Virology 442 (2013) 59–66

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Genomic analysis of vaccinia virus strain TianTan provides new insights into the evolution and evolutionary relationships between Orthopoxviruses

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ARTICLE INFO

Article history:
Received 10 January 2013
Returned to author for revisions
24 February 2013
Accepted 21 March 2013
Available online 19 April 2013

Keywords:
Vaccinia virus
TianTan
Complete genome
Orthopoxviruses
Vaccinia epidermal growth factor

ABSTRACT

Vaccinia virus (VACV) strain TianTan was used for much of China's modern history to vaccinate against smallpox, however the only genome sequence contains errors. We have sequenced additional examples of TianTan to obtain a better picture of this important virus. We detected two different subclones. One (TP03) encodes large deletions in the terminal repeats that extend into both VEGF genes and create a small plaque variant. The second clone (TP05) encodes a nearly intact complement of genes in the terminal repeats, except for an insertion of sequences resembling the telomeric 69 bp repeats. The TP05 genome spans 196,260 bp and encodes 219 genes. The revised sequence documents the integrity of all the genes in the conserved virus core. Phylogenetic methods show that TianTan belongs to a unique clade of VACV, but probably also share a common origin with strains belonging to the Copenhagen/Lister lineage and distinct from the Wyeth/Dryvax lineage.

Introduction

The Chinese National Epidemic Prevention Bureau was created in 1919 with a mandate to produce a smallpox vaccine using a seed stock of Japanese origin. In 1924 an employee, Mr. Qi Changqing (Fig. 1), was sent to Japan to study the technique for vaccine production. After his return home in 1926, Mr. Qi is reported to have isolated a sample of virus from a smallpox patient and then passaged it on the skin of monkeys, rabbits and cows. His virus, which acquired the name "TianTan" after Beijing's Temple of Heaven where the specimen was collected and the Central Epidemic Prevention department was initially housed, became the smallpox vaccine of choice for most of China's modern history (Dong, 2009; Qing, 2006). Its use spanned a period of manufacture in rabbits and cows from 1926 to 1954, a brief production hiatus while a Russian strain was used from 1955 to 1960 and then a return to production from 1960 to 1980, when smallpox was declared eradicated. Through the last part of this period (1965–1980) the vaccine was produced in chicken eggs (Zhao, 2007). Although no longer used much as a smallpox vaccine, TianTan virus is still widely used by Chinese researchers as a vaccine vector.

Mr. Qi is rightly remembered for his discovery of the virus that played such an important role in helping to eradicate smallpox in China. This is a history that spans many turbulent decades, and includes the remarkable story of how Mr. Li Yanmao later preserved the TianTan strain at great personal risk during the Japanese attack on Beijing in 1937. However, this history lacks scientific credibility, as variola virus (the causative agent of smallpox) does not exhibit a host range encompassing monkeys, rabbits, or cows (Massung et al., 1994). Based upon the DNA sequencing done to date, TianTan is clearly a vaccinia virus (VACV) and phylogenetically distinct from other Orthopoxviruses like variola, horsepox, and monkeypox. Most probably, the sample was either isolated from an individual exhibiting one of the rarer complications of smallpox vaccination (Bray, 2003), or contaminated with another strain being propagated at the time in the Center for Disease Prevention. Considering the difficulties associated with virus culture in the 1920s, and the fact that the origins of even modern viruses continues to generate controversy (Crewdson, 2002), the later explanation seems more probable. Dairen (a name of Chinese origin) and Ikeda (a Japanese name) are some of the oldest VACV strains once used in Japan (Dr. I. Arita, personnel communication), and whether they are related to TianTan would be interesting to determine.

Given the critical role that TianTan virus has served in eradicating smallpox in China, it is unfortunate that the only available complete genome sequence (Jin et al., 1997) comes from an era when DNA sequencing was far more difficult, and thus it contains a number of sequencing errors. These include at least nine frameshift mutations in what are generally believed to be essential genes (Upton et al., 2003). Because of the errors, these sequence data are often avoided in comparisons with other Orthopoxviruses. To address this concern,

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0042-6822/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2013.03.025

we have taken advantage of next generation sequencing technologies to produce more accurate genome sequences for two clonal Wuhan laboratory strains of TianTan virus, which come originally from the National Vaccine and Serum Institute. Besides providing an accurate sequence for one of the most historically important smallpox vaccines, we also describe a curious pattern of deletions and rearrangements, which may account for some of the unique properties as a vaccine. This pattern of genome rearrangements also provides further insights into the evolution of Orthopoxviruses.

Results and discussion

Virus isolation and genome sequencing and assembly

We started by randomly selecting 24 different viruses from a stock of VACV strain TianTan and plaque purifying them on BSC-40 cells. After being cloned and plated, these viruses produced two kinds of plaques. Viruses of the more abundant TP03 type (21/24 clones) formed smaller plaques with an area about half that of viruses of the TP05 type (3/24 clones). Fig. 2 illustrates this property of some representative viruses. A single step growth curve, prepared using BSC-40 cells infected with virus at a low multiplicity of infection (MOI=0.01), showed that the small plaque variety (TP03) also grew slightly slower and to lower titers

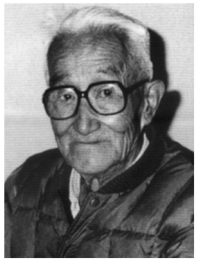

Fig. 1. Qi Changqing (1896–1992). Mr. Qi discovered and isolated the TianTan strain of vaccinia virus in 1926. The photograph was taken circa 1988 and was kindly provided by Mr. Geng Jia, Yunnan Daily.

