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A Mutation in the Gene Encoding the Vaccinia Virus 37,000-M_r Protein Confers Resistance to an Inhibitor of Virus Envelopment and Release

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Plaque formation in vaccinia virus is inhibited by the compound N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine (IMCBH). We have isolated a mutant virus that forms wild-type plaques in the presence of the drug. Comparison of wild-type and mutant virus showed that both viruses produced similar amounts of infectious intracellular naked virus in the presence of the drug. In contrast to the mutant, no extracellular enveloped virus was obtained from IMCBH-treated cells infected with wild-type virus. Marker rescue experiments were used to map the mutation conferring IMCBH resistance to the mutant virus. The map position coincided with that of the gene encoding the viral envelope antigen of M_r 37,000. Sequence analysis of both wild-type and mutant genes showed a single nucleotide change (G to T) in the mutant gene. In the deduced amino acid sequence, the mutation changes the codon for an acidic Asp residue in the wild-type gene to one for a polar noncharged Tyr residue in the mutant.

Vaccinia virus is not released from infected cells by budding through the plasma membrane or by cell lysis (23), but by a unique series of events leading to the release of an enveloped virion (15, 18, 23). This process starts with the association of fully infectious intracellular naked vaccinia (INV) virions with the Golgi apparatus, resulting in the wrapping of virions in a double-membraned structure. Wrapped virions migrate along actin-containing microfilaments (11, 12) to the cell surface, where the outer of the two Golgi membranes fuses with the plasma membrane. Exgulfment of the virion results in the release of extracellular enveloped vaccinia (EEV) virions, which are composed of the original INV virion enclosed by the inner Golgi membrane. Such enveloped particles have been shown to be responsible for virus spread in cell culture as well as in vivo (1, 3, 22). The envelope of EEV virions contains an acylated viral protein of M_r 37,000 (37K protein) (13, 14) as the major envelope-specific antigen and several glycoproteins (20, 21), one of which is the viral hemagglutinin (21, 26). The presence of an envelope increases virion infectivity and the rapidity of virus penetration into cells (4, 27).

Investigation of the molecular events leading to EEV virion release have centered on the effects of inhibitors on the release process. Correct glycosylation of vaccinia virus envelope glycoproteins is apparently required, since inhibition of glycosylation by glucosamine or 2-deoxy-D-glucose inhibits the wrapping of INV virions by the Golgi membranes (24). The closely packed membrane organization of the Golgi apparatus is necessary, since the ionophore monensin transforms the Golgi cisternae into large vacuoles and inhibits INV virion wrapping (25). The final release from the plasma membrane of already exgulfed EEV virions requires an intact cytoskeleton, since the presence of cytochalasin D causes the congregation of large numbers of EEV virions on the cell surface without any EEV virions appearing in the incubation medium (25).

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Very little is known about viral proteins required for envelope formation, and only recently, a viral polypeptide of M_r 14,000 was shown to be important for virion envelopment and egress (29).

The compound N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoylhydrazine (IMCBH) inhibits vaccinia virus release (10, 16, 23) by preventing INV virion wrapping by Golgi membranes. In this study we describe the isolation and characterization of an IMCBH-resistant mutant and show that a single amino acid change in the 37K envelope protein results in the drug resistance phenotype.

MATERIALS AND METHODS

Virus and cells. The IHD-J strain of vaccinia virus was used in this study. The temperature-sensitive vaccinia virus mutant *ts*7 (5) was obtained from R. Drillien, Strasbourg, France. The rabbit kidney cell line RK-13 was cultivated in minimal Eagle's medium with 5% fetal calf serum. African green monkey kidney cells (CV-1) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal calf serum.

Compound IMCBH was kindly provided by Hans J. Eggers (Institut für Virologie der Universität zu Köln, Cologne, Germany). The compound was dissolved at a concentration of 10 mg/ml in dimethyl sulfoxide and stored at −20°C. In experiments with IMCBH, the drug was added to cell cultures at 1 h postinfection. Control flasks were treated with an equal volume of dimethyl sulfoxide lacking IMCBH.

