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Genome Scale Evolution of Myxoma Virus Reveals Host-Pathogen Adaptation and Rapid Geographic Spread

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The evolutionary interplay between myxoma virus (MYXV) and the European rabbit (*Oryctolagus cuniculus*) following release of the virus in Australia in 1950 as a biological control is a classic example of host-pathogen coevolution. We present a detailed genomic and phylogeographic analysis of 30 strains of MYXV, including the Australian progenitor strain Standard Laboratory Strain (SLS), 24 Australian viruses isolated from 1951 to 1999, and three isolates from the early radiation in Britain from 1954 and 1955. We show that in Australia MYXV has spread rapidly on a spatial scale, with multiple lineages cocirculating within individual localities, and that both highly virulent and attenuated viruses were still present in the field through the 1990s. In addition, the detection of closely related virus lineages at sites 1,000 km apart suggests that MYXV moves freely in geographic space, with mosquitoes, fleas, and rabbit migration all providing means of transport. Strikingly, despite multiple introductions, all modern viruses appear to be ultimately derived from the original introductions of SLS. The rapidity of MYXV evolution was also apparent at the genomic scale, with gene duplications documented in a number of viruses. Duplication of potential virulence genes may be important in increasing the expression of virulence proteins and provides the basis for the evolution of novel functions. Mutations leading to loss of open reading frames were surprisingly frequent and in some cases may explain attenuation, but no common mutations that correlated with virulence or attenuation were identified.

The experimental introduction of myxoma virus (MYXV) into the European rabbit (*Oryctolagus cuniculus*) population of Australia and its unprecedented and unanticipated spread initiated one of the great natural experiments in evolution (1). The subsequent emergence of slightly attenuated viruses that were more efficiently transmitted and the natural selection of rabbits with genetic resistance to MYXV were carefully documented in real time (2). Sixty years later these studies continue to inform theory and practice in host-parasite coevolution and particularly the complex relationship between virulence and transmissibility.

MYXV is a poxvirus and the type species of the *Leporipoxvirus* genus. MYXV is native to South America, where its natural host is the tapeti (forest rabbit; *Sylvilagus brasiliensis*), in which the virus causes a largely innocuous, localized, cutaneous fibroma. MYXV is transmitted by mosquitoes or other biting arthropods probing through the fibroma and picking up virus on their mouthparts. Transmission is passive, as MYXV does not replicate in the vector. In European rabbits, which are not native to the Americas, MYXV causes the generalized lethal disease myxomatosis. As such, this represents a classic example of a pathogen that is highly virulent in a new host species with no evolutionary history of adaptation to that pathogen. Viruses closely related to MYXV are found in *Sylvilagus bachmani* (brush rabbit) on the west coast of the United States and the Baja Peninsula of Mexico (Californian myxoma viruses) and in *Sylvilagus floridanus* (eastern cottontail) in eastern and central parts of North America (rabbit fibroma virus [RFV]) (2).

European rabbits were introduced into Australia with European settlement in 1788, but the continent-wide spread of rabbits was initiated in 1859 by the introduction of 18 to 24 wild rabbits for hunting. Within 50 years these rabbits had spread over most of Australia with the exception of the wet tropics and the far north (3). The European rabbit became Australia's worst vertebrate pest, responsible for enormous ecological destruction and agricultural losses. Field trials in 1950 to assess MYXV as a biological control resulted in the mosquito-driven epizootic spread of the virus throughout much of southeastern Australia in the summer of 1950 to 1951, and it reemerged the following spring (4). Assisted by large-scale inoculation campaigns, MYXV spread and was established over the rabbit-infested areas of Australia during the next 5 years (2).

The MYXV introduced into Australia, termed Standard Laboratory Strain (SLS), was derived from an isolate made in Brazil, probably in 1910 (2, 5) and subsequently maintained by rabbit passage. Importantly, the original virus used to initiate the epizootic was available to serve as a reference for subsequent field isolates. SLS had a case fatality rate estimated at 99.8% in infected wild rabbits and similar lethality in laboratory rabbits, which are domestic breeds of *Oryctolagus cuniculus*.

It quickly became apparent that viruses with slightly lower case

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fatality rates were emerging in the field and outcompeting ongoing releases of the virulent SLS (6–8). Fenner and Marshall (9) classified the virulence of MYXV into 5 grades based on average survival times, case fatality rates, and symptomatology of groups of 4 to 6 laboratory rabbits infected with very low doses of virus. The predominant viruses in the field were of grade 3 virulence (case fatality rates of 70 to 95%), with average survival times that were prolonged compared to that for SLS (17 to 28 days versus <13 days). Mosquito transmission is a function of the titers of virus in the skin lesions induced by the virus and how long the rabbit survives. By allowing the infected rabbit to survive for longer with high titers of virus, the moderately attenuated viruses had a selection advantage over more-virulent strains. Highly attenuated grade 5 viruses (<50% case fatality rates) tended to be poorly transmitted because the infected rabbits controlled virus replication, in turn reducing transmissibility (10). Importantly, the emergence of more-attenuated virus strains may have facilitated the rapid selection of rabbits with genetic resistance to MYXV (2).

A separate strain of MYXV was released in France in 1952; the virus was obtained from the Laboratory of Bacteriology in Lausanne, Switzerland, and has hence been termed the Lausanne strain (Lu), although like SLS it was originally isolated in Brazil (in Campinas in 1949). Unlike SLS, Lu had undergone relatively few rabbit passages. Lu and SLS have indistinguishable levels of virulence in laboratory rabbits; however, Lu is considerably more virulent than SLS in genetically resistant rabbits. Despite the differences in starting virus, environmental conditions, and insect vectors, the outcome of MYXV-rabbit coevolution in Europe was remarkably similar to that in Australia, with the emergence of attenuated viruses and the selection of rabbits with genetic resistance (11).

The Lu strain of MYXV is considered the reference genome. It has a double-stranded DNA (dsDNA) genome of 161,777 bp with inverted terminal repeats (TIR) of 11,577 bp. It contains 158 unique open reading frames, 12 of which are duplicated in the TIR. Genes located toward the center of the genome tend to be conserved between poxviruses and are essential for replication and structure, whereas those toward the termini tend to be involved in subversion of the host immune response or have host range functions and are less conserved across poxviruses (12).

We have recently outlined the evolutionary patterns and dynamics of the Australian progenitor SLS virus and 19 Australian isolates sampled between 1951 to 1999, as well as two isolates of grade 1 and grade 5 virulence from the early radiation of MYXV in the United Kingdom following the introduction of MYXV there in 1953 (13). To reveal the genetic basis for the phenotypic differences between these viruses, and particularly their profound differences in virulence, we report here the detailed genome sequences of these viruses plus those of an additional five Australian viruses. In addition, we sequenced and analyzed a second strain of KM13 (KM13 2A) and the Lu virus strain produced by the Commonwealth Serum Laboratories (CSL) for release in Australia, as well as a grade 3 virus isolated in the United Kingdom in 1954. Such a rich genomic data set enabled us to obtain a more detailed picture of the evolution and geographic spread of this virus through Australia and particularly the broad range of genes involved in this evolutionary process.

Materials and Methods

Virus isolates. The isolates of MYXV used in this study are described in Table 1.

Preparation of DNA. Viruses were passaged twice in RK13 cells to prepare working stocks; viral DNA was prepared from infected RK13 cells as previously described (13).

Sequencing, assembly, and comparative analyses. The seven virus samples newly reported here were sequenced on the Illumina HiSeq 2000 platform. Demultiplexed and trimmed sequence reads were assembled with the Velvet *de novo* assembler (14) using a range of *k*-mer values from 59 to 77 and an expected coverage of 600×. Contigs containing MYXV genomic DNA were identified by BLASTX searches and were ordered into a single scaffold against the Lu genome (accession no. AF170726) using the Abacas.pl script (15). The quality of each scaffold was verified by remapping the untrimmed reads to the assembly using Smalt (www.sanger.ac.uk/resources/software/smalt/); the resulting BAM files were converted to pileup format to verify the read coverage at each site. Read coverage line plots for scaffolds at each *k*-mer value were generated in R and examined by eye. In general, we found that scaffolds generated at high *k*-mers (greater than 65) resulted in single contig assemblies of the MYXV genomes, but inspection of coverage plots revealed many low-coverage regions. Further examination of these low-coverage areas revealed that these were large insertions unique to the strain in question compared to the 23 previously sequenced strains of MYXV (13). Assemblies at lower *k*-mer values (51 to 65) were often fragmented into multiple contigs but showed even read coverage across contigs corresponding to MYXV segments. Further, these were of the expected lengths relative to the 23 previously sequenced strains (13). Gaps, single nucleotide polymorphisms (SNPs), and indels of interest were closed by Sanger sequencing of PCR products. In every case, only one complete, or nearly complete, copy of the terminal inverted repeat (TIR) was assembled at either the 5′ or the 3′ end, though up to a full read length of the complementary TIR was observed at the opposite end, allowing easy identification of the TIR junction. To further verify the position of the TIR junction, we duplicated the complete TIR, generated a reverse complement of the sequence that was added on the opposite end, and remapped the sequence reads to that assembled portion of the genome.

Genome annotation was transferred from the Lu strain to the newly sequenced MYXV genomes using the Rapid Annotation Transfer Tool (16). EMBL flat files of transferred gene models were then inspected and compared to Lu using the Artemis comparison tool (17); incorrect models were corrected, and new gene models were added where transfer had not occurred. Genes are numbered based on their location in the MYXV genome, with the direction of transcription indicated by *L* or *R* (e.g., *M010L*). Genes in the TIR are identified by *L/R* (e.g., *M007L/R*). Proteins are identified by the same number as the gene with the transcription direction omitted, e.g., M010.

To generate the heat maps for the comparative analyses of each gene to the SLS and Lu strains, we used a custom Perl script to produce multi-FASTA files containing all taxa in which this gene was present. Sequence alignments were generated using ClustalW (18), and PAUP* 4.0b10 (19) was used to remove ambiguous and gapped sites from the alignments and generate the number of SNP mutations in each gene. Columns from the distance matrix comparing viral taxa to SLS were parsed, and two subsequent matrices were generated, one for European strains compared to Lu and one for Australian strains compared to SLS.

