implementation of these regulations and to discuss how best to respond to changes in the molecular epidemiology of viruses.

Classification of B19V

Dr K. Brown [Health Protection Agency (HPA), UK] described the criteria used by the ICTV to classify viruses. Classification by the ICTV does not extend beyond species and no consideration is given to either genotypes or clades. In the case of B19V, it is classified as a member of the *Parvoviridae* family, belonging to the erythrovirus genus. While sequence comparisons are becoming increasingly important in classification, other criteria are considered including mode of replication, virus structure, genomic organization, transcriptional and biological properties. The ICTV has classified the recently identified variant viruses, specifically V9, originally identified in France [8], A6 [9] and LaLi [10] as strains of B19V [6]. The genetic diversity of the B19V strains falls into three well-recognized genotypes [5], which can be confirmed by pairwise sequence identity profiles. Nucleotide divergence is approximately 10–15% between the different genotypes. These B19V strains are clearly distinct from other erythroviruses, such as the primate parvoviruses viruses [pig-tailed macaque parvovirus (PmPV), rhesus macaque parvovirus (RmPV) and simian parvovirus (SPV)], and more distant, tentative members such as bovine parvovirus 3 and chipmunk parvovirus. Dr Brown mentioned that the two recently identified human parvoviruses, that is, human parvovirus PARV4 [11,12] and human bocavirus [13], are quite distinct from B19V and would not be discussed further during the meeting.

Regulatory issues

Dr J.-M. Spieser (EDQM, Strasbourg, France) summarized the B19V test kit meeting held at EDQM on 9 November 2006, in response to differences in the ability of laboratories to detect genotype 2 B19V. Genotype 2 B19V has been identified in plasma pools that are undergoing batch release in Europe. The assay available from Roche is suitable only for the detection of genotype 1 B19V. The Artus (Qiagen, Hamburg, Germany) assay detects genotype 2 and some of the genotype 3 viruses. Both companies are addressing the shortfalls in the current assay kits. Currently, the Official Control Authority Batch Release (OCABR) guidelines require the detection of genotype 1 B19V, and recommend the detection of viruses such as A6 and V9 [14]. The batch release advisory group have endorsed the proposal that the guideline for B19V should be updated to reflect the requirement for the detection of different virus genotypes and be mandated in the Ph. Eur.

Dr M. W. Yu (Center for Biologics Evaluation and Research; CBER, Bethesda, MD, USA) reviewed the US Food and Drug Administration's (FDA) previous discussions on NAT testing for B19V in the USA. Most source plasma fractionators perform in-process B19V NAT testing, excluding high-titre donations following mini-pool testing. Blood collection establishments voluntarily retrieve and discard in-date components from donors with high titres of B19V DNA, to prevent their use in transfusion recipients. An infusion of a coagulation factor VIII product devoid of any anti-B19V, which was derived from plasma unscreened for B19V by NAT in a mini-pool format, with an overall load of B19V DNA as low as 2×10^4 IU, has been shown to transmit in a seronegative recipient [15]. For manufacturing pool B19V NAT testing, the FDA is currently proposing a limit of $\leq 10^4$ IU/ml for all plasma-derived products. The FDA has reviewed and approved some in-house B19V NAT procedures, for mini-pools and manufacturing pools under the Biologics Licensing Applications or their supplements for plasma derivatives. B19V NAT assays are required to be validated as analytical procedures and should be capable of detecting all virus genotypes. In the future, the FDA may consider B19V testing as donor screening, because of known risks in individuals with chronic anaemia, those who are pregnant or immunocompromised. Such screening would be dependent upon the availability of suitable commercial kits and sufficient resolution time.

Prevalence and clinical properties of different genotypes of B19V

Ms K. Hokynar (Haartman Institute, Helsinki, Finland) described studies where B19V DNA was identified in skin biopsies. Sequence analysis identified more divergent viruses, now recognized as genotype 2 B19V [10]. Analysis of tissue samples from North West Europe failed to identify genotype 3 B19V; however, genotypes 1 and 2 were both readily identified individuals born prior to 1950, while those born after this date were predominantly infected with genotype 1 [16]. *In vitro* studies of the three genotypes showed no differences in infectivity or in the activity of the p6 promoter, which is most efficient in cells permissive for B19V infection, and enhanced by the expression of NS1 [17]. Serological cross-reactivity is observed between B19V genotypes 1 and 2 using recombinant antigens and sera from individuals infected with specific genotypes [17]. All three genotypes of B19V are extremely similar, constituting a single serotype, with amino acid divergence for VP1 no greater than 4%.

Professor S. Modrow (University of Regensburg, Germany) described a clinical case, where a renal transplant recipient developed transient anaemia and arthritis and was diagnosed with B19V. Detailed molecular analysis revealed that the patient was infected with a genotype 2 B19V. A review of the original assays performed showed that there was differential sensitivity for the different genotypes of B19V. The patient showed persistent, high levels of B19V DNA [$> 10^{11}$ genome equivalents (geq)/ml] and episodes of severe anaemia [18]. Treatment with intravenous immunoglobulin (IVIG) lowered

viral loads and resolved anaemia. After 4 years, B19V DNA and anti-B19V IgM antibodies were still detectable. The patient subsequently started to develop anti-B19V IgG antibodies. Both IgG reactivity and avidity were comparable in sera from genotypes 1 and 2 B19V-infected individuals, when challenged by enzyme-linked immunosorbent assay (ELISA) using antigen from the VP1-unique region from all three genotypes. It was noted that this case presented in a very similar way to ones seen with a genotype 1 B19V infection.

