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Genetic Analysis of Hepatitis C Virus with Defective Genome and Its Infectivity in Vitro▽

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Replication and infectivity of hepatitis C virus (HCV) with a defective genome is ambiguous. We molecularly cloned 38 HCV isolates with defective genomes from 18 patient sera. The structural regions were widely deleted, with the 5′ untranslated, core, and NS3-NS5B regions preserved. All of the deletions were in frame, indicating that they are translatable to the authentic terminus. Phylogenetic analyses showed self-replication of the defective genomes independent of full genomes. We generated a defective genome of chimeric HCV to mimic the defective isolate in the serum. By using this, we demonstrated for the first time that the defective genome, as it is circulating in the blood, can be encapsidated as an infectious particle by *trans* complementation of the structural proteins.

Viruses with a deletion mutation in their genome have been identified as defective interfering (DI) particles for many virus species (1, 3, 9, 16). Part of the DI virus genome is deleted, but regions indispensable for replication and packaging are preserved. Most DI viruses occur spontaneously in the course of cell culture infected with a high titer of wild-type viruses. Hepatitis C virus (HCV) with a defective genome has been found in liver and serum specimens of some HCV patients (4, 8, 15). HCV has a plus-strand RNA genome that encodes the viral core, E1, E2, and p7 structural proteins and NS2, NS3, NS4A, NS4B, NS5A, and NS5B nonstructural proteins (10). According to the reports, the deletions have been found mainly in the structural region and most of the deletions are in frame, but some deletions are out of frame (4), raising questions about whether the defective HCV genome is merely a by-product of a full genome or a self-replicating genome and whether it can be encapsidated into an infectious virus particle.

In the present study, we molecularly cloned 38 HCV isolates with defective genomes from HCV patient sera to address these questions by genetic analyses and infection experiments. As long as we explored, all of the deletions were in frame, indicating the potential to support translation from the authentic initiation codon to the termination codon, although the structural region was widely deleted, as reported previously. Phylogenetic analyses evidenced self-replication of the defective genomes independent of full genomes. We demonstrated for the first time, by *trans* complementation experiments, that the defective genome, as it is circulating in the blood, can be encapsidated as an infectious particle, designated HCV_{CCD}.

First, to amplify HCV cDNAs in 21 serum specimens from 18 HCV patients (genotype 1b), we performed three sets of long-distance reverse transcription (RT)-PCRs flanking (i) the 5′ untranslated region (UTR) to the 5′ part of the NS3 region, (ii) the remaining part of the NS3 region to the end of NS5B, and (iii) the 5′ UTR to the end of the NS5B region (Fig. 1A). The specimens were collected with informed consent. cDNA was synthesized with RNase H-deficient reverse transcriptase Superscript III (Invitrogen, Carlsbad, CA) at a higher temperature (55°C) to reduce template switching and mispriming. PCRs were performed in a (hemi)nested manner with high-fidelity polymerase KOD plus or KOD FX (Toyobo, Osaka, Japan) as described previously (5). For some target nucleotide positions, a mixture of two or three primers was used to reduce mismatches due to sequence heterogeneity (Table 1). Of the 21 specimens examined, representative results are shown in Fig. 1. An amplicon of the 5′ UTR-NS3 region of the predicted size (ca. 3.7 kb) was detected in all specimens (18/18), and representative results are shown in Fig. 1B. In addition, a shorter amplicon suggestive of a defective HCV genome was simultaneously present in four specimens from 1 (R4) of 12 cases of clinically mild hepatitis and from 3 (T5, K3, and K4-pre) of 6 cases of active hepatitis (clinical data not shown). Defective genomes were found in the patients with relatively higher copy numbers of HCV RNA (>8.1×10^5 copies/ml in the 5′ UTR, Table 2), suggesting that the coexistence of a defective genome is related to hepatitis severity. The authentic-size amplicon was poorly detected when coexisting with a defective HCV genome shorter than 2 kb (T5 and K3), presumably because of preferential amplification of the shorter amplicon. A shorter amplicon was not detected for the NS3-

