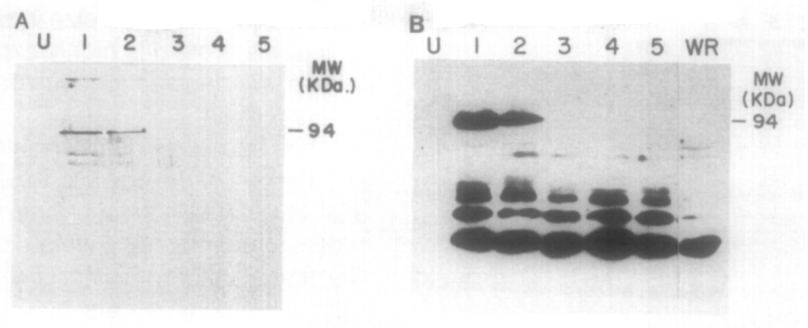

FIG. 2. (A) Immunodetection of the 94-kDa inclusion protein in cells infected with vaccinia virus mutants. Western blots of cell extracts from cells infected with different vaccinia mutants were reacted with a monospecific polyclonal rabbit antiserum against the ATIs of cowpox virus and visualized by the immunophosphatase staining method. U, uninfected cells. Lanes 1–5, cells infected with wild-type vaccinia virus (lane 1) and mutants 48-7, 101-14, 48-42, and 87-4 (lanes 2–5). (B) Immunogenicity of the 94-kDa inclusion protein in animals. Western blots of extracts from cells infected with different vaccinia virus mutants were reacted with a polyclonal vaccinia antiserum obtained after 2 weeks of immunization in mice inoculated intraperitoneally with live vaccinia virus (10^6 PFU per mouse) and visualized with ^{125}I-Protein A. U, uninfected cells. WR, purified vaccinia virus. Lanes 1–5, extracts from cells infected with wild-type virus and mutants 48-7, 101-14, 48-42, and 87-4, respectively.

sults show that this nonstructural protein is one of the major immunogens during immunization of mice with vaccinia virus, as was previously shown to be the case with several structural proteins which are modified during virus persistence.

Location of the 94K gene of vaccinia virus

It has been previously shown that the vaccinia virus gene encoding the 94-kDa inclusion protein maps in the *Eco*RI E fragment located in the middle of the *Hin*dIII A fragment of the viral genome, by hybridization with a 2-kb *Acc*I fragment and nucleotide sequence analysis of the region around the initiation codon (Patel *et al.*, 1987). Figure 3 shows the precise location of the coding sequence of the 94K gene, obtained by complete nucleotide sequencing (see below). Southern blot analysis of DNA from several mutants digested with *Eco*RI and hybridized with probe P1 (Fig. 3) showed that no major deletions occur in this region when compared with vaccinia virus wild-type DNA (data not shown), suggesting that small deletions or point mutations might be responsible for the alteration of this gene in the mutants.

Transcriptional analysis of the 94K gene of vaccinia virus

In experiments of immunoblotting with vaccinia virus mutants that do not synthesize the 94-kDa inclusion protein, the polyclonal antiserum did not recognize the 94-kDa protein or the truncated 40-kDa protein. This result could be due to a lack of recognition of a truncated polypeptide (coded by a functional gene) by the antisera, or it could be due to the lack of expression of the gene in cells infected with vaccinia virus mutants. In order to confirm the expression of the 94K gene by vaccinia mutants, the RNAs synthesized at late times after infection were examined by Northern blot and nuclease S1 analysis.

For Northern blot analysis a 1.9-kb *Acc*I fragment included in the coding region of the 94K gene was used as the radiolabeled probe (Fig. 3, P1). Figure 4A shows that a major 4.5-kb RNA was transcribed from this region, similar to that previously reported for cowpox virus. The size of the RNA and the extent of accumulation were similar with all the mutants when compared to wild-type virus.

Nuclease S1 protection experiments were used to

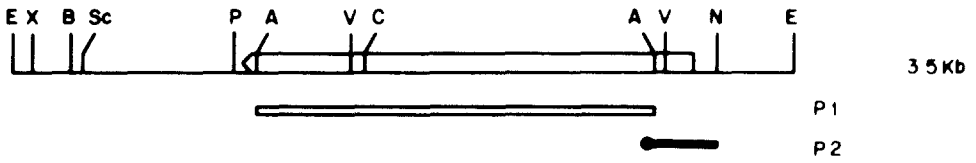

FIG. 3. Location of the 94K gene in the vaccinia virus genome. Restriction map of the *Eco*RI E fragment containing the complete coding sequence of the 94K gene. Restriction sites are abbreviated as follows: A, *Acc*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; N, *Nco*I; P, *Pst*I; Sc, *Sac*I; V, *Eco*RV; X, *Xho*I. Probes P1 and P2 used for genetic and transcriptional mapping of the 94K gene are also indicated.

