

**Protein kinase library and cell-free protein synthesis.** The construction and identity of the 404 cDNAs encoding human protein kinases used in this study were previously described (41). *In vitro* transcription and cell-free protein synthesis were performed as previously reported (42, 43). Briefly, DNA templates containing a biotin-ligating sequence were amplified by split-primer PCR with kinase cDNAs and corresponding primers and then used for protein synthesis with a fully automated protein synthesizer, a GenDecoder (CellFree Sciences, Ehima, Japan). For synthesis of FLAG-tagged full-length NS5A and domain III proteins derived from the JFH-1 isolate, DNA templates containing a FLAG sequence were generated from the NS5A expression plasmid (8) by split-primer PCR and used with a wheat germ expression kit (CellFree Sciences) according to the manufacturer's instructions.

**Amplified luminescent proximity homogeneous assay (AlphaScreen).** FLAG-tagged NS5A proteins were mixed with biotinylated kinases in 15  $\mu$ l of reaction buffer (20 mM Tris-HCl [pH 7.6], 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, and 1 mg/ml bovine serum albumin) in the wells of 384-well OptiPlates (PerkinElmer, Waltham, MA) and incubated at 26°C for 1 h. The mixture was then added to the detection mixture containing 0.1  $\mu$ l protein A-conjugated acceptor beads (PerkinElmer), 0.1  $\mu$ l streptavidin-coated donor beads (PerkinElmer), and 5  $\mu$ g/ml of the anti-FLAG M2 antibody, followed by incubation at 26°C for 1 h. AlphaScreen signals from the mixture were detected using an EnVision device (PerkinElmer) with the AlphaScreen signal detection program.

***In vitro* phosphorylation assay.** To obtain purified kinases used for *in vitro* phosphorylation assays, DNA templates containing a glutathione S-transferase (GST)-tobacco etch virus (TEV) sequence were generated by split-primer PCR with kinase cDNAs and corresponding primers and used in a cell-free production system with the wheat germ expression kit as described above. The GST-fusion recombinant proteins were purified on glutathione-Sepharose 4B (GE Healthcare, Buckinghamshire, United Kingdom) and then eluted in 40  $\mu$ l of phosphate-buffered saline (PBS) containing 5 U of AcTEV protease (Life Technologies) in order to cleave the GST tag from the protein. Biotinylated NS5A proteins were synthesized from DNA templates by using the cell-free BirA system (44). Biotinylated NS5A proteins (40  $\mu$ l) were coupled on 15  $\mu$ l of streptavidin MagneSphere Paramagnetics particles (Promega, Madison, WI) and were dephosphorylated by using 10 U of lambda protein phosphatase (New England BioLabs, Ipswich, MA). After washing three times, protein-coupled beads were incubated with 1  $\mu$ l of purified recombinant kinases at 37°C for 30 min in 15  $\mu$ l kinase buffer (50 mM Tris-HCl [pH 7.6], 500 mM potassium acetate, 50 mM MgCl<sub>2</sub>, and 0.5 mM dithiothreitol) containing 1  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP. After the reaction, the beads were washed twice with PBS and then boiled in sample buffer and separated by SDS-PAGE. Phosphorylated NS5A proteins were visualized via autoradiography, and the relative kinase activity of each kinase was determined by normalizing the band intensity of NS5A to that of NS5A incubated with dihydrofolate reductase (DHFR). The band intensities were quantified using Image J software.

**Preparation of viral stocks and virus infections.** Cell culture-derived infectious HCV particles (HCVcc) were prepared as described previously (30, 36). HCVpp consisting of HCV envelope glycoproteins, the murine leukemia virus Gag-Pol core proteins, and the luciferase transfer vector and pseudoparticles with the vesicular stomatitis virus G glycoprotein (VSV-Gpp) were generated in accordance with methods described previously (36, 45). Cells seeded onto 24-well plates were transfected with siRNA and/or plasmid DNA as described below and infected with HCVcc for 4 h at a multiplicity of infection (MOI) of 0.5 to 5 or with diluted supernatant containing HCVpp or VSV-Gpp for 3 h. After infection, the cells were washed with PBS and incubated in fresh complete growth medium for 72 h at 37°C until harvest.

**siRNA and plasmid DNA transfections.** siRNAs were purchased from Sigma-Aldrich. The sequences were as follows: CKI- $\alpha$ , 5'-GGCUAAAGG CUGCAACAAAdTdT-3' and 5'-UUUGUUGCAGCCUUUAGCCdTdT-3'; CKI- $\gamma$ 1, 5'-GAGAUGAUUUGGAAGCCCUdTdT-3' and 5'-AGGGC

UCCAAAUCAUCUCdTdT-3'; CKI- $\gamma$ 2, 5'-GCGAGAACUCCCCAGA GGAdTdT-3' and 5'-UCCUCUGGGAAGUUCUCGCdTdT-3'; CKI- $\gamma$ 3, 5'-CUUACAGGAACAGCUAGAAdTdT-3' and 5'-AUCUAGCUGU UCCUGUAAAGdTdT-3'; CKI- $\epsilon$ , 5'-GCGACUACAACGUGAUGGdTdT-3' and 5'-ACCAUCACGUUGUAGUCGCdTdT-3'; CKII- $\alpha$ , 5'-CCU AGAUCUUCUGGACAAAdTdT-3' and 5'-UUUGUCCAGAAGAUCU AGGdTdT-3'; PKAC $\beta$ , 5'-CAAUAAGAGCAUACUUUGAdTdT-3' and 5'-UCAAGUAUGCUCUAAUUUGdTdT-3'; Plk1, 5'-GUCUCAAGG CUCCUAAUAdTdT-3' and 5'-UAUUAGGAGCCUUUGAGAdTdT-3'; TSSK2, 5'-CACCUACUGACUUUGUGGAdTdT-3' and 5'-UCCAC AAAGUCAGUAGGUGdTdT-3'; ApoE, 5'-GGAGUUGAAGCCUACA AAdTdT-3' and 5'-UUUGUAGGCCUUCAACUCCdTdT-3'; CLDN1, 5'-CAGUCAAUGCCAGGUACGAdTdT-3' and 5'-UCGUACCUGGCA UUGACUGdTdT-3'; PI4K-III $\alpha$ , 5'-CCCUAAAGGCGACGAGAGAdTdT-3' and 5'-UCUCUCGUCGCCUUUAGGGdTdT-3'. The Mission siRNA universal negative control (Sigma-Aldrich), which is designed to have no homology to known gene sequences, was used as a negative control. Silencer Cy3-labeled GAPDH siRNA (Life Technologies) was used to confirm siRNA delivery efficiency. Basically, 10 nM siRNAs were transfected into cells by using Lipofectamine RNAiMax (Life Technologies) according to the manufacturer's recommended procedures. Plk1 siRNA was transfected at 5 nM because of its cytotoxic effect. Plasmid DNA transfection was carried out by using TransIT-LT1 transfection reagent (Mirus, Madison, WI) according to the manufacturer's protocol. For cotransfection of siRNA and plasmid DNA, 6 pmol of siRNA and 200 ng of plasmid DNA were transfected into Huh7.5.1 cells seeded onto a 24-well cell culture plate by using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions.

**RNA synthesis and electroporation.** HCV RNA synthesis and electroporation were basically performed as described previously (8). In the context of coelectroporation of siRNA and an *in vitro*-synthesized subgenomic reporter replicon, or full-length HCV RNA, a total of  $3 \times 10^6$  to  $5 \times 10^6$  Huh-7 cells were electroporated with 120 pmol siRNA and 3  $\mu$ g SGR-JFH1/Luc RNA or 5  $\mu$ g JFH-1 RNA at 260 V and 950  $\mu$ F. After electroporation, the cells were immediately transferred onto 24-well or 6-well culture plates or 10-cm cell culture dishes.

**Luciferase assay.** Cells harboring a subgenomic reporter replicon and HCVpp-infected cells were lysed in passive lysis buffer (Promega). The luciferase activity was determined using a luciferase assay system (Promega) as previously described (46). Secreted GLuc activity was measured in 25- $\mu$ l aliquots of cell culture supernatants by using the BioLux *Gaussia* luciferase assay kit (New England BioLabs) according to the manufacturer's recommended protocol. The luminescence signal was measured on an Infinite M200 microplate reader (Tecan, Männedorf, Switzerland).

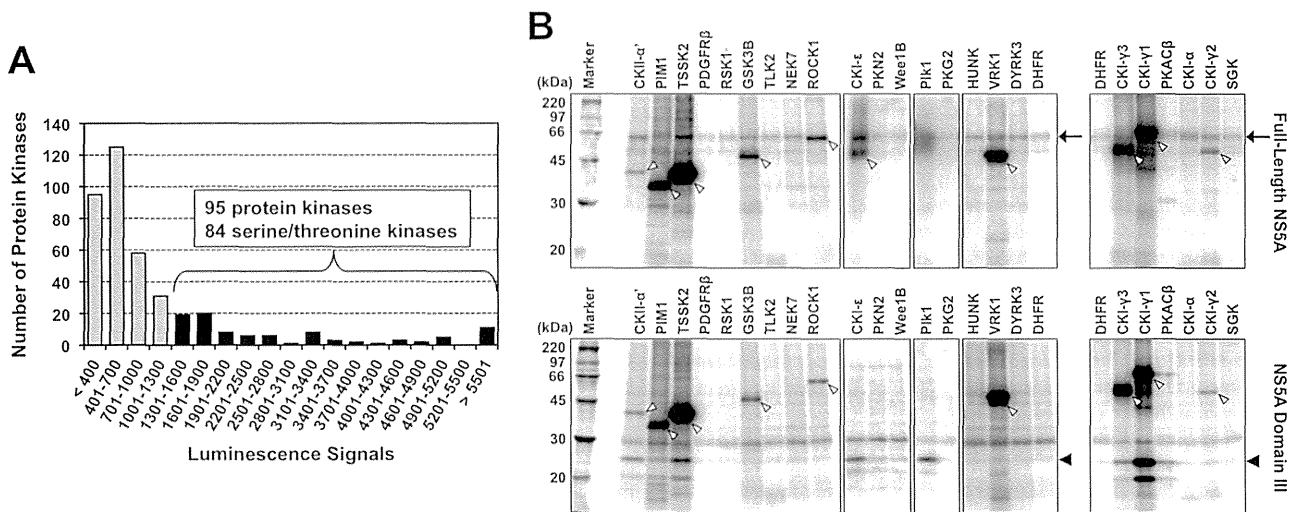
**Quantification of HCV core.** HCV core protein in cell lysates and culture supernatants was quantified by using a highly sensitive enzyme immunoassay as described previously (8).

**RNA extraction and RT-qPCR.** Total cellular RNA was extracted with TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Quantification of cellular gene expression was performed by reverse transcription-quantitative PCR (RT-qPCR) using an Applied Biosystems 7500 fast real-time PCR system (Life Technologies) as described previously (47, 48). Primer/probe sets for qPCR targeting CKI- $\gamma$ 1, CKI- $\gamma$ 2, CKI- $\gamma$ 3, and TSSK2 genes were selected from validated Assays-on-Demand products (Life Technologies).

**Intra- and extracellular infectivity assays.** Intra- and extracellular infectivities of HCVcc were determined as described previously (8). The infectious titers were expressed as focus-forming units (FFU)/ml.

**Cell viability assay.** Cell viability was determined using the CellTiter-Glo luminescent cell viability assay (Promega) according to the manufacturer's instructions.

**Expression of HCV proteins based on vaccinia virus, immunoprecipitation, immunoblotting, and silver staining.** HCV protein expression based on vaccinia virus, immunoprecipitation, and immunoblotting were performed as previously described (8). pJFH1 was transfected into



**FIG 1** Identification of NS5A-associated kinases. (A) AlphaScreen-based protein-protein interaction assay. FLAG-tagged full-length NS5A, and each of 404 biotinylated human protein kinases, which were synthesized in a cell-free protein production system, were mixed with the detection mixture containing anti-FLAG antibody, protein A-conjugated acceptor beads, and streptavidin-coated donor beads in 384-well plates. Luminescence signals from the mixture were detected. Ninety-five protein kinases were identified with luminescence signals of  $\geq 1,300$ , of which 84 were serine/threonine kinases. The assay was performed in duplicate for each sample, and data shown are mean values of duplicate experiments. (B) Exemplary autoradiographic images of NS5A phosphorylated *in vitro*. Purified kinases were mixed with biotinylated NS5A proteins coupled on streptavidin beads in kinase buffer containing  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ . After the reaction, samples were subjected to SDS-PAGE and autoradiography. The arrows, black arrowheads, and white arrowheads indicate phosphorylated full-length NS5A, phosphorylated NS5A domain III, and autophosphorylated kinases, respectively.

cells before infection with vaccinia virus expressing the T7 RNA polymerase. NS5A p58 and p56 protein levels were quantified by densitometry using Image J software. Silver staining of proteins in polyacrylamide gels was carried out using a Silver Stain MS kit (Wako Pure Chemical Industries, Osaka, Japan) in accordance with the manufacturer's protocol.

**Subcellular fractionation analysis.** Cells were suspended in homogenization buffer (10 mM HEPES-NaOH [pH 7.4], 0.25 M sucrose, and 1 mM EDTA) and disrupted by repeated passages through a 25-gauge needle. After low-speed centrifugation, postnuclear supernatants were layered on linear 11-ml iodixanol gradients from 2.5% to 30% and centrifuged at 40,000 rpm for 3 h in an SW41 rotor (Beckman, Fullerton, CA). Thirteen fractions (0.8 ml in each fraction) were collected from the top of the gradient. Each fraction was concentrated by ultrafiltration units with a 10-kDa molecular mass cutoff (Millipore, Bedford, MA), separated by SDS-PAGE, and immunoblotted with antibodies specific for NS5A, calnexin, and GM130.

