JOURNAL OF VIROLOGY, Sept. 2003, p. 10139–10146 Vol. 77, No. 18
0022-538X/03/$08.00+0 DOI: 10.1128/JVI.77.18.10139–10146.2003

Genetic Variability of Immunomodulatory Genes in Ectromelia Virus Isolates Detected by Denaturing High-Performance Liquid Chromatography

Gloria Ribas,[1]† Jose Rivera,[2]† Margarida Saraiva,[2]‡ R. Duncan Campbell,[1] and Antonio Alcami[2]*

MRC UK HGMP Resource Centre, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SB,[1] and Department of Medicine and Department of Pathology, Division of Virology, Addenbrooke's Hospital, University of Cambridge, Cambridge CB2 2QQ,[2] United Kingdom

Received 19 August 2002/Accepted 26 March 2003

The genetic variability of nine genes in 12 isolates and strains of ectromelia virus, which causes a smallpox-like disease (mousepox) in mice, was determined and allows for classification of ectromelia viruses. The low genetic variability suggests that evolutionary pressure maintains the activity of immunomodulatory genes in natural poxvirus infections.

Poxviruses are complex DNA viruses (28). Variola virus (VaV) was the causative agent of smallpox. Vaccinia virus (VV) was the vaccine agent used to eradicate smallpox (17), but VaV remains a concern because of its potential use in bioterrorism (41). Cowpox virus (CPV) is probably a rodent virus that infects other animal species. Ectromelia virus (EV) is a highly virulent natural mouse pathogen that causes mousepox, a disease that was proposed as a model of human smallpox (16, 17).

Poxviruses encode homologues of immune molecules to modulate host defense mechanisms (3, 47). The epidermal growth factor homologue VGF enhances cell proliferation, promoting virus virulence in mice (26). The complement control protein VCP blocks complement activation and inflammatory responses (23). The semaphorin homologue vSEMA interacts with a receptor in macrophages and induces cytokines and adhesion molecules (11, 46).

Other viral proteins characterized here are soluble cytokine receptors which neutralize cytokine activity (1, 27, 30, 42). VV, CPV, and EV encode soluble viral gamma interferon receptors (vIFN-γR) with unique broad species specificity (6, 29, 44). CPV encodes four soluble tumor necrosis factor receptor (vTNFR) genes, named cytokine response modifier B (*crmB*) (19), *crmC* (39), *crmD* (24), and *crmE* (36). Secreted TNF binding activity is produced by 3 of 15 VV strains due to expression of CrmC and CrmE (2, 34). *crmB* is the only vTNFR gene predicted to be active in VaV. *crmD* is active in EV isolates Moscow-3P2, MP-3, MP-4, and SF (24), whereas *crmB*, *crmC*, and *crmE* are truncated in EV Moscow. Another TNFR superfamily member, a secreted CD30 homologue (vCD30), has been identified in CPVs GRI-90 and Brighton Red and in EVs Hampstead and Naval (33, 37, 38). vCD30 binds CD30 ligand and has immunomodulatory activity (33, 37).

Viral immunomodulatory genes are frequently determinants of viral pathogenesis, and thus their genetic variability may shed light on their relative importance during natural infections. The expression of immunomodulatory molecules was investigated in VV strains, but the growth of VV in vitro and outside its natural host, which is unknown, may have allowed an accumulation of mutations (2, 4–7). In contrast, EV was isolated from outbreaks in laboratory mouse colonies, presumably transmitted from a natural reservoir (16, 17), and offered an excellent opportunity for these investigations. Expression of binding activity for cytokines is conserved among 12 EV isolates and strains, but genetic variability was not investigated previously (43–45).

The sequence variability of nine immunomodulatory genes in 12 EV isolates, totaling 108 genes, was investigated with a novel experimental approach used to identify single nucleotide polymorphisms in human genes. Denaturing high-performance liquid chromatography (DHPLC) subjects PCR products to ion-pair reverse-phase liquid chromatography (48). Under conditions of partial heat denaturation, heteroduplexes formed in samples having sequence variation display reduced retention times relative to their homoduplex counterparts. This identifies mutations in PCR products of unknown sequence when mixed with PCR products of known sequence. To our knowledge, this is the first report using this approach for the study of viral genes.

We studied a collection of EV isolates originated in temporally and geographically diverse laboratory mousepox outbreaks and virus strains derived from them by passage in corioallantoic membranes (43). These included EV Hampstead (1930), the first EV to be isolated (25); isolates from German and Austrian outbreaks (EVs MP-1, MP-2, MP-3, MP-4, and MP-5) (32); and EV Naval, isolated in the United States (12). We also analyzed the plaque-purified EV strain Moscow-3P2 (10), derived from the highly virulent EV Moscow (1947) (8); Ishibashi-I-111, originated in Japan (20); and two egg-passaged derivatives of EV Hampstead, EVs Hampstead Egg (15) and

* Corresponding author. Mailing address: Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Level 5, Box 157, Hills Rd., Cambridge CB2 2QQ, United Kingdom. Phone: 44 (1223) 763403. Fax: 44 (1223) 330158. E-mail: aa258@mole.bio.cam.ac.uk.

† Present address: Centro Nacional de Investigaciones Oncológicas, 28029 Madrid, Spain.

‡ Present address: National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom.

