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H. 知的財産権の出願・登録状況（予定を含む）
なし

厚生労働科学研究費補助金（エイズ対策研究事業）
分担研究報告書

機能的Tリンパ球反応に関する研究

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研究要旨

予防エイズワクチンにおけるウイルス抗原特異的ヘルパーTリンパ球（CD4陽性Tリンパ球）の誘導の是非については未だ明確な答えは得られていない。H25年度は、予防エイズワクチンとして開発が進展中であるGag発現センダイウイルスワクチンによって免疫賦与したアカゲサル群を対象に解析を行い、サル免疫不全ウイルス（SIV）感染前後（ワクチンブースト後およびチャレンジ後1週目）のCD4陽性Tリンパ球の機能動態を明らかにすることを目的とした。具体的には、各機能マーカー（MIP-1β, IL-2, TNF-α, IFN-γ, CD107a）の発現を指標としたSIV抗原応答性（SIV特異的CD4陽性Tリンパ球頻度）についての定量的解析を行った。その結果、ワクチンにより誘導された殆どのSIV特異的CD4陽性Tリンパ球亜集団（MIP-1β, IL-2, TNF-α, IFN-γ陽性亜集団）は、SIVチャレンジ後1週目で顕著な減耗を示したのに対し、CD107a陽性亜集団は有意な減少を示さなかった。以上のことから、CD107a陽性亜集団は他のSIV特異的CD4陽性Tリンパ球亜集団よりも高いウイルス抵抗性を示すことが示唆された。CD107a発現能を有する（細胞傷害活性を有する）HIV抗原特異的CD4陽性Tリンパ球の誘導は、今後のエイズワクチン開発において着目すべきポイントの一つとして挙げられよう。

A．研究目的

CD8陽性細胞傷害性Tリンパ球（CTL）は、慢性持続感染を成立させるHIVおよびSIVの体内複製抑制において中心的役割を担う。一方、CD4陽性ヘルパーTリンパ球は、CTLの誘導・維持に重要であるとされつつもそれ自身がウイルスの標的であり、特にウイルス抗原特異的CD4陽性ヘルパーTリンパ球は他のヘルパーTリンパ球よりも優先的にウイルスの標的となることが知られている。つまり、CTL誘導を目的としたワクチンは予防エイズワクチンの有力候補として考えられるものの、同時に免疫賦与され得る抗原特異的CD4陽性ヘルパーTリンパ球に関して、その誘導の功罪については定かではない。

そこでH25年度は、CTL誘導を主目的としたGag発現センダイウイルス（SeV-Gag）ベクターワクチンを施したアカゲサル群を解析対象とし、サル免疫不全ウイルス（SIV）感染前後（ワクチンブースト後からチャレンジ後1週目）のウイルス抗原特異的CD4陽性Tリンパ球の機能動態を明らかにすることを目的とした。

B．研究方法

SeV-Gagベクターワクチンを施したビルマ産アカゲサル（18頭）のSIVmac239チャレンジ前（ワクチンブースト後）およびチャレンジ後1週

の末梢血由来単核球（PBMC）凍結サンプルを用い、SIV 特異的 CD4 陽性 T リンパ球頻度を測定するため、VSV-G シュードタイプ SIV を感染させた同一個体由来の B lymphoblastoid cell line（SIV-BLCL）と PBMC を共培養後、CD4 陽性 T リンパ球における各機能マーカー（MIP-1β、IL-2、TNF-α、IFN-γおよび CD107a）の発現を、細胞内免疫染色後のフローサイトメトリー解析にて測定した。

（倫理面への配慮）

遺伝子組換え生物等を用いる実験については国立感染症研究所の承認あるいは文部科学大臣の確認を得ている。動物実験については国立感染症研究所および医薬基盤研究所の動物実験委員会の承認を得てから開始し、動物実験等の実施に関する基本指針を遵守した。

C．研究結果

ワクチン接種サル全個体について、SIV特異的CD4陽性Tリンパ球における各機能マーカー陽性亜集団の構成を調べた結果、SIVチャレンジ前では、TNF-α陽性亜集団が最も高い割合を示した。一方、SIVチャレンジ後1週目では、CD107a陽性亜集団の割合が最も高く、IL-2陽性亜集団の割合が最も低い傾向を示した。

続いて、ワクチン接種サル全個体について、SIV チャレンジ前後での SIV 特異的 CD4 陽性 T リンパ球頻度の変動について解析した。その結果、全 SIV 特異的細胞集団（各機能マーカーのいずれか 1 つが陽性）は SIV チャレンジ後に減少していた。各機能マーカー別にみると、SIV 特異的 MIP-1β、IL-2、TNF-αおよび IFN-γ陽性亜集団の頻度は、SIV チャレンジ後減少していた。一方、SIV 特異的 CD4 陽性 T リンパ球のうち CD107a 陽性亜集団の頻度は、SIV チャレンジ前後で有意な変動を認めなかった（図１）。

D．考察

本研究では、ワクチン接種サルにおいて、ワクチン後および SIV チャレンジ後 1 週目の CD4 陽性 T リンパ球中の SIV 特異的 MIP-1β、IL-2、TNF-α、IFN-γおよび CD107a 反応を解析した。その結果、ワクチン誘導 SIV 特異的 CD4 陽性 T リンパ球のうち、CD107a 陽性亜集団以外の SIV 特異的 MIP-1β、IL-2、TNF-αおよび IFN-γ陽性亜集団の頻度が SIV チャレンジ後に減少することを見出した。特に IL-2 陽性亜集団は SIV 感染により顕著に減耗していた。このことから、これらのワクチン誘導 SIV 特異的 CD4 陽性 T リンパ球亜集団は、SIV 感染標的となっていると考えられ、ワクチンによる SIV 特異的 CD4 陽性 T リンパ球誘導が SIV 感染標的増幅に結びついている可能性が示された。

一方、ワクチン誘導 SIV 特異的 CD4 陽性 T リンパ球のうち、CD107a 陽性亜集団は SIV 感染後でも殆ど減耗せず、優占的な構成集団へと変遷していた。この結果は、SIV 特異的 CD4 陽性 T リンパ球 CD107a 陽性亜集団が SIV 感染抵抗性であることを示すものである。

以上のことから、予防エイズワクチンにおけるウイルス抗原特異的 CD4 陽性 T リンパ球の誘導の是非について考察した場合、感染増悪に至る可能性がある IL-2 の発現能に代表されるようなヘルパーT リンパ球の誘導を抑えること、およびウイルス抵抗性の大きい CD107a 陽性細胞を効果的に誘導することが鍵であると考えられる。

E．結論

ワクチン接種アカゲサルの SIV 感染前後の解析から、ワクチン誘導 SIV 特異的 CD4 陽性 T リンパ球のうち、MIP-1β、IL-2、TNF-αおよび IFN-γ陽性亜集団は SIV 感染により顕著に減耗するのに対し、CD107a 陽性亜集団は高いウイルス抵抗性を有することを明らかにした。

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G．知的財産権の出願・登録状況（予定を含む。）

1　特許取得

なし。

2　実用新案登録

なし。

3　その他

なし。

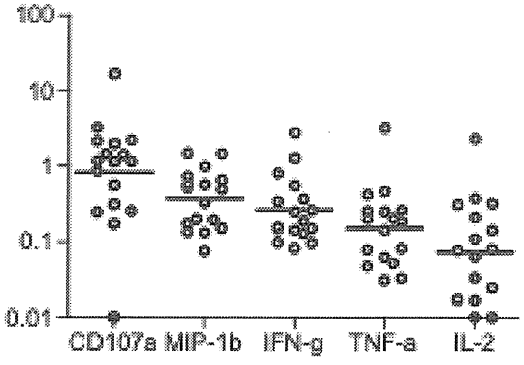

図 1．SIV 特異的 CD4 陽性 T リンパ球の各亜集団頻度の SIV 感染前後の比

ワクチン接種サル 18 頭の SIV 感染前（ワクチン後）および感染後 1 週目の SIV 特異的 CD4 陽性 T リンパ球中の CD107a、MIP-1β、IFN-γ、TNF-α、および IL-2 陽性亜集団の頻度を測定し、各々について SIV 感染後の感染前に対する比を算出した。

