Recombination plays a critical role in virus evolution. It helps avoid genetic decline and creates novel phenotypes. This promotes survival, and genome sequencing suggests that recombination has facilitated the evolution of human pathogens, including orthopoxviruses such as variola virus. Recombination can also be used to map genes, but although recombinant poxviruses are easily produced in culture, classical attempts to map the vaccinia virus (VACV) genome this way met with little success. We have sequenced recombinants formed when VACV strains TianTan and Dryvax are crossed under different conditions. These were a single round of growth in coinfected cells, five rounds of sequential passage, or recombinants obtained using leporipoxvirus-mediated DNA reactivation. Our studies showed that recombinants contain a patchwork of DNA, with the number of exchanges increasing with passage. Further passage also selected for TianTan DNA and correlated with increased plaque size. The recombinants produced through a single round of coinfection contain a disproportionate number of short conversion tracks (<1 kbp) and exhibited 1 exchange per 12 kbp, close to the ~1 per 8 kbp in the literature. One by-product of this study was that rare mutations were also detected; VACV replication produces ~1 × 10⁻⁸ mutation per nucleotide copied per cycle of replication and ~1 large (21 kbp) deletion per 70 rounds of passage. Viruses produced using DNA reactivation appeared no different from recombinants produced using ordinary methods. An attractive feature of this approach is that when it is combined with selection for a particular phenotype, it provides a way of mapping and dissecting more complex virus traits.

IMPORTANCE
When two closely related viruses coinfect the same cell, they can swap genetic information through a process called recombination. Recombination produces new viruses bearing different combinations of genes, and it plays an important role in virus evolution. Poxviruses are a family of viruses that includes variola (or smallpox) virus, and although poxviruses are known to recombine, no one has previously mapped the patterns of DNAs exchanged between viruses. We coinfected cells with two different vaccinia poxviruses, isolated the progeny, and sequenced them. We show that poxvirus recombination is a very accurate process that assembles viruses containing DNA copied from both parents. In a single round of infection, DNA is swapped back and forth ~18 times per genome to make recombinant viruses that are a mosaic of the two parental DNAs. This mixes many different genes in complex combinations and illustrates how recombination can produce viruses with greatly altered disease potential.
used to genetically modify VACV (14, 15), that this is an accurate process (16, 17), and that this process also operates in trans and can be detected using transfected DNAs (18). Poxviruses replicate in sequestered structures called factories (19), each of which derives from a single infecting particle, and it is presumed that recombinants can form within these factories only if different DNA mix in the presence of the recombination machinery. VACV uses a single-strand annealing mechanism to produce recombinant molecules in a reaction catalyzed by the E9 viral DNA polymerase and I3 single-strand DNA binding protein (20, 21), and since both proteins primarily reside within virus factories, that is presumably where recombination also occurs. We have suggested that random variations in the timing and degree of mixing of virus factories within coinfected cells could explain why recombinant frequencies proved difficult to measure reproducibly (13). If two or more viruses infect any particular cell but a portion of the factories do not mix, such a process would decrease the yield of recombinants in a stochastic manner relative to the number of nonrecombinant (i.e., fully parental) viruses produced by DNA replication.

Although much has been learned concerning the mechanism of poxvirus recombination, questions remain regarding how these processes and physical constraints might affect the patterns of DNA exchange and thus the overall genetic composition of the resulting pool of parental and recombinant viruses. How genetic linkage distances relate to the actual numbers of exchanges in recombinant viruses also remains to be established. In this study, we have used the ~1,400 SNPs that differentiate two strains of VACV, a Dryvax clone and a TianTan clone, as sequence tags that can be used to track the origins of the different DNA segments in recombinant progeny. Our study shows that VACV recombination reactions produce genomes exhibiting a “patchwork” of exchanges, some apparently derived from a succession of crossovers over the course of a single infection cycle. Interestingly, viruses produced using nongenetic reactivation methods (22) appear indistinguishable from recombinants produced in a more regular manner, showing that such viruses are likely subjected to similar replication and developmental pathways.