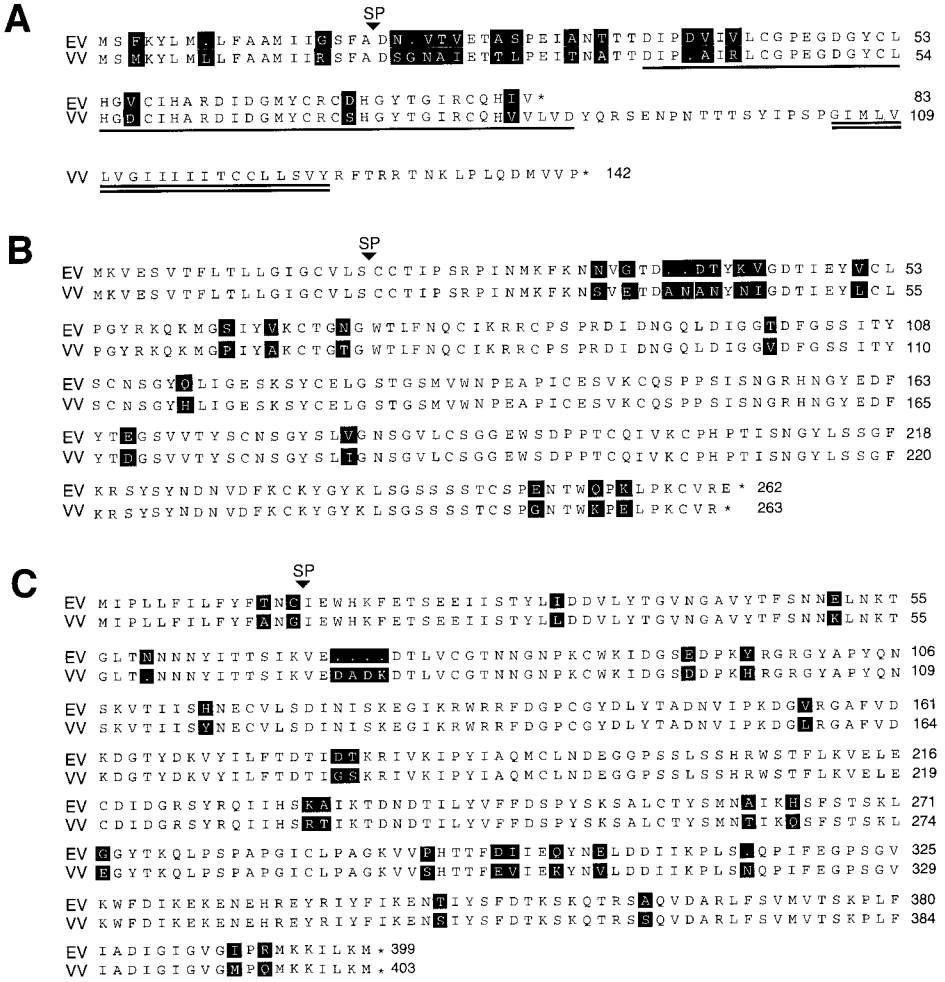


FIG. 1. Predicted amino acid sequence of the EV homologues of VGF, VCP, and vSEMA. Shown are pairwise alignments of the predicted amino acid sequences of VGF (A), VCP (B), and vSEMA (C) from EV Moscow-3P2 and VV Copenhagen (18). Solid backgrounds represent amino acid differences and stars indicate stop codons. Solid triangles show signal peptide cleavage sites (SP). In the VGF sequence (A), the regions of higher sequence similarity to host EGF are underlined and the transmembrane anchoring domain is double underlined.

Mill Hill (17). EV Hampstead Egg and EV Ishibashi-I-111 are attenuated in mice after footpad inoculation (16, 17).

Sequence information on EV genes is very limited (9, 16). EV VGF, VCP, and vSEMA genes were PCR amplified by use of oligonucleotides based on the VV sequence and were sequenced on both strands on an Applied Biosystems 377 sequencer (Perkin Elmer). The *VGF* gene in EV Moscow-3P2 encodes a truncated polypeptide that lacks the transmembrane anchoring domain (Fig. 1A). VGF is shed from the cell by proteolytic cleavage (26). Myxoma virus encodes a truncated VGF that is secreted and active (26); thus, the EV VGF may also be active. The *VCP* gene in EV Moscow-3P2 encodes a full-length polypeptide (18) (Fig. 1B). Consistent with this, supernatants from EV-infected cultures block the classical and alternative pathways of complement activation (J. Rivera and A. Alcami, unpublished data). EV Moscow-3P2 vSEMA was previously expressed for functional studies, but its sequence was not reported (11). We found that the *vSEMA* gene encodes a polypeptide of similar length to that from VV Copenhagen and longer than that from VV WR (18, 40) (Fig. 1C).

DHPLC analysis was carried out with an automated system from TransGenomic (Crewe, United Kingdom) (35). Viral genes were PCR amplified as overlapping fragments of <500 bp (13) with specific oligonucleotides (Table 1), *Taq* DNA polymerase, and 10 to 50 ng of viral core DNA (14). PCR products were mixed 1:1 with DNA from a reference EV, denatured at 95°C for 5 s, cooled to 65°C over 40 min, and loaded onto a preheated reverse-phase column based on nonporous poly(styrene-divinyl-benzene) particles (SNASep column; TransGenomic). PCR products were eluted through an acetonitrile gradient in 0.1 M triethylamine acetate buffer, pH 7, at 0.9 ml/min and were detected at 260 nm. The temperature for detection of heteroduplexes was deduced from both computational analysis (WaveMaker software; Transgenomic) and an experimental melting profile of the reference DNA. PCR samples of unknown sequence were mixed with the control DNA and run at two to four different temperatures to increase the probability of detecting mismatches that may not resolve at some temperatures due to domains with very different GC content.