Evolutionary analysis. A total of 30 genome sequences of MYXV were subjected to phylogenetic analysis, with a total alignment length of 163,555 nucleotides (nt). Sequences were aligned by MAFFT (20), then inspected by eye. Phylogenetic analysis employed the maximum likelihood (ML) method, available in PhyML 3.0 (21). Because of the very low numbers of substitutions separating these sequences, we employed the HKY85 model of nucleotide substitution (22) with subtree pruning and regrafting (SPR) branch swapping. To assess the robustness of each node on the tree, a bootstrap resampling analysis was undertaken (1,000 repli-

TABLE 1 Origin of strains of MYXV sequenced here[e] and in reference 13

Virus	Formal name	Geographic origin	Source	Reference	Virulence grade	Region sequenced[d]	Accession no.
SLS (Moses strain/strain B)	None given	Brazil	Rabbit tissue stock (Fenner)[a]	9	1	1–161777 (161,763)	JX565574
Glenfield	Aust/Dubbo/2-51/1	Central NSW	CV-1 cell stock[b]	29	1	15–161763 (161,742)	JX565567
KM13	Aust/Corowa/12-52/2	Southern NSW	Rabbit tissue stock (Fenner)	9	3	1–161777 (161,771)	JX565569
KM13 2A	**Aust/Corowa/12-52/2A**	**Southern NSW**	**Rabbit tissue stock (Fenner)**	**30**	**3**	**1–161777 (161,769)**	**KC660080**
Uriarra	Aust/Uriarra/2-53/1	Canberra District	CV-1 cell stock	29	5	1–161777 (161,768)	JX565577
SWH	Aust/Southwell Hill/9-92/1	Canberra District	Wild rabbit	31	4	1–161777 (161,797)	JX565576
BRK	Aust/Brooklands/4-93	Canberra District	Wild rabbit	31	1	1–161777 (161,701)	JX565562
Bendigo	Aust/Bendigo/7-92	Central Victoria	Wild rabbit	31	1	1–161777 (161,738)	JX565565
Meby	Aust/Meby/8-91	Tasmania	Wild rabbit	31	5	87–161691 (161,542)	JX565571
Lu	Brazil/Campinas/1949/1	Brazil	Commonwealth Serum Laboratories 1973[c]		1	1–161777 (161,778)	JX565570
Cornwall	England/Cornwall/4-54/1	Cornwall, UK	Rabbit tissue stock (Fenner)	9	1	1–161777 (161,775)	JX565566
Sussex	**England/Sussex/9-54/1**	**Sussex, UK**	**Rabbit tissue stock (Fenner)**	**9**	**3**	**1–161777 (161,778)**	**KC660084**
Nottingham attenuated	England/Nottingham/4-55/1	Nottingham, UK	Rabbit tissue stock (Fenner)	9	5	1–161777 (161,777)	JX565572
Gung/91	Aust/Gungahlin/1-91	Canberra District	Wild rabbit	31	4	151–161627 (161,443)	JX565568
Wellington	Aust/Wellington/1-91	Central NSW	Wild rabbit	31	1	29–161749 (161,688)	JX565582
BRK/12-2-93	Aust/Brooklands/2-93	Canberra District	Wild rabbit	25	ND[f]	140–161638 (161,496)	JX565563
BD23	Aust/Bulloo Downs/11-99	Southwest Queensland	Wild rabbit	49	ND	285–161555 (161,971)	JX565584
BD44	**Aust/Bulloo Downs/12-99**	**Southwest Queensland**	**Wild rabbit**	**49**	**ND**	**1–161777 (162,847)**	**KC660079**
BRK/897	Aust/Brooklands/1-95	Canberra District	Wild rabbit	25	ND	103–161675 (161,545)	JX565564
OB1/406	Aust/OB1/Hall/3-94	Canberra District	Wild rabbit	25	ND	87–161691 (161,612)	JX565573
OB2/W60	**Aust/OB2/Hall/11-95**	**Canberra District**	**Wild rabbit**	**25**	**ND**	**1–161777 (162,483)**	**KC660081**
OB3/Y317	**Aust/OB3/Hall/2-94**	**Canberra District**	**Wild rabbit**	**25**	**ND**	**1–161777 (161,748)**	**KC660083**
OB3/1120	**Aust/OB3/Hall/2-96**	**Canberra District**	**Wild rabbit**	**25**	**ND**	**1–161777 (161,722)**	**KC660082**
WS1/234	Australia/Woodstock 1/3-94	Canberra District	Wild rabbit	25	ND	1–161777 (161,754)	JX565578
WS6/1071	Aust/Woodstock 6/11-95	Canberra District	Wild rabbit	25	ND	41–161737 (161,752)	JX565580
WS1/328	Aust/Woodstock 1/3-94	Canberra District	Wild rabbit	25	ND	156–161622 (161,483)	JX565579
WS6/346	Aust/Woodstock 6/3-95	Canberra District	Wild rabbit	25	ND	140–161638 (161,430)	JX565581
SWH/8-2-93	Aust/Southwell Hill/2-93	Canberra District	Wild rabbit	25	ND	1–161777 (161,740)	JX565575
SWH/805	**Aust/Southwell Hill/11-93**	**Canberra District**	**Wild rabbit**	**25**	**ND**	**1–161777 (161,780)**	**KC660085**
SWH/1209	Aust/Southwell Hill/2-96	Canberra District	Wild rabbit	25	ND	33–161745 (162,413)	JX565583

[a] Virus stocks were originally obtained as freeze-dried rabbit tissue from Frank Fenner, John Curtin School of Medical Research, Australian National University, Canberra, ACT, Australia.

[b] Virus stocks were from viruses plaque purified as described in reference 29.

[c] Virus was from an ampoule of freeze-dried rabbit tissue powder prepared by the Commonwealth Serum Laboratories for rabbit control.

[d] Based on the Lu sequence from Cameron et al. (12), 1 to 161777, as corrected by Morales et al. (34); the actual sequence length is shown in parentheses.

[e] Boldface indicates data for isolates sequenced for this paper.

[f] ND, not determined.

cates) employing the parameters described above. To determine whether these 30 MYXV genomes contain any recombinant regions, we utilized the RDP, GENECOV, and BOOTSCAN methods available within the RDP4 package (23) and the default parameters. As with our previous study (13), no recombination was observed.

To estimate the rates of evolutionary change and times to common ancestry in these data (including those of two key nodes shown in Fig. 1), we employed the Bayesian Markov chain Monte Carlo (MCMC) method, available in the BEAST package (24). This analysis utilized both strict and relaxed (uncorrelated log normal) molecular clocks, a Bayesian skyline coalescent prior, and the HKY85 nucleotide substitution mode. The MCMC was run for 100 million generations, and convergence was observed in all parameters. Statistical uncertainly is presented as values for the 95% highest-probability density (HPD).

Nucleotide sequence accession numbers. The seven new MYXV genome assemblies have been deposited on GenBank under accession numbers KC660079 to KC660085.

RESULTS

Evolution and phylogeography of MYXV. Our phylogenetic analysis of 30 complete MYXV genomes, including 5 new Australian isolates sampled during 1993 to 1999 and an early attenuated isolate from the United Kingdom sampled in 1954, depicted the major division between the Australian and European epidemics observed previously (Fig. 1) (13), with no evidence of recombination. In addition, that all the recently sampled Australian viruses (1991 to 1999) are clearly distinct from both SLS and Lu indicates that these two viruses made no significant contribution to the later evolution of MYXV in Australia even though they were introduced multiple times over many years. Hence, these data suggest that all (sampled) Australian MYXV strains have their ancestry in the initial introduction of SLS in 1950, although the close phylogenetic relationship among the sequences means that we cannot determine whether the Glenfield (Gv) strain, which was also widely released in NSW and Victoria, made any contribution to the spread of MYXV. Our estimates of rates of nucleotide substitution—at 0.8×10^{-5} to 1.1×10^{-5} nucleotide substitutions per site per year (95% HPD values)—and times to common ancestry were also essentially identical to those observed previously (13). Hence, these data again indicate that the evolution of MYXV is both relatively rapid (for a dsDNA virus) and remarkably clocklike.

A visual overview of genome scale genetic variation, manifest as the genetic distance of each gene from the progenitor strain—SLS for the Australian isolates and Lu for the European isolates—is represented by heat maps (Fig. 2A and B, respectively). These maps reveal that the majority of genes remain highly conserved, with a few genes exhibiting more diversity. An example of the latter is *M017L*. Although the function of this gene is unknown, it has acquired mutations in the majority of the Australian strains compared to SLS (Fig. 2A; Table 2). Multiple genes (*M003.1L/R*, *M103L*, *M105L*, and *M132L*) have acquired mutations in OB3/1120/1996 and WS6/1071/1995, which are linked to the other MYXV strains by a relatively long branch (Fig. 1). However, of these, only *M103L* encodes a protein with a predicted function (structural membrane protein), while the majority of

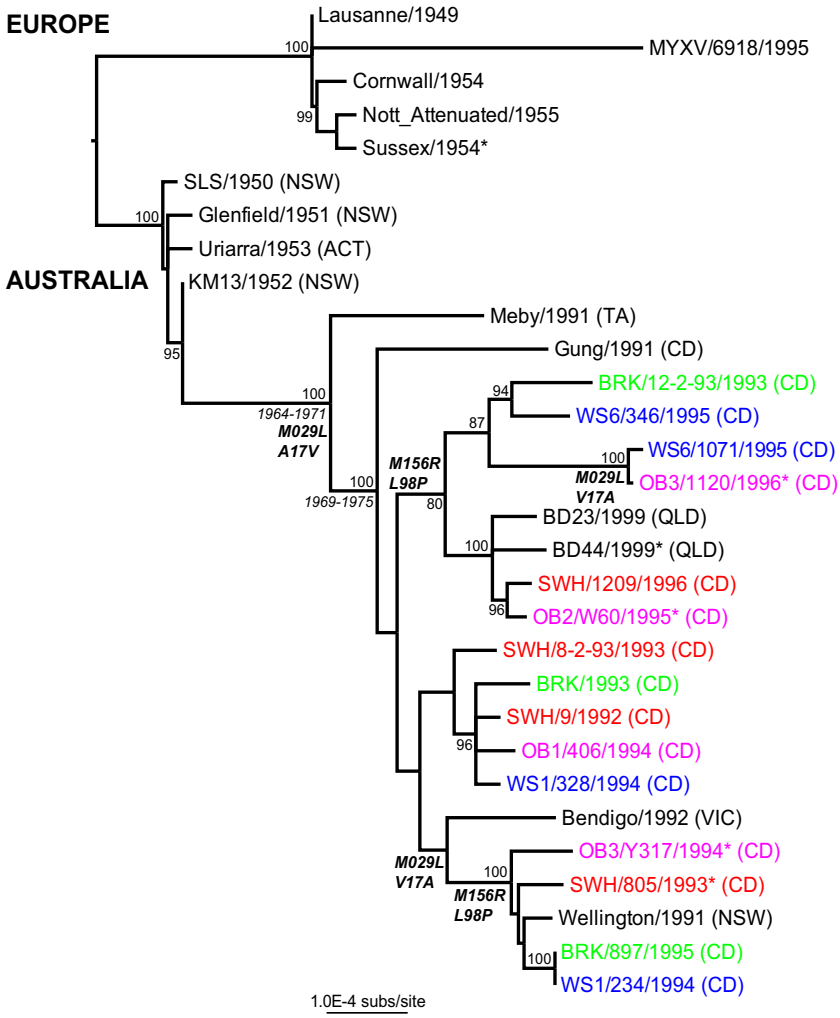

FIG 1 Phylogeny and phylogeography of MYXV isolates. Samples are color-coded according to place of sampling (BRK [Brooklands], green; OB, pink, SWH [Southwell Hill], red; WS [Woodstock], blue), while the state or region of sampling is noted in parentheses (ACT, Australian Capital Territory; CD, Canberra District; NSW, New South Wales; QLD, Queensland; TA, Tasmania; VIC, Victoria). Viruses newly sequenced here are marked with an asterisk. The phylogenetic distribution of mutation and reversion in the *M029L* gene and of mutation in the *M156R* gene is also shown. Bootstrap values are shown for key nodes, and all horizontal branches are drawn according to the number of nucleotide substitutions per year. Divergence times (95% HPD values) for two key nodes in the Australian part of the phylogeny were inferred from the BEAST analysis (see Materials and Methods).