MATERIALS AND METHODS

Cells and viruses. Vaccinia virus strains DPP17 (GenBank JN654983), TP03 (GenBank KC207810), and TP05 (GenBank KC207811) were cloned from stocks of Dryvax (DPP17) and TianTan (TP03/TP05) viruses (9, 23). They were cultured on BSC-40 cells in modified Eagle’s medium (MEM; Gibco) supplemented with 5% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, and 1% antibiotic at 37°C in a 5% CO₂ atmosphere. Two types of recombinant virus stocks were prepared. The DTM viruses (Dryvax-TianTan mixture) were generated by coinfecting cells with DPP17 and TP05 at a multiplicity of infection (MOI) of 0.02 (each 0.01), culturing the cells for 2 days, harvesting the cell-virus mixture, and releasing the virus by freeze-thawing. A sample (10 μl, 0.5% of the lysate, or ~0.02 PFU/cell) was then used to infect another fresh dish of cells, and this was repeated for a total of five rounds of passage. Individual DTM viruses were then isolated using three rounds of cloning by limiting dilution as described previously (9). The DTH viruses (Dryvax-TianTan high MOI) were produced by coinfecting cells with DPP17 and TP05 at an MOI of 10 (each 5) for 24 h, followed by three rounds of cloning. Plaque images were processed with ImageJ (24).

Virus DNA reactivation. A third collection of recombinant VACV’s were prepared using DNA reactivation reactions as described previously (22). Briefly, Buffalo green monkey kidney (BGMK) cells were grown to near confluence in 60-mm dishes and infected with Shope fibroma virus (SFV) strain Kasza at an MOI of 1 in phosphate-buffered saline (PBS).

<table>
<thead>
<tr>
<th>Amplicon size (bp)</th>
<th>Primer name</th>
<th>Sequence (5’-3’-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,120</td>
<td>DVP-209F</td>
<td>CGAAAGAAGATGATGGGAC</td>
</tr>
<tr>
<td></td>
<td>DVP-226R</td>
<td>ATAAAGGAGAAGGGACAC</td>
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<tr>
<td></td>
<td>DVP-213F</td>
<td>CTTTGGATGATTGTGATA</td>
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<tr>
<td></td>
<td>DVP-226R</td>
<td>ATAAAGGAGAAGGGACAC</td>
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<tr>
<td>1,230</td>
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<td>GACTTGGCATGATATCTT</td>
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<td></td>
<td>DVP-007F</td>
<td>TACCGGCATCAAACAC</td>
</tr>
<tr>
<td>225</td>
<td>DVX-107F</td>
<td>AACTGGAGTAGAGATAGC</td>
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<td></td>
<td>DVX-108R</td>
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<td>208F</td>
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<tr>
<td></td>
<td>209R</td>
<td>ATTCATCCCCGAGCAT</td>
</tr>
</tbody>
</table>

After 1 h at 37°C, the medium was replaced with MEM containing 10% fetal bovine serum and incubated for another hour, and the MEM was replaced with Opti-MEM (Gibco). DPP17 and TP03 VACV DNAs were extracted from sucrose gradient-purified virions using phenol-chloroform and mixed in a 1:1 ratio, and the SFV-infected cells were transfected with 5 μg of this DNA using Lipofectamine 2000 (Gibco). The cells were incubated for 4 h, the Opti-MEM medium was replaced with MEM containing 10% serum, and the cells were cultured for another 3 days. The cells were then subjected to three rounds of freeze-thawing, and 0.2 ml was used to infect BSC-40 cells (which do not support SFV growth). The reactivated VACV was cloned using three rounds of limited dilution, and recombinants were identified using PCR and the primer pairs DVP-209F and DVP-226R plus DVX-004F and DVX-007R (Table 1). The 209F/226R primers should produce a 1.1-kbp ampiclon in reaction mixtures containing DPP17 DNA, whereas TP03 DNA does not serve as a substrate. The DVX-004F/DVX-007R primer pair targets telomeric repeat sequences and should produce 665-bp and 1,230-bp products in reaction mixtures containing TP03 and DPP17 DNAs, respectively. After cloning, the viruses were purified and sequenced as described below. Four DTD clones (Dryvax-TianTan DNA reactivation) were sequenced.

Virus sequencing and genomic analysis. Stocks of 16 DTM clones, 15 DTH clones, and 4 DTD clones were prepared and purified over sucrose gradients. Viral DNAs were extracted and sequenced as described previously (9) using a Roche 454 GS Junior system. Roche GS De NovoAssembler software was used to deconvolute and assemble the raw sequencing data into contigs, and nearly full-length genomes were generated using CLC Genomics Workbench 6. The average read redundancy was 15, which permitted the assembly and mapping of all of the recombinant junctions with confidence. Multiple-sequence alignments were prepared using the program LAGAN (http://genome.lbl.gov/vista/lanag/submit.shtml) (25), and Base-By-Base software (26) was used to produce a visual summary of the whole-genome alignments.

