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A Single Point Mutation of Ala-25 to Asp in the 14,000-M_r Envelope Protein of Vaccinia Virus Induces a Size Change That Leads to the Small Plaque Size Phenotype of the Virus

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The molecular defect responsible for a structural and functional abnormality of the 14,000-molecular-weight (14K) envelope protein of vaccinia virus has been identified. Through DNA sequence analysis of the entire 14K gene from wild-type vaccinia virus and three vaccinia virus mutants, a single base change of C to A was found that resulted in the substitution of Asp for Ala-25. This mutation is responsible for protein size abnormality, as documented by cell-free translation in a rabbit reticulocyte lysate of in vitro mRNA transcripts. In addition, through marker rescue experiments we show that this mutation is responsible for the small plaque size phenotype of vaccinia virus mutants. The structural consequence of the point mutation is a possible turn in an alpha-helix domain with destabilization of a hydrophobic interaction at the N terminus, resulting in monomers and trimers of vaccinia virus 14K protein with decreased electrophoretic mobilities. The functional consequence of the point mutation is a reduction in virulence of the virus.

Vaccinia virus, a member of the genus *Orthopoxvirus*, is one of the largest and most complex animal viruses which replicate in the cytoplasm of infected cells (17). It is currently being considered as a candidate vaccine against a broad spectrum of infectious pathogens of human and veterinary importance (18). The virus has a large 187-kilobase-pair (kbp) double-stranded genome which codes for about 200 genes involved in virus penetration, transcription, DNA replication, and morphogenesis. However, little is known about the proteins involved in these events. Late in infection, the virus causes extensive destruction of cells in culture and in the spleen and liver of infected mice (6). In an immunosuppressed human host, the virus can cause generalized vaccinia (1). This was recently confirmed by the vaccination of a solider who was seropositive for human immunodeficiency virus (24). Because of the complications found in the past during vaccination campaigns with live vaccinia virus, a panel of experts from the World Health Organization has recommended the development of attenuated strains of vaccinia virus with identifiable genetic markers for the construction of future recombinant vaccines (2). Attenuated mutants of vaccinia virus have been obtained by several methods, including (i) inactivation of the viral thymidine kinase gene (*tk*) (3), (ii) generation of deletions at the left end of the viral genome (7, 19), and (iii) insertion of the interleukin-2 gene, which decreases virulence in athymic nude mice (9, 23). Ideally, vaccinia virus mutants with multiple lesions in genes which determine virulence will be desirable as candidate vaccines to minimize the risks associated with vaccination and to prevent reversion to the virulent form of the virus. We have previously reported the generation of highly attenuated mutants of vaccinia virus with several genetic lesions (7, 19). The mutants were generated during virus persistence in Friend erythroleukemia cells and had a characteristic 8-megadalton (MDa) deletion starting 2.2 MDa from the left terminus of the viral genome as well as major alterations in the sizes of three structural proteins with molecular weights of 39,000, 21,000, and 14,000 (the 39K, 21K, and 14K proteins) (20). Generation of the 8-MDa DNA deletion and alteration in size of the 14K protein were accompanied by a marked decrease in virulence (7, 19, 20). Alteration in the size of the 14K protein was a dominant trait during virus persistence, since it was present in the whole virus population by passage 48 (7, 20). The 14K protein increased in apparent size by about 1.5K, producing variants with the small plaque size phenotype (8, 20).

Structural and functional studies revealed the 14K protein to be an envelope protein, which forms trimers held by disulfide bonds and is localized on the cell surface (28). It has an important role in virus penetration at the level of fusion with the cell membrane and is capable of inducing neutralizing antibodies (27). Marker rescue experiments of vaccinia virus mutants that had altered 14K proteins with sequences containing the wild-type 14K gene suggested that a lesion on this gene is responsible for the small plaque size phenotype of mutants (8, 26).

Because the ability of vaccinia virus to form plaques in susceptible cells represents a characteristic phenotypic marker and because the virus plaque size correlates with virulence, it was important for the understanding of the role of the vaccinia virus 14K protein in viral pathogenicity to define the nature of the 14K genetic lesion. In this report, we demonstrate that substitution of aspartic acid for alanine at position 25 in the 14K envelope protein of vaccinia virus results in an electrophoretic size change of the protein and that this in turn gives rise to vaccinia virus mutants with the small plaque size phenotype.

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MATERIALS AND METHODS

Materials. The plasmid M13(+) Bluescribe transcriptional vector, pBS(+), was purchased from Stratagene, La Jolla,

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Calif. RNasin was obtained from Promega Biotec, Madison, Wis. T7 RNA polymerase, restriction endonucleases, and other Ultrapure Chemicals (enzyme grade) were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md., or from New England BioLabs, Inc., Beverly, Mass. [α-^{35}S]dATP (>1,000 Ci/mmol) was obtained from Amersham Corp., Arlington Heights, Ill. [α-^{32}P]dATP (3,000 Ci/mmol), [α-^{32}P]dCTP (3,000 Ci/mmol), and [*trans*-^{35}S] methionine (1,050 Ci/mmol) were obtained from ICN Radiochemicals, Inc., Irvine, Calif. Other materials used were commercial products of the highest grade available from Aldrich Chemical Company, Inc., Milwaukee, Wis., or Sigma Chemical Co., Inc., St. Louis, Mo. Tissue culture materials were purchased from Falcon Labware, Oxnard, Calif. Tissue culture medium and newborn calf serum were obtained from GIBCO Laboratories Life Technologies, Inc., Grand Island, N.Y. Nitrocellulose paper for Southern and Western (immuno-) blot analysis was purchased from Schleicher & Schull, Inc., Keene, N.H.