Mutant isolation. Vaccinia virus was mutagenized during replication by inclusion of 5-bromodeoxyuridine (1 μg/ml) in the growth medium of RK-13 cells infected with wild-type vaccinia virus strain IHD-J at a multiplicity of infection of 3. Mutants resistant to IMCBH (IMCBH^r) were enriched by passage of the mutagenized virus in a new RK-13 cell culture by inclusion of IMCBH (10 μg/ml) in the growth medium. The extracellular medium from this infection contained EEV virions released from infected IMCBH^r cells. This extracellular virus harvest was then used for a second enrichment

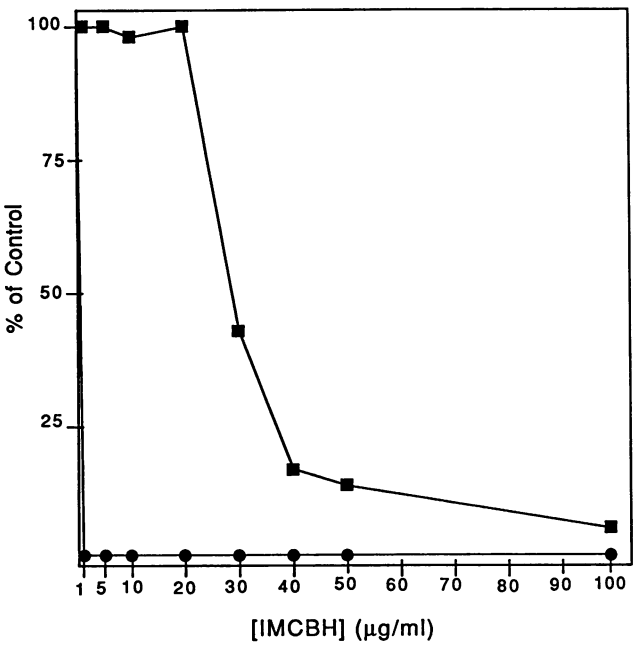

FIG. 1. Wild-type (●) and IMCBH^r mutant (■) viruses were grown in RK-13 cells at a multiplicity of 3 for 24 h in the presence of various IMCBH concentrations. The yield of extracellular virus was determined by plaque assay. Data are plotted as a percentage of the maximum yield obtained in the absence of IMCBH.

passage under the same growth conditions as the first passage. The extracellular medium from this passage was plaqued in the presence of IMCBH (10 μg/ml). Forty-seven plaques were picked. One plaque (Cl-23) was plaque purified two additional times under the same conditions as used for the first plaque purification.

Cesium chloride gradient analysis. Isotopic labeling of viral DNA was achieved by inclusion in the medium of 5 μCi of [^{3}H]thymidine per ml. At the end of infection, extracellular virus was concentrated by centrifugation at 40,000 × *g* in a Beckmann SW-27 rotor for 60 min, and the sediment was resuspended by mild sonication. Intracellular virus was released from infected cells by first swelling the cells in 1 ml of distilled water for 10 min, followed by Dounce homogenization. Virus preparations were centrifuged on CsCl gradients formed by layering 1.30 (3 ml), 1.25 (4 ml), and 1.20 (5 ml) g of CsCl per ml for 60 min at 160,000 × *g* in a Beckmann SW-40 rotor (21). After centrifugation, the gradients were harvested dropwise, the density was ascertained by refractometry, and the radioactivity was determined after 10% trichloroacetic acid precipitation of the gradient fractions.

Plaque assay. Extracellular virus released into the incubation medium during infection was assayed for infectivity after removal of free-floating cells by sedimentation at 1,500 rpm for 5 min. The cell sediments were pooled with cells scraped from the plastic flasks into 5 ml of phosphate-buffered saline. Cells were frozen, thawed, and sonicated to effect release of intracellular virus. Virus infectivity was assayed by plaque titration on RK-13 cells (26). Four petri dishes were used at each 10-fold dilution step.

Molecular cloning. DNA restriction fragments were cloned in plasmid vector pUC9 (31) according to standard proce-