Ⅲ.　研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

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Ⅳ.　研究成果の刊行物・別刷

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Emergence in Japan of an HIV-1 Variant Associated with Transmission among Men Who Have Sex with Men (MSM) in China: First Indication of the International Dissemination of the Chinese MSM Lineage

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A survey of HIV-1 strains circulating in the Tokyo-Kanagawa metropolitan area of Japan during 2004 to 2011 (n = 477) identified six Japanese males (patients 1 to 6), who harbored viruses with genome segments derived from a distinct CRF01_AE variant uniquely found among men who have sex with men (MSM) in China (designated CN.MSM.01-1). These six HIV infections were diagnosed in 2010 and 2011 among MSM (3 of 75) and men with unknown risk factors (3 of 63) and differed from the vast majority of HIV infections among MSM in Japan, which are overwhelmingly characterized by subtype B (239 of 246 [97.2%]). Approximately one-third (91 of 239 [38.1%]) of subtype B strains from MSM in Japan belong to a large monophyletic cluster (designated JP.MSM.B-1). In addition, we identified a smaller subtype B cluster (n = 8) (designated JP.MSM.B-2) that also contains strains from two Chinese MSM living in Japan. Interestingly, patients 5 and 6 were found to be coinfected with CRF01_AE (CN.MSM.01-1) and subtype B (JP.MSM.B-2 or JP.MSM.B-1) variants that are unique to the HIV-1 epidemics among MSM in China and Japan, respectively. Our study demonstrates for the first time the effect of the expanding HIV epidemic among MSM in China on transmission in neighboring countries and shows the ongoing mixing of CRF01_AE and subtype B lineages unique to HIV-1 that cocirculate in MSM populations in East Asia. This finding highlights the importance of strengthening epidemiological surveillance in the region and the need for effective measures to limit transmission among MSM in East Asia.

Along with the resurgence in HIV infection among men who have sex with men (MSM) in the Western world (1–3), there have been reports of emerging or newly identified HIV epidemics among MSM in Asia (4, 5) and other regions (6). China is the most populous country in the world, and Chinese HIV epidemics among MSM are expanding rapidly (7). According to the joint Chinese Ministry of Health and UNAIDS survey, the proportion of MSM among newly identified HIV cases in China has risen from 0.3% in 1985 to 2005 to 12.2% in 2007 and 32.5% in 2009 (7, 8). Recent cross-sectional studies of MSM populations have shown HIV prevalences ranging from 0.5% in Jinan to 8.5% in Chongqing and 9.1% in Chengdu (reviewed in reference 6). The prevalence of HIV-1 among MSM in China is increasing through time. For instance, the prevalence among MSM in Beijing increased from 0.4% in 2004 to 5.8% in 2006 (9), and in Jiangsu the prevalence rose from 0 to 5.8% in the period from 2003 to 2007 (10). A large-scale national survey conducted in 2008 in 61 cities across China, incorporating over 18,000 MSM, revealed an HIV prevalence of 4.9%, with incidences ranging from 2.6 to 5.4 per 100 person-years (11). This reaches levels of HIV incidence that are comparable to those observed in a cohort study of MSM in Bangkok (5.7 per 100 person-years) (12).

In contrast, HIV case reports remain very low in Japan compared to many other countries, even though Japan is also experiencing a similar but much smaller upsurge in HIV-1 infections among MSM (6). The numbers of newly reported HIV cases among MSM has more than doubled from 305 in 2002 to 655 in 2011 in Japan. Among a total of 1,019 new HIV cases reported in 2011, 67.2% were among MSM (13).

However, the HIV-1 strains responsible for the recent increases in HIV infection among MSM in Asia are different than those typically observed in MSM epidemics elsewhere. HIV-1 epidemics among MSM in major cities in the United States and Europe were first detected in the early 1980s (14); for these epidemics, subtype B was the responsible founder strain (14) and is also commonly found in most developed countries in the Asia-Pacific region, including Japan. According to Kato et al., who studied samples collected in February 1998 to March 2002 in Tokyo, subtype B was almost the only HIV-1 strain identified among MSM in Japan (15). A recent survey, however, began to detect a small number of CRF01_AE infections, as well as CRF01_AE/subtype B discordant strains or recombinants (the present study). In contrast, China recently witnessed a dramatic shift in genotype distribution from subtype B to CRF01_AE among MSM. The proportion of subtype

Received 13 September 2012 Accepted 17 January 2013
Published ahead of print 30 January 2013
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doi:10.1128/JVI.02370-12

B infections decreased from ~90% in 2005 to 2006 to ~20% in 2009, while CRF01_AE increased dramatically from 3.7% in 2005 to more than 50% in 2009 among MSM in Beijing (16–18). The rise of CRF01_AE infections among MSM has also been reported in other regions in China, including Liaoning (northeastern) (19), Jiangsu (eastern) provinces (10), Shijiazhuang, the capital city of Hebei (north-central) (20), and Zhengzhou, the capital city of Henan (Central) (21).

In the present study, we conducted a large-scale molecular epidemiological survey in the Tokyo-Kanagawa metropolitan areas in Japan and found the first evidence that HIV-1 strains typical of MSM in China have begun to spread internationally.

MATERIALS AND METHODS

Study subjects. Plasma samples were collected in 2004 to 2011 from a total of 477 newly diagnosed HIV-seropositive individuals of various risk groups in the Tokyo-Kanagawa metropolitan area: 2004 to 2005 ($n = 67$), 2006 to 2007 ($n = 107$), 2008 to 2009 ($n = 143$), and 2010 to 2011 ($n = 160$). HIV infections were confirmed by Western blotting with Lab Blot 1,2 kit (Bio-Rad Laboratories, Japan). No HIV-2 infections were detected. Study participants included 415 Japanese citizens and 62 foreign residents in Japan. Risk factors for the study subjects include heterosexuals ($n = 103$; Japanese, $n = 59$ [male = 46, female = 13]; foreigners, $n = 44$ [male = 19, female = 25]), MSM ($n = 261$; Japanese, $n = 246$; foreigner, $n = 15$), and an unknown-risk group ($n = 113$; Japanese, $n = 110$ [male = 108, female = 2]; foreigner, $n = 3$ [male = 3]). The countries of origin for the foreigners included Thailand ($n = 20$), Laos ($n = 6$), Brazil ($n = 5$), Peru ($n = 5$), China ($n = 5$), United States ($n = 4$), Indonesia ($n = 2$), Taiwan ($n = 2$), United Kingdom ($n = 2$), France ($n = 1$), Nigeria ($n = 2$), and Myanmar, Philippine, Senegal, Tanzania, Uganda, Zaire, Zambia, and Zimbabwe ($n = 1$ each) (Table 1). The study was approved by the institutional review boards of Kanagawa Prefectural Institute of Public Health and the respective hospitals and clinics. Informed consent was obtained from all study participants.

HIV-1 nucleotide sequence determination and data analyses. Plasma HIV-1 RNA was extracted using a High-Pure viral RNA kit (Roche Diagnostics). HIV-1 nucleotide sequences of 1.1-kb pro-RT (HXB2; 2,253 to 3,392 nucleotides [nt]) and 325-bp *env* C2/V3 (HXB2; 7,011 to 7,336 nt) regions were PCR amplified and determined as described by Kondo et al. (22). To further characterize the strains of interest, we also determined HIV-1 nucleotide sequences of 1.4-kb *env* gp120-gp41 regions (HXB2; 6,940 to 8,346 nt). HIV-1 genotypes were determined using neighbor-joining phylogenetic analysis (23) in conjunction with HIV-1 subtype/CRF reference strains relevant to the present study (http://www.hiv.lanl.gov/content/index). The analysis was performed using MEGA4 (24) and clade support was evaluated using 1,000 bootstrap replicates. Both pro-RT and *env* C2/V3 genotypes were obtained from 422 study subjects. Either only pro-RT or *env* C2/V3 genotypes were obtained from 27 and 25 subjects, respectively, and only protease (HXB2; 2,253 to 2,549 nt) or RT (HXB2; 2,550 to 3,392 nt) region genotypes were obtained from two subjects and one subject, respectively. Sequences with differently assigned genotypes for the pro-RT and *env* C2/V3 regions were considered to be discordant. Discordant genotypes were labeled by abbreviating the subtype assignment for the two regions; for instance, the pro-RT/*env* C2/V3 combination B/CRF01_AE was notated as B/01. The accession numbers for the nucleotide sequences obtained in the present study are AB735856 to AB735938.