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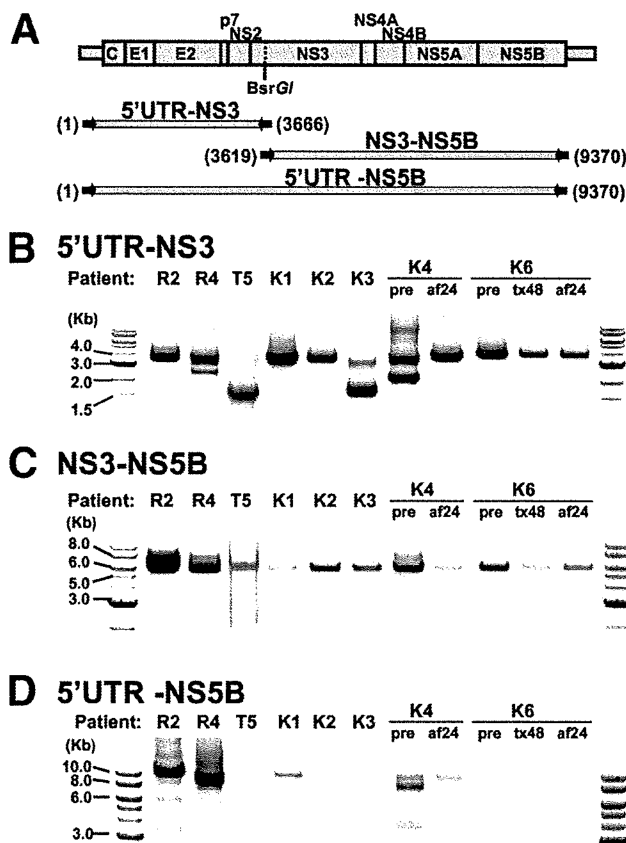


FIG. 1. Representative results of long-distance RT-PCRs for serum HCV. (A) The three sets of long-distance RT-PCR used: 5′ UTR-NS3 (5′ UTR to the 5′ part of the NS3 region), NS3-NS5B (the remaining part of the NS3 region to the end of NS5B), and 5′ UTR-NS5B (5′ UTR to the end of the NS5B region). The nucleotide positions of the 5′ and 3′ ends of each amplicon are indicated in parentheses. PCR products were electrophoresed and stained with ethidium bromide. Results of the representative 11 specimens (eight patients) are shown for 5′ UTR-NS3 (B), NS3-NS5B (C), and 5′ UTR-NS5B (D). Serum specimens were collected from patients K4 and K6 before interferon treatment (pre), at the end of the full 48-week treatment period (tx48), and 24 weeks after the full treatment period (af24). DNA molecular size markers are at both sides of panels B to D.

NS5B region, while the amplicon of the predicted size (ca. 5.6 kb) was detected in all of the specimens, albeit with various efficiencies (Fig. 1C). The 5′ UTR-NS5B region, which covers almost the whole genome, was then amplified, and an amplicon of the predicted size (9.4 kb) was detected in 10 specimens from nine patients (5 specimens in Fig. 1D). Of the successfully amplified specimens, two (R4 and K4-pre) also contained a shorter amplicon, in accordance with the results of the 5′ UTR-NS3 PCR. The NS3-NS5B region is essential for autonomous replication of HCV as an RNA replicon in vitro (5–7). It has been shown that NS5A is the only nonstructural protein that can *trans* complement HCV replication (13). They used a nonadaptive mutation of NS5A as a replication-incompetent NS5A protein instead of a deletion mutant protein. Thus, we speculate that deletion of the NS3-NS5B region cannot be complemented in *trans*. Intriguingly, the shorter amplicon was not detected after full-term interferon treatment in patient K4 (K4-af24), although it was detected prior to treatment (K4-pre) (Fig. 1B and D). The possible reasons for this are that (i) the defective genome disappeared naturally, (ii) packaging of the defective genome by the helper virus was impeded by an unknown mechanism of interferon, or (iii) replication of the defective genome is preferentially inhibited by the interferon pathway. Further studies are needed to reveal the effect of a defective HCV genome on the pathogenesis and treatment of HCV.

A total of 38 isolates with defective HCV genomes were molecularly cloned into plasmid vector pASGT (unpublished data) from the shorter amplicons of the 5′ UTR-NS3 PCR from four serum specimens (R4, T5, K3, and K4-pre in Fig. 1B) at the AscI and BsrGI restriction sites. The nucleotide sequences were determined with an autosequencer (3730 DNA analyzer; Applied Biosystems, Foster City, CA). Sequence analyses revealed that the structural region was widely deleted in all of the defective isolates and that the deletion ranges were quite diverse among the isolates (extending up to the NS2 region) (Fig. 2A). In contrast, the 5′ UTR and core regions were constantly preserved, suggesting that these regions, as well as the NS3-NS5B region, are indispensable for the production of HCV with a defective genome. Intriguingly, defective genomes with different deletion patterns coexisted in single specimens from two patients (three patterns in patient K3 and four patterns in patient K4-pre). Moreover, two deletions in a single genome were observed in five isolates from patient R4 (isolate R4S-5). As many as three deletions in a single genome were observed in the isolate from patient K3 (e.g., isolate K3S-15), in which two small deletions resulted in two tiny residual fragments. Such diversity in deletion ranges indicates flexibility of the remaining structural region for the replication of defective HCV genomes. Nevertheless, all of the deletions identified in the 38 isolates were in frame (Fig. 2B), implying that these defective HCV genomes have the potential for translation from the core to the authentic end of NS5B without a frameshift.

