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Generation of varicella-zoster virus (VZV) and viral mutants from cosmid DNAs: VZV thymidylate synthetase is not essential for replication *in vitro*

(recombination/herpesvirus/vaccine)

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ABSTRACT **Four overlapping cosmid clones were constructed that contain the complete genome of the attenuated Oka strain of VZV. Transfection of human melanoma cells with the four cosmids resulted in production of infectious VZV. A double-stranded oligonucleotide, encoding a stop codon in all three open reading frames, was inserted into one of the cosmids at the 5′ end of the viral thymidylate synthetase gene. Transfection of melanoma cells with the mutant cosmid, along with the other three cosmids, resulted in VZV that does not express the viral thymidylate synthetase protein. The mutant virus grew at a rate similar to that of the parental Oka strain virus. Production of recombinant VZV using cosmid DNAs will be useful for studying the function of viral genes in VZV replication and establishment of latency. Furthermore, manipulation of the Oka strain of VZV might allow one to produce a vaccine virus that does not establish latency in the central nervous system or a virus that encodes foreign antigens for use as a polyvalent live virus vaccine.**

VZV is the etiologic agent of chickenpox and herpes zoster. An attenuated live virus vaccine, VZV Oka, has been developed by passage of the virus in cell culture (1). This vaccine has been shown to protect healthy and immunocompromised children, as well as healthy adults, from chickenpox (for review, see ref. 2). The genetic basis for attenuation of the Oka strain of VZV is unknown.

Genetic studies of VZV have lagged behind that of the other human herpesviruses. At present, site-specific mutations have been engineered only into the deoxypyrimidine kinase gene of VZV. Cotransfection of cells with virion DNA and a plasmid containing a foreign viral gene flanked by VZV deoxypyrimidine kinase DNA resulted in VZV mutants with an insertionally inactivated deoxypyrimidine kinase gene. In both cases the foreign viral antigen was used to select for the VZV mutant (3, 4). A system in which VZV mutants could be obtained without the need to express foreign antigens, which might alter the phenotype of the resultant virus, would be useful for studying the role of viral genes in virulence, replication, and establishment of latency.

Recently, van Zijl *et al.* (5) described a method to generate recombinant pseudorabies virus using a set of overlapping cosmid DNAs. This technique has been used to insertionally inactivate specific pseudorabiesvirus genes (6) and to insert foreign genes into the virus for use as candidate vaccine strains (7). We have used this approach for manipulation of the VZV genome.

Comparison of the nucleotide sequence of herpes simplex virus 1 with VZV indicates that only five of the predicted 72 genes of VZV do not have herpes simplex virus 1 counterparts (8). One of these five genes, VZV open reading frame (ORF) 13, encodes the viral thymidylate synthetase gene (9). Thymidylate synthetase catalyzes the methylation of deoxyuridylate to thymidylate. This gene may be an important target for future antiviral agents. While other animal herpesviruses encode a thymidylate synthetase gene (10, 11), it is not known whether the viral thymidylate synthetase gene is essential for replication.

To determine whether the VZV thymidylate synthetase gene is essential, we developed a system for generating recombinant virus with site-specific mutations. Due to the difficulties in obtaining VZV virion DNA for transfection and in obtaining cell-free virus to plaque purify VZV mutants from wild-type virus, we used cosmid DNAs to generate recombinant VZV. Transfection of cells with four overlapping cosmid DNAs, spanning the entire VZV Oka genome, resulted in infectious VZV. Site-directed mutagenesis of one of the cosmids, followed by transfection, resulted in mutant VZV that grows well and does not express the viral thymidylate synthetase protein.