**Indirect immunofluorescence and microscopy analyses.** Cells incubated for 3 days after infection with HCVcc of JFH-1 were fixed with 4% paraformaldehyde for 15 min at room temperature. After washing with PBS, the cells were permeabilized with 0.05% Triton X-100 in PBS for 15 min at room temperature and subsequently incubated in PBS containing 10% goat serum for 1 h. The cells were then costained with antibodies against core and NS5A, followed by incubation with fluorescent secondary antibodies. Cells were counterstained with Hoechst 33342 (Sigma-Aldrich) to label nuclei and BODIPY 493/503 (Life Technologies) to label lipid droplets and then mounted in Vectashield (Vector Laboratories, Burlingame, CA). Subcellular localization of HCV proteins was observed on a Leica SP2 AOBs laser scanning confocal microscope (Leica, Wetzlar, Germany). Colocalization of NS5A and LDs or core was evaluated quantitatively by using the intensity correlation analysis of the Image J software. To statistically compare degrees of colocalization, we determined the intensity correlation quotient (ICQ) (49). ICQ values are distributed between  $-0.5$  and  $+0.5$ , with a value of  $\sim 0$  reflecting random staining and values between 0 and  $+0.5$  versus values between 0 and  $-0.5$  indicative of dependent versus segregated immunolabeling, respectively.

**Mass spectrometry analysis.** Immunoprecipitated NS5A bands were excised from the gels after silver staining and destained, followed by in gel digestion with trypsin in 50 mM ammonium bicarbonate overnight at  $30^\circ\text{C}$ . Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed on an LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) using Xcalibur (version 2.0.7), coupled to an UltiMate 3000 LC system (Dionex LC Packings, Sunnyvale, CA). The Proteome Discoverer software (version 1.3; Thermo Fisher Scientific) was used to generate peak lists from the raw MS data files. The resulting peak lists were subsequently submitted to a Mascot search engine (version 2.4.1; Matrix Science, London, United Kingdom) and compared against the HCV protein sequences in the NCBI nonredundant protein database (version 20 January 2013; 74,0475 sequences) to identify peptides. The Mascot search parameters were as follows: two missed cleavages permitted in the trypsin digestion; variable modifications including oxidation of methionine, propionamidation of cysteine, and phosphorylation of serine, threonine, and tyrosine; peptide mass tolerance of  $\pm 5$  ppm; fragment mass tolerance of  $\pm 0.5$  Da. A minimum Mascot peptide score of 25 was set for peptide selection.

**Statistical analyses.** Statistical analyses were performed using the Student *t* test unless otherwise noted. A *P* level of  $<0.05$  was considered significant.

## RESULTS

**A kinome-wide screening of human protein kinases for identification of NS5A-associated kinases.** It has been reported that some protein kinases directly or stably associate with HCV NS5A and phosphorylate it *in vitro* (25, 50, 51). To search comprehensively to identify novel NS5A-associated kinases, a kinome-wide screening for interactions of full-length NS5A with human kinases was initially performed. We synthesized 404 human kinases with a wheat germ cell-free protein production system and screened them in terms of their association with NS5A by using a high-throughput assay system based on AlphaScreen technology (Fig. 1A;

TABLE 1 Serine/threonine kinases that exhibited efficient phosphorylation of NS5A

Kinase	LU	Relative kinase activity <sup>a</sup>	
		FL	D3
TSSK2	8,310	4.24	14.94
CKII- $\alpha'$	5,068	2.30	8.20
Plk1	3,230	2.70	44.65
CKI- $\gamma$ 2	2,602	4.31	3.15
CKI- $\gamma$ 1	2,560	NA	138.69
CKI- $\gamma$ 3	2,218	0.23	52.51
CKI- $\epsilon$	2,012	7.95	9.48
PKAC $\beta$	1,854	0.15	69.54
CKI- $\alpha$	1,354	4.66	1.00

<sup>a</sup> LU, light units from the AlphaScreen; FL, full-length NS5A; D3, domain III of NS5A. NA, not assessed due to overlap between purified kinases and NS5A on the gel. The relative kinase activity is the fold increase of the *in vitro* activity of each kinase relative to that of DHFR.

see also Table S1 in the supplemental material). Ninety-five proteins were selected as those that possibly bind to NS5A under the cutoff condition of luminescence signals at  $\geq 1,300$ . Among them, 84 were serine/threonine kinases, and Plk1 and CKII- $\alpha'$ , the catalytic subunit  $\alpha'$  of CKII whose associations with NS5A have been reported (25, 50), were found in the group as signals at 3,230 (Plk1) and 5,068 (CKII- $\alpha'$ ). This suggested that our assay system is highly reliable for screening the NS5A-kinase interaction.

*In vitro* phosphorylation of NS5A by the identified NS5A binding serine/threonine was determined. Each kinase that was synthesized *in vitro* and purified was incubated with either full-length NS5A or domain III of NS5A in the presence of [ $\gamma$ -<sup>32</sup>P]ATP and separated by SDS-PAGE. Phosphorylated NS5A proteins were then visualized by autoradiography (Fig. 1B). The relative kinase activity was determined by normalizing the band intensity of

phosphorylated NS5A with that of NS5A incubated with DHFR, which had no kinase activity and was used as a negative control. Twenty-nine out of 84 serine/threonine kinases were not accurately assessed due to their low levels of expression. As shown in Table 1, among a total of 55 kinases tested (see Table S2 in the supplemental material), nine (CKI- $\alpha$ , CKI- $\gamma$ 1, CKI- $\gamma$ 2, CKI- $\gamma$ 3, CKI- $\epsilon$ , CKII- $\alpha'$ , PKAC $\beta$ , Plk1, and TSSK2) exhibited efficient phosphorylation of NS5A, defined as a more-than-4-fold or 8-fold increase in activity against the full-length NS5A or domain III of NS5A, respectively, compared to the negative control. Consistent with previous reports (9, 25, 50), Plk1 and CKII- $\alpha'$  showed apparent kinase activities against NS5A *in vitro*.

**Identification of an NS5A-associated kinase, CKI- $\alpha$ , that is important for the HCV life cycle.** On the basis of the *in vitro* screenings, nine candidate kinases were further tested as to whether they play roles in the HCV life cycle. We conducted siRNA-based gene silencing of each kinase and assessed its effect on virion production. Huh7.5.1 cells were transfected with siRNAs targeting the kinases and infected with JFH-1 virus at an MOI of 1, 48 h after siRNA transfection. After an additional 72-h incubation, the viral core levels and infectious virus yields in cell culture supernatants were determined. Knockdown efficiencies of the targeted genes at 72 h after JFH-1 infection are shown in Fig. 2A. Efficient knockdown was confirmed either by immunoblotting or RT-qPCR. Figure 2B indicates the effects of gene silencing on virus production (upper panel) and on cell viability by ATP-based luminescence assays (lower panel). An approximately 30-fold reduction in infectious virus yields was observed following knockdown of ApoE, which has been shown to have important roles in HCV assembly and release (52). Among the kinases tested, silencing of CKI- $\alpha$  showed the most profound inhibition of infectious HCV production ( $\sim 40$ -fold) without cytotoxicity. Knock down of CKII- $\alpha'$  and PKAC $\beta$  led to a moderate reduction in infectious virus pro-

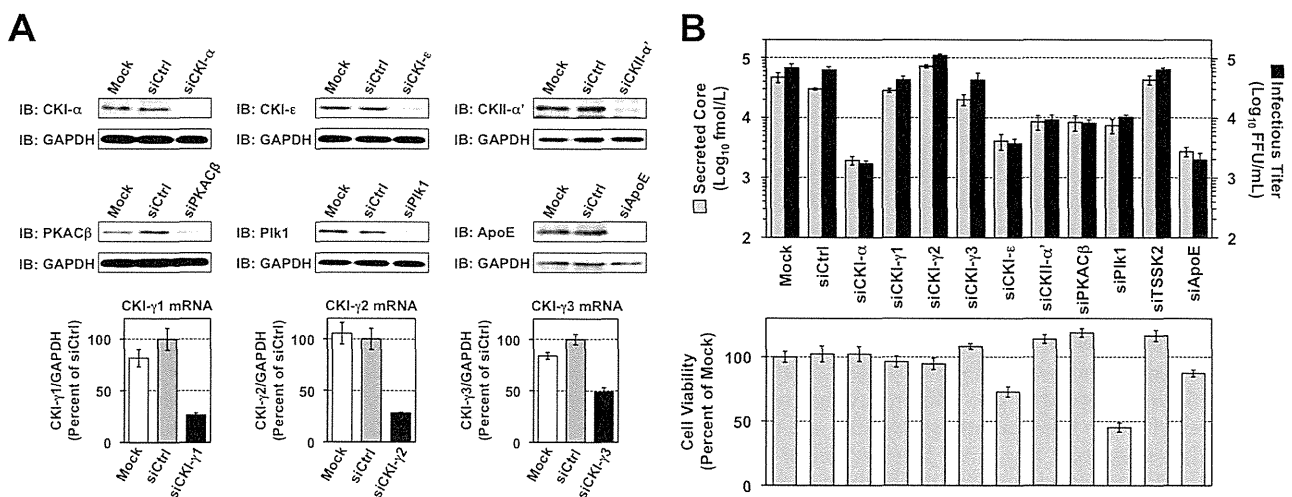
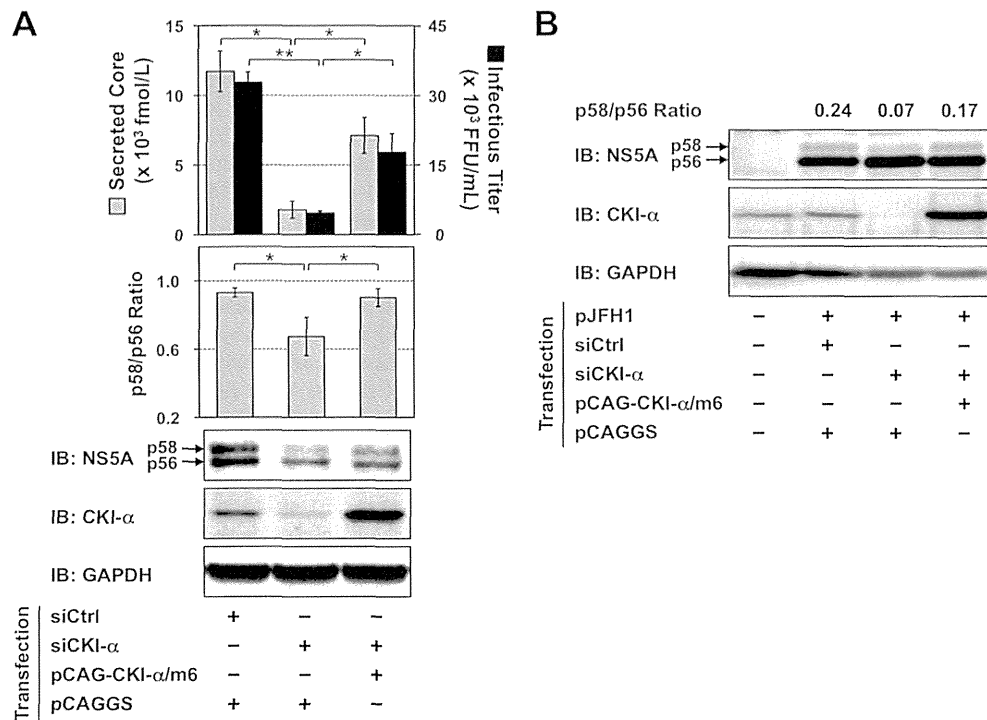


FIG 2 Identification of NS5A-associated kinases involved in the HCV life cycle. (A) siRNA-based gene silencing of NS5A-associated kinases. Huh7.5.1 cells were transfected with siRNAs targeting the indicated genes and were harvested 5 days later for immunoblotting (IB) and RT-qPCR to confirm knockdown efficiencies. mRNA levels of target genes relative to GAPDH mRNA were normalized with values for transfection of control siRNAs (siCtrl), which were set at 100%. Results represent the means  $\pm$  standard deviations from three independent transfections of siRNA. Mock represents transfection without siRNA. (B) Infectious HCV production and cell viability following knockdown of NS5A-associated kinases. Huh7.5.1 cells were infected with JFH-1 virus at an MOI of 1, 2 days after siRNA transfection. Culture supernatants and cells were harvested 3 days later to determine infectious virus yields (upper panel) and cell viability (lower panel), respectively. Cell viability for each transfection was normalized to that for mock transfection (mock), which was set at 100%. Results shown represent the means  $\pm$  standard deviations from three independent transfections of siRNA. Mock, transfection without siRNA.



**FIG 3** Restoration of NS5A hyperphosphorylation and infectious virus yields by ectopic expression of siRNA-resistant CKI- $\alpha$ . (A) Huh7.5.1 cells were cotransfected with the indicated siRNAs and plasmid DNAs. The next day, cells were infected with JFH-1 virus at an MOI of 0.5. Culture supernatants and cells were harvested an additional 3 days later for measurement of virus yields and immunoblotting (IB). The p58/p56 ratios were calculated after quantifying the band intensities of NS5A. Values shown represent the means  $\pm$  standard deviations from three replicate experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . (B) Huh-7 cells were transfected either with CKI- $\alpha$  siRNA (siCKI- $\alpha$ ) or with an irrelevant control siRNA (siCtrl). The next day, cells were retransfected with pJFH1 and pCAG-CKI- $\alpha$ /m6 or empty vector (pCAGGS), followed by infection with vaccinia virus expressing the T7 RNA polymerase at an MOI of 10. NS5A bands were quantified by densitometric analysis, and the p58/p56 ratios were calculated. Immunoblotting images and values shown are representative of two independent experiments.

duction ( $\sim 10$ -fold), which was consistent with previous reports showing that CKII- $\alpha'$  and PKA are involved in virion assembly and viral entry, respectively (9, 53). Knockdown of CKI- $\epsilon$  and Plk1 also resulted in a moderate decrease in virion production ( $\sim 20$ -fold), but they induced moderate to severe cell toxicity as well.

