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Neomycin Resistance as a Dominant Selectable Marker for Selection and Isolation of Vaccinia Virus Recombinants†

CHRISTINE A. FRANKE,[1] CHARLES M. RICE,[2] JAMES H. STRAUSS,[2] AND DENNIS E. HRUBY[1]*

Center for Gene Research, Department of Microbiology, Oregon State University, Corvallis, Oregon 97331,[1] and Division of Biology, California Institute of Technology, Pasadena, California 91125[2]

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The antibiotic G418 was shown to be an effective inhibitor of vaccinia virus replication when an appropriate concentration of it was added to cell monolayers 48 h before infection. Genetic engineering techniques were used in concert with DNA transfection protocols to construct vaccinia virus recombinants containing the neomycin resistance gene (*neo*) from transposon Tn*5*. These recombinants contained the *neo* gene linked in either the correct or incorrect orientation relative to the vaccinia virus 7.5-kilodalton gene promoter which is expressed constitutively throughout the course of infection. The vaccinia virus recombinant containing the chimeric *neo* gene in the proper orientation was able to grow and form plaques in the presence of G418, whereas both the wild-type and the recombinant virus with the *neo* gene in the opposite polarity were inhibited by more than 98%. The effect of G418 on virus growth may be mediated at least in part by selective inhibition of the synthesis of a subset of late viral proteins. These results are discussed with reference to using this system, the conferral of resistance to G418 with *neo* as a positive selectable marker, to facilitate constructing vaccinia virus recombinants which contain foreign genes of interest.

The use of cloning and expression vectors to study and experimentally manipulate individual genes independently of their normal resident environment has become a central and vital theme in molecular genetics. Due to a number of unique biological attributes, vaccinia virus (VV) would seem to offer an ideal system for such studies (12). A number of laboratories have demonstrated the feasibility of this approach by constructing recombinant VV strains which contain and express heterologous viral and cellular antigens (13, 15, 16). Such hybrid strains may prove useful in the preparation of vaccines against a variety of human and animal diseases. Unfortunately the current methods used to construct VV recombinants are slow, time-consuming, and expensive and do not facilitate genetic engineering of the foreign DNA insert. These drawbacks have thus far retarded the development of VV as a general eucaryotic expression vector. The chief limitation of this system stems from the fact that the methods used to detect and isolate VV recombinants of interest rely on unsophisticated screening procedures (20). One major improvement has been provided by Mackett et al., who developed a VV insertion vector which inserts foreign genes into the middle of the VV thymidine kinase (*tk*) gene (11). This results in functional inactivation of tk activity and converts the recombinant virus to a tk^- phenotype. In principle, it should be possible to select recombinants metabolically with bromodeoxyuridine. However, the spontaneous rate of tk^- mutants arising in the virus population is quite high, on the order of 10^{-3} to 10^{-4}, so that recombinant VV still makes up only a fraction of the total tk^- population (D. E. Hruby, unpublished data). This is particularly troublesome if one is attempting to insert a large foreign gene which will be rescued at a low frequency. Thus, although the use of bromodeoxyuridine is certainly a useful enrichment technique, it does not provide a direct selection for recombinants. These problems could be circumvented if methods were developed to employ dominant selectable markers for isolating VV recombinants.

An appropriate strategy might be the construction of tandem plasmid insertion vectors with a selectable marker linked to a foreign gene of interest. This would consist of a VV promoter (P1) linked to a polylinker region for the insertion of foreign genes, and a second VV promoter (P2) linked to a selectable marker, with the entire tandem arrangement embedded in VV DNA sequences sufficient to catalyze recombination into a nonessential region of the viral genome. Such a plasmid could be used to insert the tandem genes into the VV genome. Recombinants could then be selected directly by using biochemical agents.

There are a number of positive selection schemes which could potentially be adapted to the VV system, but the two with the greatest promise would seem to be selection of the tk^+ phenotype with methotrexate (21) and of the Neo^r phenotype with G418 (18). Methotrexate has previously been used to select VV recombinants with a tk^+ phenotype that resulted from the insertion of a functional viral *tk* gene into a VV tk^- mutant (21). In analogy to selecting for tk^- VV, it might be anticipated that the number of tk^+ revertants would be equal to or greater than the number of tk^+ VV arising from recombination. This problem could be circumvented by using a VV mutant containing a deletion in the *tk* region (11). An alternative approach would be the use of G418. The synthetic antibiotic G418, which is an effective inhibitor of protein synthesis in both eucaryotic and procaryotic systems, has been used as a selective agent for transformants that have taken up and are expressing the bacterial neomycin resistance gene, *neo* (18). This system is particularly powerful because no equivalent enzymatic activity exists in eucaryotic cells and therefore there is no background of spontaneous G418-resistant mutants. In the experiments reported here, we have sought to determine whether the G418-*neo* system could be used to select VV recombinants, specifically, whether VV replication in tissue culture cells is inhibited by the drug, and if so whether the

* Corresponding author.