mutations involved are commonplace and/or synonymous ones exhibiting no clear association with changing virulence. Similarly, with the exception of the attenuated Spanish isolate 6918, which appears as genetically distant based on this and the phylogenetic analyses, the European isolates have very few mutations compared to Lu (Fig. 2B), reflecting their sampling early in the epidemic.

To reveal aspects of the phylogeography of MYXV, we coded the Australian isolates by their state of origin (Fig. 1), in which CD delineates viruses that were sampled in close proximity to each other (within 10 to 15 km) in the Canberra District, which straddles the NSW/Australian Capital Territory (ACT) border in southeastern Australia (see below). Strikingly, BD23 and BD44, sampled from hot, dry rangelands at Bulloo Downs in southwest Queensland in 1999, are very closely related to viruses (OB2/W60/1995 and SWH/1209/1996) sampled 3 to 4 years earlier from the cool-climate, higher-rainfall Canberra district, approximately 1,000 km away. Also of interest is the Meby strain, sampled from Tasmania, which is separated from mainland Australia by the Bass Strait, which is up to 240 km wide. Although SLS was released in Tasmania in the early 1950s following its spread on the mainland, Meby is clearly descended from a mainland virus that diverged in the late 1960s and has then remained isolated since this time (Fig. 1). It is therefore possible that the virus reached Tasmania from the mainland on a mosquito inadvertently transported by ship or plane. The majority of the sequenced viruses were isolated between 1993 and 1996 from a set of seven closely situated study sites (WS1, WS6, OB1, OB2, OB3, SWH, and BRK) in the Canberra district (25, 26). From the phylogenetic analysis (Fig. 1) it is obvious that viral lineages have cocirculated at a single locality during a specific time period. In general, these results highlight the relative rapidity of MYXV movement, likely aided by mosquito transmission, including a dispersal of over 1,000 km during 1950.

Comparison of the SLS and Lu sequences. SLS was the original virus released in Australia in 1950. We compared the complete genome sequence of SLS to that of the Lu strain. These two progenitor strains have differences in symptomatology, virulence,

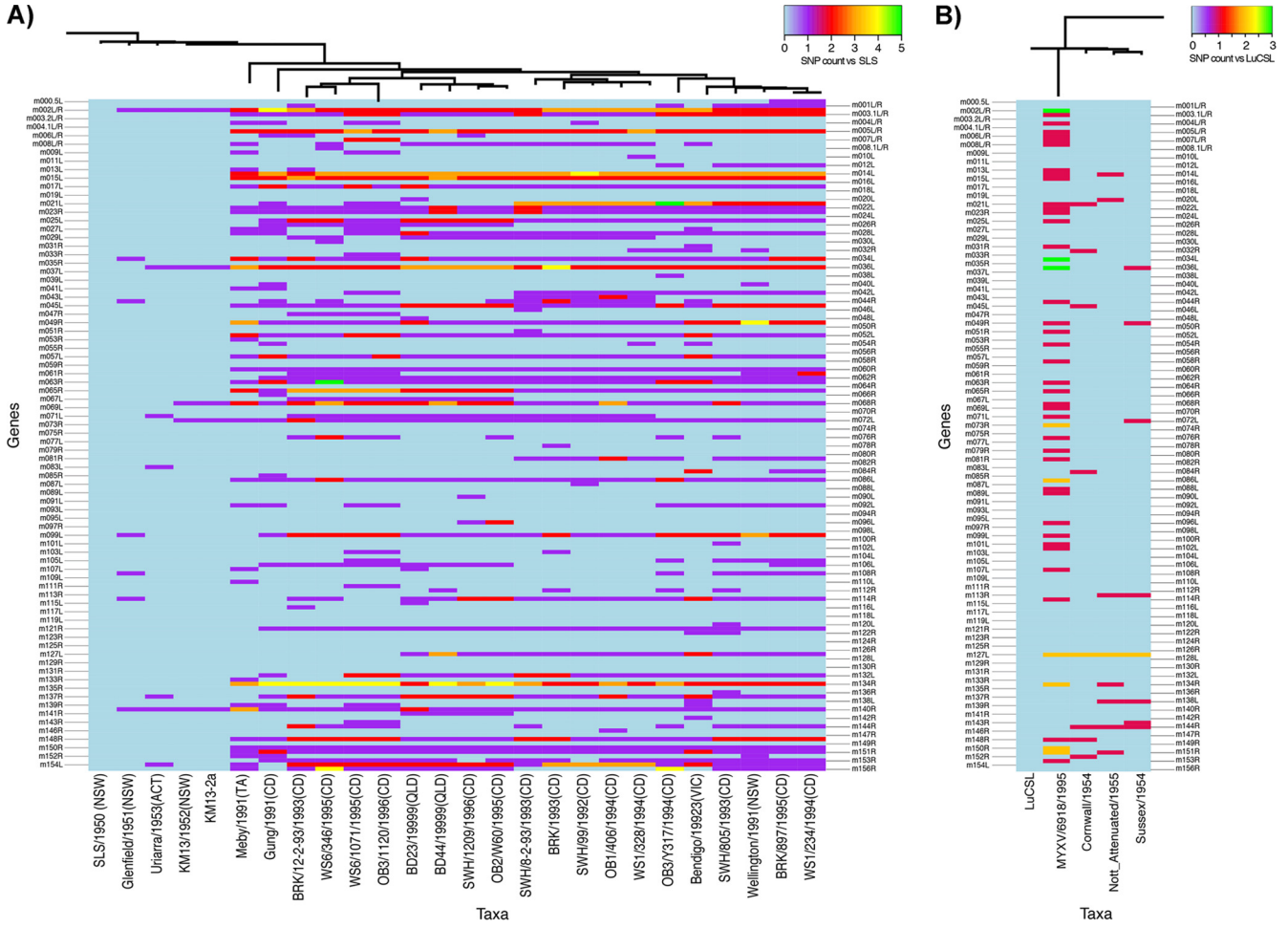

FIG 2 Heat maps showing the number of SNP mutations in each gene from the SLS (A) and Lu (B) strains. Genes are organized in rows according to their order in the reference genome, and taxa are ordered along columns by their branching order in the MYXV phylogeny.

and passage history prior to release. Overall, there are 80 nucleotide differences (0.05% difference), including indels, between SLS and Lu (72 if TIRs are counted in only one copy) (Table 3). However, frameshifts in *M005L/R*, *M083L*, and *M152R* due to indels also produce multiple amino acid changes in SLS compared to the Lu sequence: *M005L/R*, which codes for an E3 ubiquitin (Ub) ligase/apoptosis regulator, is disrupted by a C insert at nucleotide 34. It is likely that translation occurs from an alternative ATG from nucleotide 17 that does not change the ANK repeats and the C-terminal F-box domain of the M005 protein. *M083L* is disrupted by a C deletion in a homopolymer tract at nucleotide 513. M083 is homologous to rabbit carbonic anhydrase (12) and is probably a virion structural protein. Finally, there is a T deletion in a homopolymer toward the 3′ end of the *M152R* (Serp 3) gene at nucleotide 782; read-through of the Lu stop codon leads to a predicted protein of 273 amino acids in SLS rather than 266 amino acids in Lu. These indels are also present in Australian isolates of MYXV sampled between 1951 and 1953, confirming that the mutations were present in the progenitor virus. It is likely that one of these frameshift mutations explains the reduced virulence of SLS compared to that of Lu.

Comparisons of SLS with subsequent Australian isolates. We sequenced three isolates of MYXV that had been sampled within the first 3 years of the initial epizootic of myxomatosis in Australia and that had been previously characterized in terms of virulence (9): the Gv strain (Dubbo/Feb 1951; grade 1 virulence), KM13 (Corowa/Dec 1952; the prototype grade 3 virus), and Uriarra (Ur) (Uriarra/Feb 1953; grade 5 virulence [27]). Amino acid sequence changes and gene disruptions between SLS and these three viruses are summarized in Table 4.