Thus, CKI- $\alpha$ , which phosphorylates NS5A, had the highest impact on HCV production based on *in vitro* comprehensive screenings for protein kinases and a subsequent siRNA-based assay.

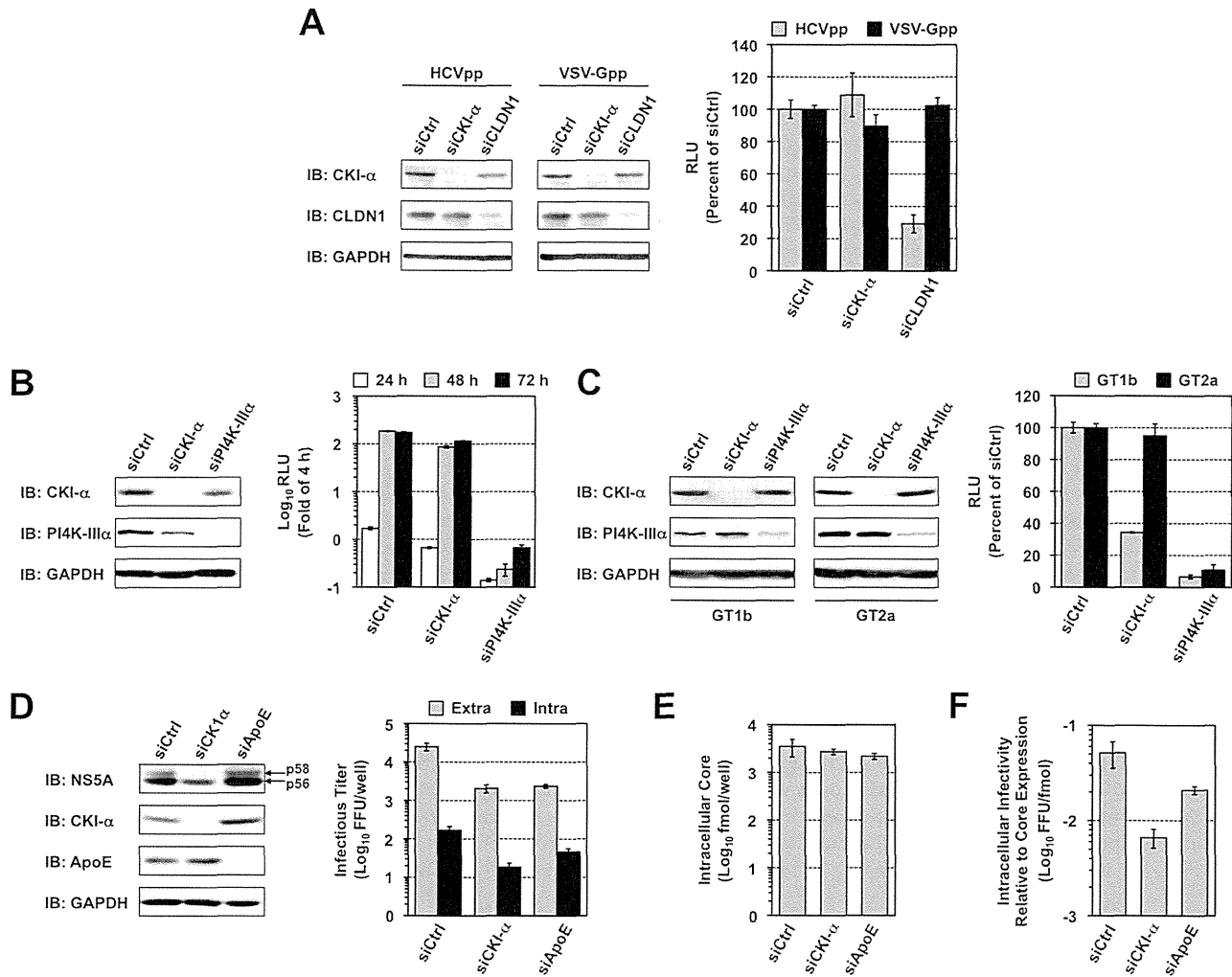
To further demonstrate that impaired virus production results specifically from CKI- $\alpha$  silencing and is not an off-target effect of the siRNA, cells were cotransfected with CKI- $\alpha$  siRNA and a mutated CKI- $\alpha$  expression vector (pCAG-CKI- $\alpha$ /m6) that contained 6 base mismatches within the site targeted by the CKI- $\alpha$  siRNA without a change in amino acids, followed by JFH-1 infection at an MOI of 0.5 on the next day. The cells and culture supernatants were harvested 3 days later for immunoblotting and titrations of virus yields, respectively (Fig. 3A). Transfection of the CKI- $\alpha$  siRNA led to a significant reduction in infectious virus yields and in the p58/p56 ratio of NS5A. Ectopic expression of the siRNA-resistant CKI- $\alpha$ /m6 apparently restored virus yields ( $P < 0.05$ ), as well as the p58/p56 ratio of NS5A ( $P < 0.05$ ). Similar results regarding the p58/p56 ratio were obtained from immunoblot analysis following vaccinia virus-T7 polymerase-mediated expression of HCV proteins (Fig. 3B). These results indicated that impaired virion production and reduced NS5A hyperphosphorylation are

specifically caused by CKI- $\alpha$  silencing. Infectious virus yields showed a closer correlation with the p58/p56 ratio of NS5A than did the expression level of CKI- $\alpha$ , suggesting that the involvement of CKI- $\alpha$  in HCV production is through hyperphosphorylation of NS5A.

**CKI- $\alpha$  is mainly involved in virion assembly in the HCV life cycle.** Although it has been reported that CKI- $\alpha$  plays roles in the regulation of HCV RNA replication through NS5A phosphorylation, experiments addressing its involvement in the viral life cycle have been performed using the subgenomic replicon system (27). To determine the basic role of CKI- $\alpha$  in the production of infectious HCV, the effect of CKI- $\alpha$  silencing on individual steps in the HCV life cycle was assessed.

First, we used an HCVpp system to analyze viral entry. Two days after the siRNA transfection, the cells were infected with HCVpp derived from JFH-1 or VSV-Gpp and were cultured for a further 3 days (Fig. 4A). Consistent with a previous report (54), CLDN1 knockdown inhibited HCVpp entry by approximately 70%, but not VSV-Gpp entry, compared to transfection with negative control siRNA. CKI- $\alpha$  silencing did not affect HCVpp or VSV-Gpp entry, suggesting that CKI- $\alpha$  is not required for HCV entry.

Second, the effect of CKI- $\alpha$  knockdown was tested using an HCV subgenomic replicon system. Three days after the siRNA transfection, the cells were coelectroporated with the identical siRNA and JFH-1 subgenomic luciferase reporter replicon RNA,



**FIG 4** Virion assembly is a primary target of CKI- $\alpha$ . (A) Effect of CKI- $\alpha$  knockdown on viral entry. Huh7.5.1 cells were transfected with the indicated siRNAs. Two days later, cells were infected with HCVpp (gray bars) or VSV-Gpp (black bars) and harvested an additional 3 days later for immunoblotting (IB) and luciferase assays. Values were normalized to the value for transfection with control siRNAs (siCtrl), set at 100%. Values shown represent the means  $\pm$  standard deviations from three independent transfections of siRNA. (B) Transient replication assay for the JFH-1 subgenomic replicon following CKI- $\alpha$  knockdown. Huh-7 cells transfected with the indicated siRNAs were coelectroporated with the identical siRNAs, and JFH-1 subgenomic luciferase reporter replicon RNA, and harvested at the indicated time points for immunoblotting (IB) and luciferase assays. The luciferase activity at each time point was corrected by the luciferase value at 4 h posttransfection to normalize transfection efficiencies. Values shown represent the means  $\pm$  standard deviations from three replicate experiments. (C) Effects of CKI- $\alpha$  knockdown on replication in replicon cell lines derived from genotypes 1b and 2a. Two cell lines harboring an HCV subgenomic luciferase reporter replicon, LucNeo#2 (genotype 1b, GT1b) and SGR-JFH1/LucNeo (genotype 2a, GT2a), were transfected with the indicated siRNAs and harvested 3 days later for immunoblotting (IB) and luciferase assays. Luciferase activities were normalized to the luciferase values for transfection with control siRNA (siCtrl), set at 100%. Values shown represent the means  $\pm$  standard deviations from three independent transfections of siRNA. (D) Effects of CKI- $\alpha$  knockdown on viral assembly and release. Huh7-25 cells transfected with the indicated siRNAs were coelectroporated with the identical siRNAs and JFH-1 RNA. Cells and supernatants were harvested 3 days later for immunoblotting (IB) and titrations of extracellular and intracellular infectious virus by focus-forming unit (FFU) assays. Values represent the means  $\pm$  standard deviations from three replicate experiments. (E) Effect of CKI- $\alpha$  knockdown on the abundance of intracellular core protein. Amounts of core in cells for which results are shown in panel D were measured. Results represent the means  $\pm$  standard deviations from three replicate experiments. (F) Effect of CKI- $\alpha$  knockdown on intracellular infectivity relative to core protein expression. Intracellular infectivity relative to core expression was determined by normalizing the yield of intracellular infectious virus (shown in panel D) with the amount of intracellular core protein shown in panel E. Results represent the means  $\pm$  standard deviations from three replicate experiments.

followed by harvesting at different time points (Fig. 4B). The reporter luciferase activity at each time point was corrected with the luciferase value at 4 h posttransfection to normalize transfection efficiencies. Although efficient knockdown was achieved with siRNAs (Fig. 4B, left panel), CKI- $\alpha$  knockdown led to a marginal but nonnegligible decrease in the luciferase activity over the indi-

cated time period. In contrast, PI4K-III $\alpha$  knockdown, as a positive control, resulted in a marked decrease (>200-fold) in activity (Fig. 4B, right panel). The effect of CKI- $\alpha$  silencing on the replication of the subgenomic replicon was further analyzed by using two cell lines derived from genotype 1b (LucNeo#2) (38, 39) and genotype 2a (SGR-JFH1/LucNeo) (Fig. 4C). Both Huh-7-based

subgenomic replicon cell lines carry a firefly luciferase reporter gene fused to the neomycin phosphotransferase gene. As shown in the right panel of Fig. 4C, knockdown of CKI- $\alpha$  resulted in a marked (~65%) decrease in replication of the genotype 1b replicon but only a slight (~10%) decrease in the genotype 2a replication, although knockdown efficiencies of CKI- $\alpha$  were sufficient and comparable in both cell lines (Fig. 4C, left panels). Our result with the genotype 1b replicon was consistent with a previous report (27). In contrast, the limited impact of CKI- $\alpha$  silencing on the replication of the JFH-1 subgenomic replicon suggests that the RNA replication step may not be a key role for CKI- $\alpha$  in the regulation of HCV JFH-1 production.

Finally, we focused on the late stages of the HCV life cycle and analyzed the involvement of CKI- $\alpha$  in virion assembly and release via a single-cycle virus production assay (55), in which Huh7-25 cells lacking CD81 expression were used. Three days posttransfection with siRNAs, the cells were cotransfected with the identical siRNAs and JFH-1 RNA by electroporation. The cells and culture supernatants were harvested after a further 3 days, and titrations of intra- and extracellular infectious virus were assessed (Fig. 4D to F). Reduced NS5A hyperphosphorylation was observed following CKI- $\alpha$  knockdown, but not following ApoE knockdown or transfection with irrelevant siRNA (Fig. 4D, left panel). Both CKI- $\alpha$  and ApoE knockdown led to an ~10-fold reduction in the yield of extracellular infectious virus compared to the negative control. Approximately a 9-fold reduction was found in the yield of intracellular infectious virus following CKI- $\alpha$  knockdown (Fig. 4D, right panel), indicating that CKI- $\alpha$  is not required for virus release from cells. Despite the marked decrease in intracellular virion yield, CKI- $\alpha$  knockdown resulted in only a 1.3-fold reduction in the abundance of intracellular core protein (Fig. 4E), supporting a limited impact for CKI- $\alpha$  knockdown on viral replication (Fig. 4B). Furthermore, CKI- $\alpha$  silencing led to approximately an 8-fold reduction in the intracellular infectivity relative to core protein expression, which represents the efficiency of viral assembly expressed as the yield of intracellular infectious virus normalized to the amount of intracellular core protein (Fig. 4F). Collectively, these observations suggest that in the HCV life cycle, CKI- $\alpha$  plays a key role most likely in the assembly of infectious viral particles.

