JOURNAL OF VIROLOGY, Mar. 1988, p. 866–874
0022-538X/88/030866-09$02.00/0

Vol. 62, No. 3

Deletion of the Vaccinia Virus Growth Factor Gene Reduces Virus Virulence

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Received 29 July 1987/Accepted 11 November 1987

The vaccinia virus growth factor (VGF) gene encodes a polypeptide with amino acid sequence homology to epidermal growth factor (EGF) and transforming growth factor alpha and is present twice, once at each end of the virus genome within the inverted terminal repetition. Recombination procedures were used to replace more than half of both VGF genes with a β-galactosidase cassette which served as a color indicator for isolating an unconditionally viable VGF$^-$ mutant. The VGF$^-$ mutant genotype and phenotype were confirmed by Southern blot analysis and assays for functional growth factor. The plaque-forming efficiencies of VGF$^-$ and wild-type (WT) viruses were similar in a variety of cell types containing low or high densities of EGF receptors, suggesting a lack of a specific requirement for either VGF or the EGF receptor in the initiation of virus infection. The yield of VGF$^-$ virus was similar to that of WT virus in growing BS-C-1 and Swiss 3T3 cells, but lower in resting Swiss 3T3 cells. The greatest differences between VGF$^-$ and WT virus occurred in vivo: higher doses of VGF$^-$ virus than WT virus were required for intracranial lethality in mice and for production of skin lesions in rabbits. Thus, expression of the VGF gene is important to the virulence of vaccinia virus.

The inverted terminal repetition (ITR) of the vaccinia virus genome contains at least three genes which are expressed early during infection (49, 50). A portion of the predicted amino acid sequence of one (47) has been shown by computer analysis to be closely related to epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) and more distantly related to proteins of the blood coagulation system (2, 3, 36). Analysis of vaccinia virus-infected culture supernatants led to the detection of an acid-stable polypeptide that competed with EGF for binding to EGF membrane receptors, induced phosphorylation of tyrosine residues in the EGF receptor, had mitogenic properties for tissue culture cells, did not cross-react with TGF-α in a radioimmunoassay, and exhibited minimal reactivity with certain antisera for native EGF (24, 45, 46). Since this EGF-like activity was present only in virus-infected cultures and was synthesized with the kinetics expected of an early virus-encoded gene product, it was termed vaccinia virus growth factor (VGF). A partial amino-terminal sequence analysis of the purified protein conclusively showed that it was indeed coded within the ITR (45). This partial amino-terminal sequence and the measured amino acid composition of the purified factor, taken together with the predicted amino acid sequence of the product of the open reading frame, suggested that this polypeptide was derived from a primary translation product by removal of putative signal and transmembrane domains, yielding a fully processed polypeptide of about 77 amino acids. Further studies suggested that the natural form of the gene product was glycosylated, which partially explained its apparent anomalous molecular weight of 22,000 to 26,000 in sodium dodecyl sulfate–polyacrylamide gels (45).

The importance of VGF to vaccinia virus replication in vitro and in vivo has not been investigated. We report here the construction of a vaccinia virus mutant which lacks a functional VGF gene. The VGF$^-$ mutant replicated less efficiently than did the wild-type (WT) virus in resting Swiss 3T3 cells and exhibited an attenuated phenotype following intracranial (i.c.) and intradermal inoculations into mice and rabbits, respectively.

MATERIALS AND METHODS

Cells and viruses. The vaccinia virus WR strain, originally obtained from the American Type Culture Collection Rockville, Md., was propagated in HeLa cells and purified as reported previously (22). CV-1 and Swiss 3T3 cells were grown in Dulbecco modified Eagle medium (DMEM; Quality Biologic Inc.) supplemented with 10% fetal bovine serum (FBS). A431 human epitheloid carcinoma cells, which have a high density of EGF receptors on the cell surface, were grown in DMEM supplemented with 5% FBS or 10% bovine serum (14). BS-C-1 cells were grown in minimum essential medium (MEM; GIBCO Laboratories) containing 10% FBS.

Enzymes and chemicals. Restriction enzymes were supplied by Bethesda Research Laboratories, Inc., Gaithersburg, Md., New England BioLabs, Inc., Beverly, Mass., or Boehringer Mannheim Biochemicals, Indianapolis, Ind., and used as specified by the manufacturers. The Klenow fragment of DNA polymerase I, T4 DNA ligase, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and isopropyl-β-D-thiogalactopyranoside (IPTG) were obtained from Boehringer Mannheim. The *Bgl*II linker was purchased from New England BioLabs, and EGF was obtained from Collaborative Research, Inc., Waltham, Mass.

Animals. Male BALB/cByJ and A/J mice between 6 and 10 weeks of age were obtained from Jackson Laboratory, Bar Harbor, Maine. Outbred black rabbits of both sexes were obtained from Spring Valley Laboratories. Guidelines in the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" were followed for animal husbandry.

Virus infectivity assays. Virus infectivity was estimated as

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described previously (7). Virus plaques were visualized after 2 days by adding 1 ml of 0.3% crystal violet stain solution containing 5% ethanol and 10% formaldehyde.

Virus infection of cells. Cultures of BS-C-1, A431, or Swiss 3T3 cells were infected for 1 h at 37°C with vSC20 or WT virus at the indicated multiplicity of infection (MOI) in 0.5 ml of MEM. Cultures were covered with 2.0 ml MEM or DMEM and the optimal concentration of FBS and harvested at the indicated times postinfection (p.i.).