Virus and cells. Monolayer cultures of African green monkey kidney cells (BSC-40) were grown in Dulbecco modified Eagle medium supplemented with 10% newborn calf serum. Wild-type vaccinia virus (strain WR) and attenuated mutants 48-7, 48-12, and 48-21 were grown in BSC-40 cells and purified by the method of Joklik (11). These mutants have a small plaque size phenotype in BSC-40 cells and are highly attenuated, and their biological properties have been previously described (7, 8, 20).

Cloning and DNA sequence analysis. Isolation of plasmid and vaccinia virus DNA and cloning were carried out by standard procedures (13). To clone the complete 14K gene, vaccinia virus wild-type and mutant DNAs were digested with the restriction enzyme *Hin*dIII, and the cleavage products were separated by electrophoresis in 0.7% agarose. Fragment A was sliced out, electroeluted, ethanol precipitated, and then digested with *Eco*RI. Plasmid pUC19 was also linearized with *Eco*RI. The *Eco*RI subfragments of vaccinia virus *Hin*dIII-A and of pUC19 DNA were phenol extracted, ethanol precipitated, and ligated, and transformation was carried out with competent *Escherichia coli* JM83 cells. After screening of the recombinant clones with a pure ^{32}P-labeled 14K gene fragment, recombinant clones were selected and found to contain a 2.5-kbp *Eco*RI fragment in the vector pUC19. After restriction mapping, a 0.42-kbp *Rsa*I fragment was subcloned into the *Sma*I site of the M13(+) Bluescribe transcriptional vector or into the *Sma*I site of the vaccinia virus insertion vector pSC11. DNA-sequencing analysis was performed with both the chemical cleavage method of Maxam and Gilbert after end labeling with Klenow fragment (15) and the dideoxy-chain termination method of Sanger et al. (30) using M13 Universal sequencing primers (New England BioLabs) and Klenow fragment.

Introduction of wild-type 14K-encoding gene into the *tk* region of mutant virus. The vaccinia virus insertion vector pSC11 (4) was linearized with the restriction enzyme *Sma*I, and the DNA was phenol extracted and ethanol precipitated. The linearized pSC11 and the 0.42-kbp *Rsa*I fragment which contains the coding sequence for the wild-type 14K envelope protein were ligated and transformed into *E. coli* JM83. The positive clones were identified with a pure ^{32}P-labeled 14K probe (320-bp *Dra*I-*Nar*I fragment), and we selected a recombinant plasmid (pSC14K7.5) containing the vaccinia virus wild-type 14K gene under the control of the vaccinia virus promoter p7.5 (5). The recombinant plasmid was then introduced into the *tk* region of attenuated vaccinia virus mutant 48-7 by homologous recombinations (4). Confluent cultures of BSC-40 cells grown in 60-mm dishes were infected with the attenuated vaccinia virus mutant 48-7 at 0.01 PFU per cell and transfected by calcium phosphate precipitation with 10 μg of pSC14K7.5 DNA and with 10 μg of calf thymus DNA as the carrier (8). Cell cultures were harvested 48 h postinfection, and recombinant viruses were isolated by plaque assay after addition of X-gal to the agar overlay (4). Blue plaques were picked and plaque purified three times, and stocks of recombinant viruses were prepared in BSC-40 cells. As an additional criterion of marker rescue, we also selected viruses with the large plaque size phenotype after staining them with 0.01% neutral red added as an agar overlay (26). Individual large plaques detected by visual inspection were picked, grown, plaque purified three times, and amplified in BSC-40 cells.

In vitro transcriptions. The coding sequences for the 14K gene of wild-type and mutant viruses contained in a 0.42-kbp *Rsa*I fragment were subcloned from the pUC vectors into the *Sma*I site of M13(+) Bluescribe transcriptional vector (Stratagene), and the corresponding plasmids pBS(+)14KT7 and pBS(+)15.5KT7 were obtained. The transcriptional plasmid, pBS(+)14KT7 or pBS(+)15.5KT7, was linearized with *Hin*dIII (which cleaves downstream of the inserts), phenol extracted, ethanol precipitated, and suspended in diethyl pyrocarbonate (Sigma)-treated water. In vitro runoff transcriptions of the M13(+) Bluescribe-14K and/or -15.5K gene constructs, pBS(+)14KT7 or pBS(+)15.5KT7, were performed basically as described by Melton et al. (16) but with T7 RNA polymerase instead of SP6 RNA polymerase. Briefly, the linearized plasmid (2 μg) was added to 100 μl of 40 mM Tris hydrochloride buffer, pH 7.5, containing 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 40 U of the ribonuclease inhibitor RNasin (Promega Biotec), 10 mM dithiothreitol, 0.5 mM each ATP, CTP, GTP, and UTP, and 10 to 20 U of T7 RNA polymerase (Bethesda Research Laboratories). Transcription was performed at 37°C for 60 min, following which 2.5 μl of 3.0 M KCl in diethyl pyrocarbonate-water was added; the DNA template was removed by incubation with 2 U of RNase-free DNase (Promega Biotec) at 37°C for 10 min, and 5 μl of 0.5 M EDTA in diethyl pyrocarbonate-water was added to stop the reaction. The mixture was extracted with phenol-chloroform and then with chloroform. The mRNA was precipitated with ethanol, and the pellet was dried and dissolved in diethyl pyrocarbonate-treated water for cell-free translation.