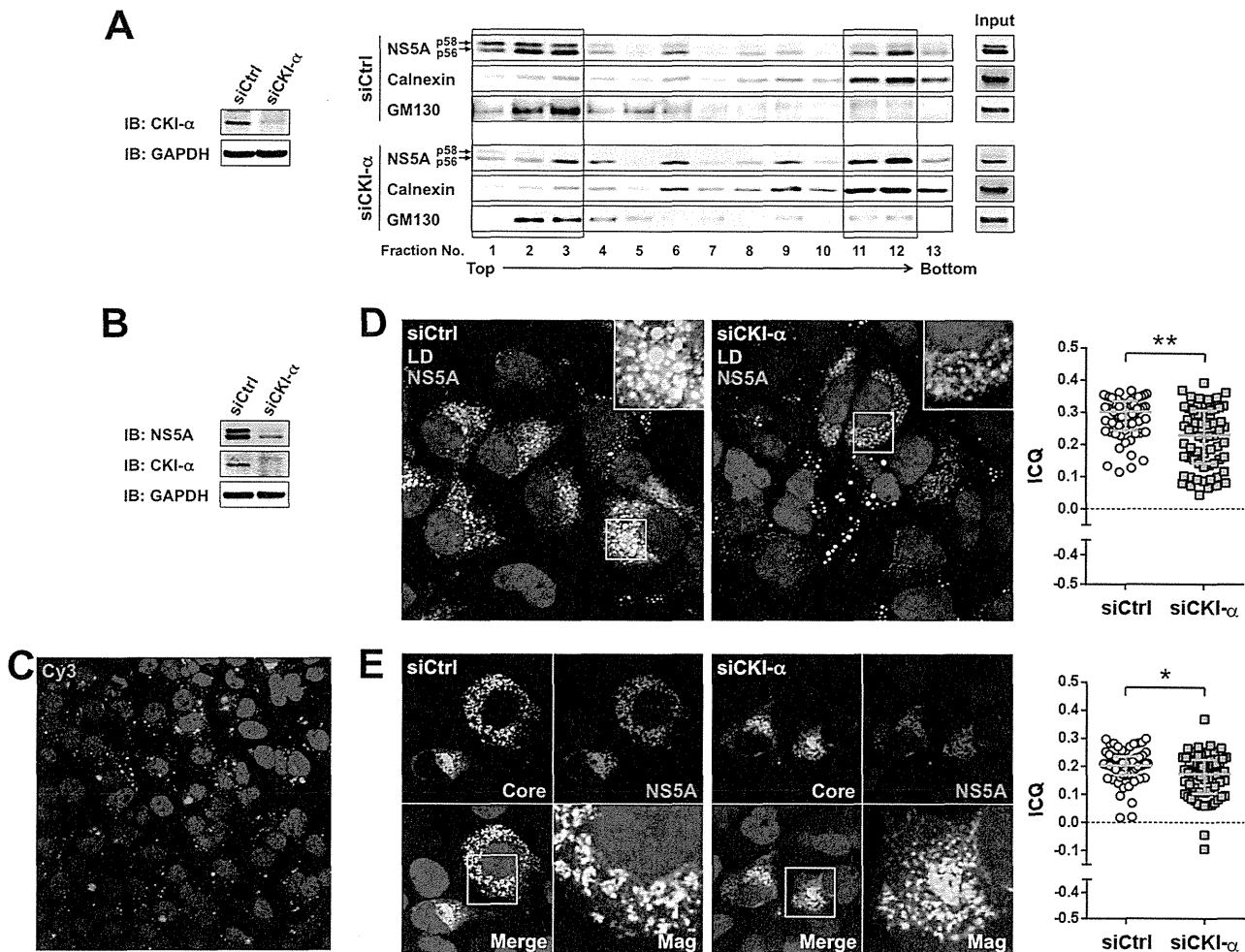
**NS5A hyperphosphorylation mediated by CKI- $\alpha$  possibly contributes to recruitment of NS5A to low-density membrane structures around LDs in infected cells.** It has been demonstrated that recruitment of NS5A to cytoplasmic low-density membrane structures surrounding LDs, and the interaction of NS5A with the core protein at the site, are essential to HCV assembly (7, 8, 56). To gain mechanistic insight into the function of CKI- $\alpha$  in virion assembly, we performed a subcellular fractionation assay and examined whether NS5A phosphorylation by CKI- $\alpha$  contributed to the subcellular localization of NS5A. Lysates of cells transfected with JFH-1 RNA in the presence or absence of CKI- $\alpha$  silencing (Fig. 5A, left panel) were fractionated with 2.5 to 30% iodixanol gradients followed by immunoblotting of the fractions (Fig. 5A, right panels). In control cells (siCtrl), hyperphosphorylated p58 NS5A predominantly resided in low-density fractions, such as fractions 1 to 3, while hypophosphorylated p56 NS5A localized not only in the low-density fractions but also in high-density fractions, such as fractions 11 and 12. In contrast, knockdown of CKI- $\alpha$  (siCKI- $\alpha$ ) decreased the abundance of hyperphosphorylated NS5A and NS5A in the low-density fractions. NS5A levels in the high-density

fractions were not reduced by CKI- $\alpha$  knockdown. These results indicate that CKI- $\alpha$  is involved in the distribution of NS5A in cells as well as in its hyperphosphorylation.

We next assessed whether the intracellular localization of NS5A and its interaction with LDs or the core protein are affected by CKI- $\alpha$  knockdown by using laser-scanning confocal immunofluorescence microscopy. Cells were transfected either with CKI- $\alpha$  siRNA (siCKI- $\alpha$ ) or with an irrelevant control siRNA (siCtrl), followed by infection with HCVcc. Efficient knockdown of CKI- $\alpha$  was confirmed by immunoblotting and was associated with decreased p58 expression (Fig. 5B). The delivery of siRNA into nearly 100% of the cells was observed with Cy3-labeled siRNA (Silencer Cy3-labeled GAPDH siRNA) (Fig. 5C). In siCtrl-transfected cells, NS5A was colocalized or closely associated with LDs. In contrast, its association with LDs was decreased following CKI- $\alpha$  depletion (Fig. 5D) ( $P < 0.0001$  by two-sided Mann-Whitney test). Similarly, NS5A and the core protein were clearly colocalized in control cells, while their colocalization was reduced in CKI- $\alpha$  knockdown cells (Fig. 5E) ( $P = 0.0110$  by two-sided Mann-Whitney test). These microscopy findings suggest that CKI- $\alpha$  and/or CKI- $\alpha$ -mediated hyperphosphorylation of NS5A is involved in the NS5A-core colocalization at or around LDs in HCV-infected cells. Taken together with the results of our subcellular fractionation assay (Fig. 5A), it is likely that CKI- $\alpha$  plays a role in recruiting NS5A to low-density membrane structures around LDs through hyperphosphorylation of NS5A, and it may facilitate the NS5A-core interaction at these sites.

**Identification of potential phospho-acceptor regions for CKI- $\alpha$ .** The above results prompted us to identify the phospho-acceptor sites for CKI- $\alpha$  by using a proteomics approach. Lysates of cells expressing the HCV JFH-1 genome, transfected with either CKI- $\alpha$  siRNA or an irrelevant control siRNA, were immunoprecipitated with an anti-NS5A antibody followed by SDS-PAGE (Fig. 6A). Immunoblotting showed a marked reduction of NS5A p58 following CKI- $\alpha$  knockdown (Fig. 6A, right panel). Silver-stained gel bands of p58 and p56 (Fig. 6A, left panel) were excised and subjected to in-gel digestion, followed by mass spectrometry analysis (Fig. 6B). A total of 629 peptides were identified from both control NS5A (siCtrl) and NS5A with CKI- $\alpha$  knockdown (siCKI- $\alpha$ ) after peptide selection with a Mascot peptide score of  $\geq 25$  (see Table S3 in the supplemental material) and yielded 53% proteome coverage in total (49.4% for control NS5A and 41.8% for NS5A with CKI- $\alpha$  knockdown), as indicated in the upper panel of Fig. 6B (red letters). Peptides corresponding to domain III in NS5A were not obtained in this analysis. We identified three kinds of phosphopeptides (1, GSPPEASSSVSQLAPSLR; 2, AP TTPPR; 3, TVGLSESTISEALQLAIK [Fig. 6B, upper panel, highlighted in green, blue, and yellow, respectively]) (see also Table S3). However, fine mapping of phosphorylation sites was not completely successful in this assay, probably due to the low abundance of immunoprecipitated NS5A. We next assessed which peptide contained the potential phospho-acceptor sites for CKI- $\alpha$  by comparing the frequencies of phosphopeptides identified with and without CKI- $\alpha$  knockdown. As shown in the lower panel of Fig. 6B, the frequency of phosphopeptide 1 relative to the total number of peptide 1 identified was decreased after CKI- $\alpha$  knockdown (from 26.2% to 19.8%). In contrast, the relative frequencies of phosphopeptides 2 and 3 were unaffected or increased by CKI- $\alpha$  knockdown. We noted that the threonine residue in peptide 2 is unlikely to be a consensus phosphorylation site of CKI



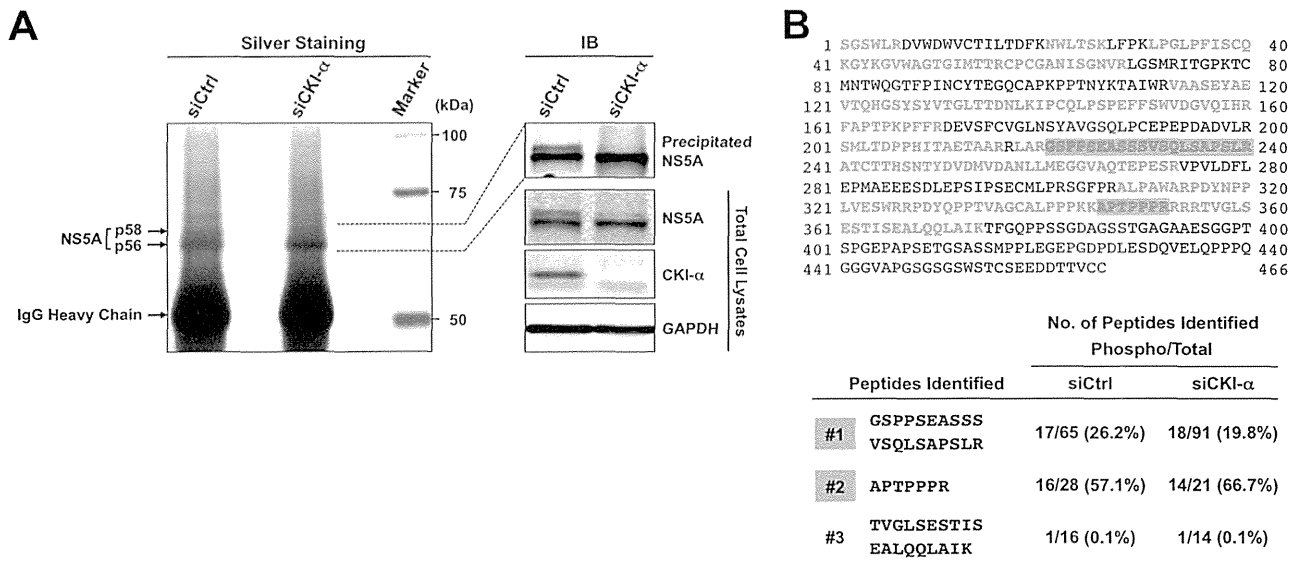


**FIG 5** Effects of CKI- $\alpha$  knockdown on the subcellular localization of NS5A and its interaction with LDs or core protein. (A) Iodixanol density gradient analysis (right). Huh7-25 cells transfected with the indicated siRNAs were coelectroporated with the identical siRNAs and JFH-1 RNA. Cell lysates were prepared 3 days after electroporation and fractionated by iodixanol gradients of 2.5% to 30%. The gradient was collected in 0.8-ml fractions for immunoblotting. Total cell lysates before fractionation were loaded as input controls. Detected bands in fractions 1 to 3 and in fractions 11 and 12 are enclosed by squares. (Left) Immunoblotting (IB) results for CKI- $\alpha$  3 days after electroporation. GAPDH was included as a loading control. (B) Immunoblot (IB) of NS5A and CKI- $\alpha$  3 days after HCVcc infection. GAPDH was included as a loading control. (C) siRNA delivery efficiency. Cy3 fluorescence (red) was observed 3 days after transfection of Silencer Cy3-labeled GAPDH siRNA. Nuclei were counterstained with Hoechst 33342 (blue). (D) Colocalization of NS5A and LDs. Confocal microscopy images show cells transfected with either CKI- $\alpha$  siRNA (siCKI- $\alpha$ ) or an irrelevant control siRNA (siCtrl), followed by infection with JFH-1 virus (left). Cells were fixed with paraformaldehyde 3 days after infection and labeled with an antibody specific for NS5A (red). Cells were counterstained with BODIPY 493/503 (green) to label lipid droplets and with Hoechst 33342 (blue) to label nuclei. Insets represent enlarged views of portions surrounded by squares. Colocalization of NS5A and LDs pixels were assessed quantitatively by intensity correlation analysis using ImageJ software (right). Plots shown represent the ICQ obtained from each of >60 NS5A/LD double-positive cells. Bars indicate the median  $\pm$  interquartile range of the plots. \*\*,  $P < 0.01$  by two-sided Mann-Whitney test. (E) Colocalization of NS5A and core protein. Confocal microscopy images show cells transfected either with CKI- $\alpha$  siRNA (siCKI- $\alpha$ ) or with a control siRNA (siCtrl), followed by infection with JFH-1 virus (left). Fixed cells were labeled with antibodies specific for NS5A (red) and core (green). Nuclei were counterstained with Hoechst 33342 (blue) in the merged images. Mag images represent enlarged views of portions surrounded by squares in the merged images. Colocalization of NS5A and core pixels was assessed quantitatively by intensity correlation analysis using ImageJ software (right). Plots shown represent ICQs obtained from each of >60 NS5A/core double-positive cells. Bars indicate the median  $\pm$  interquartile range. \*,  $P < 0.05$  by two-sided Mann-Whitney test.