Three of the nonsynonymous mutations in Gv, which is more virulent than SLS, are in enzymes involved in viral transcription and replication, M044 (RNA helicase: R606H; the R is conserved in chordopoxvirus sequences), M108 (DNA helicase: F18I; only MYXV and RFV have F at this position; other chordopoxviruses have I, M, or L), and M114 (RNA polymerase: A686V; the A is completely conserved at this position in chordopoxvirus sequences), each of which could affect replication efficiency. *M014L*, *M130R*, and *M153R* all have single-nucleotide indels that disrupt the reading frame. The single-nucleotide indel in *M014L* causes premature termination at residue 477, making the protein smaller than the 517-amino-acid SLS protein. This indel is also present in Ur and KM13, indicating that the mutation arose early on. M014 has an N-terminal BTB motif and C-terminal kelch motifs and is predicted to form an E3 ubiquitin ligase complex that targets cellular proteins to the proteosome for destruction (28). This trun-

TABLE 2 Mutations from SLS conserved in Australian MYXV isolates[a]

SLS position	Gene[k]	Protein function[e]	Mutation	No. of viruses
1968	*M002L/R*	TNF binding/apoptosis inhibition	A226V	21 (all recent)
2576	*M002L/R*	TNF binding/apoptosis inhibition	Synonymous GCG→GCA	24 (all Australian)
3168	*M003.1L/R*	PRR[e] signal inhibition?	A37V	21 (all recent)
5082	*M005L/R*	Apoptosis inhibition/Ub ligase	R434W	21 (all recent)
5756	*M005L/R*	Apoptosis inhibition/Ub ligase	S209Y	21 (all recent)
11484	Intergenic	—	A→G	13
12348	*M009L*	Putative Ub ligase	A261V	16[b]
12715	*M009L*	Putative Ub ligase	Frameshift T insert 420	20 (including Meby)[c]
16042	*M014L*	Putative Ub ligase	V175I	21 (all recent)
16201	*M014L*	Putative Ub ligase	G122W	21 (all recent)
16478	*M014L*	Putative Ub ligase	Synonymous GTC→GTT	18
16615	Intergenic	—	A deletion	13 (includes Ur)
16923	*M015L*	Ribonucleotide reductase	Synonymous GAA→GAG	21 (all recent)
17332	*M015L*	Ribonucleotide reductase	V85A	21 (all recent)
17877	*M017L*	?	E71K	21 (all recent)
18236	Intergenic	—	Multiple GTAGGTAG insert	21
18250	Intergenic	—	Multiple AGTTTAGT insert	17
18277	Intergenic	—	T→C	21 (all recent)
21578	*M021L*	EV maturation; VACF12L orthologue	D315N	14
23608	*M022L*	EV[g] protein	Synonymous GTC→GTT	21 (all recent)
24933	*M025L*	VACV F16 orthologue	M11I	20
28185	*M028L*	EV formation	S244L	18
36832	*M034L*	DNA polymerase	Synonymous TTC→TTT	21 (all recent)
38437	*M036L*	VACV O1 orthologue	C270Y	22 (all recent and KM13)
38987	*M036L*	VACV O1 orthologue	Synonymous CTG→TTG	21 (all recent)
41406	Intergenic	—	T insert	20
47167	*M045L*	Virion morphogenesis	D263N	21 (all recent)
50515	*M049R*	VACV G5 orthologue	Synonymous CTG→TTG	21 (all recent)
52256	*M052L*	Core structural protein	S29N	21 (all recent)
55900	*M057L*	Core protein	L90V	21 (all recent)
57398	*M060R*	Virion protein	Synonymous GCG→GCA	21 (all recent)
58821	*M062R*	Host range	K142T	20
59512	*M063R*	Host range	S195C	21 (all recent)
59585	Intergenic	—	T deletion	20
60567	*M065R*	Poly(A) pol[i] subunit	T98 M	21 (all recent)
64305	*M068R*	RNA pol subunit	Synonymous ACG→ACA	22
70115	*M072L*	RNA pol-associated transcription factor	I150T	22
82120	*M083L*	CA homologue/virion protein	G insert	21 (all recent)
85496	*M086L*	DNA helicase	Synonymous CGA→CGG	21 (all recent)
99168	*M099L*	Core protein precursor	Synonymous GCG→GCA	20
112683	*M114R*	RNA pol subunit	P1147H	12
115902	*M121R*	CLECT[h] EV protein	S21F	20
119939	*M127L*	Photolyase	Synonymous 1107 AGC→AGT	15
123409	*M132L*	?	C133Y	18
125935	*M134R*	Membrane protein	S84P	21 (all recent)
128748	*M134R*	Membrane protein	Synonymous GCG→GCA	21 (all recent)
131595	*M134R*	Membrane protein	AAA insert (K)	22
133151	*M137R*	VACV A51 orthologue	Synonymous GGC→GGT	20
135593	*M140R*	Putative Ub ligase	P76H	24
142764	*M148R*	Putative Ub ligase	L383F	21 (all recent)
145699	*M150R*	Putative Ub ligase	P173S	21 (all recent)
147192	*M151R*	Serpin (Serp 2)	R173G	21 (all recent)
148711	*M153R*	Ub ligase MHC-1 downregulation	Synonymous TGT→TGC	17
149127	*M153R*	Ub ligase MHC-1 downregulation	L204S	19 (24) (all Australian)[d]
149717	*M154R*	VACV M2 orthologue/NF-κB inhibition?	Y53C	19
149836	*M154L*	VACV M2 orthologue/NF-κB inhibition?	Synonymous 39 GTC→GTT	13
150280	*M156R*	eIF2α homologue (IFN resistance)	L98P	13

[a] Mutations shared by 12 or more viruses.
[b] SWH/1209, OB3/1120, BD44, and BD23 have deletions of the associated region of *M009L*.
[c] Meby was isolated in Tasmania not on mainland Australia. All other Australian isolates were from the mainland. There are 21 isolates from 1991 to 1999.
[d] All Australian isolates sequenced have the nucleotide mutation at SLS 149127, but Gv, WS6/1071, BD44, and Meby have frameshift mutations that alter the reading frame of *M153R* and Wellington has a 9-nucleotide deletion which shifts the amino acid mutation to L201S.
[e] ?, unknown; —, not applicable.
[f] PRR, pattern recognition receptor.
[g] EV, enveloped virus.
[h] CLECT, C-type lectin.
[i] pol, polymerase.
[k] Mutations duplicated in TIR are indicated by *L/R* in the gene name and are only shown at the LH end of the genome.

cation would delete the final kelch domain, potentially altering target protein recognition. However, the role of M014 in virulence is unknown, as is that of *M130R*. The large number of gene disruptions in Gv suggests that this virus may be a variant selected during previous plaque purification (29) from which this virus was obtained. The likely explanation for the attenuation of KM13 is the disruption to *M014L*. Although this mutation is also present in the virulent Gv, there is no other obvious mutation that might lead to the attenuation of KM13. To further assist in the documentation of virulence determinants, we also sequenced a laboratory variant of KM13 reported to have a lower case fatality rate (KM13 2A) (2, 30). The only difference between KM13 and KM13 2A appears to be an extra A in a noncoding sequence of KM13.

Also of note is that Ur has an extra C inserted after nucleotide

TABLE 3 Genome changes in SLS compared to Lu[a]

Lu position	Lu sequence	SLS sequence	SLS position	Gene	Mutation and/or location	Protein function
22	A	—[b]	21	Noncoding		
621	A	C	621	Noncoding		
2577	C	T	2577	*M002L*	Syn[c] GCG→GCA (A) nt 69	TNF binding/apoptosis regulator
2794	G	—	2793	Intergenic	Bw[d] M003.1 and M002	
6092	C	T	6091	*M005L*	D98N GAC→AAC nt 297	E3 Ub ligase/apoptosis regulator
6349	—	G	6349	*M005L*	Frameshift	E3 Ub ligase/apoptosis regulator
6351	A	G	6351	*M005L*	Syn CCT→CCC (P) nt 34	E3 Ub ligase/apoptosis regulator
9370	G	A	9370	*M008L*	Syn GAC→GAT (D) nt 1005	Putative E3 Ub ligase
13169	G	T	13169	Intergenic	Bw M009L and M10L	
18237–18244	GGTATGTA	—	18235	Intergenic	Bw 17L and 18L tandem repeat	
20976	C	T	20967	*M021L*	Syn CAG→CAA (Q) nt 1554	EV maturation
22645	C	T	22636	*M022L*	Syn ACG→ACA (T) nt 1032	EV protein
25039	G	A	25030	*M026R*	A9T GCA→ACA nt 25	DNA binding phosphoprotein
30013	T	C	30004	*M030L*	T10A ACA→GCA nt 28	RNA pol subunit
36188	T	C	36179	*M034L*	Y227C TAT→TGT nt 680	DNA pol
38319	G	A	38310	*M036L*	Syn TTC→TTT (F) nt 936	VACV O1L orthologue
40605–40607	TTC	—	40595	*M040L*	E 258 deleted	DNA binding phosphoprotein
48097	G	A	48085	*M046L*	Syn CCC→CCT (P) nt 201	Membrane protein
48780	A	G	48768	*M047R*	T164A ACA→GCA nt 490	Late gene expression regulator
53704	T	C	53692	*M054R*	Syn CGT→CGC (R) nt 519	Membrane fusion complex
54952	A	G	54940	Intergenic	Immediately 5′ to *M056R*	
55308	C	T	55296	*M057L*	S291N AGT→AAT nt 872	Core protein
56431	C	T	56419	*M058R*	Syn ATC→ATT(I) nt 228	Core protein
57922	A	T	57910	*M061R*	K41N AAA→AAT (K) nt 123	Thymidine kinase
60376	A	G	60364	*M065R*	Syn ACA→ACG (T) nt 90	Poly(A) pol regulatory subunit
62205	—	T	62194	Intergenic	Immediately 5′ to *M068R*	
67449	C	T	67438	*M071L*	Syn TCG→TCA (S) nt 735	Membrane protein
74445	G	A	74434	*M076R*	Syn TCG→TCA (S) nt 1740	mRNA capping enzyme
80489	A	G	80478	*M081R*	Q371R CAG→CGG nt 1112	Early transcription factor subunit
82131	G	—	82119	*M083L*	C deletion at 513 →reading frameshift	Carbonic anhydrase homology/membrane protein
82179	C	T	82167	*M083L*	Syn CTG→CTA (L) nt 462	Carbonic anhydrase homology/membrane protein
83974	A	G	83962	*M085R*	Syn GTA→GTG (V) nt 669	VACV D10R orthologue
87056	A	C	87044	*M088L*	Syn TCT→TCG (S) nt 1494	Virion protein
90140	T	C	90128	*M092L*	Syn GCA→GCG (A) nt 1790	Core protein
118290	T	C	118278	*M124R*	Syn GGT→GGC (G) nt774	Unknown
123774	T	C	123762	*M132L*	Syn GTA→GTG (V) nt 45	Unknown
128789	C	T	128777	*M134R*	S1031L TCG→TTG nt 3092	Membrane protein
129085	G	T	129073	*M134R*	A1130S GCT→TCT nt 3388	Membrane protein
130326	C	T	130314	*M134R*	Syn GGC→GGT (G) nt 4629	Membrane protein
131079	C	T	131067	*M134R*	Syn GAC→GAT (D) nt 5382	Membrane protein
131133	T	C	131121	*M134R*	Syn GCG→GCC (A) nt 5436	Membrane protein
131176	C	T	131164	*M134R*	Syn GTG→TTG (L) nt 5479	Membrane protein
131187	C	A	131175	*M134R*	Syn ACC→ACA (T) nt 5490	Membrane protein
131230	A	G	131218	*M134R*	T1845A ACG→GCG nt 5533	Membrane protein
131238	G	C	131226	*M134R*	E1847D GAG→GAC nt 5541	Membrane protein
131259	T	C	131247	*M134R*	Syn GAT→GAC (D) nt 5562	Membrane protein
131316	A	G	131304	*M134R*	Syn GCA→GCG (A) nt 5619	Membrane protein
131328	G	A	131316	*M134R*	Syn CCC→CCA (P) nt 5631	Membrane protein
131377	A	G	131365	*M134R*	T1894A ACA→GCA nt 5680	Membrane protein
131424	C	T	131412	*M134R*	Syn GAC→GAT (D) nt 5727	Membrane protein
131487	G	A	131475	*M134R*	M1930I ATG→ATA nt 5790	Membrane protein
131550	G	A	131538	*M134R*	Syn GGG→GGA (G) nt 5853	Membrane protein
132122	G	A	132110	*M135R*	Syn GCG→GCA (A) nt 420	Immune modulation/virulence
133197	G	A	133185	*M137R*	D96N GAC→AAC nt 286	VACV A51
133552	A	G	133540	*M137R*	D214G GAC→GGC nt 641	VACV A51
134435	C	T	134423	*M138L*	D106N GAC→AAC nt 316	α-2,3-Sialyltransferase
141046	T	C	141034	*M147R*	S115P TCG→CCG nt 3343	S/T-specific protein kinase
147887	G	A	147875	*M152R*	A66T GCA→ACA nt 196	Serp 3
148316	A	G	148304	*M152R*	T209A ACA→GCA nt 625	Serp 3
148375	A	G	148363	*M152R*	Syn GCG→GCA (A) nt 684	Serp 3
148472	T	—	148459	*M152R*	nt 782; readthrough	Serp 3
149140	C	T	149127	*M153*	S204L TCA→TTA nt 611	E3 Ub ligase/MHC-1 downregulation
149864	C	T	149851	*M154L*	Syn GTG→GTA (A) nt 24	NF-κB regulation