TABLE 1. Sequences of the oligonucleotides used in these studies

Gene	Fragment	Annealing temp (°C)	$MgCl_2$ (mM)	Oligonucleotide	Sequence (5′→3′)[a]
crmB	1	50	2	CKBP1	CCC*AAGCTT*ACCATTACAAATATTATTCATAATAG
				CKBP8	CGC*GGATCC*AACATCGATTCCCATATGATGG
crmC	1	50	2	CrmC2	GTGATATACAGCTATTAATTTCG
				CrmC4	GGCATTTTCTACATTGATGACATCC
	2	50	2	CrmC3	CACAAAATGTGAACGCTGCCTACC
				CrmC5	GTAATTATTAATATGTCTTCATACTG
crmD	1	50	2	CrmD1	CGAGATAATATCCAAGTTAAGCG
				CrmD6	TCTGTCCTGTGTGTTACTACACG
	2	50	2	CrmD3	AGGCAAATGTAGCAGTAATCAAG
				CrmD5	ATCTTGCACTGCGTTGTGAGGTC
	3	50	2	CrmD4	GATTGTACTCCTGTCTTTATTGG
				CrmD2	ATAACTTTCGTCATCGTTAAGTC
crmE	1	50	3	K3R2	CGGACGCGATATATTCCGACATGG
				K3R4	GGAGACAATAACTATTCGAGTCAC
	2	55	3	K3R3	CCTTGGTGTCATAGTTGTAGAGGTCC
				K3R6	GATGATTAAAAGTTAGGGAGGGGATG
vCD30	1	50	2	CD30-1	GTTCTGGATACATGCACAAAG
				CD30-2	GGAGGATAATCATTTGCAAACG
vSEMA	1	50	2	Sema-14	TTTGTCGGATTGTCTATAGTAC
				Sema-15	TGCCGTGTATAAATCATAACC
	2	50	2	Sema-16	TGATATAAACATATCAAAAGAAGG
				Sema-17	TGGTATGTGGAACAACTTTTCC
	3	50	2	Sema-18	AAAATTGGGAGGATATACAAAGC
				Sema-6	CCC*AAGCTT*AATAGCGTTGCTGTAAAAATAATTCC
IFN-γR	1	55	3	B8R-1	TATATATTCGATCGCCATGGAC
				B8R-4	CGTATACAACAAGTAGATATCAC
	2	55	3	B8R-3	CATATCAATTTGTACATCGAGC
				B8R-2	TGAGGTTATGTTGATTATGCTACG
VCP	1	55	2.5	VCP-1	AAAGTAGCTGACATAAACCATTG
				VCP-2	ATTTGATCGGTGAATCTAAATCG
	2	55	2.5	VCP-3	TTAACAGATTCACAAATAGGTGC
				VCP-4	TTATTATTTGTACGATGTCCAGG
VGF	1	55	2	VGF-3	TCCCAATCTTGTTATAAACACAC
				VGF-6	TTTTTCTGAACGTTGATAGTCTAC
	2	55	2	VGF-7	ATTGTTTACACGGTGTCTGTATC
				VGF-4	TACAGATACATTGCATAGCATAGC

[a] Restriction sites added to the oligonucleotide sequence are shown in italics.

The chromatograms of the reference strain (Moscow-3P2 or Hampstead) consisted of a single major peak at most temperatures tested, unless some denaturation occurred which broadened the peak (Fig. 2). The chromatograms were different when the reference DNA was mixed with PCR fragments from other viruses containing mutations which were detected at some but not all temperatures. To determine the accuracy of the DHPLC analysis, the DNA sequences of all the genes were determined for five EVs (Moscow-3P2, Hampstead, Naval, MP-5, and Ishibashi-I-111) and of those genes predicted by DHPLC analysis to have mutations for the other EVs.

The DHPLC analysis showed no mutations in the *vIFN-γR* gene among EV isolates (Fig. 2), consistent with the conservation of secreted IFN-γ binding activity (44). Mutations in the *vSEMA* gene were only identified in EV Hampstead Egg, which had a truncated polypeptide (Fig. 2 and 3). More genetic variability was found in the *VCP* gene (Fig. 2 and 3). The sequence of the EV Ishibashi-I-111 gene was the most divergent from that of the reference strain EV Moscow-3P2, and interestingly was almost identical to that of VV Copenhagen (18). Two point mutations in the *VCP* gene of EV Ishibashi-I-111 (G681A and A717C) result in two amino acid substitutions (D231N and K239N) compared to VCP encoded by VV Copenhagen. This was unlikely to be due to PCR or viral DNA contamination since it was confirmed in two different preparations of EV Ishibashi-I-111 DNA, and parallel amplification of a control gene showed the expected EV sequence (not shown). The *VGF* gene was identical in all EVs except for Naval and MP-5 (Fig. 2). The mutations translated into amino acid substitutions but retained the shorter polypeptide length of EV VGF (Fig. 3).