(57). Thus, the results suggest that peptide 1 (GSPSPSEASSSVSQL SAPSLR) is the peptide most likely to contain the amino acids phosphorylated by CKI- $\alpha$ .

**S225 and S232 are key residues involved in NS5A hyperphosphorylation and hyperphosphorylation-dependent regulation of infectious virus production.** Peptide 1 identified above contains eight serine residues that are highly conserved among HCV isolates and are clustered within LCS I (Fig. 7A). To identify amino

acids responsible for CKI- $\alpha$ -mediated hyperphosphorylation, we assessed the impacts of alanine or aspartic acid substitutions for these 8 serine residues on NS5A hyperphosphorylation and virus production. An HCV JFH-1 genome with the reporter luciferase, which enabled us to evaluate viral replication by measuring GLuc activity, and a series of its NS5A mutated constructs (Fig. 7A) were generated. Supernatants of cell cultures transfected with the RNA transcripts were harvested at 4, 24, 48, and 72 h posttransfection



**FIG 6** Identification of NS5A phosphopeptides by a phospho-proteome approach. (A) Silver staining and immunoblotting of immunoprecipitated NS5A. Huh-7 cells transfected with the indicated siRNAs were coelectroporated with the identical siRNAs and JFH-1 RNA. Cell lysates were prepared 3 days after electroporation and immunoprecipitated with an anti-NS5A antibody. Immunoprecipitates were subjected to SDS-PAGE, followed by silver staining and immunoblotting (IB). (B) Phosphopeptide mapping of NS5A by LC-MS/MS analysis. p58 and p56 bands of NS5A were excised from the gel and subjected to in-gel digestion, followed by mass spectrometry analysis. Red letters represent the amino acids identified. Three kinds of phosphopeptides identified are highlighted in green (phosphopeptide 1), blue (phosphopeptide 2), and yellow (phosphopeptide 3). The numbers represent amino acid positions within NS5A.

and subjected to the GLuc assay. As shown in the left panel of Fig. 7B, serine-to-alanine substitution at either aa 229 (S229A) or at aa 235 (S235A) resulted in severe reduction in the viral replication. In contrast, the replication capacities of S222A, S228A, S230A, and S238A mutant reporter viruses were comparable to that of the wild type (WT). S225A and S232A mutations led to a slight but nonnegligible reduction in replication compared to WT. The phospho-mimetic aspartic acid substitution for aa 235 (S235D) exhibited a much higher replication capacity (~100-fold) than S235A, indicating that phosphorylation of S235 is required for efficient viral replication. In contrast, the replication capacity of S229D was still more-than-10-fold lower than that of WT, suggesting that introduction of negative charge at this position is not sufficient to enhance viral replication (Fig. 7B, right panel). S225D and S232D mutations restored viral replication capacities and exhibited the same replication phenotype as WT. Interestingly, S222D and S230D resulted in a slight reduction in viral replication compared to S222A and S230A, consistent with previous reports (23, 58) (Fig. 7B, right panel).

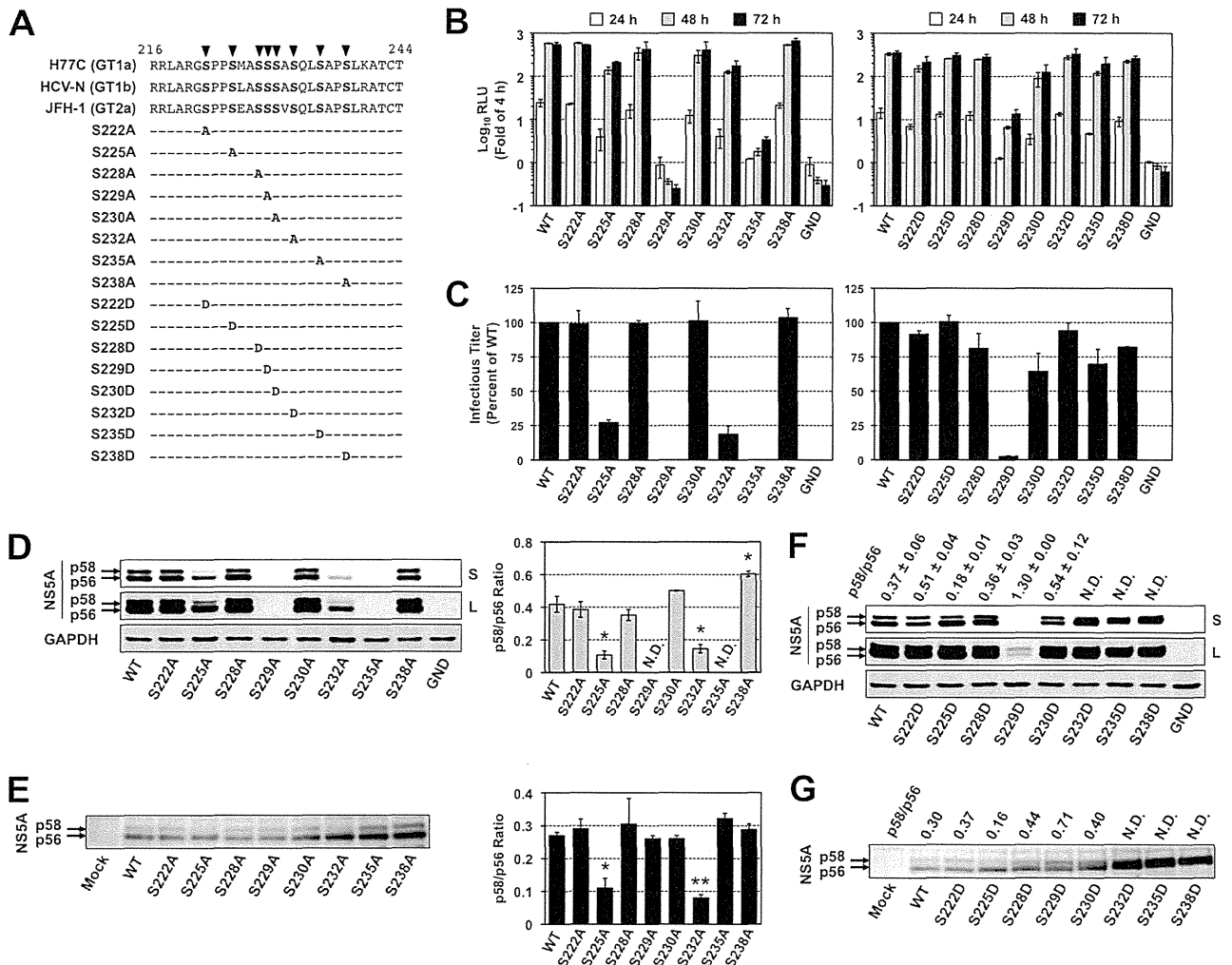
We next evaluated the effects of the NS5A mutations on infectious virus production by titrations of infectious virus in culture supernatants of cells transfected with RNA transcripts of JFH-1 viruses at 72 h posttransfection (Fig. 7C). S225A and S232A mutations resulted in 4- and 5-fold reductions in the virus infectious titer, respectively, compared to WT, while the abilities of S225D and S232D mutants to produce infectious virus were comparable to that of WT. Little or no virus production was observed with S229A, S229D, and S235A mutations, presumably because of their strong negative impacts on viral replication. A slight reduction in virus production observed with S230D and S235D mutations was most likely due to their replication capacities. S222A, S222D,

S228A, S228D, S230A, S238A, and S238D substitutions had no significant effect on virus production (>75% of the WT level).

To determine the effect of the NS5A mutations on NS5A hyperphosphorylation, cells expressing JFH-1 viruses were subjected to immunoblotting, and the p58/p56 ratio of NS5A was estimated (Fig. 7D). The hyperphosphorylated p58 band of NS5A was clearly detected in cells transfected with WT and S222A, S228A, S230A, and S238A mutants, which had mean p58/p56 ratios of 0.42, 0.38, 0.35, 0.50, and 0.60, respectively. These p58/p56 ratios were reproducible in multiple repeated experiments but much lower than the p58/p56 ratios (e.g., 0.93 in siCtrl-transfected cells) in Fig. 3A. This difference may be attributed to the difference in the way by which HCV was introduced into cells (virus infection in Fig. 3A and transfection of viral genome in Fig. 7D and F). The p58 levels were significantly reduced in cells transfected with S225A or S232A mutants, which had mean p58/p56 ratios of 0.11 and 0.15, respectively (Fig. 7D). Since the p58/p56 ratios of the S229A and S235A mutants were not determined due to low levels of NS5A expression, we reevaluated the p58/p56 ratio of each viral mutant by using a vaccinia virus-T7 polymerase-mediated protein expression system (Fig. 7E, left panel). Cells transfected with pJFH1 or a series of its NS5A mutants were infected with vaccinia virus expressing the T7 RNA polymerase and harvested for immunoblotting. Similar to the results shown in Fig. 7D, the p58/p56 ratios of S225A and S232A mutants were significantly reduced, while S229A and S235A mutations had no effects (Fig. 7E, right panel). The hyperphosphorylated band of NS5A was observed in cells transfected with the S222D, S225D, S228D, S229D, or S230D mutant in both experimental settings. Interestingly, the S232D, S235D, and S238D mutations resulted in a slight retardation of p56 mobility (Fig. 7F and G), consistent with previous reports (58, 59).

Collectively, S225 and S232 are key residues involved in





**FIG 7** Effects of mutations at potential CKI- $\alpha$  phosphorylation sites on NS5A hyperphosphorylation, viral replication, and infectious virion production. (A) Highly conserved serine residues located within the LCS I region of NS5A and the sequences of NS5A mutants used. Arrowheads represent putative phosphorylation sites replaced with alanine or aspartic acid. The numbers represent amino acid positions within NS5A. (B) Viral replication. Huh-7 cells were transfected with the indicated JFH-1-based GLuc reporter constructs, including the WT and its replication-defective mutant (GND). Culture supernatants were harvested at the indicated time points for luciferase assays. The GLuc activity at each time point was normalized with the activity at 4 h posttransfection, and the fold changes are shown. Results represent the means  $\pm$  standard deviations from three independent experiments, each performed in triplicate. (C) Infectious virion production. Huh-7 cells were transfected with the indicated JFH-1 viral RNAs, including the WT and its replication-defective mutant (GND). Culture supernatants were harvested 3 days (72 h) later for titrations of infectious virus in a focus-forming unit (FFU) assay. The infectious virus yield of each NS5A mutant was normalized with that of JFH-1 WT, which was set at 100%. Results represent the means  $\pm$  standard deviations from multiple independent experiments, each performed at least in triplicate. (D) Immunoblot of NS5A in lysates of cells transfected with JFH-1 viral RNAs carrying the indicated serine-to-alanine mutations (left). GAPDH is a loading control. The p58/p56 ratio of each virus was determined by carrying out densitometric analysis of NS5A bands (right). Results shown represent the means  $\pm$  standard deviations from multiple independent experiments. \*,  $P < 0.05$ , compared to WT. N.D., not determined, due to low levels of expression. (E) Vaccinia virus-T7 polymerase-mediated expression of NS5A in cells transfected with pJFH1 carrying the indicated serine-to-alanine mutations (left). NS5A bands were quantified by densitometric analysis, and p58/p56 ratios were calculated (right). Data shown represent the means  $\pm$  standard deviations from multiple independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (versus WT). (F) Immunoblot of NS5A in lysates of cells transfected with JFH-1 viral RNA carrying the indicated serine-to-aspartic acid mutation. GAPDH was included as a loading control. The p58/p56 ratio of each virus was determined by carrying out densitometric analysis of NS5A bands. Results shown represent the means  $\pm$  standard deviations from multiple independent experiments. N.D., not determined, due to the poor separation of p58 and p56 bands. (G) Vaccinia virus-T7 polymerase-mediated expression of NS5A in cells transfected with pJFH1 carrying the indicated serine-to-aspartic acid mutation. NS5A bands were quantified by densitometric analysis, and p58/p56 ratios were calculated. Data shown are representative of two independent experiments. N.D., not determined, due to the poor separation of p58 and p56 bands. S and L in panels D and F represent short exposure and long exposure, respectively.

NS5A hyperphosphorylation and hyperphosphorylation-dependent regulation of infectious virus production. In addition, S225A and S232A reproduced the viral phenotype following CKI- $\alpha$  knockdown more precisely than the other serine mu-

tants within the peptide 1 region. It is most likely that S225 and S232 of NS5A are important for CKI- $\alpha$ -mediated hyperphosphorylation, which is involved in the robust production of infectious HCV.

## DISCUSSION

Phosphorylation at serine and threonine residues in HCV NS5A is critical for regulation of the viral life cycle, including genome replication and infectious virus assembly (8, 9, 14, 16–18, 59–61). Several serine/threonine protein kinases have been identified as enzymes that potentially phosphorylate NS5A (9, 25, 26, 28, 50, 51). To our knowledge, however, this study is the first to identify through a kinome-wide screening protein kinases that interact with and phosphorylate NS5A. The *in vitro* AlphaScreen and phosphorylation assays, followed by RNAi screening on the HCVcc system, identified CKI- $\alpha$  as a major NS5A-associated kinase involved in NS5A hyperphosphorylation and the production of infectious virus.

