posited in that database. About 30% of the Japanese subtype B MSM variants in the JP-TK HIVDB belong to the JP.MSM.B-1 lineage, and <1 to 2% belong to each of the three minor variants (JP.MSM.B-2, JP.MSM.B-3, and JP.MSM.B-4) (10).

As shown in Table 1, in the Los Alamos HIVDB, we identified a total of seven sequences sampled outside Japan that matched the JP.MSM.B-1 lineage. These comprised four strains from Asia (06CN.LN107, 06CN.LN126, and 07CN.LN159 from MSM in Liaoning Province, China [27], and 09TW.36497 from Taiwan [28]), two strains from North America (05US.ARC_A221 from the United States [29] and 08CA.PHAC09_054 from Canada), and one strain from Europe (04DE.fd5gjGvO1zxQvv0 from Germany [30]).

Interestingly, a total of 13 JP.MSM.B-1-related variants were identified among the sequences present in the UKHIVRDB that were sampled from 2006 to 2010 (Table 1). This represents approximately 0.04% of the subtype B strains (13/~32,000) and 0.016% of all of the HIV-1 strains registered in the UKHIVRB (13/~80,000) from 1997 to 2011 (Table 1). Intriguingly, 10 out of the 13 JP.MSM.B-1 variants identified in the United Kingdom (Table 2) were part of a distinct monophyletic cluster within JP.MSM.B-1 with good statistical support (bootstrap value of 70%) (Fig. 2). Further, this cluster contained a 2004 sample from Germany (04DE.fd5gjGvO1zxQvv0) (30) and a 2005 U.S. sample (05US.ARC_A221) (29) (Fig. 2). We thus designated this the Global (UK)-JP.MSM.B-1 cluster (Fig. 2). We also noted that one of three United Kingdom sequences placed outside this cluster (a 2010 sample, 10UK.a10) was closely related to two 2008 Japanese MSM samples (08JP.Y440 and 06JP.Y308) with a bootstrap value of 95% (Fig. 2; Table 2). In addition, three infections belonging to the JP.MSM.B-1 lineage were identified among MSM in Liaoning Province in northeastern China (06CN.LN107, 06CN.LN126, and 07CN.LN159). These grouped together within the JP.MSM.B-1 lineages with a bootstrap value of 67% and are designated the CN-JP.MSM.B-1 cluster (Fig. 2) (10). These three Chinese MSM had no history of travel outside China (X. Han, personal communication).

Our survey of three HIV sequence databases revealed a small number of hits to three minor Japanese subtype B MSM lineages (JP.MSM.B-2 through JP.MSM.B-4) that were sporadically identified outside Japan and/or among foreign MSM residing in Japan (Table 1). The JP.MSM.B-2 lineage contained one 2005 United Kingdom sample (05UK.b01) and two from Chinese MSM residing in Japan (09JP.CN.GM3061 [AB735867] and 09JP.CN.Y519 [AB735896]) (10). These two Chinese MSM sequences grouped with sequences from Japanese MSM with a bootstrap score of 99% (10). In addition, the JP.MSM.B-3 lineage contained one 2011 United Kingdom sample (11UK.c01) and one sample from a United Kingdom MSM residing in Japan (05JP.UK.GM1736). In a neighbor-joining tree, these two cases were genetically similar and grouped with a bootstrap score of 89% (Fig. 2). The JP.MSM.B-4 lineage contained one sample from the United Kingdom (06UK.d01) and one from Taiwan (09TW.36025) (Fig. 2).

In contrast, the Chinese MSM subtype B lineage (CN.MSM.B-1) was rarely detected outside China; only one CN.MSM.B-1 sequence was detected in the UKHIVRDB (2005 sample 05UK.e01) (Tables 1 and 3; Fig. 2). However, a total of 20 sequences belonging to the B′/CN.MSM.B-2 lineage were identified in the UKHIVRDB from 2004 to 2010 (Tables 1 and 3; Fig. 2). This represents 0.025% (20/~80,000) of the HIV-1 strains in the UKHIVRDB and 0.06% (20/~32,000) of the subtype B strains in the UKHIVRDB (Table 2). Among these 20 sequences, 5 formed a distinct phylogenetic cluster (designated B′$_{UK}$) within B′/CN.MSM.B-2 with a bootstrap score of 90% (Fig. 2).

Time scale of the global dispersal of Asian MSM subtype B lineages. To explore when subtype B variants associated with transmission among MSM in East Asia emerged and spread in the United Kingdom and globally, we estimated divergence times and spatial dispersal by a Bayesian relaxed molecular clock analysis (25, 31). The analyses were performed with BEAST (24) on the basis of a nucleotide sequence alignment of 1.1-kb *pol* (pro-RT) regions of subtype B strains; this alignment was identical to that used for the neighbor-joining phylogeny presented in Fig. 2. The estimated evolutionary rate of the pro-RT region analyzed was 2.20 (95% highest probability density [HPD], 1.88 to 2.51) $\times 10^{-3}$ substitutions/site/year. This rate is similar to those previously estimated for subtype B (10, 32–34).

As shown in Fig. 3, the estimated time of the most recent common ancestor (tMRCA) of lineage JP.MSM.B-1 was 1989.1 (95% HPD, 1986.5 to 1992.5) and the tMRCAs of lineages CN.MSM.B-1 and B′/CN.MSM.B-2 were 1985.6 (1981.5 to 1989.8) and 1985.3 (1981.8 to 1988.8), respectively. The estimated tMRCAs for the Global (UK)-JP.MSM.B-1 and CN-JP.MSM.B-1 clusters were 1998.9 (1995.6 to 2001.7) and 2000.0 (1997.5 to 2002.5), respectively (Table 4).

In the molecular clock phylogeny, the B′$_{UK}$ cluster was placed among subtype B′ strains isolated from Thailand and the nearby Yunnan Province of China (Fig. 3). Other Chinese subtype B′ strains formed a separate cluster (designated B′$_{FPD}$) that comprised a number of subtype B′ strains from former plasma donors (FPDs). The estimated tMRCA of the B′$_{FPD}$ and B′$_{UK}$ clusters were 1991.5 (1988.7 to 1994.9) and 2002.6 (1999.5 to 2005.3), respectively (Fig. 3; Table 4). The estimated tMRCAs of the JP.MSM.B-2, JP.MSM.B-3, and JP.MSM.B-4 lineages were 1988.3 (1982.7 to 1993.3), 1987.9 (1983.7 to 1991.8), and 1989.4 (1985.1 to 1996.6), respectively. (Fig. 3; Table 4). These estimated tMRCAs are compatible with our previous estimates (10). At the root of the molecular clock phylogeny were subtype B strains from Haiti and from Trinidad and Tobago and some U.S. strains. This is in agreement with the hypothesis that the global dispersal of pandemic subtype B took place via the United States from the Caribbean region (35, 36). All of the subtype B clusters identified in this study were statistically well supported with posterior probability support values of >0.95 (Fig. 3).

FIG 3 Bayesian molecular clock reconstruction of HIV-1 subtype B evolutionary history in East Asia and the rest of the world. Shown is an MCC tree of the subtype B 1.1-kb pro-RT sequences estimated by a Bayesian MCMC approach (see Materials and Methods for details). The branch lengths in the MCC trees represent time, and the corresponding time scale is shown at the bottom of the tree. The 95% HPD intervals of the tMRCA estimates for phylogenetic clusters of interest are shown. Nodes marked with asterisks have strong statistical support (posterior probability, >0.95). The geographic origins of the sequences are represented by colors (see inset panel) or two-letter country codes (as described in the legend to Fig. 2).

TABLE 4 Summary of estimated tMRCAs of HIV-1 subtype B variants associated with transmission in East Asia and the related subclusters[a]

HIV-1 subtype B variant	Subcluster	tMRCA (95% HPD)	Remark(s) (reference[s])
JP.MSM.B-1		1989.1 (1986.5–1992.5)	Major subtype B variant associated with transmission among MSM in Japan (10)
	Global (UK)-JP.MSM.B-1	1998.9 (1995.6–2001.7)	A subcluster within JP.MSM.B-1 that was identified in the United Kingdom and contained the strains from other regions of the world (this study)
	CN-JP.MSM.B-1	2000.0 (1997.5–2002.5)	JP.MSM.B-1 variants identified among MSM in China (10)
JP.MSM.B-2		1988.3 (1982.7–1993.3)	One of the minor subtype B transmission clusters among MSM in Japan (10)
	cn-JP.MSM.B-2	1997.9 (1994.2–2001.6)	JP.MSM.B-2 variants found among Chinese MSM resident in Japan (10)
JP.MSM.B-3		1987.9 (1983.7–1991.8)	One of the minor subtype B transmission clusters among MSM in Japan (10)
JP.MSM.B-4		1989.4 (1985.1–1996.6)	One of the minor subtype B transmission clusters among MSM in Japan (10)
CN.MSM.B-1		1985.6 (1981.5–1989.8)	Subtype B transmission cluster identified among MSM in China (10)
B′/CN.MSM.B-2		1985.3 (1981.8–1988.8)	Subtype B′ (Thailand variant of subtype B) (35, 36); a fraction of the subtype B strains identified among MSM in China belong to the subtype B′ lineage (10)
	B′FPD	1991.5 (1988.7–1994.9)	Subtype B′ subcluster responsible for the epidemic among FPDs in China (33)
	B′UK	2002.6 (1999.5–2005.3)	Subtype B′ subcluster identified in the United Kingdom (mostly among MSM) (this study)

[a] Based on BEAST analysis under a relaxed uncorrelated lognormal molecular clock with the GTR nucleotide substitution model, a gamma-distribution model, and a nonparametric Bayesian skyride coalescent model (see Materials and Methods). Estimated substitution rate, 2.20 (95% HPD, 1.88 to 2.51) $\times 10^{-3}$/site/year. Effective sample size (ESS) values are >200.