Statistical analysis. Statistical comparisons of the viral subtype distributions in patients with different transmission routs were performed using Pearson χ^2 test, using SPSS v10.0 software. All statistical analyses were two sided, and $P < 0.05$ was considered to be statistically significant.

Bayesian phylodynamic inference. To estimate a time scale for the subtype B and CRF01_AE evolutionary dynamics and their spatial disper-

TABLE 1 Summary of risk categories of study subjects and distributions of HIV-1 genotypes in the Tokyo-Kanagawa metropolitan area in Japan, 2004–2011[a]

HIV-1 genotype[b]	HIV-1 cluster typical of MSM in China or Japan	No. of subjects (%)	No. of subjects (%) in risk group[c]													
			Heterosexual					MSM			Unknown					
				Japanese		Foreigner						Japanese		Foreigner		
			Total	M	F	M	F	Total	Japanese	Foreigner	Total	M	F	M	F
Subtype B		396	41	31	1	5	4	253	239	14	102	98	2	2	0
	JP.MSM.B-1	*139 (35.1)*	*8 (19.5)*	*8 (25.8)*				*91 (36.0)*	*91 (38.1)*		*40 (39.2)*	*39 (39.8)*	*1 (50.0)*		
	JP.MSM.B-2	*8 (2.0)*	*1 (2.4)*	*1 (3.2)*				*6 (2.4)*	*4 (1.7)*	*2 (14.3)*	*1 (1.0)*	*1 (1.0)*			
CRF01_AE		51	38	12	6	6	14	5	4	1	8	8	0	0	0
	CN.MSM.01-1	*4 (7.8)*						*2 (40.0)*	*2 (33.3)*		*2 (25.0)*	*2 (25.0)*			
B/01		10	5	0	0	2	3	3	3	0	2	2	0	0	0
	JP.MSM.B/CN.MSM.01-1[d]	*2 (20.0)*						*1 (33.3)*	*1 (33.3)*		*1 (50.0)*	*1 (50.0)*			
Other		20	19	3	6	6	4	0	0	0	1	0	0	1	0
Total		477	103	46	13	19	25	261	246	15	113	108	2	3	0

[a] Values in italics indicate the numbers of HIV-1 CRF01_AE and subtype B variants that belong to the cluster typical of MSM in China (CN.MSM.01-1) or Japan (JP.MSM.B-1 and JP.MSM.B-2).
[b] B/01, samples with genotype discordance (subtype B and CRF01_AE) in *pol* and *env* regions; other, other genotypes (see the text).
[c] Proportions (%) of the respective variants among each HIV-1 genotype are shown in parentheses. M, male; F, female.
[d] JP.MSM.B/CN.MSM.01-1 indicates the study subjects coinfected with HIV-1 variants typical of MSM in Japan (JP.MSM.B-1 or JP.MSM.B-2) and China (CN.MSM.01-1) (see the text and Table 2).

sal patterns in Asia, we performed Bayesian phylogeographic inference (25) using Markov Chain Monte Carlo (MCMC) sampling as implemented in BEAST (26). We specify a full probabilistic model for both subtype B and CRF01_AE pro-RT sequences, including a general time-reversible model of nucleotide substitution with a discretized gamma distribution to model rate variation among sites, an uncorrelated relaxed clock based on a lognormal distribution to model rate variation among lineages (27), a nonparametric Bayesian skyride coalescent prior for the tree (28), and a discrete diffusion model to estimate ancestral location states in the tree. For the latter, we considered various sampling locations for subtype B (n = 189)—Japan (JP; n = 83), China (CN; n = 92), Netherlands (NL; n = 1), Thailand (TH; n = 9), the United States (US; n = 3), and France (FR; n = 1)—and for CRF01_AE (n = 183)—Japan (JP; n = 45), China (CN; n = 78), Central African Republic (CF; n = 3), Hong Kong (HK; n = 2), Korea (KR; n = 3), Singapore (SG; n = 28), Thailand (TH; n = 3), Taiwan (TW; n = 7), and Vietnam (VN; n = 14). We used a Bayesian stochastic search variable selection procedure to focus on the most relevant diffusion rate parameters for the high-dimensional discrete rate matrices (25). MCMC analyses were run sufficiently long to achieve convergence and adequate effective sampling sizes, as diagnosed using Tracer (http://tree.bio.ed.ac.uk/software/tracer/). We summarized the evolutionary histories using maximum clade credibility (MCC) trees and visualized these in FigTree (http://tree.bio.ed.ac.uk/software/figtree/). To visualize the ancestral locations in the MCC tree, we grouped Chinese locations and Japanese locations and colored branches according to the sampling country for simplicity. We report evolutionary rate and date estimates using means and 95% highest density posterior (HPD) intervals.

RESULTS

HIV-1 genotype distribution in Tokyo-Kanagawa areas, 2004–2011. Using neighbor-joining phylogenetic analyses, we determined the HIV-1 genotypes of pro-RT and *env* C2/V3 regions for a total of 477 samples collected from 2004 to 2011 from various risk groups in the Tokyo-Kanagawa metropolitan area in Japan (Table 1). This resulted in the following overall HIV-1 genotype distribution: subtype B, 396 (83.0%); CRF01_AE, 51 (10.7%); B/01, 10 (2.1%); and others, 20 (4.2%) (CRF02_AG, n = 8; subtype A, n = 4; subtype C, n = 3; A/C, n = 3; subtype D, n = 1; K/C, n = 1) (Table 1). HIV-1 subtype B predominated among MSM (253 of 261 [96.9%]; 239 of 246 [97.2%] for Japanese; 14 of 15 [93.3%] for foreigners), as well as among the unknown-risk group (102 of 113 [90.3%]; 100 of 110 [90.9%] for Japanese; 2 of 3 [66.7%] for foreigners). Subtype B was less prevalent among heterosexuals (41 of 103 [39.8%]; 32 of 59 [54.2%] for Japanese [Pearson χ^2 test, $P < 0.001$]; 9 of 44 [20.5%] for foreigners) (Table 1). In contrast, CRF01_AE was detected in a significant proportion of heterosexuals (38 of 103 [36.9%]; 18 of 59 [30.5%] for Japanese [Pearson χ^2 test, $P < 0.001$]; 20 of 44 [45.5%] for foreigners). Although subtype B remains predominant among MSM in Japan, we detected a small number of CRF01_AE infections among MSM (5 of 261 [1.9%]; 4 of 246 [1.6%] for Japanese; 1 of 15 [6.7%] for foreigners), as well as among the unknown-risk group (8 of 113 [7.1%]; 8 of 110 [7.3%] for Japanese) (Table 1). In addition, a total of 10 subtype B/CRF01_AE genotype-discordant cases were detected: heterosexuals (n = 5), MSM (n = 3), and unknown-risk group (n = 2) (Table 1).

Evidence for linkage between HIV-1 epidemics among MSM in Japan and China. To explore the origins of the rare CRF01_AE strains found among MSM (and the unknown-risk group) in Japan, we scrutinized their phylogenetic relationships in the context of reference nucleotide sequences from the HIV database. In particular, we focused on nucleotide sequences previously reported from MSM in Asia. Figure 1 shows the neighbor-joining phylogenies estimated from the 1.1-kb pro-RT region (Fig. 1A and D), the 325-bp *env* C2/V3 region (Fig. 1B and E), and the 1.4-kb *env* gp120 region (Fig. 1C and F) using subtype C strains as an outgroup. Fig. 1A, B, and C and Fig. 1D, E, and F show the phylogenies for CRF01_AE and subtype B strains, respectively. In addition to subtype C strains, we also constructed the trees using group O strains as an outgroup. The tree topology and the statistical support for each node were robust to the different outgroups (data not shown).