In vitro cell-free translation of mRNA. Cell-free translation of mRNA was performed in a nuclease-treated rabbit reticulocyte lysate system (Promega Biotec). Translation was carried out in a 50-μl total volume at 30°C for 60 min. The translation products were then used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10 μl in each lane) either before or after immunoprecipitation with monoclonal antibody C3 (MAbC3). The specificity of MAbC3 for vaccinia virus 14K protein has been described previously (27). Western blots were carried out as previously described (8).

Immunoprecipitation and SDS-PAGE analysis. Typically, 50 μl of protein A-agarose (Bethesda Research Laboratories) washed three times with phosphate-buffered saline was suspended in 250 μl of 5% BLOTTO (nonfat dry milk) and 3 μl of rabbit anti-mouse immunoglobulin G (stock; 1 mg/ml) was added. The mixture was incubated at room temperature for 2 h and centrifuged; the pellet was washed 10 times with phosphate-buffered saline and suspended in 250 μl of 5% BLOTTO, and 3 μl of mouse monoclonal antibody MAbC3

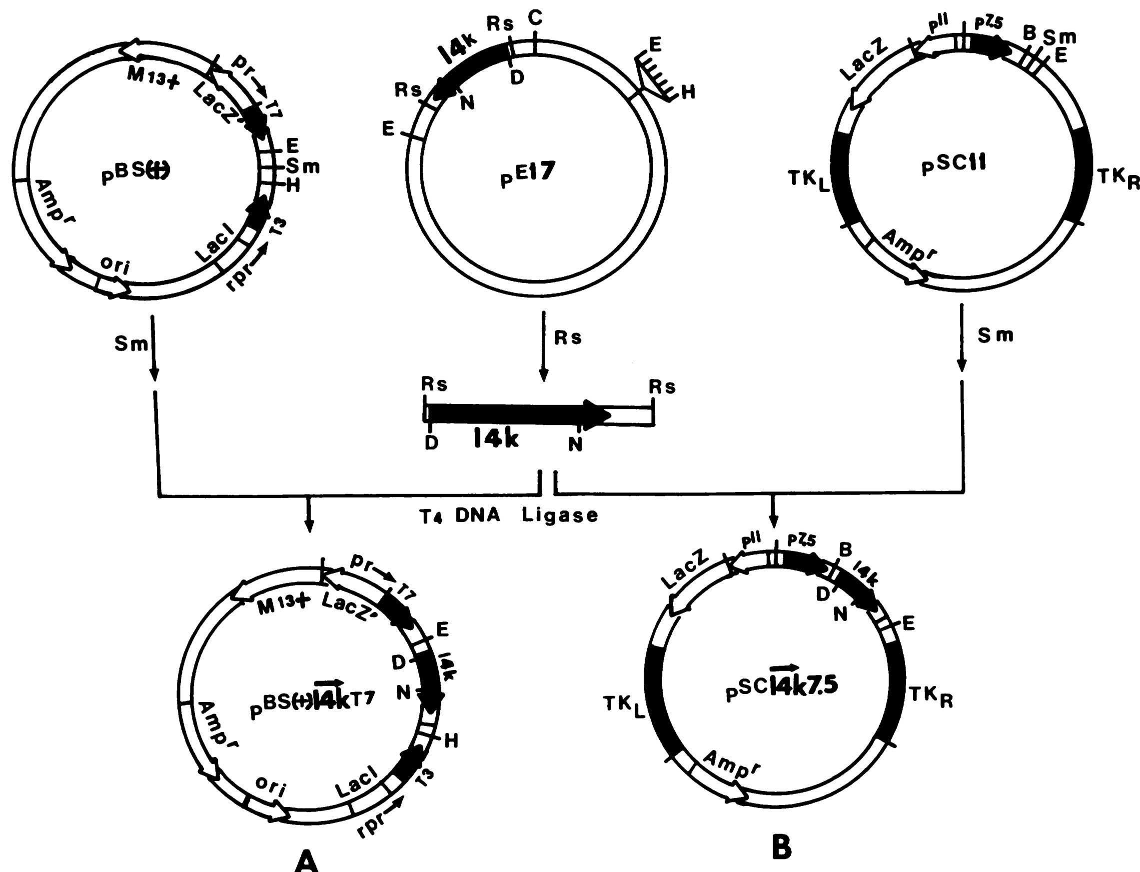

FIG. 1. Scheme for insertion of the vaccinia virus 14K gene into expression vectors. The 14K gene from pE17 (8) was subcloned either into the *Sma*I site of M13(+) Bluescribe transcriptional vector pBS(+) or into the vaccinia virus insertion vector pSC11, as described in Materials and Methods. The restriction sites indicated are *Bam*HI (B), *Cla*I (C), *Dra*I (D), *Eco*RI (E), *Hin*dIII (H), *Nar*I (N), *Rsa*I (Rs), and *Sma*I (Sm). T7 and T3 are the bacteriophage promoters, pr is the M13 universal sequencing primer, rpr is the M13 universal reverse sequencing primer, p11 and p7.5 are vaccinia virus promoters (4), and TK_L and TK_R are left and right sequences, respectively, of the vaccinia virus thymidine kinase gene.