MATERIALS AND METHODS

Cosmid and Viral DNAs. Cosmid pDVcosA2 (12) was cut with *Sal* I and *Xho* I, incubated with the large (Klenow) fragment of DNA polymerase I to produce blunt ends, and ligated to double-stranded oligonucleotides containing *Not* I or *Mst* II sites. The oligonucleotide used for insertion of a *Not* I site was AGCGGCCGCT; for *Mst* II two separate double-stranded oligonucleotides were used since the fourth nucleotide of the *Mst* II recognition site varies. The sequence TACCTTAGGTA was used for ligation of the VZV *Mst* II fragment B and TACCTCAGGTA was used for ligation of the VZV *Mst* II fragment A (see below). The attenuated Oka strain of VZV (1) was kindly provided by M. Takahashi (Osaka University, Suita, Osaka, Japan). The virus was passaged three times in MRC-5 cells and VZV DNA was purified from nucleocapsids of infected cells (13). Viral DNA was incubated with T4 DNA polymerase to produce blunt ends, *Not* I or *Mst* II linkers were ligated to the DNA, the DNA was then digested with *Not* I or *Mst* II, respectively, and the DNA was inserted into cosmids that had been linearized at the *Not* I or *Mst* II sites. The cosmids were packaged and inserted into *Escherichia coli* PLK-A. Two cosmids containing the *Not* I fragment A [VZV nt 1–45,295 (14)], *Not* I fragments B and D (nt 62,859–107,329), *Mst* II fragment A (nt 84,970–124,884), and *Mst* II fragment B (nt 33,239–70,356) of VZV Oka were selected for use in transfections (Fig. 1).

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Abbreviations: VZV, varicella-zoster virus; ORF, open reading frame; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; 5,10-MeTHF, 5,10-methylenetetrahydrofolate; PFU, plaque-forming unit(s).

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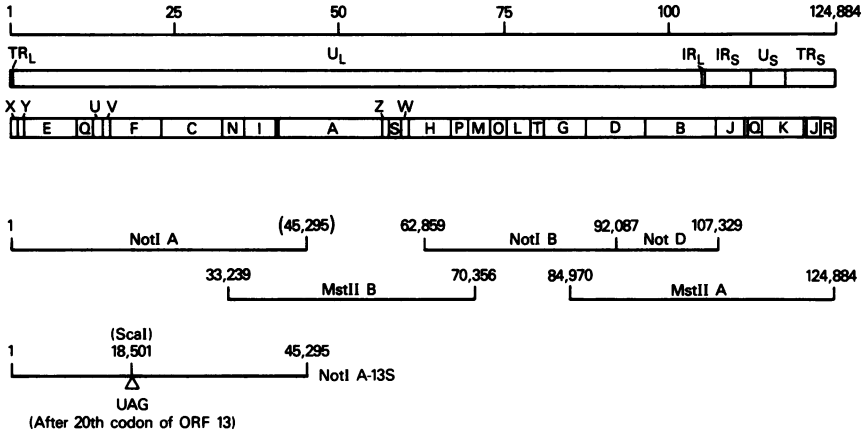

FIG. 1. Construction of recombinant VZV. The prototype VZV genome [Dumas strain (14)] is 124,884 bp long and consists of terminal repeat (TR), unique long (U_L), internal repeat (IR), and unique short (U_S) DNA domains. A *Bam*HI restriction endonuclease map (with letters indicating restriction fragments) is shown above the *Not* I and *Mst* II restriction fragments used to generate the overlapping cosmid clones. The nucleotide positions correspond to the sequence of the prototype (Dumas) strain of VZV (14). The 3′ end of VZV *Not* I A terminates within the R3 repeat region [nt 41,453–41,519 of Dumas (14)] resulting in deletion of the *Not* I site at the 3′ end of the VZV insert (indicated in parentheses). The *Not* I site at position 92,087 is present in the Oka strain but not in the Dumas strain (see text for details). Cosmid VZV *Not* I A-13S, with an oligonucleotide containing stop codons in all three ORFs inserted in ORF13 (sixth line), was used to construct mutant VZV that does not express the viral thymidylate synthetase.

To produce a cosmid with a stop codon in the VZV thymidylate synthetase gene, cosmid VZV *Not* I A was cut with *Sse*8387I (Takara Biochemicals, Berkeley, CA), which cuts the cosmid twice at VZV nt 9780 and 22,631 (14). The 12.9-kb *Sse*8387I fragment from cosmid VZV *Not* I A was cloned into plasmid pUC19 to create plasmid pUC-Sse. A partial digestion of pUC-Sse was performed with *Sca* I, which cuts the plasmid twice, and plasmid DNA cut at only one site was separated by pulsed-field electrophoresis, and a double-stranded oligonucleotide (TAGCTAGGCGCGC-CTAGCTA) was inserted into the *Sca* I site. The oligonucleotide contains an *Asc* I restriction endonuclease site and stop codons in all three reading frames. Plasmid pUC-Sse containing the oligonucleotide inserted into the *Sca* I site of the thymidylate synthetase gene was isolated and sequenced to verify that the oligonucleotide had been inserted at the correct site. The latter plasmid was cut with *Sse*8387I and the large fragment containing the mutated VZV DNA was inserted in place of the wild-type *Sse*8387I fragment in cosmid VZV *Not* I A to create cosmid VZV *Not* I A-13S. This cosmid contains the thymidylate synthetase (ORF13) gene with stop codons after the 20th codon of the gene (Fig. 1). Two independent clones of cosmid VZV *Not* I A-13S were selected. Plasmid pCMV62 contains the VZV immediate-early ORF62 gene driven by the human cytomegalovirus *IE* promoter (15).