Epidemiological studies of B19V infection in blood donors, pregnant women and children in Ghana were described by Dr D. Candotti (University of Cambridge, UK). In Ghana, approximately 8% of children have anti-B19V IgG, rising to 80% in adults. Viral loads and levels of anti-B19V IgM are higher in children. The rate of persistent infection is ~1·4%. One of the most striking observations of B19V infection in Ghana is that the circulating viruses are almost all genotype 3 [19]. DNA sequence analysis has revealed that the genotype 3 viruses can be divided into two subtypes or clusters that differ by more than 5% nucleotide identity [20]. These have been termed 3a and 3b, and the clustering is independent of the region of the B19V genome analysed. The nucleotide substitution rates were examined for B19V in Ghana and compared with V9, the prototype genotype 3 virus, identified nearly 10 years ago [8]. It was found that like genotype 1 B19V and canine parvoviruses, the genotype 3 viruses have an unexpectedly high rate of evolutionary change [21,22]. It would appear that the type 3a and 3b clusters were derived from a common ancestor approximately 500 years ago; however, there is a wide interval around this date.

Presence of different B19V genotypes in plasma products and susceptibility to inactivation

Professor A.-M. Eis-Hübinger (University of Bonn, Germany) reviewed studies performed on factor VIII and factor IX concentrates to determine the frequency of contamination of these products with genotypes 1 and 2 of B19V. A total of 202 different lots of clotting factor concentrates were examined. Older products used until the early 1980s that had not undergone viral inactivation procedures (21 lots, representing eight different products) were compared with more recent batches in use between 2000 and 2003 (181 lots, representing 13 different products). In the factor VIIIs, 81% were contaminated with genotype 1 B19V, and 14% were contaminated with genotype 2. In the more recent factor VIIIs, 46% were contaminated with genotype 1 B19V and 1·6% were contaminated with genotype 2 (two products were co-contaminated with genotype 1). The highest loads of genotype 1 and genotype 2 B19V were ~10^7 and ~10^5 geq/ml, respectively. It was suggested that the much lower frequency of detection of genotype 2 B19V is due to generally lower prevalence compared to genotype 1 [23].

Dr M. W. Yu (CBER) described a study looking at factor VIII concentrates using a consensus polymerase chain reaction (PCR) for genotypes 1–3 for B19V, followed by specific restriction endonuclease digestion of the product to discriminate genotype 1 from genotypes 2 and 3. A range of products (n = 202 lots) of differing purity produced before 1984 until 2004 were analysed. Of these, 79 lots were positive in the initial screening assay, and a single lot, from 1997, was positive for B19V genotype 2. DNA sequence analysis confirmed the genotype of this virus. This final product did not contain any genotype 1 B19V, and the load for genotype 2 was 10^3 geq/ml. In the case of more recent lots, contamination with B19V was generally less frequent, reflecting the implementation of NAT screening by the manufacturers [24].

Dr M. Nübling (Paul Ehrlich Institute; PEI, Langen, Germany) presented data on behalf of Dr J. Blümel (PEI) comparing the biological and physicochemical properties of B19V genotypes 1 and 2 (isolate IM-81) [25]. Infection of the cell line KU812Ep6 with the two B19V genotypes revealed that there were no differences in expression of the capsid at either the mRNA or protein level. Thermal inactivation occurs through the disintegration of the capsid proteins, with no differences observed between the two genotypes. Virions were heated and subjected to DNAse treatment prior to Southern blotting to analyse the integrity of the viral genomic DNA. Treatment of 5% albumin solution, spiked with B19V and heated to 56 °C, resulted in the same temporal inactivation kinetics, regardless of virus genotype. Similar inactivation profiles were observed for genotype 2 B19V, as had been shown previously for genotype 1 B19V virus, when subjected to low pH conditions [26].

Professor Jean-Pierre Allain (Cambridge) outlined a PCR inhibition method, utilizing a preamplification step to quantify B19V inactivation by photochemical treatment using amotosalen (S59). This molecular approach to measuring the inactivation effects of S59 on B19V has been established as an alternative to *in vitro* culture of the virus [27].

Experience with commercial and in-house assays for the detection and quantification of B19V DNA

The performance of two commercially available kits for the quantification of B19V DNA was reviewed by Dr S. Baylis (NIBSC, UK). The first kit, the Roche parvovirus B19 quantification kit for the LightCycler, only detects genotype 1 B19V. When equivalent copy number (10^6) were analysed for the three genotypes, no amplification plots were observed for genotypes 2 and 3 B19Vs in this real-time assay. However, analysis of amplification products by gel electrophoresis revealed that all three genotypes were amplified, with a much reduced signal for genotype 2 B19V, suggesting mismatches in primer and probe sequences. In the case of the Artus *RealArt* Parvo

LC kit, good amplification plots were observed for genotypes 1, 2 and 3a of B19V, while the genotype 3b virus was under quantified by approximately 2–3 logs generating much later threshold cycle (Ct) values, which could have an impact on the threshold concentration of 10 IU/ml applying to certain plasma pools [28–30]. Primer and probe sequences are of critical importance in the detection of variant viruses, this is further complicated with requirements to perform quantitative assays.

Dr T. Cuypers (Sanquin, Amsterdam, the Netherlands) described the experience of running two assays concurrently for B19V in a screening centre. The assays included the commercially available Roche LightCycler assay and a previously published consensus assay [28], validated in-house. Screening assays, performed during the previous 2 years, identified three instances where discrepant results occurred between the two tests. Molecular characterization was performed to identify the reasons for the discrepant results. One sample, not detected in the Roche assay was found to be a genotype 2 B19V, containing mutations in the primer and probe binding regions. A genotype 1 sample was under quantified by ~2 $\log_{10}$ in the Roche assay compared with the in-house assay, with a mutation at or near the end of the reverse primer binding region in the Roche test. In a third case, there was a single point mutation in the probe binding site of the in-house assay, which resulted in a failure to detect a genotype 1 B19V in the plasma sample [31]. Genotypes 2 and 3 for B19V appear to be very rare in Dutch and Belgian donors.