To determine the ratio of defective to full genomes, we performed quantitative PCRs targeting a relatively conserved E2 sequence, which is commonly deleted in the defective genomes, with primers listed in Table 1. Calculation of the 5′ UTR/E2 ratio, which must theoretically be 1 without the existence of the defective genome, showed higher values (1.7 to 2.45) in specimens containing the defective genomes (R4, K3, and K4pre in Table 2), indicating that the defective genome level in serum is 0.7 to 1.45 times the full genome level. However, to clarify the impact of defective genomes on pathogenesis and their effect on the treatment of HCV, accumulation of more data is needed.

The nucleotide sequence comparison of 38 defective HCV isolates showed sequence diversity. Such diversity was observed even among isolates obtained from the same specimen. Perhaps such diversity is a result of self-replication and the subsequent evolution of the defective HCV genome. To explore this possibility, phylogenetic analyses were performed on the nucleotide sequence data from patient K4. Sequences at the 5′ and 3′ maximum overlapping regions located outside the deletions were separately compared (Fig. 2A), and phylogenetic trees were created by the neighbor-joining method with GENETYX software (Genetyx Inc., Tokyo, Japan). As a re-

TABLE 1. Primers used for long-distance and quantitative RT-PCRs in this study

Test and region(s)	Direction	Primer(s)[a]	Sequence[b]	Position[c]
Long-distance PCR				
5′ UTR-NS3	RT	606R/712R	GTTTCCATAGACTC(A/G)ACGGG	3930–3949
5′ UTR-NS3, 5′ UTR-NS5B	1st forward	420	GGCGACACTCCACCATAGATCACTC	1–42
5′ UTR-NS3	1st reverse	605R/713R	ACCGGAATGACATCAGCATG(T/C)CTCGT	3741–3766
5′ UTR-NS3, 5′ UTR-NS5B	2nd forward	AscT7-420	ATCGTAGGCGCGCCTC*TAATACGACTCACTATA*GC CAGCCCCCGATTGGGGGCGACACTCCACCATAGATCACTC	1–42
5′ UTR-NS3	2nd reverse	604R/714R	CGAGGTCCTGGTCTACATT(G/A)GTGTACAT	3639–3666
NS3-NS5B, 5′ UTR-NS5B	RT	386R	AATGGCCTATTGGCCTGGAG	9390–9392
NS3-NS5B	1st forward	602/723	CCACCGCAACACAATCTTTCCT(G/A)GCGAC	3529–3556
NS3-NS5B, 5′ UTR-NS5B	1st reverse	719R/720R/721R	GAGTGTTTAGCTCCCCGTTCA(T/C/G)CGGTTGGG	9363–9392
NS3-NS5B	2nd forward	603/724	CAAAGGGTCCAATCACCCA(A/G)ATGTACAC	3619–3646
NS3-NS5B, 5′ UTR-NS5B	2nd reverse	607R/654R/722R	CGGTTGGGGAGCAGGTA(G/A/G)A(T/T/C)GCCTAC	9345–9370
Quantitative PCR				
5′ UTR	RT	738RH	ACTCGCAAGCACCCTATCAGGC	291–312
5′ UTR	Forward	736	AAGCGTCTAGCCATGGCGTTAGTA	73–96
5′ UTR	Reverse	737R	GGCAGTACCACAAGGCCTTTCG	272–293
5′ UTR	Probe	733FB	FAM-TCTGCGGAACCGGTGAGTACAC-BHQ1	147–168
E2	RT	743RH/744RH/753RH/753RH	CAACGCTCTCCTCG(A/A/G/G)GTCCA(A/G/A/G)TTGCA	2271–2296
E2	Forward[d]	751/752	GGCCTCCACATGGCAA(C/T)TGGTTCGG	1972–1993
E2	Forward[d]	739/740	CCGCCGCAAGGCAACTGGTT(C/T)GG	1974–1993
E2	Reverse	741R/742R	GCCTCGGGGTGCTTCCGGAAGCA(G/A)TCCGT	2088–2116
E2	Probe	734FB/735FB	FAM-TGGATGAA(T/C)AGCACTGGGTTCACCAAGAC-BHQ1	2001–2029

[a] Primers separated by slashes harbor a nucleotide substitution(s) (in parentheses) in the sequence in the same order.
[b] An underline and a double underline indicate recognition sequences for AscI and BsrGI, respectively, with which the PCR products were subcloned into plasmid vector pASGT5. Italics denote the T7 promoter, which was used to synthesize RNA in vitro from the T5S2 isolate (Fig. 4A).
[c] Nucleotide positions correspond to the HCV-JS sequence (12).
[d] Forward primers for E2 were mixed in the reaction mixture.