DISCUSSION

In this study, we explored the possible interplay between HIV-1 epidemics in East Asia and those in the rest of the world, with a particular focus on the United Kingdom. We carried out an extensive survey of the Los Alamos HIVDB, the UKHIVRDB, and the JP-TK HIVDB. We found that a major HIV-1 subtype B variant (JP.MSM.B-1), which is responsible for approximately one-third of the HIV infections among Japanese MSM, is also present globally. This lineage was detected in various locations in the western hemisphere and the Asia-Pacific region ($n = 20$): Asia (China and Taiwan) ($n = 4$), Europe (United Kingdom and Germany) ($n = 14$), and North America (the United States and Canada) ($n = 2$) (Table 1; Fig. 2). Intriguingly, we found that 10 of 13 JP.MSM.B-1 variants from the UKHIVRDB, together with two samples from the Los Alamos HIVDB (from Germany and the United States) formed a distinct monophyletic cluster within JP.MSM.B-1 [designated Global (UK)-JP.MSM.B-1] (Fig. 2 and 3; Table 2). Similarly, three Chinese MSM sequences were placed as a separate monophyletic cluster within JP.MSM.B-1 (designated CN-JP.MSM.B-1) (Fig. 2 and 3; Table 2). A molecular clock analysis performed with BEAST indicated that the tMRCA for the JP.MSM.B-1 lineage was ~1989 and the tMRCAs for the Global (UK)-JP.MSM.B-1 and CN-JP.MSM.B-1 clusters within it were ~1999 and ~2000, respectively (Fig. 3; Table 4). All seven United Kingdom samples with known exposure category information are from MSM (Table 2). Thus, most of the JP.MSM.B-1 infections in the United Kingdom discovered in this study likely belong to the MSM risk group. Taken together, these results suggest that JP.MSM.B-1 circulated for several years in Japan before being introduced into nearby regions of Asia (China and Taiwan) and to the western hemisphere (the United States and European countries), most likely through global MSM networks (Fig. 3).

The Chinese subtype B MSM lineage (CN.MSM.B-1) was identified only once in the UKHIVRDB and never in the rest of the world, except in Japan (Table 1). In contrast, a total of 20 B′/CNMSM.B-2 variants were detected in the UKHIVRDB among samples collected in 2005 to 2010 (Table 1). HIV-1 subtype B′ (37, 38) was originally identified among injection drug users (IDUs) in Thailand and spread into neighboring Asian countries through drug use networks. This lineage also contributed the founding strain responsible for the HIV outbreaks in central China among FPDs in the early to mid-1990s (33, 39). The exposure categories of the 20 subtype B′ infections identified in the UKHIVRDB are as follows: heterosexual exposure, $n = 9$ (45%); MSM or bisexual exposure, $n = 4$ (20%); unknown exposure, $n = 7$ (35%). These individuals originated from the following regions: Southeast Asia, $n = 4$ (25%); the United Kingdom, $n = 4$ (25%); Eastern Europe, $n = 2$ (10%); Southwest Asia, $n = 1$ (5%); unknown, $n = 9$ (45%) (Table 3). We also noted that most (18 of 20) of the United Kingdom subtype B′ infections were identified before 2009 (Table 1). While the subtype B′ lineage is thought to have originated from the Bangkok IDU epidemic in the late 1980s, the B′ samples in this study do not have IDU as a risk factor (where risk information is known). The exposure categories of the samples that belonged to the B′UK subcluster ($n = 5$) were MSM or bisexual ($n = 2$) and unknown ($n = 3$), suggesting that the B′UK cluster is most likely related to the MSM transmission network in the United Kingdom (Table 3).

By combining the various sources of information at our disposal, we illustrate in Fig. 4 the hypothesized time scales and migration pathways of the global dissemination of the subtype B lineages associated with transmission among MSM in East Asia. Figure 4 is based on the results obtained in this and previous studies (10).

The strategy used here to search for virus variants was straightforward to implement and may be useful for the investigation and

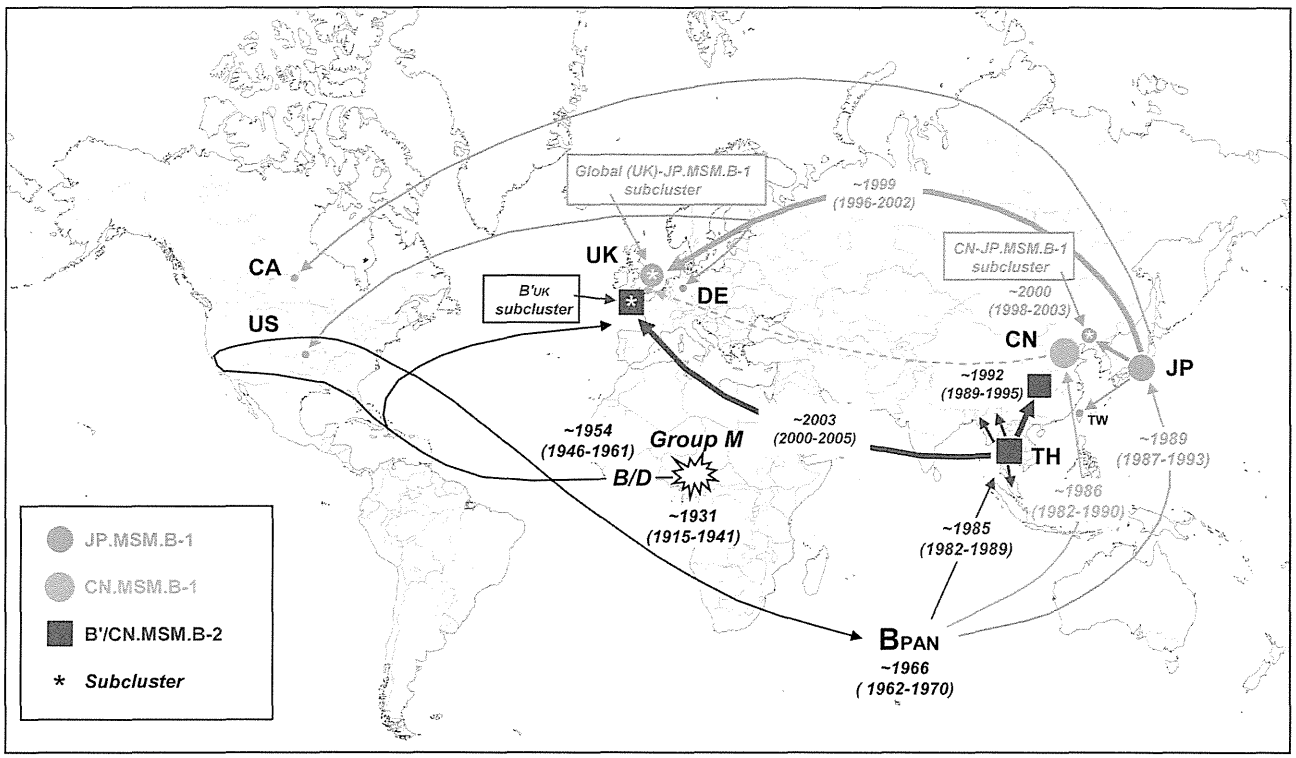

FIG 4 Illustration of the global dispersal estimated timeline of HIV-1 subtype B variants associated with MSM transmission in East Asia. JP.MSM.B-1, a major subtype B lineage among Japanese MSM, has been disseminated globally. It formed distinct subclusters in the United Kingdom [with strains from Germany and the United States; designated Global (UK)-JP.MSM.B-1] and in northeastern China (designated CN-JP.MSM.B-1). Subtype B′ was originally identified among IDUs in Bangkok, Thailand, was disseminated widely among IDUs in neighboring countries and was responsible for the explosive HIV-1 outbreak among FPDs in central China in the early to mid-1990s. It was also identified among MSM in China (designated B′/CN.MSM.B-2) (10, 12). A relatively large number of subtype B′ infections were identified in the UKHIVRDB (see text; Table 1). Arrow thickness reflects qualitative trends in viral migration. Black arrows depict the origin of pandemic subtype B and its plausible global dispersal history (33, 36). The estimated tMRCAs (95% HPD) of each subtype B lineage and its ancestor are shown (33, 36). For country code definitions, see the legend to Fig. 2.

detection of other unknown epidemiological links among HIV epidemics in different locations and risk groups. While an initial survey with the BLAST tool is robust and convenient, its results do not always correlate with the phylogenetic relationships of any two sequences, especially when the tool is applied to rapidly evolving pathogens that are sampled sequentially over time and that exhibit evolutionary rate variation among lineages. As illustrated in Fig. 5, as intracluster genetic diversity increases over time, there is an increasing chance that genetic distance comparisons do not accurately reflect whether a given sequence belongs to a specific monophyletic cluster or not (Fig. 5). As a consequence, phylogenetic cluster definitions based on monophyly are robust when applied to temporally sampled (heterochronous) sequence alignments.

Wertheim et al. recently proposed a technique for the rapid screening of a very large number of HIV-1 sequences to find "clusters" that are defined directly by sequence similarity, and they conclude that a threshold of 1% sequence similarity between sequences from different individuals was sensitive enough to identify many of the inferred transmission clusters reported in previous studies (40, 41). Such approaches are objective, computationally efficient, and robust to recombination, yet necessarily they do not use all of the information present in a phylogenetic representation. Nonetheless, phylogenetic and genetic distance-based cluster definitions are closely related; in our study, we found no identified BLAST hits to our target lineages among nucleotide sequences with <94 to 95% homology (Fig. 6). Further work is required to fully explore the advantages and disadvantages of the two approaches when applied to rapidly evolving heterochronous sequences.

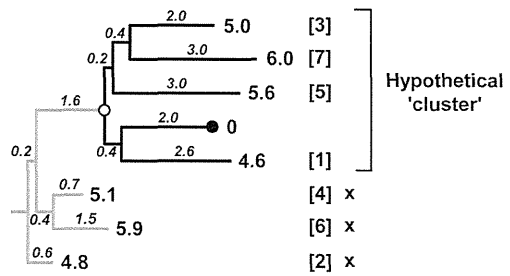

FIG 5 Relationship between genetic distance and phylogeny. In this hypothetical tree, branch lengths are shown in arbitrary units (italicized numerals). The value at the tip of each branch is the genetic distance of each sequence from the representative reference (solid circle) strain for the hypothetical cluster. These distances are also ranked from smallest to largest, and the ranks are shown in brackets. The common ancestor of the cluster of interest is marked by the open circle, and × marks sequences that do not belong to that cluster. Crucially, sequences that do not belong to the cluster may be more genetically similar to the reference strain than sequences within the cluster.