Dr Marta José (Grifols, Barcelona, Spain) described the validation of both in-house qualitative and quantitative consensus B19V assays, for the detection of all three genotypes. Validation was performed according to current guidelines. Particular attention was paid to B19V assay specificity, with no cross-reactivity observed with other blood borne viruses. A variety of genotype 2 and genotype 3 B19V-positive plasma samples were analysed, and good correlations were found with previously determined titres from other laboratories and the ones determined by the in-house quantitative assays.

Dr T. Gierman (Talecris, Raleigh, NC, USA) was unable to attend the meeting and his presentation on experience in testing for B19V genotypes was summarized by Dr Zerlauth.[1]

Three tests are utilized to reduce B19V viral loads in plasma fractionation pools: a qualitative donor sample test for testing mini-pools; a separate qualitative test for the QC of fractionation pools; and a quantitative test using dual-labelled fluorogenic detection probes for quality and technical operations investigations. As part of on-going efforts to assess the performance of this test system, the potential frequency of occurrence of variant B19V genotypes in US source plasma was examined. Archived sample pools created from 'non-elevated' plasma samples (samples containing B19V genotype 1 titres > 2×10^5 IU/ml and genotypes 2 and 3 titres > 5×10^6 copies/ml would have been excluded as a result of screening with the donor sample test) were retested using a fluorogenic detection probe capable of differentially detecting B19V genotypes 2 or 3. The testing of 242 large-scale sample pools (3840 samples) and 609 intermediate-scale pools (960 samples) failed to identify the presence of B19V genotypes 2 and 3. PCR analysis of 340 individual 'elevated' samples also failed to identify B19V genotypes 2 or 3 among them. The inability to detect B19V genotypes 2 and 3 in material representing approximately 1·5 million source plasma donations, which suggests that the prevalence of these genotypes within the US source plasma donor population is very low.

Provision of plasmid clones to OMCL network and manufacturers

Dr J. Fryer (NIBSC, UK) discussed how plasmid clones representing the main B19V genotypes would be distributed through the next EDQM PTS for B19V DNA later in 2007. High-titre DNA stocks have been prepared for near full-length plasmid clones, representing genotypes 1, 2, 3a and 3b of B19V (N8, A6, V9 and D91·1, respectively). Dilution to equal copy number gave equivalent results using a consensus in-house TaqMan assay for B19V DNA [28]. These plasmid clones will be distributed as a validation panel only for use in the PTS, until a plasma reference panel becomes available.

Availability of B19V viraemic plasma for reference panel development

Genotype 2

Several plasma units were identified that contained high titres of genotype 2 B19V. Dr M. Gessner (Baxter, Vienna, Austria) described the B19V plasma samples termed IM-81 and IM-82. IM-81 was a high-titre (~11·3 $\log_{10}$ IU/ml) genotype 2 plasma sample, which had been sequenced and characterized previously [25] and shown to be cross-neutralized by genotype 1 sera. IM-82 represents a subsequent bleed (4 days later) from the same donor with a titre of 7·4 $\log_{10}$ IU/ml. Dr S. Baylis (NIBSC) described a plasma pool, sourced in the

[1]Dr G. Zerlauth (Baxter, Vienna, Austria) summarized this special meeting at SoGAT XX, held in Warsaw, Poland, on 12–13 June 2007. At the same meeting, Drs L. Rinckel and T. Gierman (Talecris, Raleigh, NA, USA) reported that they have identified a high-titre, high-volume genotype 3 B19V plasma. Thus, in order to harmonize results obtained by control laboratories and plasma fractionators, a genotype panel containing each of the three genotypes of B19V will be jointly formulated by NIBSC and CBER. The panel will be evaluated together with additional genotype 2 samples and be calibrated against the current WHO International Standard for B19V DNA (99/800) in an international collaborative study. The presentations from the extraordinary SoGAT meeting are available at the following link: http://www.nibsc.ac.uk/partners/SoGAT/March_2007_Presentations.html.

USA containing a genotype 2 B19V. The pool was identified due to discrepant results, using different B19V NAT assays. This pool contains 6·2 $\log_{10}$ IU/ml of genotype 2 B19V DNA. The virus was not infectious in culture, and was likely to be neutralized by anti-B19V present in the pool. Despite the plasma being pooled, there was no genotype 1 B19V present. Dr M. Koppelman (Sanquin, Amsterdam, the Netherlands) described the identification of a genotype 2 B19V plasma sample (207458), with a titre of ~7 $\log_{10}$ IU/m. Sequence analysis indicated that this B19V was most closely related to the A6 virus [31]. Dr José (Grifols) described another genotype 2 B19V plasma sample. This sample was identified by using two different assays: the first specific for genotype 1 B19V and the second, a consensus assay described in her earlier presentation. The plasma gave negative results in the genotype 1 B19V assay, but was positive in the consensus assay. This led to further characterization of the plasma sample, which was found to have a titre of 7·3 $\log_{10}$ IU/ml. The sample was negative for a range of other virus markers, and was also negative for anti-B19V IgG and IgM, and likely to represent the early ramp-up phase.