PCR and Southern blotting. Southern blotting was used to confirm the rearrangement detected in clone DTM28. Virus DNA was digested with NdeI (Fermentas) and size fractionated by electrophoresis on 0.7% agarose gels. The DNA was fragmented with 0.2 M HCl, denatured with 0.4 M NaOH, transferred to a nylon membrane, and fixed with a UV cross-linker. Two primers (201F and 239R) (Table 1) and PCR were used to prepare a probe in a reaction mixture containing biotin-16-dUTP (Roche; 1093070), which was subsequently hybridized to the prepared membrane and detected using IRDye 800CW-coupled streptavidin (Li-Cor; 926-32230) and a Li-Cor imager. The DTM28 and DTM28Δ viruses were also differentiated using PCR and 208F and 209R (Table 1) primers. This was done in combination with 201F and 239R in a PCR mixture containing all four primers.
**RESULTS AND DISCUSSION**

**Virus isolation and genome sequencing and assembly.** We used three different methods to produce VACV recombinants. The first method was designed to explore the effects of repeated passage, at a low MOI, on a seed mixture initially composed of just two different genetically tagged viruses. These viruses were originally cloned from stocks of Dryvax (DPP17) and TianTan (TP05) vaccines and differ in sequence by 1 SNP per 140 bp. In contrast to TP05, DPP17 also encodes a 6-kbp deletion near the right terminal inverted repeat (TIR) boundary as well as 150 other smaller insertions and deletions (indels) distributed across the two genomes (Fig. 1). The TP05 strain forms plaques that are approximately twice the diameter of those formed by the DPP17 strain, which provided an opportunity to explore what effect a growth bias might have on the pattern of recombinants.

For this first experiment, a 1:1 mixture of the two different VACVs was used to infect BSC-40 cells at an MOI of 0.02 and cultured for 48 h, and a portion (10^4 or ~0.02 PFU/cell) of the resulting progeny was passaged again under the same conditions. This was repeated to produce a total of 5 rounds of replication. Each time, the infection produced overlapping plaques that partly cleared the entire plate. We then plated out the diluted virus in 24-well plates and identified 36 wells each containing just a single random plaque. These 36 viruses were then cloned again, also by limiting dilution, and designated DTM (Dryvax-TianTan mixture) strains. Using this method minimized the risk of picking certain plaque types, since the only criterion we used to choose a clone was that the virus had to have been diluted to the point where it was the only plaque in a well, in the first round of selection. To avoid the problem of resequencing any nonrecombinant parental strains, PCR and three different primer pairs were used to determine the genetic origin of three different sites within each genome: within the terminal inverted repeat (primers DVX-004F/DVX-007R), in the central part of the genome (primers DVX-107F/DVX-108R), and near the junction with the right terminal inverted repeat (primers DVX-213F/DVX-209F/DVX-226R) (Table 1). Fourteen clones were selected because at least one position was recombinant with respect to either of the other two sites. We also chose two additional viruses, which exhibited a pa-

**FIG 1** Patterns of DNA exchange in recombinant vaccinia viruses. The genome sequences of DTM (A) and DTH (B) recombinant clones were aligned against the parent genomes DPP17 and TP05 using the program LAGAN and edited using the program Base-By-Base. TP05 was used as the reference strain, and any differences between a given virus and TP05 are color coded to indicate insertion, substitution, and deletion mutations derived from strain DPP17. Thus, the blank regions represented fragments derived from TP05.
rental arrangement of markers at these three sites, although these viruses were subsequently determined to also be recombinants. These viruses were cloned two more times, and 16 were sequenced. After sequencing and assembly, these DTM recombinants exhibited a patchy pattern of SNPs suggesting that each virus was the product of approximately 30 exchanges over the course of virus replication.

