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Limited Expression of Poliovirus by Vaccinia Virus Recombinants due to Inhibition of the Vector by Proteinase 2A

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A recombinant vaccinia virus was constructed that expressed poliovirus coat precursor protein P1 fused to about two-thirds of the 2A proteinase. The truncated 2A segment could be cleaved away from the P1 region by coinfecting with poliovirus type 1, 2, or 3 or with human rhinovirus 14 but not with encephalomyocarditis virus. Further cleavage of the vector-derived P1 to yield mature poliovirus capsid proteins was not observed. Attempts to isolate vaccinia virus recombinants containing portions of the poliovirus genome that encompassed the complete gene for proteinase 2A were unsuccessful, unless expression of functional 2A was abolished by insertion of a frameshift mutation. We conclude that an activity of the 2A proteinase, probably its role in translational inhibition, prevented isolation of vaccinia virus recombinants that expressed 2A.

Poliovirus RNA is translated in one open reading frame into precursors that require proteolysis to release the viral proteins that generate and encapsidate progeny virus (32). Of the many site-specific cleavages required, all but the final maturation cleavage of coat protein VP0 to VP4 and VP2 are attributed to two viral proteins, 2A and 3C (10, 12, 26, 38). Proteinase 3C cleaves the precursor P1 into three capsid proteins, VP0, VP3 and VP1, and is thought also to catalyze all other essential cleavages in the P2 and P3 regions of the genome (Fig. 1A), except that which separates P1 from P2. For enteroviruses (e.g., poliovirus) and rhinoviruses, that is the function of 2A, which is thought also to inhibit host cell protein synthesis by indirectly inactivating P220, the large subunit of the cellular cap-binding protein complex, thus blocking translation of capped mRNAs (7, 16, 18, 19, 37). Poliovirus mRNA is uncapped (13, 24) and thus is unaffected by this change.

Because proteinases 2A and 3C are essential to virus production, it is of interest to determine conditions under which they will operate not only in vivo but also in vitro. As all or most picornaviruses share this characteristic dependence on precursor cleavage by virus-encoded proteinases, active 2A and 3C are necessary for most experiments involving expression of more than one picornavirus gene from a cDNA. For example, experiments that aim at synthesizing isolated capsid proteins for assembly into antigenically authentic structures will need to provide for correct cleavage of the capsid precursor. Our underlying purpose in studying poliovirus expression in vaccinia virus was to determine whether this expression vector system could, in fact, be used to synthesize such particles.

Due to the length and monocistronic nature of the poliovirus RNA, recombinant vaccinia virus vectors seemed particularly suitable for picornavirus expression because of the lack of mRNA splicing, the ample size of inserts accepted, and the relative ease with which vaccinia virus can be manipulated (20, 36, 39). Recombinant vaccinia viruses have proved exceptionally useful as vectors for both cellular and viral genes (1, 27, 35). In spite of these apparent advantages and the active interest in recombinant manipulation of picornavirus cDNAs, only one other report of a picornavirus gene expressed in vaccinia virus has appeared (22). We report here the expression of the poliovirus capsid protein precursor by recombinant vaccinia virus vectors, the cleavage of the poliovirus polyprotein between P1 and P2 by homologous and heterologous picornaviruses, and evidence that expression of poliovirus protein 2A inhibits vaccinia virus replication.

Recombinant vaccinia viruses were produced by standard methods (21). Transfection of confluent monolayers of BSC-40 or HeLa cells by the method of calcium phosphate precipitation (11) used 2.5 μg of insertion plasmid DNA per ml per 60-mm plate 1 h after infection with 0.01 to 0.05 PFU of vaccinia virus (WR strain) per cell. Cells, which were incubated at 37°C in Eagle minimal essential medium in Earle saline containing 0.1 mM nonessential amino acids and