Resting cultures were prepared by the method of Jamieson et al. (21). Briefly, confluent monolayers were washed once with prewarmed MEM and maintained in MEM–1% FBS (BS-C-1 cells) or in DMEM–5% FBS (Swiss 3T3 cells) for 5 to 7 days at 37°C. The conditioned medium was removed and the cultures were infected as above with virus in DMEM (no FBS). The monolayers were covered with the conditioned medium and harvested at the indicated times p.i. DNA synthesis was measured by [^{3}H]thymidine incorporation into material that bound to DE81 filter disks (Whatman Ltd., Maidstone, England) (28).

Restriction endonuclease and DNA hybridization analyses of virus genomic DNA. DNA was isolated from purified virus (16) and cleaved with restriction endonucleases. DNA fragments were resolved by electrophoresis in a 0.7% agarose gel, stained with ethidium bromide, and photographed by UV transillumination.

DNA fragments were transferred from the agarose gel bidirectionally to two nitrocellulose membranes by a modification of the procedure of Southern (44). Nick-translated recombinant DNAs were hybridized to the immobilized DNA as described previously (34, 37). Autoradiographs were made by placing the nitrocellulose sheets in contact with X-ray film for 1 to 4 days at −70°C.

Deletion of the VGF gene sequence responsible for EGF receptor binding. A 1.6-kilobase-pair *Bgl*II restriction endonuclease fragment containing the complete nucleotide sequence of the VGF gene was isolated from pVG3 (47). The Klenow polymerase repaired fragment was ligated into *Hin*cII-cleaved pUC13, and the mixture was used to transform competent *Escherichia coli* JM109 (Fig. 1). β-Gal$^-$ colonies (white versus β-Gal$^+$ blue) were isolated by using the X-Gal–IPTG screening system. A plasmid (pSC16) was obtained that contained the entire VGF gene with the direction of viral gene transcription from the plasmid *Hin*dIII site toward the *Bam*HI site. This plasmid was cleaved with *Acc*I and then subjected to bidirectional BAL 31 digestion and Klenow enzyme repair. The DNA was modified by the addition of *Bgl*II linkers, circularized with T4 DNA polynucleotide ligase, and used to transform competent HB101 bacteria. Minipreparations of plasmids from isolated colonies were characterized by digestion with *Bgl*II and *Hin*dIII, and a plasmid, pSC18, was identified in which the *Acc*I site had been destroyed by a deletion of approximately 250 base pairs and a *Bgl*II site had been inserted. This plasmid was treated sequentially with *Bgl*II and calf intestinal phosphatase and ligated to an appropriately modified *Xba*I-*Sma*I fragment from pSC10 (11), which contained the vaccinia virus 11K promoter coupled to the *E. coli lacZ* gene (β-galactosidase cassette). Competent JM109 cells were transformed, and blue colonies (β-Gal$^+$) were isolated. One colony yielded a plasmid (pSC20) which, on characterization with *Hin*dIII and *Cla*I restriction endonucleases, showed a pattern consistent with the β-galactosidase cassette's having been inserted into the disrupted coding region of the VGF gene and in the same transcriptional orientation.

Transfection of plasmid and isolation of recombinant virus. A 25-cm^2 flask of CV-1 cells infected with WT vaccinia virus (0.05 PFU per cell) was transfected with calcium phosphate-precipitated plasmid, and a recombinant virus (blue plaque) was isolated as described previously (11, 22).

β-galactosidase assay. Quantitation of β-galactosidase was performed as described previously (11). Enzyme activity was expressed as nanomoles of *o*-nitrophenol produced per 3×10^6 cells.

EGF receptor-binding assay. A431 cells (10^3 per well) were fixed prior to assay on 24-well plates (Linbro or Flow Laboratories, Inc., McLean, Va.) with 10% formaldehyde in phosphate-buffered saline. Competition assays measuring ^{125}I-EGF binding (1.2×10^{10} cpm/nmol saturated the binding assay at 3 nM) were performed at 10% of the saturation value. VGF activities were expressed as a percentage of EGF competition (46).

Measurement of tyrosine phosphorylation of EGF receptors. Cultures of A431 cells which were 50% confluent on 35-mm plastic dishes were prelabeled with $^{32}P_i$ and infected with vaccinia virus as previously described. EGF receptors were isolated by precipitation with antibodies followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The phosphoamino acid content of the EGF receptor was determined by partial acid hydrolysis and two-dimensional thin-layer electrophoresis at pH 1.9 (24).

Histopathology. Tissues were fixed in either Bouin or Tellyesnizky fixative, paraffin embedded, sectioned at 5 μm, stained with hematoxylin and eosin, and viewed by light microscopy.