One expects that when viruses are passed five times under these conditions, the passing should provide an opportunity for repeated rounds of replication and recombination. We also examined what the virus progeny would look like if they were permitted just a single round of infection, although it is expected that this would still involve multiple rounds of replication. To do this, we coinfected BSC-40 cells with DPP17 and TP05 viruses at an MOI of 10 (5 PFU/cell of each virus) and cultured the viruses for just 24 h. These viruses were cloned and designated DTH strains (Dryvax-TianTan high MOI). After the first round of cloning, 43 viruses were randomly selected and PCR was used to identify putative recombinants as described above. Thirteen hybrid viruses were cloned twice more, along with two additional viruses (DTH13 and DTH14) that exhibited a parental pattern of markers at the three positions tested by PCR. DTH14 was subsequently identified as being identical to the TP05 parent virus, while DTH13 proved to be a recombinant. Ultimately, 15 DTH clones were sequenced and assembled as described above. These recombinants exhibited a mean of 18 exchanges per genome.

We should note one caveat regarding these methods: single plaques isolated in the first round of purification were not always pure, and this provided some limited opportunity for additional rounds of replication and recombination. For example, when a plaque initially identified as recombinant DTM22 was cloned a second time and the subsidiary plaques were reanalyzed by PCR, it was realized that the two daughter plaques (DTM22.1 and DTM22.2) were not identical. However, they are clearly "sibs," viruses sharing a common genetic origin as judged by a shared pattern of exchanges in the center of the two genomes (Fig. 1). We also noted one case in which a single apparently recombinant starting plaque resolved into two clearly unrelated recombinant clones upon replating (clones DTH10 and DTH10.2) (Fig. 1). For simplicity, our analysis has treated these particular clones as being the same as the other recombinants isolated in the study, although they may have experienced some additional limited opportunities to undergo recombination.

Crossovers in DTM and DTH viruses. After assembly, the sequences were aligned with program LAGAN and the alignment was corrected manually using Base-By-Base. Inspecting these sequences, we could readily identify the origin of each SNP-tagged segment of DNA as belonging to either the DPP17 or TP05 parent (Fig. 1). What was remarkable was the very low frequency of observed mutation even though numerous SNPs and small indels commonly differentiate clones isolated from a viral stock like Dryvax (9). No mutations were detected in any of the DTH clones, compared with the two parent viruses, and just two mutations were detected in two of the DTM clones. One was a small deletion in DTM29 at alignment position 900, which removed 2 nucleotides (nt) (Fig. 2A) just 6 nt upstream of the ORF001 start codon. Although most small deletions are associated with repeats (9, 27), this event was not. It was located immediately adjacent to an SNP that differentiates DPP17 from TP05. We also discovered a point mutation in DTM27 at alignment position 70493 (Fig. 2B). This causes a C-to-T transition mutation and an alanine-to-valine substitution in gene DVX_088 (RNA helicase). A crude estimate of the VACV replicative error rate can be calculated from the following observations and assumptions. We note that there were only two independent mutations detected in 16 DTM viruses over the course of 5 rounds of infection, and there are ~200,000 nt copied per genome per each round of infection. Each round of infection typically expands the VACV titer ~10,000-fold (i.e., between 2^{14} and 2^{15} doublings of the genome), and thus, the error rate is very crudely estimated as 2/(16 × 200,000 × 5 × 14.5) or ~1 × 10^{-8} mutation per nucleotide copied per cycle of replication. Alternatively, this is 2/(16 × 5 × 14.5) or ~1 mutation per 600 genomes per cycle of replication. By a similar method, the absence of any mutations detected among the 15 DTH clones over the course of a single round of infection suggests an error rate of <5 × 10^{-8}. The VACV E9L gene encodes a typical B-family proofreading DNA polymerase of a type encoded by a variety of viruses and bacteriophage, and this error frequency resembles that reported for phage (28). Although some drug-resistant E9L alleles cause altered spontaneous mutation rates in vivo (29–31), for comparison purposes these cannot be converted into absolute mutation rates given the uncertainties in the size or number of the genetic target(s). We could find no other reported absolute error rates for poxviruses in the literature.

Beyond these two rare mutations, the remainder of the sequences in the recombinant genomes could be ascribed to having been inherited from one or the other of the two parent viruses. In total, 1,399 SNPs (single nucleotide polymorphisms) can be used to differentiate DPP17 from TP05, and we used these SNPs to track the origins, and thus the sites of crossing over, in the hybrids. The relative abundance of these variant sites (1,399 scattered across ~200 kbp) allowed us to map the site(s) of crossing over with an average resolution of ~140 nt. In general, each hybrid virus encoded variable-length blocks of DNA derived from each of the two parent strains, and no uniquely conserved block (a hallmark of a highly selected patch of DNA) was detected in all of the viruses. The lengths of these blocks of recombined sequences varied, ranging from one to several hundred SNPs. We detected none of the large gene duplications that have been described by other authors (32, 33) but would not have expected to do so given that these structures are stable only in the presence of strong selection pressure.