As shown in the pro-RT region phylogeny (Fig. 1A), CRF01_AE strains circulating among MSM in China formed at least two distinct phylogenetic clusters (designated here CN.MSM.01-1 and CN.MSM.01-2) with bootstrap values of >80%. The CN.MSM.01-1 and CN.MSM.01-2 clusters contained totals of 25 and 12 CRF01_AE sequences, respectively, almost exclusively from MSM in China. CN.MSM.01-1 included CRF01_AE sequences from MSM in Beijing (n = 15) (16–18), Shijiazhuang (the capital city of Hebei, near Beijing; n = 6) (20), and Zhengzhou (the capital city of Henan, near Beijing; n = 3) (21), as well as one injecting drug user (IDU)/MSM sequence from Jiangsu in eastern China (10), while CN.MSM.01-2 contained CRF01_AE sequences from MSM in Beijing (n = 6) (16–18), Liaoning (near Beijing; n = 4) (19), Zhengzhou (21), and Shijiazhuang (n = 1 each) (20), respectively (Fig. 1A).

Interestingly, among a total of 51 CRF01_AE strains identified in the present study (Table 1), we found that four CRF01_AE strains from Japan (patients 1 through 4 [listed in Table 2]) belong to the Chinese MSM cluster CN.MSM.01-1 (Fig. 1A). These strains were found among the HIV-1-seropositive individuals newly diagnosed in 2010 and 2011, either from Japanese MSM (patients 1 and 2) or from men in an unknown-risk group (patients 3 and 4) (Table 2); this lineage was not observed among Japanese samples collected before 2009. In contrast, the remaining CRF01_AE strains (n = 48, including B/01; heterosexuals [n = 37]; MSM [n = 5]; unknown-risk group [n = 6]) were dispersed throughout the CRF01_AE radiation (Fig. 1A). The two Chinese CRF01_AE clusters CN.MSM.01-1 and CN.MSM.01-2 did not contain strains reported from any other MSM group outside China to date or strains from other risk populations in China or other Asian countries (Fig. 1A). The clusters CN.MSM.01-1 and CN.MSM.01-2 were also distinct from the previously reported CRF01_AE clusters identified among MSM in Singapore (designated SG.MSM.01-1 and SG.MSM.01-2 here) (29), IDUs in Northern Vietnam (VN) and Guangxi (GX) in southeastern China (designated VN-GX.IDU.01), and IDUs/heterosexuals in Guangxi (designated GX.01) (30–32), as shown in Fig. 1A.

The frequency of the CN.MSM.01-1 variants among CRF01_AE strains identified in Japanese populations (n = 30) were 40.0% (2 of 5) for MSM and 25.0% (2 of 8) for the unknown-risk group; no strains belonging to this group were detected among heterosexuals (0 of 38) (Table 1).

Phylogenetic analyses of *env* region. As shown in Fig. 1B, the phylogenetic analysis of the *env* C2/V3 region identified two additional samples (patients 5 and 6) (Table 2) that harbor genome segments of the CRF01_AE (CN.MSM.01-1) variant found among MSM in China. A total of 6 CRF01_AE strains formed a distinct monophyletic cluster (with a bootstrap value of 100%) within CN.MSM.01-1 (Fig. 1B). The bootstrap value for the

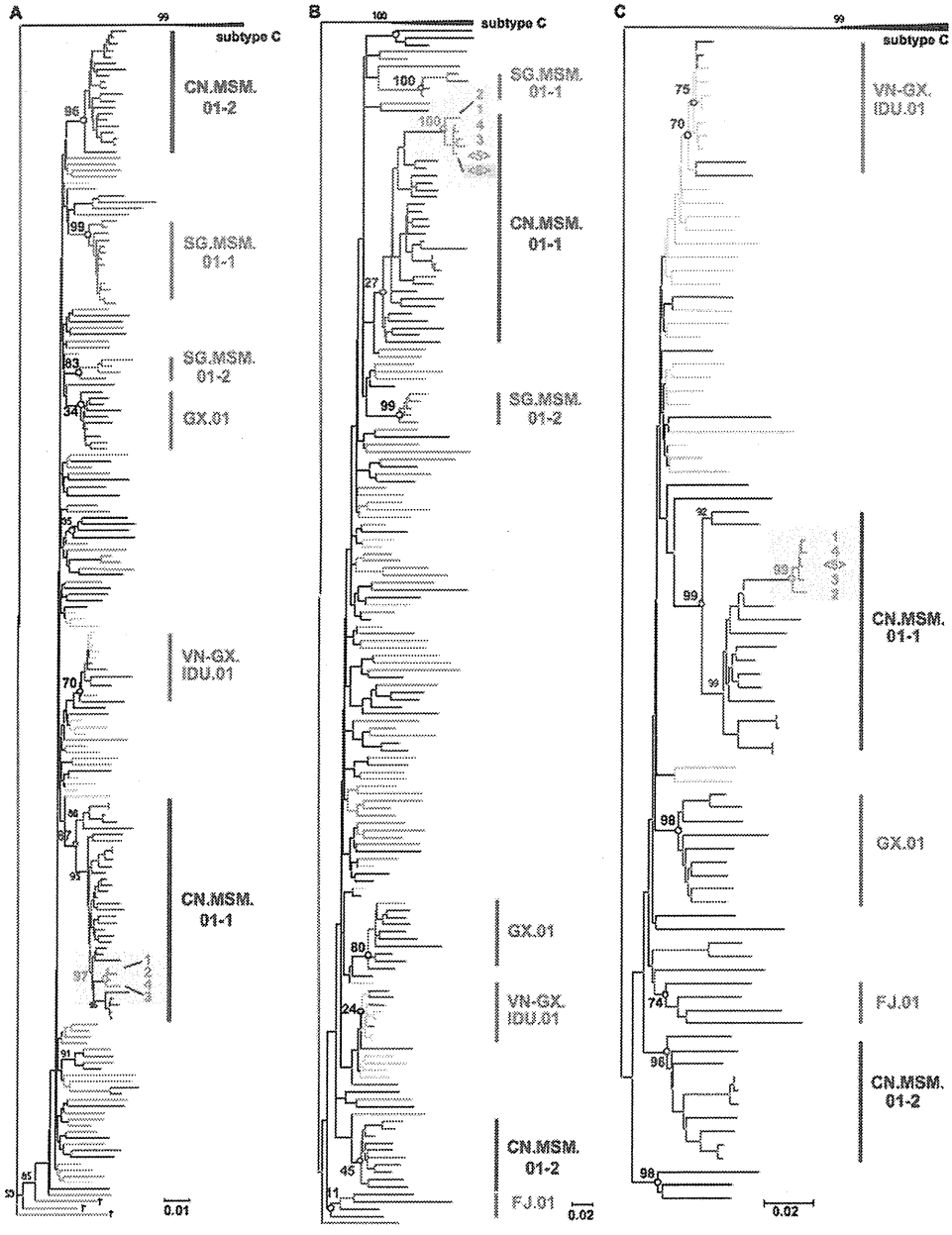

FIG 1 Neighbor-joining phylogenetic analyses. Neighbor-joining phylogenies were estimated from HIV-1 nucleotide sequences for the 1.1-kb pro-RT (HXB2; 2,253 to 3,392 nt) region (A and D), the 325-bp *env* C2/V3 (HXB2; 7,011 to 7,336 nt) region (B and E) and the 1.4-kb *env* gp120 (HXB2; 6,940 to 8,346 nt) region (C and F). Panels A, B, and C and panels D, E, and F show the phylogenetic relationships of CRF01_AE and subtype B strains, respectively. HIV-1 subtype C sequences were used as an outgroup. Bootstrap support values relevant to the present study (>80) are shown on corresponding nodes, except in the *env* C2/V3 phylogenies, where we kept all bootstrap support values even if they were low. HIV-1 subtype/CRF designations are indicated to the right of the phylogeny. The Chinese CRF01_AE MSM clusters (designated CN.MSM.01-1 and CN.MSM.01-2) and the Japanese subtype B MSM clusters (designated JP.MSM.B-1 through JP.MSM.B-4) are indicated. Samples from patients 1 to 6, who carried genome segments derived from CN.MSM.01-1, are denoted using the numbers 1 to 6. The symbols "<5>" (shaded white) and "<6>" (shaded pink) are the subjects with genotype discordance (see Table 2). Subjects belonging to JP.MSM.B-2 ($n = 8$) are indicated as x (x = a, b, c, d, f, and h) for Japanese and y(CN) (y = e and g) for Chinese MSM resident in Japan (see Table 3). The geographic origins of sequences are color-coded in the corresponding branches: Japan (JP), red; China (CN), blue; Vietnam (VN), orange; Singapore (SG), violet; Thailand (TH), green; and Taiwan (TW), Korea (KR), Hong Kong (HK), and others, black. Symbols for the study subjects (1 through 6; a through h) in red and blue indicate Japanese and Chinese nationality, respectively. Other CRF01_AE and subtype B variants that appear to be region- (and risk factor)-specific in Asia are indicated on the right of each tree. Regions: FJ, Fujiang province of eastern China; GX, Guangxi province of southeastern China. Asterisks in panel D indicate three subtype B sequence from Liaoning, China (06CN.LN107, 06CN.LN126, and 07CN.LN159) (19) that belong to the JP.MSM.B-1 cluster (see the text). The bottom three sequences marked with daggers (†) in panel A were found to be subtype B/CRF01_AE recombinants that harbored small subtype B segment in 5′ part of the pro-RT regions. Therefore, they were removed from the data set for BEAST analysis. We constructed the tree using all CRF01_AE strains of the interest, while we use only randomly selected CRF01_AE strains from the Thailand radiation for simplicity.