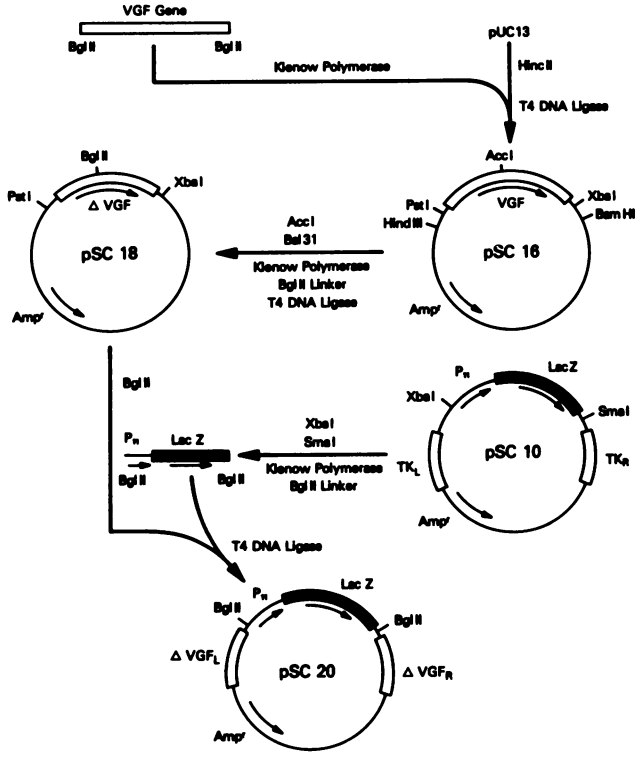

FIG. 1. Deletion of the DNA sequence coding for the EGF receptor-binding site of the vaccinia virus VGF gene. The EGF receptor-binding site of the VGF gene cloned in a pUC13 plasmid was deleted by BAL 31 enzyme digestion from a unique *Acc*I site and replaced by the *E. coli lacZ* gene controlled by a vaccinia virus late 11K promoter (see Materials and Methods).

RESULTS

Construction of a VGF$^-$ mutant of vaccinia virus. The procedures outlined in this section resulted in the replacement of a large segment of each copy of the VGF gene with a β-galactosidase expression cassette. The VGF gene has a single *Acc*I site approximately 258 nucleotides downstream from the translational start codon. This restriction endonuclease site lies within, and toward the 3′ side of, the 150-base-pair DNA sequence which has amino acid homology with the EGF–TGF-α domains presumably responsible for binding to the EGF receptor. This region of the VGF gene was deleted and replaced by a β-galactosidase cassette, composed of the *E. coli lacZ* gene coupled to an 11K late vaccinia virus promoter. This plasmid was named pSC20 and contained the β-galactosidase cassette flanked by the remaining left and right remnants of the VGF gene designated as ΔVGF_L and ΔVGF_R (Fig. 1; see Materials and Methods).

The mutated VGF gene of pSC20 was introduced into infected cells by the calcium phosphate precipitation method and recombined into the virus genome. Recombinant progeny viruses were detected as blue plaques which resulted from the action of the β-galactosidase enzyme on an indicator substrate, X-Gal (11). Because the VGF gene is present in the vaccinia virus WR genome in two copies, an isolated recombinant virus expressing β-galactosidase activity was likely to have only one of the VGF genes inactivated; however, the work of McFadden and Dales (29) predicted that a change in DNA sequence within the ITR of one terminus had a probability of being transferred to the other terminus during subsequent rounds of DNA replication. Thus, from a single blue plaque, a mixture of blue (one or two mutated VGF genes) and white (two WT VGF genes) plaques could be derived during the subsequent infection cycles. By selecting only blue plaques during additional passages, an isolate in which both ITRs contained a mutated VGF gene should be obtained, provided that such a virus is viable. Experimentally, by the fourth round of plaque purification all of the plaques observed were blue, suggesting that the parent virus (picked in the third round) had the β-galactosidase cassette in both ITRs.

Southern blot analysis of the vSC20 genome. Characterization of the vSC20 genome with four different restriction endonucleases confirmed that the *lacZ* gene had been inserted into the VGF locus in both ITRs (Fig. 2). In each case, DNA fragments from mutant and WT virus were separated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to ^{32}P-labeled VGF and *lacZ* probes. Since *Sal*I cuts within the ITR on one side of the VGF gene and in unique sequences on the other, different-sized fragments were obtained from each end of the genome. The increase in size of both fragments and their hybridization to both probes indicated that both VGF genes were mutated. A similar result was obtained with *Hin*dIII. The faster minor *Hin*dIII bands were presumably a result of variations in the number of tandem repeats at the ends of the genome (35). Because the *lacZ* gene is flanked by two *Bam*HI sites, *Bam*HI digestion of vSC20 genomic DNA resulted in four fragments that hybridized to the VGF probe: the two larger are unique fragments, one from each end of the genome; the third fragment includes the *lacZ* gene (which also hybridizes to the *lacZ* probe); and the fourth double-molar fragment is composed of the 11K promoter fused to ΔVGF_L sequences from within the ITR. It should be noted that the two larger *Bam*HI fragments from vSC20 DNA are smaller than the corresponding WT fragments by approximately the amount