To examine the pattern of crossing over in greater detail, we used Base-By-Base software to produce a table ascribing each of
of experimental factors (13), from our measurements of the con-

this number is difficult to estimate with precision due to a number

infection (akin to the method used to produce the DTH viruses),

half-maximal recombination was detected in a single round of

map of the virus. From these studies we derived an estimate that

and attempted to correlate these data with the known physical

analysis

14 to 44 (mean ± standard deviation [SD] = 30 ± 11)

results of this analysis. We had expected that viruses given

more opportunities for recombination would exhibit a greater

number of exchanges, and this was supported by these measure-

ments. The number of crossovers in the DTH viruses ranged from

numbers of exchanges suffered by each of the recombinant

viruses, we also examined the lengths of the DNA segments ex-

changed between viruses (i.e., the conversion track length) in the

DTH group. To do this, the calculation assumed that the start and

end of each exchange lay midway between the SNPs flanking the

two sites of exchange. The resolution of the method varies de-

pending upon the local SNP density, but with an average of 1 SNP

per 140 bp, we could detect exchanges ranging in size from 55 to

92,000 bp. An interesting feature of VACV recombination is illus-

trated by this analysis, which showed that there were relatively

more short conversion tracks than long ones (Fig. 4). Thus, while

the mean length of a conversion track was 12 kbp, the median was

only 2.6 kbp. The abundance of short conversion tracks would

help favor intragenic recombination events, which can be detected

between markers spaced only 54 bp apart (34). We should note that

this estimate of the recombination frequency is lower than has been

previously reported. For example, we detected a loss of linkage at

distances exceeding 350 bp in one study (35). However, this ear-

lier experiment measured the yield of recombinants when DNA

was transfected into Shope fibroma virus-infected cells, and it is

possible that the nonspecific DNA replication that is seen under

these circumstances (36) also exposes transfected DNAs to higher

levels of recombination than is normally experienced by

viruses.

Crossing over is not the only process that could produce this

abundance of short exchanges. Poxvirus replication and recombi-

nation reactions also produce hybrid (or heteroduplex) DNA

(37). Such molecules would contain mismatched bases wherever

the sequences differ, and if a subset of mismatches were subjected

to the distances between most VACV markers. When one consid-

ers that our estimate of 1 crossover per 12 kbp is associated with a

standard deviation of 19 kbp (i.e., 12 ± 19 kbp), the source of this

problem is clearly apparent.

Average length of the conversion tracts. Besides measuring the

numbers of exchanges suffered by each of the recombinant

viruses, we also examined the lengths of the DNA segments ex-

changed between viruses (i.e., the conversion track length) in the

DTH group.
paired and that all G·T mismatches are converted to G·C prior to replication, then these single marker exchanges should be biased 2:1 in favor of forming (or retaining) a G or C. We detected 51 single exchanges at sites containing base substitutions (82% of all single exchanges) in the DTM and DTH viruses. Of these, 29 retained a G or C and 22 retained an A or T, which is not significantly different from a 1:1 split ($\chi^2 = 0.96; P = 0.33$). Although we cannot disprove the hypothesis that biased mismatched repair created some of the short conversion tracts, the simplest explanation for these data is that poxvirus recombination reactions produce an abundance of short conversion tracks through a process formally akin to crossing over.

**Biased genetic origins in progeny viruses.** An interesting difference between the TianTan and Dryvax clones used in this study is that TP05 forms plaques twice the size of DPP17 plaques on BSC-40 cells. We wondered how this phenotype might segregate among the recombinants deriving from either the DTH or DTM crosses. We used the SNPs to determine what fraction of each genome derived from TP05 or DPP17 and plated all of the cloned viruses on BSC-40 cells, at the same time, to determine the average plaque size. The DTH viruses, passed just once, showed no particular compositional bias, comprising about equal portions (50% ± 27%) of each of the parental viruses (Fig. 5A). In contrast, the DTM hybrids bear a diminished (19% ± 11%) fraction of the genome derived from DPP17 SNPs (Fig. 5A). Oddly, there seems to be a simple linear relationship between plaque size and the proportion of the genome derived from each parent strain, with larger plaque sizes associated with a greater proportion of TP05-derived DNA (Fig. 5B). These data suggest that the TP05-derived DNA may confer a selective growth advantage in multiple rounds of culture (i.e., DTM viruses), but one round of growth (i.e., DTH viruses) provides insufficient time or selective pressure to bias the composition of the recombinants.