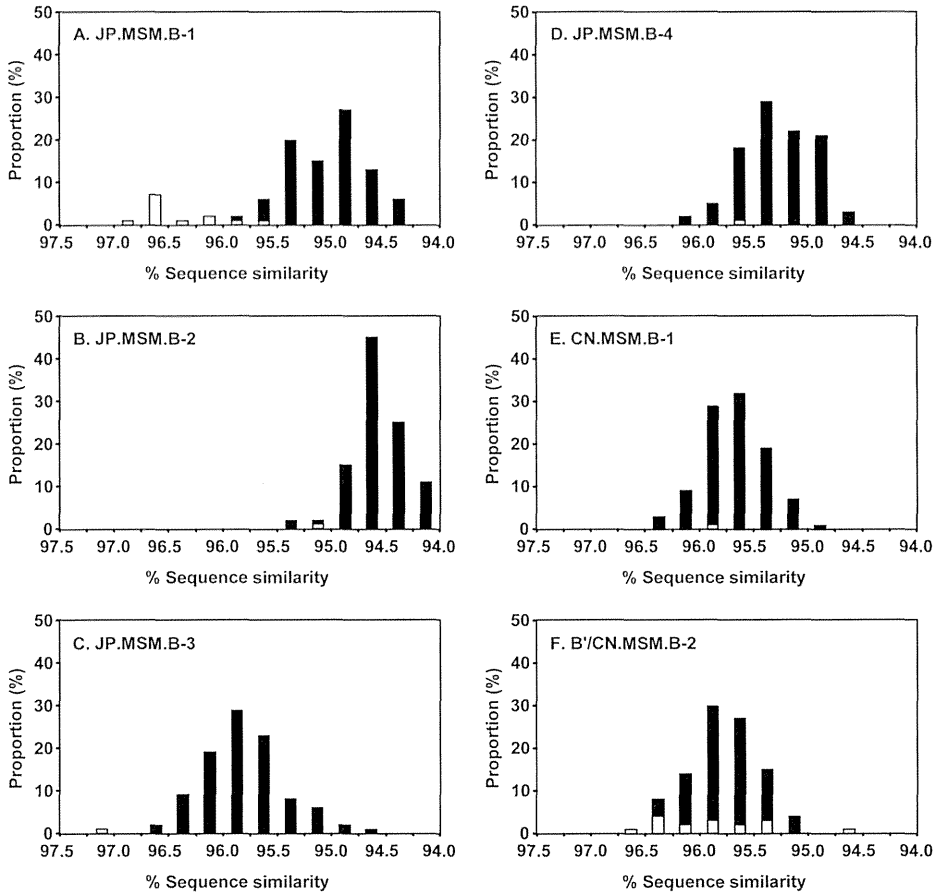

FIG 6 Distribution of sequence similarities (to the query sequence) of the sequences in the UKHIVRDB with the 100 highest BLAST scores. Each histogram (solid bars) shows the frequency distribution of the sequence among the 100 nucleotide sequences in the UKHIVRDB with the highest BLAST score of similarity to each query sequence. The query sequence used is shown in the upper left corner of each graph. The similarity values of sequences that clustered with each query in neighbor-joining phylogenies are shown as open bars. Open bars are superimposed on solid bars.

Our results provide new insights into the global spread of HIV-1 strains that warrant further study to elucidate the interrelationships of HIV epidemics on different continents. In the past, the HIV-1 epidemics in Japan and China have been considered as founded by "imported strains" from other locations. However, the results reported here show that virus migration is bidirectional and these countries are also exporting specific HIV lineages. This observation is particularly important for China, where the HIV epidemic continues to grow; furthermore, socioeconomic ties and travel between China and the rest of the world have increased rapidly.

Our study highlights the urgent importance of strengthening HIV monitoring efforts and the need to implement effective control measures to reduce HIV transmission on a global scale.

ACKNOWLEDGMENTS

This study was supported by a grant-in-aid for AIDS research from the Ministry of Health, Labor, and Welfare of Japan, as well as by the United Kingdom Medical Research Council (grant G0900274) and the European Community's 7th framework program (FP7/2007-2013) under the Collaborative HIV and Anti-HIV Drug Resistance Network (CHAIN; project 223131), and in part by international grants from the Daiwa Anglo-Japanese Foundation (O.G.P. and Y.T.) and from the Japan China Medical Association (Y.T.).

The collaborators contributing to the UKHIVRDB are as follows: steering committee members Celia Aitken (Gartnavel General Hospital, Glasgow), David Asboe, Anton Pozniak (Chelsea and Westminster Hospital, London), Patricia Cane (Public Health England, Porton Down), Hannah Castro, David Dunn (cochair), David Dolling, Esther Fearnhill, Kholoud Porter, Anna Tostevin (MRC Clinical Trials Unit at University College London, London), David Chadwick (South Tees Hospitals NHS Trust, Middlesbrough), Duncan Churchill (Brighton and Sussex University Hospitals NHS Trust), Duncan Clark (St. Bartholomew's and The London NHS Trust), Simon Collins (HIV i-Base, London), Valerie Delpech (Centre for Infections, Public Health England), Samuel Douthwaite (Guy's and St. Thomas' NHS Foundation Trust, London), Anna Maria Geretti (Institute of Infection and Global Health, University of Liverpool), Antony Hale (Leeds Teaching Hospitals NHS Trust), Stéphane Hué (University College London), Steve Kaye (Imperial College, London), Paul Kellam (Wellcome Trust Sanger Institute and University College London Medical School), Linda Lazarus (Expert Advisory Group on AIDS Secretariat, Public Health England), Andrew Leigh Brown (University of Edinburgh), Tamyo Mbisa (Virus Reference Department, Public Health England), Nicola Mackie (Imperial NHS Trust, London), Chloe Orkin (St. Bartholomew's Hospital, London), Deenan Pillay (cochair), Andrew Phillips, Caroline Sabin (University College

London Medical School, London), Erasmus Smit (Public Health England, Birmingham Heartlands Hospital), Kate Templeton (Royal Infirmary of Edinburgh), Peter Tilston (Manchester Royal Infirmary), Daniel Webster (Royal Free NHS Trust, London), Ian Williams (Mortimer Market Centre, London), Hongyi Zhang (Addenbrooke's Hospital, Cambridge), and Mark Zuckerman (King's College Hospital, London). The centers contributing data are the Clinical Microbiology and Public Health Laboratory, Addenbrooke's Hospital, Cambridge (Jane Greatorex); Guy's and St. Thomas' NHS Foundation Trust, London (Siobhan O'Shea, Jane Mullen); PHE-Public Health Laboratory, Birmingham Heartlands Hospital, Birmingham (Erasmus Smit); PHE-Virus Reference Department, London (Tamyo Mbisa); Imperial College Health NHS Trust, London (Alison Cox); King's College Hospital, London (Richard Tandy); Medical Microbiology Laboratory, Leeds Teaching Hospitals NHS Trust (Tracy Fawcett); Specialist Virology Centre, Liverpool (Mark Hopkins, Lynn Ashton); Department of Clinical Virology, Manchester Royal Infirmary, Manchester (Peter Tilston); Department of Virology, Royal Free Hospital, London (Claire Booth, Ana Garcia-Diaz); Edinburgh Specialist Virology Centre, Royal Infirmary of Edinburgh (Jill Shepherd); Department of Infection and Tropical Medicine, Royal Victoria Infirmary, Newcastle (Matthias L. Schmid, Brendan Payne); South Tees Hospitals NHS Trust, Middlesbrough (David Chadwick); Department of Virology, St. Bartholomew's and The London NHS Trust (Spiro Pereira, Jonathan Hubb); Molecular Diagnostic Unit, Imperial College, London (Steve Kaye); University College London Hospitals (Stuart Kirk); and the West of Scotland Specialist Virology Laboratory, Gartnavel, Glasgow (Alasdair MacLean, Celia Aitken, and Rory Gunson). The coordinating center was the Medical Research Council Clinical Trials Unit (Hannah Castro, Kate Coughlin, David Dunn, David Dolling, Esther Fearnhill, Lorraine Fradette, Kholoud Porter, Anna Tostevin, and Ellen White).

We have no conflicts of interest to report.

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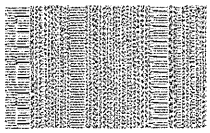

Identification of TL-Om1, an Adult T-Cell Leukemia (ATL) Cell Line, as Reference Material for Quantitative PCR for Human T-Lymphotropic Virus 1

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Quantitative PCR (qPCR) for human T-lymphotropic virus 1 (HTLV-1) is useful for measuring the amount of integrated HTLV-1 proviral DNA in peripheral blood mononuclear cells. Many laboratories in Japan have developed different HTLV-1 qPCR methods. However, when six independent laboratories analyzed the proviral load of the same samples, there was a 5-fold difference in their results. To standardize HTLV-1 qPCR, preparation of a well-defined reference material is needed. We analyzed the integrated HTLV-1 genome and the internal control (IC) genes of TL-Om1, a cell line derived from adult T-cell leukemia, to confirm its suitability as a reference material for HTLV-1 qPCR. Fluorescent *in situ* hybridization (FISH) showed that HTLV-1 provirus was monoclonally integrated in chromosome 1 at the site of 1p13 in the TL-Om1 genome. HTLV-1 proviral genome was not transferred from TL-Om1 to an uninfected T-cell line, suggesting that the HTLV-1 proviral copy number in TL-Om1 cells is stable. To determine the copy number of HTLV-1 provirus and IC genes in TL-Om1 cells, we used FISH, digital PCR, and qPCR. HTLV-1 copy numbers obtained by these three methods were similar, suggesting that their results were accurate. Also, the ratio of the copy number of HTLV-1 provirus to one of the IC genes, RNase P, was consistent for all three methods. These findings indicate that TL-Om1 cells are an appropriate reference material for HTLV-1 qPCR.

Human T-lymphotropic virus 1 (HTLV-1) was the first retrovirus to be found in humans (1, 2). HTLV-1 is a cause of adult T-cell leukemia (ATL), HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and HTLV-1-associated uveitis (3). Areas where HTLV-1 is endemic are distributed across several different regions, including southern Japan, the Caribbean, South America, and tropical Africa (4, 5). A recent report has shown that the area affected by this infection has expanded from the southern part of Japan to the entire country, particularly the Tokyo metropolitan area (6). Diagnostic tests for HTLV-1 infection are performed mainly with serological assays, such as enzyme-linked immunoabsorbent assay, particle agglutination assay, and Western blotting. Recently, another diagnostic test has been developed. Quantitation of integrated proviral DNA in peripheral blood (proviral load [PVL]) can be performed by quantitative PCR (qPCR) as a risk assessment for ATL or HAM/TSP (7, 8).