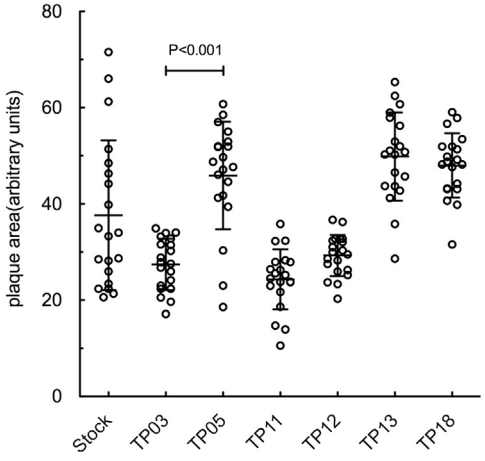

Fig. 2. Plaques formed by viruses cloned from a stock of TianTan virus. Each of the plaque-purified viruses was separately plated on BSC-40 cells, cultured for 2 days under liquid overlay, fixed, stained with crystal violet, and photographed. ImageJ (Schneider et al., 2012) was then used to measure the sizes of 20 plaques per virus, randomly selected from each dish. Viruses of the TP05 type form plaques that are significantly ($P<0.001$) larger than viruses of the TP03 type and the original stock appears to contain a mix of both large plaque and small plaque viruses. Neither of these two strains of TianTan virus produces high levels of extracellular enveloped virus, as judged by the lack of "comets" that arise through secondary infections in liquid overlay (not shown).

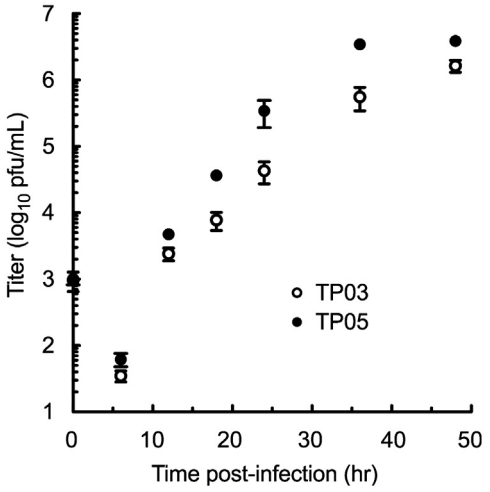

Fig. 3. Small plaque TianTan strains grow to lower titers in culture. BSC-40 cells were infected with the indicated viruses at MOI=0.01, incubated at 37 °C, harvested by freeze-thaw, and the yield of virus determined by titration on BSC-40 cells. The TP03 viruses grow somewhat more slowly, and to lower titers, than the larger plaque forming TP05 strain. The difference between the two data sets is statistically significant. Beyond the 10 h time point, TP05 produces ~5-fold more virus than TP03 ($P=0.007$, 95% CI=1.9–8.6 fold, 2-tailed ratio t test).

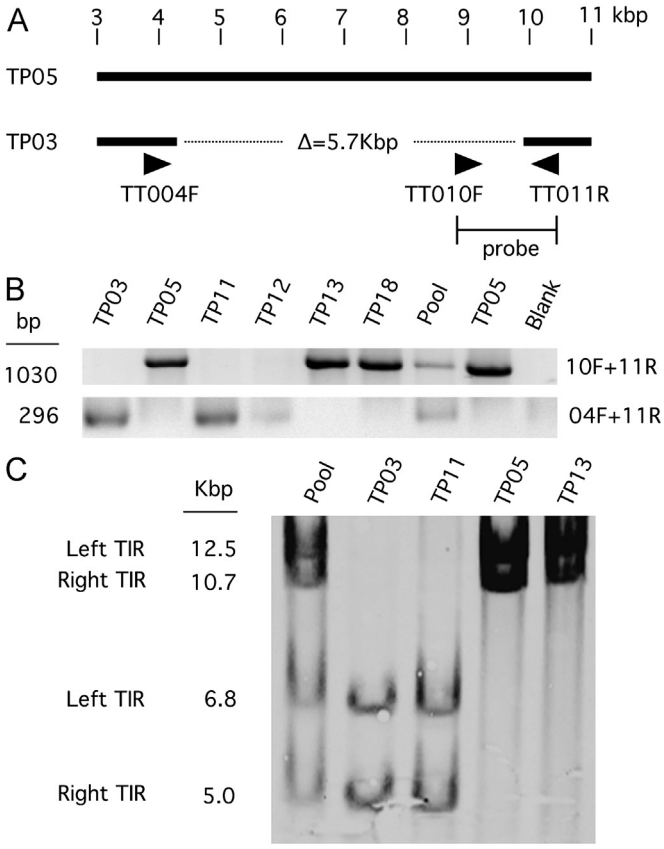

Fig. 4. PCR and Southern blotting methods confirm the presence of telomeric deletions. Panel A shows the PCR primer binding sites relative a map of the TP03 and TP05 left telomeres. The TT004F and TT011R primer pair are located sufficiently far apart (on the TP05 genome) that they can serve as PCR primers only if the distance between the primers is reduced by the deletion in TP03. The deletion eliminates the TT010F binding site, thus preventing any DNA amplification using primers TT010F and TT011R. Panel B shows that clones TP03, TP11, and TP12 encode the 5.7 kb deletion. Panel C shows a Southern blot of *Sca*I digested VACV DNAs. The probe encodes DNAs spanning the right side of the deletion boundary (Panel A). The deletion of 5.7 kb greatly shortens the *Sca*I fragments encoding the left and right TIRs in clones TP03 and TP11.

than did the large plaque variety (TP05) (Fig. 3). This suggested that our stock contained at least two variant forms of viruses.