was added as ascitic fluid. The mixture was incubated for 1 to 2 h at room temperature and centrifuged, and the pellet was washed with phosphate-buffered saline. The immunoadsorbant suspended in 150 μl of 5% BLOTTO was mixed with 150 μl of a mixture of 20 μl of reticulocyte lysate translated product and 130 μl of RIPA buffer (20 mM Tris hydrochloride, pH 8.0, 200 mM NaCl, 5 mM EDTA, 0.3 mg of phenylmethylsulfonyl fluoride per ml, 1% Nonidet P-40). The mixture was incubated overnight at 5°C and centrifuged, and the pellet was washed five times each with RIPA buffer and with phosphate-buffered saline. After the final wash, 20 μl of 2× sodium dodecyl sulfate (SDS)-reducing sample buffer (62.5 mM Tris hydrochloride, pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) was added, and the mixture was boiled for 3 min. Insoluble materials were removed by centrifugation, and the supernatants as well as samples of the nonimmunoprecipitated translational samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) with a 3% stacking gel and a 12% separating gel (12). The gel was dried in a slab gel dryer (model 1125B; Bio-Rad Laboratories), and autoradiography was carried out with XR-5 film (Eastman Kodak Co., Rochester, N.Y.).

Computer methods. The analysis, translation, and hydropathy and codon bias analyses of sequences were accomplished by using the International Biotechnologies program. The Robson-Garnier predictions of protein structure were also used.

RESULTS

(i) Nature of the genetic lesion in vaccinia virus mutants with the small plaque size phenotype. We have previously shown that changes in size of the 14K envelope protein of vaccinia virus lead to mutants with small plaque size and reduced virulence (8). Because size changes could result from point mutations, substitutions, insertions, deletions, or rearrangements of the coding sequence, we determined here the entire nucleotide sequence of the 14K gene from three mutants of vaccinia virus. The cloning and sequencing strategy is outlined in Fig. 1. DNA from virus-infected cells was digested with *Hin*dIII, the large *Hin*dIII-A was isolated and digested with *Eco*RI, and a 2.5-kbp *Eco*RI fragment containing the 14K gene (8) was cloned in pUC19 (referred to as pE17 for the wild-type viral gene). After digestion with *Rsa*I, a 0.42-kbp fragment was used for sequence analysis. This fragment contains the entire sequence of the wild-type 14K gene, starting 7 nucleotides upstream from the start codon ATG and terminating 84 nucleotides downstream from the termination codon TAA (25). The *Rsa*I fragments of the wild type and mutants 48-7, 48-12, and 48-21 were subcloned into a *Sma*I-linearized M13(+) Bluescribe transcriptional vector,

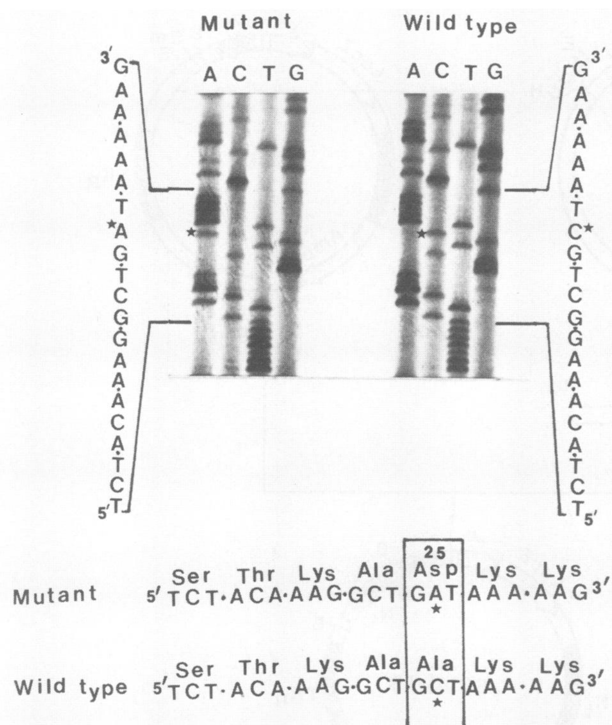

FIG. 2. DNA sequence analysis of the 14K gene from wild-type and mutant viruses. The cesium chloride-purified plasmid DNAs from pBS(+)14KT7 and pBS(+)15.5KT7 were sequenced by the dideoxy-chain termination method using M13 universal sequencing primers. Nucleotide substitutions are indicated (★), and the amino acid change at position 25 of the 14K protein is enclosed in a box. The complete nucleotide sequence of the 14K gene of wild-type and mutant 48-7 virus was deduced in both orientations.