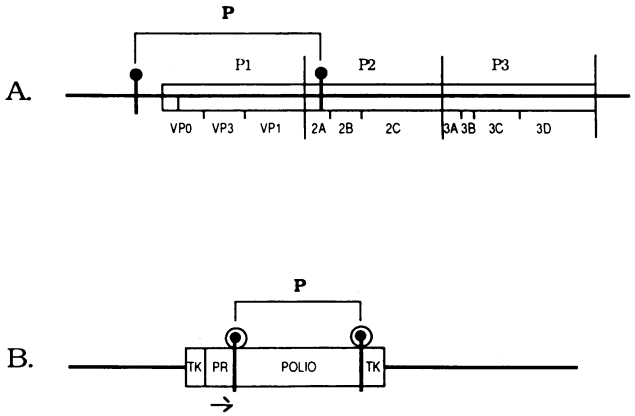

FIG. 1. Poliovirus sequences in VVPO-31. (A) Fragment P between *Bal*I sites (♀) 630 and 3684 (29) includes the 5′ portion of the poliovirus genome as shown (31). (B) P that had been inserted into the plasmid pGS20 at the *Bam*HI site (♀) was introduced into the vaccinia virus genome by recombination within the virus TK gene (TK). Arrow shows direction of message sense transcription of the poliovirus insert from the 7.5K promoter (PR).

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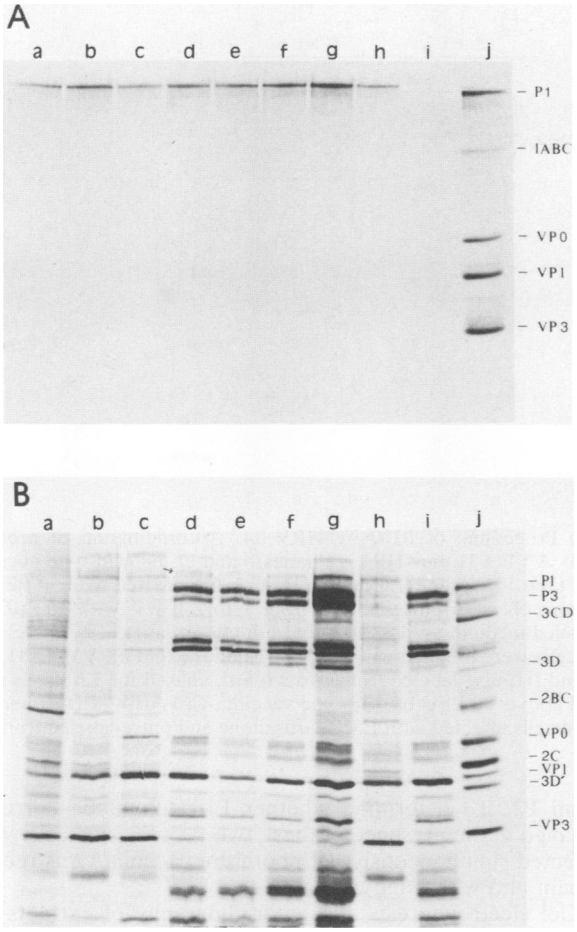

FIG. 2. Protein synthesis by recombinant VVPO-31. (A) Immunoprecipitations of infected cells pulse-labeled at 1 h intervals during infection: a, 0 h; b, 1 h; c, 2 h; d, 3 h; e, 4 h; f, 5 h; g, 9 h. Cells in lane h were treated with 40 μg of cytosine arabinoside per ml before and during infection with VVPO-31 and labeled at 5 h p.i. Other lanes: i, wild-type vaccinia virus 3 h p.i.; j, poliovirus marker sample infected at 10 PFU per cell and labeled from 3.5 to 4 h p.i. Each vaccinia virus sample was the cytoplasmic extract of approximately 4×10^5 cells, 90% of which was immunoprecipitated and 10% of which was used for the gel shown in panel B. (B) Complete cell lysates of samples in panel A. Comparison of lanes f and h shows how cytosine arabinoside treatment, which inhibited vaccinia virus DNA replication, also inhibited synthesis of late viral proteins. Electrophoresis was in 10% acrylamide (resolving gel), with a 4% stacking gel.

