

## Ectromelia Virus RING Finger Protein Is Localized in Virus Factories and Is Required for Virus Replication in Macrophages

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**We have previously described a gene of ectromelia virus (EV) that codes for a 28-kDa RING zinc finger-containing protein (p28) that is nonessential for virus growth in cell culture but is critical for EV pathogenicity in mice (T. G. Senkevich, E. V. Koonin, and R. M. L. Buller, *Virology* 198:118–128, 1994). Here, we show that, unlike all tested cell cultures, the expression of p28 is required for in vitro replication of EV in murine resident peritoneal macrophages. In macrophages infected with the p28<sup>−</sup> mutant, viral DNA replication was not detected, whereas the synthesis of at least two early proteins was observed. Immunofluorescence and biochemical analyses showed that in EV-infected macrophages or BSC-1 cells, p28 is associated with virus factories. By use of a vaccinia virus expression system to examine different truncated versions of p28, it was shown that the disruption of the specific structure of the RING domain had no influence on the intracellular localization of this protein. When viral DNA replication was inhibited with cytosine arabinoside, p28 was found in distinct, focal structures that may be precursors to the factories. We hypothesize that in macrophages, which are highly specialized, nondividing cells, p28 substitutes for an unknown cellular factor(s) that may be required for viral DNA replication or a stage of virus reproduction between the expression of early genes and the onset of DNA synthesis. In the absence of p28, the attenuation of EV pathogenicity can be explained by a failure of the virus to replicate in macrophage lineage cells at all successive steps in the spread of virus from the skin to its target organ, the liver.**

Virus pathogenesis is shaped by a complex interaction between the expression of viral genes and the host's response to infection. In the case of poxviruses, a number of genes important for virus pathogenicity have been identified, some of which inhibit host defense mechanisms at different levels, while others activate resting cells or overcome cell-specific blocks to virus replication (for a review, see reference 3). Because ectromelia virus (EV), an orthopoxvirus closely related to vaccinia virus (VV), is a natural mouse pathogen which causes a generalized infection termed mousepox, it is the ideal choice for the study of poxvirus virulence genes.

Recently, we described an EV gene encoding a RING zinc finger-containing protein with a molecular mass of 28 kDa (p28) that is nonessential for virus growth in several tissue culture cell lines but is critical for EV pathogenicity in the A strain of mice (24). Under conditions in which the EV infection caused 100% mortality by 7 days postinfection (p.i.), infection with the p28<sup>−</sup> mutant showed little or no morbidity, and no mortality was observed at virus doses 10,000-fold higher than the EV 50% lethal dose. The dramatic difference in the 50% lethal dose between the EV and mutant virus correlates with the difference in their replication in livers and spleens following footpad inoculation. Interestingly, the difference in titers between the two viruses was most pronounced in livers and less significant in footpads. The effect of the p28 gene disruption on EV pathogenicity has been reproduced with three independent mutants, two of which contained deletions in different portions of the gene, and was completely reversed in a revertant ob-

tained by homologous recombination with the wild-type p28 gene (23b, 24).

On the basis of the known functions of the numerous RING finger proteins (2, 6, 7, 11), we hypothesized that p28 might be involved in the interaction with viral or cellular DNA directly or through the interaction with other proteins (24). Detailed biochemical analyses of p28 were hampered by the insolubility of the protein when it was overexpressed in *Escherichia coli* and in eukaryotic cells. Therefore, to gain further insights into the possible role of this protein in EV virulence, we sought to find an in vitro cell culture system that would mimic the inefficient mutant virus replication in vivo and could be used to determine the stage in the virus replication cycle that required p28 gene function. In this study, we show that expression of p28 is required for EV replication in murine resident peritoneal macrophages and that in the absence of this protein, early virus proteins are expressed, whereas viral DNA is not synthesized. We also demonstrate that in EV-infected cells, p28 is localized in viral factories and that this localization is not dependent on the integrity of the RING finger domain. The requirement of p28 for the replication of the viral genome in macrophages in vitro may suggest the same role for this protein during interaction of the virus with cells of monocyte/macrophage origin in the host organism.

### MATERIALS AND METHODS

**Cells and viruses.** BSC-1 cells (African green monkey kidney cells [ATCC CCL 26]), Raw 264.7 cells (monocyte/macrophage, cells from mice [ATCC TIB 69]), Yac-1 cells (lymphoma cells from mouse strain A [ATCC TIB 160]), Neuro-2a cells (neuroblastoma cells from mouse strain A [ATCC CCL 131]), LA-4 cells (lung adenoma cells from mouse strain A [ATCC CCL 196]), primary mouse embryo fibroblasts (mouse strain A), and primary mouse ovary cells (mouse strain A) were grown in Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per

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ml, and 100 µg of streptomycin per ml. Ovarian fibroblast cultures and primary mouse embryo cells from strain A mice were prepared as described previously for B6 mice (24). Primary peritoneal macrophages were prepared from untreated strain A mice or mice previously injected 4 to 6 days earlier with 0.5 ml of sterile oil or 1.5 ml of sterile thioglycolate broth. The mouse peritoneal cavities were washed with Hanks' balanced salt solution, the cells were concentrated by centrifugation and were washed twice with Hanks' balanced salt solution prior to seeding in 24-well plates in RPMI plus 500 µM β-mercaptoethanol and were further supplemented as described above for the MEM. The cells were incubated for 3 h, washed three times with growth medium, and incubated overnight in fresh medium prior to infection. All cell culture reagents were obtained from GIBCO (Grand Island, N.Y.).

The Moscow strain of EV was a second passage of Mos-3-P1 (4). BSC-1 cell cultures were used to propagate EV and the p28<sup>-</sup> mutant virus, as described in a previous report (24). Virus infection of cells and virus infectivity assays of cell cultures or animal tissues were performed as described previously (4).

**Animals.** Specific-pathogen-free, female, ANCR (A) mice (Charles River Laboratories, Wilmington, Mass.; procured through the National Cancer Institute, Frederick, Md.) were used at 6 to 10 weeks of age. Severe combined immunodeficiency (SCID) mice (strain C.B.17) that were 4 to 6 weeks of age were obtained from Taconic (Germantown, N.Y.). Athymic outbred mice were procured through the National Cancer Institute.

New Zealand White rabbits (2.2 to 2.7 kg of body weight) were obtained from Hazleton Laboratories (Kalamazoo, Mich.) and maintained at Spring Valley Laboratories, Inc. (Sykesville, Md.) for antibody production.