All EVs tested express secreted TNF binding activity (43). CrmB was found to be truncated in EV Moscow-3P2 due to a deletion (24). The length of the PCR products amplified from

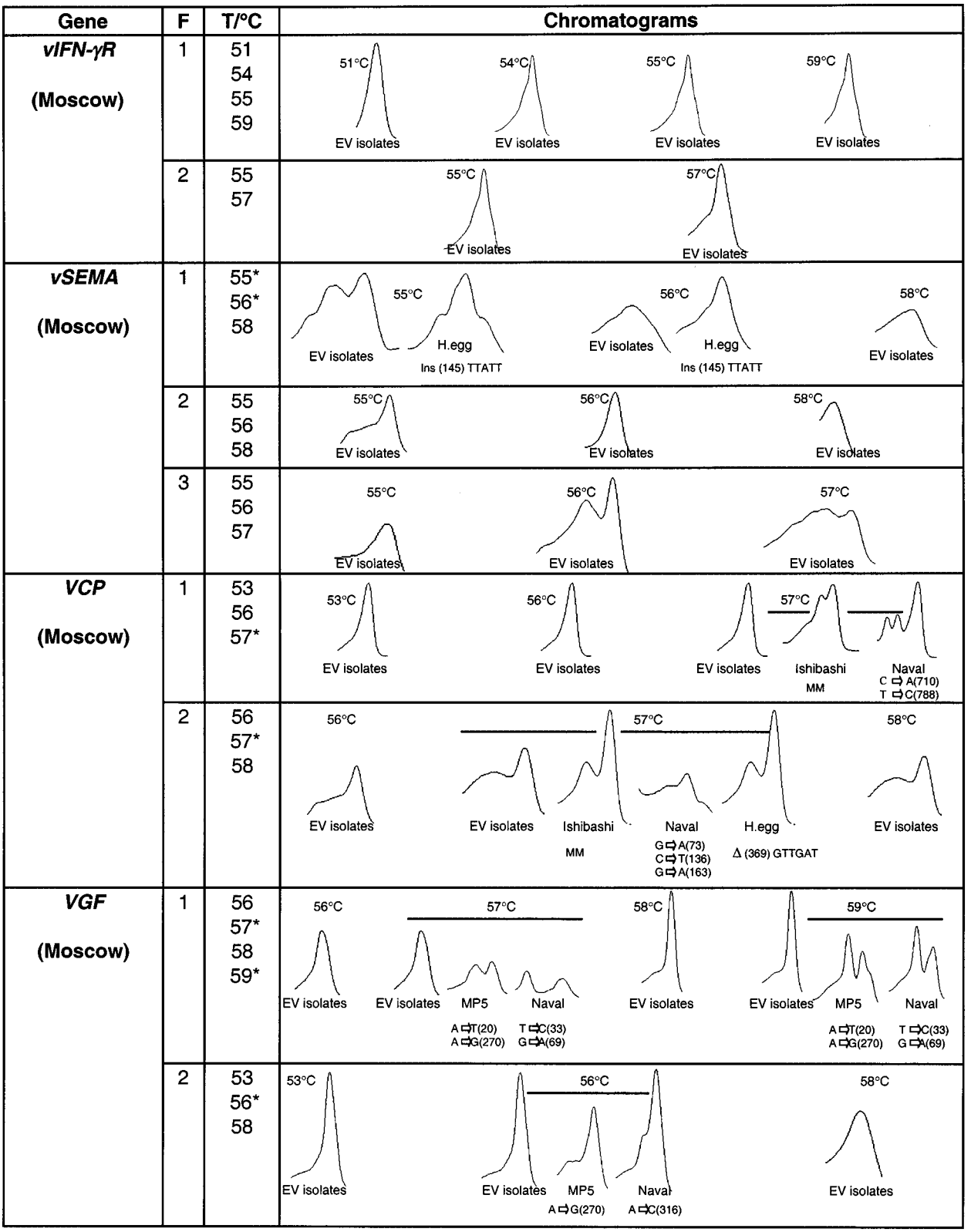


FIG. 2. DHPLC and sequence analysis of EV genes. The genes were PCR amplified under the following conditions: 30 cycles consisting of 95°C for 1 min, the indicated annealing temperature for 1 min, and 72°C for 1 min, followed by 72°C for 10 min. The PCR fragments were mixed with the reference DNA and subjected to DHPLC analysis. Elution profiles associated with the DHPLC analysis of PCR fragments from the indicated genes are shown. *x* and *y* axes depict retention times in minutes after injection and intensity of UV fluorescence at 260 nm, respectively. The fragment number (F) and melting temperature used (T) are indicated, with an asterisk marking those melting temperatures for which mutations were detected. The reference EV used for each gene is shown in parentheses. The results obtained with the reference DNA alone and when it was mixed with DNA from viruses that showed no sequence variation are labeled "EV isolates." The results from those DNA fragments in which mutations were detected are represented. The base change and the position number of the base affected are given for each chromatogram for samples containing mutations. The presence of multiple mutations (MM), insertions (Ins), and deletions (Δ) is indicated.