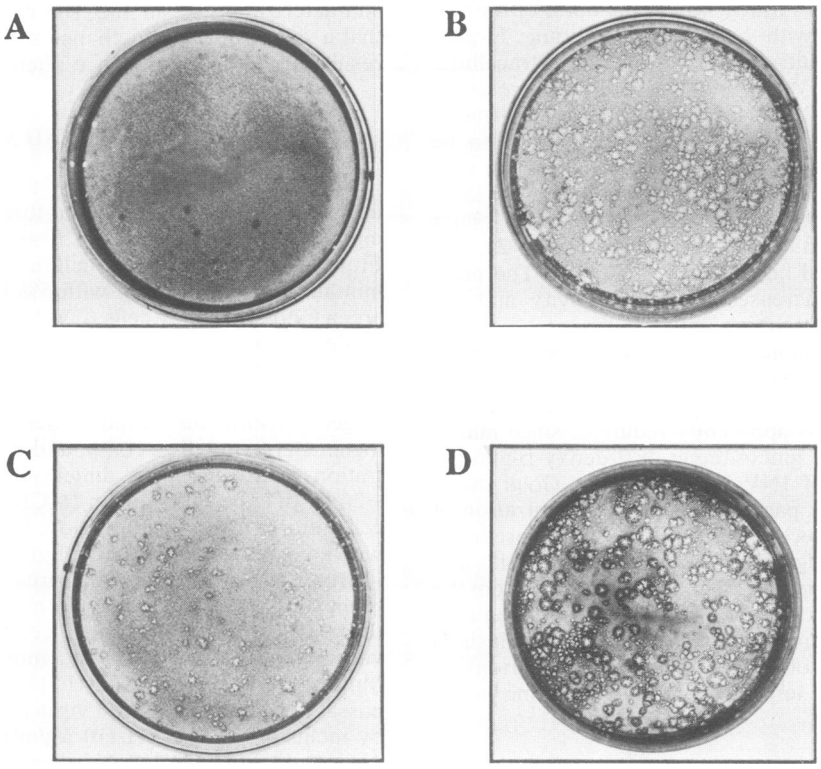

FIG. 2. Effect of IMCBH on plaque formation. Wild-type virus (A and B) and an IMCBH^r mutant (C and D) were plaqued in the presence (A and C) or absence (B and D) of IMCBH (10 μg/ml). The cells were fixed with crystal violet and photographed.

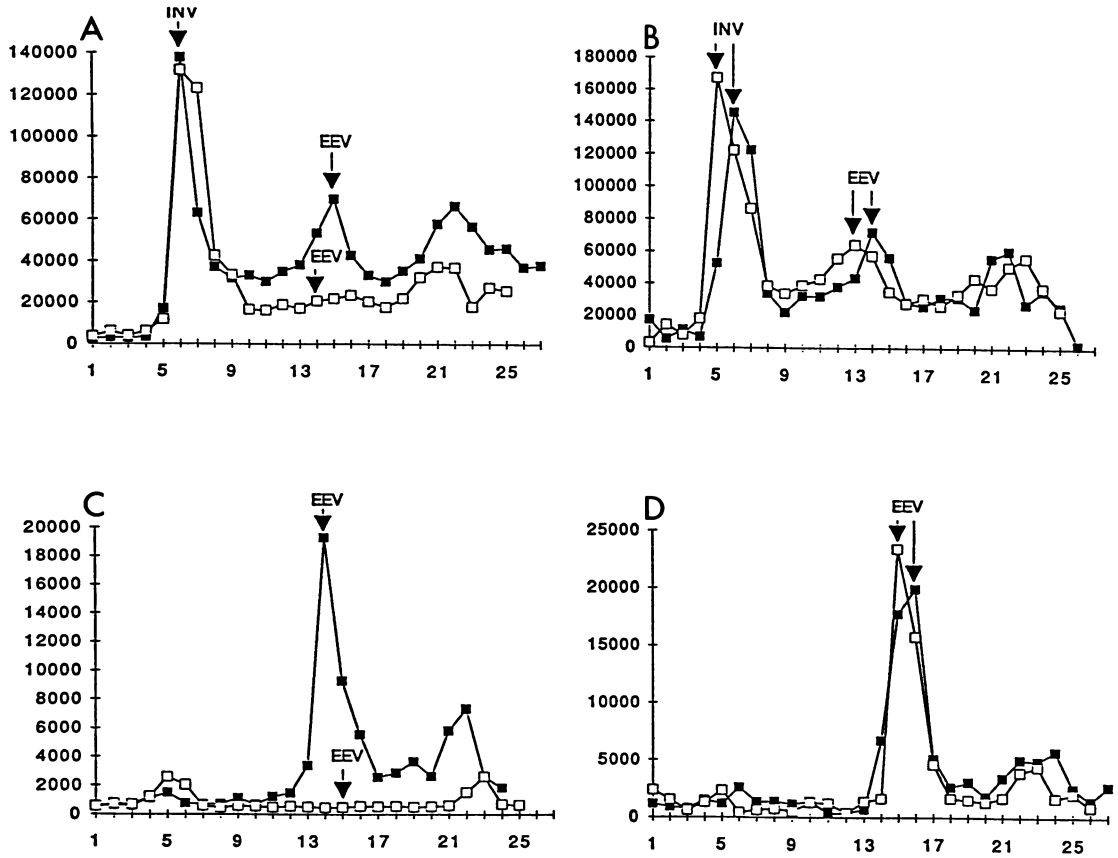

FIG. 3. Production of INV and EEV virions by wild-type and mutant virus. RK-13 cells were infected with the wild-type (A and C) or IMCBH^r mutant (B and D) virus and labeled with 5 μCi of [^{3}H]thymidine per ml. Cells were untreated or treated with IMCBH (10 μg/ml). At 24 h postinfection, extracellular and intracellular virus was subjected to CsCl density gradient centrifugation and analyzed as described in Materials and Methods. The *x* axis and *y* axis show fraction numbers and radioactivity (counts per minute), respectively. The arrows indicate the position of EEV and INV virions as determined by refractometry. (A) Wild-type and (B) mutant INV virions without (■) and with (□) IMCBH; (C) wild-type and (D) mutant EEV virions without (■) and with (□) IMCBH.

dures (17). Recombinant plasmids containing Cl-23 virus *Hin*dIII DNA restriction fragments were identified by restriction analysis or colony hybridization with appropriate radioactivity-labeled cloned *Hin*dIII fragments of the vaccinia virus WR strain as probes.