† Oregon State University Agricultural Experiment Station technical paper no. 7439.

inhibition could be overcome by a VV recombinant which contained and expressed a functional *neo* gene.

MATERIALS AND METHODS

Cells and virus. VV (WR strain) was grown and its titers were determined on monolayers of BSC-40 cells maintained in Eagle minimal essential medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 10 μg of gentamicin sulfate per ml (7). Transfections were carried out on Ltk$^-$ cells grown in the same medium. Media containing G418 (GIBCO Diagnostics) were prepared by dissolving an appropriate amount of the drug in the medium and then passing the solution through a filter (0.22-μm pore size; Millipore Corp.).

Plasmid construction. The construction of pVV5.1 will be described in detail elsewhere (manuscript in preparation). Briefly, the *Xba*I-*Xho*I fragment of pGS20 (11) which contains the VV 7.5-kilodalton (7.5Kd) gene promoter region embedded within the VV *tk* coding region was ligated into pMT21, a small ampicillin-resistant (Amp^r) derivative of pBR322 (obtained from Henry V. Huang). The *Xho*I and *Xba*I sites flanking the VV insert, as well as the *Cla*I site in the *tk* gene, were removed during the construction. pVV5.1 contained the following restriction sites, beginning 40 nucleotides downstream from the start of VV 7.5Kd promoter early transcription: *Xho*I, *Eco*RI, *Sma*I, *Bam*HI, *Pst*I, *Sma*I, and *Eco*RI. This plasmid was linearized with *Bam*HI, treated with calf intestinal alkaline phosphatase, and ligated to the *Bam*HI-*Bgl*II fragment of pBRNEO, which contained the neomycin resistance gene from Tn*5* (3). Plasmids were obtained with the gene in both orientations and were designated pVV:$\overrightarrow{NEO}$ (correct orientation) and pVV:$\overleftarrow{NEO}$ (opposite orientation).

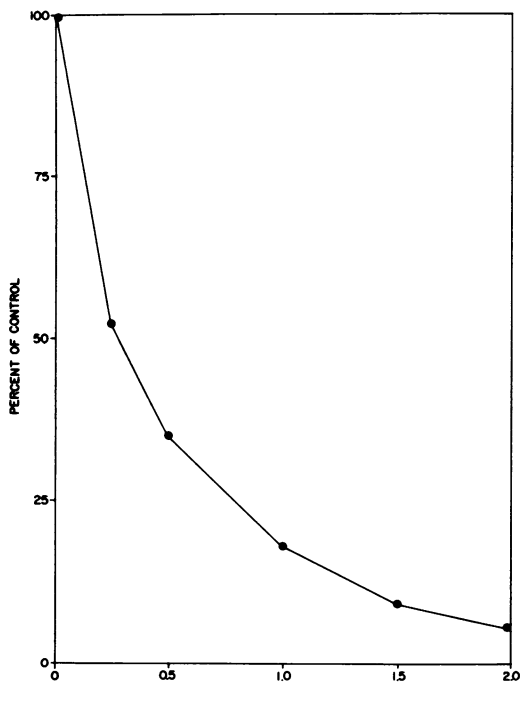

FIG. 1. Effect of increasing concentrations of G418 on VV replication. G418 was added to the cells 48 h before infection and maintained throughout the experiment. Cells were infected with VV at a multiplicity of 5 PFU/cell. After 24 h of infection, the progeny virions were harvested and the titer was determined in the absence of drug. The control titer in the absence of drug was 1.22×10^8 PFU/ml.

TABLE 1. Effect of adding G418 at different times before VV infection[a]

G418 concn (mg/ml)	Time of addition (h preinfection)	VV titer (PFU/ml)	% Inhibition of growth
0 (control)		2.45×10^7	
1	0	7.82×10^6	68.1
	12	7.20×10^6	70.6
	24	5.54×10^6	77.4
	36	5.39×10^6	78.0
	48	5.14×10^6	79.0
2	0	3.92×10^6	84.0
	12	2.55×10^6	89.6
	24	1.69×10^6	93.1
	36	1.57×10^6	93.6
	48	1.20×10^6	95.1

[a] Cells were pretreated wtih G418 for the indicated periods. The monolayers were infected with wild-type VV at a multiplicity of 5 PFU/cell. Drug-containing medium was returned to the infected cells, and infection was allowed to proceed at 37°C for 24 h. Progeny VV were then harvested and titers were determined.