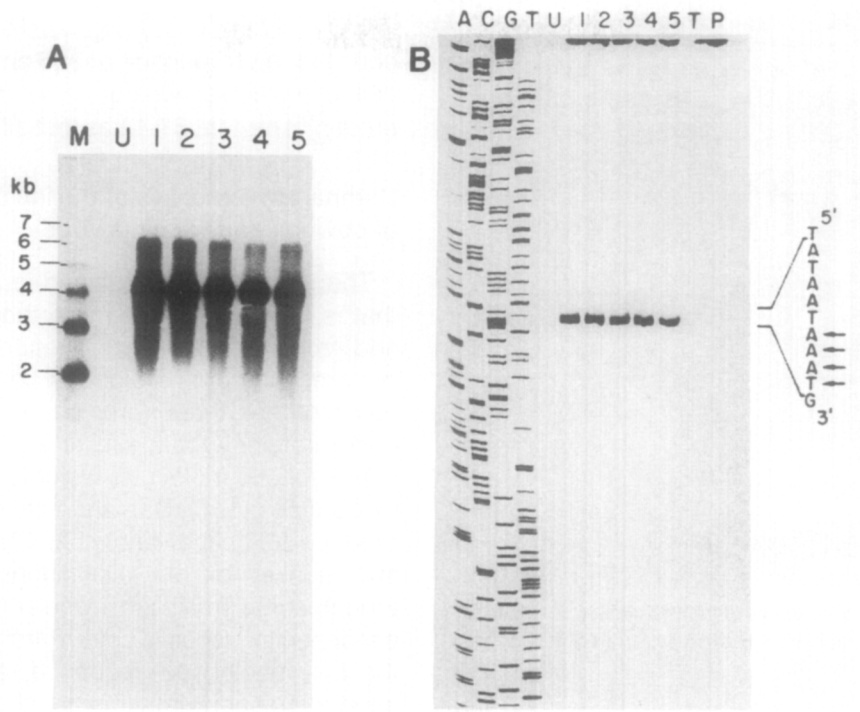

FIG. 4. Transcriptional analysis of the 94K gene of vaccinia mutants. (A) Northern blot analysis of the 94K gene transcripts. A 1.9-kbp *Acc*I fragment (probe P1, Fig. 3) was used as a probe for hybridization to blots of RNA that had been extracted from cells infected with wild-type vaccinia virus (lane 1) or mutants 48-7, 101-14, 48-42, and 87-4 (lanes 2–5), 22 hr after infection. U, RNA from uninfected cells. M, molecular weight markers. (B) Nuclease S1 analysis of the extent of complementarity between the 94K gene and the 5′ ends of its transcripts. A 313-bp *Nco*I–*Acc*I fragment (probe P2, Fig. 3) was 5′ end-labeled at the *Acc*I cleavage site. This probe was annealed to RNA that had been extracted from vaccinia virus-infected cells 22 hr after infection. After nuclease S1 treatment, the S1-resistant nucleic acids were electrophoresed in a 6% polyacrylamide sequencing gel. Lanes A, C, G, T contain size markers consisting of the products of dideoxynucleotide chain termination reactions with a template of known sequence (M13) and the M13 sequencing primer (−40). The other lanes contain nuclease S1-resistant DNA by hybridization with RNA from uninfected cells (U) or cells infected with wild-type vaccinia virus and mutants 48-7, 101-14, 48-42, and 87-4 (1–5), and tRNA (T). Lane P represents the untreated probe. The sequence of the coding strand of DNA is presented. The arrows indicate the 3′ ends of the labeled DNAs protected.

map the extent of complementarity between the 5′ end of the mRNA and the viral DNA in wild-type virus and mutants. A *Nco*I–*Acc*I fragment (probe P2, Fig. 3) was 5′ end-labeled at the *Acc*I cleavage site. The results shown in Fig. 4B indicated that the 5′ ends of the transcripts fall in the TAAAT motif, just upstream of the initiation codon within all the mutants analyzed.