To explore this question we sequenced 12 of the viruses (clones designated TP01-to-TP06 and TP08-to-TP13) and assembled the genomes using standard methods (Qin et al., 2011). Interestingly these TianTan clones all seemed to be essentially identical except for symmetrical 5.7 kbp deletions located in the telomeric inverted repeats (TIRs) in viruses of the small plaque TP03 type. To confirm that this was not an artifact of the assembly algorithm, we used the PCR and Southern blotting methods to examine the DNA structure in this region of the genome (Fig. 4). These methods provided independent evidence of 5.7 kb deletions in clones TP03, TP11, and TP12 and the presence of the correspondingly larger fragment in clones TP05, TP13, and TP18. The small plaque phenotype correlated perfectly with the presence of the deletions (Fig. 2). As would have been expected, we also noted that the unpurified stock of virus contained viruses of both types (Fig. 4).

These mutations delete or inactivate several genes of mostly still unknown function from the small plaque variants (Table 1). The deletion of the homolog of the Copenhagen strain C12L gene from clone TP03 (TT_009 in clone TP05) excises the SPI-1 serpin gene, but that is not expected to alter the growth of the virus in culture as suggested by studies using SPI-1 mutants of VACV strain WR (Kettle et al., 1995). However, it is striking that these mutations would delete the promoters and N-termini of both copies of the epidermal growth factor gene homolog from TP03 (TT_011 in clone TP05). Depending upon the cell type and the growth state of the host cells, deleting the VACV epidermal growth factor gene (VEGF) can affect the growth of VACV in culture (Buller et al., 1988), and it is likely the major reason why the TP03-like clones produce smaller plaques than the TP05-like clones. Interestingly several different variants of a VACV strain called "vvDD", containing a double deletion of the thymidine kinase and VEGF genes, have been tested for more cancer-specific oncolytic activity (McCart et al., 2001). The TP03-like TianTan viruses seem to have naturally anticipated an element of this strategy (i.e. deletion of the growth factor genes).

Table 1
Genes located in a 5.7 kbp deletion in TianTan clone TP03 terminal inverted repeats.

Gene function or feature	Copenhagen gene number	TianTan TP05		TP03
		Gene number	Size (bp)	Size (bp)
Ankyrin	C17L/B23R	TT_005	528	0
Unknown	C16L/B22R	TT_006	546	0
Unknown	C15L/B21R	TT_007	264	0
Unknown	–	TT_008	156	0
Unknown	C14L	TT_009	573	0
Serpin (SPI-1)	C12L	TT_010	1062	0
Epidermal growth factor	C11R	TT_011	426	253[a]

[a] C-terminal fragment.

The genome of TianTan clone TP05

The TP03-like variants are probably an artifact of passage in culture, which likely occurred sometime after the Wuhan strains were plaque purified in the 1990s. We therefore focused our attention on characterizing the larger TP05-like isolates, which aside from the 5.7 kbp insertions relative to the TP03-like viruses, are nearly identical. (Other differences between TP03 and TP05 include four single-nucleotide substitutions and three small insertion/deletions or indels.) In our final assembly TP05 spans 196,260 bp and encodes 219 open-reading frames, including the genes duplicated in the TIRs. The TIRs comprise approximately 12 kbp each and duplicate 12 genes.

The revised sequence of TianTan clone TP05 validates and extends some of the concerns expressed previously (Upton et al., 2003), in that a number of genes that are typically found intact in vaccinia virus stocks contain frameshift mutations in the original VACV TianTan genome sequence (referred to as "TT00" herein). However, not all the sequence differences that we have identified are necessarily a result of sequencing errors in TT00. We noted several small indels, where the longer sequence encoded two flanking and duplicated copies of a sequence found only once in the shorter sequence (Fig. 5A). These are typically unstable sites in poxviruses (Coulson and Upton, 2011; Qin et al., 2011) and likely reflect real differences between different clonal isolates. Where these mutations create in-frame deletions, they characteristically alter the lengths of the encoded proteins by only a few amino acids. We also noted a great many single-nucleotide polymorphisms (SNPs); far too many to be explained as sequencing errors and in numbers (~0.5% differences between TT00 and TP03/05) consistent with these viruses being different clonal isolates of an original vaccine stock. Table 2 summarizes the genes that exhibit significant size differences between the two sequences. It also highlights some small open-reading frames that were not previously annotated, but are now recognized as true genes due to their conservation across many different strains.

We also noted that TP05 differs significantly from TT00 in that much of the sequences comprising the TT00 TIR have been excised by a deletion spanning the homologs of the DVX002-12 genes. Unlike the 5.7 kbp deletion in the TP03 clone, the larger deletion