In a previous study, CKI- $\alpha$  was reported to be involved in the replication of the subgenomic replicon derived from genotype 1b, with evidence that attenuation of CKI- $\alpha$  expression inhibited viral RNA replication up to 60% 5 days after the CKI- $\alpha$  knockdown (27). However, our study with the HCVcc system, as well as detailed analyses dissecting individual steps in the HCV life cycle, revealed that virion assembly is more affected by CKI- $\alpha$  silencing than is viral genome replication. It is highly likely that the CKI- $\alpha$ -mediated hyperphosphorylation of NS5A plays a role in recruiting NS5A to low-density membrane structures around LDs, leading to the acceleration of the early step(s) of virus particle formation. Mutagenesis analyses of putative CKI- $\alpha$  phosphorylation sites identified by a phospho-proteomic approach demonstrated that serine-to-alanine substitution at aa 225 or aa 232 in NS5A did to some extent reproduce the viral phenotype following CKI- $\alpha$  knockdown, indicating that S225 and S232 may be key residues for CKI- $\alpha$ -mediated NS5A hyperphosphorylation and regulation of virion assembly.

It is commonly held that HCV replication is regulated through the tight and delicate control of the ratio between p58 and p56 levels. Adaptive mutations or kinase inhibitors, which reduce NS5A hyperphosphorylation, enhance the HCV RNA replication of genotype 1 isolates, possibly by modulating its interaction with the host vesicle-associated membrane protein-associated protein subtype A (VAP-A), which is an essential factor for HCV replication (17–19, 24). In contrast, reduction of NS5A hyperphosphorylation by RNAi targeting protein kinases results in inhibition of the replication of genotype 1 adaptive replicons, indicating a role for p58 in efficient viral replication (25, 27). Impaired RNA replication resulting from reduced NS5A hyperphosphorylation has also been reported in the case of JFH-1 or JFH-1-based recombinant virus (25, 58–60). Consistent with a previous report (27), we found that CKI- $\alpha$  depletion inhibited the replication of the genotype 1b subgenomic replicon LucNeo#2, which carries the adaptive S2204R mutation in NS5A (38, 39) (Fig. 4C). Our transient-replication assay with the JFH-1 subgenomic replicon showed a slight but significant reduction in replication following CKI- $\alpha$  depletion (Fig. 4B). However, CKI- $\alpha$  silencing did not affect RNA replication in SGR-JFH1/LucNeo cells, where the JFH-1 subgenomic replicon stably replicates (Fig. 4C). Thus, the involvement of CKI- $\alpha$  in HCV RNA replication might be genotype or isolate dependent. We observed a difference in the replication capacity following CKI- $\alpha$  knockdown between transient and stable replication of JFH-1 replicons (Fig. 4B and C). A moderate reduction of replication was also detected when the JFH-1 genome carrying the S225A or S232A mutation was transiently transfected

(Fig. 7B). One may infer that CKI- $\alpha$  is involved in the initiation of viral RNA replication rather than in its maintenance.

Our intra- and extracellular infectivity assays following CKI- $\alpha$  depletion suggested that CKI- $\alpha$  primarily targets virion assembly in the HCV life cycle (Fig. 4D to F), although a slight but nonnegligible negative effect of CKI- $\alpha$  knockdown on viral replication was observed (Fig. 4B). It is accepted that the assembly of HCV particles requires recruitment of NS proteins, including NS5A as well as structural proteins, to cytoplasmic membrane structures around LDs, leading to an interaction between NS5A and the core, which is important for efficient encapsidation of the viral genome (7, 8, 56). To understand how CKI- $\alpha$  is involved in virion assembly, we performed a subcellular fractionation assay and immunofluorescence confocal microscopy. Our subcellular fractionation assay clearly showed that hyperphosphorylated NS5A, p58, is mainly localized in low-density membrane fractions, while hypophosphorylated NS5A, p56, prefers high-density fractions. NS5A abundance in lighter fractions was decreased following CKI- $\alpha$  depletion (Fig. 5A). These results are supported by microscopic analyses that demonstrated that CKI- $\alpha$  silencing reduced colocalization of NS5A with LDs and the core (Fig. 5D and E). We tried to confirm the interaction of NS5A and core in HCVcc-infected cells that had been transfected with CKI- $\alpha$  siRNA or irrelevant siRNA. However, the interaction was not observed under this condition, presumably because immunoprecipitated NS5A and/or core was not abundant enough to assess their coimmunoprecipitation in siRNA-transfected cells. Alternatively, we assessed the interaction in Huh-7 cells coexpressing core and NS5A, although no p58 form (no functional NS5A) was detected in this setting. We clearly detected the interaction of NS5A and core in this experiment and found that CKI- $\alpha$  depletion had no significant effect on this interaction (data not shown). Taken together with the results of confocal microscopy (Fig. 5E), this finding might suggest that both (i) phosphorylation of serine residues in the C terminus of NS5A, which is involved in the generation of basally phosphorylated NS5A, as shown previously (8), and (ii) CKI- $\alpha$ -mediated hyperphosphorylation, in which serine residues in the LCS I region are mainly involved, are important for an efficient interaction between NS5A and core in HCV replicating cells. Collectively, CKI- $\alpha$ -mediated hyperphosphorylation of NS5A may contribute to an increase in the local concentration of NS5A at low-density membrane structures around LDs rather than in facilitating the physical interaction of NS5A and core. The relationship between the phosphorylation status of NS5A and its localization on cellular membranes has been previously reported. Miyanari et al. showed that mutated NS5A expressed from JFH-1 variants (JFH1<sup>AAA99</sup> and JFH1<sup>AAA102</sup> in their report), whose p58/p56 ratios were lower than that of wild-type virus, was not recruited to LDs (56). Qiu et al. fractionated lysates from replicon cells and demonstrated that a substantial amount of hyperphosphorylated NS5A was detected in lighter fractions. Treatment with an NS5A inhibitor, BMS-790052, reduced hyperphosphorylation of NS5A and concomitantly decreased the overall amount of NS5A in low-density membrane fractions (62). These findings raise questions about the regulatory mechanism(s) of the subcellular localization of NS5A, especially at low-density membrane structures. The above-mentioned NS5A mutants, JFH1<sup>AAA99</sup> and JFH1<sup>AAA102</sup>, have triple alanine substitutions for the APK sequence at aa 99 to 101 and the PPT sequence at aa 102 to 104 in NS5A, respectively, but neither is likely to be a CKI recognition site. In addition, the NS5A inhibitor

has no inhibitory effect on CKI activity (62). Thus, it appears that two or more kinds of serine/threonine-specific protein kinases, including CKI- $\alpha$ , participate in NS5A phosphorylation that is important for the regulation of its subcellular distribution. It is tempting to speculate that host factors involved in membrane trafficking or lipogenesis possibly interact with NS5A in a phosphorylation-dependent manner and facilitate recruitment of NS5A to low-density membrane structures surrounding LDs. Although further study is needed to validate this speculation, NS5A-interacting factors, such as VAP-A and diacylglycerol acyltransferase-1 (19, 63, 64), might be candidates for involvement in the regulation of this process.

To identify potential phospho-acceptor sites for CKI- $\alpha$ , a phospho-proteome analysis was carried out with NS5A isolated from HCVcc-infected cell lines with and without CKI- $\alpha$  knockdown (Fig. 6). Three kinds of phosphopeptides were identified out of a total of 629 peptides after peptide selection, although fine mapping of the phosphorylation sites was not completely successful. Among them, only the relative frequency of phosphopeptide 1 (GSPPEASSSVSLSAPSLR) was decreased after CKI- $\alpha$  knockdown, suggesting the possibility that peptide 1 contains amino acids phosphorylated by CKI- $\alpha$ . Serine residues in peptide 1, which are well conserved among HCV genotypes, matched the consensus sequences for CKI- $\alpha$ -mediated phosphorylation (57). In contrast, a threonine residue in peptide 2 is not conserved and does not match the consensus sequences for phosphorylation by CKI- $\alpha$ . The frequency of phosphopeptide 3 was unchanged with and without CKI- $\alpha$  knockdown (0.1% versus 0.1%). Further mutagenesis analyses targeting the peptide 1 region suggested that S225 and S232 are possible CKI- $\alpha$  phosphorylation sites involved in NS5A hyperphosphorylation and infectious virus production, because alanine substitution for either of these serine residues reproduced the viral phenotype after CKI- $\alpha$  knockdown more accurately than the other mutants within the peptide 1 region (Fig. 7B to E). The S229A and S235A mutations severely impaired viral replication, suggesting that phosphorylation at S229 and S235 is essential for efficient viral replication (Fig. 7B). However, the HCV protein expression assay using vaccinia virus expressing the T7 RNA polymerase revealed that phosphorylation of these residues is not involved in NS5A hyperphosphorylation (Fig. 7E). In the case of genotype 1 isolates, NS5A mutations that reduce hyperphosphorylation enhanced viral RNA replication (17, 18). However, this was not the case for the S225A or S232A mutation in genotype 2a. In addition, S229A and S235A mutations in genotype 1b (Con1) markedly enhance viral replication (18), but the same mutations are lethal in genotype 2a. S232 has been shown to be a potential phosphorylation site for CKI- $\alpha$  by a peptide-based kinase assay *in vitro* (28). However, the present study is the first to demonstrate the significance of phosphorylation at S225 and S232 in infectious virus production. The sequence coverage of NS5A by our mass spectrometry analysis was less than 60% and was especially low in domain III of NS5A (Fig. 6B). We cannot exclude the possibility of the presence of additional CKI- $\alpha$  phosphorylation sites in NS5A.

Recently, two cellular kinases involved in the regulation of NS5A phosphorylation have been identified. PI4K-III $\alpha$  is essential for HCV replication (65–70) and catalyzes the synthesis of phosphatidylinositol 4-phosphate accumulating in HCV replicating cells through its enzymatic activation resulting from an interaction with NS5A (71, 72). PI4K-III $\alpha$  directly interacts with the

C-terminal end of NS5A domain I, and NS5A–PI4K-III $\alpha$  binding is essential for viral replication. Its depletion resulted in a relative increase of p58 abundance, while overexpression of enzymatically active PI4K-III $\alpha$  increased the relative abundance of p56 (73). Plk1 has been shown to play a role in viral replication through hyperphosphorylation of NS5A (25). Plk1 was coimmunoprecipitated with NS5A, and knockdown of Plk1 or treatment with a specific inhibitor decreased both NS5A hyperphosphorylation and HCV replication. Since the recognition sites for CKI- $\alpha$ , PI4K-III $\alpha$ , and Plk1 have been assumed to be spatially close to each other (25, 28, 73), it is interesting to analyze their interactive actions in regard to regulation of NS5A phosphorylation.

The exquisite balance between the two different phosphorylated forms of NS5A has been proposed to regulate the HCV life cycle; basally phosphorylated p56 abundance is hypothetically involved in viral RNA replication, and hyperphosphorylated p58 is required for virion assembly (9, 17, 18, 73, 74). However, this hypothesis has not yet been fully proven. Our results here provide strong evidence supporting the involvement of NS5A hyperphosphorylation in viral assembly and that of CKI- $\alpha$  in mediating this process. These results not only contribute to a better understanding of the regulatory details of the HCV life cycle but also illuminate targets for potential antiviral strategies.

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# Intercontinental Dispersal of HIV-1 Subtype B Associated with Transmission among Men Who Have Sex with Men in Japan

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## ABSTRACT

Transmission clusters of HIV-1 subtype B uniquely associated with the epidemic among men who have sex with men (MSM) in East Asia have recently been identified. Using the Los Alamos HIV sequence database and the UK HIV drug resistance database, we explored possible links between HIV MSM epidemics in East Asia and the rest of the world by using phylogenetic and molecular clock analyses. We found that JP.MSM.B-1, a subtype B MSM variant that accounts for approximately one-third of the infections among Japanese MSM, was detected worldwide, in the United Kingdom ( $n = 13$ ), mainland China ( $n = 3$ ), the United States, Germany, Canada, and Taiwan ( $n = 1$  each). Interestingly, 10 United Kingdom samples plus two from Germany and the United States formed a distinct monophyletic subgroup within JP.MSM.B-1. The estimated divergence times of JP.MSM.B-1 and the latter subgroup were  $\sim 1989$  and  $\sim 1999$ , respectively. These dates suggest that JP.MSM.B-1 was circulating for many years in Japan among MSM before disseminating to other countries, most likely through global MSM networks. A significant number of other Asian MSM HIV lineages were also detected in the UK HIV drug resistance database. Our study provides insight into the regional and global dispersal of Asian MSM HIV lineages. Further study of these strains is warranted to elucidate viral migration and the interrelationship of HIV epidemics on a global scale.

## IMPORTANCE

We previously identified several transmission clusters of HIV-1 subtype B uniquely associated with the epidemic among men who have sex with men (MSM) in East Asia. Using the Los Alamos HIV sequence database and the UK HIV drug resistance database, we explored the possible interplay of HIV MSM epidemics in the different geographic regions and found previously unrecognized interrelationships among the HIV-1 epidemics in East Asia, the United Kingdom, and the rest of the world. Our study provides insight into the regional and global dispersal of Asian MSM HIV lineages and highlights the importance of strengthening HIV monitoring efforts and the need for implementing effective control measures to reduce HIV transmission on a global scale.

HIV-1 transmission and infection among men who have sex with men (MSM) continues worldwide (1, 2). While the HIV-1 incidence in the general adult population is decreasing in most countries, the MSM risk group continues to be disproportionately affected by HIV-1 infection. MSM were approximately 19 times more likely to become infected than average individuals in the global general population in 2000 to 2006 (3). East Asia is no exception to this trend, and HIV-1 epidemics among MSM in China have expanded rapidly (4). According to the Chinese Ministry of Health, the proportion of MSM among newly identified HIV cases in China has risen from 0.3% in 1985 to 2005 to 8.6% in 2009 and to 17.5% as of October 2012 (4, 5). Further, a large cross-sectional study of MSM in 61 Chinese cities in 2008 found that the average HIV prevalence in this group reached 4.9% (6). The highest HIV prevalences among MSM were found in cities in central-western and southwestern China: 8.5% in Chongqing, 9.1% in Chengdu (reviewed in reference 1), and 10.8% in Kunming (7, 8).