Genotype 3

While several high-titre, high-volume plasma samples have been identified for genotype 2 B19V, there is limited material available for genotype 3. Dr D. Candotti (University of Cambridge) summarized a series of clinical samples, comprising both genotypes 3a and 3b viruses. None of the available samples exceeded ~6 $\log_{10}$ IU/ml of B19V DNA. It was proposed that B19V samples might be sought prospectively, by identifying persistent infections in blood donors (which may have titres as high as 4–5 $\log_{10}$ IU/ml). However, several thousand donations would have to be screened. Additional sources of genotypes 2 and 3 B19V have been examined and these include the screening of anti-B19V IgM-positive sera from Brazilian patients presenting with rash-like illness by Dr K. Brown (HPA). In a recently published study from Brazil [32], clinical samples from patients with B19V-like symptoms were tested for B19V DNA and the virus genotype determined. All three genotypes of B19V were identified. Dr K. Brown outlined the approach taken in his study using biotinylated PCR products and pyrosequencing to determine the genotype of each B19V-positive sample. The method was validated using previously identified variant viruses [30]. Of 50 B19V IgM-positive samples studied by this approach, 29 were positive for B19V DNA by PCR, ranging in concentration from 10^2 to 10^{10} geq/ml. These PCR-positive viruses were all genotype 1, with three unique point mutations being identified. A small study was presented by Dr S. Baylis on behalf of Dr D. York (Molecular Diagnostic Services Pty Ltd, South Africa) and Mr D. Stubbings (National Bioproducts Institute, South Africa). High-titre B19V plasma donations (n = 9) were genotyped and in contrast to the findings on the West Coast of Africa, these B19V-positive samples were all genotype 1.

Conclusions and recommendations

Overall, based upon the classification by the ICTV and in terms of what is currently know about the biological and serological properties of the different genotypes of B19V, these genotypes clearly represent strains of the same virus. The more recently identified variants appear not to be so well represented in Europe and North America as genotype 1 B19V. However, different genotypes of B19V have been found in donor plasma that has led to batch release issues and based upon recent PTS studies, some assays have proved ineffective in detecting genotype 2 B19V DNA [7]. In order to harmonize the results obtained for the detection and quantification of B19V DNA between control laboratories and the manufacturers of plasma derivatives, it was agreed that standardization of assays using well-characterized reference materials would be the way forward.

The consensus opinion at the meeting was to produce a genotype panel of plasma samples representing the different genotypes of B19V. As B19V DNA testing has a quantitative limit (10 IU/ml), any reference panel would be required to reflect the need for accuracy around this threshold concentration. Future collaborative studies used to evaluate candidate plasma samples for a reference panel would need to be calibrated against the WHO International Standard for B19V DNA [4]. In the absence of sufficient genotype 3 B19V material, it was felt that cloned DNAs may be suitable for preparing a panel, until a plasma reference panel becomes available. The European common technical specifications for *in vitro* diagnostic medical devices permits the use of materials such as cloned DNAs (independently quantified by spectrophotometry) where a suitable source of native material is absent. What was emphasized throughout the meeting is the importance of depositing DNA sequence for B19V strains in the databases, to ensure that as much information is available as possible to enable good assay design. However, genetic variation was to be expected in the future, including genotype 1 B19V variants, and robust assay design is essential to deal with inevitable genetic changes.

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Appendix

The meeting participants were from regulatory/research/reference laboratories, kit manufacturers, and plasma derivative manufacturers. The following is a list of speakers at the meeting: Dr G. Zerlauth, Baxter, Austria; Dr K. Brown, HPA, UK; Dr J.-M. Spieser, EDQM, France; Dr M. W. Yu, CBER, Bethesda, MD, USA; Ms K. Hokynar, Haartman Institute, Finland; Professor S. Modrow, University of Regensburg, Germany; Dr D. Candotti, University of Cambridge, UK; Professor A.-M. Eis-Hübinger, University of Bonn, Germany; Dr M. Nübling, Paul Ehrlich Institute, Germany; Professor J.-P. Allain, University of Cambridge, UK; Dr S. Baylis, NIBSC, UK; Dr T. Cuijpers, Sanquin, the Netherlands; Dr M. José, Grifols, Barcelona, Spain; Dr J. Fryer, NIBSC, UK; Dr M. Gessner, Baxter, Austria; Dr M. Koppelman, Sanquin, the Netherlands. The meeting was chaired by Dr P. Minor (NIBSC, UK).

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識別番号・報告回数			報告日	第一報入手日 2008 年 2 月 26 日	新医薬品等の区分 該当なし	厚生労働省処理欄
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研究報告の概要

メルケル細胞癌（Merkel cell carcinoma: MCC）は、稀ではあるが進行の早いヒトの皮膚がんであり、主に高齢者や免疫抑制者にみられる。われわれは今回、digital transcriptome subtraction (DTS)法を用いて MCC 検体について調べ、新種のポリオーマウイルスを同定し、メルケル細胞ポリオーマウイルス（MCV または MCPyV）と命名した。このウイルスは、MCC 腫瘍の 10 検体のうち 8 検体（80%）で検出されたが、体内のさまざまな部位から採取した対照組織では 59 検体中 5 検体（8%）、対照皮膚組織では 25 検体中 4 検体（16%）でしか検出されなかった。MCV 陽性であった 8 MCC 検体のうち 6 検体では、ウイルス DNA は腫瘍ゲノム内に組み込まれており、そのパターンより、MCV の感染と組み込みにより腫瘍細胞のクローン増殖となったことが示唆された。したがって、MCV は MCC の発生に寄与する因子であると考えられる。