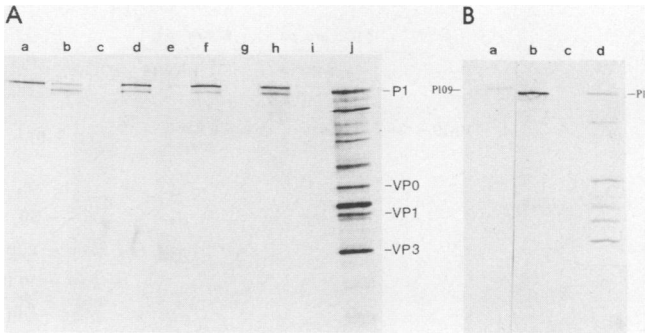

FIG. 3. Processing of P109 in poliovirus-coinfected cells. (A) Autoradiogram of immunoprecipitated proteins at 3 h p.i. from 1 h of pulse-labeling followed by 1 h of chase of HeLa cells coinfected with the following (lanes): b, VVPO-31 plus Sabin type 1 poliovirus; d, VVPO-31 plus Sabin type 2; f, VVPO-31 plus Sabin type 3; h, VVPO-31 plus Mahoney type 1 poliovirus. Other lanes: a, P109 immunoprecipitated from cells infected only with VVPO-31; c, wild-type vaccinia virus plus Sabin type 1 poliovirus; e, wild-type vaccinia virus plus Sabin type 2 poliovirus; g, wild-type vaccinia virus plus Sabin type 3 poliovirus; i, wild-type vaccinia virus plus Mahoney poliovirus; j, lysate of poliovirus (Mahoney)-infected cells labeled 3 to 3.5 h p.i. (B) Cells were subjected to 1 h of pulse-labelling, 3 h of chase, and harvested at 5 h p.i. Lanes: a, VVPO-31; b, VVPO-31 plus Sabin 3 poliovirus; c, wild-type vaccinia virus plus Sabin 3 poliovirus; d, immunoprecipitated products from poliovirus (Mahoney) lysate. Amounts of virus added were 30 PFU of wild-type vaccinia virus and VVPO-31 per cell, 20 PFU of poliovirus (Sabin) types 1, 2, and 3 per cell, and 5 PFU of poliovirus (Mahoney) type 1 per cell. [^{35}S]methionine (100 μCi/ml) was added 1 h p.i. At 2 h p.i., medium was removed, cells were washed, and incubation was continued in fresh unlabeled medium at 37°C for 1 h (A) or 3 h (B). Then cells were lysed, and cytoplasmic extracts were treated with poliovirus antiserum as described in the legend to Fig. 2.

10% calf serum, were collected about 40 h postinfection (p.i.), lysed by three cycles of freezing and thawing, treated with 25 μg of trypsin per ml for 15 min at 37°C, and stored at −70°C. Selection of thymidine kinase-negative (TK^-) virus was by plaque formation on monolayers of TK-143B cells (30) in medium containing 5% fetal calf serum, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.1), and 25 μg of bromodeoxyuridine per ml. Screening for recombinants was by hybridization of viral DNA dot-blotted on nitrocellulose filters directly from infected cells by NaI lysis (4), with nick-translated insertion plasmid DNA as a probe. Positive isolates were further plaque purified and screened by hybridization until two consecutive rounds (12 to 24 plaques per round) yielded 100% positive plaques.

High-titer stocks of vaccinia virus used for radioimmune-precipitation experiments were purified and concentrated by two 1,1,2-trichlorotrifluoroethane extractions on ice of infected cells that had been collected into 10 mM Tris (pH 9) (14). Virus was pelleted through 36% sucrose and suspended in 10 mM Tris (pH 9).