A few studies reported that several samples were positive for viral DNA when tested by PCR even though those samples had been found seroindeterminate for HTLV-1 when tested by Western blotting (9, 10). Their results suggest that HTLV-1 qPCR could be used as an additional test to confirm infection in seroindeterminate samples.

Although many laboratories have developed qPCR methods for HTLV-1 detection in Japan, a wide variety of testing methods are used. For example, the target region, primers and probes, and internal control (IC) genes vary among the laboratories (8, 11–15). These variations lead to significant differences in HTLV-1 PVL when these laboratories measure the same samples (16). As a consequence of these differences, comparison of quantitative data between laboratories will continue to be difficult without standardization.

One possible solution is to establish a reference material, which is indispensable for standardizing multicenter test results. The target material for HTLV-1 qPCR is genomic DNA (gDNA) from peripheral blood mononuclear cells (PBMCs). Therefore, HTLV-1-infected cells would be an ideal source for a reference material. To date, many cell lines from ATL patients have been established, but few of them have been well characterized for the genomic features associated with reference materials for HTLV-1 qPCR.

In this study, we investigated the genomic structure of one of these ATL cell lines, TL-Om1, to establish it as a reference material for HTLV-1 nucleic acid amplification techniques (NATs), namely, HTLV-1 clonality, karyotyping, proviral sequencing, integration sites, and determination of gene copy number of HTLV-1 and cellular genes for IC.

Received 5 August 2014 **Returned for modification** 23 September 2014
Accepted 5 December 2014

Accepted manuscript posted online 10 December 2014

Citation Kuramitsu M, Okuma K, Yamagishi M, Yamochi T, Firouzi S, Momose H, Mizukami T, Takizawa K, Araki K, Sugamura K, Yamaguchi K, Watanabe T, Hamaguchi I. 2015. Identification of TL-Om1, an adult T-cell leukemia (ATL) cell line, as reference material for quantitative PCR for human T-lymphotropic virus 1. J Clin Microbiol 53:587–596. doi:10.1128/JCM.02254-14.

Editor: A. M. Caliendo

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.02254-14.

doi:10.1128/JCM.02254-14

TABLE 1 Primers used for qPCR of HTLV-1 and IC genes

Target gene	Forward name	Forward sequence	Reverse name	Reverse sequence	Size (bp)	Primer correction factor	
						Plasmid	gDNA
HTLV-1 gene	LTR202F	ACAATGACCATGAGCCCCAAA	LTR202R	TTAGTCTGGGCCCTGACCT	101	0.9869	
	LTR215F	GCTCGCATCTCTCCTTCAC	LTR215R	AGTTCAGGAGGCACCACA	102	0.9942	
	LTR005F	CCTGACCCTGCTTGCTCAAC	LTR005R	TCAGTCGTGAATGAAAGGGAAAG	99	0.9917	
	056F	TAGTCCCACCCTGTTCGAAATG	056R	GCCAGGAGAATGTCATCCATGT	105	1.0013	
	084F	CCTGCCCCGCTTACTATCG	084R	GGCATCTGTGAGAGCGTTGA	102	0.9922	
	153F	TTGTCGCGCTACTCCTTCTTG	153R	AGGGATGACTCAGGGTTTATAAGAGA	118	0.9792	
	pX2-S[a]	CGGATACCCAGTCTACGTGTT	pX2-AS[a]	CAGTAGGGCGTGACGATGTA	100	0.9944	
RNaseP (RPPH1) gene	RPPH1-05F	TATGCACAATTATGTAATCCCCAAA	RPPH1-05R	CCAGCTCCCTATAACCTGCACTT	100	1.0025	1.0012
	RPPH1-08F	GCCGGAGCTTGGAACAGA	RPPH1-08R	AATGGGCGGAGGAGAGTAGTCT	109	0.9956	0.9937
	RPPH1-12F	AGGAAGCCCACGAAAATTCTAATT	RPPH1-12R	GTCCCCATACTCGGTGATTCTC	101	1.0019	1.0052
Albumin (ALB) gene	ALB-07F	TGCAATGAACACAGGAGAGCTACTA	ALB-07R	CCACCCAGGTAACAAAATTAGCAT	103	0.9971	0.9964
	ALB-19F	CCTGATGCTTCTCAGCCTGTT	ALB-19R	TCCATTTAAGAGTGTGTGTGGTAGGT	100	1.0019	1.0045
	ALB-26F	TGCATTGCCGAAGTGGAAA	ALB-26R	CCTCAGCATAGTTTTTGCAAACA	100	1.0038	1.0078
β-Actin (ACTB) gene	ACTB-06F	TCTGGTGTTTGTCTCTCTGACTAGGT	ACTB-06R	CCGCTTTACACCAGCCTCAT	100		0.9965
	ACTB-12F	TCCTGGGTGAGTGGAGACTGT	ACTB-12R	CCATGCCTGAGAGGGAAATG	107		1.0016
	ACTB-21F	AGCATCCCCCAAAGTTCACA	ACTB-21R	GGACTTCCTGTAACAACGCATCT	101		1.0106
CD81 gene	CD81-01F	GACACATCCCAAGGGTGCTT	CD81-01R	GGACTCAGTTCTCAATGCTTTGC	107		1.0015
	CD81-10F	ACCACGCCTTGCCCTTCT	CD81-10R	GAATCACGCCACTTCCATAACTG	111		1.0021
	CD81-21F	GGTGCACACAGCATGCATTT	CD81-21R	GTGCGCCTCTGGGTAATCAT	102		1.0009
β-Globin (HBB) gene	HBB-11F	TTGGACCCAGAGGTTCTTTGAG	HBB-11R	GGCACCGAGCACTTTCTTG	103		1.0021
	HBB-15F	AGCAGCTACAATCCAGCTACCAT	HBB-15R	GAGGTATGAACATGATTAGCAAAAGG	105		1.0033
	HBB-24F	CCCACCCCAAATGGAAGTC	HBB-24R	AGCACCATAAGGGACATGATAAGG	104		1.0111
RAG-1 gene	RAG1-03F	GCAATCCCATTTGTCCACTTTT	RAG1-03R	TCCCACTGGCCTGCATTACTA	100		1.0045
	RAG1-27F	GAAGTTTAGCAGTGCCCCATGT	RAG1-27R	ACGGGCAGTGTTGCAGATG	100		1.0006
	RAG1-32F	TCAAAGTCATGGGCAGCTATTGT	RAG1-32R	AGGGAATTCAAGACGCTCAGAA	100		0.9993

[a] Primer sequences were previously reported in reference 11.

MATERIALS AND METHODS

Cells and gDNA preparation. Jurkat clone E6-1 cells were obtained from the American Type Culture Collection. HUT102 and SLB-1 cells, which are HTLV-1-infected cell lines, were a kind gift from Masahiro Fujii (Division of Virology, Niigata University Graduate School of Medical and Dental Sciences). PBMCs were kindly provided by the Japanese Red Cross or purchased from AllCells (Alameda, CA, USA). TL-Om1 cells, an ATL-derived cell line established by Sugamura et al. (17), were maintained in RPMI 1640 (Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) supplemented with 100 U/ml penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA), 2 mmol/liter L-glutamine, and 10 ng/ml interleukin-2 (PeproTech, London, United Kingdom). Jurkat, HUT102, and SLB-1 cells were maintained in RPMI 1640 containing 10% FBS supplemented with 100 U/ml penicillin-streptomycin and 2 mmol/liter L-glutamine. DNA was extracted using a QIAamp DNA blood mini or maxi kit (Qiagen, Valencia, CA, USA).

Southern blotting. Southern blotting was performed by SRL Inc. (Tokyo, Japan). DNA was digested with EcoRI and PstI and separated on a 0.8% agarose gel as previously reported (18, 19). DNA was transferred onto nylon membranes (Roche, Mannheim, Germany). The membrane was hybridized with digoxigenin (DIG)-labeled HTLV-1 probe at 42°C overnight. DNA fragments for HTLV-1 probes were obtained from Oncor Inc. (Gaithersburg, MD, USA). Sense and antisense HTLV-1 DNA probes were prepared by random primed labeling using a DIG-High Prime kit (Roche). After the membrane was washed, HTLV-1 probe signals were obtained using a DIG luminescent detection kit (Roche).

FISH analysis. To stop the cell cycle at M phase, Colcemid (Sigma) was added to the cell culture medium at a concentration of 0.02 μg/ml and incubated for 1 h. Cells were harvested and washed with phosphate-buffered saline (PBS). After treatment with 0.075 M KCl hypotonic solution at 37°C for 1 h, cells were fixed with a solution containing acetic acid and methanol (3:1). Cells were fixed to a glass slide and dried. The complete HTLV-1 genome inserted in pUC18 (15) was used as a probe for provirus, bacterial artificial chromosome (BAC) clone RP11-919G18 was used as a probe for the albumin (ALB) gene, and BAC clones CTD-2326H15 and RP11-203M5 were used as probes for the RNase P (RPPH1) gene. BAC clones were selected from NCBI (http://www.ncbi.nlm.nih.gov/clone/) and were purchased from Advanced Geno Techs Co. (Tsukuba, Japan). The probe for 1q44 was commercially prepared by Chromosome Science Labo Inc. (Sapporo, Japan). For the detection of ALB and RPPH1 genes, the BAC clones were labeled with cyanine 3 (Cy3) and Cy5, respectively. For the detection of provirus, the DIG-labeled probe was prepared by the nick translation method. The probe was hybridized to the sample at 70°C for 5 min, followed by incubation at 37°C overnight. The probe was stained with anti-DIG-Cy3 antibody. Signals were detected by a Leica DMRA2 system and analyzed with Leica CW4000 fluorescent *in situ* hybridization (FISH) software (Wetzlar, Germany).

Splinkerette PCR analysis. Splinkerette PCR was performed as previously reported (20). The first-round PCR was performed as indicated in reference 20. The second-round, nested PCR was performed using the HTLV-1 long-terminal-repeat (LTR)-specific primer. The nested PCR product was loaded onto 3% Tris-acetate-EDTA buffer (TAE) agarose gels. Two distinct DNA bands were cut from the agarose gel and purified using a QIAquick gel extraction kit (Qiagen). After thymine and adenine (TA) cloning, each band was sequenced by the Sanger method (21).