Marker rescue. The insertion of the *neo* gene into the VV genome was performed essentially by the method described elsewhere (submitted for publication). The recombinant plasmids were coprecipitated with wild-type VV DNA and carrier salmon sperm DNA by using calcium orthophosphate (6). The precipitated DNAs were transfected into Ltk$^-$ cells 3 h after they were infected with VV at a multiplicity of 0.05 PFU/cell. The infected-cell monolayers were shocked with glycerol for 40 s to facilitate DNA uptake (10) and then incubated at 37°C for 48 h. The progeny of this initial marker rescue step were then passaged through Ltk$^-$ cells in the presence of bromodeoxyuridine (25 μg/ml) to amplify the number of tk$^-$ VV in the population. Recombinant VV containing the *neo* gene were then detected by plaque hybridization with a nick-translated *Bgl*II-*Bam*HI *neo* DNA fragment as the probe (3). Individual recombinant plaques were punched out from replica nitrocellulose filters and inoculated into 24-well dishes of BSC-40 cells. After 48 h, cell extracts were prepared by trypsinizing the infected cells. A portion of each extract was subjected to dot blot analysis (10) to confirm that VV containing the *neo* gene were present. Virus from extracts scored as positive were then subjected to an additional round of plaque purification and grown on a larger scale.

DNA analysis. DNA was extracted and purified from cytoplasmic extracts of BSC-40 cells infected at a multiplicity of 10 PFU/cell with putative VV recombinants (submitted for publication). The DNA was digested with various restriction endonucleases, and the fragments were resolved by electrophoresis in 0.8% agarose gels (14). After being stained with 0.5 μg of ethidium bromide per ml, the gels were photographed, blotted onto nitrocellulose, and hybridized with appropriate nick-translated DNA fragments as probes (17). In all cases, wild-type VV DNA was run as a control. Reference was made to previously published restriction maps of VV DNA (4), as well as to unpublished data from our own laboratory, to determine the genomic structure of the VV recombinants.