Transfections. Cosmids were digested with *Not* I or *Mst* II to release the VZV insert DNA, extracted with phenol, and precipitated. Cosmids VZV *Not* I A, *Not* I BD, and *Mst* II B (each at 250 ng); 125 ng of cosmid *Mst* II A; 50 ng of plasmid pCMV62; and 2 μg of sheared salmon sperm DNA were used to transfect 60-mm dishes containing human melanoma (MeWo) cells (16) by using the calcium phosphate procedure (17). Five days after transfection, the cells were seeded into 75-cm^2 flasks and monitored for cytopathic effects. Virus was subsequently grown in whole human fibroblast (WHF) cells (BioWhittaker) for further analysis.

Southern Blots. VZV DNA was purified from nucleocapsids of infected cells, cut with *Eco*RI, *Bam*HI, or *Eco*RI plus *Asc* I, fractionated on 0.6% agarose gels, and blotted onto nylon membranes. Cosmid DNAs, representing the complete VZV genome, were radiolabeled by random priming with [^{32}P]dCTP and used to hybridize to viral DNA on the membranes. The cosmid vector (pDVcosA2) was also radiolabeled separately and used to hybridize to viral DNA.

Radiolabeling of Thymidylate Synthetase and Immunoblot Analysis. Thymidylate synthetase was detected by preparing sonicated extracts from VZV-infected and uninfected cells as described (9). Sonicated cell extracts were incubated with 5-fluoro-2′-[^{3}H]deoxyuridine 5′-monophosphate (FdUMP; Moravek Biochemicals, La Brea, CA) and 5,10-methylenetetrahydrofolate (5,10-MeTHF) for 30 min at 37°C. The reaction mixture was boiled in sample buffer and fractionated on an SDS/12% polyacrylamide gel, and autoradiography was performed. Immunoblot analysis with rabbit antibody to VZV ORF10 was performed as described (18).

Growth Characteristics of Recombinant VZV. VZV was titered on MeWo cells by modification of the method of Grose and Brunell (16). Serial 1:10 dilutions of WHF cells infected with Oka or recombinant-derived VZV were used to infect MeWo cells. Seven days after infection, the cells were fixed and stained with crystal violet, and plaques were counted. Based on the results of these titrations, aliquots of infected cells containing 50–100 plaque-forming units (PFU) were used for subsequent experiments.

Growth curves of VZV mutants were performed by infecting 25-cm^2 flasks containing MeWo cells with VZV-infected WHF cells containing 50–100 PFU of virus. One, 2, 3, 4, and 6 days after infection, the infected MeWo cells were trypsinized and serial dilutions of the infected cells were used to inoculate fresh MeWo cells. Plaques were counted 8 days after infection as described above.

The sensitivity of VZV mutants to acyclovir was assayed by inoculating MeWo cells in 10-cm^2 wells with 50–100 PFU of virus in the presence of acyclovir at 0, 25, 50, or 100 μM in the medium. Plaques were counted 8 days after infection.

RESULTS

Generation of Infectious VZV Using Cosmid DNAs. Two independent clones of cosmids VZV *Not* I A, *Not* I BD, *Mst* II B, and *Mst* II A were obtained and digested with *Bam*HI, *Eco*RI, *Not* I, and *Mst* II to verify that the DNA had the expected pattern on restriction digest. Each of the cosmids selected had the expected restriction endonuclease pattern based on the sequence of strain Dumas (14) except for cosmids VZV *Not* I A and *Not* I BD. The VZV DNA in each clone of VZV *Not* I A had a different 3′ end terminating within the R3 repeat region (14) resulting in deletion of the *Not* I site at the 3′ end of the VZV insert. *Not* I BD had an

unexpected *Not* I site located at nt 92,087 due to a guanosine to adenosine difference at position 92,092 in the sequence of VZV Oka as compared to that of VZV Dumas.