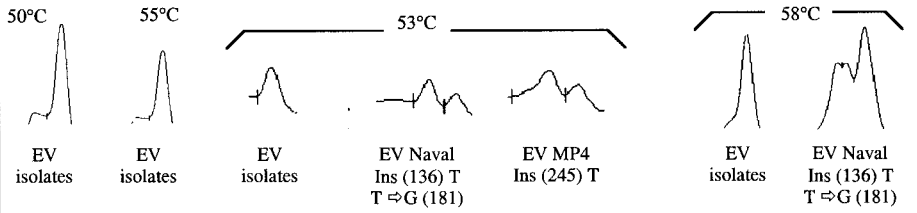
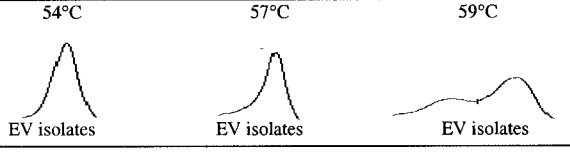
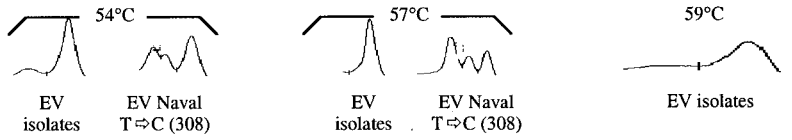
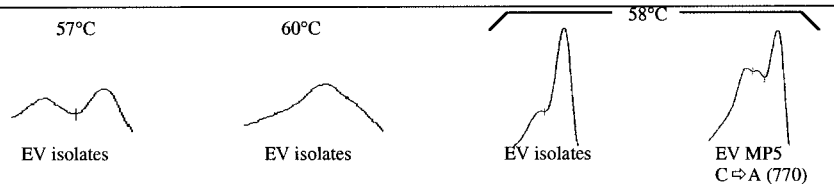
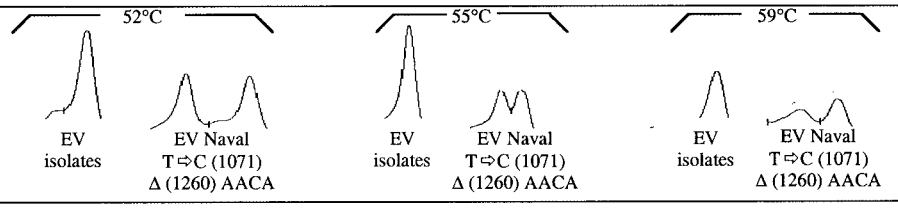
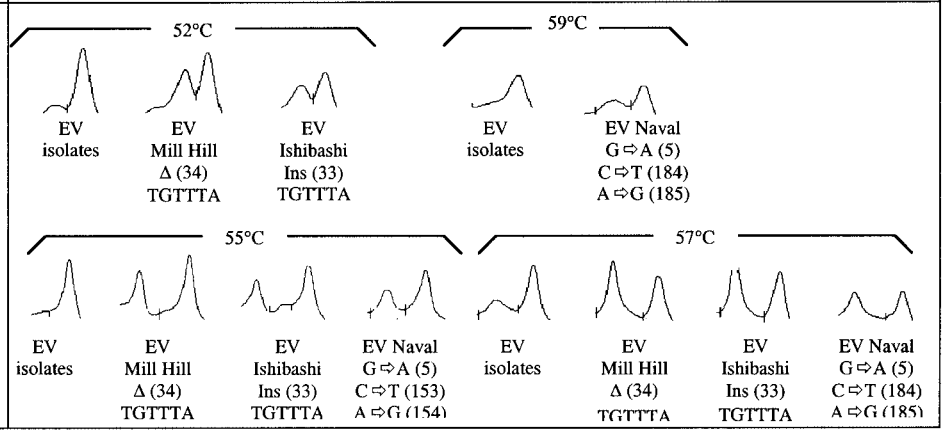
Gene	F	T / °C	Chromatogram
crmB **(EV Moscow)**	1	50 53* 55 58*	
crmC **(EV Moscow)**	1	53 59	
	2	54 57 59	
crmD **(EV Moscow)**	1	54* 57* 59	
	2	57 58* 60	
	3	52* 55* 59*	
crmE **(EV Hampstead)**	1	55 59	
	2	56 58	
CD30 **(EV Hampstead)**	1	52* 55* 57* 59*	

FIG. 2—*Continued.*

Genes / Isolates	vCD30 */[1]	CrmB [1]	CrmC [2]	CrmD **/[3]	CrmE [2]	vIFN-γR **/[2]	vSema **/[3]	VCP */[2]	VGF */[2]
Hampstead									
Hampstead Egg							√(1) Truncated protein of 27aa	√(2) Q115H ^{2} LI116-7	
Mill Hill	√(1)								
Moscow-3P2									
Moscow									
MP-1									
MP-2									
MP-3									
MP-4		√(1)							
MP-5				√(2)					√(1,2) I77V
Ishibashi I-111	√(1)							√(1,2) Protein related to VV VCP	
Naval	√(1) M70T	√(1) Extended protein of 44aa		√(1,3) F263L				√(1,2) D229Y K258E	√(1,2) A10T

FIG. 3. Variability of genes encoded by EV isolates and strains. The number of fragments analyzed for each EV gene is shown in brackets. The genes that are predicted from sequence analysis to be active or for which biological activity has been demonstrated are indicated with one or two asterisks, respectively. The variability at the DNA level is marked (√), with the fragment shown in parentheses. Mutations and deletions (^{2}) introduced in the amino acid sequence are indicated. All genes were analyzed by DHPLC, and those that were also sequenced are indicated with a shaded cell.

all EVs indicated a similar deletion (not shown), and DHPLC analysis showed genetic variability in EVs Naval and MP-4 (Fig. 2 and 3). CrmC was also found to be truncated in EV Moscow-3P2 due to mutations (24), and DHPLC analysis indicated identical sequences in all EVs tested (Fig. 2). CrmD was previously identified as an active vTNFR in EV, and the sequence of the *crmD* gene was reported for EVs Moscow-3P2, MP-3, and MP-4 (24). DHPLC analysis showed that the most divergent gene was encoded by EV Naval (Fig. 2 and 3), and mutations were detected in that of EV MP-5. Mutations in the *crmD* gene encoded by EVs MP-3 and MP-4 have been identified (24), but they were not detected by DHPLC. DNA sequencing confirmed the absence of mutations in our isolates (not shown). The *crmE* gene encodes a truncated 81-amino-acid polypeptide in EVs Hampstead and Moscow (36), and the DHPLC analysis showed high conservation among the EV genomes (Fig. 2). Mutations in the *vCD30* gene were detected in EVs Naval, Ishibashi-I-111, and Mill Hill, but only that in EV Naval translated into an amino acid substitution (Fig. 2 and 3).