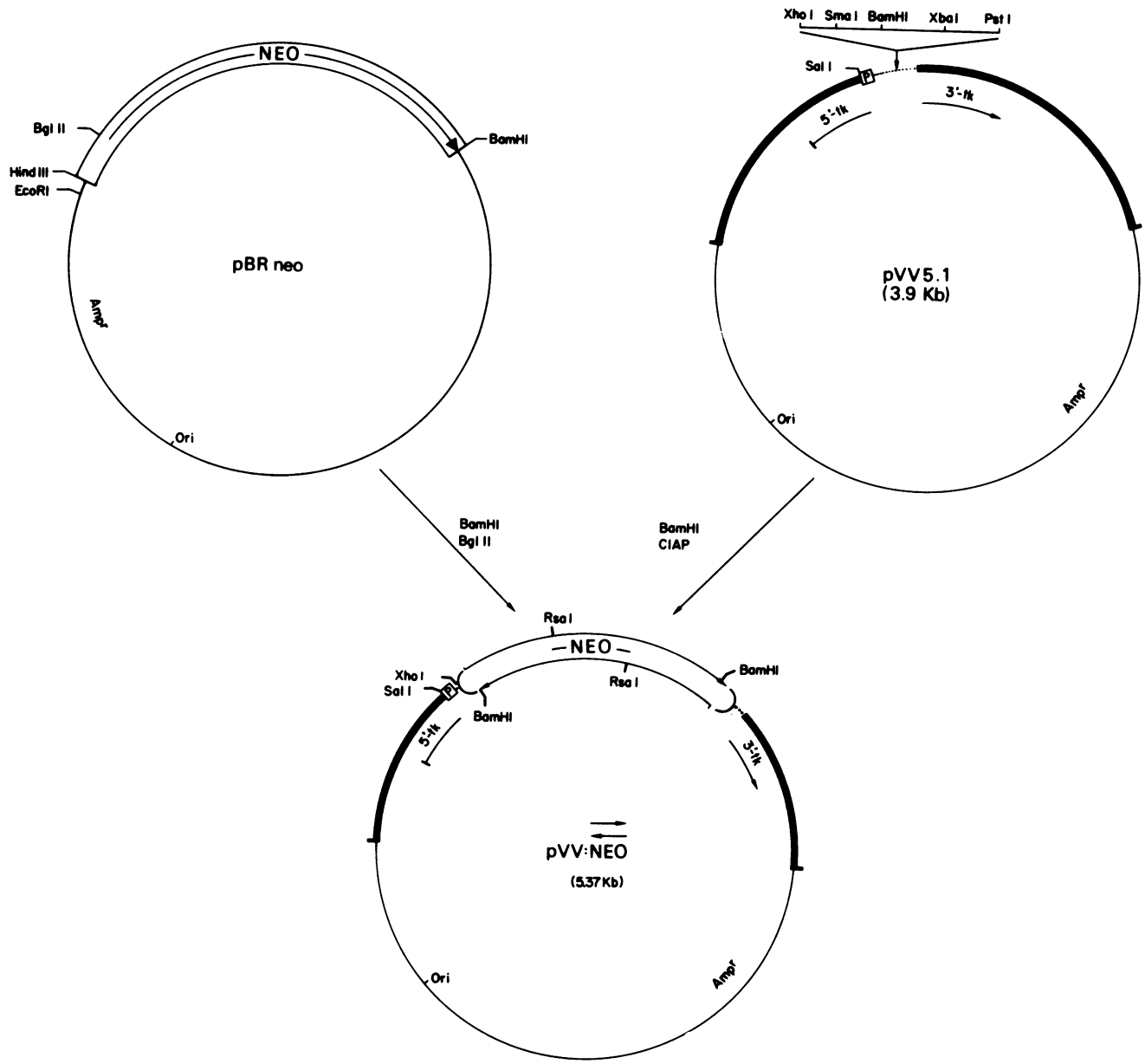

FIG. 2. Construction of the VV:NEO recombinant plasmids. VV DNA sequences are indicated by heavy lines. The position of the VV 7.5Kd gene promoter (P) is indicated. CIAP, Calf intestinal alkaline phosphatase; Ori, origin of replication.

Protein synthesis. Monolayers of BSC-40 cells in 60-mm dishes were infected at a multiplicity of 10 PFU/cell with wild-type or recombinant VV. The infected cells were either pulse-labeled for 30 min with 20 μCi of L-[^{35}S]methionine (1,140 Ci/mmol; New England Nuclear Corp.) at the indicated times postinfection or continuously labeled by having 5 μCi of radioactive label per ml present throughout the infection. The radioactively labeled infected cells were harvested and collected by centrifugation. The pellets were suspended in 100 μl of 1 mM Tris-hydrochloride (pH 9), and a small portion was used to determine the hot trichloroacetic acid-precipitable counts. Equivalent amounts of radioactively labeled cell lysates were then analyzed on 12% sodium dodecyl sulfate–polyacrylamide slab gels (19). The gels were then impregnated with 2,5-diphenyloxazole and fluorographed at −70°C on Kodak XAR-5 film (1).

RESULTS

G418 inhibition of VV growth. Previous experiments have shown that high levels of G418 (1 to 2 mg/ml) must be present for a substantial period of time (24 to 48 h) to inhibit the replication of mammalian cells and that different cell lines differ widely in their sensitivity to this drug (18). Since VV macromolecular synthesis occurs within the cytoplasmic compartment of infected cells and utilizes the host cell translational apparatus (8), it seemed likely that these considerations would also apply to G418 inhibition of viral growth. Therefore, the effect of increasing concentrations of G418 on the yield of VV progeny from one-step infections carried out on BSC-40 cell monolayers was assayed (Fig. 1). VV replication was sensitive to G418-mediated inhibition, being reduced 95% by a concentration of 2 mg/ml. Under

these conditions, VV plaque formation was completely abolished. The time of treatment required to effectively inhibit VV growth was also examined (Table 1). Although the effects observed were not dramatic, there was a general reduction of VV growth which correlated with the time the cells had been treated with G418, from 0 to 48 h before infection. Based on these results, the standard conditions used for the rest of the experiments reported here were G418 at 2 mg/ml added 48 h before infection and maintained at this concentration throughout the infection. Under these conditions, the cells appeared normal and VV replication was essentially blocked. Similar results were obtained in other host cells. For example, a 48-h treatment of Ltk$^-$ cells with 2 mg of G418 per ml resulted in 97.3% inhibition of VV growth.

Construction of VV:NEO recombinants. To ascertain whether the inhibition of VV replication by G418 could be overcome by the neomycin resistance gene (*neo*), marker rescue techniques were used to isolate VV recombinants containing this gene. Figure 2 shows the recombinant plasmids that were constructed to mobilize the *neo* gene into the VV genome. The *neo* gene from transposon Tn*5* (3) was inserted into the VV insertion vector pVV5.1 in both the correct (→) and incorrect (←) orientation relative to the VV 7.5Kd gene promoter, which is expressed both early and late during infection (11). The flanking VV DNA sequences allowed these chimeric genes to be recombined into the resident VV *tk* gene by transfecting them into the cytoplasm of VV-infected cells (submitted for publication). Individual VV recombinants were detected and isolated by plaque hybridization and several rounds of plaque purification. The genomic analysis of these recombinants is shown in Fig. 3.