Expression of poliovirus-specific polypeptide by recombinant vaccinia virus VVPO-31. The recombinant vaccinia virus VVPO-31 contained a 3,054-base-pair poliovirus insert that included 113 bases of the 5'-noncoding region, the P1-coding region, and two-thirds of the 2A gene (Fig. 1A). The insert was obtained by partial *Bal*I digestion of pPO193, which contains poliovirus type 1 (Mahoney) cDNA from approximately base 100 to 6011 in pBR322 and was obtained from M. Collette, University of Minnesota; *Bgl*II linkers were attached, and the insert was ligated into the *Bam*HI site of pGS20, which contains the vaccinia virus promoter 7.5K inserted within the vaccinia virus TK gene and was obtained from B. Moss, National Institutes of Health (20). Figure 1B shows the placement and orientation of the poliovirus sequences relative to the 7.5K promoter within the recombinant virus. The predicted mass of the protein synthesized from transcripts originating at this promoter, calculated from the nucleic acid sequence of the insert, is about 109 kilodaltons. The predicted poliovirus product would include P1 (97 kilodaltons) plus two-thirds of protein 2A (11.3 kilodaltons) and another 7 amino acids of irrelevant sequence from the vector. Termination is at a fortuitous stop codon due to the reading frame of the poliovirus insert being out of phase for the TK gene segment that follows it.

Radioimmune precipitation of HeLa cell lysates prepared

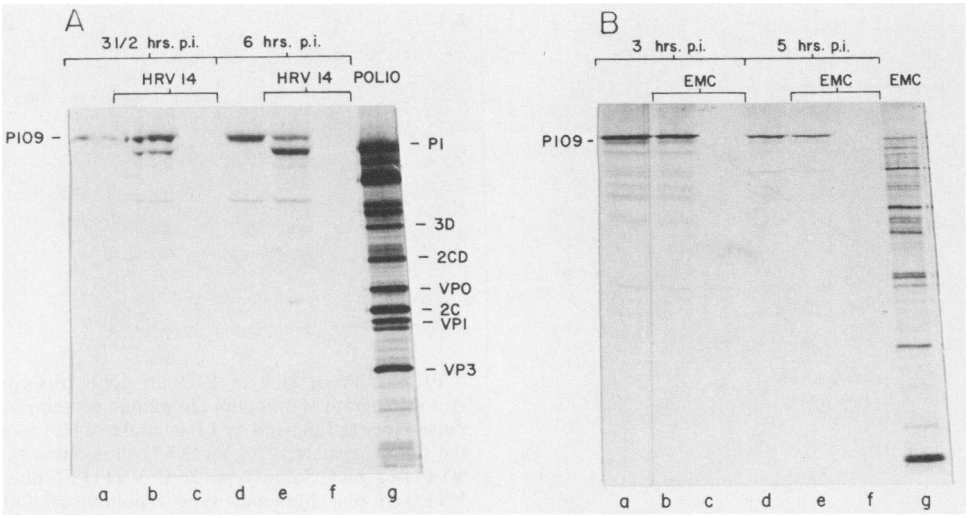

FIG. 4. Coinfections with VVPO-31 and HRV 14 or EMC virus. (A) Processing of P109 by HRV 14. Autoradiogram of proteins immunoprecipitated from cells infected with VVPO-31 alone (lanes a and d), VVPO-31 plus HRV 14 (lanes b and e), or wild-type vaccinia virus plus HRV 14 (lanes c and f). Lane g is a reference lysate of poliovirus type 1 (Mahoney) proteins. HeLa cells infected with 50 PFU of wild-type vaccinia virus or VVPO-31 per cell and 50 PFU of HRV 14 per cell were pulse-labeled from 1 to 2 h p.i. with 100 μCi of [^{35}S]methionine per ml, washed, and chased by incubation at 35°C in unlabeled medium until 3.5 h p.i. (1.5-h chase; lanes a, b, and c) or 6 h p.i. (4-h chase; lanes d, e, and f). (B) Coinfection with EMC virus. HeLa cells were infected with VVPO-31 (lanes a and d), VVPO-31 plus EMC virus (lanes b and e), or wild-type vaccinia virus plus EMC (lanes c and f), pulse-labeled from 1 to 2 h p.i., chased for 1 h (lanes a, b, and c) or 3 h (lanes d, e, and f), lysed, and immunoprecipitated as described above; 30 PFU of wild-type vaccinia virus or VVPO-31 per cell and 30 PFU of EMC per cell were used. Lane g is a cytoplasmic extract from cells infected with EMC virus alone and pulse-labeled from 4.5 to 5 h p.i.