MeWo cells were transfected with the four overlapping cosmids and the cell monolayers were split 5 days after transfection, at which time no cytopathic effect was noted. Eight to 10 days after transfection, cytopathic effects typical of VZV were observed. Similar numbers of plaques were obtained with both sets of the four cosmids; however, the number of plaques obtained varied considerably each day a transfection was performed. To improve the reliability of transfections, an uncut plasmid, pCMV62, was added to the transfection mixture in all subsequent experiments. pCMV62 encodes the VZV ORF62 protein, which is a potent transactivator of putative immediate-early, early, and late VZV genes (15), and enhances the infectivity of VZV DNA (H. Moriuchi, M. Moriuchi, S. Straus, and J.I., unpublished data). Transfection of the four cosmids (or the thymidylate synthetase mutant cosmid and the other three cosmids) without pCMV62 yielded plaques in 3 of 10 experiments (median, 20 plaques; range, 14–62 plaques); transfections with the cosmids and pCMV62 resulted in plaques in 3 of 4 experiments (median, 13 plaques; range 11–20 plaques).

Transfection-derived VZV was passaged in WHF cells and Southern blot analysis of viral DNA cut with *Bam*HI or *Eco*RI showed that the genome of recombinant-derived Oka VZV (ROka VZV) was identical to that of the parent Oka VZV from which it had been cloned (Fig. 2 *A* and *B*). Hybridization of the viral DNA with the cosmid vector showed no evidence of cosmid vector sequences in the recombined viral DNA (data not shown).

VZV Thymidylate Synthetase Is Dispensable for Viral Replication *in Vitro*. An *Sse*8387I fragment [VZV nt 9780 and 22,631 (14)] was subcloned from cosmid VZV *Not* I A into a plasmid vector and digested with *Sca* I, and a double-stranded oligonucleotide was inserted. The modified *Sse*8387I fragment was then inserted into the VZV *Not* I A cosmid in place of the wild-type sequence. The resulting cosmid, VZV *Not* I A-13S (Fig. 1), encodes the VZV thymidylate synthetase (ORF13) gene with a 20-bp insertion immediately after codon 20 of the gene. This insertion results in a stop codon in all three ORFs and an *Asc* I site that is not normally present in the Oka VZV genome. To generate VZV with the mutation in the thymidylate synthetase gene, cosmids VZV *Not* I A-13S, *Not* I BD, *Mst* II B, and *Mst* II A, and plasmid pCMV62 were transfected into MeWo cells. The cells were split 5 days after transfection, and 3–5 days later cytopathic effects similar to those seen with recombinant-derived Oka VZV were observed.

The recombinant-derived Oka VZV with stop codons in the thymidylate synthetase gene (ROka13S VZV) was passaged in WHF cells and viral DNA was isolated and cut with *Eco*RI, *Bam*HI, or *Eco*RI plus *Asc* I. Southern blot analysis of the viral DNA indicated that the *Eco*RI and *Bam*HI restriction patterns of ROka13S DNA were the same as that for Oka or ROka DNA (Fig. 2 *A* and *B*). Digestion of ROka13S DNA with *Eco*RI and *Asc* I verified that the oligonucleotide with the stop codons and *Asc* I site had been inserted into the thymidylate synthetase gene. The 5.2-kb *Eco*RI M band was cut by *Asc* I into two smaller bands of 3.8 and 1.4 kb (Fig. 2*C*).

To verify that cells infected with ROka13S VZV were unable to express viral thymidylate synthetase, protein extracts from VZV-infected cells were incubated with [^{3}H]-FdUMP and 5,10-MeTHF. This results in a ternary complex in which [^{3}H]FdUMP is covalently bound with 5,10-MeTHF to cellular or viral thymidylate synthetase. The ternary complex containing the cellular enzyme migrates as a 35-kDa protein, whereas the complex containing the VZV enzyme migrates as a 32.5-kDa protein (9). Analysis of cells infected with Oka or ROka VZV showed both the cellular (35 kDa) and viral (32.5 kDa) thymidylate synthetase. In contrast, cells infected with ROka13S VZV, with a mutation in the thymidylate synthetase gene, contained only the cellular (35 kDa) enzyme (Fig. 3*A*). The stop codons, inserted after codon 20 of the thymidylate synthetase gene, should result in a truncated protein that lacks the putative FdUMP binding site at the cysteine of codon 183 (9).