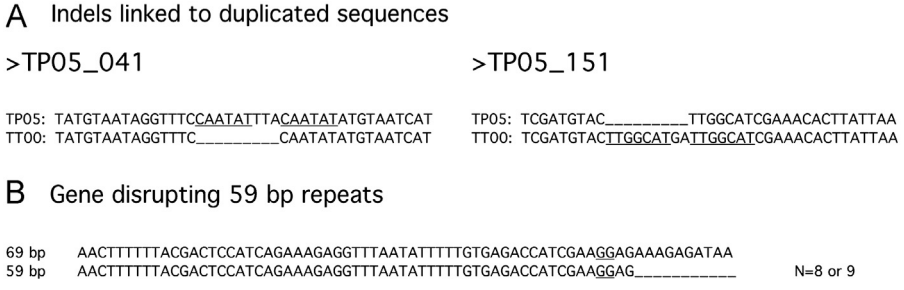

Fig. 5. Special sequence features associated with mutations in TianTan strains. Panel A illustrates two of the small indels that differentiate the original TianTan genome sequence (TT00) from clone TP05. These indels in VACV strains are typically flanked by small duplications (underlined). Panel B illustrates the 59 bp repeats, which disrupts genes in the left and right TIRs of clones TP03 and TP05. The upper row shows the sequence of the 69 bp repeats found elsewhere in the telomeres of VACV strain TT00 (Genbank accession AF095689), the lower row shows the 59 bp repeats which disrupt genes in the TP03 and TP05 clones. Eight or nine copies of the 59 bp repeat are found in different TianTan clones. Note that VACV strain WR encodes an extra "A" between the two underlined "G's" in a 70 bp repeat (Baroudy et al., 1982). Many of our sequencing reads detected fragments of sequence derived from the virus telomeric repeats, and also encoding the 69 bp repeats reported previously (Jin et al., 1997).

Table 2
Gene differences between TianTan strains TP05 and TT00 (Genbank accession number AF095689).

Gene function or feature	ORF[a] number (TP05)	Size (bp)	
		TP05	TT00
Chemokine-binding protein (Cop-C23L/B29R)	TT_001 TT_002	318 414	735 –
EGF growth factor (Cop-C11R)	TT_011	426	423
Ubiquitin ligase (WR012/207)	TT_014	189	156
Ankyrin-like protein (WR015)	TT_018	399	348
Ankyrin-like protein (WR017)	TT_020	216	231
α-Amanitin sensitivity (Cop-N2L)	TT_033	528	291[b]
Ankyrin (Cop-M1L)	TT_034	1413	1419
NFκB inhibitor (Cop-M2L)	TT_035	663	591
Ankyrin/NFkB inhibitor (Cop-K1L)	TT_036	855	570
Putative monoglyceride lipase (Cop-K5L)	TT_040	366	405
Putative monoglyceride lipase (Cop-K6L)	TT_041	255	246
Cytoplasmic protein (Cop-F8L)	TT_050	198	153
IEV associated (Cop-F12L)	TT_054	1908	1095[b]
IMV protein (WR053.5)	TT_057	150	150[c]
IFN resistance/PKR inhibitor (Cop-E3L)	TT_063	573	342[b]
Virosome component (Cop-E5R)	TT_065	1026	774
Putative ORF (Cop-E5R fragment)	–	–	321
F10L kinase substrate, core protein (Cop-E8R)	TT_068	822	825
DNA polymerase (Cop-E9L)	TT_069	3018	3021
Virulence factor (Cop-G6R)	TT_089	498	267[b]
RNA polymerase (RPO147) (Cop-J6R)	TT_103	3861	2958
RNA polymerase co-factor (RAP94) (Cop-H4L)	TT_107	2388	1812[b]
Decapping enzyme (Cop-D10R)	TT_120	747	507[b]
NPH-I/helicase (Cop-D11L)	TT_121	1896	1710[b]
Disulfide bond pathway (Cop-A2.5L)	TT_126	231	–[b]
Core protein (Cop-A4L)	TT_128	852	510
IMV membrane protein (Cop-A14L)	TT_138	273	228
Entry and cell–cell fusion (Cop-A21L)	TT_145	354	351
Cowpox A-type inclusion protein (Cop-A26L)	TT_151	684	693
P4c precursor (Cop-A26L)	TT_153	1509	690
Unknown (YMTV-120.5L) (WR153.5)	TT_158	129	–
Unknown (Cop-A31R)	TT_159	375	426
ATPase/DNA packaging protein (Cop-A32L)	TT_160	813	1176
EEV membrane protein (Cop-A33R)	TT_161	558	438[b]
Unknown (WR161)	TT_166	195	195[c]
Semaphorin (Cop-A39R)	TT_168 TT_169	774 933	687 429
Membrane glycoprotein (Cop-A43R)	TT_173	582	585
IL-1/TLR signaling inhibitor (Cop-A46R)	TT_176	711	633
TNF receptor (CrmC) (Cop-A53R)	TT_183	561	372
Unknown (Cop-B11R)	TT_198	219	231
IFN α/β receptor (Cop-B19R)	TT_206	1056	1062
Ankyrin (Cop-B20R)	TT_207	1794	1842

[a] Open-reading frame. We generally annotated any open-reading frame (> 150 bp) that had been previously identified as encoding a VACV gene. Where a gene was disrupted by a frame shift mutation (e.g. Cop-C23L/B29R) the open reading frames are indicated next to gene they are proposed to derive from.

[b] Error noted and corrected previously (Upton et al., 2003). The column labeled "TT00" shows the original reported gene size, the sequences we report in the column labeled "TP05" are identical to the corrected values reported by Upton et al.

[c] Not previously annotated.

in TT00 spares the virus copy of the VEGF gene. The TIRs of TP05 thus most closely resemble those of other extant VACV strains, suggesting that TP05 is probably more representative of the original TianTan lineage than are the other two TianTan clones.