[a] Mutations in the TIR are only shown at the left hand TIR.
[b] —, nucleotide deleted.
[c] Syn, synonymous.
[d] Bw, between.

30 in *M005L/R*. This means that the alternative ATG, which we predict to be used by SLS and all other Australian viruses sequenced here, does not create a sense open reading frame (ORF) in Ur. The only downstream ATG that is compatible with an ORF is at nt 308; translation from this ATG would produce a 382-residue protein with 5 ANK repeats and the C-terminal F-box compared to 7 ANK repeats in the 478-residue SLS protein. However, there is no convincing promoter sequence upstream of this ATG. This is likely the main attenuating mutation in Ur. Ur also has an A insert in a homopolymer tract toward the 3′ end of *M134R* at nucleotide 5911, the same location as the 3A insert in Gv and KM13 (Table 4). This leads to a predicted truncated protein of 1,973 amino acid residues, rather than the 2,000 residues of the SLS and Lu proteins, which retains the predicted C-terminal transmembrane domain that is conserved across the *Chordopoxvirinae*.

TABLE 4 Coding changes and indels in viruses from 1951 to 1953 compared to SLS

Gene	Protein function (no. of aa[b])	Change[a] for virus:		
		Glenfield (1951, grade 1)	KM13 (1952, grade 3)	Ur (1953, grade 5)
M005L/R	Host range/E3 Ub ligase (478)			ORF disrupted by C nt ins[c] at 30
M014L	E3 Ub ligase (517)	ORF disrupted by C nt ins at 1405	ORF disrupted by C nt ins at 1405	ORF disrupted by C nt ins at 1405
M036L	VACV O1 orthologue (680)		C270Y*	F293L
M044R	RNA helicase (678)	R606H		
M071L	Virion protein (324)			E172K
M072L	RNA pol-associated transcription factor (796)		I150T*	
M108R	DNA helicase (478)	F18I		
M114R	RNA pol subunit (1,155)	A686V		
M130R	Unknown (122)	ORF disrupted by G ins at 30 f		
M134R	Surface glycoprotein (2,000)	AAA nt ins* K	AAA ins* K	A nt ins→premature stop at 1973
M137R	Orthologue to VACV A51 (310)			A308T
M140R	E3 Ub ligase (553)	P76H*	P76H*	P76H*
M141R	OX-2 homologue (218)	S45 insert		
M153R	RING CH E3 Ub ligase (206)	ORF disrupted by G nt deletion at 329	L240S*	L240S*

[a] *, present in all modern isolates from Australia.
[b] aa, amino acids.
[c] ins, insert.

Recent Australian isolates. In total, we determined sequences for 21 Australian viruses isolated between 1991 and 1999, 6 of which had been characterized by virulence assays: Bendigo, Wellington, BRK (grade 1), SWH, Gung (grade 4), and Meby (grade 5) (31). All of these viruses have the C insertion at 35 in *M005L/R* and the T deletion in *M152R* seen in SLS. However, the indel in *M083L* present in SLS, Ur, Gv, and KM13 has reverted in every Australian isolate sequenced from the 1990s. Similarly, the indel disrupting the *M014L* gene found in Gv, Ur, and KM13 is not present in any of the more recent isolates. All the isolates have the 3A indel in *M134R* seen in Gv and KM13. However, the underlying sequence reads that map to that genomic region indicate that there is a subpopulation of viruses in OB3/1120 that have a 2A insertion, rather than a 3A insertion; this 2A indel would lead to disruption of the *M134R* ORF. A similar subpopulation with the 2A insert was seen in Ur, which has a majority population with a single A insert. Homopolymer sequences such as those in *M134R* are common in MYXV, and poly(A) or poly(T) tracts are common at the 3′ ends of genes and in the intergenic sequence, where they are frequently part of promoter structures for the downstream gene or the T_5NT early transcription termination signal. In the Australian isolates, 13 of 16 single-base indels that occur in coding sequences (Table 5) occur in homopolymer tracts of 4 or more bases, and there are 17 positions with single-base indels in intergenic homopolymers involving one or more viruses (positions in TIRs have been counted only once). Polymerase slippage leading to read-throughs or premature termination (e.g., SLS *M152R*) may facilitate evolutionary plasticity, allowing slight changes in protein sequences. Indels either in homopolymers or repeat sequence can also lead to gene disruption, in turn affecting virulence (32, 33), and also function to repair ORFs, as in the case for *M083L*.

All but one of the recent isolates have a frameshift mutation due to a single nucleotide insertion in a homopolymer tract in *M009L*, a member of a three-gene family (*M006L/R*, *M008L/R*, and *M009L*) (12) that are predicted to encode E3 ubiquitin ligases with N-terminal BTB domains followed by kelch motifs (28). The insertion at nucleotide 420 produces a truncated protein of 146 rather than 509 residues. In addition, four viruses have further mutations that disrupt the reading frame, and *M009L* is also disrupted in viruses that have gene duplications from the right hand (RH) end of the genome (see below), implying that this gene is nonessential. BRK has a 92-bp deletion in the *M036L* gene, which leads to a truncated protein of only 212 residues rather than 680 in the SLS protein. The function of this gene (an orthologue of vaccinia virus [VACV] *O1L*) in MYXV is unknown, but in VACV the O1 protein enhances signaling via Erk1/2 by the viral epidermal growth factor (VGF) homologue and increases virulence (60). As BRK is of grade 1 virulence (31), *M036L* is unlikely to be crucial for virulence in this virus. In this respect, the attenuated United Kingdom isolates Sussex (1954; grade 3) and Nottingham (1955; grade 5) also have a common indel that disrupts the *M036L* ORF, and the attenuated Spanish isolate 6918 has an independent disruption in *M036L* (34). Both Nottingham and 6918 possess other mutations that explain attenuation. However, the disruption in *M036L* is the only one in Sussex, suggesting that it may play some role in virulence.

ORF-disrupting mutations were also common in *M153R*, which encodes a protein with an N-terminal RING-CH domain, which is predicted to form an E3 Ub ligase complex and which downregulates major histocompatibility complex class 1 (MHC-1), CD4, ALCAM/CD166, and Fas/CD95 on the membranes of infected cells, potentially inhibiting $CD8^+$ T lymphocyte recognition and death signaling. Deletion of this gene in the T1 Lu-derived strain reduced the case fatality rate from 100% to 30% (35–37). Meby, a grade 5 virus, has a 73-bp deletion between repeat sequence blocks (AATACG) in the region of *M153R* encoding the C-terminal conserved region (CR) (31, 35) of the 206-amino-acid protein, which leads to read-through of the normal stop signal and a completely changed C-terminal protein sequence after residue 168. A single nucleotide deletion at nt 469 in WS6/1071 and OB3/