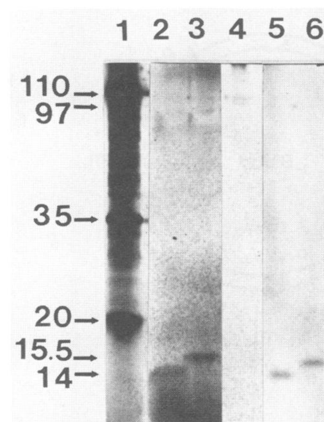

FIG. 3. Cell-free translation products of the 14K gene from wild-type and mutant viruses. Transcripts of the 14K gene from wild-type and mutant 48-7 virus cloned in pBS(+)14KT7 and pBS(+)15.5KT7 (Fig. 1A) were translated in the rabbit reticulocyte cell-free system. The ^{35}S-labeled proteins before (lanes 1 to 3) and after (lanes 4 to 6) immunoprecipitation with MAbC3 were analyzed on a 12% SDS-polyacrylamide gel and detected after autoradiography. Translation products are from brome mosaic virus mRNA (lanes 1 and 4), wild-type 14K mRNA (lanes 2 and 5), and mutant 15.5K mRNA (lanes 3 and 6). The translation products of brome mosaic virus mRNA were used as molecular mass markers (indicated at left in kilodaltons).

pBS(+) (Fig. 1A). This vector contains sequences complementary to the universal M13 sequencing primer and reverse primer in opposite orientation flanking a polylinker region for dideoxy DNA sequencing. With this vector we carried out both the Sanger and the Maxam and Gilbert sequencing analyses to verify the sequence in both orientations. We sequenced the complete 0.42-kbp *Rsa*I fragments of the wild type and of three mutants. Part of the N-terminal sequences of the 14K gene of the wild type and of mutant 48-7 is shown in Fig. 2. While no other alterations were observed in the sequence, a point mutation at position 74 was found in the 14K-coding sequence of vaccinia virus mutants. The mutation results in a change of C to A and results in substitution of Asp for Ala at position 25. We conclude that the point mutation of Ala-25 to Asp is the lesion in the 14K gene of vaccinia virus variants.

(ii) A point mutation in the 14K gene is responsible for a 1.5K increase in the size of the viral envelope protein in vaccinia virus mutants. The apparent M_w of the 14K protein expressed in vaccinia virus mutants 7, 12, and 21 was 1.5K larger than in the wild-type virus when examined by SDS-PAGE (8). To prove that a point mutation is the cause of the apparent protein size alteration, RNA was synthesized in vitro and translated in a rabbit reticulocyte cell-free system. The M13 vector containing the wild-type gene encoding a polypeptide with an apparent M_w of 14,000 (shown in Fig. 1A) and the M13 vector containing the mutant gene encoding a polypeptide with apparent M_w of 15,500 (not shown) were linearized with the restriction enzyme *Hin*dIII, and transcripts were synthesized by T7 RNA polymerase under the control of a T7 promoter. The transcripts were translated in a rabbit reticulocyte lysate, and the products were analyzed by SDS-PAGE before and after immunoprecipitation with MAbC3 specific for the 14K vaccinia virus protein (27). The results are shown in Fig. 3. Transcripts made from the vector with the wild-type gene synthesized a protein with an apparent M_w of 14,000 (lanes 2 and 5); transcripts made from the vector with the 15.5K gene synthesized a 15.5K polypeptide (lanes 3 and 6). The translation products of brome mosaic virus mRNA are shown for comparison of molecular weights (lane 1). The size of in vitro translation products was found to be the same as the size of in vivo 14K and 15.5K products (not shown). From these results we conclude that a charged point mutation of Ala-25 to Asp is responsible for an anomalous migration of the 14K polypeptide on SDS-PAGE.

(iii) A point mutation is responsible for the small plaque size phenotype of vaccinia virus mutants. We had previously reported that plasmids containing a complete copy of the gene encoding the wild-type 14K protein but not a deleted gene could rescue the apparent M_w of the mutated 15.5K protein to a M_w of about that of the 14K protein and the plaque size phenotype of the mutant virus changed from small to large (8). In these experiments we generated homozygous viruses. Although the nucleotide sequence of the rescued gene was not determined, we inferred that the mutant viruses had acquired the wild-type sequence. To extend these studies and to establish beyond doubt the role of the point mutation in the virus plaque size phenotype, we inserted the 14K gene in another region of the viral genome. We reasoned that dual expression of a wild-type 14K gene and a mutated gene might lead to recombinants with an intermediate plaque size phenotype (heterozygous for 14K and 15.5K genes). Thus, we generated a plasmid (pSC14K7.5) from the insertional vector pSC11 (22) containing the wild-type 14K gene under the control of the early-late promoter p7.5 (5) and flanked with vaccinia virus *tk* sequences (Fig. 1B). This vector, pSC14K7.5, was then intro-