from 0 to 9 h after infection with 30 PFU of VVPO-31 per cell revealed a single large product with an electrophoretic mobility expected for a 109-kilodalton protein (Fig. 2A). Cytoplasmic extracts of cells pulse-labeled for 1 h with 100 μCi of [^{35}S]methionine (1,000 μCi/mmol) per ml at the times postinfection indicated in Fig. 2 were treated with rabbit anti-poliovirus serum and formaldehyde-fixed *Staphylococcus aureus* cells (IGSORB). Immuneprecipitates were analyzed by polyacrylamide gel electrophoresis (17) and autoradiography. Expression of the poliovirus genes was detected within the first hour after infection (Fig. 2A, lane a) and continued throughout the entire 10-h interval examined, consistent with the properties of the 7.5K promoter, which transcribes message both before and after viral DNA replication (6).

P109 could not be reliably detected without immunoprecipitation from lysates, in part because it migrated very near one of the major vaccinia virus proteins (compare Fig. 2A and B) and in part because it represented only a small fraction of the total protein synthesized, on the order of 0.1 to 0.5%, estimating from band intensity in the autoradiograms (note that the immunoprecipitated samples shown in Fig. 2A were ninefold greater than the complete lysate samples analyzed in Fig. 2B).

Cleavage of recombinant product by coinfecting poliovirus. A preliminary experiment showed that, in cells infected with both wild-type vaccinia virus and poliovirus, vaccinia virus proteins were detectable during the first 3 h, but poliovirus proteins were not (data not shown). Since P109 was synthesized during this early stage of infection by the recombinant (Fig. 2A), it could be labeled during that time without labeling poliovirus-derived P1, thus allowing any subsequent processing of P109 to be monitored.

Vaccinia virus vector VVPO-31 encodes neither the poliovirus proteinase 3C required to cut P1 into VP0, VP3, and VP1 nor the intact 2A proteinase needed for cleaving P1 from P2. To determine whether P109 could be correctly cleaved in the presence of these two proteinases, cells were infected simultaneously with both the vaccinia virus recombinant and with poliovirus.

Gel electrophoresis and autoradiography of extracts immunoprecipitated with poliovirus antiserum showed evidence of processing of P109 during coinfection, but only to P1 (Fig. 3). Thus cells infected only with VVPO-31 synthesized P109 (Fig. 3A, lane a), but coinfected cells (lane b) contained two immunoprecipitable bands—P109 as well as a protein which comigrated with P1 in the Mahoney marker (lane j). The same two bands were obtained whether the coinfecting poliovirus was type 1 Mahoney (lane h) or any of the oral vaccine (Sabin) strains of the three serotypes of poliovirus (lane b, d, or f). When coinfection was prolonged for an additional 2 h (Fig. 3B, lane b), only the P1 product was detected, indicating that nearly all of P109 was cleaved. Lanes c, e, g, and i are controls in which cells were coinfected with the same poliovirus strains and with wild-type vaccinia virus in place of the recombinant. They synthesized neither P109 nor the cleavage product of P109, verifying that the P1 bands in lanes b, d, f, and h were derived from P109 and were not the products of the coinfecting polioviruses. Although chase periods of up to 6 h were allowed (data not shown), no further processing of the vaccinia virus-derived P1 to capsid proteins VP0, VP1, and VP3 was observed.