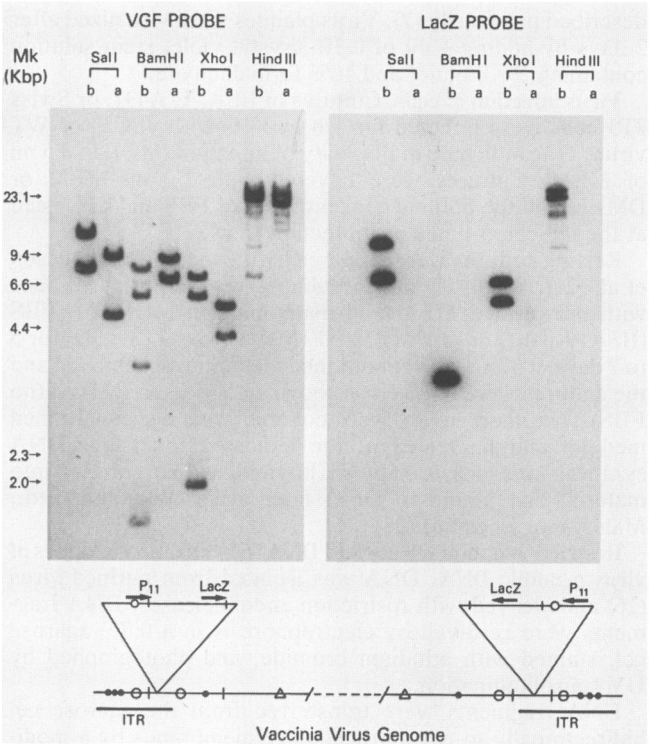

FIG. 2. Structure of the VGF gene in mutant vSC20. Wild-type (lanes a) and vSC20 (lanes b) DNA was digested with the indicated restriction endonucleases, and the reaction products were separated in a 0.7% agarose gel, bidirectionally transferred onto nitrocellulose paper, and hybridized with either ^{32}P-labeled VGF or *lacZ* DNA. A restriction endonuclease map of the terminal regions of the vaccinia virus genome is shown at the bottom. Symbols: △, *Hin*dIII; |, *Bam*HI; ○, *Xho*I; ●, *Sal*I.

of the deletion in the VGF gene. Similarly, *Xho*I digestion of vSC20 DNA produced two unique terminal fragments and a third double band, which was a fusion between ΔVGF_L sequences and the 11K promoter that hybridized to the VGF probe. Only the two larger fragments detected by the VGF probe were also reactive with the *lacZ* probe. This analysis demonstrated that both VGF genes of vSC20 had been mutated, and therefore a protein capable of binding to EGF receptors should not be made.

Absence of EGF-like activity in vSC20 virus infections. Since VGF produced in a WT infection is released from cells (45, 46), culture supernatants from WT or vSC20 virus infections were analyzed in EGF receptor-binding assays. Direct analysis of acidified culture supernatants (Fig. 3) and further parallel purification of acid–ethanol-solubilized peptides from WT- and VGF$^-$-infected BS-C-1 culture supernatants (250 ml) demonstrated the absence in VGF$^-$ infections of a polypeptide with the biochemical and functional characteristics of VGF. These results were not explained by the failure of the VGF$^-$ mutant to infect the culture, since cell-associated β-galactosidase activity was detected at the levels, and with the kinetics, previously reported for another β-galactosidase-producing recombinant vaccinia virus (11).

A second analysis made use of the observation that vaccinia virus infection specifically stimulated a kinase activity, resulting in tyrosine phosphorylation of the EGF receptor (24). A431 cells, which have a high density of EGF receptors on the cell surface, were infected with WT and vSC20 viruses. The EGF receptors were isolated and sub-

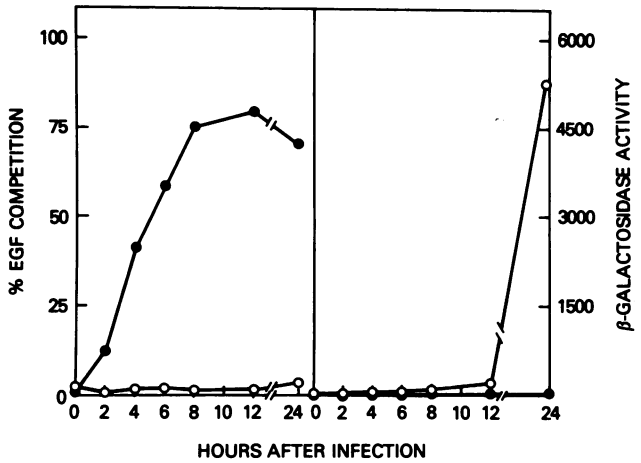

FIG. 3. EGF receptor-binding activity in supernatants from vSC20 and WT virus-infected cultures. BS-C-1 monolayers were infected at a MOI of 15 PFU per cell with WT (●) or vSC20 (○) virus and maintained in MEM–0.2% FBS until harvested. At the indicated time points, serial dilutions of acidified medium were analyzed in an EGF-binding competition assay, and the cell lysate was tested for β-galactosidase activity (see Materials and Methods).

sequently analyzed for phosphotyrosine content (Fig. 4). In contrast to the WT infection, vSC20 did not induce specific tyrosine phosphorylation of EGF receptors, indicating that no ligands had bound to the EGF receptor during the infection. Both experiments indicated that a vSC20 infection did not produce any EGF receptor-binding activity. Consequently, all of the EGF receptor-binding activity detected in WT virus-infected culture supernatants must result from expression of the two VGF genes located in the ITR.

Effect of the VGF mutation on initiation of infection in vitro. The function of VGF in vaccinia virus replication could be explained in at least two ways: (i) a membrane form of the VGF molecule is a component of the virion and mediates binding of the virus to cells via the EGF receptor (3, 13), and/or (ii) VGF stimulates cellular metabolism, resulting in a larger number of metabolically active cells capable of supporting efficient replication and spread of the virus (3). Experiments were carried out to differentiate between these two possibilities.

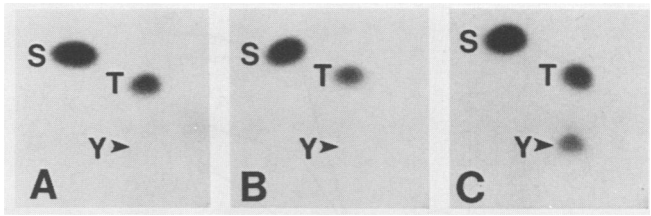

FIG. 4. Phosphotyrosine content of EGF receptors after infection with WT and vSC20 viruses. $^{32}P_i$-labeled A431 cells were infected at 37°C with an inoculum containing no virus (A) or 20 PFU of vSC20 (B) or WT virus (C). EGF receptors were then isolated after 4 h of incubation by immunoprecipitation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) content was estimated.