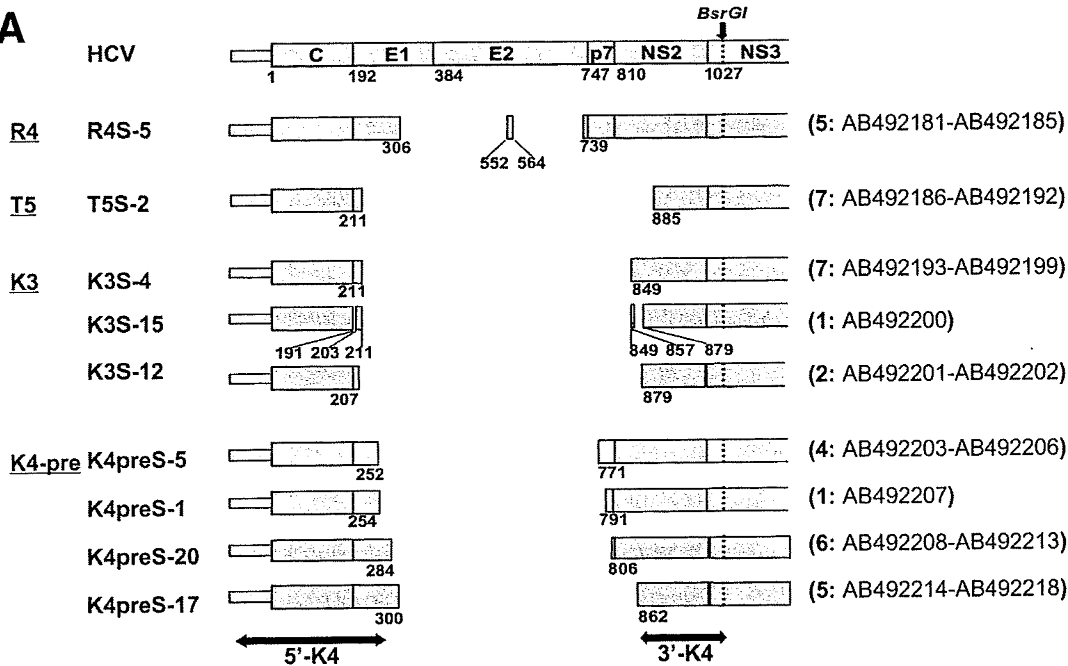
sult, isolates with the same deletion pattern formed genetic clusters that were distinct from each other, as well as from those of nondefective HCV isolates (Fig. 3A and B). Similar results were obtained for the other patients with defective HCV genomes (data not shown). These results suggest that a defective HCV genome is capable of replication to accumulate mutations and to evolve independently of the nondefective HCV genome.

TABLE 2. Quantitative PCRs for the 5′ UTR and E2 regions of HCV[a]

Region for quantification	No. of copies/ml		5′ UTR/E2 ratio
	5′ UTR	E2	
R2	2.0×10^6	1.7×10^6	1.17
R4	5.3×10^6	2.2×10^6	2.44
T5	ND[b]	ND[b]	
K1	8.3×10^5	8.4×10^5	0.99
K2	3.6×10^5	3.5×10^5	1.01
K3	8.6×10^5	5.1×10^5	1.7
K4pre	8.1×10^5	3.3×10^5	2.45
K4af24	4.5×10^5	4.4×10^5	1.01

[a] For quantification of the 5′ UTR and E2 regions, the TaqMan Fast PCR Universal mixture and the 7500 Fast Real-Time PCR system (Applied Biosystems) were used in a two-step method with the primers and probes shown in Table 1 according to the manufacturer's protocol. The copy number of HCV was determined by the standard-curve method with serial dilutions of the synthesized full-length HCV RNA.
[b] ND, not determined due to sample shortage.

Next, the ability of the defective HCV genome to be encapsidated and released from cells as HCV_{CCD} was examined. A genotype 1b replicon RNA lacking the structural region was synthesized by using defective isolate T5S-2 from patient T5 (Fig. 2 and 4A) as the template in an in vitro transcription system (MEGAscript T7 kit; Ambion, Inc., Austin, TX) under the control of the T7 promoter. Also, capped mRNA encoding the genotype 1b structural proteins from the same patient (designated C-NS2 in Fig. 4A) was synthesized in vitro with the mMessage mMachine T7 kit (Ambion). Both synthesized RNAs were cotransfected into Huh7.5 hepatoma cells. However, HCV_{CCD} was not obtained, presumably because of low replication or virus productivity of genotype 1b HCV per se. In fact, we transfected the defective RNA alone and observed the replication and protein expression of HCV, but with low efficiency (data not shown). Thus, to augment virus productivity, a JFH1-based chimeric HCV genome (genotype 1b/2a) and its deletion mutant were generated to mimic isolate T5S-2 (designated TNS2J1 and TNS2J1ΔS, respectively, Fig. 4A). JFH1 is genotype 2a HCV isolate that can produce high levels of infectious virus (14). To verify the virus productivity of TNS2J1, Huh7.5 cells (10-cm plate) were transfected with 10 μg of in vitro-synthesized RNA from TNS2J1 or JFH1 by lipofection with TransMessenger transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. Two days later, the culture medium was concentrated 10-fold and inoculated into naïve Huh7.5 cells (four-well chamber