**Plasmid constructions.** The p28K gene (24) was amplified by PCR with oligonucleotide primers (5'-GGGGGGCCATGGAATTCGATC-3') and (5'-GGGGGGGAGCTCCGACATACATCGTCG-3'), which created a *Nco*I site and a *Sac*I site at the 5' and 3' termini of the gene, respectively. The PCR-amplified DNA was inserted between the *Nco*I and *Sac*I sites of pPG-174 (9). The resulting plasmid, pPG-p28, was used for transient expression of p28 under the T7 promoter in mammalian cells infected with recombinant VV vTF-7-3 expressing the T7 RNA polymerase (8). The plasmid pPG-p24 containing the truncated p28 gene was constructed by cutting pPG-p28 at the unique *Nsp*I site (see Fig. 8B) and by removing the protruding 3' terminus using the exonuclease activity of T4 DNA polymerase. The truncated p28 gene was liberated by *Nco*I digestion, gel purified, and ligated into pPG-174 cut with *Nco*I and *Sac*I. The plasmid pPG-p18 was constructed by digesting pPG-p28 at the unique *Eco*RV and *Hpa*I sites (see Fig. 8B) that are located upstream and downstream of the finger domain-coding region, respectively; the resulting large fragment, containing the vector and N-terminal part of the p28, was gel purified and self-ligated.

**Transient expression of p28 and its truncated derivatives in eukaryotic cells.** Monolayers of BSC-1 cells on coverslips, placed into 24-well Costar tissue culture plates, were infected with recombinant VV vTF-7-3 (8) with a multiplicity of 10 PFU per cell and, after adsorption for 1 h, were transfected with plasmids containing p28 or its derivatives by using the reagent lipofectin (GIBCO/BRL Inc., Gaithersburg, Md.) as recommended by the manufacturer.

**Slot blot hybridization.** Macrophages were cultured in wells of 48-well Costar tissue culture plates, infected with EV or p28<sup>-</sup> for 0, 8, and 24 h, and disrupted by using proteinase K and sodium dodecyl sulfate (SDS) and then by performing phenol extraction and ethanol precipitation of the DNA. Total DNA from each well was immobilized on nylon membranes (Hybond-N<sup>+</sup>; Amersham, Arlington Heights, Ill.) in 0.5 N NaOH with a dot blot apparatus. Viral DNA was detected by hybridization of a <sup>32</sup>P-labeled EV DNA probe by a standard procedure (23).

**Expression of the p28 protein of EV in bacteria.** It was previously shown that p28 expressed in *E. coli* DH5α cells as a fusion protein with glutathione-S-transferase was insoluble, accumulating as inclusion bodies (24). In the present study, p28 overexpression in *E. coli* was achieved with the pET system (Novagen, Madison, Wis.). The p28 gene was isolated from pPG-p28 by digestion with *Nco*I and *Xho*I at the respective unique sites and was ligated into similarly digested pET15b. A clone was isolated, and p28 was expressed in *E. coli* BL21(DE3) under the control of the T7 promoter. Although the protein turned out to be insoluble and also toxic for bacterial cells, it was still possible to isolate large amounts of this protein from inclusion bodies and to raise polyclonal antibody in rabbits.

**Rabbit polyclonal antibody to the p28 protein.** Inclusion bodies were purified by several washes of insoluble bacterial fraction with 1% Triton X-100 in phosphate-buffered saline (PBS) and 1% sodium deoxycholate in PBS and then with 5 M urea. p28 was purified by SDS-polyacrylamide gel electrophoresis (PAGE). Gel slices containing p28 were pooled in 0.5 ml of PBS, emulsified with 0.5 ml of complete Freund's reagent, and injected (0.1 ml per site) intradermally into 10 sites on the shaved back and flanks of each of two New Zealand White rabbits. At 2 and 4 weeks later and at approximately 2-month intervals, the rabbits were given booster injections with similar preparations of p28 combined with incomplete Freund's reagent. Sera from immunized rabbits were utilized after four successive antigen booster injections.

**Immunofluorescence.** BSC-1 cells, primary ovarian fibroblasts, or peritoneal macrophages were grown overnight on glass coverslips in 24-well plates. Cultures were infected with various multiplicities of infection of virus for 1 h at 37°C, washed twice with MEM or RPMI complete medium, and incubated in fresh medium for an additional 15 h, at which time the cells were fixed with 3% paraformaldehyde and then permeabilized with 0.05% saponin in PBS. Macro-

TABLE 1. Multiplication of EV and the p28<sup>-</sup> mutant in organs of athymic and SCID mice

Tissue or organ	Log <sub>10</sub> of virus titers ± SD <sup>a</sup>			
	Athymic		SCID	
	EV	p28 <sup>-</sup>	EV	p28 <sup>-</sup>
Footpad	5.65 ± 0.59	3.78 ± 0.37 <sup>b</sup>	8.33 ± 0.22	7.87 ± 0.64
Spleen	6.10 ± 0.30	3.35 ± 0.39 <sup>b</sup>	7.43 ± 0.29	6.11 ± 0.73 <sup>b</sup>
Liver	4.38 ± 0.56	<2.00 <sup>c</sup>	6.11 ± 0.32	4.44 ± 0.33 <sup>b</sup>

<sup>a</sup> Four mice from each group were infected subcutaneously in their footpads with 5 × 10<sup>4</sup> PFU of EV or the p28<sup>-</sup> mutant and were sacrificed at 6 days p.i. for assays of virus infectivity in tissues.

<sup>b</sup> Significantly different from value for EV by the Student *t* test (*P* < 0.05).

<sup>c</sup> Limit of detection of virus infectivity.

phages were stained with Mac-1 (clone M1/70; kindly supplied by Kevin Holmes, National Institutes of Health, Bethesda, Md.) or F4/80 (clone CLA3-1; BioSource, Camarillo, Calif.), both of which were rat monoclonal antibodies directly conjugated to fluorescein. The Fc receptor present on the surfaces of the macrophages was blocked with an anti-Fc monoclonal rat antibody (clone 2.46Z, Pharmingen, San Diego, Calif.) for 30 min before the addition of polyclonal rabbit antibodies. The cells were incubated with polyclonal rabbit antiserum against p28 or VV E3L protein (kindly provided by B. Jacobs, Arizona State University, Tempe, Ariz.) in PBS-0.05% saponin at a 1:500 dilution and then with Texas red (1:100 dilution; Jackson ImmunoResearch Laboratory, Inc., West Grove, Pa.)-conjugated anti-rabbit antibody. All incubations were carried out at room temperature in a moist atmosphere. Cells were stained for DNA with Hoechst 33258 stain (5 µg/ml) and mounted in Fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, Ala.).