Inverse PCR analysis. TL-Om1 gDNA was digested with BamHI or XbaI. Digested DNA was purified by phenol-chloroform extraction followed by ethanol precipitation. Briefly, 1/10 volume of 3 M sodium acetate and 2.5 volume of 100% ethanol were added to the sample. After centrifugation at $2 \times 10^4 \times g$ for 15 min, the DNA pellet was washed with 70% ethanol and then air dried. Purified DNA was self-ligated using a Ligation-Convenience kit (Nippon Gene, Tokyo, Japan). Ligated DNA was purified again by phenol-chloroform extraction followed by ethanol precipitation. PCR was performed with KOD FX (Toyobo, Osaka, Japan). The PCR mixture contained 20 ng gDNA, 0.4 mM forward and reverse primers, 1 mM deoxynucleoside triphosphate (dNTP), 1× KOD FX buffer, and 0.5 U KOD FX in a total volume of 25 μl, in duplicate. The forward primer sequence was 5′-ACAAATACACCTTGCAATCCTATG G-3′, and the reverse primer sequence was 5′-CGCTTGGGAGACTTCT TGCT-3′. PCR mixtures were denatured at 94°C for 2 min, followed by 34 cycles of 98°C for 10 s and 68°C for 10 min. PCR products were loaded onto 0.8% agarose gels and detected by LAS-3000 (Fujifilm, Tokyo, Japan).

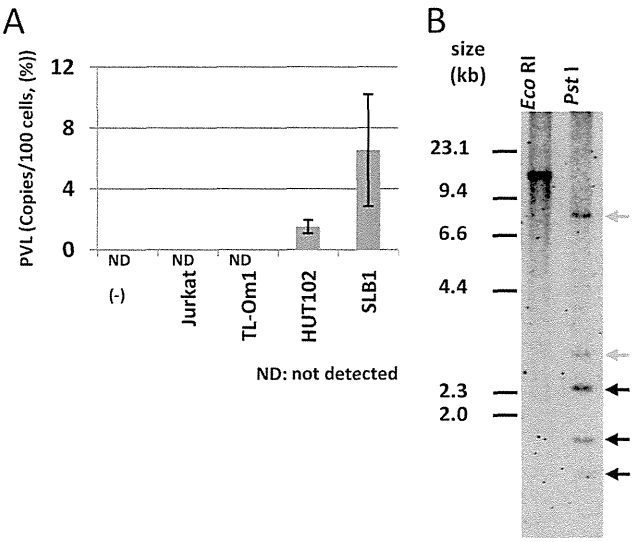
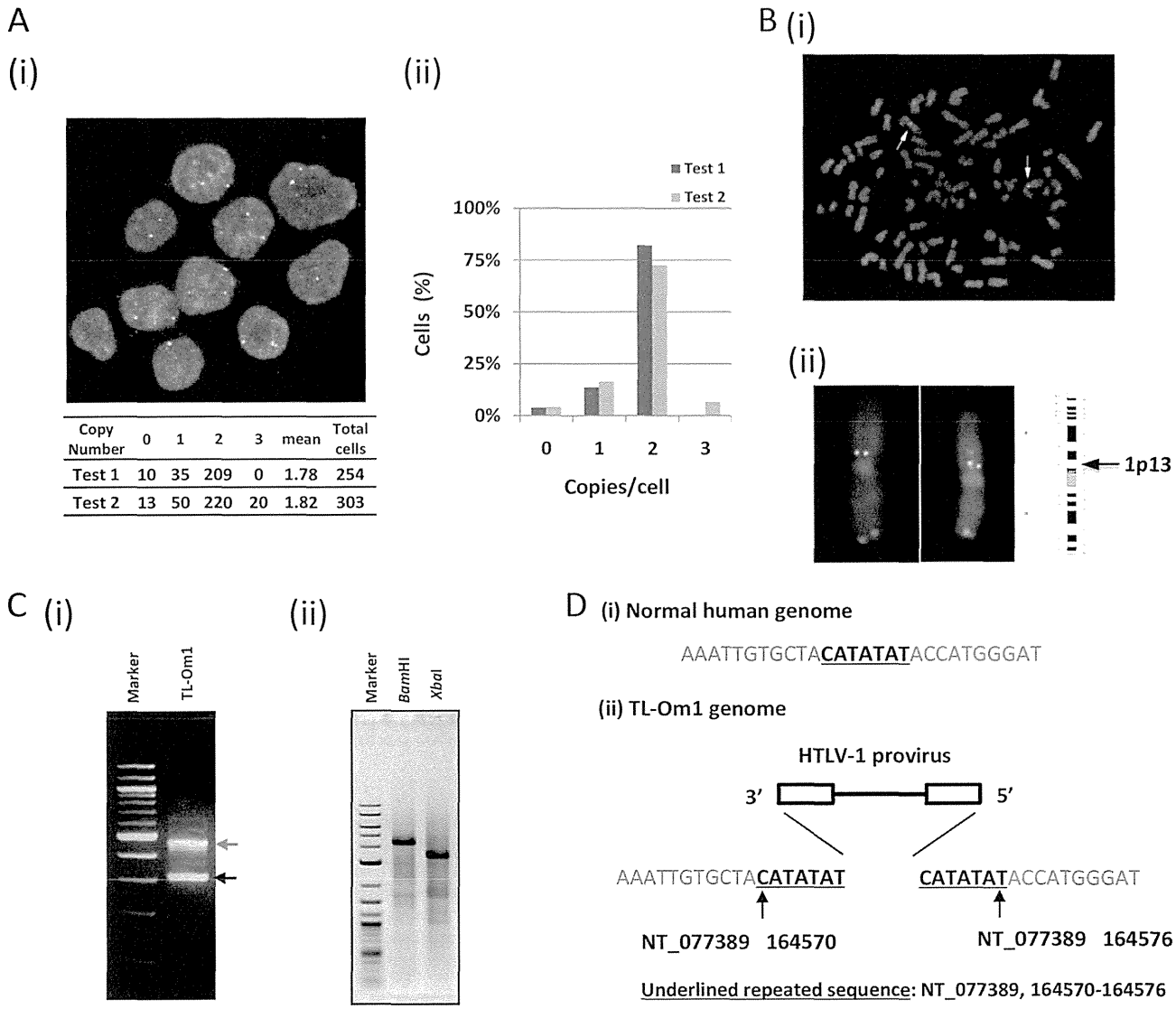


FIG 1 Infectivity and clonality of HTLV-1 provirus in TL-Om1 cells. (A) Mitomycin C-treated Jurkat, TL-Om1, HUT102, and SLB1 cells were cocultured with Jurkat cells. PVL (%) was measured 2 weeks later by qPCR. (B) gDNA from TL-Om1 cells digested with EcoRI or PstI was subjected to Southern blotting probed by the full HTLV-1 genome. Three black arrows show bands for typical HTLV-1 genomic sequences; two gray arrows show bands for host genomic sequences ligated to the HTLV-1 genome. Because the EcoRI site is not included in the HTLV-1 sequence, the number of bands indicates the number of clones in the cells. Detection of two gray bands indicates that there is a pair of 5′ and 3′ HTLV-1 genomes conjugated with the host genome, signifying that the HTLV-1 provirus is monoclonal. On the other hand, detection of more than two gray bands indicates that it is multiclonal.

Genomic long PCR. Genomic long PCRs were performed using KOD FX (Toyobo). Primers are listed in Table S1 in the supplemental material. The conditions for the PCR mixture and thermal cycling program were the same as those for the inverse PCR analysis.

DNA sequencing analysis. The genomic long PCR and inverse PCR products were purified by a GenElute PCR Clean Up kit (Sigma). Direct sequencing was performed using a BigDye Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequence primers are listed in Table S2 in the supplemental material. Sequences were read and analyzed using a 3120× genetic analyzer (Applied Biosystems).

Synchronized qPCR analysis. The primers used for the synchronized qPCR amplification are listed in Table 1. The PCR mixture was prepared with SYBR premix *Ex Taq* II (TaKaRa, Tokyo, Japan) containing 100 ng gDNA and 0.4 mM forward and reverse primers in a total volume of 15 μl, in triplicate. PCR was performed according to the manufacturer's protocol. The ΔC_T(RPPH1) value (where C_T is threshold cycle) was calculated by the following equation: ΔC_T(RPPH1) = average C_T of target gene primer results − average C_T of RPPH1. The gene copy number was calculated by the following equation: target gene copy number (N) = copy number determined by FISH × $2^{-\Delta C_T(\mathrm{RPPH1})}$. Using normal PBMCs or plasmids, the primer correction factor, which can compensate for small differences in amplification efficiency among different primers, was calculated. The correction factor was determined by the difference of each C_T



Copy Number	0	1	2	3	mean	Total cells
Test 1	10	35	209	0	1.78	254
Test 2	13	50	220	20	1.82	303

FIG 2 Clonality, copy number, and integration site of HTLV-1 in TL-Om1 cells. (A) HTLV-1 proviral copy number per cell was determined by FISH using an HTLV-1 full-genome probe. (i) Yellow signals indicate the HTLV-1 probe. Lower table shows the counts of HTLV-1 signals per cell. (ii) Vertical axis indicates percentage counts of each fraction in relation to total cells. Data were the results from two independent analyses. (B) Number of HTLV-1 integrated chromosomes was determined in metaphase cells with the HTLV-1 and 1q44 probes. (i, ii) Yellow signals indicate the HTLV-1 probe, and red signals indicate the 1q44 probe. All HTLV-1 signals were located on chromosome 1. HTLV-1 signals on chromosome 1 were positioned at 1p13. (C) Determination of the HTLV-1 integration site in TL-Om1 cells. (i) The 3′ integration site was determined by Splinkerette PCR with an HTLV-1-specific primer. PCR products were subjected to agarose gel electrophoresis. (ii) BamHI- or XbaI-digested TL-Om1 genomes were self-ligated and subjected to inverse PCR with an HTLV-1-specific primer set. PCR products were subjected to agarose gel electrophoresis. (D) 5′ and 3′ HTLV-1 integration sites were determined by a sequencing analysis of DNA fragments from both Splinkerette and inverse PCR. (i) Normal human sequence; (ii) determined HTLV-1 integration site. HTLV-1 was inversely integrated at chromosome 1: NT_077389, 164570 to 164576.

value of target gene primers from the average C_T value of RPPH1 primers (Table 1). The correction value was calculated as follows: correction C_T value = correction factor × actual C_T value. By applying the correction factors, we reduced the limits of error of the C_T values to 0.1 cycles with normal PBMCs (data not shown).