We used these and other Orthopoxvirus genome sequences to assemble updated phylogenetic trees using an alignment of the conserved core region from gene homologs of DVX058 to DVX155 (VACV Copenhagen genes F9L-to-A24R). Different methods assign more distantly related viruses to different branches of these trees (Fig. 6), with rabbitpox being assigned varying degrees of relatedness to other VACV strains. Horsepox virus regularly trees with viruses belonging to the Wyeth/Dryvax cluster but the sequence has drifted substantially judging by the branch lengths. However, regardless of the method used, TP03 and TP05 always cluster with TT00 and in a group that always forms a clade comprising VACV strains WR, Lister, and Copenhagen. VACV strain Copenhagen is also always assigned as a more basal root to this cluster (Fig. 6). The depth of the branches separating TP03/05 from TT00 is much greater than the branches encompassing the Dryvax-derived clones (Duke, DPP9-21, Acambis 2000, CL3, etc.), reflecting the fewer SNPs that differentiate the Dryvax clones.

One rather inexplicable feature of these phylogenetic trees is that the VACV WR strain persistently maps within the Lister/Copenhagen cluster, regardless of the method used to assemble the trees. VACV strain Western Reserve is reported to derive from the same New York City Board of Health virus stock as does Wyeth/Dryvax [summarized in Qin et al., 2011] and logically should associate with that cluster. We have not explored whether the contradictions between the reported history of the virus and these phylogenetic trees relates to the particulars of the alignment we have used, or the choice of core genes, or perhaps reflects something unknown about the long and complex passage history of strain WR.

Other features of TianTan clones TP03 and TP05

Another notable feature of both TP05 and TP03 is that the TIRs encode a 59 bp repeat sequence clearly related to the 69 or 70 bp repeats (Baroudy et al., 1982) found in the telomeres of VACV strains (Fig. 5B). The acquisition of this sequence is linked to the deletion of genes that probably would have been homologs of DVX004, -5, and -6 (Copenhagen C19L-to-C21L). The length of the 59 bp repeat region is too long to be determined by pyro-sequencing and so we used Sanger sequencing and the PCR and primers anchored in the flanking sequences to determine the cumulative lengths and number of repeats. This analysis showed that TP03 and TP05 encode nine copies of the repeat whereas other sub-cloned viruses (e.g. TP04 and TP08) encoded one less copy (data not shown). This feature appears to be an example of DNA having been accidentally captured through illegitimate recombination with another virus telomere, but why the repeat should be resistant to deletion in less certain. One possible explanation is that, once these sequences have been accidentally incorporated into the TIRs, the processes responsible for maintaining the inverted duplications also helps to stabilize the inserts. The homologous region in TT00 has been excised by the large deletion, thus providing no insights into whether this mutation is a characteristic feature of all TianTan strains.

Large deletions help define the evolutionary trajectory of VACV strains

If one compares the sequence of the TianTan strains with other sequenced VACV, one also sees specific patterns of gene deletions that can help define different lineages and identify genes essential for passage in culture. Moreover, if it is assumed that unique assemblages of genes are generally lost as virus strains evolve under culture pressure (Hendrickson et al., 2010), such analyses can help to identify the modern strains that likely still resemble ancestral VACV strains. For example, it has been suggested that VACV shares a common ancestry with a virus resembling horsepox virus (Baxby, 1981; Tulman et al., 2006), although based upon the analysis of conserved core genes, this hypothesis is only supported by the simplest of the phylogenetic tests we have used (Fig. 7). To gain a better understanding of the relationships between different VACV strains, we ran a BLASTN search using ~20 kbp of sequence spanning the left end of the horsepox (HPXV) genome (Fig. 8). The sequenced VACV strains fall into various groupings that are defined by different deletion and TIR boundaries and show