**Western blot (immunoblot) analysis.** Proteins were separated by SDS-PAGE and electrophoretically transferred (100 mA for 2 h) to nitrocellulose in 25 mM Tris-192 mM glycine-0.1% SDS-20% methanol. The filters were preincubated for 1 h at room temperature in PBS-0.1% Tween-5% nonfat dry milk to block nonspecific binding sites. After preincubation, the filters were incubated for 1 h at room temperature with anti-p28 serum (1:500) or antiserum against D12L protein (the small subunit of capping enzyme) of VV (1:500; kindly provided by N. Harris, National Institutes of Health) in PBS with 0.1% Tween and 1% nonfat dry milk. After washing with PBS-0.1% Tween, the filters were incubated with peroxidase-conjugated donkey anti-rabbit immunoglobulin G (1:4,000; Jackson ImmunoResearch Laboratory) in the same solution. Antibody binding was detected by chemiluminescence by using the ECL detection kit (Amersham) and was registered by exposure to X-ray film.

## RESULTS

**Comparison of the growth of the p28<sup>-</sup> mutant and EV in vivo.** Because of the dispensability of the p28 gene for virus replication in cell cultures as well as the sequence similarity between p28 and RPT-1—the purported down-regulator of interleukin-2-receptor gene expression—one of the possible explanations of the importance of p28 for virus replication in vivo might be the influence of p28 on the host immune response (24). To address this possibility, we compared the replications of EV and the p28<sup>-</sup> mutant in athymic nude mice with a severely depressed number of T cells and in SCID mice in which the numbers of both T and B cells were depressed (Table 1). The results were very similar to those with normal A strain mice (24). We observed a significant difference between the titers of the EV and p28<sup>-</sup> viruses in the liver and the spleen. Although in SCID mice the absolute titers of both viruses were much higher than in athymic and A strain mice, the p28<sup>-</sup> mutant replicated much less efficiently than EV in the liver and spleen, and death occurred at 14 days p.i., which was 5 days later than after infection with EV. These findings suggest that p28 is important for replication of the virus in certain organs and that various degrees of immunosuppression of the mouse cannot fully compensate for the disruption of this gene in the mutant virus.

In the experiments with footpad inoculation of normal strain A, athymic, and SCID mice, we consistently observed that the

TABLE 2. Multiplication of EV and the p28<sup>-</sup> mutant in organs of strain A mice following i.v. inoculation

Organ	Log <sub>10</sub> of virus titers ± SD <sup>a</sup>			
	31 h		96 h	
	EV	p28 <sup>-</sup>	EV	p28 <sup>-</sup>
Spleen	5.15 ± 0.19	4.79 ± 0.22 <sup>b</sup>	8.22 ± 0.86	4.82 ± 1.43 <sup>b</sup>
Liver	4.02 ± 0.28	3.27 ± 0.20 <sup>b</sup>	6.51 ± 0.32	<2.00 <sup>b,c</sup>
Lung	<2.30 <sup>c</sup>	<2.30	6.07 ± 0.28	3.62 ± 0.91 <sup>b</sup>
Thymus	<2.30	<2.30	5.21 ± 1.27	3.49 ± 1.18

<sup>a</sup> Two groups of 12 mice were inoculated i.v. with 10<sup>5</sup> PFU of EV or the p28<sup>-</sup> mutant, and 4 mice from each group were sacrificed at 31 and 96 h p.i. The remaining 4 mice from each group were observed for mortality.

<sup>b</sup> Significantly different from the value for EV by the Student *t* test (*P* < 0.05).

<sup>c</sup> Limit of detection of virus infectivity.

difference between the EV and p28<sup>-</sup> mutant titers was greater in the liver and the spleen than at the site of inoculation. This may be due to an impediment of the spread of the mutant virus from the site of inoculation to the liver and the spleen and/or less-efficient replication in these organs compared with EV infection. To examine this possibility, we directly infected livers and spleens by inoculating mice intravenously (i.v.) with relatively high doses of the viruses (14). Table 2 shows that the difference between EV and p28<sup>-</sup> mutant virus replication in the liver and the spleen was reproducible and was even more pronounced after i.v. inoculation than by the footpad route (24). At 31 h after an i.v. infection, the titer of the mutant virus in the liver was only 1.27 log units greater than the minimal detectable level, and after 96 h, the mutant was undetectable. Neither EV nor the p28<sup>-</sup> mutant virus could be detected in the lung and the thymus at 31 h after infection. By 96 h p.i., the disparity between EV and p28<sup>-</sup> mutant infectivity was highly significant in the liver, spleen, and lung, with the greatest difference observed in the liver. In the thymus, the difference was not statistically significant. The EV-infected mice died with a mean time-to-death of 7 days, whereas all of the p28<sup>-</sup> mutant virus-infected mice recovered from infection and were virus free by 16 days p.i., when the experiment was terminated. It is known from the classic work of Mims that more than 95% of an i.v. EV inoculum was taken up by the Kupffer cells of the liver and that the replication in these cells was the initial and critical step for infection of the hepatocytes (14–16). A similar role in EV infection was proposed for macrophages lining the sinusoids of the spleen (16). Thus, our results may be explained by an inability of the mutant to replicate in phagocytic reticuloendothelial cells.

**p28 is required for virus replication in murine peritoneal macrophages in vitro but is nonessential for EV replication in all other cell cultures tested.** To investigate the possibility described above, we obtained macrophages from the most convenient source, the peritoneal cavity of the mouse. It has been

TABLE 3. In vitro multiplication of EV and the p28<sup>-</sup> mutant in peritoneal macrophages from susceptible (strain A) mice

Virus type	Log <sub>10</sub> of virus titers ± SD <sup>a</sup>	
	0 h	24 h
EV	4.06 ± 0.07	5.53 ± 0.16
p28 <sup>-</sup>	3.95 ± 0.13	3.66 ± 0.15 <sup>b</sup>

<sup>a</sup> The arithmetic mean values of four replica cultures infected with 5 PFU of each virus per cell are indicated.

<sup>b</sup> Significantly different from the value for EV by the Student *t* test (*P* < 0.005).