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                              1260▼1996                                                2033▼2556
R4      R4S-5       ACCTTCTCGCCTCGTCGGCACGAAACAGTACAGGACTGCAACTGCT ATACATGGATGAATAGCACTGGGTTCACCAAGACGTGT CTGCTGATAGCCCAGGCTGAGGCCGCCTTGGAAAGCCTA
                     T  F  S  P  R  R  H  E  T  V  Q  D  C  N  C   Y  T  W  M  N  S  T  G  F  T  K  T  C   L  L  I  A  Q  A  E  A  A  L  E  S  L
                                                               306 552                                       564 739

                              974▼2994
T5      T5S-2       GTGTCCGGGGTCTACCATGCCACGAGCGACTGCTCCAACTTAAGC ATCTTTGACATCACCAAACTCTTGCTCGCCATATTCAGCCCGGTCTTGGTGCTCCAGGCTGGCTTAGTGAGAGTGCCG
                     V  S  G  V  Y  H  A  T  S  D  C  S  N  L  S   I  F  D  I  T  K  L  L  L  A  I  F  S  P  V  L  V  L  Q  A  G  L  V  R  V  P
                                                               211 885

                              974▼2886
K3      K3S-4       GGGGTGTACCATGTCACGAACGACTGCTCCAACTCAAGC TTCGTCACCAGAGTCGAGGTACAATTGCAGGCGTGGGCTCCCCCCCTCAACGTTCGGGGGGGCCGCGATGCCATCGTCCTCCTC
                     G  V  Y  H  V  T  N  D  C  S  N  S  S   F  V  T  R  V  E  V  Q  L  Q  A  W  A  P  P  L  N  V  R  G  G  R  D  A  I  V  L  L
                                                         211 849

                    915▼949                     974▼2886                        2911▼2975
        K3S-15      CCGGCTTCCGCT TTCACGAGCGACTGCTCCAACTCAAGC TTTGTCATCAGAGTCGAGGTACAATT TGCGGTCCACCCAGAGCTAATCTTTGACATCACCAAGATCTTGCTCGCCATATTC
                     P  A  S  A   F  T  S  D  C  S  N  S  S   F  V  I  R  V  E  V  Q  F   A  V  H  P  E  L  I  F  D  I  T  K  I  L  L  A  I  F
                           191  203                          211 849                       857 879

                                                                  962▼2975
        K3S-12      ACCATACCGGCTTCTGCTCACGAGGTGCGCAACGCGTCTGGGGTGTACCATGTCACGAACAACTG TGCGGTCCACTCAGAGCTAATCTTTGACATCACCAAGATCCTGCTCGCCATATTCGGC
                     T  I  P  A  S  A  H  E  V  R  N  A  S  G  V  Y  H  V  T  N  N  C   A  V  H  S  E  L  I  F  D  I  T  K  I  L  L  A  I  F  G
                                                                                       207 879

                              1098▼2653
K4-pre  K4preS-5    TGGGTAGCGCTCACTCCCACGCTCGCGGCCACGAACAACAGCGTC TTCTGTGCCGCCTGGTATATTAAGGGCGGGCTGGTTCCTGGGGCGGCATACGCTCTCTATGGCGTATGGCCGCTGCTC
                     W  V  A  L  T  P  T  L  A  A  T  N  N  S  V   F  C  A  A  W  Y  I  K  G  G  L  V  P  G  A  A  Y  A  L  Y  G  V  W  P  L  L
                                                               252 771

                              1104▼2713
        K4preS-1    GTAGCGCTCACTCCCACGCTCGCGGCCAGGAACAACAGCGTCCCCA ATGGCGTATGGCCGCTGCTCCTGCTCCTGTTGGCGTTACCACAACGAGCTTACGCCACGGACCGGGAGATGGCTGCA
                     V  A  L  T  P  T  L  A  A  R  N  N  S  V  P   N  G  V  W  P  L  L  L  L  L  L  A  L  P  Q  R  A  Y  A  T  D  R  E  M  A  A
                                                               254 791

                              1193▼2757
        K4preS-20   ACTCTCTGCTCCGCTATGTACGTGGGGGATCTCTGCGGATCCGTC CGAGCTTACGCCACGGACCGGGAGATGGCTGCATCGTGCGGAGGCGCGGTTTTTGTAGGTCTGGCAATCTTGACCTTG
                     T  L  C  S  A  M  Y  V  G  D  L  C  G  S  V   R  A  Y  A  T  D  R  E  M  A  A  S  C  G  G  A  V  F  V  G  L  A  I  L  T  L
                                                               284 806