使用上の注意記載状況・その他参考事項等

代表として静注用ヘブスブリン－IH の記載を示す。
2. 重要な基本的注意
(1)本剤の原材料となる血液については、HBs抗原、抗HCV抗体、抗HIV-1抗体、抗HIV-2抗体陰性で、かつALT（GPT）値でスクリーニングを実施している。更に、プールした試験血漿については、HIV-1、HBV及びHCVについて核酸増幅検査(NAT)を実施し、適合した血漿を本剤の製造に使用しているが、当該NATの検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した高力価の抗HBs抗体を含有する血漿を原料として、Cohnの低温エタノール分画で得た画分からポリエチレングリコール4000処理、DEAEセファデックス処理等により抗HBs人免疫グロブリンを濃縮・精製した製剤であり、ウイルス不活化・除去を目的として、製造工程において60℃、10時間の液状加熱処理及びろ過膜処理（ナノフィルトレーション）を施しているが、投与に際しては、次の点に十分注意すること。

報告企業の意見	今後の対応
メルケル細胞癌の発生に寄与すると考えられる新規のポリオーマウイルスを同定したとの報告である。 ポリオーマウイルスは、直径 40nm のエンベロープを有しない DNA ウイルスである。 静注用ヘブスブリン-IH については、万一本剤の原料血漿にポリオーマウイルスが混入したとしても、EMC 及び CPV をモデルウイルスとしたウイルスバリデーション試験成績から、本剤の製造工程において十分に不活化・除去されると考えている。 ヘブスブリンについては、EMC及びCPVをモデルウイルスとしたウイルスバリデーション試験成績からは、製造工程において不活化・除去が十分であるとは説明困難であるため、ポリオーマウイルスの原血漿への混入が判明した場合にはその事実を総合機構及び厚生労働省に報告する。	ポリオーマウイルスに関連する情報については、今後も注視することとする。

mains of TRF1 and TRF2, these structural variations emphasize that the TRFH domain is a versatile framework for interactions with different proteins.

The crystal structure of the $TRF2_{TRFH}$-$Apollo_{TBM}$ complex is corroborated by mutagenesis. Mutations of the conserved hydrophobic residues of Apollo (F504, L506, and P508) or TRF2 (*F120*) completely abolished the interaction both in vitro and in vivo (Fig. 4, F and G). We further assayed the cellular localization of wild-type and mutant Apollo by expressing hemagglutinin (HA)–tagged proteins in human telomerase reverse transcriptase (hTERT)–immortalized human BJ fibroblasts. Although wild-type Apollo showed the expected telomere localization, the L506E/P508A double mutant was distributed throughout the nucleoplasm with no obvious accumulation at telomeres (Fig. 4H). This result confirms the structural information and indicates that the binding of Apollo to the TRFH domain of TRF2 is required for the telomeric localization of Apollo.

We next asked whether other shelterin-associated proteins might contain the F/Y-X-L-X-P motif suggestive of an interaction with the TRFH domain of TRF1 or TRF2. We identified this motif in PinX1, originally identified as a TRF1-interacting protein in a yeast two-hybrid screen (*6*). An 11-residue fragment of PinX1 (R287-D-F-T-L-K-P-K-K-R-R297), referred to as $PinX1_{TBM}$, closely resembles $TIN2_{TBM}$ (fig. S12A), suggesting that it may bind to $TRF1_{TRFH}$ in the same fashion as does $TIN2_{TBM}$. ITC data confirmed the $TRF1_{TRFH}$-$PinX1_{TBM}$ interaction, whereas no measurable interaction was observed between $TRF2_{TRFH}$ and $PinX1_{TBM}$ (fig. S12B). Mutagenesis studies showed that PinX1-L291 and TRF1-*F142* are critical for the interaction, whereas PinX1-P293 is not (fig. S12C). These results are consistent with those of the $TRF1_{TRFH}$-$TIN2_{TBM}$ interaction (Fig. 2D) and indicate that PinX1, like TIN2, binds the TRFH domain of TRF1 but not TRF2. Protein sequence database searches showed many instances of telomere-associated proteins containing the F/Y-X-L-X-P motif (fig. S13). Future studies are needed to address whether this motif mediates the TRF1/TRF2 binding of these telomere-associated proteins in vivo.

Our results indicate that binding to the TRFH docking site involves the sequence F/Y-X-L-X-P in shelterin-associated proteins, which contacts the same molecular recognition surface of the TRFH domains of TRF1 and TRF2 with distinct specificities. Because TRF1 and TRF2 play different roles in telomere length homeostasis and telomere protection (*1*), we propose that the TRFH domains of TRF1 and TRF2 function as telomeric protein docking sites that recruit different shelterin-associated factors with distinct functions to the chromosome ends.

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17. Materials and methods are available as supporting material on *Science* Online.
18. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Materials and Methods
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Clonal Integration of a Polyomavirus in Human Merkel Cell Carcinoma

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Merkel cell carcinoma (MCC) is a rare but aggressive human skin cancer that typically affects elderly and immunosuppressed individuals, a feature suggestive of an infectious origin. We studied MCC samples by digital transcriptome subtraction and detected a fusion transcript between a previously undescribed virus T antigen and a human receptor tyrosine phosphatase. Further investigation led to identification and sequence analysis of the 5387–base-pair genome of a previously unknown polyomavirus that we call Merkel cell polyomavirus (MCV or MCPyV). MCV sequences were detected in 8 of 10 (80%) MCC tumors but only 5 of 59 (8%) control tissues from various body sites and 4 of 25 (16%) control skin tissues. In six of eight MCV-positive MCCs, viral DNA was integrated within the tumor genome in a clonal pattern, suggesting that MCV infection and integration preceded clonal expansion of the tumor cells. Thus, MCV may be a contributing factor in the pathogenesis of MCC.