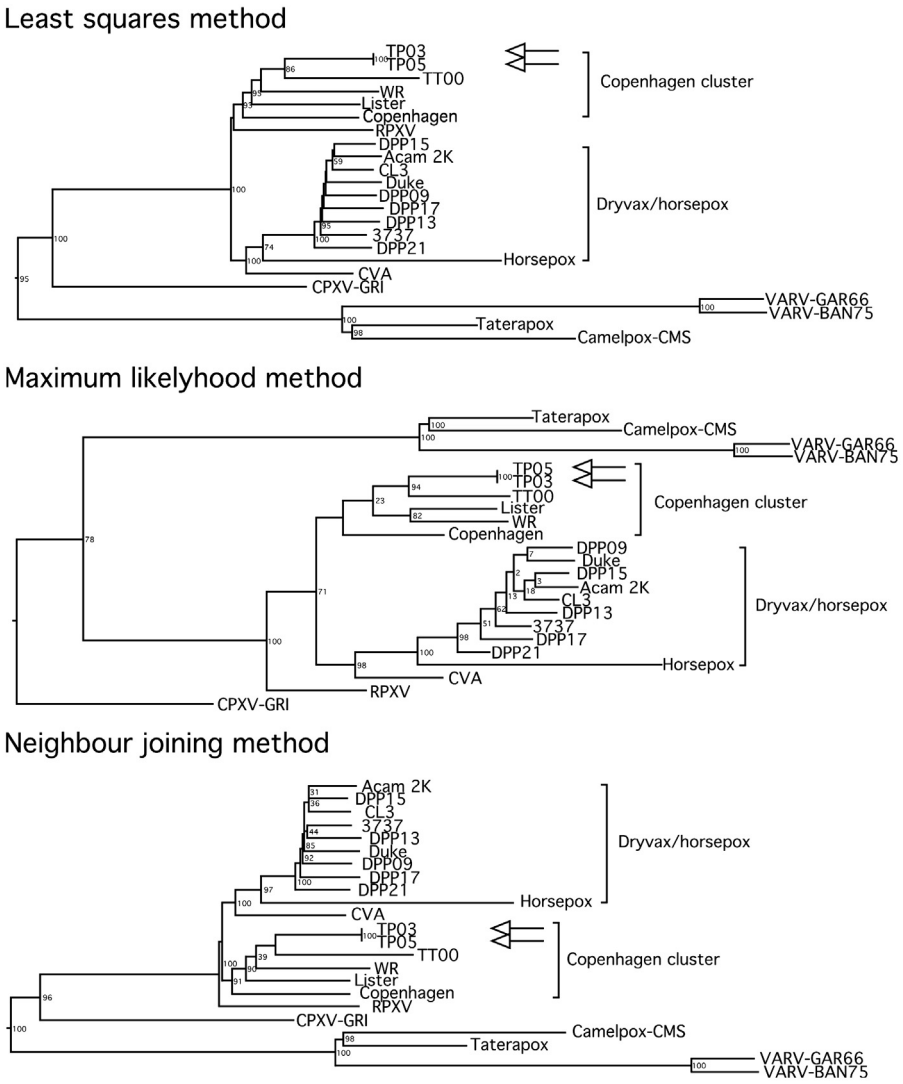

Fig. 6. Phylogenetic relationships between different Orthopoxviruses. We prepared an alignment of virus sequences lying between the gene homologs of genes DVX058 to DVX155 (VACV Copenhagen genes F9L-to-A24R) for the indicated viruses, and then used three different phylogenetic methods to examine the relationship between the TianTan clones and other Orthopoxviruses. The three methods all produced very similar trees, with the TianTan clones always falling in a "Copenhagen" cluster. Cowpox (strain Brighton Red), ectromelia, and monkeypox viruses were included in the original alignment, but always mapped outside of these groupings and have been removed for clarity. White arrows indicate the two newly sequenced strains.

evidence of previously noted evolution through gene deletion (Hendrickson et al., 2010). All the VACV strains are distinguished from HPXV by a common 10.7 kbp deletion (DNA encoding the mostly fragmented HSPV007-015b genes (Tulman et al., 2006), gray bar in Fig. 8) that juxtaposes the SPI-1 and VEGF genes in VACV. Many genomes have been cloned from vaccine stocks (e.g. Lister and ACAM2K), which encode a full complement of the remaining genes in this part of the genome, although the location of the left TIR boundary (Fig. 8, circled vertical bars) varies between these strains due to the presence of different deletions in the region surrounding the right TIR boundary (see below). TP05 resembles these more complete VACV strains, aside from the disruption of genes homologous to DVX004–6 by the insertion of repeat elements near the left telomere. TP03 and TT00 exhibit more extensive deletions, much like those that have accumulated in VACV strain WR. An interesting feature of these viruses is that nearly all, except TP03, retain at least one functional copy of DVX014, the VEGF gene homolog discussed above. This strongly suggests that the gene is under positive selection pressure in culture.

A similar pattern of gene deletions is also seen at the right end of the different VACV genomes (Fig. 9), although this part of the genome appears to be more dynamic and subject to rearrangement. Here the "Acambis clone 3" (CL3) isolate is an anomaly, as it is the only type of VACV to retain DNAs homologous to 1.1 kbp of HPXV sequence in the region near genes DVX214-15. Related sequences are also detected in ectromelia, camelpox, and variola viruses. The CL3-like viruses are a rare type of VACV clone found in Dryvax stocks (Qin et al., 2011), which exhibits unusually high virulence compared to strains like ACAM2K (Weltzin et al., 2003). These sequences have been deleted from all other known VACV isolates including the TianTan strains and it is possible that they play some role in CL3's unusual virulence. Otherwise, there is little to differentiate (or relate) the pattern of gene deletions in TianTan virus from/to most other VACV strains although the lack of alignment to some portions of the Lister TIR (Fig. 9, gray bar) is not supportive of a direct relationship between the viruses. Assuming that VACV strains have undergone progressive deletion of genes in culture, this analysis of the telomeres suggests that they have followed an evolutionary path involving HPXV-like and then CL3-like viruses, after which different strains have evolved along different pathways. Viruses that have been cultured for part of their history in eggs (like CVA/MVA and TianTan) seem to have suffered further deletions.

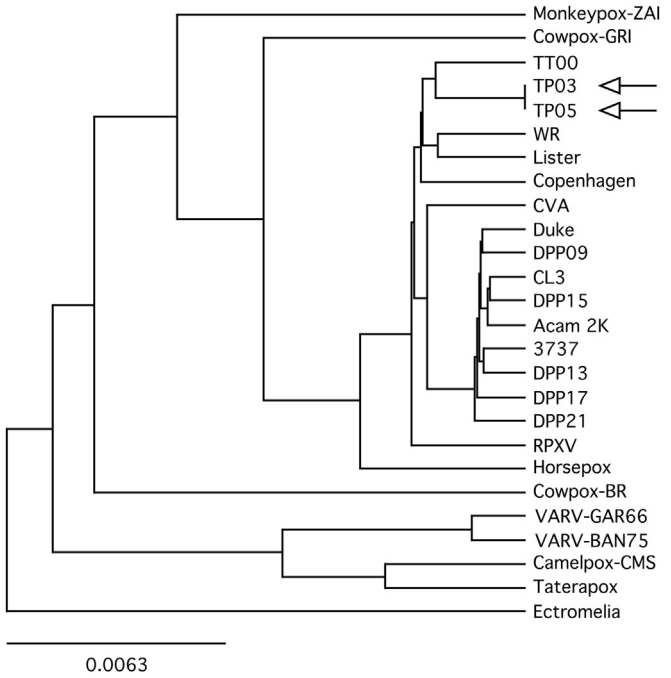

Fig. 7. Phylogenetic relationships between different Orthopoxviruses. A simple UPGMA (unweighted pair group method with arithmetic mean) approach was also used, along with the alignment prepared for Fig. 6, to explore the relationship between Orthopoxvirus strains. The method again groups TianTan strains with VACVs like Copenhagen, but now places horsepox virus on a branch distinct from other VACV-like strains. Tulman et al. (2006) similarly located horsepox virus in an early, but distinct branch of the VACV family.