Digital PCR analysis. Primers and probes for digital PCR analysis of HTLV-1 were previously reported (11, 15). In brief, the primers and probe for HTLV-1 were as follows: forward, 5′-CGGATACCCAGTCTACGTG TT-3′; reverse, 5′-CAGTAGGGCGTGACGATGTA-3′; probe, FAM-5′-CTGTGTACAAGGCGACTGGTGCC-3′-TAMRA (where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine). The primers and probe for albumin were as follows: forward, 5′-TGTCAT CTCTTGTGGGCTGT-3′; reverse, 5′-GGTTCTCTTTCACTGACATC TGC-3′; probe, FAM-5′-CCTGTCATGCCCACACAAATCTCTCC-3′-TAMRA. The mixture of primers and probe for RPPH1 was purchased from Applied Biosystems. The PCR mixture was prepared using 2× digital droplet PCR (ddPCR) supermix for probes (Bio-Rad, Hercules, CA, USA). Droplets were prepared on a QX100 droplet generator (Bio-Rad). PCR was performed with a LifePro thermal cycler (Bio-Rad) and detected with a QX100 droplet reader (Bio-Rad). Data were means of triplicate analysis.

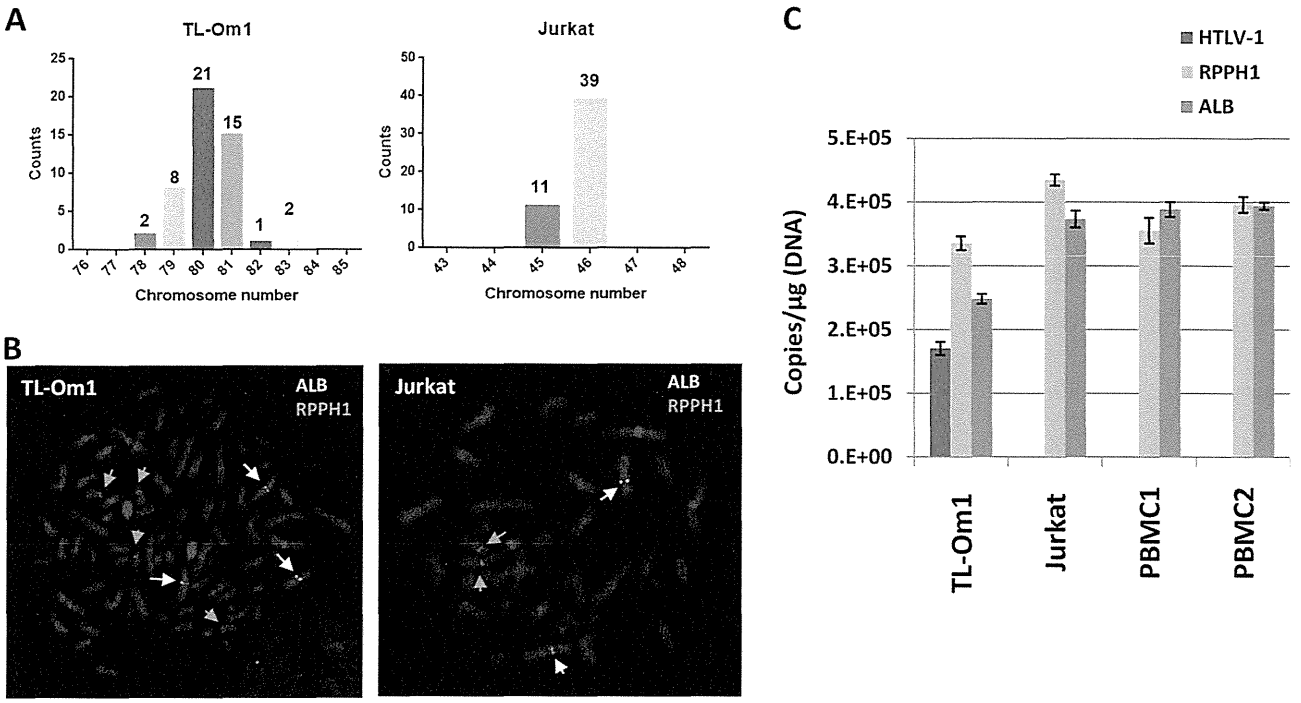


FIG 3 Gene copy number of IC cellular genes for HTLV-1 qPCR. (A) The number of chromosomes in TL-Om1 and Jurkat cells at metaphase was counted. Horizontal line indicates the number of chromosomes per cell. (B) Representative FISH images of TL-Om1 and Jurkat cells at metaphase. Yellow and red arrows indicate signals for ALB and RPPH1 probes, respectively. Left panel shows three signals for ALB and four for RPPH1; right panel shows two signals for ALB and two for RPPH1. (C) Determination of the gene copy number of HTLV-1, RPPH1, and ALB genes by digital PCR. gDNA of TL-Om1 and Jurkat cells and of PBMCs from two healthy donors were subjected to digital PCR. Data show the absolute copy number of HTLV-1, RPPH1, and ALB genes per microgram of gDNA. Bars are means from triplicate analyses.

***In vitro* HTLV-1 infectivity test.** Frozen cells were thawed and immediately cultured for a week. Exponentially growing cells were used for the assay. Jurkat, TL-Om1, SLB1, and HUT102 cells were treated with 50 μg/ml mitomycin C (Kyowa Hakko Kirin, Tokyo, Japan) and incubated for 1 h at 37°C. After being washed twice with 2% FBS-PBS, 1×10^5 cells were added to culture medium containing 1×10^6 Jurkat cells. Mitomycin C was used to block the growth of ATL cell lines added to Jurkat cells. Cells were cocultured for 2 weeks and then subjected to qPCR to determine PVL, as described previously (11).

RESULTS

HTLV-1 infectivity in TL-Om1 cells. We investigated the production potential of infective virus to ascertain the clonal stability of HTLV-1 integration *in vitro*. Mitomycin C-treated TL-Om1 cells were cocultured with Jurkat cells for 2 weeks. At the end of the 2 weeks, no HTLV-1 integration was observed in the Jurkat cells that were cocultured with TL-Om1 cells, while HTLV-1 integration was observed when Jurkat cells were similarly cocultured with SLB-1 and HUT102 cells (Fig. 1A). These findings suggested that the production of infective HTLV-1 particles from TL-Om1 cells was low or diminished; thus, the increase in copy number over the course of cell culture was thought to be negligible. If TL-Om1 cells had infectious potential, the clonality of HTLV-1 provirus in them would vary because of the mutual HTLV-1 infections between cells. To evaluate the clonality of HTLV-1 provirus in TL-Om1 cells, TL-Om1 gDNA was analyzed by Southern blotting. EcoRI-digested gDNA showed a single band, while PstI digestion produced five DNA bands that contained an HTLV-1 sequence (Fig. 1B). Three of the five DNA bands were HTVL-1 internal sequences. The other two DNA bands contained either 5′ or 3′ HTLV-1 sequences ligated with the host genome (Fig. 1B). These fragment patterns indicated that HTLV-1 provirus integration in TL-Om1 cells was monoclonal.

Determination of copy number and integration site of HTLV-1 provirus by FISH. To confirm the clonality and copy number of HTLV-1 provirus and of IC genes in detail, we performed a FISH analysis. There were one or two signals of HTLV-1 provirus in the cells. The mean proviral copy number was calculated at 1.8 copies/cell from the count of signals with >250 cells in two independent analyses (Fig. 2Ai and ii). Double-staining of the TL-Om1 genome with both HTLV-1 and 1q44 probes in meta-

TABLE 2 Gene copy number of IC genes determined by FISH

Karyotype	Gene copy no.			
	TL-Om1 (20 analyzed cells)		Jurkat (20 analyzed cells)	
	RPPH1 gene	ALB gene	RPPH1 gene	ALB gene
2N	0	0	20	20
3N	1	20	0	0
4N	19	0	0	0
Average	3.95	3	2	2
Ratio to the RPPH1 gene	1	0.76	1	1

TABLE 3 Summary of ratio of gene copy numbers to the RPPH1 gene

Method	Cell line	Gene copy no. ratio to the RPPH1 gene							
		RPPH1 gene	ALB gene	ACTB gene	CD81 gene	HBB gene	RAG-1 gene	HTLV-1 gene	LTR gene
FISH	TL-Om1	1.00	0.76					0.46	
	Jurkat	1.00	1.00						
Digital PCR	TL-Om1	1.00	0.74					0.51	
	Jurkat	1.00	0.86						
	PBMC1	1.00	1.09						
	PBMC2	1.00	0.99						
qPCR (plasmid)	TL-Om1	1.00	0.74					0.48	1.02
	Jurkat	1.00	0.92						
qPCR (gDNA)	TL-Om1	1.00	0.74	1.18	0.99	0.92	0.94		
	Jurkat	1.00	0.95	1.07	0.99	0.90	1.08		
	PBMC 1	1.00	0.99	1.00	0.98	0.99	1.00		
	PBMC 2	1.00	1.01	1.01	0.99	1.00	1.01		

phase showed that all HTLV-1 DNA signals were located on chromosome 1 (Fig. 2Bi). When the number of copies of chromosome 1 was 1, 2, 3, or 4 per cell, the number of HTLV-1 proviruses per cell was 1, 1, 2, and 2, respectively (data not shown). HTLV-1 signals on chromosome 1 were positioned on the band of 1p13 (Fig. 2Bii). These results correlated well with the Southern blotting results that showed monoclonal integration.