Furthermore, these mRNAs, when translated by an *in vitro* rabbit reticulocyte lysate (Fig. 5, TOTAL), synthesized proteins of 94 kDa for the wild-type virus and mutant 48-7 (lanes 1, 2) and of 40 kDa for mutants 101-14 and 87-4 (lanes 3, 4), similar to the results obtained *in vivo*. Immunoprecipitation experiments confirmed the synthesis of the 94-kDa protein and the lack of recognition of the truncated form by a monospecific ATI antiserum (Fig. 5, IP). These results indicate that this lack of recognition is not due to very low steady-state levels of the 40-kDa protein, as might be suggested from the reduced accumulation observed.

These results indicate that the 94K gene is properly expressed by all the mutants in infected cells and suggest that although the 40-kDa protein is not recognized by a polyclonal antiserum it may be the most likely truncated form of the inclusion protein synthesized by these mutants.

Nucleotide sequence analysis of the 94K gene of wild-type vaccinia virus and mutant 87-4

In order to define a gene of the inclusion body family corresponding to a member of the orthopoxvirus group that does not produce typical ATIs and the kind of alterations that occur in this gene during virus persistence *in vitro*, we obtained the nucleotide sequence of the 94K gene of vaccinia virus and the mutant 87-4. Figure 6 shows the complete nucleotide sequence of the 94K gene of vaccinia virus, WR strain, plus 613 nt upstream

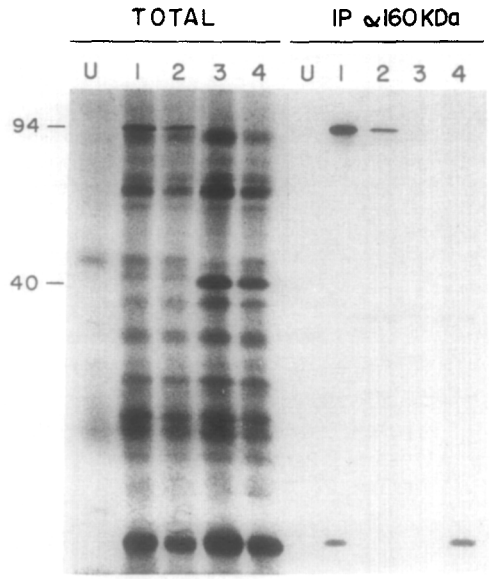

FIG. 5. *In vitro* translational analysis of the 94K gene transcripts of vaccinia virus mutants. Total RNA was extracted from cells infected with wild-type vaccinia virus (lane 1) or mutants 48-7, 101-14, and 87-4 (lanes 2–4), 22 hr after infection, and translated in a message-dependent rabbit reticulocyte cell-free system. The translation products were resolved directly by SDS–PAGE (TOTAL) or after immunoprecipitation with a monospecific antiserum against the 160-kDa major component of the ATIs of cowpox virus (IPα160 kDa). U, Translation products of RNA from uninfected cells. The migrations of the 94- and 40-kDa polypeptides are indicated.

of the initiation codon (spanning from the *Eco*RI right site of the map) and 367 nt downstream of the termination codon. The open reading frame (ORF) contains 2175 nt and encodes for a polypeptide of 725 amino acids with a predicted molecular weight of 84,410 Da. The carboxyl-terminal region of the 94-kDa protein is interesting because the 160-kDa ATI protein of cowpox virus contains 10 slightly variable tandem repeats of about 30 amino acids each, which have been suggested to be involved in the secondary structure of the protein because of a conserved cysteine present downstream of each repetition and the conservation of several charged amino acidic positions (Funahashi *et al.*, 1988). The 94-kDa protein of vaccinia virus still conserves four of these repeats. The difference in molecular weight between the predicted 84.4 kDa and the observed 94 kDa may reflect aberrant mobility possibly caused by the presence of the tandemly repeated sequence.

The comparison of this sequence with that obtained from mutant 87-4 showed a 99.5% homology. The insertion of an extra cytidilate residue in a run of five C in vaccinia virus ORF (nt 1706–1710) produces a frameshift mutation and premature translational termination (TAA, nt 1711–1713) (Fig. 7). The ORF now only encodes for a polypeptide of 366 amino acids with a predicted molecular weight of 42,022 Da. The 40-kDa protein of mutant 87-4 has lost all the tandem repeats.