Marker rescue. For marker rescue experiments, CV-1 cells were grown in 35-mm dishes. Subconfluent cultures were infected with 0.1 PFU of the temperature-sensitive vaccinia virus mutant *ts*7 per cell (5). After 1 h at room temperature, 2 ml of medium was added and the cells were incubated at 33°C for 2 h. The medium was then removed, and 250 μl of calcium phosphate-precipitated DNA (9) containing 1 μg of carrier DNA, 1.25 μg of IHD-J virus DNA, and 0.25 μg of recombinant plasmid DNA was added. For controls, the precipitate contained 2.25 μg of carrier DNA and either 0.25 μg of Cl-23 virus DNA (positive control) or 0.25 μg of IHD-J virus DNA (negative control). After 1 h at room temperature, 2 ml of DMEM containing 2% fetal calf serum was added, and the cells were incubated at 39.5°C for 2 h. Cells were then treated for 1 min with DMEM containing 10% glycerol, washed twice with 1 ml of phosphate-buffered saline, covered with 2 ml of DMEM containing 8% fetal calf serum, and incubated at 39.5°C for 48 h. Cells and growth medium were then frozen, thawed, and sonicated, and 200 μl of a 10^{-2} dilution was used to inoculate fresh CV-1 cells. Cells were treated with IMCBH (10 μg/ml) or incubated in the absence of the drug. After 48 h, cultures were stained with crystal violet and plaques were counted.

DNA sequencing. DNA was sequenced by the dideoxy chain termination method (30) with a kit purchased from Pharmacia (Uppsala, Sweden).

Nucleotide sequence accession numbers. The nucleotide sequences discussed herein have been assigned GenBank accession numbers M60412 (wild-type IHD-J) and M60413 (mutant Cl-23).

RESULTS

Resistance of Cl-23 to IMCBH. The relative sensitivity of wild-type vaccinia virus and the drug-resistant mutant to IMCBH inhibition was determined by titration of extracellular progeny virus from cells treated with various IMCBH concentrations (Fig. 1). Virus release was reduced by 99% for wild-type virus-infected cells incubated with 1 μg of the drug per ml. In contrast, virus release by the IMCBH^r mutant was unaffected by as much as 20 μg of IMCBH per ml, whereas higher inhibitor concentrations did result in considerable reductions in virus yields. Subsequent experiments were therefore performed with IMCBH at 10 μg/ml.