Confirmation of integration site of HTLV-1 in TL-Om1 cells. To identify the integration site of monoclonal HTLV-1 provirus, Splinkerette PCR was performed with TL-Om1 gDNA. Two specific PCR products were obtained by gel electrophoresis (Fig. 2Ci). The DNA fragments were analyzed by direct sequencing. Sequencing analysis of the lower-molecular-weight DNA fragments (Fig. 2Ci, lower band) showed that they were provirus genomic sequences. Sequencing analysis of the higher-molecular-weight band showed that it contained host gDNA ligated to the 3′ LTR of HTLV-1. We also performed inverse PCR with TL-Om1 gDNA that was digested with BamHI or XbaI followed by self-ligation. Single DNA bands were obtained from both BamHI and XbaI self-ligated templates (Fig. 2Cii). Sequencing analysis demonstrated that both bands contained the same sequences. A BLAST search revealed that the sequence was located on chromosome 1. The integration site was identified, and the HTLV-1 provirus was integrated inversely in between the CATATAT repetitive sequences at the region of NT_077389 from nucleotides (nt) 164570 to 164576 on chromosome 1 (Fig. 2Di and ii).

We determined the full-length sequence of HTLV-1 provirus in TL-Om1 cells by genomic long PCR followed by direct sequencing. The length of HTLV-1 provirus was determined to be 8,941 bp (GenBank accession no. AB979451; see also Text S1 in the supplemental material). The percent identity to the HTLV-1 genomic sequence of the ATK-1 strain (accession no. J02029) was 98.7%. Compared with the full-length HTLV-1 genomic sequence of ATK-1, there was a 93-nt deletion in the *env* gene. The region that was deleted was equivalent to nt 5547 to 5669 of ATK-1. The deduced amino acid sequence of the deletion was 31 in-frame amino acids (Δ125–155 of Env). The deleted region was located on the receptor binding domain of Env (see Fig. S1 in the supplemental material).

Calculation of chromosome and gene copy numbers of HTLV-1, RPPH1, and ALB genes in TL-Om1 and Jurkat cells. We counted the chromosome number in TL-Om1 and Jurkat cells by FISH analysis. Jurkat cells were analyzed as one of the control cell lines. The chromosome number differed from 78 to 83 in TL-Om1 cells (Fig. 3A). The mean chromosome number was estimated at 80.2, which indicated that the karyotype of TL-Om1 cells was about 4*N*. There were 45 or 46 chromosomes in Jurkat cells, indicating that their karyotype is near that of normal human diploid cells (Fig. 3A and B and Table 2).

The absolute gene copy number of HTLV-1 provirus and IC genes was measured using digital PCR. gDNA from TL-Om1 cells, Jurkat cells, and PBMCs from two healthy donors was subjected to digital PCR and used to calculate the copy numbers of these genes (Fig. 3C). Although the ALB-to-RPPH1 gene copy number ratios in the two PBMC samples were 1.09 and 0.99, the ALB-to-RPPH1 gene copy number ratio in TL-Om1 cells was low (ratio of 0.74) (Table 3). The provirus-to-RPPH1 gene copy number ratio in TL-Om1 cells was 0.51 (Table 3). These results were consistent with the provirus- and ALB-to-RPPH1 gene copy number ratios estimated by FISH, which were 0.46 and 0.76, respectively (Table 3). The usefulness of TL-Om1 as a reference standard is strongly supported by the consistent results from the FISH and digital PCR analyses (Table 4).

Estimation of the gene copy number of HTLV-1 and IC genes by synchronized qPCR. We previously developed a method to determine inherited allelic deletions by using qPCR with primer sets that can amplify fragments synchronously, even though the

TABLE 4 Absolute gene copy number per microgram gDNA determined by digital PCR

Cell line	Gene copy no./μg gDNA[a]		
	HTLV-1 gene	RPPH1 gene	ALB gene
TL-Om1	170,171.1	335,452.3	248,410.8
Jurkat	NT	434,529.6	373,423.9
PBMC1	NT	355,116.1	388,650.0
PBMC2	NT	397,260.3	394,520.5

[a] Data are means of triplicate analysis. NT, not tested.

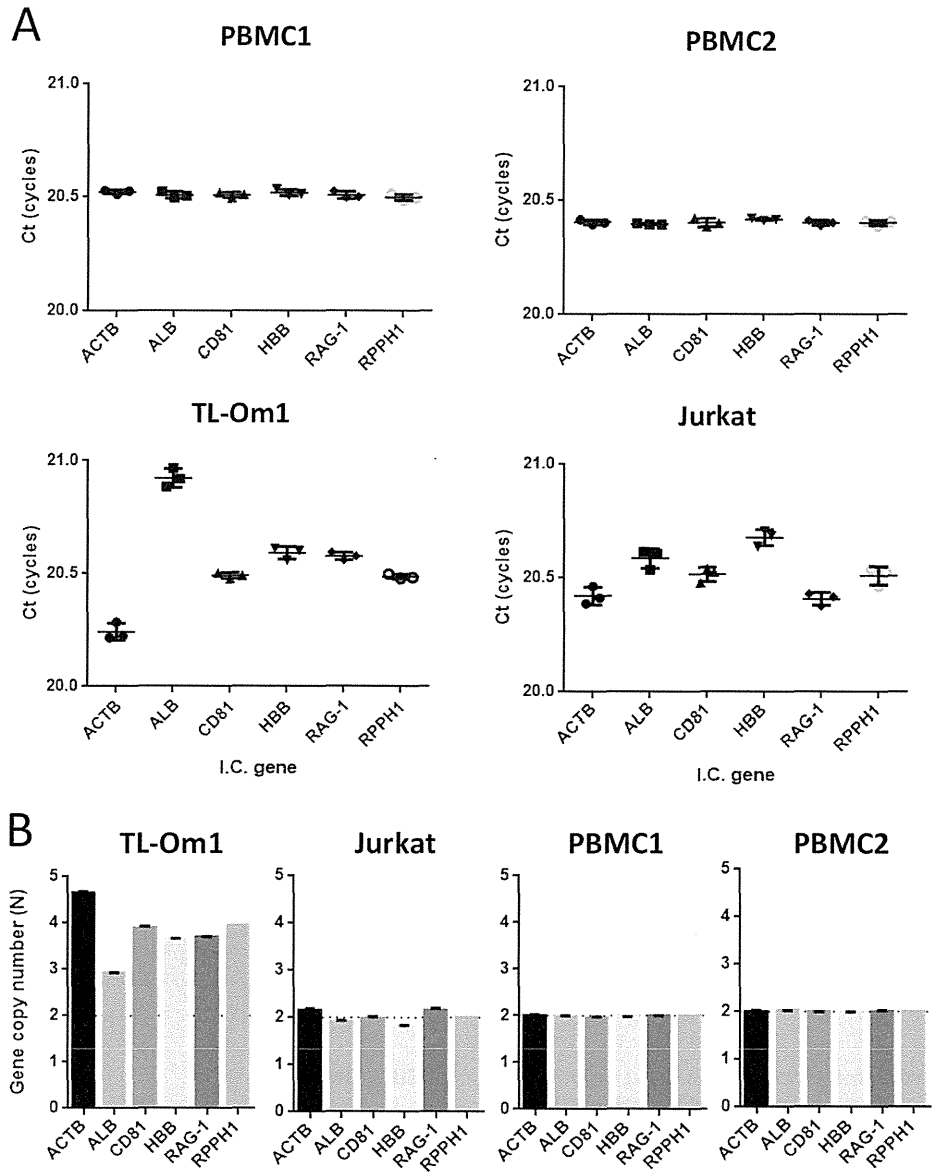


FIG 4 Estimation of gene copy number of IC genes in TL-Om1 cells by qPCR. gDNA of TL-Om1 and Jurkat cells and of PBMCs from two healthy donors was tested by qPCR with synchronous amplification primer sets for IC genes. (A) C_T scores (cycles) of each primer set for IC genes. Each dot indicates the mean from triplicate analyses. The C_T scores in the graph were the results of correction by the factors described in Table 1. (B) Estimated gene copy number of IC genes calculated using the difference in C_T scores from RPPH1. The copy numbers of IC genes of TL-Om1 and Jurkat cells were calculated based on FISH analysis for the RPPH1 gene. RPPH1 gene copy number from PBMCs was set as 2*N*. Equation for the estimation of gene copy number was as follows: gene copy number (N) = RPPH1 gene copy number determined by FISH analysis $\times 2^{-\Delta CT}$, $\Delta C_T = C_T$(target gene) $- C_T$(RPPH1).

target genes are different. The method shows that the difference in C_T value determines the difference in gene copy number. We used primer sets for HTLV-1 genes (LTR and coding regions) and ACTB, ALB, CD81, HBB, and RAG-1 IC genes (Table 1). To increase the specificity, we used primer correction factors, which compensate for the slight difference in PCR amplification efficiency between different primers for target genes. As shown in Fig. 4A, TL-Om1 and Jurkat cells did not show the complete synchronized amplifications that were observed in normal PBMCs. By setting the PCR amplification efficiency of all primer sets per cycle to approximately 2-fold, the ratio of the gene copy number against the RPPH1 gene was estimated using the difference in the mean C_T scores of the IC gene primer sets from the mean of those for the RPPH1 gene. The ratios of the gene copy number of the ALB gene to that of the RPPH1 gene in TL-Om1 and Jurkat cells were 0.74 and 0.92, respectively (Table 3). When the copy number of the RPPH1 gene in TL-Om1 cells was set at 3.95, which was determined by FISH analysis, the copy number of the IC genes was at least 2.9 (ALB gene) and at most 4.7 (ACTB gene) (Fig. 4B and Table 3).