Ability of heterologous picornaviruses to cleave P109. We performed similar experiments with human rhinovirus 14 (HRV 14) and encephalomyocarditis (EMC) virus as the coinfecting viruses to test 2A cleavage by picornaviruses from genera other than *Enterovirus*. HRV 14 cleaved P109 to P1, but EMC virus did not (Fig. 4). Cells simultaneously infected with HRV 14 and VVPO-31 synthesized P109 (Fig. 4A, lanes b and e); it was processed by 3.5 h p.i. (lane b) and increasingly so by 6 h p.i. (lane e), and the product was again

a protein that comigrated with P1 of poliovirus type 1 Mahoney. In contrast, cells infected with VVPO-31 and EMC virus did not show any change in P109 (Fig. 4B); even after 5 h of coinfection with EMC, P109 persisted without cleavage (lane e).

Inhibition of vaccinia virus by 2A. To provide active 2A proteinase in the recombinants and thus obtain P1 cleavage without coinfection, an insertion plasmid pJJ50, containing the entire 5′ half of the poliovirus genome, was prepared (Fig. 5A). However, no recombinants (0 of 234 TK⁻ plaques derived from four separate transfections in three cell lines) could be isolated from cells transfected with pJJ50. Since this was an unusually low occurence of recombinants, we investigated the possibility that the viral sequences present in this plasmid that were not present in pJJ31, either from the 5′ end of the genome or those encoding the complete 2A protein, were inhibitory to vaccinia virus growth.

To test the effect of the 2A protein, we abolished its expression by making a 4-base insertion in pJJ50 near the sequences that encode the carboxy terminus of VP1. A *Bcl*I restriction site was cut, filled by treatment with DNA polymerase large fragment, and religated, thus adding four nucleotides and shifting the reading frame of 2A (Fig. 5B). Recombinant vaccinia viruses were then readily obtained by transfecting cells with the modified plasmid pJJ50B (6 of 35 TK⁻ plaques tested contained the poliovirus insert). Furthermore, isolates of the recombinant virus VVPO-50B synthesized an appropriately sized protein, which was immunoprecipitated with poliovirus antiserum (Fig. 6). This indicated the evident lack of inhibition by the 5′-noncoding region on vaccinia virus protein synthesis as well as translation from the correct poliovirus AUG. This AUG is 743 bases downstream from the 5′ end of the poliovirus sequence. Bases introduced by cloning of pPV16, introduction of an *Eco*RI linker, and the nucleotides derived from transcription of p11K sequences resulted in a putative transcript in which poliovirus nucleotide number 1 was preceded by 31 or 32 nucleotides (there are two adjacent start sites in the vaccinia virus 11K protein mRNA [3]). These include an AUG 27 nucleotides before the first poliovirus base, but a termination codon occurs in this reading frame after 48 poliovirus nucleotides, as in all three reading frames in the poliovirus noncoding regions, thus precluding translation of P1 from any of the nine upstream AUGs in this vaccinia virus mRNA. It is not known whether VVPO-50B synthesizes any polypeptides from the short upstream reading frames, but synthesis of the poliovirus P1-related protein shown in Fig. 6 must occur from a vaccinia virus mRNA, which presumably contains a leader of 775 nucleotides, including 9 upstream AUGs. It is also interesting to note that this aberrant P1 protein, in which the last 55 amino acids of VP1 were replaced with a different sequence of 80 residues as the result of the introduced frameshift, remained immunoprecipitable by our poliovirus antiserum.

This is, to our knowledge, the first report of success in isolating recombinant vaccinia viruses capable of expressing poliovirus proteins. Although it is now evident that certain problems exist in using vaccinia virus as a vector for picornaviruses, we found that coinfection experiments with