Polyomaviruses have been suspected as potential etiologic agents in human cancer since the discovery of murine polyoma virus (MuPyV) by Gross in 1953 (*1*). However, although polyomavirus infections can produce tumors in animal models, there is no conclusive evidence that they play a role in human cancers (*2*). These small double-stranded DNA viruses [~5200 base pairs (bp)] encode a variably spliced oncoprotein, the tumor (T) antigen (*3*, *4*), and are divided into three genetically distinct groups: (i) avian polyomaviruses, (ii) mammalian viruses related to MuPyV, and (iii) mammalian polyomaviruses related to simian virus 40 (SV40) (*5*). All four known human polyomaviruses [BK virus (BKV), JCV, KIV, and WUV (*6*, *7*)] belong to the SV40 subgroup. In animals, integration of polyomavirus DNA into the host genome often precedes tumor formation (*8*).

Merkel cell carcinoma (MCC) is a neuroectodermal tumor arising from mechanoreceptor Merkel cells (Fig. 1A). MCC is rare, but its incidence has tripled over the past 2 decades in the United States to 1500 cases per year (*9*). It is one of the most aggressive forms of skin cancer; about 50% of advanced MCC patients

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live 9 months or less. Gene expression profiling studies indicate that MCC may comprise two or more clinically similar diseases with distinct etiologies (*10*). Like Kaposi's sarcoma (KS), MCC occurs more frequently than expected among immunosuppressed transplant and AIDS patients (*11*). These similarities to KS, an immune-related tumor caused by KS-associated herpesvirus (*12*), raise the possibility that MCC may also have an infectious origin.

To search for viral sequences in MCC, we used digital transcriptome subtraction (DTS), a methodology we developed that can identify foreign transcripts by using human high-throughput cDNA sequencing data (*13*). We generated two cDNA libraries from a total of four anonymized MCC tumors. One library was prepared with the use of mRNA from a single tumor (MCC347), and the other was prepared with mRNA pooled from three tumors (MCC337, 343, and 346) to increase the likelihood of detecting rare viral sequences (table S1).

From these two libraries, we respectively pyrosequenced 216,599 and 179,135 cDNA sequences (~150 to 200 bp). These 395,734 cDNA sequences were trimmed with LUCY stringency equivalent to PHRED scores of 20 or higher (*14*). Copolymers of adenine or thymidine [poly(A) and poly (T), respectively], dust (low-complexity), human repeat, and primer adaptor sequences were then removed, leaving 382,747 sequences to form a high-fidelity (HiFi) data set. Of these, 380,352 (99.4%) aligned to human RefSeq RNA, mitochondrial, assembled chromosomes, or immunoglobulin sequences in National Center for Biotechnology Information (NCBI) databases. Of the remaining 2395 HiFi candidate sequences, one transcript (DTS1) from MCC347 cDNA aligned with high homology to African green monkey (AGM) lymphotropic polyomavirus (LPyV) and to human BK polyomavirus T antigen sequences. A second DTS transcript (DTS2) had no homology to deposited polyomavirus sequences but was subsequently identified by aligning HiFi candidates to the full-length viral genome (see below). These two sequences define a previously unknown human polyomavirus that we call Merkel cell polyomavirus (MCV or MCPyV) because of its close association with MCC.

Rapid amplification of cDNA ends (3′-RACE) extended DTS1 to three different cDNAs (Fig. 1B): One transcript terminated at a poly(A) site in the T antigen sequence, and two cDNAs read through this weak poly(A) site to form different length fusions with intron 1 of the human receptor tyrosine phosphatase type G gene (*PTPRG*) (GenBank:18860897) on chromosome 3p14.2. Viral integration at this site was confirmed by sequencing DNA polymerase chain reaction (PCR) products with the use of a viral primer and a human *PTPRG* primer. The same three RACE products were independently cloned from MCC348, a lymph node metastasis from the MCC347 primary tumor, indicating that this tumor was seeded from a single tumor cell already positive for the T antigen–PTPRG fusion transcript.