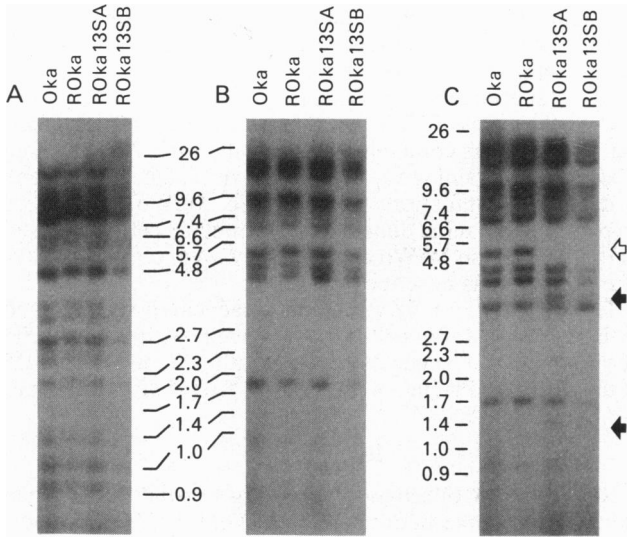

FIG. 2. Southern blot of DNAs from Oka and recombinant VZV. Virion DNA, isolated from nucleocapsid preparations, was digested with *Bam*HI (*A*), *Eco*RI (*B*), or *Eco*RI plus *Asc* I (*C*) and probed with all four radiolabeled cosmid DNAs. Restriction endonuclease patterns for *Bam*HI or *Eco*RI digests are identical for each of the viral DNAs. Digestion of two clones of ROka13S VZV DNA with *Asc* I and *Eco*RI cuts the 5.2-kb *Eco*RI fragment M (open arrow) into two bands of 3.8 and 1.4 kb (solid arrows), due to the inserted *Asc* I site. Numbers refer to size of DNAs in kilobase pairs.

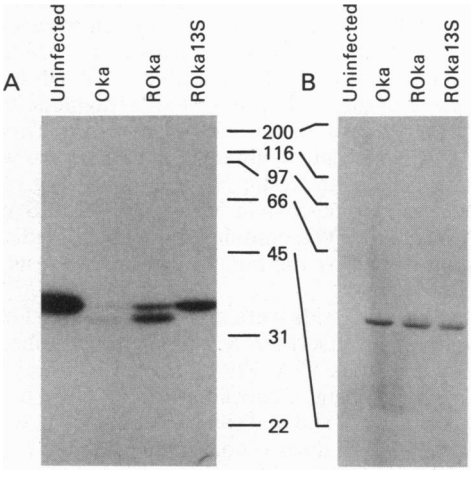

FIG. 3. (*A*) Autoradiograph of radiolabeled thymidylate synthetase in extracts of VZV-infected cells. Incubation of protein extract from uninfected cells (lane 1) with [^{3}H]FdUMP and 5,10-MeTHF labels a 35-kDa protein corresponding to cellular thymidylate synthetase. Incubation of extracts from Oka (lane 2) or ROka VZV (lane 3) infected cells yields 35- and 32.5-kDa proteins, corresponding to cellular and viral thymidylate synthetase, whereas incubation of extracts from ROka13S VZV (lane 4) infected cells yields only a 35-kDa protein due to absence of viral thymidylate synthetase. (*B*) Immunoblot of protein extracts from uninfected (lane 1) or VZV-infected (lanes 2–4) cells with rabbit antibody to VZV ORF10 shows the 50-kDa ORF10 protein in VZV-infected cells. Numbers refer to the mass of proteins in kilodaltons.

Immunoblot analysis of another aliquot of protein extract from the same pool used for the thymidylate synthetase experiments verified that cells infected with Oka, ROka, or ROka13S VZV expressed the virion protein VZV ORF10 (Fig. 3*B*). Thus, the absence of viral thymidylate synthetase in cells infected with ROka13S was not due to the absence of VZV gene expression. Similar results were seen with both clones of ROka13S (data not shown). Thus these results indicate that VZV thymidylate synthetase is dispensable for VZV replication *in vitro*.