duced by marker rescue into the *tk* region of the small plaque size vaccinia virus mutant 48-7, and virus recombinants were selected on the basis of blue plaque appearance. This is because the *tk* insertional vector contains the *E. coli lacZ* gene under the control of the vaccinia virus late promoter p11 (4). For comparison, we also generated 14K homozygous viruses by using plasmid pE17 (Fig. 1) in the marker rescue experiments of mutant 48-7 and selecting for viruses that acquired large plaque size after staining them with neutral red (26). Several of the plaque-purified viruses isolated after marker rescue with either pSC14K7.5 or pE17 were tested for the following markers: plaque size phenotype, apparent size of the 14K protein, and DNA site of integration. The plaque size of a representative heterozygous recombinant virus (Fig. 4A, plate 3) is much larger than that of the parental mutant virus (plate 2) and only slightly smaller than that of the wild-type virus (plate 1). Homozygous viruses (those viruses that had only rescued the mutated 15.5K gene) had a plaque size phenotype similar to that of the wild-type virus (not shown). When, on the other hand, the mutant 15.5K gene (*Rsa*I 420-bp fragment) was introduced into the *tk* region of the wild-type virus, we observed a plaque size phenotype smaller than that of the parental wild-type virus and similar to that of the heterozygous recombinant virus shown in Fig. 4A, plate 3. The plaque size of the heterozygous recombinant virus is not due to a TK^- phenotype, because TK^- recombinant vaccinia viruses with the luciferase gene or human immunodeficiency virus type 1 *env* gene had plaque sizes similar to that of the wild-type virus (not shown). As determined by Western blot analysis with MAbC3, recombinant viruses are heterozygous, since they synthesized two immunoreactive 14K and 15.5K polypeptides (Fig. 4B, lanes 3 to 7), whereas the rescued homozygous viruses only synthesized a 14K protein (not shown). By Southern blot hybridization analysis of *Hin*dIII DNA digests, we established that the heterozygous viruses (Fig. 4C) had two copies of the gene encoding the 14K protein, one copy in *Hin*dIII-A (the naturally occurring locus) and the other in *Hin*dIII-J (the recombinant locus). As a result of plasmid insertion in *Hin*dIII-J, this DNA fragment increased in size. To prove that the isolated recombinants are derived from a parental mutant with an 8-MDa deletion at the left end of the viral genome (19), Southern blot hybridization analysis was carried out with a 1-kb *Sal*I DNA probe contained within the inverted terminal repetition (21). As indicated in Fig. 4D, this probe hybridized with the right end (*Hin*dIII-B) and the left end (*Hin*dIII-C) of wild-type vaccinia virus DNA (lane 1) and only with *Hin*dIII-B of the vaccinia virus mutant (lane 2) because the deletion spans the left inverted terminal repetition (19, 21). The *Sal*I probe hybridized only with *Hin*dIII-B in all DNAs prepared from recombinant virus-infected cells (lanes 3 to 7). A lower hybridization band seen in Fig. 4D, lanes 3 to 7, was due to plasmid sequences contained in the *Sal*I probe (cloned in pUC9) that recognized homologous sequences in the plasmid (pSC11) used as insertional vector.

The above results demonstrate that the point mutation in the 15.5K gene is the cause of the vaccinia virus small plaque size phenotype.

(iv) Structural and functional consequences of the substitution of Asp for Ala-25 in the 14K protein of vaccinia virus. Computer-assisted predictions of alpha helices, beta sheets, and possible turns of the 14K vaccinia virus envelope protein were determined from the nucleotide sequences of the wild-type gene and of the mutant gene (Fig. 2). The 110-amino-acid protein appears as a rigid structure with internal