TABLE 4. Multiplication of EV and the p28<sup>-</sup> mutant in organs of susceptible and resistant mice

Tissue or organ	Log <sub>10</sub> of virus titers ± SD <sup>a</sup>			
	Strain A (susceptible)		Strain B6 (resistant)	
	EV	p28 <sup>-</sup>	EV	p28 <sup>-</sup>
Footpad	7.96 ± 0.32	6.27 ± 1.55	8.18 ± 0.34	7.00 ± 0.97
Spleen	8.22 ± 0.13	3.22 ± 0.44	4.27 ± 0.77	5.05 ± 0.94
Liver	7.60 ± 0.23	3.15 ± 0.60	5.73 ± 1.13	5.60 ± 0.22

<sup>a</sup> Four mice from each group were infected subcutaneously in their footpads with 1.5 × 10<sup>3</sup> PFU of EV or p28<sup>-</sup> mutant and were sacrificed at 6 days p.i. for assays of virus infectivity in tissues.

shown by several workers and by several criteria that more than 90% of the mouse peritoneal cells adhering to tissue culture plates after an incubation of several hours with subsequent extensive washing are macrophages (13, 18). We confirmed the macrophage identity of the cells in our preparations by direct staining with macrophage-specific monoclonal rat antibodies Mac-1 and F4/80 (1) conjugated to fluorochrome. More than 95% of peritoneal cells in different preparations stained with Mac-1 and F4/80 but not with unrelated rat monoclonal antibodies, whereas BSC-1 cells and primary ovarian cells (a fibroblast-enriched population) from strain A mice showed no staining for macrophage-specific antigens (data not shown). The resident peritoneal macrophages as well as oil- and broth-elicited macrophages were obtained from strain A mice and were tested directly for their ability to support EV and p28<sup>-</sup> mutant replication. In all three types of macrophages, EV replicated more efficiently than the p28<sup>-</sup> mutant. Table 3 shows a typical result with resident peritoneal macrophages. No replication of mutant virus was detected in these cells, in contrast with a 10- to 20-fold increase in the titer of EV by 24 h p.i. The same result was observed with the other clonally independent p28<sup>-</sup> mutant, suggesting that this phenomenon is indeed due to the deletion in the p28 gene rather than another, unidentified mutation (data not shown). In oil- or thioglycolate broth-induced macrophages, the difference in titers between the mutant and wild-type viruses varied somewhat from experiment to experiment (data not shown). Since this could be explained by variation in the activation state of the macrophages, resident peritoneal macrophages were used throughout the subsequent study. We found previously that p28 was nonessential for EV replication in four different cell cultures (24). Since macrophages may be an important (but not the only) cell type in which a p28<sup>-</sup> mutant would not grow, we extended the studies of comparative reproduction of the EV and mutant to several additional cell cultures from strain A mice. Taken together, our data indicate that in the following cell lines and primary cultures, EV and p28<sup>-</sup> mutant replicated efficiently and to identical titers: BSC-1 (kidney cells from African green monkeys), primary mouse embryo

TABLE 5. In vitro multiplication of EV and the p28<sup>-</sup> mutant in peritoneal macrophages from resistant (B6) mice

Virus type	Log <sub>10</sub> of virus titers ± SD <sup>a</sup>	
	0 h	24 h
EV	3.41 ± 0.27	4.13 ± 0.04
p28 <sup>-</sup>	3.51 ± 0.22	4.05 ± 0.13

<sup>a</sup> The arithmetic mean values of four replica cultures infected with 5 PFU of each virus per cell are indicated.

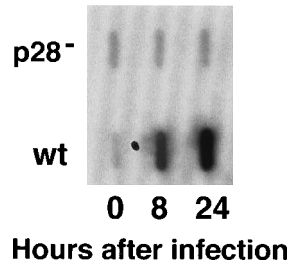


FIG. 1. Viral DNA synthesis in EV or p28<sup>-</sup> mutant-infected peritoneal macrophages. Total DNA was extracted from triplicate cultures at 0, 8, and 24 h after infection with EV or the p28<sup>-</sup> mutant, and viral DNA was detected by slot blot hybridization of total infected cell DNA with a <sup>32</sup>P-labeled EV DNA probe. A typical result of one of three experiments is presented. wt, wild type.

fibroblasts (mouse strains A and C57BC/6), primary mouse ovary cells (mouse strains A and C57BC/6), RAW 264.7 (macrophage-like cells from mice), Yac-1 cells (lymphoma cells from mouse strain A), Neuro-2a (neuroblastoma cells from

mouse strain A), and LA-4 (lung adenoma cells from mouse strain A).

Thus, we have so far found only one cell type, namely, peritoneal macrophages from strain A mice, which mimicked the inefficient replication of the p28<sup>-</sup> mutant in vivo.

**Experiments with mice resistant to mousepox confirm the correlation between virus replication in macrophages in vitro and in target organs in vivo.** The experiments described above show a clear correlation between the inability of the p28<sup>-</sup> mutant to replicate in peritoneal macrophages from strain A mice in vitro and in the target organs, namely, the spleen and especially the liver, which is rich in tissue-fixed macrophages (which are called Kupffer cells). In order to further explore this correlation, we compared the replication of the wild-type EV and the mutant in macrophages and organs from C57BL/6NCR (B6) mice that are genetically resistant to EV infection. Resistance to mousepox in B6 mice is best explained by an early immunological response to infection (3). Gamma interferon was also shown to be critical in recovery of B6 mice from mousepox (10). As anticipated, the titers of wild-type EV in