If virus entry into cells is mediated in large part by the binding of virus-associated VGF to the EGF receptor, the VGF$^-$ mutant should have altered plaque-forming efficiencies compared with WT virus. Moreover, the relative plaque-forming efficiency of VGF$^-$ and WT virus could be expected to depend on the EGF receptor density of the host cell. With active BS-C-1 cells which exhibit low to moderate EGF binding (20), VGF$^-$ and WT virus preparations gave particle-to-PFU ratios of 26 and 16, respectively, indicating that both viruses infected the host cell with a similar efficiency. The small difference which was noted was within the range of experimental error; however, a consistent finding was the 10% smaller plaque diameter of the VGF$^-$ mutant compared with WT virus. Virus plaque-forming efficiency in additional cell lines was then examined under conditions of high cell density and active or resting cellular metabolism. In no case, whether cells had high or low densities of EGF receptors, did VGF$^-$ virus have a dramatically lower relative plaque-forming efficiency than WT virus (Table 1). Indeed, the VGF$^-$ virus appeared to form plaques slightly better than WT virus in resting BS-C-1 and active A431 cells. Furthermore, when A431 cells were treated with anti-EGF receptor monoclonal antibody 528 (23, 41), both WT and VGF$^-$ virus plaque formation was significantly reduced from that of the controls (Student's t test, $P = 0.004$), but to a similar degree (the percentage of control plaques detected in the presence of monoclonal antibody 528 was 0.74 and 0.54

TABLE 1. Relative plaque-forming efficiency of WT and VGF$^-$ viruses

Cell line	Cell metabolic state	Relative plaque-forming efficiency[a,b]		EGF binding
		WT	VGF$^-$	
BS-C-1	Active	1	1	Low to moderate[c]
BS-C-1	Resting	4.7[d]	6.9[d]	Low[c]
Swiss 3T3	Active	0.024	0.014	No data
Swiss 3T3	Resting	0.011[e]	0.009[e]	Moderate[f]
A431	Active	1.8[d]	2.7[d]	High[g]

[a] For each cell line, six 0.5-ml aliquots of a dilute suspension of WT or VGF$^-$ virus were plated on active (90 to 100% confluent) or resting (see Materials and Methods) monolayers and incubated for 48 h. Monolayers were stained, and plaques were counted. For each cell line and virus, the geometric mean of individual plaque values was divided by the geometric mean of the values obtained with active BS-C-1 cells. The WT and VGF$^-$ virus ratios for each cell line were tested for equality.

[b] Particle/PFU ratio calculations involved the use of the following factor derived from reference 22: 1 optical density unit at 260 nm = 1.2×10^{10} particles.

[c] From Holley et al. (20), 19.3 and 4.5 fmol of EGF for low-density (active) and high-density cell monolayers, respectively.

[d] Relative WT plaque-forming efficiency was significantly different (Student's t test) from that of VGF$^-$ virus in A431 active cells ($P < 0.001$, 20 df) and resting BS-C-1 cells ($P = 0.01$; 20 df), but not in active Swiss 3T3 cells ($P > 0.2$, 10 df).

[e] Based on a single analysis.

[f] From Brown et al. (4) and Collins and Rozengurt (12), 82.7 and 70 fmol of EGF, respectively.

[g] From Haigler et al. (19), 4,000 fmol of EGF; from Gill and Lazar (17), 500 to 5,700 fmol of EGF for low- and high-density culture conditions, respectively.

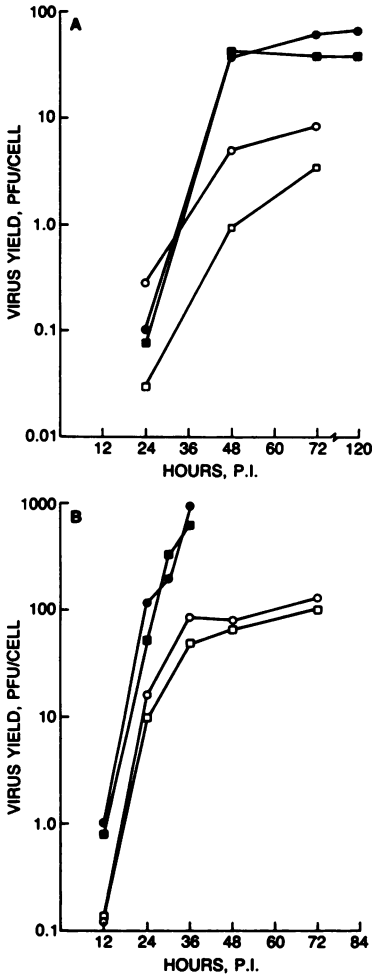

FIG. 5. WT and VGF$^-$ virus yield per cell in low MOI of Swiss 3T3 and BS-C-1 cell lines. Cultures of resting (open symbols) and growing (solid symbols) Swiss 3T3 cells (A) and BS-C-1 cells (B) were infected with a MOI of 0.005 to 0.01 BS-C-1 PFU of WT (○, ●) and VGF$^-$ (□, ■) viruses and harvested at the indicated times p.i. Virus infectivity was assayed and expressed as progeny virus yield per cell. Control experiments indicated that uninfected resting BS-C-1 and Swiss 3T3 cells showed 6 and 14% of the [^{3}H]thymidine incorporation into DNA of uninfected growing cells, respectively. The addition to resting cells of EGF to a final concentration of 10 ng/ml increased the [^{3}H]thymidine incorporation by 1.3- and 4-fold for BS-C-1 and Swiss 3T3 cells, respectively.

for WT and VGF$^-$ virus, respectively; $P = 0.11$). These results were consistent with the lack of a specific requirement for either VGF or the EGF receptor in the initiation of virus infection in tested cell lines.