Comparative analysis of the inclusion protein genes of cowpox and vaccinia virus

The 94K gene is contained in a region of the DNA that is highly conserved in orthopoxviruses (Mackett and Archard, 1979). Hybridization analysis has shown the presence of homologous genes in a similar position in other orthopoxvirus genomes. These genes encode proteins with a similar antigenic specificity and characteristic molecular weights. Cowpox (160 kDa), raccoonpox (155 kDa), and ectromelia (130 kDa) can produce ATIs, and variola (96 kDa), vaccinia (94 kDa), and monkeypox (92 kDa) cannot produce ATIs, although some small and irregular inclusions have been described for vaccinia virus (Patel *et al.*, 1986). To date the only nucleotide sequence reported is the 160K gene of cowpox virus (Funahashi *et al.*, 1988). To explain whether the great differences in molecular size were due to deletions, duplications, or frameshift mutations, the attainment of a nucleotide sequence of a gene encoding a protein of lower molecular mass was necessary. Once we obtained the nucleotide sequence of the 94K gene of vaccinia virus, the comparison of the 94K and 160K genes was done by DNA star program computing (ALIGN file version 5.87). We aligned the 3155 nt sequenced by us with the 4644 nt reported by Funahashi *et al.* (1988) containing the coding sequence of the 160K gene plus surrounding sequences. The homology was 95.687% and the overlapping region was 3251 nt long. We found 125 base changes distributed over the entire sequence (94 in the 94K ORF). A deletion of two consecutive adenylate residues (between nt 2772 and 2773) occurs in the 94K ORF of vaccinia virus that produces a frameshift mutation that leads to premature translational termination a few nucleotides after (TAG, nt 2789–2791). It is interesting that this deletion occurs in a region where tandemly repeated sequences have been previously described in the 160K gene of cowpox virus. The 94K gene maintains 4 of 10 tandem repeats (Fig. 8). Furthermore, we found a deletion of 26 of 28 confirmed repeats of the triplet GAT in the sequence present upstream of the 94K gene, as described previously (Patel *et al.*, 1987), and deletions of the triplet AGT (between nt 1191 and 1192) and of four consecutive triplets GAACAAGAACAA (between nt 1684 and 1685) in the coding region. In the same sequence, we found insertions of the triplets CCC (nt 1707–1709) and GATAAA