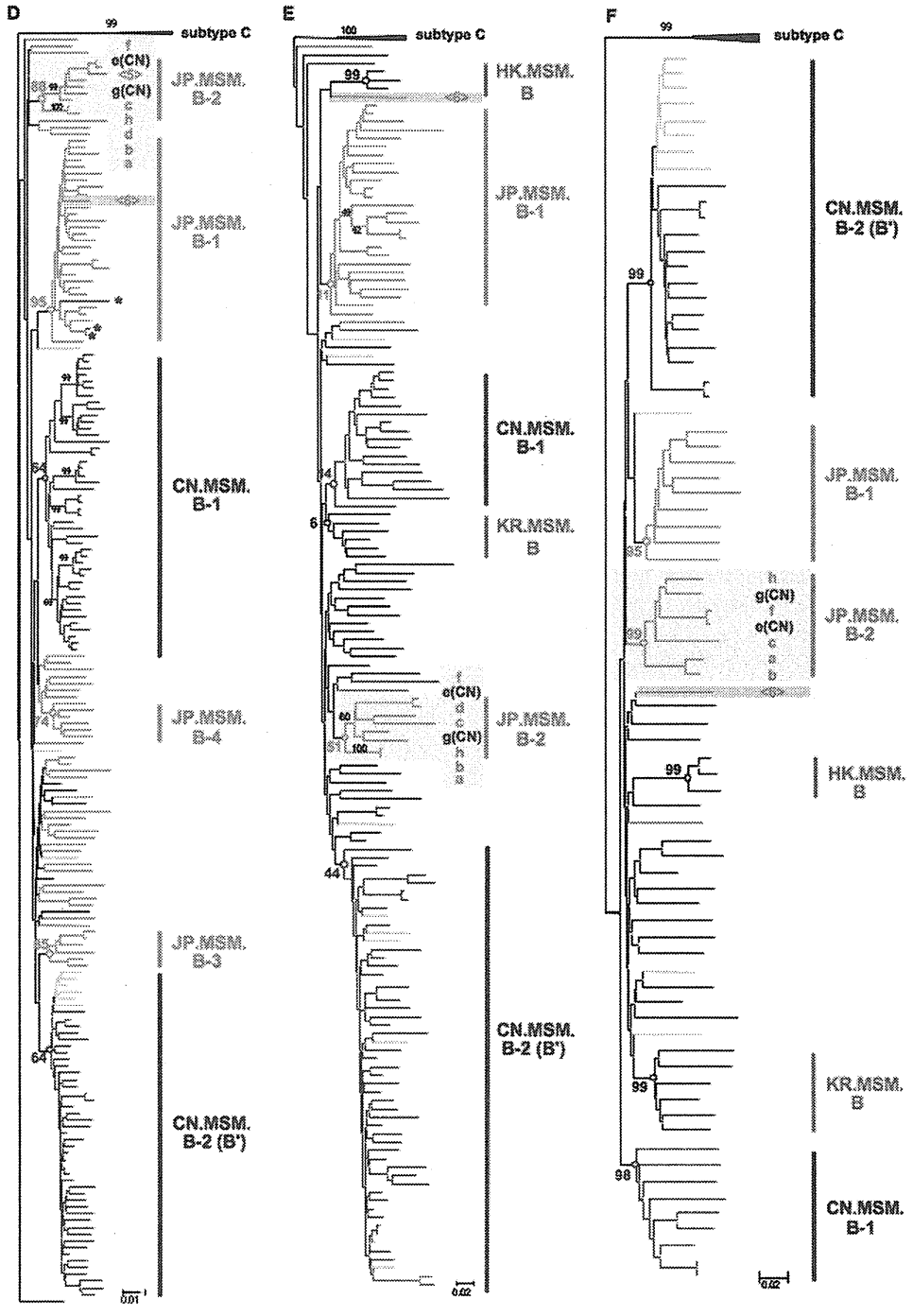

FIG 1 continued

CN.MSM.01-1 cluster in the *env* C2/V3 phylogeny was low (27%), likely due to the short nucleotide sequence length for that region (325 nt). Therefore, to further confirm and strengthen our findings, we determined the nucleotide sequences of the 1.4-kb *env* gp120-gp41 regions of these samples. As illustrated in the *env* gp120-gp41 phylogeny (Fig. 1C), all CN.MSM.01-1-related samples, except patient 6 (see below), belonged to CN.MSM.01-1 cluster. Using these longer sequences, the bootstrap support for the CN.MSM.01-1 was very high (99%) (Fig. 1C).

Identification of subtype B clusters and coinfection with the strains characteristic for MSM populations in Japan and China. Although the *env* C2/V3 genotypes of patients 5 and 6 were classified as CRF01_AE (CN.MSM.01-1) (Fig. 1B), we found that the nucleotide sequences of their pro-RT regions were placed in among subtype B strains of U.S.-European origin (Fig. 1D). Patients 5 and 6 thus exhibited genotype discordance between the pro-RT region (subtype B) and the *env* (CRF01_AE: CN.MSM.01-1) regions (Table 2). Furthermore,

TABLE 2 Summary of epidemiologic and genotype information of six Japanese male cases infected with CRF01_AE variant (CN.MSM.01-1) uniquely found among MSM in China[a]

Patient[b]	Strain	Geographic origin	Yr of sample collection	Age (yr)	Risk factor	HIV-1 genotype		
						1.1-kb pro-RT (HXB2; 2,253–3,392 nt)	325-bp *env* C2/V3 (HXB2; 7,011–7,336 nt)	1.4-kb *env* gp120-gp41 (HXB2; 6,940–8,346 nt)
1	10JP-GM3217	Kanagawa	2010	27	MSM	CN.MSM.01-1	CN.MSM.01-1	CN.MSM.01-1
2	10JP-GM3345	Tokyo	2010	28	MSM	CN.MSM.01-1	CN.MSM.01-1	CN.MSM.01-1
3	11JP-GM3527	Tokyo	2011	29	–[c]	CN.MSM.01-1	CN.MSM.01-1	CN.MSM.01-1
4	11JP-GM3557	Tokyo	2011	29	–	CN.MSM.01-1	CN.MSM.01-1	CN.MSM.01-1
5	10JP-GM3289	Tokyo	2010	26	MSM	JP.MSM.B-2	CN.MSM.01-1	CN.MSM.01-1
6	10JP-GM3426	Tokyo	2010	38	–	JP.MSM.B-1	CN.MSM.01-1/B(US)	B(US)

[a] CRF01_AE and subtype B clusters uniquely found among MSM in China (CN.MSM.01-1) and Japan (JP.MSM.B-1and JP.MSM.B-2), respectively. B(US), HIV-1 subtype B of U.S.-European origin; CN.MSM.01-1/B(US), coexistence of the respective HIV-1 strains (see the text and Fig. 1).
[b] Patients 1 to 6 were all male. Patients 5 and 6 showed genotype discordance between the *pol* and *env* regions: they were most likely to be coinfected with CRF01_AE (CN.MSM.01-1) and subtype B (JP.MSM.B-2 or JP.MSM.B-1) variants uniquely found among MSM in China and Japan, respectively (see the text).
[c] –, Unknown.

for patient 6, the genotyping of the *env* C2/V3 and gp120-gp41 regions was inconsistent. Namely, the nucleotide sequence of the *env* C2/V3 region amplicon was genotyped as CRF01_AE (Fig. 1B), whereas the nucleotide sequence of the *env* gp120-gp41 amplicon was genotyped as subtype B (Fig. 1C). These results suggested that patient 6, as well as patient 5, were most likely to be coinfected with CRF01_AE (CN.MSM.01-1) and subtype B of U.S.-European origin. Table 2 summarizes the epidemiological and genotyping information for these six Japanese male cases who harbored genome segments derived from the Chinese CRF01_AE MSM variant (CN.MSM.01-1).