                              1240▼2924
        K4preS-17   CTCATTTCCCAGCTGTTCACCTTCTCACCTCGCCGGCATGAGAC CCCCCCTCTCAACGTTCGGGGAGGCCGCGGTGCCATCATCCTCCTCATGTGCGCGGTTCACCCAGAGCTGATTTTTGAC
                     L  I  S  Q  L  F  T  F  S  P  R  R  H  E  T   P  P  L  N  V  R  G  G  R  G  A  I  I  L  L  M  C  A  V  H  P  E  L  I  F  D
                                                               300 862
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FIG. 2. Sequence analysis of the defective HCV genomes. A total of 38 isolates were molecularly cloned into a plasmid vector and sequenced. Data from representative isolates are presented. Nucleotide positions and deduced amino acid positions correspond to those of genotype 1b strain HCV-JS (12). (A) Defects located in the structural region were compared. The remaining regions are illustrated as shaded boxes. Below the boxes are numbers indicating amino acid positions at the end of each remaining region. At the top of the panel is the HCV genome with the amino acid position at the N terminus of each HCV protein below. The BsrGI restriction site that was used to clone the PCR products is shown as a dotted line. Each value in parentheses at the right is the number of isolates showing the same deletion pattern, followed by the GenBank accession number(s). The two-headed arrows indicate the 5′ and 3′ maximum overlapping regions among the defective HCV isolates in the K4-pre specimen that are compared in the following phylogenetic analyses (5′-K4 and 3′-K4; see Fig. 3). (B) Deletion breakpoints and their adjacent nucleotides and deduced amino acid sequences are indicated. Solid triangles denote breakpoints, and numbers indicate the nucleotide positions (above) and amino acid positions (below) at the junctions.

slide). Cells inoculated with the culture medium from TNS2J1 RNA-transfected cells markedly expressed HCV protein, as shown by immunofluorescent staining (Fig. 4B). The percentage of HCV-positive cells in chimera-infected cells, 40% (565/1,240), was greater than that of JFH1, 3% (37/1,210), demonstrating that the chimeric genome TNS2J1 can produce infectious HCV more robustly than JFH1 can ($P < 0.0001$).

Taking advantage of this chimeric genome, we conducted *trans* complementation experiments. To mimic the T5S-2 iso-