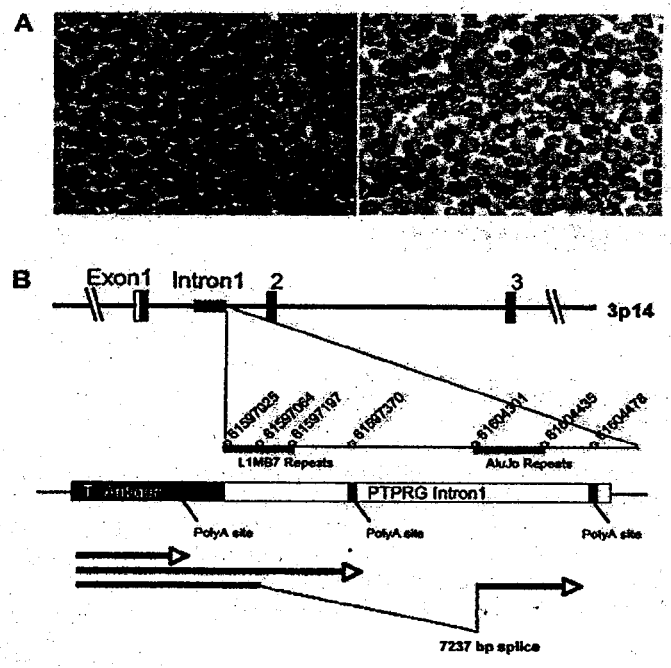

Fig. 1. (A) MCC is an aggressive skin cancer derived from Merkel mechanoreceptor cells that expresses neuroendocrine and perinuclear cytokeratin 20 markers, distinguishing it from other small round cell tumors (MCC349, left, hematoxylin and eosin; right, cytokeratin 20 staining, 40×. Scale bar represents 10 μm). (B) Discovery of Merkel cell polyomavirus transcripts in (MCC). 3′-RACE mapping of an MCC fusion transcript between the MCV T antigen and human *PTPRG*. A cDNA corresponding to a polyomavirus-like T antigen transcript was found by DTS analysis of MCC. This T antigen cDNA was extended by 3′-RACE to map three mRNA sequences (arrows), one of which terminates at a viral polyadenylation site and two of which extend into flanking human sequence and terminate in intron 1 of the human *PTPRG* gene on chromosome 3p14, indicative of viral DNA integration into the tumor cell genome. The two viral-human chimeric transcripts were generated by read-through of a weak polyadenylation signal in the viral T antigen gene. Identical RACE products were also sequenced from a lymph node metastasis of this primary tumor.

By viral genome walking, we sequenced the complete closed circular genome of MCV (5387 bp, prototype) from tumor MCC350. A second genome, MCV339 (5201 bp), was then sequenced by using MCV-specific primers. The sequences of MCV350 and MCV339 have GenBank accession numbers EU375803 and EU375804, respectively. Both viruses encode sequences with high homology to polyomavirus T antigen, VP1, VP2/3, and replication origin sequences (Fig. 2A). MCV has an early gene expression region [196 to 3080 nucleotides (nt)] containing the T antigen locus with large T and small T open reading frames and a late gene region containing VP1 and VP2/3 open reading frames between 3156 and 5118 nt. The T antigen locus has features conserved with other polyomavirus T antigens, including cr1, DnaJ, pRB1-binding Leu-X-Cys-X-Glu (LXCXE) motif, origin-binding, and helicase/adenosine triphosphatase (ATPase) domains. Mutations in the C terminus of MCV350 and 339 large T open reading frames are predicted to truncate large T protein but are unlikely to affect small T antigen protein expression. The replication origin is highly conserved with that of other polyomaviruses and includes features such as a poly(T) tract and conserved T antigen binding boxes (fig. S1). MCV has highest homology to viruses belonging to the MuPyV subgroup and is most closely related to AGM LPyV (Fig. 2B) (*15*). It is more distantly related to known human polyomaviruses and SV40. The principal differences between MCV350 and MCV339 are a 191-bp (1994 to 2184 nt) deletion in the MCV339 T antigen gene and a 5-bp (5216 to 5220 nt) insertion in the MCV339 late promoter. Excluding these sites, only 41 (0.8%) nucleotides differ between MCV350 and 339.

To investigate the association between MCV infection and MCC, we compared tumors from 10 MCC patients to two tissue control groups. The first control group was composed of unselected tissues from various body sites (including nine skin samples) from 59 patients without MCC (table S2). These samples were taken consecutively on a single surgical day and tested for MCV positivity with two PCR primer sets in the T antigen locus (LT1 and LT3) and one in the VP1 gene (VP1). These primers do not amplify cloned human BKV or JCV genomic DNA or SV40 genome from COS-7 cells. A second control group composed of skin and skin tumor samples from 25 immunocompetent and immunosuppressed patients without MCC were tested with LT1 and VP1 primers (table S2). Samples were randomized and tested in a blinded fashion. Southern blotting of PCR products was performed to increase sensitivity (fig. S2).

Of the 10 MCC tumors from different patients, 8 (80%) were positive for MCV sequences by PCR (Table 1 and table S1). Seven tumors showed robust amplification, and one tumor was positive only after PCR–Southern hybridization. MCC348 (metastasis from MCC347) and

MCC338 (infiltrating tumor from MCC339) were also positive. Two tumors, MCC343 and 346, remained negative after testing with 13 PCR primer pairs spanning the MCV genome. None of the 59 control tissues, including nine skin samples, was positive by PCR alone, but five gastrointestinal tract tissues tested weakly positive after PCR–Southern hybridization (8%, $P < 0.0001$, table S2). Viral T antigen sequences were recovered from three of these samples, confirming low copy number infection. Similarly, only 4 of 25 (16%, $P = 0.0007$, table S2) additional skin and non-MCC skin tumor samples from immunocompetent and immunosuppressed patients tested positive for MCV sequences (Table 2 and table S2).

To determine whether MCV DNA was integrated into the tumor genome, we examined MCC samples by direct Southern blotting without PCR amplification. When MCV DNA in MCC tumor is digested by single-cutter restriction endonucleases, such as EcoRI or BamHI, and probed with viral sequence, four possible patterns are predicted to occur: (i) if the viral DNA exists as freely replicating circular episomes, then a ~5.4 kilobase (kb) band will be present (integrated-concatenated virus will also generate a ~5.4 kb band); (ii) if MCV DNA integrates polyclonally, as might occur during secondary infection of the tumor if MCV is a passenger virus, then diffuse hybridization from different band sizes is expected; (iii) if MCV DNA integrates at one or a few chromosomal sites, then the tumors will have identical or near-identical non-5.4-kb banding patterns; or (iv) if MCV DNA integrates at different chromosomal sites before clonal expansion of the tumor cells, then distinct bands of different sizes will be present (monoclonal viral integration).