The pattern of VV DNA fragments generated with *Sal*I showed that the 5.2-kilobase (kb) *Sal*I M fragment present in wild-type DNA was replaced in the two VV-NEO recombinants by two new fragments of 5.8 and 0.6 kb (Fig. 3A). The new fragments were expected as a result of the insertion of the 1.2-kb chimeric 7.5Kd promoter:*neo* gene, which contained a single new *Sal*I site within the promoter region. The identity of the new fragments was confirmed by Southern analyses. Nick-translated VV *Hin*dIII-J DNA, which contained the VV *tk* gene, hybridized to *Sal*I M fragment as well as to the two new VV:NEO-derived bands (Fig. 3B). In contrast, when labeled *neo* DNA was used as the probe, no hybridization to parental VV DNA or to the 0.6-kb fragment, which should contain only VV sequences, was detected, whereas the 5.8-kb band apparently contained the *neo* gene (Fig. 3C). The orientation of *neo* within the recombinants was determined by *Xho*I-*Rsa*I double digestion. If the gene were in the correct orientation this would liberate a 469-base-pair fragment of *neo*, whereas in the opposite orientation a 700-bp fragment would be generated (Fig. 2). The data (Fig. 3D) confirmed that isolated VV:NEO recombinants contained the *neo* gene in both orientations.

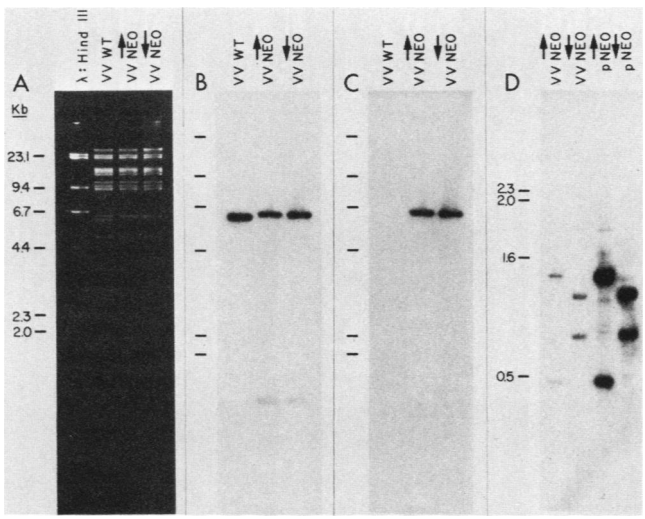

FIG. 3. Genomic structure of VV:NEO recombinants. DNAs from wild-type (WT) VV, VV:NEO, and VV:NEO were restricted with *Sal*I and analyzed by agarose gel electrophoresis (A). The DNA fragments were then transferred to nitrocellulose and probed with nick-translated VV *Hin*dIII-J DNA (B) or *neo* DNA (C). To confirm the orientations, the parental plasmid and viral DNAs were double-digested with *Xho*I-*Rsa*I and subjected to Southern blot analyses with nick-translated *neo* DNA as the probe (D). The positions of lambda DNA fragments digested with *Hin*dIII run as markers are indicated to the left of each panel.

TABLE 2. One-step growth of VV:NEO in the presence of G418[a]

VV strain	Titer (PFU/ml)			% Inhibition of growth
	Initial	Without G418	With G418	
Wild type	4.8×10^6	5.6×10^8	5.2×10^6	99.1
VV:NEO	3.0×10^6	4.6×10^8	4.4×10^8	4.3
VV:NEO	1.9×10^6	3.7×10^8	4.6×10^6	98.8

[a] Infections were carried out at an input of 5 PFU/cell on monolayers of BSC-40 cells (7) which had been incubated for the previous 48 h with or without G418 (2 mg/ml). After a 24-h infection, the progeny VV were harvested and counted.

Growth of VV:NEO in the presence of G418. The VV:NEO recombinants were then tested for their ability, relative to that of wild-type VV, to replicate in the presence of G418. Since the treated cells were presumably in a fully inhibited state, it was unclear whether sufficient *neo*-encoded phosphotransferase would be expressed to enable NEO to replicate. However, with G418 treatment sufficient to inhibit the replication of wild-type VV by 99.1%, VV:NEO was essentially unaffected (Table 2). In contrast, VV:NEO was unable to grow, indicating the necessity for the *neo* gene to be driven by a VV promoter for it to be functional in this situation. Similarly, wild-type VV and VV:NEO were plated on BSC-40 cells in the presence and absence of G418 (Fig. 4). In the absence of drug, both wild-type VV and VV:NEO formed plaques of normal size and morphology. In the presence of G418, wild-type VV could not form plaques, whereas VV:NEO formed almost the same number of plaques as on the control plate, although they were slightly smaller. This would indicate that the progeny VV:NEO virions were able to spread from cell to cell and overcome the G418-mediated inhibition in second- and third-generation infections. VV:NEO behaved identically to wild-type VV in these assays (data not shown).