Absence of VZV Thymidylate Synthetase Does Not Affect the Growth Rate of Virus *in Vitro*. To determine whether the absence of thymidylate synthetase expression affects the ability of VZV to grow in cell culture, we infected MeWo cells with VZV-infected cells containing 50–100 PFU of Oka, ROka, or two independent clones of ROka13S VZV. At various times after infection, cells were trypsinized and the yield of infectious virus was determined by titration on MeWo cells. Analysis of the growth of the four viruses indicated that there was little or no difference in their growth rates over time (Fig. 4). Thus, recombinant-derived virus and the parent virus had similar growth characteristics, and lack of expression of VZV thymidylate synthetase did not affect the growth of virus *in vitro*.

Absence of Viral Thymidylate Synthetase Does Not Affect VZV Sensitivity to Acyclovir. Inhibition of the VZV-encoded ribonucleotide reductase has been shown to alter the level of cellular deoxynucleoside triphosphates and to enhance the sensitivity of VZV to acyclovir (19). Since the absence of VZV thymidylate synthetase might also result in a change in levels of cellular deoxynucleoside triphosphates, we determined whether ROka13S had altered sensitivity to acyclovir. Analysis of the growth of Oka, ROka, and two clones of ROka13S VZV at different concentrations of acyclovir showed that the absence of viral thymidylate synthetase had little or no effect on the growth of the virus in the presence of acyclovir (Fig. 5).

DISCUSSION

We have developed a procedure to make infectious VZV by transfecting cells with a set of four overlapping VZV cosmid DNAs. As a test of this methodology and to explore the role of a unique VZV gene, we performed site-directed mutagenesis of one of the cosmids to generate mutant VZV that was unable to express the viral thymidylate synthetase protein.

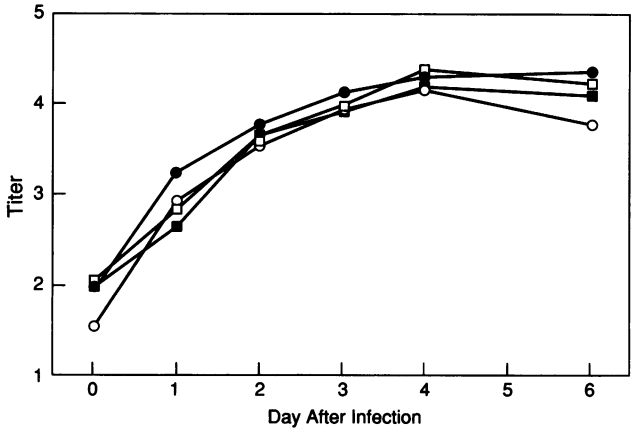

FIG. 4. Growth of Oka and recombinant VZV. MeWo cells were inoculated with VZV-infected cells and aliquots were harvested on days 1, 2, 3, 4, and 6 after infection and titered on MeWo cells. Day 0 indicates the titer of virus in the VZV-infected cell inocula. The experiment was performed in duplicate and the titer (logarithm of the mean number of PFU per dish) at each time point is indicated. ■, Oka; ○, ROka; □, ROka13SA; ●, ROka13SB.

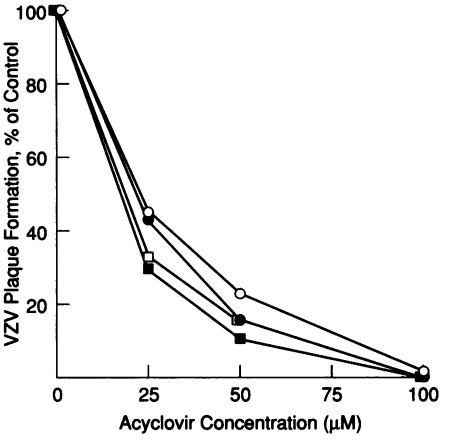

FIG. 5. Growth of Oka and recombinant VZV in the presence of acyclovir. MeWo cells were inoculated with VZV-infected cells in the presence of various concentrations of acyclovir and plaques were counted 8 days after infection. VZV plaque formation is expressed as the percentage of the number of plaques in the presence of acyclovir divided by the number of plaques in the absence of acyclovir. The experiment was performed in duplicate and the mean values are shown. ■, Oka; ○, ROka; □, ROka13SA; ●, ROka13SB.