TABLE 5 Insertion and deletions in coding regions of Australian MYXV isolates

SLS position[a]	Gene	Protein function	Mutation and context[c]	Effect	Virus(es) (virulence grade if known)
408	*M000.5L/R*	?[b]	G del	Frameshift from aa 58 and read-through stop codon	BD44
5533	*M005L/R*	Apoptosis inhibition/host range	C insert (homopol)	Stop after aa 317	WS6/346
6352	*M005L/R*	Apoptosis inhibition/host range	C insert (homopol)	ORF disruption	Ur (5)
10663	*M008.1L/R*	Serpin	CC insert (homopol)	ORF disruption	BD44
11626	*M009L*	Putative Ub ligase	A del (homopol)	ORF disruption	SWH/8-2-93
12170	*M009L*	Putative Ub ligase	TA insert (TA repeat)	ORF disruption	BRK (1)
12715	*M009L*	Putative Ub ligase	A insert (homopol)	ORF disruption	All recent Australian strains except Bendigo (1)
12809	*M009L*	Putative Ub ligase	A del	ORF disruption	WS6/1071, OB3/1120
14397	*M012L*	dUTP pyrophosphatase	13 nt del	ORF disruption	OB3/Y317
15164	*M014L*	Putative Ub ligase	G insert (homopol)	ORF disruption	Gv (1), KM13 (3), Ur (5)
18324	*M018L*	Cytoplasmic protein; VACV F8L orthologue	TT insert	Frameshift from aa 60; read-through adds 20 aa	OB3/Y317
22511	*M021L*	EV maturation; VACV F12L orthologue	9-nt insert (duplication)	Duplicates LLG aa 4–6	OB3/Y317
38589–38680	*M036L*	VACV O1 orthologue	92-base deletion	ORF disruption	BRK (1)
40596	*M040L*	DNA binding phosphoprotein	TCT duplication	E258 inserted	Bendigo (1)
58322	*M061R*	Thymidine kinase	T insert (homopol)	Read-through adds LKY to C terminus	WS1/234
59143	*M063R*	Host range	ACC duplication	H72 duplicated	Gung/91 (4)
59554–59568	*M063R*	Host range	15 base del of repeat sequence	Deletes TEEEE from a repeat at the C terminus	WS6/346
60122–60124	*M064R*	Host range?	AGA del (tandem repeat)	E deleted	OB1/406; BRK/12-2-93
60122–60124	*M064R*	Host range?	AGA inserted (tandem repeat)	E 168 inserted	Well (1); WS1/234; BRK/897; SWH/805; OB3/Y317
82120	*M083L*	CA homologue/structural	G insert (homopol)	Corrects G deletion in SLS	All recent Australian strains
92163	*M093L*	Core protein	GGAAAC duplication	VP duplication	SWH (4)
113080	*M115L*	Fusion protein	CTTCGT del	66D 67E deleted	Gung/91 (4)
122397	*M130R*	?	G insert (homopol)	ORF disrupted	Gv (1)
131595	*M134R*	Transmembrane protein	AAA insert (homopol)	K insert	All Australian strains except Ur (A) and SLS; OB3/1120 has 2A subpopulation
131595	*M134R*	Transmembrane protein	A insert (homopol)	Truncates ORF, early stop	Ur (5)
137195	*M141R*	OX-2 homologue; downregulation of macrophage activation	AGT insert (tandem repeats)	S insert in repeat sequence	Gv (1)
141092	*M147R*	S/T-specific protein kinase	GT del (tandem repeat)	Repeat sequence disrupts ORF	BD23
148485	*M152R*	Serp 3	A del (homopol)	Premature stop 271 (SLS 273)	WS6/1071; OB3/1120
148845	*M153R*	Ub ligase/MHC-1 downregulation	G del (homopol)	ORF disruption, early stop after aa 118	GV (1)
148845	*M153R*	Ub ligase/MHC-1 downregulation	G insert (homopol)	ORF disruption, early stop after aa 124	BD44
148985	*M153R*	Ub ligase/MHC-1 downregulation	T del	ORF disruption, stop after aa 161	WS6/1071; OB3/1120
149018	*M153R*	Ub ligase/MHC-1 downregulation	73 bp del between repeats	Sequence read-through replaces CR domain of M153	Meby (5)
149062–149070	*M153R*	Ub ligase/MHC-1 downregulation	9 bp del in duplicated sequence	VEE repeat deleted from CR domain	Well (1)
150294	*M156R*	eIF2α homologue; IFN resistance	T del (homopol)	Read through stop, extra EG at C terminus	WS6/346; OB3/Y317

[a] Sequence positions are for the Australian progenitor SLS; mutations in genes within the TIRs are shown only for the left-hand TIR.
[b] ?, unknown.
[c] del, deletion; homopol, homopolymer.

1120 leads to a stop after residue 161 and removes the CR region but retains the N-terminal RING-CH domain and two putative transmembrane domains at 95 to 115 and 135 to 153 (35). Gv and BD44 both have truncated proteins of 118 and 124 residues due to an independent indel at nt 329. Other indels causing significant disruption to ORFs were found only in single viruses: *M000.5L/R* (BD44; unknown function), *M008.1L/R* (BD44; secreted serine proteinase inhibitor; virulence function), *M005L/R* (WS6/346; host range; virulence function), *M012L* (OB3/Y317; dUTPase), and *M147R* (BD23; Ser/Thr-specific protein kinase) (Table 5). The disruptions to *M008.1L/R* in BD44 and *M005L/R* in WS6/346 might be expected to attenuate these viruses (38, 39).

Gene duplications. The inverted terminal repeat regions of poxviruses contain noncoding terminal regions essential for replication but also contain different numbers of genes, depending on the location of the TIR boundary. These genes typically have virulence or host range functions, but the number of genes can vary greatly. This region also appears to be a potential recombination hot spot, as shown by recombination and deletions, including those in malignant rabbit virus, a recombinant between MYXV and RFV (40), or the MYXV SG33 vaccine strain (41).

Two genes, *M154L* and *M156R*, which are normally found as single copies outside the RH TIR, with *M156R* overlapping the TIR boundary, have been duplicated at the left hand (LH) TIR in the common ancestor of SWH/1209, OB2/W60, BD44, and BD23. In addition, the *M153R* gene has been partially duplicated (Fig. 3). This duplication is essentially an expansion of the TIR by 1,635 bp, 36 nt downstream of the *M153R* ATG start codon, meaning that *M153R* now overlaps the TIR junction (25). At the LH end of the genome, this duplicated sequence has replaced 923 bp of the *M009L* gene, leaving only the 5′ 608 bp; however, the *M009L* ORF is disrupted after codon 146 due to a T insert at nt 420. M156 is an orthologue of the VACV K3 protein and is predicted to inhibit the action of type 1 interferon (IFN) (42). M154 is an orthologue of VACV M2 and so may inhibit NF-κB (12, 43). At the LH end, *M153R* lacks the 5′ 36 nucleotides and upstream promoter, and

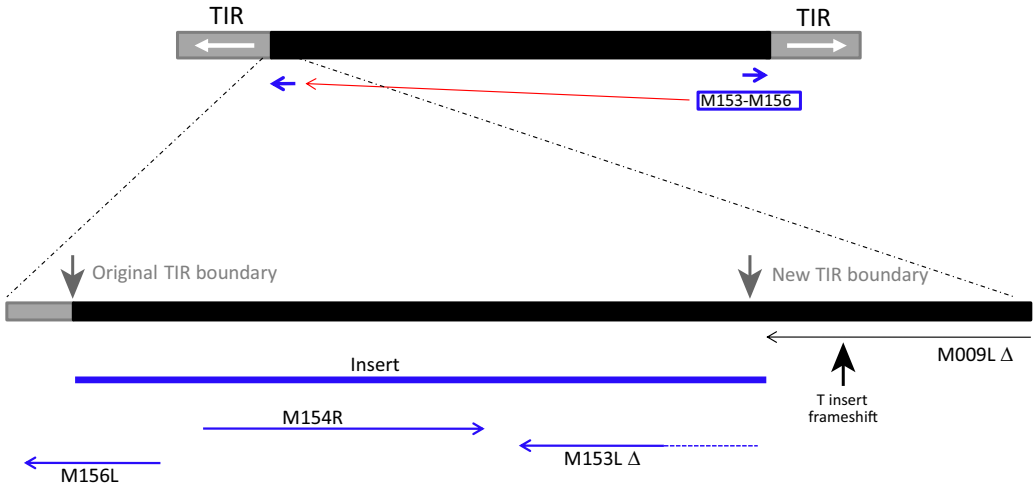

FIG 3 Expansion of the TIR boundaries in SWH/1209, OB2/W60, BD23, and BD24. Duplication of the 1,635-nucleotide region outside the right-hand (RH) TIR containing *M153R* to *M156R* and inversion and insertion of this sequence at the left-hand (LH) TIR are shown. This replaces the 3′ 923 nucleotides of *M009L* at the LH end; there are now complete copies of *M156* and *M154* at each end of the genome, but *M153* has lost the 5′ 36 nucleotides and promoter at the LH end of the genome. *M156R* originally spanned the TIR boundary at the RH end of the genome. The sequence in the TIR was present at the LH end as noncoding DNA. Note that the Lu genome annotation does not use the M155 gene number.

the insert is not in frame with the *M009L* sequence. Interestingly, this duplication was observed in the Canberra region in viruses isolated in 1995 and 1996 and is also present in viruses isolated from southwest Queensland in 1999; that viruses with this deletion occupy such a wide geographic area means that the deletion is unlikely to have an adverse effect on fitness. Whether this duplication of two potential virulence genes increases virulence or compensates for other mutations by increasing expression of these proteins is not known.

Finally, two intergenic repeat sequence regions have been defined as being variable in Australian field isolates (25, 31), one between *M017L* and *M018L*, with 2 to 8 extra copies of a GTAT GTAG repeat compared to SLS and 1 or 2 extra copies of an AGT TTAGT repeat (Fig. 4A), and the other immediately upstream of the M002 gene in the TIR, with 27 or 39 nucleotides deleted in 10 recent Australian isolates (Fig. 4B). That the latter duplication occurs on multiple branches of the phylogenetic tree indicates that it has been gained or lost in different viral lineages.

Promoter sequences. Alterations in gene expression and potentially virulence could occur due to changes in promoters. The poxvirus early (E), intermediate (I), and late (L) promoter sequences are conserved in the leporipoxviruses (44). Six viruses have mutations with a potential impact on putative promoters. WS6/346 has a T deletion in the upstream T tract of the *M008.1L/R* L promoter (Fig. 5A); OB1/406 has an extra T in the upstream T tract of the L promoter for *M057L* (Fig. 5B). G91 has a mutation in a putative weak L promoter for *M000.5L/R*, but whether this ORF is expressed has not been determined (Fig. 5C). SWH/8-2-93 has an extra A inserted in the potential E promoter for *M138L* (Fig. 5D), which might be predicted to enhance the promoter structure based on consensus early promoter sequences (44). WS6/1071 and OB3/1120 have an A deleted in the 3′ end of the potential E promoter of *M153R* (Fig. 5E), which could have an impact on promoter activity. However, both viruses also have a deletion at nt 321 in *M153R*, which disrupts the ORF.

Pathways to attenuation and virulence in Australian isolates. Overall, nine viruses derived from SLS and sequenced here have previously defined virulence phenotypes. The coding changes from SLS in these viruses are summarized in Table 6. Three attenuated viruses were sequenced from the 1990s, of which only the grade 5 Meby has a probable explanation for its attenuated phenotype. Strikingly, the grade 4 SWH/9/1992 is closely related to the grade 1 BRK. Excluding the disruption of the *M036L* ORF in BRK, only three coding differences exist to explain the attenuated phenotype of SWH: a P227S mutation at the C terminus of M004, a P33L mutation in M087 (mRNA capping enzyme; P at this position is conserved in most poxviruses), and a VP duplication in the M093 viral core protein. BRK has one unique mutation outside M036, A47V in M112, a Holliday junction resolvase. The A in this position is not conserved outside the leporipoxviruses. A similar analysis with the three grade 1 viruses sequenced revealed only two shared mutations for two viruses: Bendigo and Wellington both share I481V in M032 and Y302H in M099, the major core protein precursor. This suggests that attenuation and virulence mutations may be subtle or involve multiple epistatic effects.