In order to further characterize the origins of subtype B sequences detected in patients 5 and 6, we analyzed their phylogenetic relationships in more detail. As shown in pro-RT region tree (Fig. 1D) and summarized in Table 1, approximately one-third (91 of 239 [38.1%]) of the subtype B strains found in MSM in Japan formed a large monophyletic cluster (designated JP.MSM.B-1; bootstrap value > 80%) within the wider diversity of subtype B of U.S.-European origin. The subtype B sequence detected in patient 6 belonged to this Japanese MSM subtype B cluster (JP.MSM.B-1). In contrast, the subtype B sequence detected in the patient 5 fell into a separate small cluster (designated JP.MSM.B-2; bootstrap value = 88%) (n = 8). Intriguingly, the JP.MSM.B-2 cluster contained sequences from two Chinese MSM living in Japan [patient codes e(CN) and g(CN) in Fig. 1] in addition to Japanese MSM (n = 4): one man with an unknown risk factor and one heterosexual (Fig. 1). We also note that subtype B sequences from patients a (04JP-Y201) and b (06JP-GM1986) consistently occupied basal positions on the JP.MSM.B-2 branches (Fig. 1D to F), suggesting that JP.MSM.B-2 have most likely emerged among Japanese MSM population and then transmitted to Chinese MSM living in Japan. Table 3 summarizes the epidemiological and genotypic information for the study subjects harboring the JP.MSM.B-2 variant that appears to be unique to the MSM population in Japan.

As shown in Fig. 1D to F, JP.MSM.B-1 and JP.MSM.B-2 were indeed distinct from other subtype B variants reported previously, including MSM-related subtype B clusters from Korea (33) (designated KR.MSM.B) and Singapore (29) (designated SG.MSM.B-1 and SG.MSM.B-2). We also note that

TABLE 3 Epidemiologic and genotype information of study subjects infected with HIV-1 subtype B (JP.MSM.B-2) variant that appears to be unique to MSM and related populations in Japan[a]

Patient code	Strain	Geographic origin	Yr of sample collection	Nationality	Age (yr)	Risk factor[b]	HIV-1 genotype			Remarks
							1.1-kb pro-RT (HXB2; 2,253–3,392 nt)	325-bp *env* C2/V3 (HXB2; 7,011–7,336 nt)	1.4-kb *env* gp120-gp41 (HXB2; 6,940–8,346 nt)	
a	04JP-Y201	Kanagawa	2004	Japan	30	MSM	JP.MSM.B-2	JP.MSM.B-2	JP.MSM.B-2	
b	06JP-GM1986	Tokyo	2006	Japan	35	MSM	JP.MSM.B-2	JP.MSM.B-2	JP.MSM.B-2	
c	06JP-Y281	Kanagawa	2006	Japan	24	Hetero	JP.MSM.B-2	JP.MSM.B-2	JP.MSM.B-2	
d	09JP-Y449	Kanagawa	2009	Japan	33	MSM	JP.MSM.B-2	JP.MSM.B-2	NA	
e(CN)	09JP.CN-Y519	Tokyo	2009	China	26	MSM	JP.MSM.B-2	JP.MSM.B-2	JP.MSM.B-2	Likely infected in Japan, seroconverted in 2009
f	09JP-GM3050	Kanagawa	2009	Japan	37	Unknown	JP.MSM.B-2	JP.MSM.B-2	JP.MSM.B-2	
g(CN)	09JP.CN-GM3061	Tokyo	2009	China	27	MSM	JP.MSM.B-2	JP.MSM.B-2	JP.MSM.B-2	Likely infected in Japan, seroconverted in 2009
h	11JP-GM3473	Tokyo	2011	Japan	33	MSM	JP.MSM.B-2	JP.MSM.B-2	JP.MSM.B-2	
5	10JP-GM3289	Tokyo	2010	Japan	26	MSM	JP.MSM.B-2	CN.MSM.01-1	CN.MSM.01-1	Coinfection of two variants

[a] CRF01_AE and subtype B variants unique to MSM populations in China (CN.MSM.01-1) and Japan (JP.MSM.B-1 and JP.MSM.B-2). NA, data not available. Patient codes e(CN) and g(CN) represent Chinese MSM living in Japan. Patient 5 here is identical to patient 5 in Table 2. All of the subjects were male.
[b] Hetero, heterosexual contact; –, unknown.

subtype B strains from MSM in China formed two distinct phylogenetic subclusters (designated CN.MSM.B-1 and CN.MSM.B-2) within subtype B (Fig. 1D to F). CN.MSM.B-2 was found to be highly similar to the subtype B′ variant (Thailand variant of subtype B) (34, 35) originally identified among IDUs in Thailand. Subtype B′ strains from Thailand were indeed clustered together with CN.MSM.B-2 (Thailand subtype B′ strains are depicted as branches in green in the CN.MSM.B-2 clusters in Fig. 1D to F). JP.MSM.B-1 and JP.MSM.B-2 variants are thus not related to any of subtype B variants identified to date (Fig. 1D to F).

Of note, in addition to JP.MSM.B-1 and JP.MSM.B-2, at least two additional statistically well-supported subtype B clusters (designated JP.MSM.B-3 and JP.MSM.B-4) were recognized among Japanese MSM and men with unknown risk factor (Fig. 1D). Bootstrap values for JP.MSM.B-3 and JP.MSM.B-4 nodes were 85 and 74%, respectively (Fig. 1D). This observation suggests the presence of multiple HIV-1 transmission networks among MSM in Japan.

Spatiotemporal history of the distinct CRF01_AE and subtype B clusters identified in Asia. To explore when CRF01_AE and subtype B variants in East Asia emerged, we inferred divergence times and spatial dispersal patterns using a Bayesian phylogeographic analysis (25) incorporating a relaxed molecular clock model (27). The analyses were performed using BEAST v1.7.4 (26) based on the two nucleotide sequence data sets for the 1.1-kb *pol* (pro-RT) regions of CRF01_AE (Fig. 2A) and subtype B (Fig. 2B) strains, similar to those that were used for neighbor-joining tree analyses (see Fig. 1). The estimated evolutionary rates were 2.81 (95% HPD, 2.42 to 3.22) $\times 10^{-3}$ and 1.81 (1.46 to 2.15) $\times 10^{-3}$ substitutions/site/year for CRF01_AE and subtype B, respectively. The results were essentially similar to those previously estimates for CRF01_AE and subtype B (31, 36, 37).

As shown in Fig. 2A, the estimated times to the most recent common ancestor (tMRCAs) for CN.MSM.01-1 and CN.MSM.01-2 were 1996.5 (1993.9 to 1999.1) and 1999.0 (1996.5 to 2001.1), respectively. The tMRCA for the Japanese CN.MSM.01-1 subcluster (designated JP-CN.MSM.01-1 subcluster) containing four strains from Japanese MSM and men with unknown risk factor (patients 1 through 4) (Table 2) was estimated to be 2008.6 (2006.9 to 2009.6), indicating that the JP-CN.MSM.01-1 subcluster indeed emerged very recently as a descendant of the CN.MSM.01-1 lineage. The estimated tMRCAs for SG.MSM.01-1, SG.MSM.01-2, GX.01, FJ.01, and VN-GX.IDU.01 were 2000.3 (1998.3 to 2002.2), 1996.2 (1993.8 to 1998.5), 1999.2 (1997.3 to 2001.1), 1998.1 (1994.8 to 2000.5), and 1994.0 (1992.9 to 1995.4), respectively.