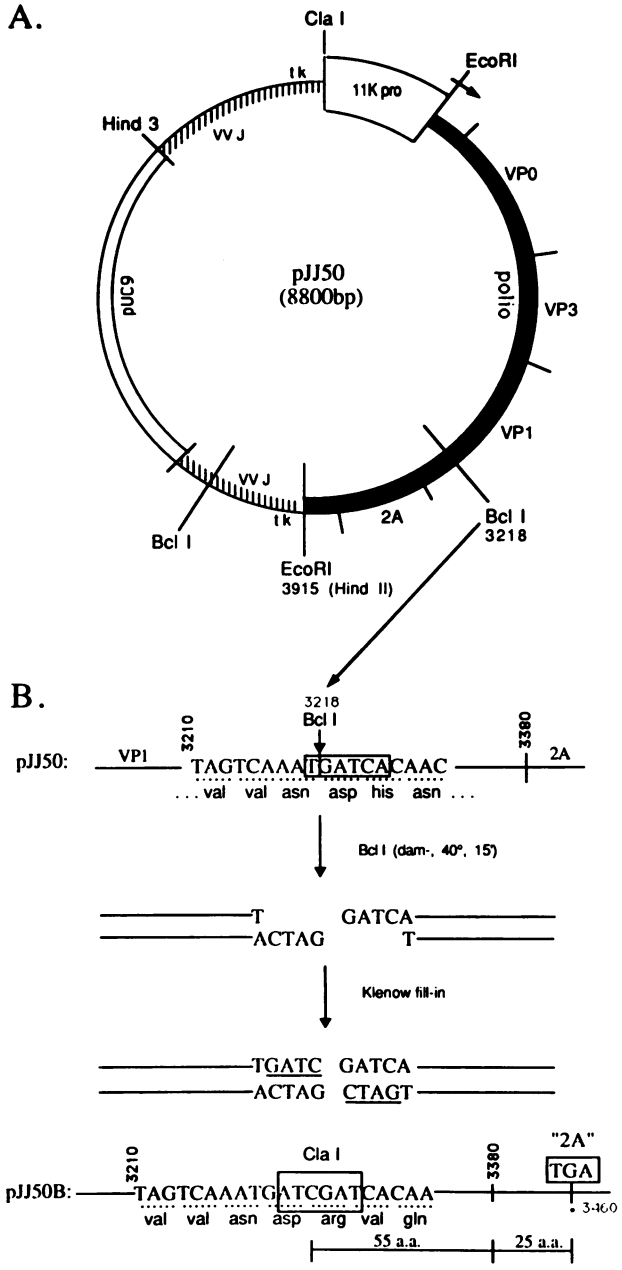

FIG. 5. Construction of insertion plasmids containing 2A. (A) pJJ50. A 3,913-base-pair *Eco*RI-*Hin*dII fragment encoding the 5′ half of the poliovirus genome was removed from the infectious poliovirus clone pPV16 (33) (the generous gift of B. Semler), modified at the *Hin*dII end by the addition of *Eco*RI linkers, and ligated to the vaccinia virus insertion plasmid pH3JCla11K containing the vaccinia virus late promotor 11Kpro (3), obtained from M. Mackett. Insertion of the poliovirus fragment at the *Eco*RI site in pH3JCla11K (arrow) places the polio sequences 8 to 9 nucleotides downstream from the mRNA start site and immediately adjacent to the ATG initiation codon of the 11-kilodalton vaccinia virus protein. (B) pJJ50B. A four-base insertion was introduced into pJJ50 upstream of the 2A gene by partial digestion with *Bcl*I, treatment with DNA polymerase large fragment (Klenow fill-in), religation, and transformation of *Escherichia coli* JM110 cells (Dam⁻) for ampicillin resistance. Screening for plasmids correctly modified at the VP1 *Bcl*I site (position 3218) was by restriction digest analysis for fragments of appropriate sizes with *Bcl*I and *Cla*I. Correct polymerization and ligation of either *Bcl*I site in pJJ50 converts it to a *Cla*I site. The change in amino acid sequence, which begins immediately after the frame shift, is indicated as is the TGA stop codon, which occurs 80 residues downstream.