What would produce this effect is not clear. Plaque size is likely determined by many different genetic factors, and there are many differences in the gene compositions of the two parent strains. For example, DPP17 contains a large deletion in the right TIR compared to TP05, and this deletion bears a number of different genes (Table 2). However, this deletion is not completely responsible for plaque variation, since DPP25, containing all the genes in this region, forms plaques only slightly larger than DPP17 (9). We subsequently annotated all of the hybrid genomes using GATU (39) and evaluated the differences (Table 3). There are many mutant genes segregating in complex ways between the different viruses, including mutant forms of I4L (40), F3L (41), E5R (42), M11C, A51R, and C23L, but no obvious distribution patterns could be discerned by inspection beyond the fact that the more presumably functional genes the virus showed (Table 3, gray cells), the

![FIG 5 Biased selection for sequences associated with the TianTan parent. The percentage of DNA derived from each of the parental viruses was determined from the fraction of SNPs derived from each parent. Panel A shows how the composition varied in viruses passaged just once (DTH hybrids) or five times (DTM hybrids) prior to cloning. Passage appeared to select for SNPs linked to the TP05 TianTan parent, as the percentage of Dryvax DNA decreased from 50% ± 27% to 19% ± 11% with continued passage. Panel B illustrates how the plaque size is related to the genetic origins of the hybrid. The viruses forming smaller plaques more closely resemble the DPP17 parent. To measure the plaque size, each of the cloned hybrids was plated on BSC-40 cells (in parallel), cultured for 2 days, stained with crystal violet, and scanned, and the plaque area was determined using ImageJ (24). Twenty randomly selected plaques were measured for each virus.](http://jvi.asm.org/)

<table>
<thead>
<tr>
<th>Protein or feature</th>
<th>Gene</th>
<th>Nucleotide length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1-β receptor</td>
<td>Cop-B16R</td>
<td>DVPX_209</td>
</tr>
<tr>
<td></td>
<td>Cop-B17L</td>
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</tr>
<tr>
<td>IFN-α/β receptor</td>
<td>Cop-B20R</td>
<td>DVPX_213</td>
</tr>
</tbody>
</table>
better it grew. The nontranscribed repeats in VACV telomeres cannot be assembled into contiguous sequences, due to the redundancies in the repeats, but some of the fragments of junction sequences differ enough in TianTan and Dryvax to deduce the origins of the telomeres. This analysis detected a trend suggesting that viruses bearing TP05-like telomeres also form larger plaques. However, all that could really be concluded from this analysis is that TianTan-derived sequences generally contribute greater advantage in culture than does Dryvax DNA.

**Large deletions formed through illegitimate recombination.**

Poxviruses are also known to suffer deletion mutations during passage. The most extreme example is probably modified vaccinia virus Ankara (MVA), which accumulated six large deletions, and many smaller ones, when it was passaged >570 times in chicken embryo fibroblasts (43). Over the course of these experiments, we did detect one such large deletion mutation when we sequenced clone DTM28. The deletion spans 21 kbp and encompasses gene DVX_201 to DVX_239 (Fig. 6). In the initial assembly, we found 11 sequence reads that started in gene DVX_201 and terminated in gene DVX_239 (Fig. 6A), along with sequence reads derived from all of the intervening genes. The deletion spans the right TIR boundary, but among the reads were some from the unique genes DVX_202 to DVX_209, suggesting that we had sequenced a stock of virus containing the DTM28 parent contaminated by a virus bearing the deletion (DTM28Δ). To confirm this interpretation of the data, we prepared primers 201F and 239R (which are located 21 kbp apart in genes DVX_201 and DVX_239, respectively [Fig. 6B]) and used PCR to detect the novel 1.2-kbp amplicon that was predicted to be formed in this process (Fig. 6C). We also tested DNAs extracted from the virus stocks that had been archived during the process of passaging these viruses 5 times, before cloning, as well as DTM27, another independent clone that was purified in parallel. Only the purified DTM28 stock contained a virus bearing the deletion (Fig. 6C), suggesting that DTM28Δ arose during the expansion of the stock. Finally, we used the 1.2-kbp amplicon to probe a Southern blot of NdeI-cut virus DNA and showed that the DTM28 stock contains viruses contributing a 6.7-kbp band characteristic of the deletion-containing fragment as well as a 5.4-kbp band, which derives from the two 5.4-kbp NdeI fragments that encode the boundaries of the deletion (Fig. 6D). We subsequently subcloned this stock and separately isolated the two viruses, confirming the viability of DTM28Δ and the fact that none of the deleted genes are essential (Fig. 6E) in cell culture.