An example of the complexity of possible virulence determinants involves two genes that encode proteins that are functionally conserved in poxviruses and inhibit type 1 interferon responses: *M029L*, an orthologue of VACV *E3L*, and *M156R*, an orthologue of VACV *K3L* (Fig. 1). Eleven of the recent Australian isolates have an A17V mutation in *M029L*, the only one in this gene; based on a molecular clock dating analysis, this mutation was fixed between 1969 and 1975 (Fig. 1) and coincides with the introduction and spread of the European rabbit flea, which altered the epizootology of myxomatosis in temperate Australia. Interestingly, this mutated sequence has reverted to the original sequence twice on independent branches of the tree and in viruses isolated from widely separated geographic regions. M029 has been shown to function similarly to VACV E3, binding double-stranded RNA, inhibiting protein kinase R (PKR) activation, and inhibiting IFN-β, tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6) expression (45); it is a critical virulence factor in

A) Intergenic repeat sequence between M017L and M018L

```
Lu  (18237)  GTATGTAGGTATGTAG----------CTTAGTAGTTTAGT----------GTAA (18263)
SLS (18236)  GTATGTAG----------CTTAGTAGTTTAGT----------GTAA (18254)
GV           GTATGTAG----------CTTAGTAGTTTAGT----------GTAA
KM13         GTATGTAG----------CTTAGTAGTTTAGT----------GTAA
Ur           GTATGTAG----------CTTAGTAGTTTAGT----------GTAA
BRK          GTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGT--------GTAA
BEND         GTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGT--------GTAA
WELL         GTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGTAGTTTAGTGTAA
GUNG         GTATGTAGGTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGT--------GTAA
SWH          GTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGT--------GTAA
Meby         GTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGT----------GTAA
BRK/12-2-93  GTATGTAGGTATGTAG----------CTTAGTAGTTTAGT----------GTAA
BRK/897      GTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGTAGTTTAGTGTAA
OB1/406      GTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGT--------GTAA
SWH/1209     GTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGT--------GTAA
BD23         GTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGT--------GTAA
SWH/8-2-93   GTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGT--------GTAA
WS1/234      GTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGTAGTTTAGTGTAA
WS1/328      GTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGT--------GTAA
WS6/1071     GTATGTAGGTATGTAGGTATGTAGGTATGTAGGTATGTAGGTATGTAGGTATGTAGGTATGTAGCTTAGTAGTTTAGT----------GTAA
WS6 346      GTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGTAGTTTAGTGTAA
BD44         GTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGT--------GTAA
OB2/W60      GTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGT--------GTAA
OB3/Y317     GTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGTAGTTTAGTGTAA
OB3/1120     GTATGTAGGTATGTAGGTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGT----------GTAA
SWH/805      GTATGTAGGTATGTAGGTATGTAG----------CTTAGTAGTTTAGTAGTTTAGTAGTTTAGTGTAA
```

B) Intergenic deletions between M002L/R and M003L/R

```
Lu  (2727)   CCGAATTAGAGAGGAGCCAATTAGGAGCCGAATTAGGAGCCGAATTAGAGAGGAGCCGAATTAGGAGCCGAATTAGAGAGGAGCCA (2822)
SLS (2736)   CCGAATTAGAGAGGAGCCAATTAGGAGCCGAATTAGGAGCCGAATTAGAGAGGAGCC-AATTAGGAGCCGAATTAGAGAGGAGCCA (2820)
BEND         CCGAATTAGAGAGGAGCCAATTAGGAGCCG----------AATTAGGAGCCGAATTAGAGAGGAGCCA
WELL         CCGAATTAGAGAGGAGCCAATTAGGAGCCG----------AATTAGGAGCCGAATTAGAGAGGAGCCA
GUNG         CCGAATTAGAGAGGAGCCAATTAGGAGCCG----------AATTAGGAGCCGAATTAGAGAGGAGCCA
BRK/807      CCGAATTAGAGAGGAGCCAATTAGGAGCCG----------AATTAGGAGCCGAATTAGAGAGGAGCCA
SWH/8-2-93   CCGAATTAGAGAGGAGCCAATTAGGAGCCG----------AATTAGGAGCCGAATTAGAGAGGAGCCA
WS1/234      CCGAATTAGAGAGGAGCCAATTAGGAGCCG----------AATTAGGAGCCGAATTAGAGAGGAGCCA
SWH/805      CCGAATTAGAGAGGAGCCAATTAGGAGCCG----------AATTAGGAGCCGAATTAGAGAGGAGCCA
OB3/Y317     CCGAATTAGAGAGGAGCCAATTAGGAGCCG----------AATTAGGAGCCGAATTAGAGAGGAGCCA
WS6/346      CCGAATTAGAGAGGAGCC----------AATTAGGAGCCGAATTAGAGAGGAGCCA
OB3/1120     CCGAATTAGAGAGGAGCC----------AATTAGGAGCCGAATTAGAGAGGAGCCA
```

FIG 4 Insertions and deletions in noncoding repeat sequence regions in MYXV isolates.

rabbit infections but also has a second function of binding RNA helicase A (RHA; DHX9), which promotes virus replication in some cell lines, so it has been described as a dual-function virulence and host range factor (46). The amino acid sequence from the related leporipoxvirus RFV is conserved from amino acid 1 to 8 and is identical to that from MYXV from amino acid 20 to 55 but is poorly conserved between residues 9 and 19; this region is also divergent in the Californian MSW strain of MYXV (47). If we consider the mutation and reversion in MYXV and the divergence in 3 leporipoxviruses with different natural hosts, then we find it possible that this region is involved in species specificity and host adaptation.

M156 is a homologue of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α) and competes with eIF2α for phosphorylation by PKR (42). The L98P mutation in M156 is present in 13 of the modern isolates and most likely has appeared twice on independent branches of the tree (Fig. 1) (although a lack of bootstrap support at key nodes means that we cannot formally exclude a single origin of this mutation). Two of the viruses with this mutation, WS6/346 and OB3/Y317, which are phylogenetically distinct, possess an additional T deletion in a homopolymer at the 3′ end of *M156R* that allows read-through of the stop codon and the addition of EG at the C terminus of the protein. Based on its nuclear magnetic resonance (NMR) structure, the 102-amino-acid M156 protein is predicted to be a 5-stranded antiparallel β barrel (42). The L98P mutation occurs in the β5 sheet, with the L side chain predicted to form part of the interior of the barrel, while it has been proposed that residues D97 and R99 are involved in binding PKR (42). Interestingly, *M156R* has been duplicated in the common ancestor of four of the viruses sequenced here, all of which have the L98P mutation. In VACV undergoing artificial selection, *K3L* has been shown to expand and reduce in copy numbers while acquiring adaptive mutations (48).

The virulent Lu CSL virus, released in Australia from the 1970s to the 1990s. The Lu virus was widely released in Australia from the 1970s to the 1990s. However, our phylogenetic analysis, coupled with previous studies (25, 31, 49), demonstrates that Australian field isolates are derived from SLS and that, if Lu has left descendant viruses in Australia, they have not been sampled. The Lu virus sequenced here is from a vial supplied for release and has only a single difference from the originally published Lu sequence (12, 34): a C insert at nucleotide 142 in a homopolymer tract in *M127L*, causing a frameshift mutation. The mutation may have been present in a single plaque or pock used to produce the original seed virus for release (50).

DISCUSSION

MYXV evolution is characterized by a relatively high rate of nucleotide substitution, frequent changes of virulence, and a rapid

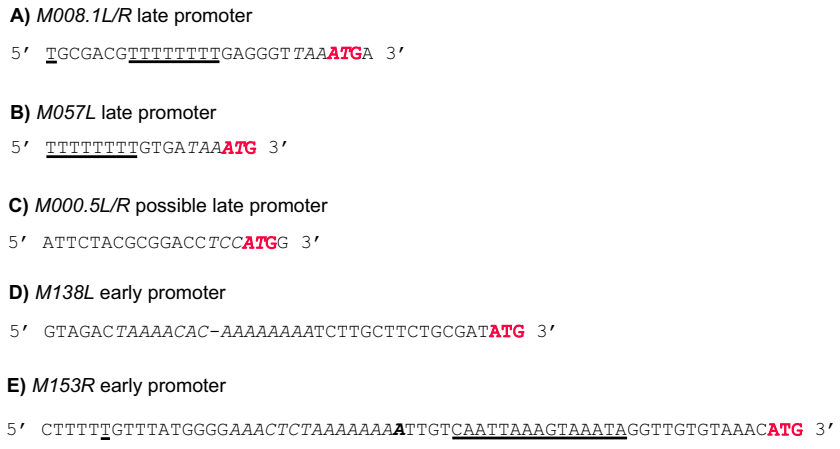

FIG 5 Sequence mutations in 5 potential promoter regions. (A) *M008.1L/R* late promoter. There is a TAAAT late promoter motif (italicized) incorporating the ATG start codon (in red). This is preceded by a 6-nt spacer and then a run of 8 Ts (underlined), which is typical of strong late promoters (spacer of 4 to 10 nt and then a T-rich tract of 5 to 15 nt). A number of isolates have a mutation at the underlined upstream T (T→C), but this seems unlikely to affect the promoter structure. WS6 346 and OBY317 have a T deletion in the 8-T tract. At the LH end of the genome this promoter is in a noncoding sequence, but at the RH end it is within the 3′ end of *M156R*. (B) *M057L* late promoter. A TAAAT motif incorporates the ATG, a 4-nucleotide spacer, and then 8 Ts. OB1/406 has an extra T in the T tract, making 9 Ts. (C) *M000.5L/R* possible late promoter. The putative promoter structure is italicized. The 2 C residues are almost invariably A in late promoters. There is no upstream T-rich domain for 100 nt upstream. G-91 has a C→T mutation in the putative promoter structure, yielding TTCATG. There is no sign of an upstream A-rich region that could act as an early promoter. (D) *M138L* early promoter. SWH 8/2/93 has an extra A. The dash is in the poly(A) tract of the italicized potential promoter. (E) *M153R* early promoter. A potential early promoter sequence is italicized. All Australian isolates lack the upstream T (underlined). This seems unlikely to have any impact on the promoter. WS6 1071 and OB31120 lack an A in the homopolymer tract (boldface). A possible alternative promoter is underlined but seems too close to the ATG.

spread in geographic space. This was exemplified by the initial mosquito-borne epizootic in 1950 to 1951, during which SLS spread across an area approximately 1,600 km south to north and 1,800 km east to west in 3 months (4). Indeed, our phylogeographic analysis clearly shows that viruses from geographically disjunct regions of Australia can still be remarkably closely related, indicative of frequent viral traffic. The success of MYXV and, subsequently, rabbit hemorrhagic disease virus (RHDV) as biological controls, combined with changes in land management, means that modern rabbit populations are likely to be less connected than in 1950 (51, 52). The key vectors for viral transmission are the mosquito, which is predominantly a spring to autumn vector and requires water for breeding, and rabbit fleas, *Spilopsyllus cuniculi* (in temperate Australia) and *Xenopsylla cunicularis* (in arid Australia), which were introduced into Australia in 1970 and 1994, respectively. Fleas provide the potential for local transmission year round, whereas mosquitoes are seasonal but have the potential for longer-distance spread. Virus may also be spread by dispersing migrating rabbits—predominantly juvenile males—that are either incubating the disease or immune and carrying fleas with the virus. In addition, large-scale rabbit migrations out of dry country during droughts may bring high numbers of susceptible animals into contact with virus, providing opportunities for spread. Accidental or deliberate translocation of infected rabbits could possibly also occur. Work in the Canberra district also suggests that viral spread is rapid, as shown by the multiple viral lineages that can cocirculate within a single community, with no apparent dominance of one lineage over any other. Such lineage cocirculation also tentatively suggests that these viruses do not differ greatly in long-term fitness despite their possible differences in virulence, although this will need to be confirmed with additional data. Indeed, in our analysis as a whole, there was no obvious signal for major fitness differences across multiple genotypes within a small geographic range.