Eight of 11 MCC DNA samples (including MCC348 metastasis from MCC347) digested with either BamHI or EcoRI showed robust MCV hybridization, and these corresponded to the same tumors positive by PCR analysis with multiple primers (Fig. 3A and fig. S3). Monoclonal viral integration (pattern iv) was evident with one or both enzymes in six tumors: MCC339, 345, 347, 348, 349, and 352 (solid arrowheads). EcoRI digestion of MCC339, for example, produced two distinct 7.5- and 12.2-kb bands that would arise only if MCV is integrated at a single site in the majority of tumor cells. MCC344 and 350 bands have episomal or integrated-concatemeric bands (open arrowhead, pattern i). MCC352 has a monoclonal integration pattern (solid arrowheads, pattern iv) on BamHI digestion as well as an intense 5.4-kb band (open arrowhead), consistent with an integrated concatemer. All three tumors negative by PCR with ethidium bromide staining (MCC337, 343, and 346) were also negative by direct Southern blotting.

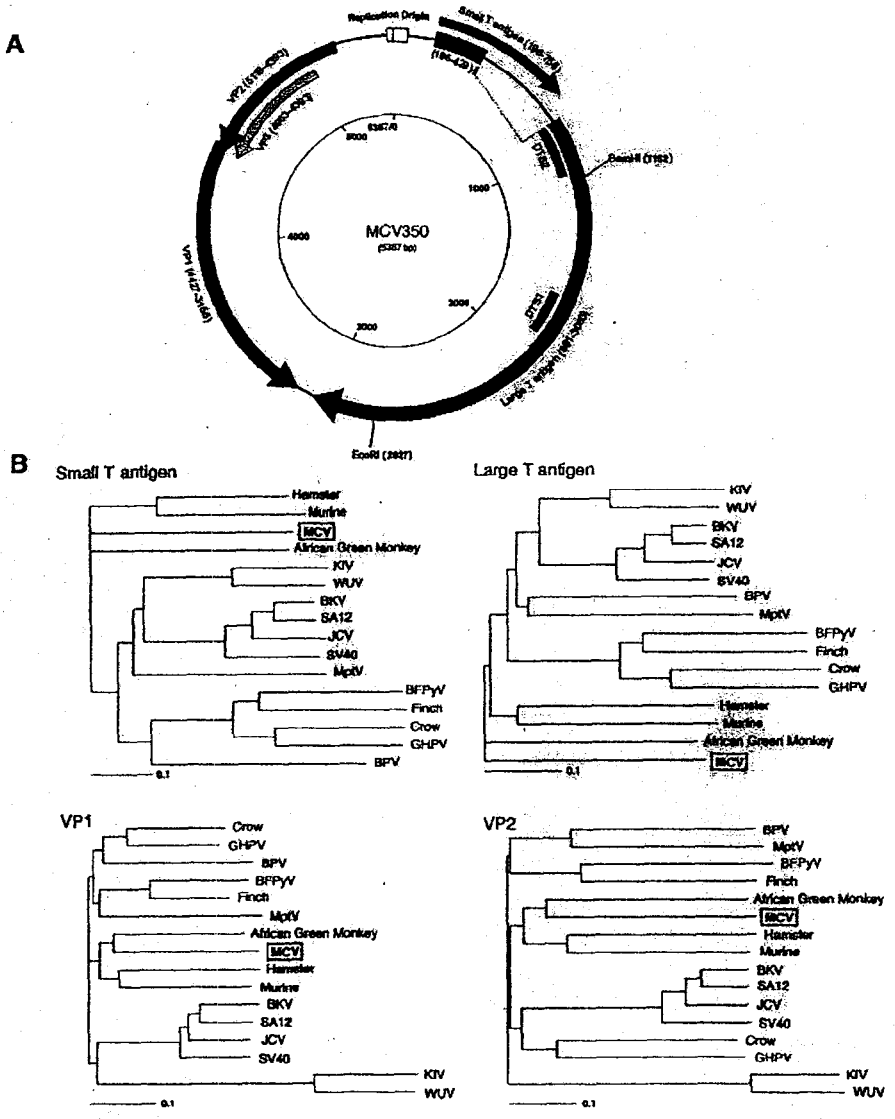

Fig. 2. **(A)** Schematic of MCV genome. Genome walking was used to clone the full MCV genome from tumor MCC350. The genome encodes typical features of a polyomavirus, including large T (purple) and small T (blue) open reading frames. Also shown are predicted VP1 (green) and overlapping VP2 (orange) and VP3 (yellow) genes. DTS1 and DTS2 (red) represent cDNA fragments originally identified by DTS screening. The former was used to identify MCV, and the latter is a spliced transcript with no homology to known polyomavirus sequences. **(B)** Neighbor-joining trees for putative MCV large T, small T, VP1, and VP2 proteins. The four known human polyomaviruses (BKV, JCV, KIV, and WUV) cluster together in the SV40 subgroup (blue), whereas MCV is most closely related to MuPyV subgroup viruses (red). Both subgroups are distinct from the avian polyomavirus subgroup (orange). Scale bars indicate an evolutionary distance of 0.1 amino acid substitutions per position in the sequence.

Table 1. PCR for MCV DNA in MCC tissues. A plus symbol indicates that the sample was strongly positive by ethidium bromide staining only with one or more primers. A minus symbol indicates that the tissue was negative for all primers. Entries with both plus and minus symbols indicate that the sample was negative by ethidium bromide staining but positive after Southern hybridization of PCR products.

	MCC cases (n = 10)	
Patient	Tissue ID	MCV positivity
1	MCC337	−/+
2†	MCC338	+
2	MCC339	+
3	MCC343	−
4	MCC344	+
5	MCC345	+
6	MCC346	−
7	MCC347	+
7‡	MCC348	+
8	MCC349	+
9	MCC350	+
10	MCC352	+
	Total (%)	8/10 (80)

†MCC338 was from an infiltrating tumor in skin tissue adjacent to MCC339 tumor. ‡MCC348 taken from a metastatic lymph node from MCC347.