Effect of VGF mutation on virus yield. Although some effects of EGF and, by analogy, VGF occur within minutes of binding to the EGF receptor (8–10, 18), the initiation of other EGF-induced processes such as DNA and protein synthesis require hours. In cells synchronously infected with a high MOI of virus, similar biological effects of VGF could be blocked by the virus-induced inhibition of certain host cell macromolecular processes (32). A low MOI of virus, however, would allow secreted VGF produced during the first infection cycle to act on adjacent cells prior to subsequent rounds of virus infection. Since studies with ectromelia virus (closely related to vaccinia virus) have shown that in nature, infection of the mouse occurs through the epidermal layer of the skin, of which a proportion of the cells are resting, the ability of WT and VGF$^-$ viruses to replicate in two cell lines sensitive to contact inhibition was evaluated (15, 38). Swiss 3T3 cells are known to be highly responsive to the exogenous addition of EGF (39, 40), whereas BS-C-1 cells are relatively refractile (20). These cells were inoculated with low MOI of virus, and the per-cell progeny virus yield was assayed at different times p.i. (Fig. 5). This experiment demonstrated a number of points. (i) Using a paired-sample Student t test and all time points in the growth curve, we found that the per-cell progeny virus yields from WT and VGF$^-$ virus infections of growing BS-C-1 cells, on average, were not significantly different ($P > 0.1$; 8 df), whereas with growing Swiss 3T3 cells, yields of VGF$^-$ virus were slightly less than that of WT virus ($P < 0.001$; 38 df). (ii) Both WT and VGF$^-$ viruses yielded less progeny virus per cell in resting than in growing cells. (iii) The yield of VGF$^-$ virus was marginally lower than that of WT virus ($P < 0.05$, 5 df) in resting BS-C-1 cells, but significantly lower in resting Swiss 3T3 cells ($P < 0.001$, 12 df). This difference noted in Swiss 3T3 cells was not due to an altered rate of infection of the VGF$^-$ mutant, since both WT and VGF$^-$ viruses formed plaques with similar efficiencies on Swiss 3T3 cells, although these efficiencies were 50- to 100-fold lower than on BS-C-1 cells (Table 1). (iv) The magnitude of the difference in per-cell yields between WT and VGF$^-$ viruses was greatest in the resting-cell line that was most responsive to EGF and presumably VGF (Fig. 5 legend; Table 1, column 5). These experiments indicated that VGF was important to varying degrees for the replication of virus in resting 3T3 and BS-C-1 cells and growing 3T3 cells. The next series of experiments were carried out to determine whether VGF was important in virus pathogenesis.

Effect of the VGF mutation on virus replication in vivo. We have previously shown that infection of inbred mice via i.c. inoculation provides a relatively sensitive method for analyzing the effect of mutations on vaccinia virus replication in vivo (6). BALB/cByJ male mice were inoculated with serial dilutions of WT and VGF$^-$ viruses, and both the level of virus infectivity in the brain and the average lethal dose for 50% of the population (LD_{50}) was measured. The VGF$^-$

TABLE 2. Replication of VGF$^-$ and WT viruses in brains of BALB/cByJ mice

Virus	Dose (PFU)[a]	Virus yield (PFU/brain)	LD_{50}
WT	3.0×10^3	$9.6 \times 10^6 \times^{\div} 1.5$	$1.2 \times 10^1 \times^{\div} 2.5$
WT	3.0×10^4	$3.1 \times 10^7 \times^{\div} 1.6$	
VGF$^-$	1.5×10^3	$5.5 \times 10^4 \times^{\div} 1.3$[b]	$2.5 \times 10^4 \times^{\div} 2.5$[b]
VGF$^-$	1.5×10^4	$5.7 \times 10^5 \times^{\div} 1.3$[b]	

[a] Serial 10-fold dilutions of virus (30 μl) purified from cells in 1 mM Tris hydrochloride (pH 9.0) were injected with a tuberculin syringe and 26-gauge needle into the right cerebral hemispheres of six anesthetized 6-week-old BALB/cByJ male mice. The Spearman-Karber method was used to calculate the LD_{50} (30). Only mice dying between days 2 and 14 after inoculation were used in the calculations. Additional mice were sacrificed at 5 days p.i. to quantitate virus infectivity and for histologic examination. Data are expressed as geometric means and relative standard errors.

[b] Significantly different from animals inoculated with WT virus ($P < 0.05$, Student's t test).

mutant replicated to lower levels of infectivity in the brain and had a greater LD_{50} than did the WT virus (Table 2).