The outcome of infection with MYXV depends on the interaction of multiple viral immune evasion and immunosuppression proteins and proteins and cells of the host innate and adaptive immune systems, together with the proteins required for virus replication, assembly, and infection. The emergence of slightly attenuated viruses during the early radiation in Australia and Europe means that mutations that enhanced transmission were selected because the infected rabbit survived longer than rabbits infected with grade 1 strains.

Most of these early (slightly) attenuated viruses still had case fatality rates of 90 to 99%, but with prolonged survival times compared to those for SLS and Lu (6, 9, 53). Experimentally, grade 4 viruses (case fatality rate of 50 to 70%; average survival times of 29 to 50 days) had the highest rates of mosquito transmission (10), but in field surveys from 1951 to 1981, these viruses were always less prevalent than grade 3 viruses (54). The rapid selection of rabbits with resistance to myxomatosis, which appears to operate through an enhanced innate immune response rather than resistance to infection (55, 56), is likely to have driven virus evolution toward increased virulence and hence to maintain transmissibility and competitiveness, and this may explain the preponderance of grade 3 viruses since all these virulence measurements were done in laboratory rabbits with no resistance. Viruses with a grade 1 phenotype in laboratory rabbits appear as grade 4 or 5 in wild rabbits with genetic resistance, while some such as BRK are found to be more virulent than the progenitor SLS when tested in wild rabbits (57, 58).

The pathway to virulence reversion and enhancement could involve reversal of attenuating mutations. For example, reversal of the indel in *M083L* has occurred in the common ancestor to all the modern isolates that we sequenced, while that in *M014L* was common to all three early viruses sequenced, although whether this is a reversal is not clear. Similarly, mutations could compensate for attenuating mutations, such as the disruption of *M036L*, which

TABLE 6 Coding changes from SLS in viruses of defined virulence

Protein	Function	Change(s) for virus[a]: Glenfield/1951 (1)	KM13/1952 (3)	Uriarra/1953 (5)	Gung/1991 (4)	Wellington/1991 (1)	Meby/1991 (5)	Bendigo/1992 (1)	SWH/9/1992 (4)	BRK/1993 (1)
M001	Chemokine binding					S213N				
M002	TNF inhibition/antiapoptosis				A226V, T188A, Q117R	A226V	A226V	A226V, Q117R	A226V	A226V
M003.1	PRR signal inhibition?				A37V	A37V	A37V	A37V, L76V	A37V	A37V
M004	Antiapoptosis						N138K		P227S	
M005	Antiapoptosis			G insert disrupts reading frame	R434W, S209Y	R434W, S209Y	R434W, S209Y	R434W, S209Y	R434W, S209Y	R434W, S209Y
M006	E3 Ub ligase?				D171G					
M009	E3 Ub ligase?				A261V, T insert at 420	A261V, T insert at 420	P343L, T insert at 420, TGG→TGA at codon 348, A140T	A261V	A261V, T insert at 420	A261V, T insert at 420, TA insert at 968
M012	dUTP nucleotidohydrolase					S129N				
M014	E3 Ub ligase?	C insert at 1406	C insert at 1406	C insert at 1406	V175I, G122W	V175I, G122W	V175I, G122W	V175I, G122W	V175I, G122W	V175I, G122W
M015	Ribonucleotide reductase small subunit				V85A	V85A	V85A	V85A	V85A	V85A
M017	?[e]				E71K	E71K	E71K	E71K	E71K	E71K
M021	VACV F12/EEV maturation				D315N	R328C, D315N		R328C, D315N	R328C, D315N	R328C, D315N
M025	VACV F16?				M11I	M11I		M11I	M11I	M11I
M027	Poly(A) pol catalytic subunit				D143N			D143N		
M028	VACV E2 EV formation				S244L	S244L	R33C	S244L	S244L	S244L
M029	IFN resistance PKR inhibitor								A17V	A17V
M032	Virion protein?					I481V		I481V		
M034	DNA pol						H222Y			
M036	VACV O1L/Leu zipper motif		C270Y	F293L	C270Y	C270Y	P278S, C270Y	C270Y	C270Y	92 nt deleted, C270Y,[b] E125K
M040	DNA binding phosphoprotein/virion							E 258 insert		
M041	VACV I5 structural				V49A					
M043	VACV I7 core/cys proteinase?								A299T	A299T
M044	RNA helicase	R606H			S361L			S361L	V350A	V350A
M045	Core enzyme, morphogenesis				D263N	D263N	D263N	D263N	D263N	D263N
M049	Core protein					E21K	T314A			
M052	Fusion complex				S29N	S29N	S29N	D290G, S29N	S29N	S29N
M057	Core protein				L90V	L90V	L90V	L90V	L90V	L90V
M062	Host range				K142T	K142T		K142T	K142T	K142T
M063	Host range				S195C, H72 insert	S195C	S195C	S195C	S195C	S195C
M064	Host range					E163 insert				
M065	Poly(A) pol regulatory subunit				T98 M	T98 M	T98 M	T98 M	T98 M	T98 M
M068	RNA pol subunit						D1012G			
M071	VACV H3L membrane protein			E172K					V113I	V113I
M072	RNA pol-associated transcription factor		I150T		I150T	I150T	I150T	I150T	I150T	I150T
M081	VETF-1[c]					R234C				
M083	Carbonic anhydrase homologue/structural protein				C nt insert 513 restores ORF	C nt insert 513 restores ORF	C nt insert 513 restores ORF	C nt insert 513 restores ORF	C nt insert 513 restores ORF	C nt insert 513 restores ORF
M084	VACV D9							S109L		
M087	mRNA capping enzyme/VITF[d]								P33L	
M092	Major core protein						M119I			
M093	Core protein								V92P93 insert	

M099	Major core protein precursor					Y302H		Y302H		
M106	Fusion complex				M136I					
M107	VACV A17 membrane protein						A172T			
M108	DNA helicase	F18I				P38S				
M112	Holliday junction resolvase									A47V
M114	RNA pol subunit	A686V						P1147H	P1147H	P1147H
M115	IMV[g] surface protein				66D67E deleted					
M121	EV group CLECT family; NK evasion?				S21F	S21F		S21F	S21F	S21F
M127	DNA photolyase							A343T		
M130	?	G nt 30 inserted ORF disrupted								
M132	?				C133Y	C133Y		C133Y	C133Y	C133Y
M134	Membrane? EV? VARV[f] B22R	K1970 insert	K1970 insert	A nt inserted at 5911, premature stop	S84P, K1970 insert, R600K	S84P, K1970 insert	S84P, K1970 insert, E1763A	S84P, K1970 insert	S84P, K1970 insert	S84P, K1970 insert
M137	VACV A51			A308T				A201T		
M138	Sialyltransferase							M25L		
M139	TLR[h] signal inhibition?						R210S			
M140	E3 Ub ligase?	P76H	P76H	P76H	P76H	P76H	P76H; T371A	P76H	P76H	P76H
M141	OX-2 homologue	S45 insert								
M142	Ser/Thr-specific protein kinase							Y183C		
M144	VACV B5R orthologue					D106G				
M148	E3 Ub ligase?				L383F	R41Q, L383F	L383F	L383F	L383F	L383F
M150	NF-κB inhibition				P173S	P173S	P173S	P173S	P173S	P173S
M151	SERP 2				P140S, R173G	R173G	R173G	P140S, R173G	R173G	R173G
M152	SERP 3				V106A	R181H				
M153	MHC downregulation	G nt 329, deleted, ORF disrupted	L204S	L204S	R40L, L204S	V182-E183-E184 deleted, L204S	nt 509–573 deleted	L204S	L204S	L204S
M154	NF-κB inhibition?					Y53C		Y53C	Y53C	Y53C
M156	IFN resistance					L98P				

[a] The year of isolation and virulence grade (in parentheses) are given.

[b] BRK has the nucleotide mutation at this position, but the earlier 92-bp deletion in M036L means that the reading frame is disrupted here.

[c] VETF-1, virus early transcription factor 1.

[d] VITF, virus intermediate transcription factor.

[e] ?, unknown.

[f] VARV, variola virus.

[g] IMV, intracellular mature virion.

[h] TLR, Toll-like receptor.

appears to be attenuating in Sussex but not in BRK, or mutations could increase virulence by new pathways. The duplication of virulence genes and the fragmentation of some reading frames also provide the raw material for further evolution of new functions, as has occurred, for example, in cowpox virus, where a gene fragment has evolved a new function in immunosuppression (59). While this might suggest that field isolates should now be of higher virulence for laboratory rabbits, the reality appears more nuanced, with grade 4 and 5 viruses present in our samples, indicating that many factors at the local level influence the effective virulence and successful transmission. In addition, the widespread establishment of the European rabbit flea in Australia, which was credited with enhancing the impact of myxomatosis by providing a year-round vector and increasing transmission, may have altered the selection pressures on both virus and rabbit.

The large and complex genome of MYXV has provided the plasticity for multiple routes to attenuation and multiple and complex routes back to virulence. The accumulation of mutations in more-recent virus isolates makes it difficult to identify single mutations that are critical for phenotype, whether virulent or attenuated. In particular, we have shown here that it is difficult to define possible roles for single amino acid changes or potentially even synonymous changes in this evolutionary process. Indeed, there has been remarkably little characterization of Australian field viruses in rabbits or even in cell culture since the 1980s. Importantly, characterization of the sequenced viruses in rabbits will provide opportunities for matching virulent and attenuated viruses that are phylogenetically closely related and for using reverse genetics to define these pathways.

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