Inclusion of IMCBH in a titration experiment had a

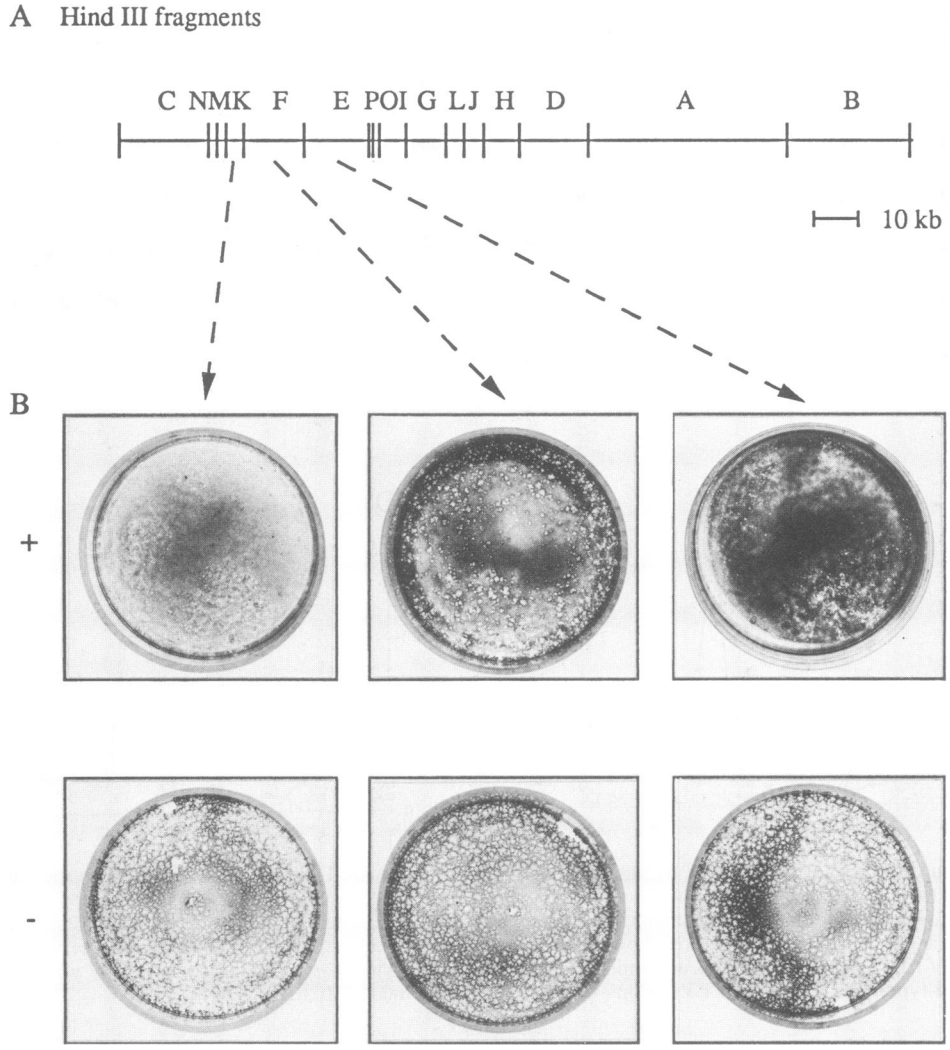

FIG. 4. Marker rescue with cloned *Hin*dIII fragments of IMCBH^r mutant DNA. (A) *Hin*dIII map of IHD-J DNA. Individual *Hin*dIII fragments were transfected into vaccinia virus-infected cells. Progeny virus was titrated in the presence (+) or absence (−) of IMCBH. (B) Results obtained with transfected *Hin*dIII fragments K, F, and E. Note that wild-type plaques in the presence of IMCBH are only obtained with progeny virus from cells transfected with the *Hin*dIII F fragment.

profound effect on plaque formation (Fig. 2). In the presence of the drug, wild-type virus produced minute plaques which were barely visible. These tiny plaques are more readily seen in the marker rescue experiment shown in Fig. 4 (upper row, plates left and right). In contrast, the IMCBH^r mutant showed similar plaque morphology and plaque efficiency in the presence and absence of IMCBH.

IMCBH has been shown to normally block virus release by inhibiting the envelopment of virions, as determined by electron microscopy (23). The lack of any IMCBH effect on the plaquing efficiency or plaque morphology of the IMCBH^r mutant does not reveal whether the plaques are formed by EEV virions released by the usual mechanism, INV virions released by cell degeneration, or both EEV and INV virions. This was resolved by cesium chloride density gradient centrifugation of virus cultivated with and without IMCBH (Fig. 3). Intracellular virus from wild-type-infected cells in the absence of IMCBH showed distinct INV and EEV virion peaks. The presence of EEV virions in this material does not indicate an intracellular origin for this virus form but rather the release of large numbers of EEV virions from the surface of infected cells by Dounce homogenization, as described previously (23–25). Centrifugation of extracellular virus showed a single peak of activity at a density typical of EEV virions. Addition of IMCBH during infection did not affect the appearance of INV virions but did eliminate EEV virions from both the intracellular and extracellular compartments. Cesium chloride centrifugation of material from the IMCBH^r mutant-infected cells showed the same INV and EEV virion distribution in the absence of IMCBH as for the wild-type virus. On the other hand, the presence of IMCBH during virus growth did not, in contrast to that seen for wild-type virus, change the occurrence or distribution of INV or EEV virions from infected IMCBH^r cells. Thus, the IMCBH^r mutation results in normal INV and EEV virion yields from virus-infected cells in the presence of IMCBH.

Mapping of the gene conferring IMCBH resistance. The mutation conferring IMCBH resistance to the Cl-23 virus was mapped by marker rescue experiments. First, the *Hin*dIII fragments except fragments A, B, and C of Cl-23