Mechanism of G418 inhibition of VV replication. G418 is an aminoglycoside antibiotic which inhibits eucaryotic protein synthesis by interfering with 80S ribosome function (2). Thus it was of interest to investigate the mechanism by which this drug interfered with VV replication and how expression of the *neo* gene alleviated the inhibition. One simple method useful for monitoring the course of a VV infection is to examine the kinetics of viral protein synthesis. From the patterns observed, inferences can be drawn about the expression of early genes, viral DNA synthesis, late gene expression, and protein-processing events. Therefore,

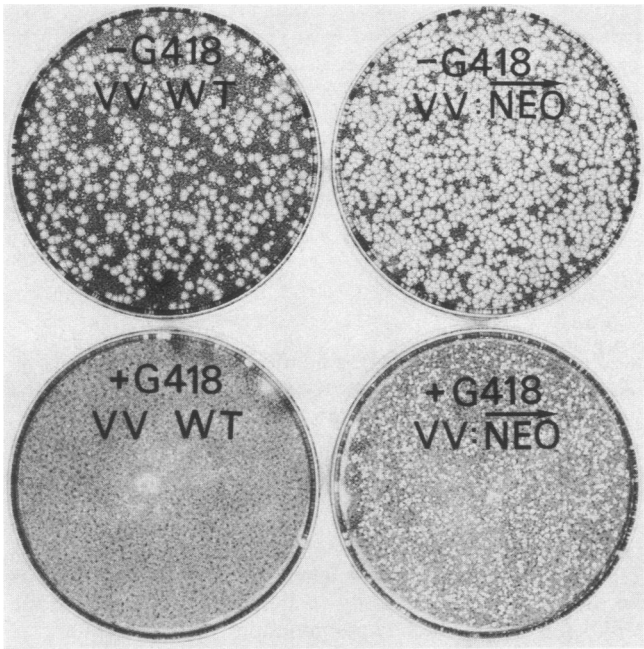

FIG. 4. VV plaque assays. Plaque assays were carried out in the presence or absence of G418 with wild-type (WT) VV and the two VV:NEO recombinants. After 48 h the plaques were visualized by staining the monolayers with methylene blue. Only results for wild-type VV and VV:$\overrightarrow{NEO}$ are shown.

monolayers of cells were infected with VV, VV:$\overrightarrow{NEO}$, or VV:$\overleftarrow{NEO}$ in the presence of [^{35}S]methionine, and the radioactively labeled polypeptides were examined by polyacrylamide gel electrophoresis. Pulse-labeling was done both early (before DNA synthesis) and late (after DNA synthesis) during infection on cells that had been mock infected or infected with wild-type VV, VV:$\overrightarrow{NEO}$, or VV:$\overleftarrow{NEO}$. The results (Fig. 5A) were immediately surprising. First, the mock-infected cell extracts showed that there was very little difference in the pattern of nascent polypeptides translated with and without G418, even though previous experiments had shown that cell growth was inhibited under these conditions. Second, both VV and the VV:NEO recombinants were able to express both early and late viral genes, which suggests that viral DNA synthesis had also occurred. However, a close examination of the pattern of viral proteins made at late times revealed that although some VV late proteins were made in normal amounts in VV- and VV:$\overleftarrow{NEO}$-infected cells, there was a distinct subset of late gene products which were either absent or greatly reduced in amount in the presence of G418. These differences were even more pronounced when continuous labeling was used (Fig. 5B). Note in both cases that VV:$\overrightarrow{NEO}$ was able to overcome this defect and synthesize those polypeptides even in the presence of G418. It is unclear whether G418 inhibition of VV late gene expression was expressed directly

TABLE 3. Effect of G418 on marker rescue efficiency[a]

G418 treatment during:		Total VV PFU	No. (%) of positive recombinants
Marker rescue	Amplification		
−	−	1.0×10^8	4.0×10^5 (0.4)
−	+	2.5×10^4	4.0×10^2 (1.6)
+	−	3.0×10^7	2.0×10^5 (0.7)
+	+	5.0×10^3	1.4×10^3 (28.0)

[a] pVV:$\overrightarrow{NEO}$ plasmid DNA was transfected into monolayers of VV-infected BSC-40 cells (multiplicity of infection, 5 PFU/cell) with or without G418 (2 mg/ml). This initial marker rescue crude stock was amplified by a subsequent passage (multiplicity of infection, 0.01 PFU/cell) with (for 48 h) and without drug. The progeny virus titers were determined, and the viruses were subjected to plaque hybridization with a *neo*-specific probe to detect *neo*-containing recombinants.