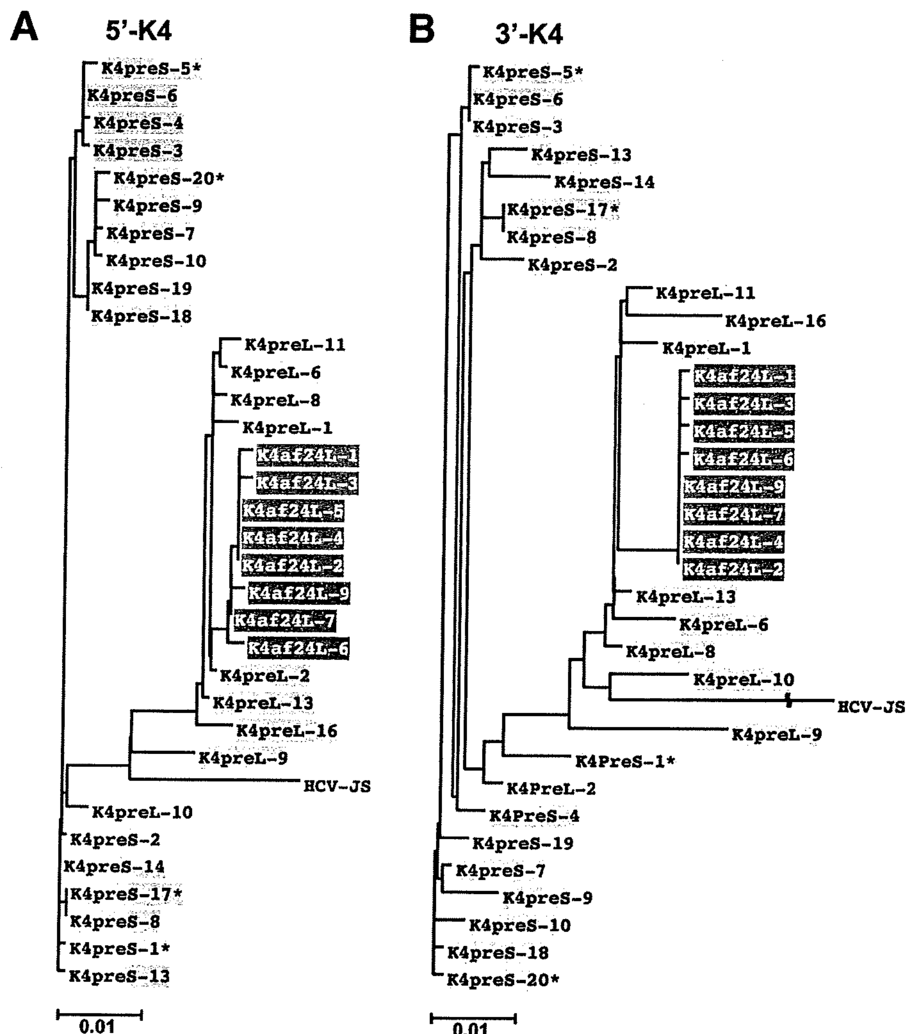

FIG. 3. Phylogenetic analyses of defective HCV genomes. Nucleotide sequence data from 33 isolates from patient K4 were used for phylogenetic analyses. The defective HCV genome (16 isolates) and the nondefective HCV genome coexisting before interferon treatment (9 isolates; GenBank accession no. AB492219 to AB492227) and those after treatment (8 isolates; GenBank accession no. AB492228 to AB492235) were compared in the 5′ and 3′ maximum overlapping regions separately (5′-K4 and 3′-K4 in Fig. 2A). Phylogenetic trees were created for the respective regions (A and B). In the isolate designations, pre and af24 stand for before and after interferon treatment and S and L stand for defective and nondefective HCV genomes, respectively. Isolates with the same deletion pattern (according to K4-pre in Fig. 2) are shaded in the same color. Asterisks denote the representative isolates illustrated in Fig. 2.

late, the region corresponding to the defect found in T5S-2 was identically deleted from the TNS2J1 genome (designated TN2J1ΔS, Fig. 4A). Ten micrograms of synthesized RNA of TN2J1ΔS was cotransfected into Huh7.5 cells (10-cm plate) together with 10 μg of synthesized capped mRNA encoding the structural region, including part of the nonstructural region of TNS2J1, designated C-NS2 or C-NS3P (Fig. 4A). Two days later, the culture medium was concentrated and inoculated into naïve Huh7.5 cells as previously described. HCV protein was expressed when cells were inoculated with the medium of cells cotransfected with TN2J1ΔS RNA and C-NS2 or C-NS3P mRNA, whereas no expression was observed in the case of TN2J1ΔS RNA alone (Fig. 4C). To stably provide the structural proteins in *trans*, packaging cell lines were established by retroviral transduction (2) of Huh7.5 cells with genes encoding the C-NS2 or C-NS3P region (Fig. 4A). These packaging cell lines were transfected with TN2J1ΔS RNA, and HCV protein was expressed in cells inoculated with the culture medium from the RNA-transfected packaging cells (Fig. 4D). Notably, the construct C-NS2 helped to produce HCV_{CCD} more efficiently than C-NS3P did (Fig. 4C). We observed less expression of the structural proteins with the C-NS3 construct than with the C-NS2 construct in a transient expression experiment (data not shown). One possible reason for this is that the C-NS3 construct needs one additional process, i.e., cleavage between NS2 and NS3, to produce NS2 and may affect the other proteins. Otherwise, it is simply because of the difference in the lengths of the constructs. These results indicate that a defective HCV genome lacking the structural region can be encapsidated by *trans* complementation of the structural proteins, thus conferring infectivity in vivo. Recently, a *trans*-packaging system consisting of an HCV subgenomic replicon and a reporter gene