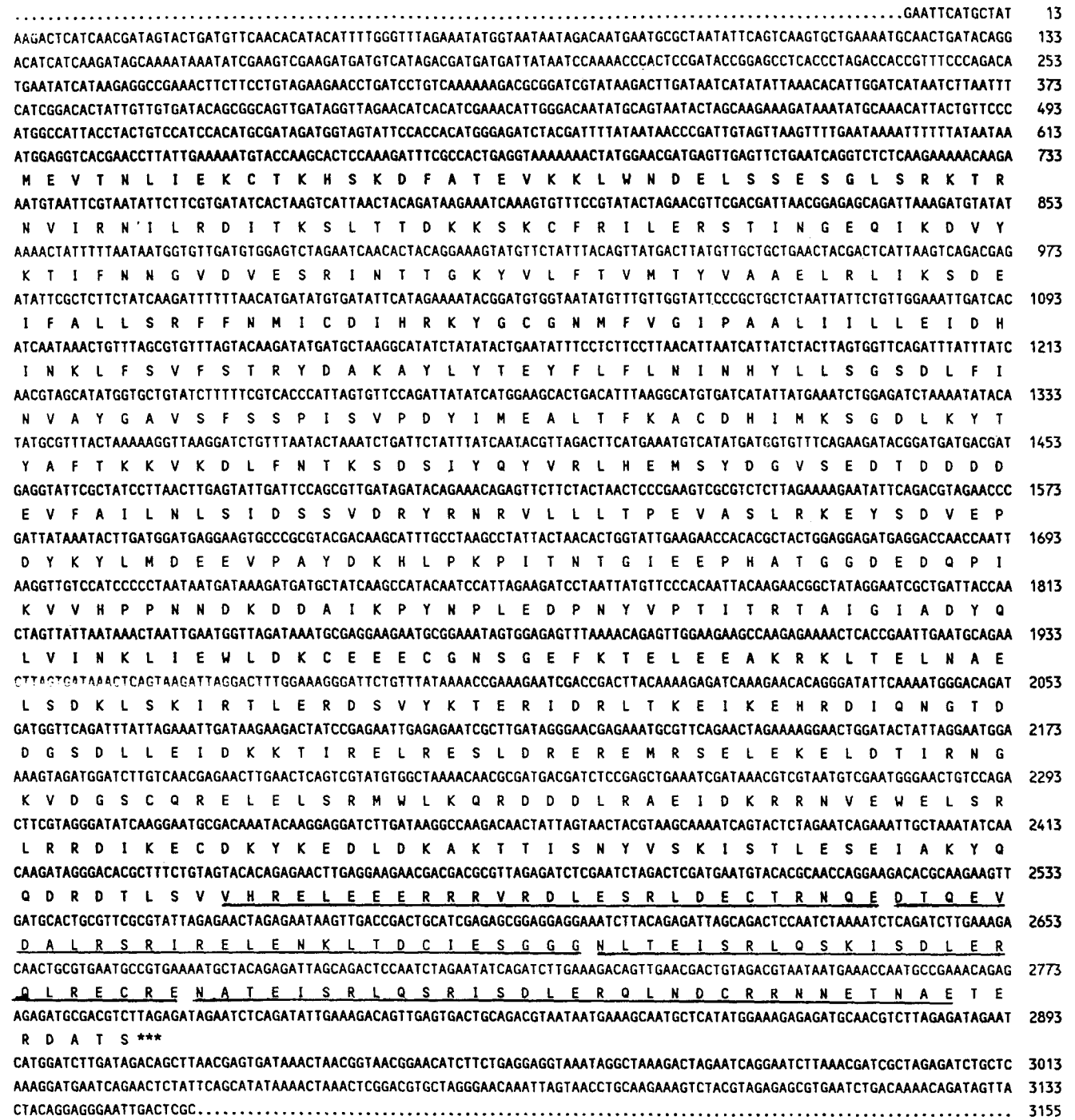

FIG. 6. Nucleotide sequence of the 94K gene and its flanking sequences. The 3155-nt sequence shown starts in the *Eco*RI restriction site located upstream of the 94K ORF (Fig. 3). The amino acid sequence encoded by the 94K gene (nt 614–2787) is shown below the nucleotide sequence. The four slightly variable tandem repeats conserved in the 94K gene are underlined as suggested by Funahashi *et al.* (1988).

(nt 1718–1723), when compared with the 160K gene of cowpox virus. The region between nt 1678 and 1738 of the vaccinia virus gene accumulated the most significant alterations. It is in this region that the insertion of a cytosine induces a frameshift mutation and premature translational termination of the 94K gene of mutant 87-4. As a result of this mutation the 94K gene of mutant 87-4 has lost all the tandemly repeated sequences (Fig. 8).

DISCUSSION

In order to complete our initial goal of defining the genetic stability of vaccinia virus during persistent in-

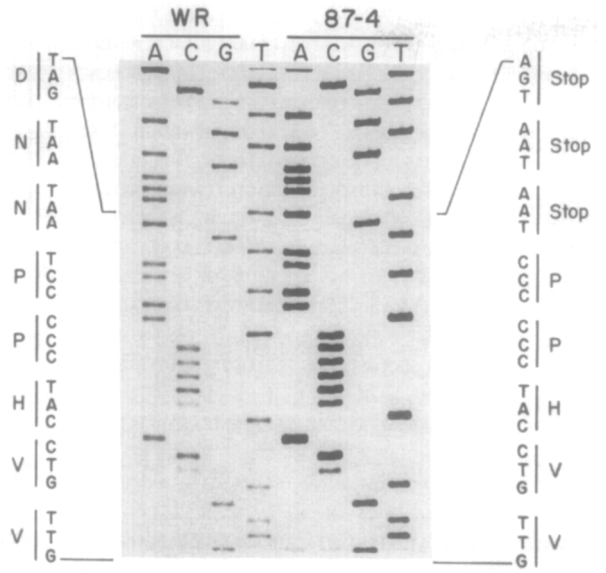

FIG. 7. Identification of the mutation responsible for the alteration in size of the 94-kDa protein in vaccinia virus mutant 87-4. The region of the nucleotide sequence of the 94K gene from vaccinia virus (WR) and mutant (87-4) between nt 1697 and 1718 (Fig. 6) and the predicted amino acid sequences are shown. An insertion of a cytidilate residue occurs in the coding strand of DNA of mutant 87-4.

fections, we have analyzed alterations in virus-induced proteins of vaccinia virus mutants isolated from persistently infected FEL cells. We have found that these mutants contain, in addition to an 8-MDa deletion in the left end of the viral genome and alterations in 7 structural proteins (Paez *et al.*, 1985, 1987), alterations in electrophoretic mobility of at least 14 nonstructural viral proteins (unpublished data). All these modified proteins are synthesized late during infection.