While the HIV incidence remains very low in Japan compared with that in many other countries, Japan is also experiencing a similar but smaller increase in HIV-1 infections among MSM (1). By the end of 2012, a cumulative total of 20,036 HIV/AIDS cases had been reported in Japan, of which approximately one-third

were associated with MSM (Ministry of Health, Japan; <http://api-net.jfap.or.jp/status/>). The number of newly reported HIV cases among MSM has more than doubled during the past decade, from 305 in 2002 to 655 in 2012 (9). Despite extensive HIV prevention and education programs targeted at MSM, many MSM in Japan appear to engage in high-risk sexual behaviors, predisposing them to HIV-1 infection and transmission (Ministry of Health, Japan; <http://api-net.jfap.or.jp/status/>).

In a recent survey of HIV-1 strains circulating among newly diagnosed infected patients in various risk groups in the Tokyo-Kanagawa metropolitan area of Japan in 2004 to 2011, it was found that Japanese MSM were infected predominantly with subtype B HIV-1

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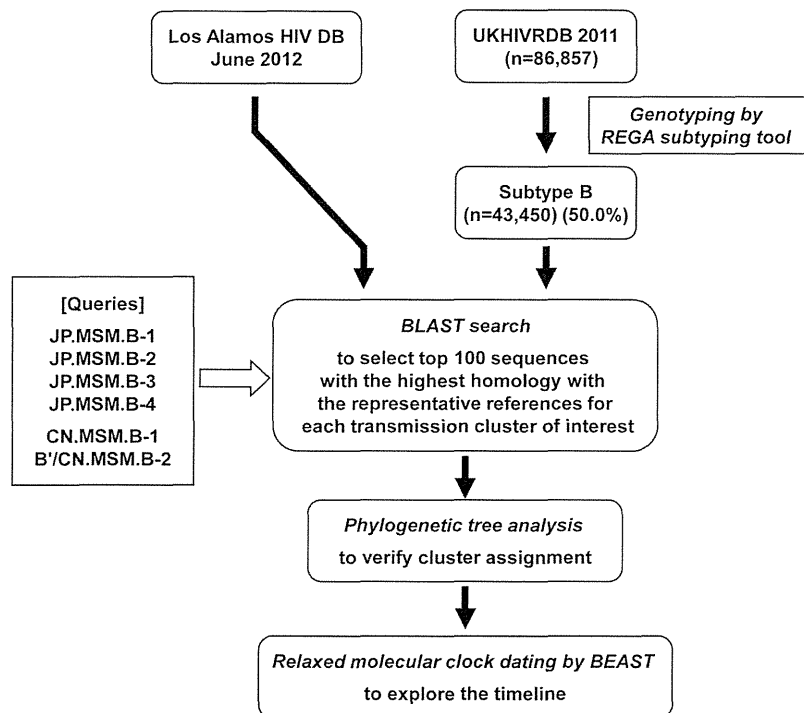
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**FIG 1** Flow chart of the procedure used to identify region- and risk factor-specific HIV-1 subtype B variants. To identify HIV sequences of interest, a BLAST search (18) against the Los Alamos HIVDB and the UKHIVRDB was undertaken, by using as queries the  $\sim 1.1$ -kb pro-RT nucleotide sequences of key subtype B MSM variants in Japan and China (listed in the leftmost box; see text). The 100 nucleotide sequences with the highest BLAST score to each query sequence were retained. We then verified the phylogenetic relationships of the database sequences to confirm possible matches to the East Asian HIV lineages of interest.

strains of U.S.-European origin (96.9%). Detailed phylogenetic analysis revealed that approximately one-third of these subtype B strains formed a single monophyletic cluster (designated JP.MSM.B-1) (10), followed by a number of minor subtype B variants, each accounting for less than 1 to 2% of MSM infections, termed JP.MSM.B-2 through JP.MSM.B-4 (10). While CRF01\_AE infections are uncommon among Japanese MSM (1.9%), 30 to 50% of these cases were found to be caused by CRF01\_AE variants (11, 12) uniquely associated with transmission among MSM in China (designated CN.MSM.01-1) (10). Virus movement between the two countries appears to be bidirectional, as we also observed that the largest Japanese MSM subtype B variant (JP.MSM.B-1) was detected among MSM in China (10). These findings suggest the presence of previously unknown interactions among the HIV epidemics in China, Japan, and the rest of the world.

Phylogenetic and phylogeographic analyses of HIV genome sequences have revealed the routes by which the virus has been disseminated in many different regions, including the Americas (13), Europe (14), and Africa (15, 16). However, despite the epidemiological importance of HIV-1 subtype B among MSM in Asia, including in Japan and other countries, the global profile of these transmission clusters and their epidemic history remains largely unknown.

In this study, we systematically searched for East Asian subtype B variants by using both the Los Alamos HIV sequence database (Los Alamos HIVDB) and the UK HIV drug resistance database (UKHIVRDB) in order to explore possible linkages between the epidemics in East Asia and those in the rest of the world. We found

that HIV-1 subtype B variants associated with transmission among MSM in East Asia have dispersed globally, giving rise to a new chain of infection in the United Kingdom through a founder effect.

## MATERIALS AND METHODS

**HIV sequence databases.** HIV sequences representing subtype B protease and partial reverse transcriptase coding regions (pro-RT region,  $\sim 1.1$  kb long) were obtained from (i) the Los Alamos HIVDB (<http://www.hiv.lanl.gov>; accessed in June 2012), (ii) the UKHIVRDB (<http://www.hivrd.org>; accessed on 20 March 2013, containing sequences up to the end of 2010) of strains from treatment-naive patients (17), and (iii) our in-house HIV database comprising sequences of strains from study participants residing in the Tokyo-Kanagawa metropolitan area of Japan (designated the JP-TK HIVDB) (10). Ethics approval for the use of anonymized data in the UKHIVRDB was given by the London Multicenter Research Ethics Committee. This study project was approved by the Ethics Committee of the National Institute of Infectious Diseases in Japan.

**Sample collation and phylogenetic analysis.** We searched the above three HIV databases for the global occurrence of subtype B variants associated with transmission among MSM in East Asia. Figure 1 shows a flow chart of the phylogenetically informed searching procedure undertaken in this study. To identify HIV sequences of interest, we first performed a BLAST (Basic Local Alignment Search Tool) search (18) by using the  $\sim 1.1$ -kb pro-RT nucleotide sequences of key subtype B MSM variants in Japan and China as queries (this genome region typically corresponds to nucleotide positions 2253 to 3392 of the HXB2 reference strain). The query sequences used were as follows: 06JP.Y306 (accession number AB735881) for the JP.MSM.B-1 lineage, 09JP.Y449 (AB735865) for the JP.MSM.B-2 lineage, 06JP.GM2086 (AB864057) for the JP.MSM.B-3 lin-



TABLE 1 Summary of the presence of regional HIV-1 subtype B variants from East Asia (Japan and China) in the databases searched<sup>a</sup>

Transmission cluster, country of origin, risk group(s), and database searched	No. of hits (country of origin)										n	Total	
	1997-2003	2004	2005	2006	2007	2008	2009	2010	2011				
JP.MSM.B-1, Japan, MSM													
Los Alamos HIVDB		1 (DE)	1 (US)	2 (CN)	1 (CN)	1 (CA)	1 (TW)				7	20	
UKHIVRDB				1	4		2	6			13		
JP-TK HIVDB											0		
JP.MSM.B-2, Japan, MSM													
Los Alamos HIVDB											0	3	
UKHIVRDB			1								1		
JP-TK HIVDB							2 (CN)				2		
JP.MSM.B-3, Japan, MSM													
Los Alamos HIVDB											0	2	
UKHIVRDB									1		1		
JP-TK HIVDB			1 (UK)								1		
JP.MSM.B-4, Japan, MSM													
Los Alamos HIVDB							1 (TW)				1	2	
UKHIVRDB				1							1		
JP-TK HIVDB											0		
CN.MSM.B-1, China, MSM													
Los Alamos HIVDB											NA <sup>b</sup>	1	
UKHIVRDB			1								1		
JP-TK HIVDB											0		
B'/CN.MSM.B-2, China, IDU/FPD/heterosexual/MSM													
Los Alamos HIVDB											NA	20	
UKHIVRDB			1	3	6	4	4	1	1		20		
JP-TK HIVDB											0		
Total	0	1	5	7	11	5	10	7	2		48		
No. of HIV-1 subtype B sequences in UKHIVRDB	11,299	3,168	4,033	4,522	4,725	5,020	4,569	4,470	1,644		43,450		
Total no. of HIV-1 sequences in UKHIVRDB	17,609	5,592	7,911	9,453	10,019	11,340	10,925	9,985	4,023		86,857		
% of HIV-1 subtype B sequences in UKHIVRDB	64.2	56.7	51.0	47.8	47.2	44.3	41.8	44.8	40.9		50.0		

<sup>a</sup> For the Los Alamos HIVDB, we tabulated only the number of hits found outside the country of origin. For the JP-TK HIVDB, we tabulated only the number of hits among foreigners resident in Japan. For the Los Alamos HIVDB and the JP-TK HIVDB, the country of origin is indicated in parentheses as follows: Brazil, BR; Canada, CA; China, CN; France, FR; Germany, DE; Japan, JP; Taiwan, TW; United Kingdom, UK; United States, US.

<sup>b</sup> NA, not applicable.

age, 06JP.Y287 (AB864025) for the JP.MSM.B-4 lineage (10), 07CN.BJ07635 (JF906694) for the CN.MSM.B-1 lineage, and 07CN.BJ061217 (JF906568) for the B'/CN.MSM.B-2 lineage (19). The choice of the specific query sequence used to represent each lineage did not significantly influence the search results. The 100 nucleotide sequences with the highest BLAST scores (E values) for each query sequence were retained. When more than one nucleotide sequence appeared to have been reported from the same infected individual, then only one sequence was retained. The top 100 sequences typically showed >94 to 95% sequence homology to the query sequences (see below). To confirm these possible matches to the East Asian HIV lineages of interest, we then verified the phylogenetic relationships of the database sequences. Trees were initially estimated by the neighbor-joining method (20) with the Tamura-Nei 93 (TN93) nucleotide substitution model (21), followed by more rigorous maximum-likelihood estimation based on the Hasegawa-Kishino-Yano nucleotide substitution model (22) and a gamma distribution model for rate variation among sites. Three subtype C strains (95IN21068, 86.ETH2220, and 92.BR025-d) were used as the outgroup.

These analyses were undertaken with MEGA 5.05 (23). The geographical origins of the subtype B sequences ( $n = 272$ ) used in this study were Argentina (AR) ( $n = 1$ ), Australia (AU) ( $n = 1$ ), Canada (CA) ( $n = 1$ ), China (CN) ( $n = 93$ ), France (FR) ( $n = 1$ ), Gabon (GA) ( $n = 1$ ), Germany (DE) ( $n = 1$ ), Haiti (HT) ( $n = 5$ ), Japan (JP) ( $n = 93$ ), Myanmar (MM) ( $n = 1$ ), Netherlands (NL) ( $n = 1$ ), Spain (ES) ( $n = 1$ ), Taiwan (TW) ( $n = 3$ ), Thailand (TH) ( $n = 15$ ), Trinidad and Tobago (TT) ( $n = 5$ ), the United Kingdom (UK) ( $n = 38$ ), and the United States (US) ( $n = 11$ ). We also estimated phylogenies after deleting sites linked to drug resistance. The tree topology was very similar to that estimated when these sites were included.