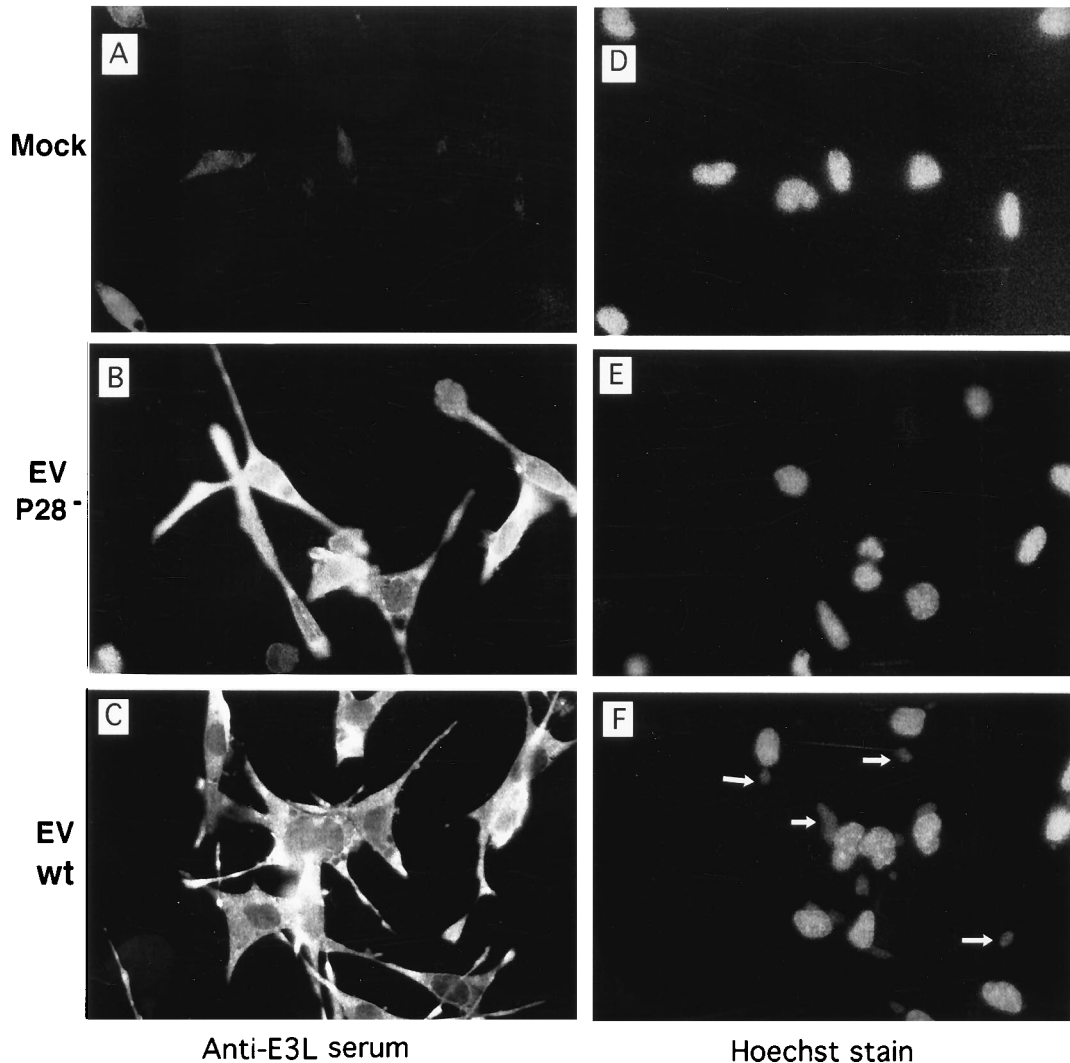


FIG. 2. Synthesis of an early protein (E3L) and formation of factories in EV- or p28<sup>-</sup> mutant-infected macrophages. Indirect immunofluorescence analysis of mock-infected (A), p28<sup>-</sup> mutant-infected (B), or EV-infected (C) macrophages with anti-E3L serum at 16 h after infection. The same samples stained with Hoechst dye are also shown (D, E, and F, respectively). White arrows indicate virus factories. wt, wild type. Magnification,  $\times 295$ .

the livers and spleens of resistant mice were 2 to 4 orders of magnitude lower than those in the respective organs of susceptible mice (Table 4); a difference in the efficiency of wild-type EV replication, even though it was less significant than that in vivo, was observed also in the peritoneal macrophages from susceptible and resistant mice (compare Tables 3 and 5). Unexpectedly, it was found that the p28<sup>-</sup> mutant replicated both in macrophages of the resistant mice and in vivo (Tables 4 and 5). Specifically, the titers of the mutant in the livers and spleens of B6 mice were 2 orders of magnitude higher than in those of the susceptible strain A mice (Table 4). Moreover, there was no measurable difference in the titers of the p28<sup>-</sup> mutant and the wild-type EV both in peritoneal macrophages and in the target organs of the resistant mice (Tables 4 and 5). Thus, the correlation between the replication of both the wild-type EV and the p28<sup>-</sup> mutant in macrophages in vitro and in target organs in vivo was also observed in these experiments, despite the difference in virus yields and the different genotypes of the host. On the other hand, and perhaps unexpectedly, the results indicate that the requirement of p28 for EV replication in macrophages and in target organs may depend on the host genotype.

**p28 is important for viral DNA replication but not for the synthesis of at least two early proteins in mouse peritoneal macrophages.** To determine the stage in the mutant virus replication cycle, which is blocked in mouse peritoneal macrophages, EV-specific DNA synthesis was monitored by slot blot hybridization of total DNA extracted from EV-infected and p28<sup>-</sup> mutant-infected peritoneal cells with an EV DNA probe. No virus-specific DNA synthesis was detected in p28<sup>-</sup> mutant-infected cells at 8 and 24 h after infection, in contrast with the marked accumulation of viral DNA in EV-infected cells (Fig. 1). Since the DNA<sup>-</sup> phenotype of the mutant may result from impairment at different stages of virus replication preceding DNA synthesis, early protein synthesis was monitored by using polyclonal antibody against the VV early RNA-binding protein E3L (28). Immunofluorescence analysis with this antibody showed good cross-reactivity with the homologous EV protein and revealed a comparable bright staining in mutant- and EV-infected macrophages, in contrast to uninfected cells (Fig. 2A through C).

In these experiments, cells were triple stained with anti-E3L antibody (Fig. 2A through C), Mac-1 or F4/80 antibody (not shown), and Hoechst dye (Fig. 2D through F). The Hoechst staining detects DNA in nuclei and specific poxvirus inclusion bodies, factories, or viroosomes which are the sites of viral DNA synthesis (17). No factories were found in most of the mutant-infected cells (Fig. 2E), in contrast to EV-infected cells (Fig. 2F). By an independent experimental approach, these findings confirmed that in mutant-infected macrophages, viral DNA synthesis was impaired. By using the triple staining, it was possible to demonstrate that one and the same cell from mutant-infected cell cultures (i) was stained with the macrophage-specific antibody (data not shown); (ii) was actually infected and synthesized at least one early virus-specific protein, as shown by the anti-E3L antibody staining (Fig. 2B); and (iii) in contrast to EV-infected cells, contained no factories (compare Fig. 2E with F). These results appeared to rule out the possibilities that the observed effects were due to a small subpopulation of cells other than macrophages, that the E3L gene was expressed in a subpopulation of the mutant-infected cells synthesizing DNA, or that the mutant virus-infected macrophages that had no factories were, in fact, not infected. In addition, using Western blotting with a polyclonal antibody against the early VV protein D12L (small subunit of the capping enzyme [19]), we showed that the EV homolog of this protein was

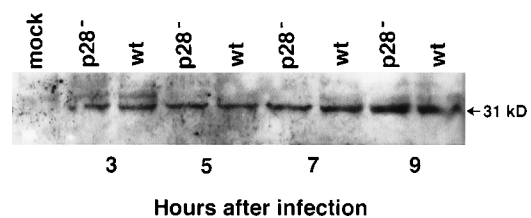


FIG. 3. Expression of the small subunit of the capping enzyme (D12L) in EV- or p28<sup>-</sup>-infected macrophages. Total cell proteins from EV-infected or p28<sup>-</sup>-infected macrophages at the indicated times after infection were resolved by SDS-PAGE, and the 31K subunit of the capping enzyme was detected by Western blotting analysis with antiserum against the D12L protein of VV. wt, wild type.

synthesized both in EV- and p28<sup>-</sup> mutant-infected macrophages (Fig. 3). Thus, the synthesis of at least two early, virus-specific proteins was not impaired in cells infected with the p28<sup>-</sup> mutant. These results indicated that the critical stage in EV replication in macrophages, for which p28 was essential, is either the DNA synthesis stage as such or a stage between the synthesis of early proteins and the onset of DNA synthesis.