Figure 3 summarizes the genetic variability of nine genes in 12 EVs. The results predicted by the DHPLC analysis in 108 genes were confirmed by DNA sequencing of 52 genes. The immunomodulatory genes are well conserved. The EV isolates that showed more variability when compared to EV Moscow-3P2 or Hampstead were Naval, Ishibashi-I-111, MP-5, and Hampstead Egg. The most variable genes were those encoding vCD30 and VCP.

We examined the sensitivity and specificity of DHPLC and concluded that DHPLC offers a reliable approach for the detection of genetic variability in viral genomes, providing the following advantages: (i) automation following temperature optimization, (ii) no requirement for special primers, (iii) no need for purification of PCR products, (iv) running time of 5 min per sample, and (v) low cost. This technique needs to be combined with DNA sequencing to determine the nature of the mutation, but initial DHPLC analysis reduces the need to sequence all samples. Here we illustrate that DHPLC analysis of 108 EV genes indicated only 13 genes that presented mutations relative to a reference gene.

Most mutations were detected by using the optimal melting temperature (T_m) suggested by the WaveMaker software, in agreement with Jones et al. (21), who described that T_m and T_m + 2°C are sufficient to detect point mutations. Only the presence of multiple melting domains of T_m at >4°C apart requires that more temperatures be used. This was the case for

5 of 18 fragments for this study. This problem may be easily avoided by dividing the gene into smaller fragments with more homogeneous melting temperatures. No correlation of DHPLC profiles with mutation type was observed. This is expected since the degree of denaturation depends on the nature of both the mismatched and flanking sequences (22, 31).

The EV immunomodulatory genes encoded by EV isolates are highly conserved and the mutations do not translate into major amino acid changes (Fig. 3). A mutation in the *vSEMA* gene of EV Hampstead Egg was the only one that inactivated the gene. Expression of secreted binding activity for interleukin-18 (IL-18), IL-1β, TNF, chemokines, IFN-γ, and IFN-α/β is highly conserved among EV isolates and strains (43–45). The only exceptions were the lack of IL-18 binding protein in EVs Ishibashi-I-111 and Hampstead Egg and of chemokine binding activity in EV Mill Hill. EVs Hampstead Egg and Mill Hill were derived from EV Hampstead by serial passage in chorioallantoic membranes. This may have allowed the introduction of mutations and caused the attenuation of EV Hampstead Egg in mice (16, 17). The only immunomodulatory gene inactivated in EV isolates was the IL-18 binding protein encoded by EV Ishibashi-I-111, a virus that is attenuated after footpad inoculation (16, 17). This suggests that there is strong selective pressure to keep these genes active in natural EV infections.

This study sheds light on the evolutionary origin of EV isolates, which is largely unknown (16). EVs isolated from London, Central Europe, or Moscow had identical sequences, with the exception of minor differences in EVs MP-4 and MP-5. The EV isolate from Japan, Ishibashi-I-111, is closely related to the European isolates, with the exception of the *VCP* gene, which suggests recombination with a VV-related poxvirus. The most divergent virus is EV Naval, which was isolated from a 1995 outbreak in the Naval Medical Research Institute (Bethesda, Md.) that was initiated after inoculation of mice with a contaminated commercially prepared mouse serum of U.S. origin (12). The United States is considered free of mousepox, and the outbreaks in the United States have been traced to the importation of infected mice or mouse tissue from Europe (49). We show that EV Naval is unrelated to the European EV isolates and therefore may have originated elsewhere, consistent with the suggestion that EV-infected mice exist in the United States (12).

In conclusion, the analysis of EV genetic variability by DHPLC combined with DNA sequencing has enabled us to investigate the presence of mutations in 108 EV genes and to determine the evolutionary relationship among EV isolates. We provide a detailed characterization of genetic variability in a poxvirus that has not been passaged extensively in tissue culture. The high conservation of immunomodulatory genes among EV isolates suggests the presence of evolutionary pressure to keep these genes active in natural poxvirus infections.

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences reported in this paper are as follows: CD30 gene (four EV isolates), AJ567675 to AJ567678; CrmB gene (five EV isolates), AJ567679 to AJ567683; CrmC gene (five EV isolates), AJ567684 to AJ567687 and AJ567726; CrmD gene (seven EV isolates), AJ567688 to AJ567693 and AJ567727; CrmE gene (three EV isolates), AJ567694 to AJ567696; vIFN-γR (four EV isolates), AJ574797 to AJ574800; SEMA gene (six EV isolates), AJ574801 to AJ574806; VCP gene (seven EV isolates), AJ574807 to AJ574813; and VGF gene (five EV isolates), AJ574814 to AJ574818.

G.R., J.R., and M.S. contributed equally to this work and are shown in alphabetical order.

This project was funded by the Medical Research Council, the Wellcome Trust (grant 051087/Z97/Z), and the European Union. G.R. was funded in part by a postdoctoral fellowship from the European Union. M.S. is funded by Fundacao para a Ciencia e Tecnologia-Praxis XXI (grant BD-18081/98). A.A. is a Wellcome Trust Senior Research Fellow.