The DNA surrounding the vaccinia virus right TIR boundary is a well-established hot spot for large deletion mutations (23). The mechanism is probably the same as that which drives the formation of small deletion mutations, starting with the misalignment of regions containing imperfect repeats (9, 27). If one aligns the reads spanning the junction boundary between DVX_201 and DVX_239, one sees several small blocks of homology (Fig. 6A, boxed) that could have stabilized the first step in an illegitimate recombination reaction. It is difficult to establish an exact rate by which such mutations arise, but this stock was plaque purified three times, following bulk-up, and only 1 virus in 16 DTM viruses passaged in parallel suffered a deletion of this type. This creates a rate of ~0.06 deletion per 4 passages, or 1 deletion per 70 passages. The six large deletions introduced into MVA over 570 passages are thus quite consistent with this estimate, although, of course, the selection pressures were very different in the two experiments.

**Recombination in SFV-reactivated vaccinia viruses.** A third small collection of recombinant viruses was also produced using Shope fibroma virus-mediated DNA reactivation assays and DNAs extracted from DPP17 and TianTan strain TP03 (22). (We used TP03, instead of the TP05 used for the preceding experiments, to test whether viruses could also be produced containing all three of the large TP03 and DP17 telomeric deletions.) This method relies upon a replicating helper virus (SFV) to rescue or “reactivate” fragments of transfected virus DNA (VACV). The SFV is subsequently eliminated by passage on a cell line that supports only VACV growth. Figure 7 shows the maps of the viruses that were recovered by this method. There were just four viruses obtained, and two (DTD03 and DTD11) are so similar that they are probably “sibs” sharing a mostly common history. These viruses were too few in number, and the passage history too complicated, to derive much in the way of statistics about recombination patterns, but the pattern of exchanges generally resembled the lesser numbers and longer conversion tracks seen in the DTH viruses. The method did also produce clones bearing the three large telomeric deletions (DTD03 and DTD18) (Fig. 7), which left the virus with TIRs just 7.3 kbp long. An important caveat is that the DTD viruses were recovered from cells that had been transfected for a few hours and then incubated for 3 days, so whether

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**TABLE 3 Gene complements in parent and hybrid viruses**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Plaque area (%)</th>
<th>Telomere</th>
<th>Right Telomere</th>
<th>TIR</th>
<th>F4L</th>
<th>C23L</th>
<th>M1L</th>
<th>F3L</th>
<th>ESR</th>
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<td>D</td>
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<td>101</td>
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<td>D</td>
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* D, DPP17-like telomere repeats; T, TP05-like telomere repeats. A minus sign indicates that the gene or genes are truncated or deleted. Shading indicates that the gene appears intact.
the recombinants were produced during the reactivation stage or during subsequent rounds of reinfection and replication is difficult to deduce.

Overall, there were no strikingly unique features of these reactivated viruses that would differentiate them from any other type of recombinant poxvirus. Perhaps the most important conclusion that could be drawn from this brief study is that this process is very accurate (no mutant viruses were recovered), and no Shope fibroma virus DNA sequences were detected in any of the reactivated VACVs. The two most similar genes in SFV and VACV are S068R and J6R, respectively (44), which share only 73% nucleotide sequence identity, with no blocks of perfect alignment of >17