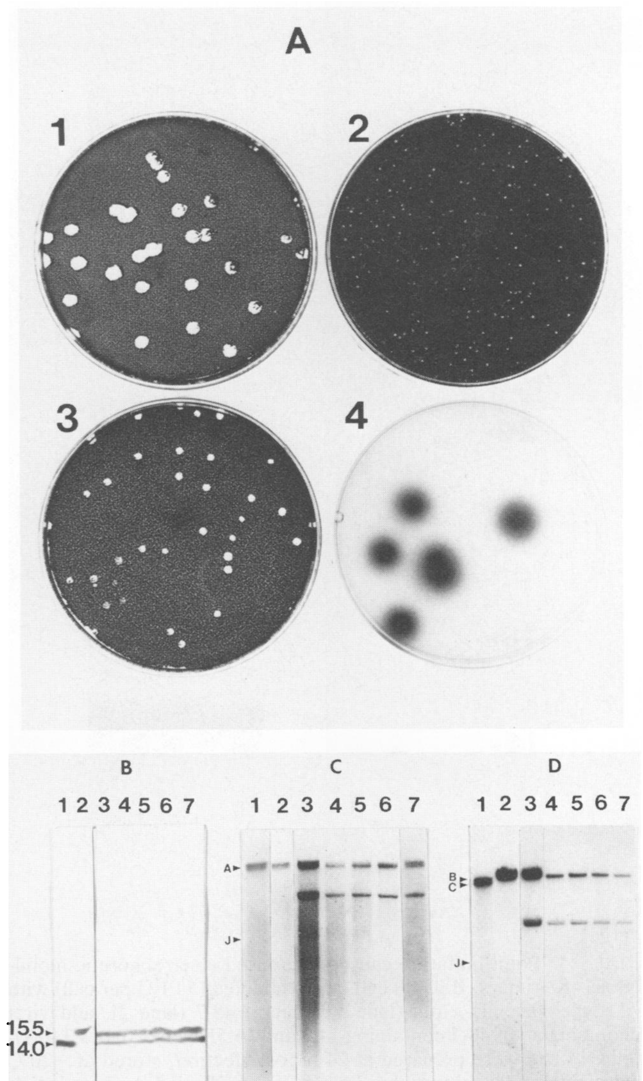

FIG. 4. Characterization of vaccinia virus recombinants generated after insertion of the wild-type 14K gene into the *tk* region of mutant virus. (A) Typical sizes of virus plaques stained with crystal violet from wild-type virus (plate 1), mutant 48-7 virus (plate 2), and recombinant 48-7 virus (plate 3). The β-galactosidase-producing plaques of recombinant 48-7 virus are also shown (plate 4). (B) Western blot showing the size of the 14K protein in recombinant virus. Extracts (40 μg of protein) of BSC-40 cells infected (1 PFU per cell) for 24 h with wild-type virus (lane 1), mutant 48-7 (lane 2), and several plaque-purified vaccinia virus recombinants from mutant 48-7 which acquired the large plaque size phenotype (lanes 3 to 7) were subjected to 12% SDS-PAGE, the Western blot was reacted with MAbC3, and proteins were revealed after immunoperoxidase staining (8). (C) Site of integration of the 14K gene. DNA from virus-infected cells was digested with *Hin*dIII, and the blot was hybridized with a ^{32}P-labeled 320-bp *Dra*I-*Nar*I fragment of the 14K gene. DNA used was from wild-type virus (lane 1), mutant 48-7 (lane 2), and several recombinants from mutant 48-7 (lanes 3 to 7). (D) Occurrence of a left-end deletion in recombinant viruses. Lanes are the same as in panel C, but the blot was hybridized with a ^{32}P-labeled 1-kb *Sal*I fragment located 2.3 MDa from both ends of vaccinia virus DNA (21).

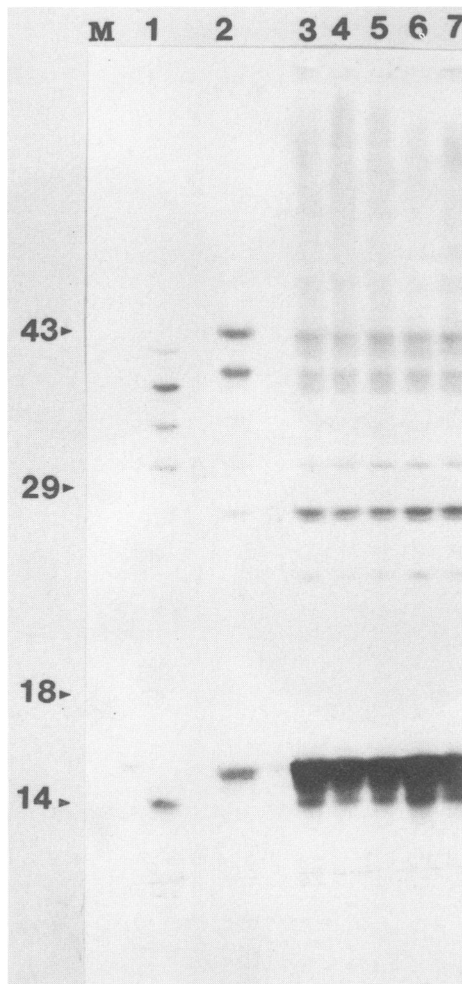

FIG. 5. Point mutation caused decreased electrophoretic mobility of 14K trimers. BSC-40 cells were infected (5 PFU per cell) with wild-type vaccinia virus (lane 1), mutant 48-7 (lane 2), and viral recombinants of 48-7 containing 14K and 15.5K genes (lanes 3 to 7). Cell extracts were prepared at 24 h postinfection, stored at −20°C overnight, and analyzed by 12% SDS-PAGE under nonreducing conditions after 5 min of boiling. The Western blot was reacted with the 14K-specific antibody MAbC3, and proteins were visualized after immunoperoxidase staining. Protein bands with sizes of about 41 and 43 kilodaltons (indicated at left) represent trimers of 14K and 15.5K respectively. The other immunoreactive bands represent protein aggregates (between 43 and 29 kilodaltons), dimers, and monomers. Lane M, protein markers of molecular mass.

alpha helices (amino acids 15 to 55, 64 to 70, and 76 to 99) and small hydrophobic N and C termini, with beta sheets and several possible turns along the protein. The two cysteines (amino acids 71 and 72) are responsible for disulfide-linked trimerization of the protein (28). The computer prediction revealed that as a consequence of the mutation of Ala-25 to Asp there is an introduction of a possible turn in the alpha helix. If so, this turn is likely to promote a conformational change at the N terminus and alter the electrophoretic mobility of the mutated 15.5K protein. In fact, a conformational change of the mutated protein was shown experimentally on the basis of the behavior of trimers on SDS-PAGE when analyzed under nonreducing conditions (Fig. 5). When trimers from mutant virus-infected cells (lane 2) were compared with those from wild-type virus-infected cells (lane 1), a clear distinction in apparent trimer size was observed. Trimers from wild-type virus-infected cells had an apparent M_w of about 41,000, while trimers from mutant virus had an apparent M_w of about 43,000. These differences in apparent M_w of trimers were also observed in aggregates of 14K and 15.5K proteins (see immunoreactive proteins between the 43K and 29K protein size markers). Similar findings were observed with trimers from cells infected with vaccinia virus recombinants expressing both genes encoding 14K and 15.5K proteins (Fig. 5, lanes 3 to 7). In this latter system some mixing between 14K and 15K monomers to oligomers appears to occur, and hence small differences in electrophoretic mobility are noted between trimers of recombinant viruses and of parental viruses.

DISCUSSION

In this paper, we provide evidence that a single point mutation of Ala-25 to Asp in the gene encoding the vaccinia virus 14K envelope protein increases the apparent size of this protein by 1.5K as determined by SDS-PAGE. This mutation in turn alters a biologically important property of the virus, i.e., its ability to form plaques with cultured cells. Since we had previously shown by one- and two-dimensional SDS-PAGE analysis that vaccinia virus mutants 7, 12, and 21 increased the size of the 14K protein by about 1.5K and that these mutants differ in isoelectric point (8), the observed point mutation of Ala-25 to Asp must account for this abnormality of migration on SDS-PAGE. This was documented by translation of 14K transcripts in the rabbit reticulocyte cell-free system (Fig. 3). These differences in protein size were also confirmed in a high-level procaryotic expression system (manuscript in preparation).