**p28 is an early viral protein and is localized in virus factories.** In order to gain further insight into the function of p28, polyclonal antibodies were raised against p28 expressed in *E. coli* and were used for detection of this protein in EV-infected BSC-1 cells as well as peritoneal macrophage cells. Western blot analysis showed that the protein appeared as early as 2 h after infection of BSC-1 cells and continued to accumulate until 24 h (Fig. 4). In cells treated with cytosine arabinoside (araC), which blocks DNA synthesis, p28 accumulated with similar kinetics but in slightly smaller amounts (Fig. 4). The sequence of the putative promoter of the p28 gene was consistent with the consensus sequence of early and intermediate gene promoters of orthopoxviruses (24). Taken together, these observations indicate that p28 is an early viral protein and may also be expressed at later stages of infection. p28 was not detected in purified EV virions by Western blot analysis (data not shown).

Intracellular distribution of p28 was studied by immunofluorescence microscopy with the same polyclonal antibody against the whole protein. It was found that the protein partitioned exclusively to virus factories both in peritoneal macrophage cells, in which it was important for virus growth (Fig. 5), and BSC-1 cells, in which it was nonessential for virus repro-

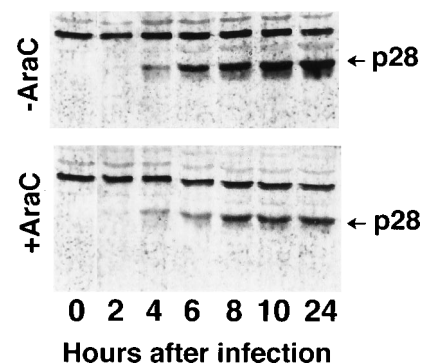


FIG. 4. Time course of p28 synthesis in EV-infected BSC-1 cells. Cell extracts at the indicated times after infection with EV in the presence or absence of araC were resolved by SDS-PAGE, and p28 was detected by Western blotting analysis with the anti-p28 antiserum. araC (40  $\mu$ g/ml) was added 2 h prior to infection.

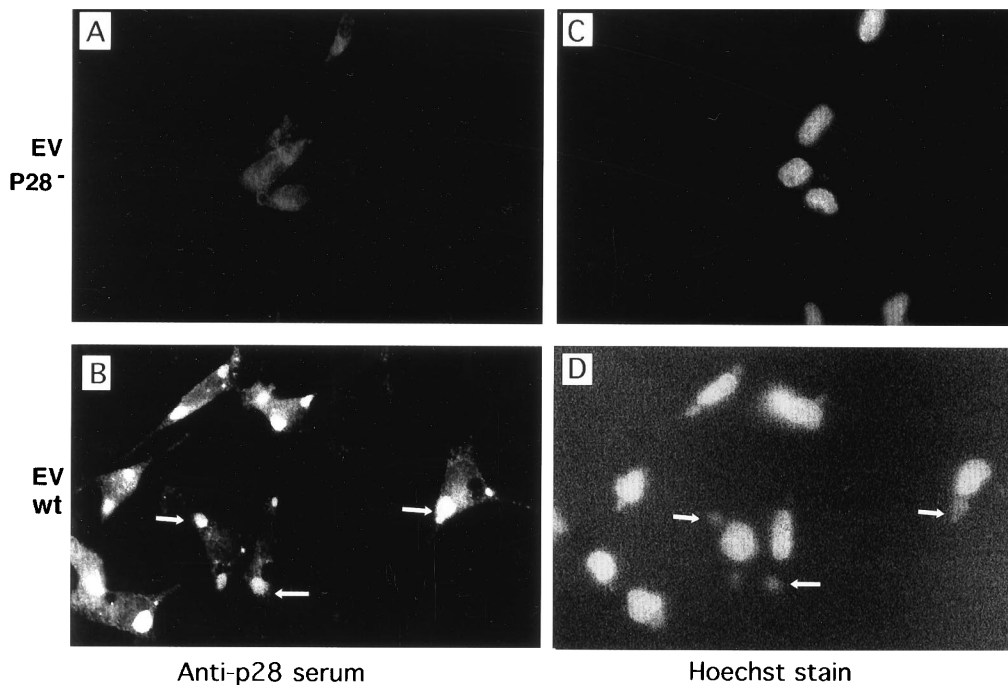


FIG. 5. Subcellular localization of p28 in EV-infected macrophages. Indirect immunofluorescence analysis of p28<sup>-</sup> mutant (A)- or EV (B)-infected macrophages with anti-p28 antiserum at 16 h after infection. The same samples stained with Hoechst dye are also shown (C and D, respectively). White arrows indicate virus factories. Magnification,  $\times 279$ . wt, wild type.

duction (Fig. 6). It should be noted that EV infection of BSC-1 cells produced syncytia and, accordingly, the appearance of factories in these cells was different (Fig. 6 and 7B) from that of macrophages infected with EV (Fig. 5) or that of BSC-1 cells infected with VV and transiently expressing p28 (see Fig. 9B). Figures 5 and 6 demonstrate the complete colocalization of immune staining for p28 with the Hoechst dye staining for virus factories in peritoneal mouse macrophages and BSC-1 cells, respectively. As shown in Fig. 2, the cells in Fig. 5 were triple stained; the third staining with Mac-1 was used to prove the macrophage origin of these cells (data not shown). No anti-p28 staining and no factories were found in mutant-infected macrophage cells (Fig. 5A and C). Preliminary results of cell fractionation also showed that p28 was tightly associated with virus factories and could be solubilized only after DNase treatment (data not shown).

As shown above, p28 was synthesized in the presence of araC, under conditions in which DNA synthesis and therefore the formation of the factories were completely prevented. Thus, it was interesting to study the localization of p28 in the presence of this inhibitor. In this case, p28 was detected as very bright dots in the BSC-1-infected cell cytoplasm (Fig. 7C), with the number of dots and the fluorescence intensity directly correlated with the multiplicity of infection (data not shown). Thus, in the absence of factories, p28 was not diffuse in the cytoplasm but rather accumulated in specific structures that probably were the precursors of factories containing the parental virus DNA. When araC was added later in infection, allowing some limited DNA synthesis to proceed, the foci containing p28 became larger (Fig. 7D), showing the complete colocalization of p28 with viral DNA at any stage of the development of the factories.