REFERENCES

1. **Alcami, A.** 2003. Viral mimicry of cytokines, chemokines and their receptors. Nat. Rev. Immunol. **3:**36–50.
2. **Alcami, A., A. Khanna, N. L. Paul, and G. L. Smith.** 1999. Vaccinia virus strains Lister, USSR and Evans express soluble and cell-surface tumour necrosis factor receptors. J. Gen. Virol. **80:**949–959.
3. **Alcami, A., and U. H. Koszinowski.** 2000. Viral mechanisms of immune evasion. Immunol. Today **21:**447–455.
4. **Alcami, A., and G. L. Smith.** 1996. Receptors for gamma-interferon encoded by poxviruses: implications for the unknown origin of vaccinia virus. Trends Microbiol. **4:**321–326.
5. **Alcami, A., and G. L. Smith.** 1992. A soluble receptor for interleukin-1 beta encoded by vaccinia virus: a novel mechanism of virus modulation of the host response to infection. Cell **71:**153–167.
6. **Alcami, A., and G. L. Smith.** 1995. Vaccinia, cowpox, and camelpox viruses encode soluble gamma interferon receptors with novel broad species specificity. J. Virol. **69:**4633–4639.
7. **Alcami, A., J. A. Symons, P. D. Collins, T. J. Williams, and G. L. Smith.** 1998. Blockade of chemokine activity by a soluble chemokine binding protein from vaccinia virus. J. Immunol. **160:**624–633.
8. **Andrewes, C. H., and W. J. Elford.** 1947. Infectious ectromelia: experiments on interference and immunisation. Br. J. Exp. Pathol. **28:**278–285.
9. **Chen, N., R. M. Buller, E. M. Wall, and C. Upton.** 2000. Analysis of host response modifier ORFs of ectromelia virus, the causative agent of mousepox. Virus Res. **66:**155–173.
10. **Chen, W., R. Drillien, D. Spehner, and R. M. Buller.** 1992. Restricted replication of ectromelia virus in cell culture correlates with mutations in virus-encoded host range gene. Virology **187:**433–442.
11. **Comeau, M. R., R. Johnson, R. F. DuBose, M. Petersen, P. Gearing, T. VandenBos, L. Park, T. Farrah, R. M. Buller, J. I. Cohen, L. D. Strockbine, C. Rauch, and M. K. Spriggs.** 1998. A poxvirus-encoded semaphorin induces cytokine production from monocytes and binds to a novel cellular semaphorin receptor, VESPR. Immunity **8:**473–482.
12. **Dick, E. J., Jr., C. L. Kittell, H. Meyer, P. L. Farrar, S. L. Ropp, J. J. Esposito, R. M. Buller, H. Neubauer, Y. H. Kang, and A. E. McKee.** 1996. Mousepox outbreak in a laboratory mouse colony. Lab. Anim. Sci. **46:**602–611.
13. **Ellis, L. A., C. F. Taylor, and G. R. Taylor.** 2000. A comparison of fluorescent SSCP and denaturing HPLC for high throughput mutation scanning. Hum. Mutat. **15:**556–564.
14. **Esposito, J., R. Condit, and J. Obijeski.** 1981. The preparation of orthopoxvirus DNA. J. Virol. Methods **2:**175–179.
15. **Fenner, F.** 1949. Studies in mousepox (infectious ectromelia of mice). VI. A comparison of the virulence and infectivity of three strains of ectromelia virus. Aust. J. Exp. Biol. Med. Sci. **27:**31–43.
16. **Fenner, F., and R. M. L. Buller.** 1997. Mousepox, p. 535–553. *In* N. Nathanson (ed.), Viral pathogenesis. Lippincott-Raven, Philadelphia, Pa.
17. **Fenner, F., R. Wittek, and K. R. Dumbell.** 1989. The orthopoxviruses. Academic Press, Inc., London, United Kingdom.
18. **Goebel, S. J., G. P. Johnson, M. E. Perkus, S. W. Davis, J. P. Winslow, and E. Paoletti.** 1990. The complete DNA sequence of vaccinia virus. Virology **179:**247–266, 517–563.
19. **Hu, F., C. A. Smith, and D. J. Pickup.** 1994. Cowpox virus contains two copies of an early gene encoding a soluble secreted form of the type II TNF receptor. Virology **204:**343–356.
20. **Ichihashi, Y., and S. Matsumoto.** 1966. Studies on the nature of Marchal bodies (A-type inclusion) during ectromelia virus infection. Virology **29:**264–275.
21. **Jones, A. C., J. Austin, N. Hansen, B. Hoogendoorn, P. J. Oefner, J. P. Cheadle, and M. C. O'Donovan.** 1999. Optimal temperature selection for mutation detection by denaturing HPLC and comparison to single-stranded conformation polymorphism and heteroduplex analysis. Clin. Chem. **45:**1133–1140.
22. **Ke, S. H., and R. M. Wartell.** 1993. Influence of nearest neighbour sequence