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**FIG 6** Illegitimate recombination detected during the cloning and sequencing of hybrid DTM28. During the sequencing of DTM28, 11 reads were detected linking gene DVX_201 to gene DVX_239. Panel A shows an alignment of these reads to sequences within the two genes, which are normally spaced 21 kbp apart. We have also identified sequence identities (circles), short patches of homology (boxed), and a simple repeat (underlined) common to sequences flanking the fusion site. The sequence in these reads transitions cleanly from one gene to the next, with no evidence of any unrelated additional sequences having been added in the process. Panel B showed a way to form this deletion. Illegitimate recombination between identical parents (DTM28) excised 21 kbp and created the virus we subsequently called DTM28Δ. Panel C shows the results of a PCR analysis using primers targeting sites flanking the fusion site. These are located too far apart in the parent viruses (e.g., DTM27) to amplify 21 kbp of intervening sequence. The DTM28Δ virus was probably formed during the expansion of the clone prior to sequencing, as it is not detected in intermediary viruses during the course of passages. Panel D shows a Southern blot of NdeI-digested virus DNA showing that the sequenced virus stock contained two viruses. These are the DTM28 hybrid (indicated by a 5.4-kbp fragment common to both parent strains), and DTM28Δ (indicated by a 6.7-kbp fragment containing the fusion junction). Panel E shows that DTM28Δ is independently viable. Six randomly selected viruses were separately plaque purified from the sequenced stock, and PCR was used to detect sequences found only in the deleted region in DTM28 (primers 208F + 209R) or capable of being amplified only if the intervening sequences are deleted (primers 201F + 239R).

**FIG 7** Patterns of DNA exchange in recombinant vaccinia viruses produced using leporipoxvirus-mediated reactivation reactions. The genome sequences of the DTD recombinant clones were aligned against the parent genomes DPP17 and TP03 using the program LAGAN and edited using the program Base-By-Base. Because this experiment used TP03 DNA, and TP05 was always used as the reference strain in all of our analyses (Fig. 1), the telomeric deletion mutations that differentiate TP03 from TP05 show up as additional red blocks in the TP03 alignment.
There is even less similarity between VACV and fowlpox virus, another virus that has also been used to reative orthopoxviruses (45). This is probably insufficient sequence similarity to support frequent recombination between the helper and reactivated viruses. Additionally, VACV hybrids may well be rare and difficult to isolate in the absence of selection, if not simply inviable. Such data support the long-standing suspicion that using a heterologous helper virus, like SFV or fowlpox virus, to reactivate orthopoxviruses can be done without mutation and does not produce hybrid strains.

Conclusions. Next-generation DNA sequencing technologies are greatly improving our understanding of the genome structures and genes in large DNA viruses. Here we show that these methods can also be used to characterize the structures of recombinant poxviruses. These studies show that recombinant VACVs are not surprisingly, composed of a patchwork of DNA fragments derived from the parent viruses. The numbers of exchanges varies depending upon the passage history, but if one uses methods like those classically used to produce VACV recombinants (a high multiplicity of infection [10] and 1 day of cocultivation), one detects about 1 physical crossover per 12 kbp in the DTH viruses, a number only slightly higher than the ~8 kbp we have estimated from a review of the older genetic literature. However, there is a lot of noise observed in this number (12 ± 19 kbp), perhaps explaining why accurate classical recombination maps were never produced for VACV.

Interestingly, the lengths of the recombinant patches (i.e., the conversion tracts) are heavily biased toward shorter sizes, something that would favor intragenic recombination. What mechanism would produce such an effect is difficult to identify, although we have previously used genetic methods to show that VACV replication and recombination are intimately linked processes (18, 46), probably because the VACV E9 DNA polymerase exhibits properties characteristic of a recombinase both in vitro (21) and in vivo (20). Thus, recombination may just be an indirect byproduct of virus replication, conceivably associated with the DNA polymerase-catalyzed repair of broken replication structures. Regardless of the mechanism, this process could have interesting genetic consequences for virus evolution, as it would create a lot of diversity within recombinant genes, not just diverse combinations of different genes. This becomes of critical importance when one considers the challenge posed to viruses by rapidly evolving responses to biological features like immunodominant epitopes. Short conversion tracks offer a selective advantage for a virus, as they provide a mechanism for rearranging and eliminating peptide epitopes while still retaining gene function.

These studies also show how sequencing could be used to characterize more complex virus traits than those regulated by single genes. Continued passage of the DTM viruses selected for viruses bearing greater proportions of the TianTan genome, and this was considered in some still-unclear manner to plaque size. By producing recombinants, applying a selection strategy (perhaps in an iterative manner), and then sequencing clones bearing the desired traits, it should be possible to map genes that collectively regulate the phenotype of interest. This is not a novel approach, of course; related methods have been used for decades to map complex genetic traits in many different organisms. However, the widespread availability of next-generation sequencing technologies creates a tool that could easily be used by many more laboratories studying gene families and gene interactions in large DNA viruses.

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