**The RING finger is not important for the association of p28 with virus factories.** In order to determine the role of the RING finger in the association of p28 with virus factories, we

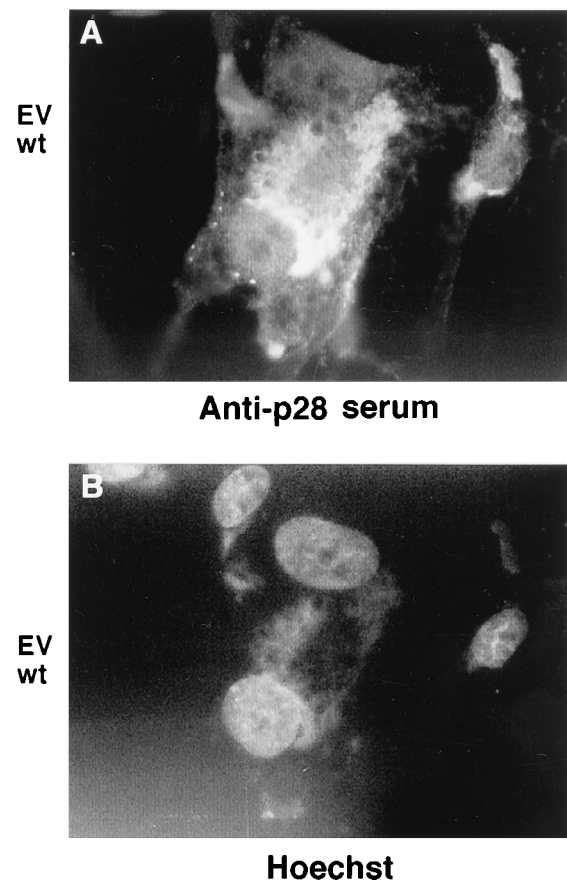


FIG. 6. Subcellular localization of p28 in EV-infected BSC-1 cells. (A) Indirect immunofluorescence analysis with anti-p28 antiserum at 16 h after infection; Hoechst dye staining of the same sample (B). Magnification,  $\times 729$ . wt, wild type.

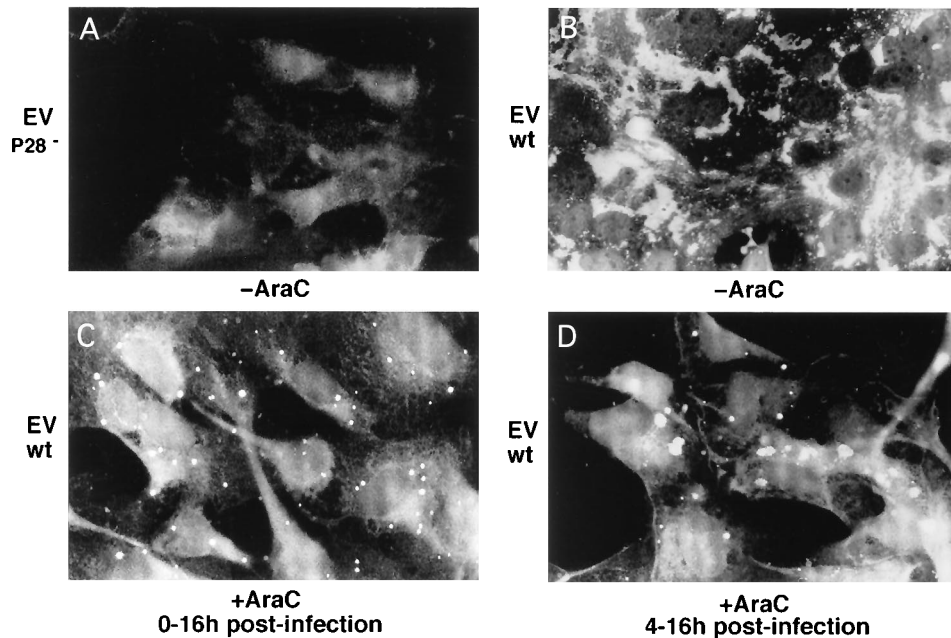


FIG. 7. Subcellular localization of p28 in EV-infected BSC-1 cells in the presence of AraC. Indirect immunofluorescence analysis of infected cells with anti-p28 antiserum at 16 h after infection is shown for p28<sup>-</sup> mutant-infected cells (A), EV-infected cells (B), EV-infected cells, with araC added before infection (C), and EV-infected cells, with araC added 4 hours after infection (D). wt, wild type.

constructed a set of plasmids that could express the complete p28 gene or its truncated versions in VV-infected cells (Fig. 8). When overexpressed in VV-infected cells under the control of a T7 promoter, p28 localized exclusively in virus factories, in a good correlation with its localization during EV infection (Fig. 9B). Elimination of the whole RING domain resulted in diffuse cytoplasmic localization of the truncated 18,000-molecular-weight (18K) protein (Fig. 9C); however, unexpectedly, removal of the three distal cysteines from the Zn finger domain

that is likely to disrupt the entire RING domain structure (Fig. 8A and B) had no influence on the intracellular localization of the predicted 24K protein (Fig. 9D). These results, together with the diffuse localization of the homologous 21.7K protein of the WR strain of VV that lacks the RING domain (27), suggest that an N-terminal portion of p28 containing more than 182 but fewer than 203 amino acid residues is sufficient for localization of the protein to the factories (Fig. 8B and C). Thus, the specific structure of the RING finger domain of p28 is not important for its association with the factories and probably has a different function.

## DISCUSSION

The p28 protein of EV and particularly its RING finger domain are highly conserved throughout the orthopoxvirus genus. The predicted sequences of the EV, cowpox virus (24), variola virus (12, 25), and VV strain IHD (27) proteins show greater than 90% identity, with about 97% identity in the RING domain. Interestingly, the RING finger protein gene is disrupted or completely deleted in the WR and Copenhagen strains of VV, respectively (24, 27). We also observed the truncation of this protein in one of the isolates of the IHD strain of VV (23a). Shope fibroma virus (genus leporipoxvirus) and the closely related myxoma virus also encoded similar RING finger proteins (Shope fibroma virus open reading frame N1R) that shared 29% identity with EV p28 (27). Thus, it seems possible that the RING finger protein is important for the replication of virulent poxviruses in vivo.

Our previous study showed that disruption of the p28 gene had no appreciable effect on the multiplication of EV in cell culture but abolished its lethality for the disease-susceptible strain A mice, as well as its ability to replicate in different organs of mice (24). Similarly low levels of EV p28<sup>-</sup> mutant infectivity in the organs of immunocompromised and normal animals observed in this study were consistent with a direct

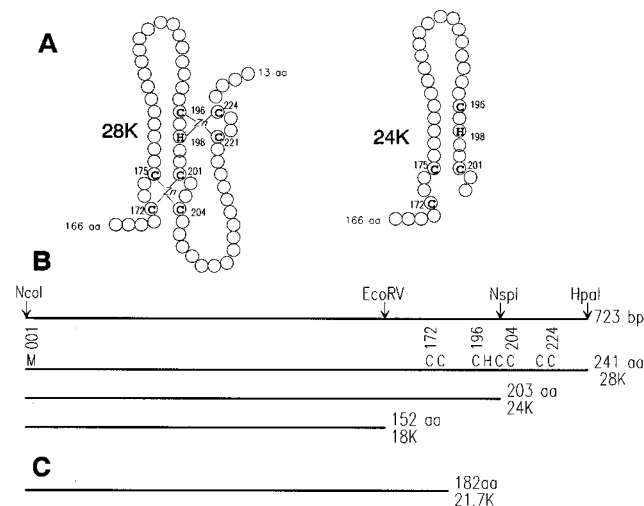


FIG. 8. The structure of p28 and its truncated derivatives. (A) Schematic representation of the structure of the C3HC4 RING finger domain in EV p28 and its disruption in the truncated 24K derivative, based on the analogy with the RING domain of the herpesvirus IPC0 protein (2); (B) restriction sites used to generate truncated derivatives of p28 and linear structures of the truncated polypeptides; (C) linear structure of the homologous protein of VV. aa, amino acid.