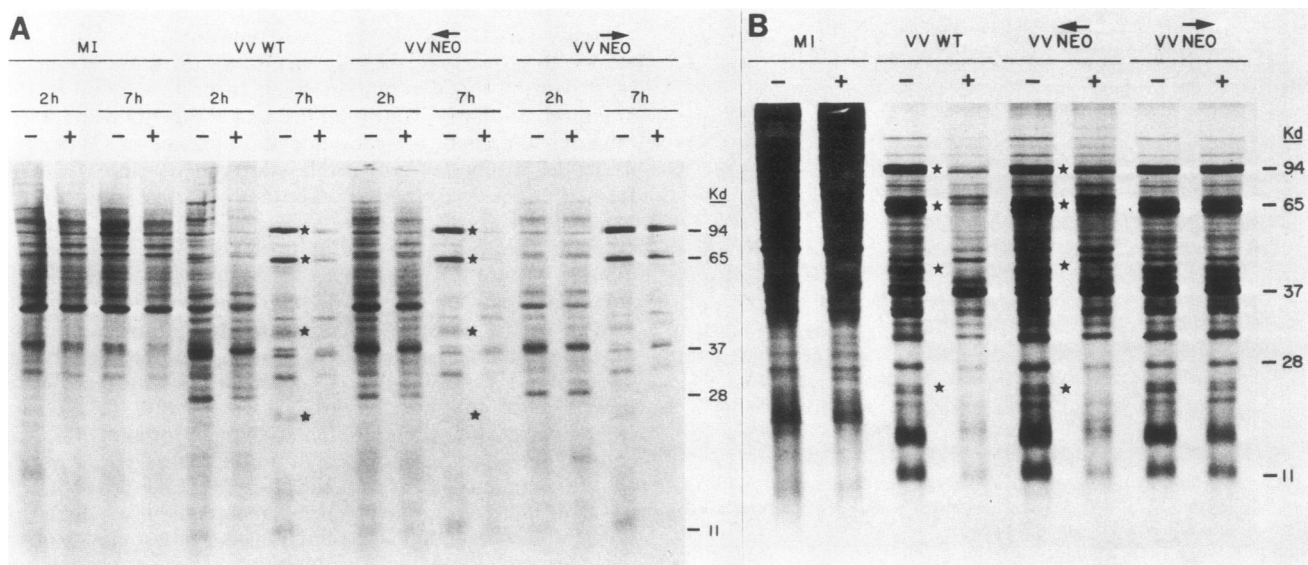

FIG. 5. Effect of G418 on VV protein synthesis. Monolayers of infected cells were either continuously labeled or pulse-labeled at the indicated time postinfection with [^{35}S]methionine. Radioactive proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. (A) Pulse label; (B) continuous label. The positions of VV proteins run as molecular weight markers are indicated (in kilodaltons). ★, Late gene products that were either absent or greatly decreased in the presence of G418. MI, Mock infected.

at the translational level or indirectly at the posttranslational level (i.e., cleavage).

G418 enhancement of marker rescue efficiency. To ascertain whether G418 selection could be used during the initial marker rescue steps to raise the percentage of recombinants obtained, the following experiment was performed. The VV:NEO plasmid was transfected into recipient cells with and without G418, the progeny of the initial rescue were passaged one additional time (with and without drug selection), and the percentage of recombinant virions was determined by a combination of plaque assay and hybridization protocols. Having G418 present during either the marker rescue or amplification step resulted in a modest increase in the efficiency of marker rescue, twofold and fourfold, respectively (Table 3). However, when the drug was present during both steps, a synergistic 70-fold increase in the percentage of recombinants was observed.

DISCUSSION

These experiments have demonstrated that the antibiotic G418 is an effective and potent inhibitor of VV replication in mammalian cells. When cell monolayers are treated for 48 h before infection with 2 mg of G418 per ml, the growth of VV is inhibited by 95 to 99% and the ability of the virus to form visible plaques is totally blocked. The G418-mediated inhibition is apparently due to a reduction in the expression of a subset of VV late genes. In any case, the inhibition can be overcome by introducing a bacterial neomycin resistance gene into the VV genome so that it is regulated and expressed by a VV promoter. Such a recombinant (VV:NEO) is able to replicate, assemble, form plaques, and express its entire genetic program in the presence of high levels of G418.

This information should be of immediate consequence as far as using *neo* as a positive selectable marker to facilitate the selection and isolation of VV recombinants containing foreign genes of interest. There are at least two potential avenues to pursue. First, it is possible that the pVV5.1:NEO plasmid could be cotransfected into VV-infected cells along with another gene which is in a different VV insertion vector plasmid in the presence of G418. The second insertion vector could contain either similar or dissimilar flanking VV DNA sequences so long as they resulted in insertion in a nonessential region. Cells which are competent for DNA uptake should take up both plasmids. The *neo* gene will be expressed in those cells and allow a productive infection and concomitant marker rescue to occur. Conversely, VV which infect noncompetent cells will be unable to replicate in the presence of G418. This procedure would use the *neo* gene to convect other genes into the VV genome while reducing the background of wild-type VV replication. Convection procedures have previously been applied successfully in cellular transformation studies (5) and to temperature-sensitive VV mutants (9). A second approach would be to construct dual-insertion vectors with the *neo* gene linked to a second gene. This latter approach would have the potential advantage of allowing VV recombinants to be selected directly after the marker rescue procedure. Any virus able to form plaques in the presence of G418 should contain the *neo* gene as well as the passenger gene of interest. Since *neo* is a bacterial gene and probably not significantly homologous to any VV DNA sequences, such insertion vectors could be tailored by virtue of their homologous VV DNA flanking sequences to insert the foreign gene in any nonessential region of the viral genome. Such plasmid constructions with a variety of VV promoters and assayable foreign genes are currently in progress.