Direct examination of brain sections from both WT and VGF^- virus-infected mice sacrificed 5 days following i.c. inoculation yielded a similar histologic picture, which has been described previously by Mims (31) and Simon and Werner (42). Briefly, animals infected with either virus developed an acute choriomeningitis, ependymitis, and vasculitis associated with disseminated inflammatory cell infiltrates and focal degeneration or lysis. The development of lesions was similar in both WT and VGF^- infections. This attenuated character of the VGF^- virus in mice was also observed by the footpad route of inoculation, in which both virus infectivity levels in the foot and anti-vaccinia virus neutralizing responses were lower in VGF^- than comparable WT infections (unpublished results).

To determine whether this attenuated phenotype of the VGF^- mutant was specific to infections of the mouse or could be generalized to other in vivo infections, we inoculated outbred black rabbits with approximately matching doses of VGF^- and WT viruses along the left and right sides of the dorsal midline. In this and additional experiments, between 10- and 100-fold more VGF^- than WT virus was required to achieve a visible lesion over the course of the infection (Fig. 6). At all doses of virus the severity of the lesion, as measured by ulceration of the epidermis, edema, and inflammatory cell infiltration of the dermis, was always greatest with the WT virus (Table 3). A further difference between a WT and VGF^- virus infection was the degree of proliferation (hyperplasia) of the epidermal cells at the margin of the lesion (Table 3, column 4).

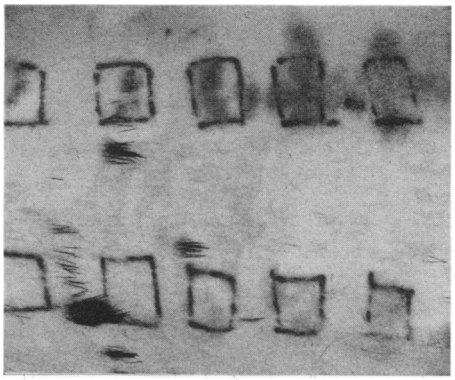

FIG. 6. Intradermal inoculation of rabbit skin with WT and vSC20 viruses. The back of a black outbred rabbit was shaved, and increasing doses (Table 3) of VGF^- and WT viruses were inoculated intradermally with a bifurcated needle onto the left (bottom row) and right (top row) sides of the dorsal midline. At 5 days p.i., the lesions were photographed. Lesions resulting from the largest doses of virus are located at the right-hand side of the photograph.

DISCUSSION

A VGF^- virus was constructed by removing approximately half of both virus VGF genes and replacing them with a cassette expressing a color selection marker, β-galactosidase. It was shown that this mutant no longer produced a factor(s) which competes with EGF for binding to the EGF receptor, stimulates the anchorage-independent growth of appropriate rodent cells in the presence of TGF-β (D.

TABLE 3. Intradermal inoculation of rabbit skin with WT and VGF^- viruses

Virus and dose[a] (PFU)	Gross lesion severity[b]	Histopathological examination of lesions			
		Epidermis			Infiltration into dermis[f]
		No. of cells thick[c]	No. of mitotic figures[d]	Severity of ulcer[e]	
WT					
0.5×10^0	0	4	1	0	+1
0.5×10^1	0	5	1	+1	+1
0.5×10^2	+1	8	2	+3	+2
0.5×10^3	+2	8	1	+1	+2
0.5×10^4	+3	9	1	+3	+3
0.5×10^5	+4	8	1	+3	+3
0.5×10^6	+4	–[g]	–	+4	+3
VGF^-					
1.1×10^0	0	2	1	0	0
1.1×10^1	0	2	2	0	0
1.1×10^2	0	2	0	0	0
1.1×10^3	0	2	0	0	+1
1.1×10^4	+1	3	–	0	0
1.1×10^5	+1	2	0	0	0
1.1×10^6	+2	4	1	+1	+2

[a] A black outbred rabbit was inoculated as described in the legend to Fig. 6. The animal was sacrificed on day 4 and observed for gross pathology. Tissue samples were excised, fixed, and processed for light microscopy.

[b] The relative severity of the lesion was scored on a scale from 0 to +4, with 0 being no detectable lesion and +4 being severe ulceration with edema.

[c] The thickness of several areas of epidermis undergoing proliferation were estimated and measured by counting the number of cells of epidermal origin between the surface of the epidermis and the boundary with the dermis. The control epidermis was 2 cells thick.

[d] The number of mitotic figures was counted in a ×400-magnified field of an area showing proliferation of epidermal cells.

[e] The relative depth of the erosion of the epidermis into the dermis is shown on a scale from 0 to +4, with 0 being no detectable erosion and +4 being complete destruction of the integrity of the epidermal layer.

[f] The degree of infiltration of inflammatory cells into the dermis was scored on a scale of 0 to +4, with 0 being no infiltration, +1 being limited perivascular cuffing, and +4 being extensive infiltration of macrophages and neutrophils which formed a compact mass of cells beneath lesions.

[g] –, No observation possible.

Twardzik, unpublished results), or triggers phosphorylation of tyrosine residues in the EGF receptor. From these observations, it can be inferred that no other region of the vaccinia virus genome codes for a protein capable of specific binding to EGF receptors. The insertion of the β-galactosidase cassette into the virus genome of the VGF$^-$ mutant did not affect virus replication under optimal cell growth conditions in BS-C-1 cells. In addition, VGF$^-$ virus replicated as efficiently as WT virus in HeLa cell cultures used to produce the purified seed stocks for all of the experiments (N. Cooper, unpublished results). Thus, the VGF gene (like other DNA sequences from the terminal regions of the genome [5, 6]) was not essential for replication of virus in vitro.