**Molecular clock analysis.** To determine the time scale of the global dispersal of subtype B, especially in the context of HIV epidemics in East Asia, we undertook a molecular clock phylogenetic analysis by the Bayesian Markov Chain Monte Carlo (MCMC) approach implemented in the BEAST v1.7.4 package (24). To model sequence evolution, we used the generalized time-reversible (GTR) nucleotide substitution model with a gamma distribution model to account for rate variation among sites. We

**TABLE 2** Summary of epidemiologic and demographic information about subjects identified in this study who harbored HIV-1 subtype B variants associated with transmission among MSM in Japan

Japanese MSM subtype B variant and strain name or sample ID	Country of origin or birth <sup>a</sup>	Ethnicity or nationality	Sampling yr	Age (yr)	Sex	Risk factor or origin <sup>d</sup>	Source	Accession no.	Transmission subcluster	Reference or remark
JP.MSM.B-1										
04DE.fd5gjGvO1zxQvv0	Germany	NA <sup>b</sup>	2004	NA	NA	NA	Los Alamos HIVDB	GQ400617	Global (UK)-JP.MSM.B-1	30
05US.ARC_A221	US	NA	2005	NA	NA	BD	Los Alamos HIVDB	JN697357	Global (UK)-JP.MSM.B-1	29
06CN.LN107	China (Liaoning)	Chinese	2006	28	M <sup>c</sup>	MSM	Los Alamos HIVDB	FJ531410	CN-JP.MSM.B-1	27
06CN.LN126	China (Liaoning)	Chinese	2006	17	M	MSM	Los Alamos HIVDB	FJ531423	CN-JP.MSM.B-1	27
07CN.LN159	China (Liaoning)	Chinese	2007	22	M	MSM	Los Alamos HIVDB	FJ531457	CN-JP.MSM.B-1	27
08CA.PHAC09_054	Canada	NA	2008	NA	NA	NA	Los Alamos HIVDB	JQ675213	-	Data submission only
09TW.36497	Taiwan	Taiwanese	2009	NA	NA	NA	Los Alamos HIVDB	HQ657934	-	28
06UK.a01	UK	Caucasian	2006	NA	M	MSM/bi	UKHIVRDB	AB906905	Global (UK)-JP.MSM.B-1	
07UK.a02	UK	Caucasian	2007	NA	M	MSM/bi	UKHIVRDB	AB906906	Global (UK)-JP.MSM.B-1	
07UK.a03	UK	Caucasian	2007	NA	M	MSM/bi	UKHIVRDB	AB906907	Global (UK)-JP.MSM.B-1	
07UK.a04	US	Caucasian	2007	NA	NA	NA	UKHIVRDB	AB906908	Global (UK)-JP.MSM.B-1	
07UK.a05	SE Asia	Other Asian	2007	NA	M	MSM/bi	UKHIVRDB	AB906909	-	
09UK.a06	SE Asia	Other Asian	2009	NA	M	NA	UKHIVRDB	AB906910	-	
09UK.a07	US	Caucasian	2009	NA	M	MSM/bi	UKHIVRDB	AB906911	Global (UK)-JP.MSM.B-1	
10UK.a08	E Europe	Caucasian	2010	NA	M	MSM/bi	UKHIVRDB	AB906912	Global (UK)-JP.MSM.B-1	
10UK.a09	UK	Caucasian	2010	NA	M	MSM/bi	UKHIVRDB	AB906913	Global (UK)-JP.MSM.B-1	
10UK.a10	NA	NA	2010	NA	NA	NA	UKHIVRDB	AB906914	-	Closely related to 2008 Japanese MSM samples 08JP.Y440 and 06JP.Y308
10UK.a11	NA	NA	2010	NA	NA	NA	UKHIVRDB	AB906915	Global (UK)-JP.MSM.B-1	
10UK.a12	NA	NA	2010	NA	NA	NA	UKHIVRDB	AB906916	Global (UK)-JP.MSM.B-1	
10UK.a13	NA	NA	2010	NA	NA	NA	UKHIVRDB	AB906917	Global (UK)-JP.MSM.B-1	
JP.MSM.B-2										
09JP.CN.GM3061	China	Chinese	2009	27	M	MSM	JP-TK HIVDB	AB735867		Chinese MSM resident in Japan Chinese MSM resident in Japan for last 20 years
09JP.CN.Y519	China	Chinese	2009	26	M	MSM	JP-TK HIVDB	AB735896		
05UK.b01	NA	Other Asian	2005	NA	M	MSM/bi	UKHIVRDB	AB906918		
JP.MSM.B-3										
05JP.UK.GM1736	UK	Caucasian	2005	39	M	MSM	JP-TK HIVDB	AB863991		British MSM resident in Japan, likely infected by Japanese partner
11UK.c01	NA	Not known	2011	NA	NA	NA	UKHIVRDB	AB906919		Closely related phylogenetically to 05JP.UK.GM1736
JP.MSM.B-4										
09TW.36025	Taiwan	NA	2009	NA	NA	NA	Los Alamos HIVDB	HQ657878		
06UK.d01	UK	Caucasian	2006	NA	M	MSM/bi	UKHIVRDB	AB906920		

<sup>a</sup> US, United States; SE, Southeast; UK, United Kingdom; E, Eastern.<sup>b</sup> NA, not available.<sup>c</sup> M, male.<sup>d</sup> BD, blood donation; bi, bisexuality.

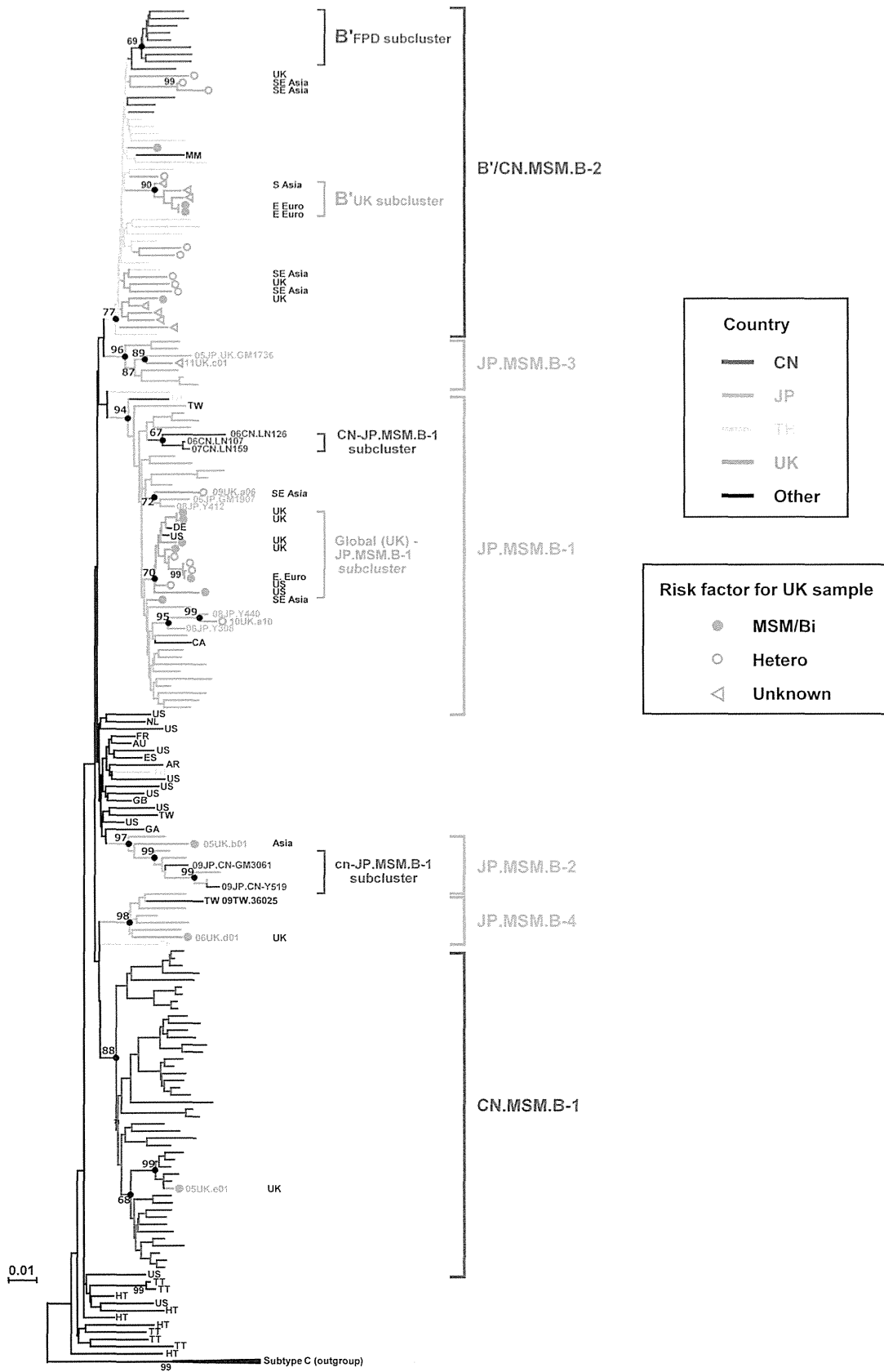


TABLE 3 Summary of epidemiologic and demographic information about subjects identified in UKHIVRDB who harbored HIV-1 subtype B variants uniquely associated with the epidemic in China<sup>a</sup>

Chinese HIV-1 subtype B variant and strain code	Country or region of birth <sup>d</sup>	Sampling yr	Sex <sup>b</sup>	Risk factor <sup>c</sup>	Source	Accession no.	Transmission subcluster <sup>d</sup>
CN.MSM.B-1, 05UK.e01	UK	2005	M	MSM/bi	UKHIVRDB	AB906921	
B'/CN.MSM.B-2							
05UK.f01	UK	2005	F	Hetero	UKHIVRDB	AB906922	
06UK.f02	SE Asia	2006	F	Hetero	UKHIVRDB	AB906923	
06UK.f03	SE Asia	2006	F	Hetero	UKHIVRDB	AB906924	
06UK.f04	UK	2006	F	Hetero	UKHIVRDB	AB906925	
07UK.f05	UK	2007	M	MSM/bi	UKHIVRDB	AB906926	
07UK.f06	NA <sup>c</sup>	2007	F	Hetero	UKHIVRDB	AB906927	
07UK.f07	E Europe	2007	M	MSM/bi	UKHIVRDB	AB906928	B'UK
07UK.f08	E Europe	2007	M	MSM/bi	UKHIVRDB	AB906929	B'UK
07UK.f09	SE Asia	2007	F	Hetero	UKHIVRDB	AB906930	
07UK.f10	NA	2007	NA	NA	UKHIVRDB	AB906931	B'UK
08UK.f11	S Asia	2008	M	NA	UKHIVRDB	AB906932	B'UK
08UK.f12	SE Asia	2008	M	Hetero	UKHIVRDB	AB906933	
08UK.f13	NA	2008	M	Hetero	UKHIVRDB	AB906934	
08UK.f14	NA	2008	NA	NA	UKHIVRDB	AB906935	
09UK.f15	UK	2009	M	MSM/bi	UKHIVRDB	AB906936	
09UK.f16	NA	2009	F	Hetero	UKHIVRDB	AB906937	
09UK.f17	NA	2009	NA	NA	UKHIVRDB	AB906938	
09UK.f18	NA	2009	NA	NA	UKHIVRDB	AB906939	
10UK.f19	NA	2010	NA	NA	UKHIVRDB	AB906940	
11UK.f20	NA	2011	NA	NA	UKHIVRDB	AB906941	B'UK

<sup>a</sup> UK, United Kingdom; SE Asia, Southeast Asia; S Asia, southern Asia (India, Bangladesh, Pakistan); E Europe, Eastern Europe).

<sup>b</sup> M, male; F, female.

<sup>c</sup> Hetero, heterosexual; bi, bisexual.

<sup>d</sup> B'UK, subtype B' transmission subcluster identified in the United Kingdom and most likely associated with MSM/bisexual population in the United Kingdom (see the text) (Fig. 2).

<sup>e</sup> NA, not available.

used a relaxed uncorrelated lognormal molecular clock to model rate variation among lineages (25) and a nonparametric Bayesian skyride coalescent model as a prior distribution for phylogeny shape and size (26). The MCMC analyses were run for 50 million generations with sampling every 5,000th state. Multiple chains were executed to ensure that adequate mixing and convergence had been achieved, as assessed with the Tracer software (<http://tree.bio.ed.ac.uk/software/tracer/>). The posterior distribution of phylogenies was summarized in a maximum clade credibility (MCC) tree, which was calculated with TreeAnnotator (<http://tree.bio.ed.ac.uk/software/annotator/>). Lineages in the MCC tree were colored according to their countries of origin with FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>). While there are a number of nucleotide sites in our sequences that were represented by ambiguity codes, this uncertainty is marginalized during maximum-likelihood/Bayesian inference in a statistically correct manner.

**Identification of clusters.** The initial identification of transmission clusters was based on the neighbor-joining phylogeny. All clusters of three or more patients with a bootstrap value of >70% were selected. Subsequently, the clusters were verified by Bayesian phylogenetic inference.

Only clusters with a posterior probability of 1.0 were considered to be transmission clusters.

## RESULTS

**Global occurrence of HIV-1 variants associated with MSM in East Asia.** To explore possible epidemiological linkages between the HIV-1 MSM subtype B epidemics in East Asia and those in the rest of the world, we searched three sequence databases for HIV-1 strains that are related to the HIV lineages uniquely associated with transmission among MSM in Japan and China (see Materials and Methods). Our phylogenetically informed search (Fig. 1) identified a total of 48 HIV-1 sequences ("hits") that were closely related to the transmission clusters identified in East Asia (Table 1). As expected, we found a number of sequences in the Los Alamos HIVDB that belonged to the CN.MSM.B-1, CN.MSM.B-2, JP.MSM.B-1, and JP.MSM.B-2 lineages because many sequences from Chinese and Japanese MSM have already been de-

**FIG 2** Phylogenetic analysis of HIV-1 subtype B variants identified outside East Asia. Neighbor-joining phylogenies were estimated from nucleotide sequences of the 1.1-kb pro-RT region (HXB2, nucleotides 2253 to 3392) of HIV-1 subtype B strains that were identified by our screening procedure (see Fig. 1 and Materials and Methods for details). HIV-1 subtype C sequences were used as the outgroup. Bootstrap support values that are relevant to this study are shown at the corresponding nodes. Ten out of 13 JP.MSM.B-1 variants found in the UKHIVRDB, together with one sample each from Germany and the United States, formed a distinct monophyletic subcluster [designated Global (UK)-JP.MSM.B-1] within JP.MSM.B-1. In addition, seven JP.MSM.B-1 variants were identified outside Japan in the Los Alamos HIVDB. The clusters of interest in our study are highlighted. The country of origin of each sequence is indicated by the ISO 3166 two-letter code ([http://www.iso.org/iso/country\\_names\\_and\\_code\\_elements](http://www.iso.org/iso/country_names_and_code_elements)) as follows: CA, Canada; CN, China; DE, Germany; JP, Japan; TH, Thailand; TW, Taiwan; UK, United Kingdom; US, United States. Region abbreviations: E Euro, Eastern Europe; SE Asia, Southeast Asia; S Asia, South Asia. The risk factor for each United Kingdom sample (if available; see also Tables 2 and 3) (denoted in the right lower box) is indicated at the tip of each branch. Asian subtype B variants and their subclusters are indicated at the right of the tree. The countries of birth for United Kingdom samples (if available) (see also Tables 2 and 3) and the names of the strains of interest in this study are shown at the tips of the respective branches.