In principle, the abnormality in protein size could be due to posttranslational modifications or to folding of the protein. We favor the latter possibility because (i) we have not been able to detect the presence of sugars after metabolic labeling, lectin binding, or glycosidase treatment, and (ii) we observed differences in protein size in systems in which posttranslational modifications do not occur, such as the rabbit reticulocyte cell-free system (Fig. 3) and after expression in *E. coli* (unpublished observations).

The change in apparent size of the 14K envelope protein has a dramatic effect on the plaque size morphology of vaccinia virus. This was documented through marker rescue experiments. When we reset the wild-type 14K gene into the *tk* region of the mutant virus, we generated recombinants that acquired larger plaque size phenotypes than that of the parental virus, although the plaque size was still smaller than that of the wild-type virus (Fig. 4A). When we reset the mutant 15.5K gene into the *tk* region of the wild-type virus, we generated recombinants with plaque sizes similar to those described above (data not shown). However, when the mutant virus with the 15.5K gene was rescued with a plasmid (pE17) containing only the entire wild-type 14K gene (Fig. 1), we then restored a plaque size phenotype similar to that of the wild-type virus (26). The reason coexpression of 14K and 15.5K proteins did not lead to complete restoration of the plaque size phenotype could be that the two proteins mix during trimer formation, resulting in an inefficient virus maturation process. This may explain why trimers are slightly reduced in size in cells infected with recombinant viruses expressing both 14K and 15.5K genes compared with trimers from cells infected with parental viruses (Fig. 5). In

fact, we had previously reported that vaccinia virus mutants (48-7, 48-12, and 48-21) had defects in virus maturation, resulting in decreased virus yields in various cell lines, and that this maturation defect could be rescued with the 14K wild-type gene (7, 8).

A possible consequence of substituting an acidic amino acid (Asp) for a neutral amino acid (Ala) at position 25 is a conformational change of the 14K protein. This is suggested by the decreased electrophoretic mobility of monomers and trimers on SDS-PAGE (Fig. 4 and 5). Computer analysis of the sequence predicted that a conformational change could be caused by a possible turn in the alpha helix. The two large internal alpha helices (amino acids 15 to 55 and 76 to 99), the turn close to the two cysteines that are involved in trimerization, and the beta sheets at both hydrophobic N and C termini all suggest that this protein could establish a hydrophobic interaction within the termini. Since the 14K protein has a role in virus-induced cell fusion (28), this hydrophobic interaction might favor fusion of the virus with the cell membrane, cell-cell fusion, or both. Introduction of a negatively charged Asp in place of Ala-25 might alter the fusogenic properties of the 14K protein. In fact, we know that trimers of the mutant 15.5K protein had decreased electrophoretic mobility on SDS-PAGE (Fig. 4 and 5) and that cells infected with mutant 48-7 virus develop strong cell-cell fusion (20). In spite of a possible conformational change, the mutated 15.5K protein is expressed on the surfaces of infected cells with kinetics similar to that of the wild-type protein (data not shown). The model that we propose is compatible with our additional findings that N-terminal deletions of the 14K protein abolish the fusogenic properties of vaccinia virus mutants (manuscript in preparation). Thus, alterations in conformation of the 14K protein can have a dramatic effect on the ability of vaccinia virus to cause fusion.

The biological consequence of the point mutation in the 14K envelope protein of vaccinia virus is virus attenuation. This is based on our previous observations that vaccinia virus mutants with a 15.5K apparent protein size are attenuated and that the rescued viruses gained virulence after the mutation was rescued with the wild-type 14K-encoding gene (8, 26). Because we showed that the defect of the attenuated mutants was at a late step during virus maturation (7), we proposed that alterations in the conformation of the 14K protein might cause a decrease in the availability of the mutated protein during virus assembly and that this, in turn, led to decreased virus yields and to virus attenuation (8). Moreover, using luciferase as a reporter gene (29), we have found that virus dissemination in tissues is slower in mice inoculated with mutant 48-7 luciferase recombinant virus with the 15.5K gene than in mice inoculated with mutant luciferase recombinant virus with the 14K gene (unpublished observations). Thus, our findings are consistent with the point mutation of Ala-25 to Asp in the 14K protein being responsible for a reduction in virulence of vaccinia virus.

It is a well-known phenomenon that point mutations can change the conformation and function of proteins. Alterations in protein conformation can lead to transcriptional activation (10), loss of antibody-binding capacity (27), changes in temperature-sensitive characteristics (22), decreased enzyme activity (14), and changes in virulence and pathogenicity (31). The findings reported here and those reported earlier (8) revealed a pleiotropic effect caused by a single point mutation of Ala-25 to Asp in the 14K envelope protein of vaccinia virus which increased the apparent size of the 14K protein, diminished the virus plaque size morphology, and decreased the virulence of the virus. The protein itself provides a convenient new genetic marker for the development of suitably modified attenuated recombinants of vaccinia virus for vaccination purposes. This point has been recently confirmed through the generation of attenuated recombinants of vaccinia virus expressing the complete envelope and *gag* genes of the human immunodeficiency virus type 1 (29).

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