In view of these results it is likely that genetic engineering procedures may be used to construct VV insertion vectors that use any of a number of selectable or easily detectable marker systems. The availability of such plasmids should greatly facilitate the use of VV as a general eucaryotic cloning and expression vector. The ability to rapidly construct a series of VV recombinants containing a foreign gene of interest and genetically manipulated derivatives should prove invaluable in this regard.

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LITERATURE CITED

1. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–91.
2. **Davies J., and A. Jimenez.** 1980. A new selective agent for eukaryotic cloning vectors. Am. J. Trop. Med. Hyg. **29**: 1089–1092.
3. **Davies, J., and D. I. Smith.** 1978. Plasmid determined resistance to antimicrobial agents. Annu. Rev. Microbiol. **32**:469–518.
4. **DeFillipes, F. M.** 1982. Restriction enzyme mapping of vaccinia virus DNA. J. Virol. **43**:136–149.
5. **Gazit, A., H. Igarashi, I. Chiu, A. Srinivasan, A. Yaniv, S. Tronock, K. C. Robbins, and S. G. Aaronson.** 1984. Expression of the normal human sis/PDFG-2 coding sequence induces cell transformation. Cell **39**:89–97.
6. **Graham, F. L., and A. J. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
7. **Hruby, D. E., L. A. Guarino, and J. R. Kates.** 1979. Vaccinia virus replication. I. Requirement for the host cell nucleus. J. Virol. **29**:705–715.
8. **Hruby, D. E., L. L. Lynn, and J. R. Kates.** 1979. Vaccinia virus replication requires active participation of the host cell transcriptional apparatus. Proc. Natl. Acad. Sci. U.S.A. **76**: 1887–1890.
9. **Kieny, M. P., R. Lathe, R. Drillen, D. Spehner, S. Skory, D. Schmitt, T. Wiktor, H. Koprowski, and J. P. Lecoq.** 1984. Expression of rabies virus glycoprotein from a recombinant vaccinia virus. Nature (London) **312**:163–166.
10. **Lapata, M. A., D. W. Cleveland, and B. Sollner-Webb.** 1984. High expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. Nucleic Acids Res. **12**:5707–5717.
11. **Mackett, M., G. L. Smith, and B. Moss.** 1984. General method for production and selection of infectious vaccinia virus recombinants expressing foreign genes. J. Virol. **49**:857–864.
12. **Moss, B.** 1974. Reproduction of poxviruses, p. 405–474. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, Vol. 3. Plenum Publishing Corp., New York.
13. **Paoletti, E., B. R. Lipinskas, C. Samsonoff, S. Mercer, and D. Panacali.** 1974. Construction of live vaccines using genetically engineered poxviruses: biological activity of vaccinia virus recombinants expressing the hepatitus B virus surface antigen and the herpes simplex virus glycoprotein D. Proc. Natl. Acad. Sci. U.S.A. **81**:193–197.
14. **Sharp, P. A., B. Sugden, and J. Sambrook.** 1973. Detection of two restriction endonuclease activities in Haemophilus parainfluenzae using analytical agarose. Biochemistry **12**:3055–3061.

15. **Smith, G. L., M. Mackett, and B. Moss.** 1983. Infectious vaccinia virus recombinants that express hepatitis B virus surface antigen. Nature (London) **302**:490–495.
16. **Smith, G. L., B. R. Murphy, and B. Moss.** 1983. Construction and characterization of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. Proc. Natl. Acad. Sci. U.S.A. **80**:7155–7159.
17. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
18. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
19. **Studier, F. W.** 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. **79**:237–248.
20. **Villarreal, L. P., and P. Berg.** 1977. Hybridization in situ of SV40 plaques: detection of recombinant SV40 virus carrying specific sequences of nonviral DNA. Science **196**:183–186.
21. **Weir, J. P., G. Bajszar, and B. Moss.** 1982. Mapping of the vaccinia virus thymidine kinase gene by marker rescue and by cell-free translation of selected mRNA. Proc. Natl. Acad. Sci. U.S.A. **79**:1219–1214.