As for the subtype B variants, the estimated tMRCA for CN.MSM.B-1 and CN.MSM.B-2 were 1984.6 (1979.7 to 1989.4), and 1987.8 (1983.7 to 1992.2), respectively. The CN.MSM.B-2 lineage formed a monophyletic cluster within the typical subtype B′ strains from Thailand and Yunnan province of China (Fig. 2B). This indicates that CN.MSM.B-2 lineage (tMRCA~1988) is a descendant of the ancestral subtype B′ distributed in Thailand and Yunnan (tMRCA~1985). The estimated tMRCAs for JP.MSM.B-1, JP.MSM.B-2, JP.MSM.B-3, and JP.MSM.B-4 were 1987.7 (1983.9 to 1991.3), 1986.9 (1980.6 to 1992.9), 1986.1 (1980.9 to 1990.8), and 1990.5 (1986.2 to 1994.0), respectively (Fig. 2B). In this particular analysis, the MCC tree is based on subtype B sequences almost exclusively from Asia with a few subtype B strains of U.S.-European origin for simplicity. In contrast to our expectation, the root was assigned to subtype B from Japan and not from U.S.-Europe, but that is simply a result from the over-representation of Japanese relative to U.S. sequences.

In addition, the tMRCA for the JP.MSM.B-1 subcluster containing three sequences from Liaoning province of northeastern China (asterisked in Fig. 2D) (designated CN-JP.MSM.B-1 subcluster) was estimated to be 1999.1 (1996.2 to 2001.8) (posterior probability of 1.0) (Fig. 2B). Similarly, the tMRCA for the subcluster containing 2 Chinese resident in Japan [patient code e(CN) and g(CN) (marked with blue triangles at the tips of the respective branches in Fig. 2B)] (see also Table 2 and Fig. 1D) (designated cn-JP.MSM.B-2 subcluster) was estimated to be 1996.7 (1991.9 to 2001.7) (posterior probability of 0.92) (Fig. 2B). All CRF01AE and subtype B clusters identified in the present study were statistically well supported: the posterior probability support values for the clusters of interest are > 0.95, except for cn-JP.MSM.B-2 (0.92) and VN-GX.IDU.01 (0.76) (Fig. 2).

DISCUSSION

In the present study, we provided the first evidence for the international dissemination of HIV-1 CRF01_AE lineages that had previously only been found among MSM in China. As summarized in Table 1, we identified a total of 6 Japanese male subjects who harbored genome segments derived from a distinct CRF01_AE variant (CN.MSM.01-1) uniquely associated with the HIV-1 epidemic among MSM in China. As depicted in Fig. 1, CRF01_AE strains circulating among MSM in China formed at least two distinct monophyletic clusters (CN.MSM.01-1 and CN.MSM.01-2). Both variants almost exclusively contained strains from MSM in China and accounted for >95% of CRF01_AE sequences from among MSM in China (Fig. 1).

Unlike in China, the prevalence of CRF01_AE among MSM in Japan is still very low (1.6% [4/246] for MSM and 3.7% [13/354] for MSM plus unknown-risk group in our study) (Table 1). However, among a total of 12 CRF01_AE strains identified in Japanese MSM and in men with an unknown risk, 4 (33.3%) belong to CN.MSM.01-1 (Table 1). Furthermore, CN.MSM.01-1 variants in Japan were identified only among newly diagnosed individuals in 2010 and 2011 ($n = 160$) and not in 2004 to 2009 ($n = 317$). This suggests that the CN.MSM.01-1 variant has emerged very recently in Japan. The extremely short branch length of CN.MSM.01-1 sequences from Japanese MSM (and men with an unknown risk factor) (Fig. 1) corroborates this notion. The Bayesian molecular clock analysis indicated that the timing of the emergence of this JP-CN.MSM.01-1 subcluster is estimated to be ~2009, significantly younger than that of ancestral CN.MSM.01-1 (~1997) (Fig. 2A). Taken together, these results strongly suggest that CN.MSM.01-1 strains found in Japanese MSM and men with unknown risk factor were indeed introduced from the Chinese MSM population very recently.

Of note, the estimated divergence times (tMRCAs) for the respective CRF01_AE variants (after the mid-1990s) are significantly younger than those for subtype B variants (mostly the mid- to late 1980s) (Fig. 2). This reflects the differences in epidemic history of the subtype B and CRF01_AE strains, with the former being introduced much earlier into the Asian MSM population. However, since then, a dramatic shift in genotype distribution from subtype B to CRF01_AE has been documented in China (16–18; see also the introduction above).

JP.MSM.B-1 and JP.MSM.B-2 appeared to be primarily asso-

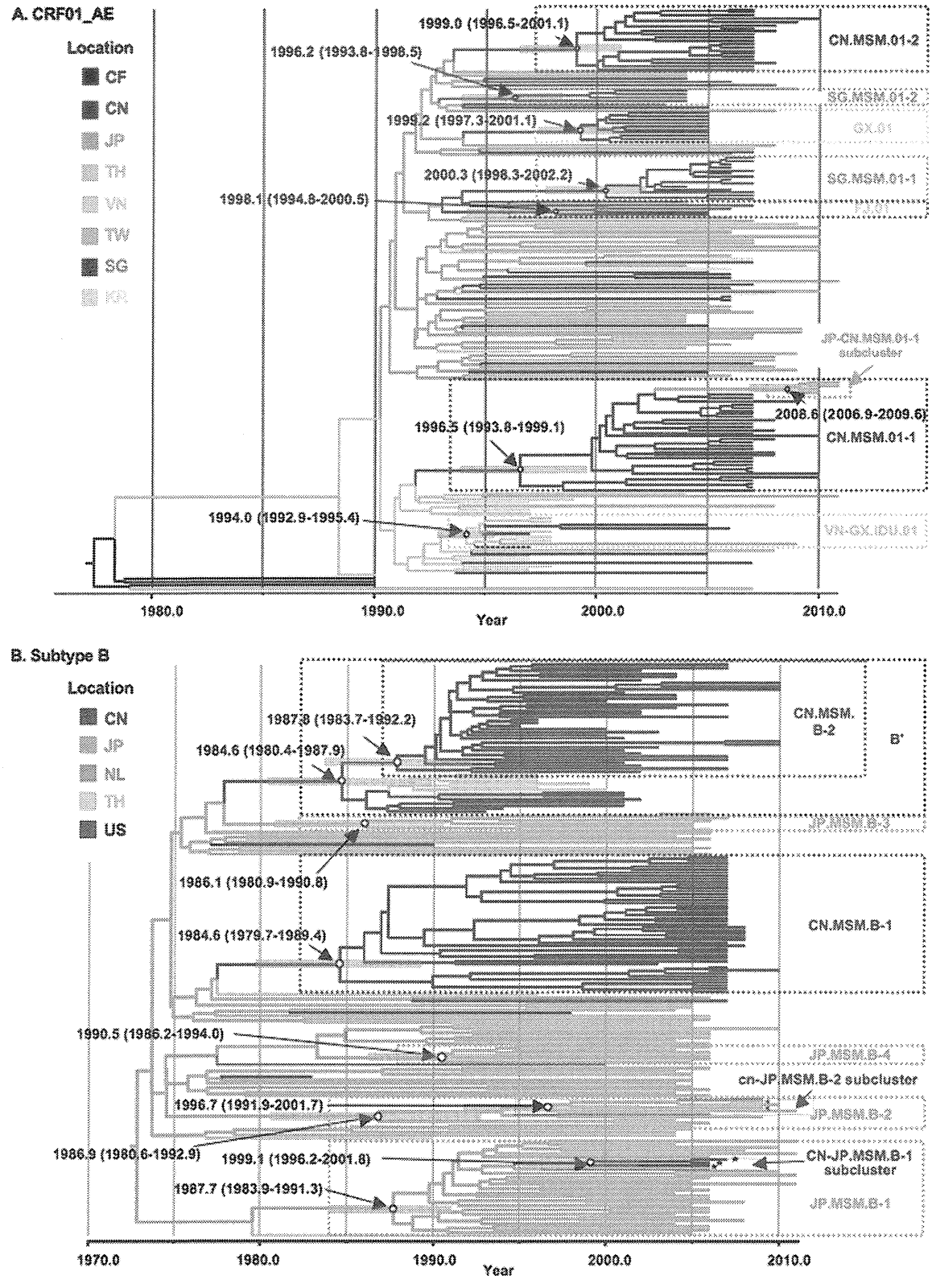

FIG 2 Bayesian reconstruction of the HIV-1 CRF01_AE and subtype B evolutionary history in Asia. Maximum clade credibility (MCC) trees of the 1.1-kb pro-RT sequences obtained by the Bayesian MCMC analysis for the CRF01_AE (A) and subtype B (B) strains are shown (see the text for details). The branch lengths in the MCC trees reflect the time, and the corresponding time scale is shown at the bottom of the tree. The 95% highest probability density (HPD) intervals for the tMRCA estimates of the clusters of interest are >0.95, except for cn-JP.MSM.B-2 (0.92) and VN-GX.IDU.01 (0.76). The geographic origins (denoted with two-letter country code) are represented by color codes (listed in the left-hand panel) for the respective branches. The tMRCA means and 95% HPDs for the key nodes are indicated.