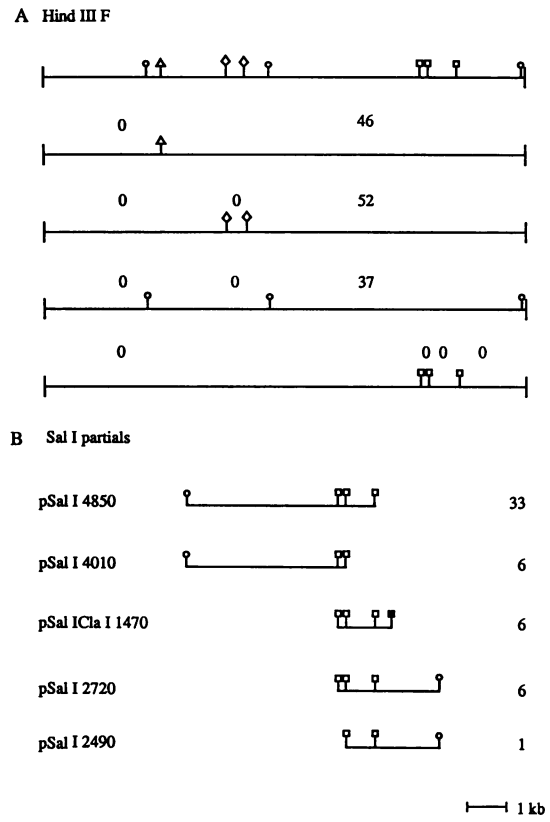

FIG. 5. Marker rescue experiments with DNA fragments derived from the *Hin*dIII F fragment. (A) The top line shows a restriction map of the *Hin*dIII F fragment. The number of wild-type plaques obtained with progeny virus titrated in the presence of IMCBH from cells transfected with cloned restriction fragments is indicated. (B) Various cloned *Sal*I partial digestion products were also used for marker rescue experiments. The number of wild-type plaques is shown at the right. The values in parts A and B represent mean values for two independent transfection experiments in which 10^{-2} dilutions of transfected cell lysates were titrated on a total of eight petri dishes. Symbols for restriction sites: ⍋, *Bgl*I; ⍒, *Bam*HI; ⍓, *Eco*RI; ⍗, *Sal*I; ▼, *Cla*I.

virus DNA were cloned in plasmid vectors. This DNA was coprecipitated with IHD-J wild-type DNA and transfected into CV-1 cells which had been infected with a temperature-sensitive vaccinia virus mutant 1 h earlier. The use of the temperature-sensitive mutant greatly reduces the background of nonrecombinant progeny virus, because virus multiplication at the nonpermissive temperature occurs only in cells that are successfully transfected by wild-type DNA and therefore most likely also contain the recombinant plasmid DNA (2). After 48 h at the nonpermissive temperature, the cells were lysed and progeny virus was titrated in the presence or absence of IMCBH. This experiment showed that of the *Hin*dIII fragments D to P, only the F fragment was able to confer IMCBH resistance to wild-type virus (Fig. 4). For illustration, the negative results obtained with the two neighboring fragments are also shown.

More detailed map information was obtained by marker rescue experiments with various fragments derived from the *Hin*dIII F fragment (Fig. 5A). The first set of cloned *Bgl*I, *Bam*HI, and *Eco*RI fragments allowed mapping of the mutation to the right-hand side of the *Hin*dIII F fragment. However, when the cloned *Sal*I fragments from this region were used for marker rescue, no wild-type plaques were seen. This can best be explained by assuming that the mutation conferring drug resistance is located close to a *Sal*I restriction site, thus leaving little flanking DNA for homologous recombination.

To test this possibility, several partial *Sal*I digestion products were cloned and used for marker rescue (Fig. 5B). The largest *Sal*I fragment produced plaque numbers similar to those obtained with the first set of positive fragments. When smaller partial digestion products were used, the number decreased but was clearly above background except for the partial fragment p*Sal*I-2490. The positive partials all had a common ~230-bp sequence, which strongly suggests that this fragment contains the mutation conferring IMCBH resistance but that additional DNA, either to the left or right, is required for efficient marker rescue.

The 230-bp sequence contains part of the coding sequences of the 37K major envelope antigen. This gene was previously mapped and sequenced in the WR strain of vaccinia virus (14). The 37K protein genes of wild-type IHD-J and the Cl-23 mutant viruses were therefore sequenced (Fig. 6). The two viruses differed only at position 927, the wild-type gene having a G residue and the mutant a T. In the derived amino acid sequence, this changes an acidic Asp in the wild-type protein to a polar noncharged Tyr in the mutant. The mutation is located in the middle of the 230-bp sequence, in agreement with the marker rescue experiments.

The sequence of the IHD-J 37K protein gene was also compared with that of the genes from the WR and Copenhagen strains (Table 1). Several differences were noted at the nucleotide level, but only one each in the IHD-J and Copenhagen strains also resulted in an amino acid change compared with the WR protein.