The absence of viral thymidylate synthetase did not alter the growth characteristics of VZV *in vitro*. In addition to thymidylate synthetase, VZV also encodes a deoxypyrimidine kinase that catalyzes the phosphorylation of thymidine to thymidylate. The presence of two enzymes that promote synthesis of thymidylate in VZV-infected cells has been postulated to be related to the high (54%) A+T content of VZV DNA. Previous experiments showed that the viral deoxypyrimidine kinase gene was not essential for VZV replication *in vitro* (3, 4). Here, we show that the expression of VZV thymidylate synthetase is also dispensable for replication of virus in cell culture.

The VZV thymidylate synthetase gene is one of only five VZV genes that does not have an herpes simplex virus 1 counterpart. The only other herpesviruses known to possess a thymidylate synthetase gene are the animal T-lymphotrophic viruses, herpesvirus saimiri (10) and herpesvirus ateles (11). Herpesvirus saimiri can transform simian and human T cells; the herpesvirus saimiri thymidylate synthetase gene was the only polyadenylylated viral transcript expressed in a T-cell line transformed by the virus (20). In addition, circular episomes of the herpesvirus saimiri genome persisting in transformed T cells lost up to 73% of the coding DNA, but the viral thymidylate synthetase gene was always preserved (21). While VZV has been detected in human lymphocytes during primary viral infection (22), the virus does not transform lymphocytes. Thus, although we have shown that the thymidylate synthetase gene of VZV is not essential for replication *in vitro*, it is unknown whether the gene plays an important role during VZV infection of lymphocytes or other cells *in vivo*.

Our procedure for mutagenesis of the VZV genome has a number of advantages over the previous methods used. (*i*) Our method does not require a selectable marker for isolation of VZV mutants. Previously, VZV mutants were generated by cotransfecting virion DNA with plasmid DNA expressing a foreign antigen; cells infected with mutant VZV were selected by the expression of the antigen (3, 4). The foreign antigen has the potential to alter the phenotype of the resultant virus and could complicate the genetic analysis of the mutant. (*ii*) Our method does not require the laborious process of isolating mutant VZV from the background of parental virus. VZV is highly cell-associated, and it is relatively difficult to obtain cell-free virus for plaque purification.

Since VZV is generated solely from cosmid DNAs, any virus produced by transfection of cells with a mutant cosmid should have a mutation at the corresponding site in the viral genome. (*iii*) In previous studies, the viral deoxypyrimidine kinase (a thymidine kinase) gene was insertionally inactivated and the resulting viruses were presumably resistant to acyclovir. Our method does not alter the viral deoxypyrimidine kinase gene and the recombinant VZV produced remained sensitive to acyclovir (Fig. 5). Acyclovir sensitivity may be important if such viruses are ultimately to be considered as candidate live virus vaccines (see below).

The ability to produce infectious VZV using cosmid DNAs will be useful for studying the biology of VZV. (*i*) Inactivation of individual VZV genes may allow one to determine which gene products are critical for virulence and the establishment of, or reactivation from, latency. While the Oka vaccine strain can protect both healthy and immunocompromised children from chickenpox, it still is capable of reactivating and causing herpes zoster, albeit to a lower apparent frequency than wild-type virus (23). Thus, inactivation of genes that are critical for latency or reactivation could yield an improved vaccine strain that might still be immunogenic but lacks the ability to cause herpes zoster at a later date.

(*ii*) The cosmid DNAs that we have described might be used as a stable repository for the attenuated Oka strain of VZV. Oka VZV can be stored as infectious cosmid DNAs, which upon transfection into cells yields Oka virus. These cosmid DNAs could ensure an unlimited supply of genetically homogeneous live Oka virus for the future.

(*iii*) The VZV Oka strain is the only live DNA virus that is acceptable for use in immunocompromised individuals. Since we have identified a gene that is not essential for VZV replication, other genes encoding viral antigens could be inserted in this site or other sites in the genome; vaccination with the resultant recombinant virus could produce immunity both to VZV and to other viruses.

M. Takahashi provided VZV Oka, B. Tomkinson provided cosmid pDVcosA2, L. Perera provided plasmid pCMV62, J. Hay provided rabbit antibody to VZV ORF10, and A. Chou, N. deWind, H. Moriuchi, M. Moriuchi, T. Russo, and S. Straus provided helpful advice.

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