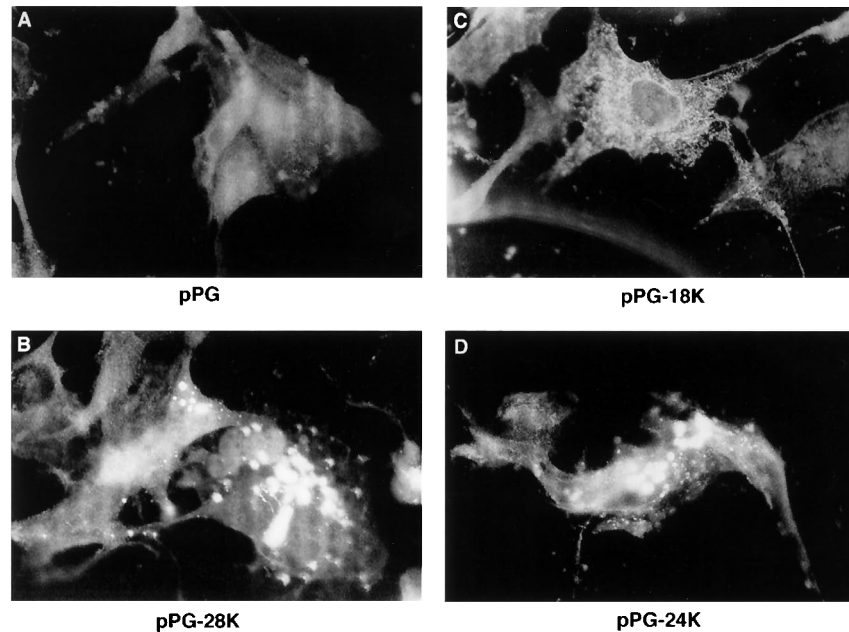


FIG. 9. Subcellular localization of p28 and its truncated derivatives transiently expressed in VV-infected BSC-1 cells. Indirect immunofluorescence analysis with anti-p28 antiserum of VV-infected cells transfected with the pPG plasmid without insert (A); pPG-28K expressing complete p28 (B); pPG-18K expressing a truncated protein, with the whole RING domain deleted (C); and pPG-24K (D) expressing a truncated protein, with the portion of the RING domain encompassing the three distal cysteine residues deleted (Fig. 8).

role for p28 in virus replication, rather than in evasion of the host immune response to infection.

Our results indicate that in at least one cell type, resident peritoneal macrophages, p28 is essential for virus replication *in vitro*. We showed here that in these cells, expression of p28 was required for EV DNA synthesis and the formation of the factories but not for early protein synthesis. The actual role of p28 in virus infection of peritoneal macrophages remains uncertain; however, the possibilities are now narrowed down to DNA replication *per se* or a stage between the expression of early proteins and the onset of DNA synthesis, e.g., the second uncoating step (17). p28 colocalized with the viral DNA throughout the course of infection, as shown by experiments with araC added at different times. Thus, we believe that p28 is likely to participate in viral DNA synthesis proper. It is tempting to speculate that p28 may substitute for an unidentified host factor involved in EV DNA replication that is not expressed in resident peritoneal macrophages from EV-susceptible strain A mice but may be present in macrophages from resistant B6 mice. Recently, participation of a host factor in VV transcription has been demonstrated; the host factor was active in certain cell lines, but in others, expression of the VV K1L gene product was required to activate it (22).

Poxvirus infection of murine peritoneal macrophages *in vitro* has been studied in several laboratories. While EV replicated in resident macrophages (5, 21), in accordance with our findings, the conclusions regarding the outcome of VV infection varied between different studies (see reference 18 and references therein). The latest and most detailed study has reported only abortive replication, even though expression of early proteins has been observed (18). Thus, in peritoneal macrophages, the observed properties of the p28<sup>-</sup> mutant of EV were similar to those of VV. It will be of interest to elucidate whether the same virus function is impaired in VV and the p28<sup>-</sup> mutant of EV and whether the inability of VV to

replicate in macrophages correlates with the absence of an intact p28 gene.

In contrast with the results described by Upton et al. (27), we were unable to observe significant staining of the cell nuclei with polyclonal rabbit antibody against complete p28. This may be due to a less sensitive immunofluorescence assay with our antibody compared with the anti-tag mouse monoclonal antibody used in the study by Upton et al. Another promoter and plasmid vector were used in our studies for transient expression of p28, perhaps resulting in a lower level of expression. In addition, it cannot be ruled out that the tag used by Upton and coworkers could affect the subcellular localization of the virus protein. Moreover, these workers studied the cell localization of the very similar but not identical protein of the IHD strain of VV and of the distantly related (29% identical amino acids) SFV protein.

The most important question is how the requirement of p28 for EV replication in mouse peritoneal macrophages contributes to its crucial role in the EV multiplication *in vivo* and therefore to its pathogenicity in mice. It is well documented that EV replicates in different cells of macrophage lineage *in vivo* and that its reproduction in Kupffer liver cells is the initial and crucial step for subsequent infection of hepatocytes (16). The efficiency of virus reproduction in these cells may determine the outcome of the infection, since severe damage of the liver is thought to be the main cause of death in mousepox (16, 20, 26). The virulent Hampstead mouse strain of EV appeared to replicate *in vivo* in Kupffer cells better than the avirulent egg strain (20); this correlates with the less-efficient growth of the avirulent virus in murine peritoneal macrophages *in vitro* (21). Our observation of the correlation between the p28<sup>-</sup> mutant virus reproduction in macrophages *in vitro* and in spleens and livers *in vivo*, which was revealed with both susceptible and resistant mouse strains, supports the same idea. Thus, it seems plausible that the highly attenuated phenotype of the EV p28<sup>-</sup>

mutant may be explained by a failure of the virus to replicate in macrophage lineage cells at all successive steps in the spread of the virus from skin to the target organ, the liver.

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