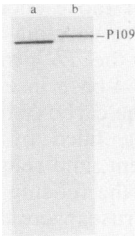

FIG. 6. Immunoprecipitated protein synthesized by VVPO-50B. Autoradiogram of immunoprecipitates from cells infected with VVPO-50B (a) or VVPO-31 (b). BSC-40 cells were infected with 25 PFU/cell and metabolically radiolabeled from 4.5 to 8.5 h p.i. in medium containing 100 μCi of [^{35}S]methionine per ml. Lysates were prepared and immunoprecipitated as described in the text.

the recombinant VVPO-31 were useful in examining the cross-specificity of 2A proteinases. 2A cleavage of the viral polyprotein between P1 and 2A may well occur intramolecularly during normal viral replication, but it occurs in *trans* during the coinfections described here and has also been shown to take place in *trans* in in vitro systems (15, 23). We found that the vector-derived P1-2A product was cleaved not only by coinfection with all three serotypes of poliovirus but also by human rhinovirus 14, in which the 2A protein is only 45% identical to poliovirus type 1 (Mahoney) 2A at the amino acid level (5, 29). However, a region near the carboxy terminus of several picornavirus 2As which is thought to encode the proteolytic active site is conserved between poliovirus and HRV 14 (2, 19). This perhaps accounts for the interchangeability of proteinase 2A observed here and would predict other picornaviruses of substantial sequence similarity to poliovirus that have the conserved region in 2A, such as coxsackievirus B3 (34), might interact as well.

The 2A region of EMC virus, however, differs from that of poliovirus both in sequence and functional strategy (25, 28). This is consistent with its inability to react with the poliovirus protein in our coinfection experiments. The first precursor normally cleaved from the EMC polyprotein is not P1 as in poliovirus and HRV 14, but L-P1-2A, i.e., the precursor of the four capsid proteins (P1) with a leader (L) protein upstream and protein 2A still connected at the carboxy end (28). Both L and 2A are subsequently cut from P1 by the viral proteinase 3C, whereas the agent of the primary cleavage between 2A and 2B has not been identified.

Although proteinase 2A cleaved the recombinant product P109, no further processing of the resultant P1 to coat proteins VP0, VP3, and VP1 by proteinase 3C was observed. This cannot be explained by a vaccinia virus proteinase inhibitor, since all of the poliovirus strains tested were able to replicate in cells infected with vaccinia virus (data not shown). Proteinase 3C or an active precursor acts on free P1 in normal poliovirus replication and in cell-free systems (23); thus the lack of 3C cleavage of the recombinant vaccinia virus-derived P1 is presumably not due to an inability of 3C to act in *trans*. It is possible that the sequence of the DNA inserted into vaccinia virus vector VVPO-31 may include one or more capsid region coding errors that inhibit reaction with 3C, but direct confirmation by sequencing was not undertaken, nor was VVPO-31 reconstructed with poliovirus sequences taken from the infectious poliovirus clone.

DNA sequences encoding complete 2A were recovered in viable vaccinia virus vectors only if they no longer correctly encoded protein 2A, suggesting that it is the expression of 2A which interferes with the replication of vaccinia virus. This apparent inhibition is consistent with other observations on the effect of 2A expression on protein synthesis (37), as well as other observations we have made that poliovirus suppresses vaccinia virus replication. For example, yields of vaccinia virus from coinfections with poliovirus were generally less than 10% of those from control vaccinia virus infections when titers of infectious progeny were determined by standard plaque assay (data not shown). Furthermore, a comparison by gel electrophoresis and autoradiography of proteins synthesized at various times during vaccinia virus-poliovirus coinfections showed that by 4.5 h p.i. vaccinia virus protein synthesis was no longer detectable (data not shown). The known involvement of 2A in poliovirus-induced cleavage of cellular P220 (16, 19, 37) suggests a plausible mechanism by which this may occur, since vaccinia virus mRNAs are capped and therefore may be as susceptible as cellular mRNA to inhibition by inactivation of the cap-binding complex. Thus, use of vaccinia virus as a vector for poliovirus or related picornavirus genomes will require some means of avoiding the inhibiting effect of 2A, perhaps by the use of a cell line whose P220 is not susceptible to 2A-induced cleavage or by using inducible expression systems such as those described by Fuerst et al. (8, 9).

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