TABLE 1. Comparison of 37K protein genes among three vaccinia virus strains[a]

Nucleotide position[b]	Codon (amino acid)		
	WR	IHD-J	Copenhagen
105	TCG (Ser)	**CCG (Pro)**	TCG (Ser)
275	GGA (Gly)	**GGC** (Gly)	GGA (Gly)
648	TTG (Leu)	**CTG** (Leu)	**CTG** (Leu)
713	CCG (Pro)	**CCA** (Pro)	CCG (Pro)
746	GAT (Asp)	**GAC** (Asp)	GAT (Asp)
749	ACC (Thr)	**ACT** (Thr)	ACC (Thr)
776	TCG (Ser)	TCG (Ser)	**TCA (Arg)**
854	CCC (Pro)	**CCT** (Pro)	CCC (Pro)
857	GAC (Asp)	GAC (Asp)	**GAT** (Asp)

[a] Nucleotide and amino acid changes with respect to the WR sequence are indicated in boldface letters. Nucleotide numbering is the same as in Fig. 5.
[b] Nucleotide position refers to the nucleotide that is different in either the IHD-J or Copenhagen strain compared with the WR strain.

DISCUSSION

The present study describes the isolation of a vaccinia virus mutant that is resistant to the inhibitory effect of IMCBH, which blocks virus release from infected cells by preventing the wrapping of INV virions by Golgi apparatus membranes (23). EEV virions are normally released when double-membrane-wrapped INV virions migrate to the cell surface and fuse the outer wrapping Golgi membrane with the plasma membrane, resulting in the exgulfment of EEV