We have focused our study on a 94-kDa nonstructural late protein because this protein was highly expressed at late times after infection and was modified in spontaneous mutants isolated from persistently infected FEL cells (Fig. 1). We have found that this 94-kDa protein is the component of the vaccinia virus cytoplasmic inclusions described by Patel *et al.* (1986). This protein is not essential for virus replication in tissue culture (Patel *et al.*, 1988) and our results with vaccinia virus mutants with a modified 94-kDa protein support this conclusion. A role has been proposed for the poxvirus ATIs in virus dissemination (Ichihashi *et al.*, 1971). The vaccinia virus 94-kDa protein forms small and irregular inclusions that are clearly distinct from the typical ATIs of cowpox and other poxviruses (Patel *et al.*, 1986). This effect is most likely a problem of aggregation of this truncated protein, because large amounts of the 94-kDa protein are produced (Patel *et al.*, 1986). It has been suggested that the 94-kDa protein may be a nonfunctional derivative of the gene encoding the major component of ATIs. However, the high accumulation of this protein in infected cells (about 4% of total cellular protein) and the apparent conservation of "inclusion protein" coding genes in the genomes of several orthopoxviruses that do not produce typical ATIs, including field isolates and several vaccine strains (i.e., variola, monkeypox, and vaccinia viruses), suggest that this protein might have some additional role other than directing the production of the ATIs. We have observed that the vaccinia virus 94-kDa inclusion protein is highly immunogenic in animals (Fig. 2B). We have also verified that the 94K gene of vaccinia virus is a truncated form of the 160K gene of cowpox virus, occurring by a small deletion in a region where sets of tandemly repeated amino acids are located. The fact that all the orthopoxviruses which do not produce typical ATIs encode for inclusion proteins with similar molecular weights suggests a common way of generating these truncated gene products by mutations leading to translational termination in the

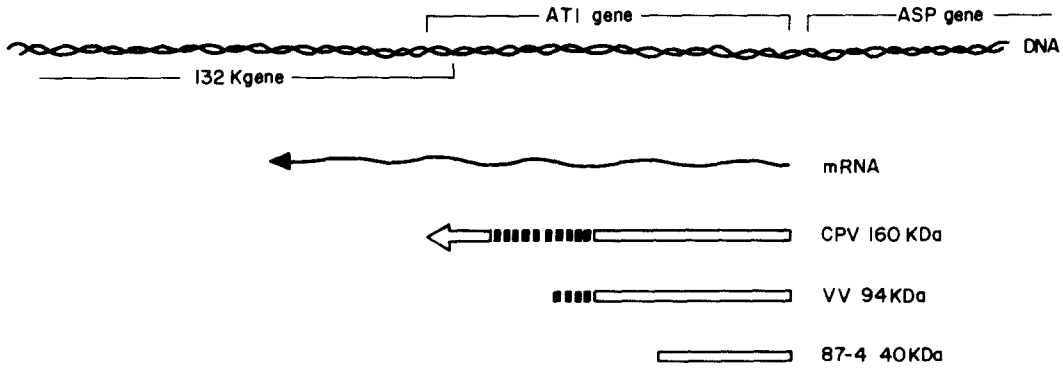

FIG. 8. Diagrammatic representation of the inclusion protein genes of cowpox and vaccinia virus. The ATI gene is flanked by the second largest subunit of the DNA-dependent, RNA polymerase (132K gene) and a gene of unknown function that contains a run of 28 aspartic residues in the amino acid sequence (ASP gene). The mRNA transcribed from the ATI gene and the coding regions of this gene in cowpox (CPV) and vaccinia virus wild type (VV) or mutant (87-4) are shown. Black boxes indicate the number of tandem repeats contained in each coding sequence.

tandem repeat region. However, this suggestion does not explain why truncated, possibly nonfunctional, but highly immunogenic proteins are maintained during evolution of viral genomes.