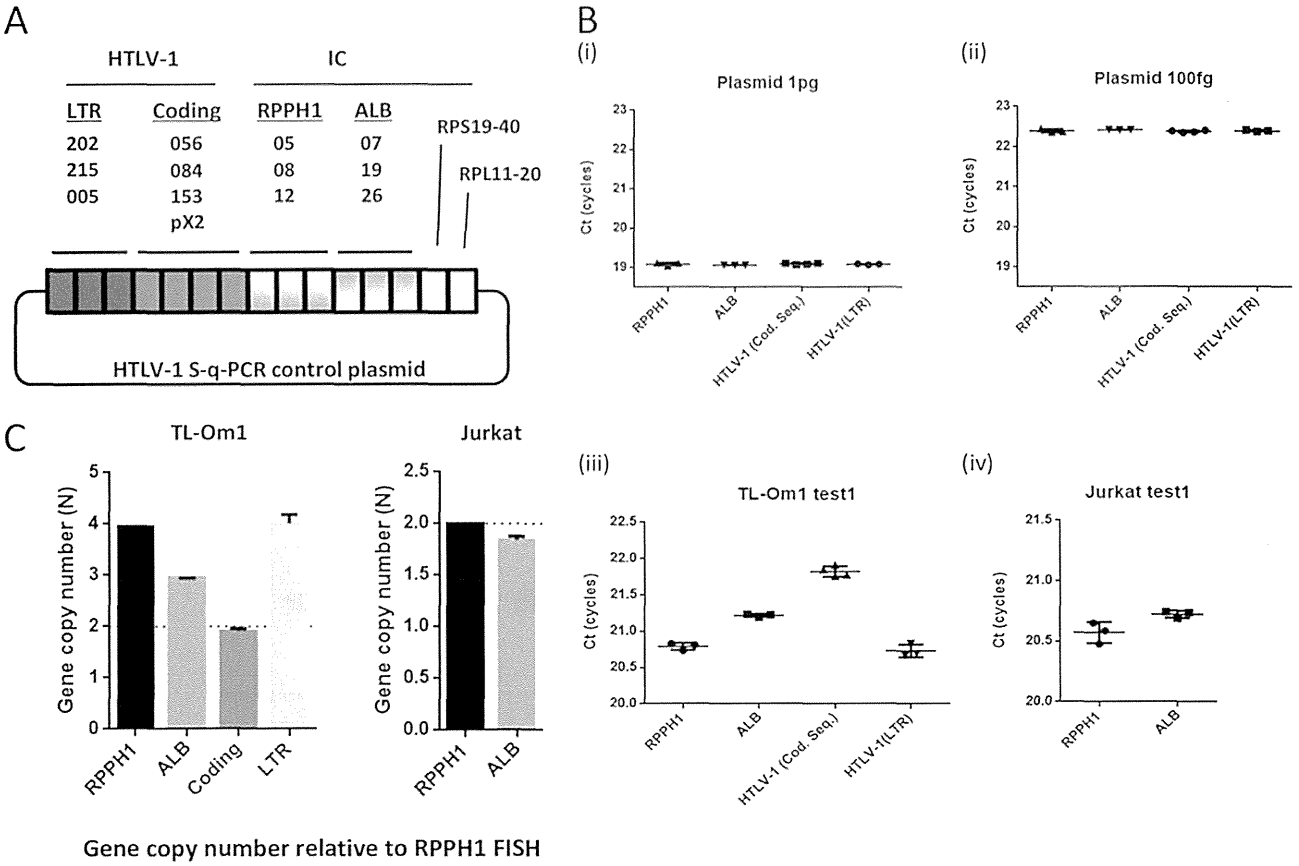


FIG 5 Estimation of the HTLV-1 gene copy number in TL-Om1 cells by synchronized qPCR. gDNA of TL-Om1 and Jurkat cells and of PBMCs from two healthy donors was tested for qPCR with synchronous amplification primer sets for HTLV-1, RPPH1, and ALB genes. (A) Construction of control plasmid with a single copy of each target sequence; (B) data indicate C_T scores of HTLV-1, RPPH1, and ALB genes for control plasmid at 1 pg, 100 fg/reaction, and for TL-Om1 and Jurkat cells. qPCR with the plasmid showed synchronous amplification of all primer sets. Each dot indicates the mean from triplicate analyses. The C_T scores in the graph are the results of correction by the factors described in Table 1. (C) Estimated HTLV-1 and ALB gene copy number in TL-Om1 and Jurkat cells. Data were estimated using the difference in C_T scores between target genes and RPPH1 genes.

Additionally, we tried to determine the HTLV-1 copy number in TL-Om1 cells using a synchronized qPCR method. We prepared a plasmid that had one copy of every target PCR amplicon (Fig. 5A). The plasmid had the same copy number as all the target regions. Using the plasmid as a template, we performed qPCR and confirmed the synchronized amplification of primer sets for HTLV-1, RPPH1, and ALB genes (Fig. 5B). The difference in mean C_T scores for the HTLV-1 gene to the RPPH1 gene was 1.05 cycles on average in TL-Om1 cells (Fig. 5C and Table 3). As with the sequencing analysis, use of the synchronized qPCR method also estimated the copy number of the LTR to be 4.01, indicating that TL-Om1 cells have two LTRs (Fig. 5C and Table 3).

Comparison of HTLV-1 copy number from different calculation methods. We compared the results of HTLV-1 and ALB gene copy number obtained from FISH, digital PCR, and synchronized qPCR. The copy number ratios of the HTLV-1 gene to the RPPH1 gene in TL-Om1 cells were 0.46, 0.51, and 0.48, from FISH, digital PCR, and synchronized qPCR, respectively, and those for the ALB gene were 0.76, 0.74, and 0.74 (Fig. 6 and Table 3). The results from these varied assays strongly support one another, indicating that TL-Om1 cells are suitable for use as a reference material for HTLV-1 qPCR.

DISCUSSION

Recently, NAT reference materials have been established for the safety of blood and blood products, such as international standards for HIV, hepatitis B virus, and hepatitis C virus (22–24). These materials have been frequently used for the purpose of calibration and validation of test systems, preparation of secondary reference materials, and comparison of multicenter results, which have helped improve the consistency of the results. Most international standards for blood-transmitted viruses use plasma from infected human blood, because the test target is extracted from human plasma. With regard to HTLV-1 NAT, it may be better to use a cell line as a reference material to standardize the qPCR results, because this test uses cells obtained from peripheral blood. An example of NAT reference material using cell lines is reported in a test for quantitation of BCR-ABL mRNA. Panels of K562 cells combined with HL60 cells were set as standards, which have been approved by the WHO Expert Committee of Biological Standardization (25). Although a variety of cell lines harboring HTLV-1 provirus in their genomes has been established, detailed characterization of the candidate cell lines with regard to their suitability as reference materials for HTLV-1 NATs has not yet been per-

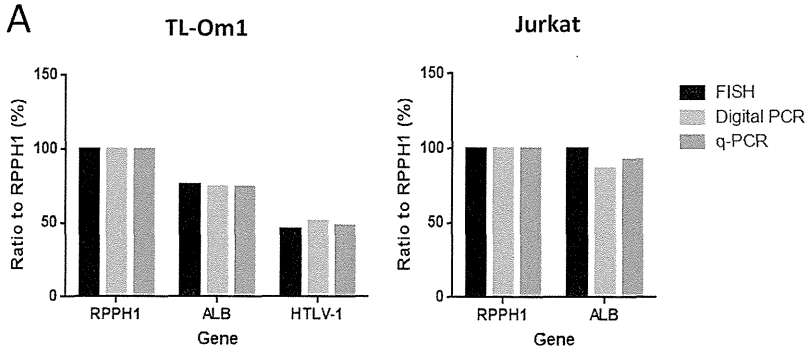


FIG 6 HTLV-1- and ALB-to-RPPH1 gene copy number ratios. (A) Comparison of the HTLV-1- and ALB-to-RPPH1 gene copy number ratios determined by FISH, digital PCR, and qPCR. Data indicate percentages of gene copy number ratio to the RPPH1 gene.

formed. Among the HTLV-1 cell lines, TL-Om1 is well known to be latently infected with HTLV-1 and is thought to be stable for HTLV-1 clonality (17, 26). Transcription from HTLV-1 provirus in TL-Om1 cells is blocked by the highly methylated LTR (27).

In this study, we evaluated the distinct genomic properties of HTLV-1 and IC genes in TL-Om1 cells with regard to their suitability as reference materials for HTLV-1 NATs. Precise information about HTLV-1 infectivity, karyotype, and absolute copy number of HTLV-1 and cellular control genes of TL-Om1 is useful for applying TL-Om1 as a reference material for HTLV-1 qPCR. As such, for this use, TL-Om1 has advantages over other cell lines, such as the human ATL cell line MT2 and the rat T-cell line TARL-2. A recent study of HTLV-1 testing in Japanese blood donor screening revealed that virus prevalence is not limited to areas where HTLV-1 is endemic but has shifted to the entire country, especially the Tokyo metropolitan area (6). Nationwide HTLV-1 tests have been performed on pregnant women in Japan since the end of 2010. The frequent occurrence of seroindeterminate results after Western blotting is one weakness of the HTLV-1 antibody tests. HTLV-1 qPCR is thought to be a solution for decreasing the number of seroindeterminate results; therefore, an accurate measurement of HTLV-1 proviral DNA by qPCR is needed. Additionally, a PVL value of >4% in PBMCs is reported to be a risk factor for ATL development from HTLV-1 asymptomatic carriers, which emphasizes the importance of measuring PVL by qPCR (7). PVL monitoring also provides a risk indicator for HAM/TSP (8).

An attempt to minimize the differences between laboratories by using a common plasmid that included the pX region has been reported. When standard curves were constructed by utilizing the common plasmid in all participating laboratories, the differences in median intralaboratory coefficient of variation (CV) could be reduced by about half (16). Although the attempt worked well among participating laboratories with in-house qPCR methods, the transferability of utilizing common plasmids for standard curves to other methods for PVL determination, for example, digital PCR, is uncertain.

To standardize HTLV-1 qPCR, we advocate the use of TL-Om1 cells with finely elucidated HTLV-1 genomic information as reference material. A previous report showed that PVL values of males and females, on average, are 1.39% and 2.10%, respectively (7). Thus, a dilution or a serial dilution of TL-Om1 with PBMCs or Jurkat cells at a PVL value of around 2% would be an appropriate material for the standardization of HTLV-1 qPCR. These kinds of references can be easily prepared, because the absolute gene copy number is determined from the dilution rate of TL-Om1. TL-Om1 cells were also used as a control in a deep-sequencing-based method for the quantification of the clone size of HTLV-1-infected cells in HTLV-1 carrier or ATL patients (28).

We conclude that TL-Om1 cells can be used as a useful reference material for HTLV-1 NATs. By using TL-Om1 cells, researchers will be able to define the exact values of HTLV-1 by quantifying the copy numbers of provirus and IC genes. In the future, we hope that other laboratories will utilize the features of TL-Om1 cells to standardize the HTLV-1 qPCR.

ACKNOWLEDGMENTS

This work was supported by grants-in-aid for scientific research and by Health and Labor Sciences research grant H23-shinkou-ippan-016 from the Ministry of Health, Labor and Welfare of Japan.

We thank all the members of the HTLV-1 qPCR standardization group for their useful discussions about this research.

We declare that we do not have any competing interests.

Ethical approval was not required for this study.

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