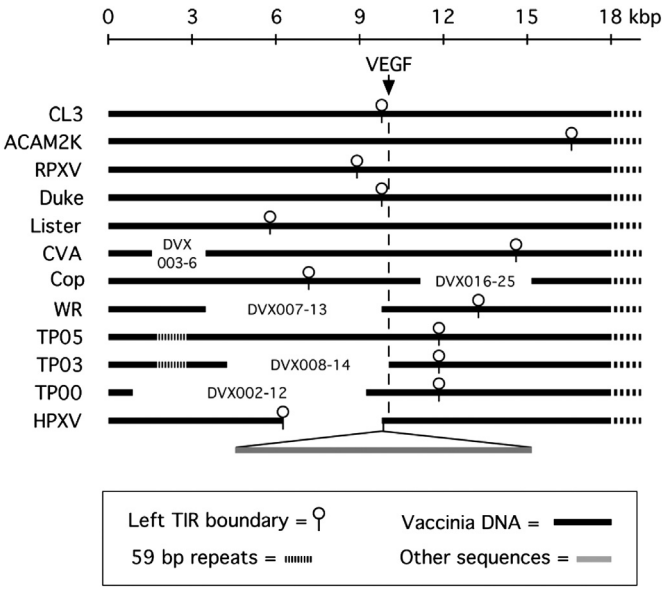

Fig. 8. Relationship between deletions mapping in the left telomere of common VACV strains. The TP05 strain encodes the longest and most complete TIR in the TianTan lineage. Note that the VEGF gene seems to be a privileged site, which is sometimes duplicated, and nearly always retained in VACV strains. The deletion extending into the VEGF gene promoter and N-terminus in the TP03 genome is unusual in this regard. Horsepox virus (HPXV) encodes an additional 10.7 kbp insert upstream of the VEGF gene (gray bar). The labels (e.g. DVX007-13) refer to genes present in a consensus of VACV Dryvax clones, which would have been located in the region spanned by the deletion (Qin et al., 2011).

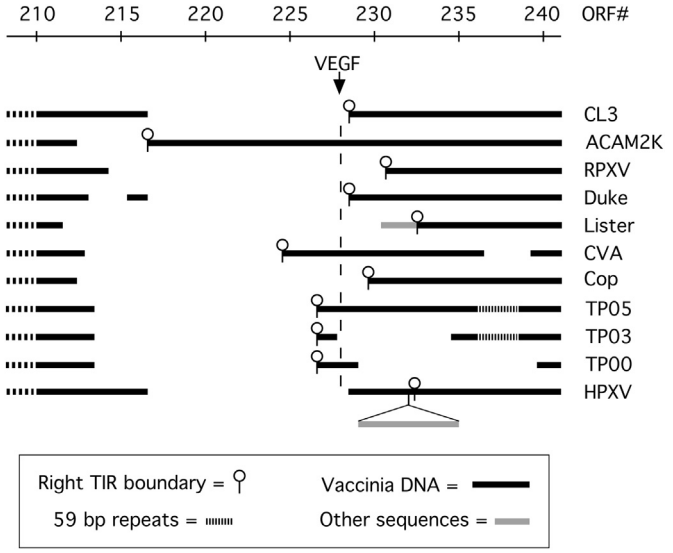

Fig. 9. Relationship between deletions mapping in the right telomere of common VACV strains. The TP05 strain encodes a complement of genes in the right telomere similar to most other VACV, apart from the disruption of three genes in the TIRs by the 59 bp repeats. The left end of the deletion extending to the left of the TIR boundary (barred circle) does not extend into the DVX212/TT_206 gene (a secreted IFNα/β receptor homolog) in the TianTan strains, in contrast to strains like ACAM2K where this gene is partially truncated by a larger deletion. Note that because of the complexity of the maps in this region of the different genomes, we have used the open-reading frames (ORF#) to align the different sequences instead of true map distances. We have also omitted strain WR, due to the additional complexity of the rearrangements in this portion of the WR genome.

Conclusions

Our sequencing of viruses belonging to the TianTan lineage clearly documents its relationship to other widely used VACV viruses, and suggests that TianTan virus most likely shares a common origin with a strain also ancestral to VACV strain Copenhagen. This relationship is clearly supported by all available phylogenetic methods and is compatible with the patterns of deletions. However, the genome of the virus has clearly also been affected by its separate passage history, with additional mutations and deletions that have further altered the TIRs. Although the virus we obtained must have been plaque purified at some point in its recent history, it still contained two types of viruses that exhibited significantly different growth properties due to deletions into the region encompassing the VACV epidermal growth factor gene homologs.