On the other hand, establishment of persistent infections in cell cultures is a good method to test genetic stability of poxviruses *in vitro.* We have isolated vaccinia mutants from persistently infected FEL cells which produce an altered 94-kDa inclusion protein. Transcriptional analysis of the 94K gene in vaccinia mutants has shown normal levels of RNA transcription and that all the 94K mRNAs are similar in length and contain the same 5′ ends as the wild type (Fig. 5). Nucleotide sequence comparison of the 94K gene between wild-type virus and mutant 87-4 has shown a homology index greater than 99.5%. The alteration of the 94-kDa protein in these mutants during the process of virus persistence is the result of mutations producing premature termination of translation. Several vaccinia virus mutants isolated independently produce similar 40-kDa truncated inclusion proteins. This effect could be explained by the possibility that the similar mutants might be siblings. Siblings represent a difficult practical problem in cell lines persistently infected for long periods of time. However, our results demonstrate that the introduction of a vaccinia virus mutation leading to the production of a 40-kDa protein occurs at different times in untreated and IFN-treated, persistently infected cells. These results indicate that mutants 101-14 and 87-4 are not siblings. It is suggested that independent of the IFN treatment, similar mutations leading to premature translational termination within a region of the 94K gene that accumulates the most significant alterations when compared with the 160K gene of cowpox virus might be responsible for generating these truncated gene products during virus persistence. The fact that the 40-kDa protein, synthesized by the vaccinia virus mutants, did not accumulate in infected cells and was not recognized by a specific polyclonal antiserum might be due to some properties of protein stability and antigenic epitopes that the carboxyl terminus confers to this protein. Recently, the complete nucleotide sequence of the vaccinia virus strain Copenhagen has been reported. This sequence contains a deletion in the region where the ATI gene is located, the result of which is a fusion protein that only includes remnants of the carboxyl terminus of the 160-kDa inclusion protein (Goebel *et al.,* 1990).

Several proteins containing tandemly repeated amino acid sequences are highly immunogenic (e.g., Plasmodium circumsporozoite antigen, streptococal M protein). Recently, similar observations have been made with the 39-kDa immunodominant structural antigen of Fowlpox virus (Binns *et al.,* 1990). The ATI protein is also highly immunogenic in orthopoxvirus and for a long time has been used as a marker for classification of this group of viruses. With vaccinia virus, we have shown that this protein is one of the major antigens during virus infection *in vivo* (Fig. 3B). Our results suggest that the partial or total absence of 10 tandem repeats present near the carboxy terminus of the 160-kDa protein may be responsible for the loss of some or all of the typical characteristics of this protein, namely protein aggregation and immunogenicity. The fact that these mutants have lost the strong immunogenicity provided by this protein (one of the major immunogens of vaccinia virus) may be beneficial in the construction of virus recombinants by stimulating increased humoral response to foreign antigens cloned in vaccinia virus.

ACKNOWLEDGMENTS

This investigation was supported by Grant BIO88-0266 from Comision Interministerial de Ciencia y Tecnología. A.d.C. was supported by a fellowship from Plan de Formación del Personal Investigador, Spain. We thank R. Bablanian for critically reading the manuscript.

REFERENCES

BERK, A. J., and SHARP, P. A. (1977). Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 digested hybrids. *Cell* **12,** 721–732.

BINNS, M., MASON, C., and BOURSNELL, M. (1990). A 39000 M_r immunodominant protein of fowlpox virus contains multiple copies of a 12 amino acid repeat sequence. *J. Gen. Virol.* **71,** 2883–2888.

BULLER, R. M. L., CHAKRABARTI, S., COOPER, J. A., TWARDZIK, D. R., and MOSS, B. (1988). Deletion of the vaccinia virus growth factor gene reduces virulence. *J. Virol.* **62,** 886–874.

BULLER, R. M. L., SMITH, G. L., CREMER, K., NOTKINS, A. L., and MOSS, B. (1985). Decreased virulence of recombinant vaccinia virus expression vector is associated with a thymidine kinase-negative phenotype. *Nature (London)* **317,** 813–815.

CARRASCO, L., and BRAVO, R. (1986). Specific proteins synthesized during the viral lytic cycle in vaccinia virus-infected HeLa cells: Analysis by high-resolution, two-dimensional gel electrophoresis. *J. Virol.* **58,** 569–577.

CHIRGWIN, J. M., PRZYBYLA, A. E., MACDONALD, R. J., and RUTTER, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18,** 5294–5299.

DALLO, S., and ESTEBAN, M. (1987). Isolation and characterization of attenuated mutants of vaccinia virus. *Virology* **159,** 408–422.