To rule out absolutely any effect of the β-galactosidase cassette on the phenotype of the vSC20 mutant, construction of a derivative of vSC20, which maintained the VGF-deleted genes but lacked the β-galactosidase cassette, was attempted several times. The procedure consisted of transfecting cells infected with vSC20 with a plasmid that contained the deletion within the VGF gene but lacked the β-galactosidase insert. Since there are two copies of the β-galactosidase gene, only double recombinants would appear as white plaques. Because the frequency of obtaining a single recombinant is 10^{-3} and the frequency of isolating a double recombinant is on the order of 10^{-6}, it was not surprising that no white plaques were observed, even under conditions which routinely yielded white-plaque virus from virus recombinants which contained only one copy of the β-galactosidase cassette. Nonetheless, it is unlikely that the vSC20 mutant attenuated phenotype in animals was a direct result of the β-galactosidase cassette, since a vaccinia virus recombinant which contained the β-galactosidase cassette in the *Eco*RI C fragment of the terminal *Hin*dIII C fragment was shown not to differ pathogenically from WT virus by the i.c. route in the mouse and the intradermal route in the rabbit (C. Flexner, personal communication).

During vaccinia virus infection, VGF could function as a ligand for binding virions to the EGF receptor of host cells or as a growth-stimulating factor or both. For the following reasons, it is not likely that VGF mediates virus binding to, and subsequent entry into, cells. (i) With the EGF competition binding assay, no VGF was detected on purified vaccinia virus virions (Twardzik, unpublished results). (ii) Plaque formation studies indicated that the efficiency of initiation of infection for either WT or the VGF$^-$ mutant did not correlate with the reported abundance of EGF receptors on the host cell. (iii) The anti-EGF receptor monoclonal antibody 528 reduced only slightly and to the same extent the plaque formation efficiency of both WT and VGF$^-$ viruses, even though it blocks EGF (41) and VGF (24) binding. (iv) Stroobant et al. (45) observed that NR-6 cells which lacked EGF receptors produced WT virus yields similar to those observed in a receptor-positive L929 cell line. Although Eppstein et al. (13) argue that the EGF receptor is involved in virus entry, they could obtain only a modest reduction of WT vaccinia virus plaque formation through prior blocking of EGF receptors with synthetic peptides.

Since purified VGF has been shown to stimulate [^{3}H] thymidine incorporation into resting diploid human fibroblasts (46) and Swiss 3T3 cells (45), the effect of the VGF mutation on virus replication in resting cells was investigated. After a low-multiplicity infection, the yield of VGF$^-$ virus was substantially lower than that of WT virus in resting Swiss 3T3 cells. A similar result was obtained in resting BS-C-1 cells, although the difference between VGF$^-$ and WT yields was smaller. These results correlated with the greater responsiveness to EGF of Swiss 3T3 cells than of BS-C-1 cells. The effect of VGF on virus replication may be mediated through a generalized stimulation of the cellular metabolism, which permits more efficient production of progeny virus.

Although the VGF gene is not essential for replication in vitro, it evidently has an important role in vivo. In mice, the i.c. LD_{50} of VGF$^-$ virus is higher than that of WT virus. In addition, smaller amounts of VGF$^-$ progeny virus than of WT progeny virus are recovered from brain tissue; however, by histologic examination, the development of lesions was similar upon infection with either virus. We found, as have others (31, 42), that orthopoxvirus lesions in the brain lack focal hyperplasia, making it difficult to explain the higher virulence of the WT virus by growth-promoting activity of VGF. Nevertheless, EGF receptors have been detected on astroyctes and, to a limited degree, on small neurons during primary cultures (1, 27). These EGF receptors do appear to be functional, since EGF has a mitogenic effect in dispersed cell cultures of both primary astrocytes (26, 43) and a glial cell line (48). It is likely, then, that EGF brain receptors are important for WT virus virulence when the virus is administered by the i.c. route. The contribution of VGF to vaccinia virus virulence was not confined to the i.c. route of inoculation, since similar findings were observed when the footpad route was used.

By a number of criteria, intradermal inoculations of the rabbit with WT virus resulted in lesions which were more severe than those caused by the VGF$^-$ mutant. It was further observed that at almost all doses of virus used, the WT virus appeared to induce localized cellular hyperplasia at the advancing margins of the lesion, whereas the mutant did not (Table 3, column 4). This cellular hyperplasia could have resulted from the direct action of VGF synthesized in infected cells or, alternatively, from the host-encoded growth factors released from infected cells or as part of the wound-healing process. To differentiate between these two explanations, one would have to isolate virus lesions very early in the infection process, before extensive necrosis occurred, and this is technically very difficult to accomplish in the two animal models used in our work.

Analysis of virulence genes such as VGF has taken on renewed importance with the potential application of vaccinia virus as a universal vaccine vehicle (33). For the safe use of these recombinant vaccinia virus vaccines, the virus virulence may need attentuation to reduce the risks of postvaccinial complications (25). The VGF$^-$ mutation may be a desirable attenuation marker for inclusion in the vaccinia virus recombinant vaccine strains.

ACKNOWLEDGMENTS

We thank Cornelio Duarte, Connie King, and Jane Ranchalis for technical assistance; D. Alling for statistical assistance; Carol McLeod for her gift of the anti-EGF receptor monoclonal antibody 528; Norman Cooper for growing and purifying the virus stocks; T. Fredrickson for histologic interpretation of sections; and Brenda Marshall for accurate and efficient manuscript production.

J. A. Cooper thanks the American Cancer Society and the National Institutes of Health (Public Health Service grant CA 28151) for support.

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