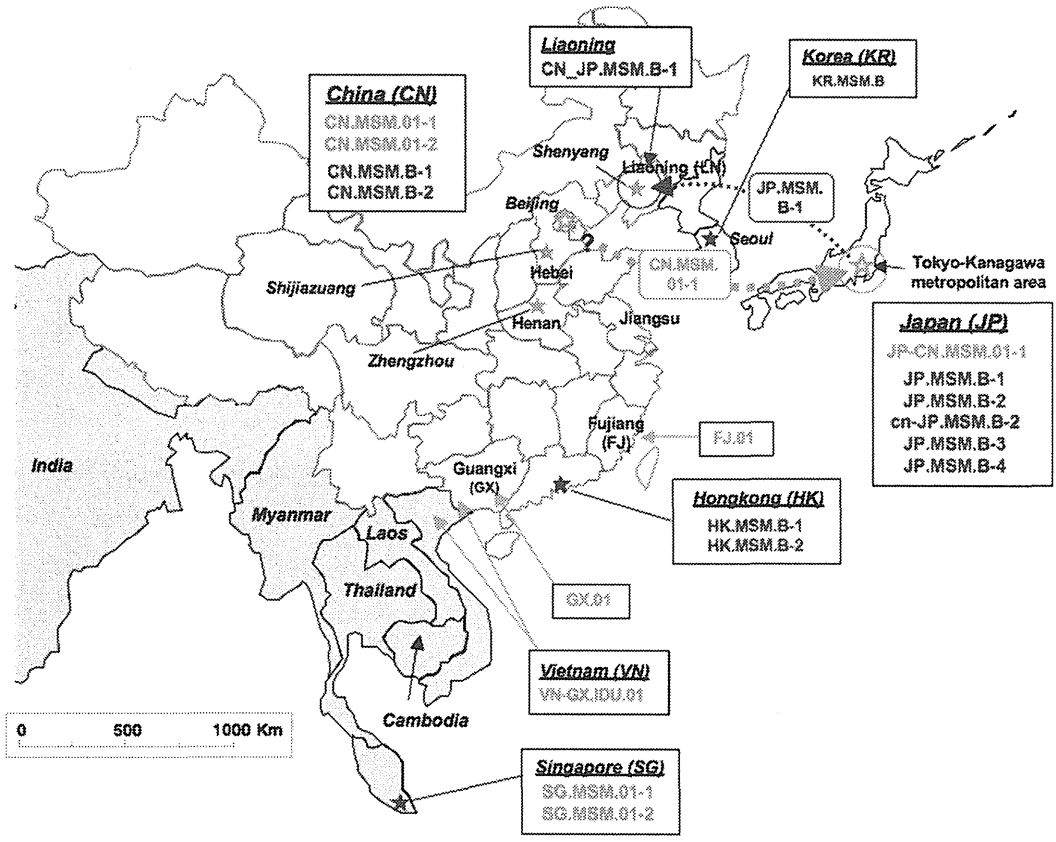

FIG 3 Map of Asia showing the distribution of CRF01_AE and subtype B variants characteristic in this region. The map depicts the geographic distribution of study sites (origins of HIV-1 sequence data set used in the present study) (yellow shading) and of the distinct lineages of CRF01_AE (in red) and subtype B (in blue) strains identified in the present study (and in previous reports). CN.MSM.01-1 and CN.MSM.01-2 are CRF01_AE variants associated with MSM in China. HIV-1 subtype B variants associated with the MSM epidemic include JP.MSM.B-1 through JP.MSM.B-4 in Japan and CN.MSM.B-1 and CN.MSM.B-2 (subtype B′) in China. CN.MSM.01-1 was probably introduced into the MSM population in Japan very recently (indicated as a broken red arrow; see the text). Other subtype B variants uniquely found among Asian MSM include KR.MSM.B in Korea, HK.MSM.B-1 and B-2 in Hong Kong. CRF01_AE variants in the non-MSM population in Asia include FJ.01 among heterosexuals in Fujian (FJ) of eastern China, GX.01 among IDU/heterosexuals in Guangxi (GX) of southeastern China, and VN-GX.IDU.01 in northern Vietnam (NV)-Guangxi (GX) IDUs. Three JP.MSM.B-1 sequences (asterisked in Fig. 1D) were identified among MSM in Liaoning of northeastern China. This suggests that the dissemination of HIV from Japanese MSM to Chinese MSM is also possible (indicated as a thin broken blue arrow; see the text).

ciated with MSM in Japan (Fig. 1). Intriguingly, however, the extensive database search revealed that three previously reported HIV-1 subtype B strains from MSM in Liaoning of northeastern China (06CN.LN107, 06CN.LN126, and 07CN.LN159) (19) appear to belong to JP.MSM.B-1 (asterisked in Fig. 1D). This suggests that the spread of virus from MSM in Japan to MSM in China may also have occurred. Figure 3 schematically illustrates the geographic distribution of CRF01_AE and subtype B variants identified among MSM and other risk populations and the hypothesized route of virus dissemination in MSM networks in East Asia.

Our discovery of two patients (patients 5 and 6) coinfected by virus variants uniquely associated with MSM transmission in China (CN.MSM.01-1) and Japan (JP.MSM.B-1 or JP.MSM.B-2) is of particular relevance to public health. Increased international travel and socioeconomic relationships between Japan and China are possibly facilitating more frequent interaction between the MSM populations of both countries.

Our study did not have complete risk factor information, and many strains had to be coded as an unknown-risk group; the high proportion in this category (113 of 477 [23.7%]) may complicate the interpretation of the results. However, the genotype distributions of the self-identified MSM group and in men with an unknown risk factor were similar and very different from the distribution in heterosexuals (Table 1). Moreover, the frequency of the major Japanese MSM subtype B cluster (JP.MSM.B-1) was almost the same in both categories (38.1% [91 of 239] for MSM versus 39.8% [39 of 98] for the unknown-risk group, respectively) (Table 1). Negative social attitudes toward MSM may be preventing some study subjects from describing their sexual preferences during interview. Thus, it is possible that some Japanese men among those in the unknown-risk group do in fact have MSM and/or bisexual risk factors. We also noted that the JP.MSM.B-1 variant was found among Japanese heterosexual men (8 of 31 [25.8%]) and not among female heterosexuals (Table 1). Although we do not have an appropriate explanation for this, we speculate that a significant proportion of (self-described) heterosexuals are probably bisexual but may not have revealed their actual sexual orientations and that the rates of partner exchange and probability of transmission per contact will be higher for bisexual men compared to heterosexual women.

Our study highlights the urgent importance of strengthening HIV monitoring efforts and the need for implementing effective measures to reduce HIV transmission among high risk groups in Asia, especially as HIV prevalence in China grows and socio-economic links between China and the rest of the world continue to expand.

ACKNOWLEDGMENTS

We thank Oliver Pybus and Yoshiyuki Nagai for critical reading of the manuscript, Yuki Naito and Shigeru Kusagawa for valuable advice in the database search and for technical help, and Wataru Sugiura for support.

This study was supported by a Grant-in-Aid for AIDS research from the Ministry of Health, Labor, and Welfare of Japan.

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