DALLO, S., RODRIGUEZ, J. F., and ESTEBAN, M. (1987). A 14K envelope protein of vaccinia virus with an important role in virus host–cell interactions is altered during virus persistence and determines the plaque size phenotype of the virus. *Virology* **159,** 423–432.

ESPOSITO, J., CONDIT, R., and OBIJESKI, P. (1981). The preparation of Orthopoxvirus DNA. *J. Virol. Methods* **2,** 175–179.

FLEXNER, C., HUGIN, A., and MOSS, B. (1987). Prevention of vaccinia virus infection in immunodeficient mice by vector-directed IL-2 expression. *Nature (London)* **330,** 259–262.

FUNAHASHI, S., SATO, T., and SHIDA, H. (1988). Cloning and characterization of the gene encoding the major protein of the A-type inclusion body of cowpox virus. *J. Gen. Virol.* **69,** 35–47.

GOEBEL, S. J., JOHNSON, G. P., PERKUS, M. E., DAVIS, S. W., WINSLOW, J. P., and PAOLETTI, E. (1990). The complete DNA sequence of vaccinia virus. *Virology* **179,** 247–266.

HATTORI, M., and SAKAKI, Y. (1986). Dideoxy sequencing method using denatured plasmid templates. *Anal. Biochem.* **152,** 232–238.

ICHIHASHI, Y., and DALES, S. (1973). Biogenesis of poxviruses: Relationship between a translation complex and formation of A-type inclusions. *Virology* **51,** 297–319.

ICHIHASHI, Y., MATSUMOTO, S., and DALES, S. (1971). Biogenesis of poxviruses: Role of A-type inclusions and host cell membranes in virus dissemination. *Virology* **46,** 507–532.

JOKLIK, W. K. (1962). The purification of four strains of poxvirus. *Virology* **18,** 9–18.

MACKETT, M., and ARCHARD, L. C. (1979). Conservation and variation in orthopoxvirus genome structure. *J. Gen. Virol.* **47,** 683–701.

PAEZ, E., DALLO, S., and ESTEBAN, M. (1985). Generation of a dominant 8 MDa deletion at the left terminus of vaccinia virus DNA. *Proc. Natl. Acad. Sci. USA* **82,** 3365–3369.

PAEZ, E., DALLO, S., and ESTEBAN, M. (1987). Virus attenuation and identification of structural proteins of vaccinia virus that are selectively modified during virus persistence. *J. Virol.* **61,** 2642–2647.

PAEZ, E., and ESTEBAN, M. (1988). Stability of vaccinia virus DNA during persistent infections: Accumulation of left-end deletions and of tandem repeats at both ends of the viral genome and prevention by interferon. *Virology* **163,** 145–154.

PATEL, D. D., and PICKUP, D. J. (1987). Messenger RNAs of a strongly expressed late gene of cowpox virus contain 5′ terminal poly-A sequences. *EMBO J.* **6,** 3787–3794.

PATEL, D. D., PICKUP, D. J., and JOKLIK, W. K. (1986). Isolation of cowpox A-type inclusions and characterization of their major protein component. *Virology* **149,** 174–189.

PATEL, D. D., RAY, C. A., DRUCKER, R. P., and PICKUP, D. J. (1988). A poxvirus derived vector that directs high levels of expression of cloned genes in mammalian cells. *Proc. Natl. Acad. Sci. USA* **85,** 9431–9435.

RODRIGUEZ, J. F., PAEZ, E., and ESTEBAN, M. (1987). A 14,000-M_r envelope protein of vaccinia virus is involved in cell fusion and forms covalently linked trimers. *J. Virol.* **61,** 395–404.

SAMBROOK, J., FRITSCH, E. F., and MANIATIS, T. (1989). "Molecular Cloning. A Laboratory Manual." 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

SHEDLOVSKY, T., and SMADEL, J. E. (1942). The LS antigen of vaccinia. II. Isolation of a single substance combining both L- and S-activity. *J. Exp. Med.* **75,** 165–178.

SHIDA, H., HINUMA, Y., HATANAKA, M., MORITA, M., KIDOKORO, M., SUZUKI, K., MARUYAMA, T., TAKAHASHI-NISHIMAKI, F., SUGIMOTO, M., KITAMURA, R., MIYAZAWA, T., and HAYAMI, M. (1988). Effects and virulence of recombinant vaccinia viruses derived from attenuated strains that express the human T-cell leukemia virus type I envelope gene. *J. Virol.* **62,** 4474–4480.