A comparison of our sequence with the published TT00 sequence still detected far too many single-nucleotide polymorphisms and indels for all to be a product of simple sequencing errors. Indeed, many of the indels illustrate the same pattern of repeat driven mutations (Fig. 5) seen in Dryvax clones (Qin et al., 2011). Our strain (s) and the TT00 isolate are likely just several examples of the viruses found in the original TianTan stocks, and the divergent sequences further illustrates the complexity of the viruses comprising the quasispecies that makes up a traditional vaccine. Although it is not widely used anymore as a smallpox vaccine, VACV continues to be used as a vector in diverse applications. The complexity detected by DNA sequencing technologies shows the importance of characterizing any clones randomly “fished” out of a traditional stock of smallpox vaccine, because viruses encoding or lacking a gene as critical as the VACV growth factor gene will likely exhibit dramatically different safety and efficacy profiles.

Methods

Virus and cell culture

Vaccinia virus (strain TianTan) was obtained from the China Center for Type Culture Collection (Wuhan, Hubei). The passage

history is obscure, although publications and dissertations indicate that it comes originally from the Institute of Virology, Chinese Academy of Preventive Medicine, and before that the Chinese National Vaccine and Serum Institute. These records suggest it would have been previously cultured on chick embryo chorioallantoic membranes and primary chick embryo fibroblasts; possibly also Vero cells. This stock has also been plaque purified at some point in its recent history, but when is also unknown. Monkey kidney BSC-40 cells from the American Type Culture Collection (Manassas, VA). The cells and virus were cultured 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% CO_2 atmosphere. The cells were tested periodically and shown to be free of mycoplasma.

Virus sequencing and genomic analysis

The virus were plaque purified, cultured, purified using sucrose gradients, and sequenced as described previously using a Roche 454 GS FLX Titanium sequencer platform and multiplex technology (Qin et al., 2011). Twelve different clones were sequenced, to read redundancies ranging from 9- to 42-fold, but discovered to be nearly all identical apart from sequence features described below. Pyro-sequencing methods produce indel-type sequencing errors within homopolymeric base runs. The true sequence can usually be deduced from inspection of the sequence of the aligned high quality replicate reads, but where that was not possible we assumed that the correct read was the one that maintained the open-reading frame in alignment with many other sequenced VACV strains. (In our previous study, 6/6 of these problematic sites were Sanger sequenced and all them were found to be pyro-sequencing artifacts.) The viruses discussed in detail in this communication were designated TP03 and TP05 (i.e. TianTan plaque 3 or 5) and have been assigned GenBank accession numbers KC207810 and KC207811, respectively. For purposes of gene comparison we reference the gene numbering system initially established for Acambis clone 2000 (AY313847) and Acambis clone 3 (AY313848) viruses and subsequently used to label other Dryvax clones (Qin et al., 2011). We did not sequence far into the repeated elements located in the virus telomeres, the first nucleotide in each of the assemblies was defined as the first nucleotide in one of the four 54 bp repeats preceding the first open-reading frame.

The other VACV and accession numbers used in this study are: Ankara strain CVA (AM501482), Copenhagen (M35027), DPP9 (JN-654976), DPP13 (JN654980), DPP15 (JN654981), DPP17 (JN654983), DPP21 (JN654986), Duke (DQ439815), Lister (AY678276), Western Reserve (NC_006998), and 3737 (DQ377945). Other Orthopoxviruses included: camelpox strain CMS (AY009089), cowpox strains GRI-90 (X94355) and Brighton Red (NC_003663), ectromelia strain Moscow (NC_004105), horsepox (DQ792504), monkeypox strain Zaire (AF-380138), rabbitpox (AY484669), taterapox (NC_008291), and variola strains Bangaladesh (L22579) and Garcia-66 (Y16780). For simplicity, we refer to the original TianTan virus sequence (AF095689) as "TT00" throughout this communication. Genome assemblies were prepared using CLC Genomics Workbench (v4.6) and annotated using GATU (Tcherepanov et al., 2006), also as described previously (Qin et al., 2011). Bioinformatic analyses were performed using Viral Genome Organizer (Lefkowitz et al., 2005; Upton et al., 2000) and Poxvirus Orthologous Clusters (Ehlers et al., 2002). These and additional bioinformatics tools can be accessed at http://www.virology.ca. Phylogenies were assembled using RDP3 (Martin et al., 2010).

Other methods

To determine the lengths of the repeat elements in a 1.2 kbp region corresponding to the genes previously designated DVX-004 to DVX-006, we used the PCR and "repeat forward" (5′ GCAG-TAGGCTAGTATCTT 3′) plus "repeat reverse" (5′ TACCGGCATCA-TAAACAC 3′) primers. The products were then sequenced using dideoxy-sequencing methods. To confirm the size and presence of the larger deletions we used the PCR and either of two forward primers (TT010F: 5′ TTTTTGTAGGAAGGAGGC 3′ and TT004F: 5′ TGGATGGCCGTATTGATT 3′) in combination with a single reverse primer (TT011R: 5′ CCGGGAGATGGGATATATGA 3′). For Southern blots virus DNAs were digested with *Sca*I, fractionated on a 0.7% agarose gel, transferred to nylon membranes, and blotted with a biotin-labeled probe prepared using primers TT010F and TT011R (above) and TP05 virus DNA as a template.

Acknowledgments

We thank Nicole Favis and Carmit Teicher for excellent technical support and the staff at the Genome Québec Innovation Centre for their assistance with DNA sequencing. We also thank Profs. Zhao Kai and Isao Arita for providing their insights into the history of Asian vaccinia virus strains. This work was supported by operating grants from the Canadian Institutes for Health Research and the Alberta Cancer Foundation and by an infrastructure award from the Canada Foundation for Innovation.

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