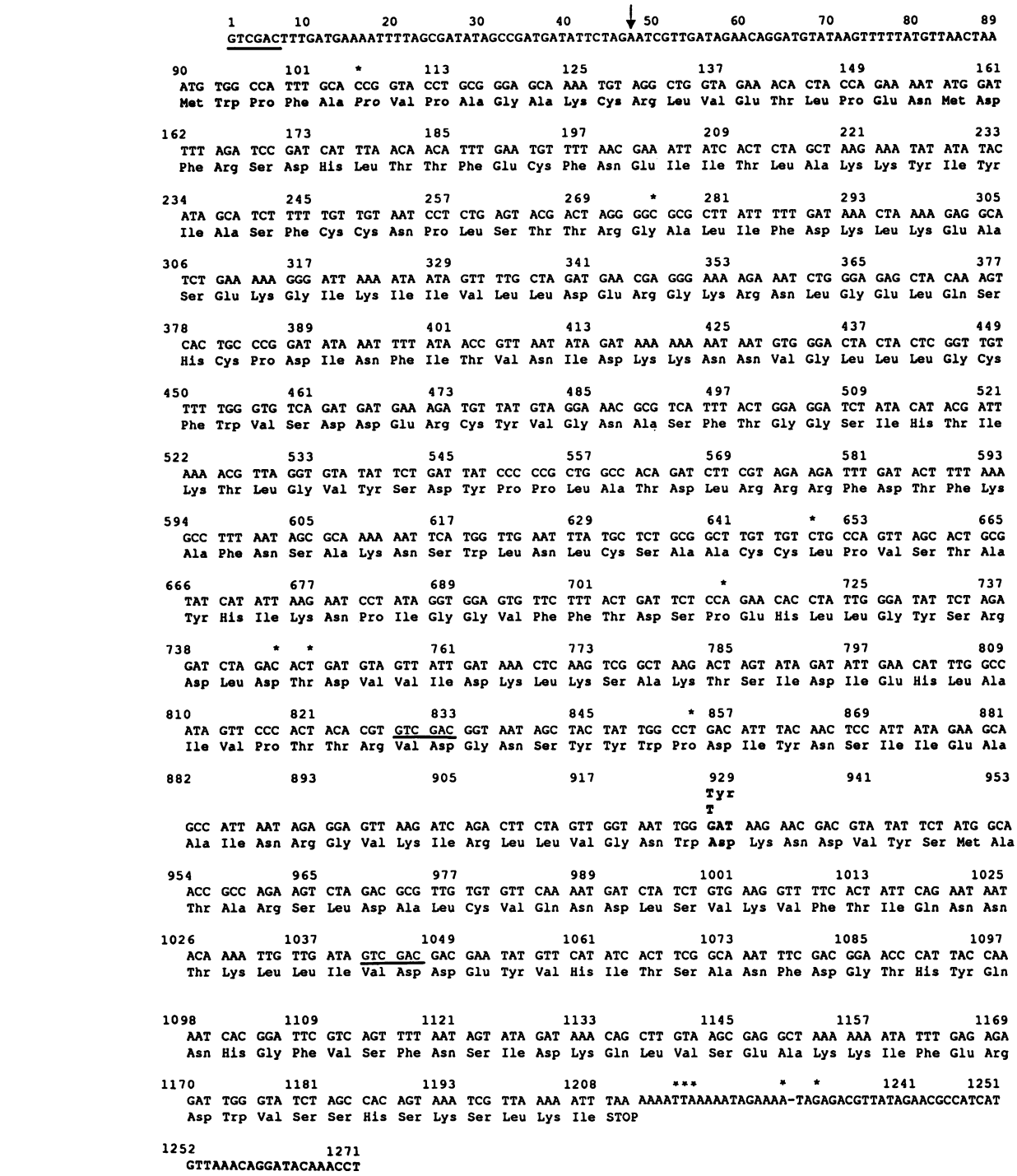

FIG. 6. Nucleotide sequence of the 37K protein gene. The nucleotide sequence of the 37K protein gene and flanking regions of the IHD-J strain of vaccinia virus is shown. The deduced amino acid sequence is also shown. The mutation in Cl-23 (nucleotide 927) and the resulting amino acid change are indicated in boldface letters, as is the corresponding region of the wild-type gene. Differences between the WR strain and IHD-J strain (see Table 1) are shown by asterisks. Except for position 105 (WR, Ser; IHD-J, Pro), these differences do not result in amino acid changes. The arrow at position 47 indicates an additional A residue in the IHD-J strain compared with the sequence of the WR strain. The *Sal*I sites are underlined.

virions composed of the original INV virions wrapped in the inner Golgi membrane (15, 18, 23).

Wild-type virus forms minute plaques in the presence of IMCBH, which prevents EEV virion formation without affecting INV virion yields (23; this study). Together, these results permit two conclusions. First, virus spread in vitro, both to neighboring cells and to distant cells, is mediated by EEV virions, in agreement with previous work (22). Second, the minute plaques formed in the presence of IMCBH are presumably the result of low-grade release of INV virions through cell degeneration. This release is not only very inefficient but insignificant compared with EEV virion release.

Marker rescue experiments and DNA sequencing were used to map the mutation conferring IMCBH resistance to the Cl-23 virus. The mutation was located in the gene encoding the major envelope antigen, previously mapped and sequenced in the WR strain of vaccinia virus (14). In previous studies this protein was referred to as the 37K protein, although its molecular weight as deduced from the gene sequence is 42,000 M_r. The 37K protein is the most abundant protein in the envelope of EEV virions (10) and contains palmitic acid (13).

Comparison of the nucleotide sequences of the wild-type and mutant 37K protein genes of the IHD-J strain revealed a single nucleotide difference. A G residue in the wild-type gene was replaced by a T residue in the mutant. This was rather surprising, since mutagenesis with 5-bromodeoxyuridine is expected to produce A-T to G-C transitions. The most likely explanation is that Cl-23 virus did not arise as a direct consequence of mutagenesis but either was present in the initial virus stock or was produced and selected for during repeated passage in the presence of IMCBH. The mutation in Cl-23 changes an Asp in the wild-type protein to a Tyr in the mutant. The change from an acidic to an uncharged polar amino acid could result in a conformational change in the 37K protein. Indeed, three different computer programs (6, 7, 19) used to examine secondary structure predict conformational changes as a result of the observed amino acid change.

The nucleotide sequences of the 37K protein genes of three vaccinia virus strains are now available: WR (14), Copenhagen (8), and IHD-J (this study). Comparison of the coding sequences showed several differences at the nucleotide level (Table 1). However, only one change in the IHD-J and Copenhagen strains also results in an amino acid change compared with the protein of the prototype WR strain. Interestingly, the amount of EEV virions produced by different vaccinia virus strains varies greatly (21, 22). Whether this is the consequence of differences in the amino acid composition of the 37K protein can be tested, for instance, by replacing the 37K protein gene of the WR strain, a poor EEV virion producer, with that of a good producer, such as the IHD-J strain.

The mode of action of IMCBH is not understood, and therefore the mechanism by which this drug inhibits wild-type virus envelopment and the reason why a mutation in the 37K protein confers drug resistance is not known. One possibility is that IMCBH binds to the wild-type but not to the mutant protein and inhibits its correct targeting to the Golgi apparatus. A defect in correct targeting is consistent with a study in which immunofluorescence microscopy was used to detect the 37K protein (10). In the absence of IMCBH, discrete foci of fluorescence were seen in the cytoplasm of infected cells, whereas in IMCBH-treated cells, weak general cytoplasmic fluorescence was seen.

The present study assigns an important role to the 37K envelope protein in virus envelopment and release. This complex process also requires a 14K viral protein (29). When expression of this protein is inhibited, infectious INV virions accumulate in the cells but no EEV virions are released and no plaques are formed. Thus, prevention of 14K protein expression and treatment of cells with IMCBH have very similar effects on the release of EEV virions. This may be due to a need for an interaction between the 14K protein, which is present on the surface of INV virions (28), and the 37K envelope protein in the complex process leading to the release of EEV virions.

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