on the stability of base pair mismatches in long DNA: determination by temperature-gradient gel electrophoresis. Nucleic Acids Res. **21:**5137–5143.
23. **Kotwal, G. J.** 2000. Poxviral mimicry of complement and chemokine system components: what's the end game? Immunol. Today **21:**242–248.
24. **Loparev, V. N., J. M. Parsons, J. C. Knight, J. F. Panus, C. A. Ray, R. M. Buller, D. J. Pickup, and J. J. Esposito.** 1998. A third distinct tumor necrosis factor receptor of orthopoxviruses. Proc. Natl. Acad. Sci. USA **95:**3786–3791.
25. **Marchal, J.** 1930. Infectious ectromelia. A hitherto undescribed virus disease of mice. J. Pathol. Bacteriol. **33:**713–728.
26. **McFadden, G.** 1995. Poxvirus growth factor, p. 17–27. *In* G. McFadden (ed.), Viroceptors, virokines and related immune modulators encoded by DNA viruses. R. G. Landes Co., Austin, Tex.
27. **McFadden, G., and P. M. Murphy.** 2000. Host-related immunomodulators encoded by poxviruses and herpesviruses. Curr. Opin. Microbiol. **3:**371–378.
28. **Moss, B.** 2001. *Poxviridae*: the viruses and their replication, p. 2849–2883. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 3rd ed., vol. 2. Lippincott Williams and Wilkins, Philadelphia, Pa.
29. **Mossman, K., C. Upton, R. M. Buller, and G. McFadden.** 1995. Species specificity of ectromelia virus and vaccinia virus interferon-gamma binding proteins. Virology **208:**762–769.
30. **Nash, P., J. Barrett, J. X. Cao, S. Hota-Mitchell, A. S. Lalani, H. Everett, X. M. Xu, J. Robichaud, S. Hnatiuk, C. Ainslie, B. T. Seet, and G. McFadden.** 1999. Immunomodulation by viruses: the myxoma virus story. Immunol. Rev. **168:**103–120.
31. **O'Donovan, M. C., P. J. Oefner, S. C. Roberts, J. Austin, B. Hoogendoorn, C. Guy, G. Speight, M. Upadhyaya, S. S. Sommer, and P. McGuffin.** 1998. Blind analysis of denaturing high-performance liquid chromatography as a tool for mutation detection. Genomics **52:**44–49.
32. **Osterrieder, N., H. Meyer, and M. Pfeffer.** 1994. Characterization of the gene encoding the A-type inclusion body protein of mousepox virus. Virus Genes **8:**125–135.
33. **Panus, J. F., C. A. Smith, C. A. Ray, T. D. Smith, D. D. Patel, and D. J. Pickup.** 2002. Cowpox virus encodes a fifth member of the tumor necrosis factor receptor family: a soluble, secreted CD30 homologue. Proc. Natl. Acad. Sci. USA **99:**8348–8353.
34. **Reading, P. C., A. Khanna, and G. L. Smith.** 2002. Vaccinia virus CrmE encodes a soluble and cell surface tumor necrosis factor receptor that contributes to virus virulence. Virology **292:**285–298.
35. **Ribas, G., M. J. Neville, and R. D. Campbell.** 2001. Single-nucleotide polymorphism detection by denaturing high-performance liquid chromatography and direct sequencing in genes in the MHC class III region encoding novel cell surface molecules. Immunogenetics **53:**369–381.
36. **Saraiva, M., and A. Alcami.** 2001. CrmE, a novel soluble tumor necrosis factor receptor encoded by poxviruses. J. Virol. **75:**226–233.
37. **Saraiva, M., P. Smith, P. G. Fallon, and A. Alcami.** 2002. Inhibition of type 1 cytokine-mediated inflammation by a soluble CD30 homologue encoded by ectromelia (mousepox) virus. J. Exp. Med. **196:**829–839.
38. **Shchelkunov, S. N., P. F. Safronov, A. V. Totmenin, N. A. Petrov, O. I. Ryazankina, V. V. Gutorov, and G. J. Kotwal.** 1998. The genomic sequence analysis of the left and right species-specific terminal region of a cowpox virus strain reveals unique sequences and a cluster of intact ORFs for immunomodulatory and host range proteins. Virology **243:**432–460.
39. **Smith, C. A., F. Q. Hu, T. D. Smith, C. L. Richards, P. Smolak, R. G. Goodwin, and D. J. Pickup.** 1996. Cowpox virus genome encodes a second soluble homologue of cellular TNF receptors, distinct from CrmB, that binds TNF but not LT alpha. Virology **223:**132–147.
40. **Smith, G. L., Y. S. Chan, and S. T. Howard.** 1991. Nucleotide sequence of 42 kbp of vaccinia virus strain WR from near the right inverted terminal repeat. J. Gen. Virol. **72:**1349–1376.
41. **Smith, G. L., and G. McFadden.** 2002. Science and society. Smallpox: anything to declare? Nat. Rev. Immunol. **2:**521–527.
42. **Smith, G. L., J. A. Symons, A. Khanna, A. Vanderplasschen, and A. Alcami.** 1997. Vaccinia virus immune evasion. Immunol. Rev. **159:**137–154.
43. **Smith, V. P., and A. Alcami.** 2000. Expression of secreted cytokine and chemokine inhibitors by ectromelia virus. J. Virol. **74:**8460–8471.
44. **Smith, V. P., and A. Alcami.** 2002. Inhibition of interferons by ectromelia virus. J. Virol. **76:**1124–1134.
45. **Smith, V. P., N. A. Bryant, and A. Alcami.** 2000. Ectromelia, vaccinia and cowpox viruses encode secreted interleukin-18-binding proteins. J. Gen. Virol. **81:**1223–1230.
46. **Spriggs, M. K.** 1999. Shared resources between the neural and immune systems: semaphorins join the ranks. Curr. Opin. Immunol. **11:**387–391.
47. **Tortorella, D., B. E. Gewurz, M. H. Furman, D. J. Schust, and H. L. Ploegh.** 2000. Viral subversion of the immune system. Annu. Rev. Immunol. **18:**861–926.
48. **Underhill, P. A., L. Jin, A. A. Lin, S. Q. Mehdi, T. Jenkins, D. Vollrath, R. W. Davis, L. L. Cavalli-Sforza, and P. J. Oefner.** 1997. Detection of numerous Y chromosome biallelic polymorphisms by denaturing high-performance liquid chromatography. Genome Res. **7:**996–1005.
49. **Whitney, R. A.** 1974. Ectromelia in United States mouse colonies. Science **184:**609.