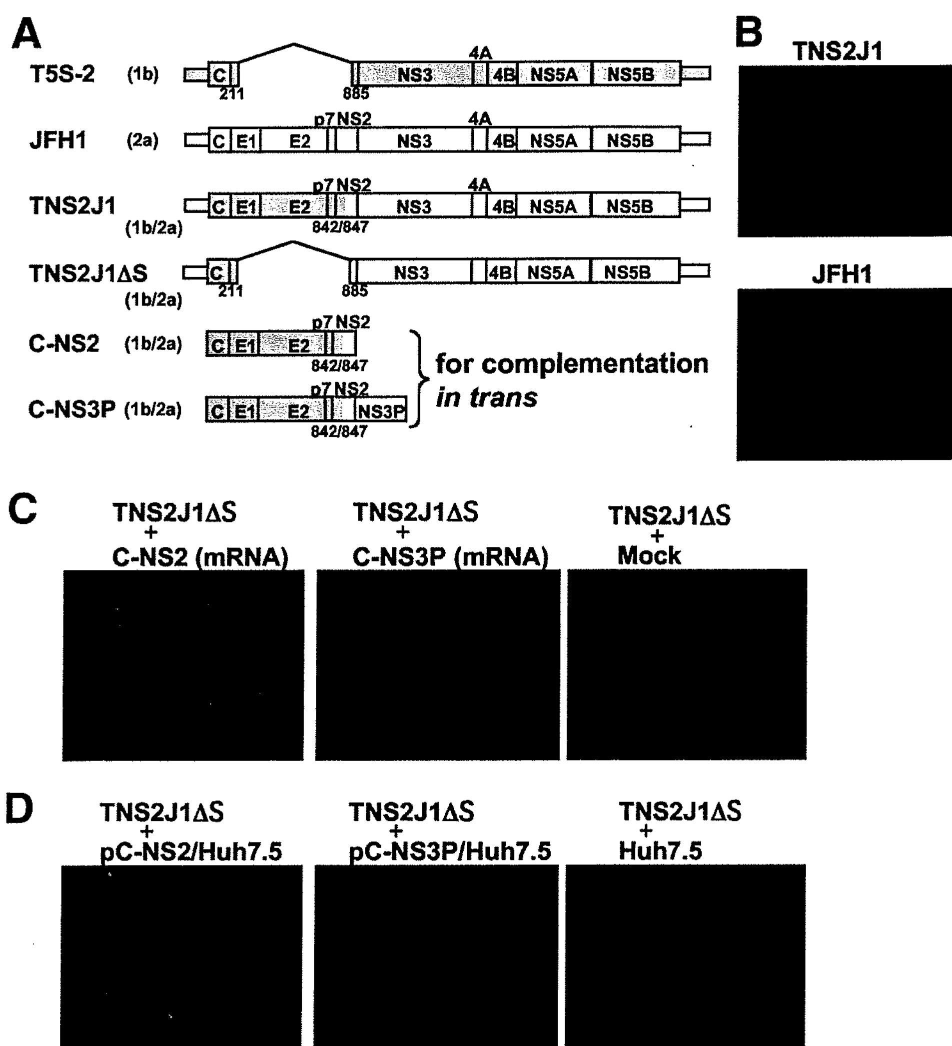

FIG. 4. In vitro infectivity of deletion mutant of chimeric HCV conferred by *trans* complementation of structural proteins. (A) Schematics of the following HCV genomic constructs: the defective HCV isolate (T5S-2), JFH1, chimeric virus of genotypes 1b and 2a (TNS2J1), and its deletion mutant (TNS2J1ΔS). C-NS2 and C-NS3 are fragments encoding the region from the core to the C terminus of the NS2 region and to the C terminus of the serine protease moiety in NS3, respectively. For the *trans* complementation experiments, the latter two constructs were inserted into pcDNA3.1 (Invitrogen) to synthesize capped mRNAs or into retroviral vector pCX4bsr (GenBank accession no. AB086384) to establish packaging cell lines stably expressing the proteins. Shaded and open boxes represent genotypes 1b (isolate from patient T5) and 2a (JFH1), respectively. The numbers below the boxes are amino acid positions at deletion breakpoints or at PCR-based recombination junctions. Naïve Huh7.5 cells were inoculated with the culture medium from cells transfected with JFH1 or TNS2J1 RNA (B), from cells cotransfected with TNS2J1ΔS RNA together with the structural region mRNA (C-NS2 or C-NS3P) or TNS2J1ΔS RNA alone (C), and from the packaging cell line (C-NS2/Huh7.5 or C-NS3P/Huh7.5) transfected with TNS2J1ΔS RNA and parental Huh7.5 cells transfected with TNS2J1ΔS RNA (D). HCV protein was detected by human HCV serum (1:500) by the indirect immunofluorescent method with Alexa Fluor 568 goat anti-human immunoglobulin G (1:200; red; Invitrogen). Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI; blue).

was also reported in which an intragenotypic chimera (2a/2a) was used as the most efficient packaging construct (11). Our packaging system used an efficient intergenotypic chimera (1b/2a) to encapsidate a genome mimicking a naturally occurring deletion (1b). Thus, although its efficiency may be different, our system could be a useful tool for the study of HCV_{CCD} of chimeric genome 1b/2a or genotype 1b.

Taken together, genetic analyses of the defective HCV genome showed the potential of its translation and self-replication. These defective genomes can be encapsidated into infectious virus-like particles by *trans* complementation of the structural proteins in vitro. The 5′ UTR and core regions, which are preserved in defective HCV genomes, are targets for the clinical quantification of HCV. Therefore, measured values may represent additive values for defective and nondefective HCVs and the method used for HCV quantification should be reevaluated.

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Impact of age at marriage and migration on HIV and AIDS epidemics in Japan

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Abstract

The causes of wide variation in the rates of HIV and AIDS epidemics among Japanese and non-Japanese nationals are not well understood. So, this paper examines the associations and assesses the potential roles of mean age at marriage, and migration in the HIV and AIDS epidemics in Japan. For the purpose, bivariate and multivariate regression analysis have been performed using epidemiological panel data to build up the relationships among overall HIV and AIDS prevalence, mean age at marriage, and migration. The same analyses have done for non-Japanese nationals living with HIV and AIDS separately. These indicators were significantly correlated with mean age at marriage, and migration. Multivariate linear regression analysis identified non-Japanese nationals' HIV and AIDS prevalence and mean age at marriage as the two most prominent factors linked with the national HIV and AIDS epidemics. The findings of this study supported the hypotheses that a high average age at marriage in the population leads to long period of premarital sex and the non-Japanese nationals' high prevalence facilitating the spread of the HIV and AIDS epidemics in Japan.

Introduction

Since the identification of the human immunodeficiency virus (HIV) in the early 1980s, much has been learned about how the virus is transmitted and how it attacks the body's immune system and causes the acquired immune deficiency syndrome (AIDS). The AIDS epidemic has grown on an unprecedented scale in three decades since it was first recognized, and is now considered a global crisis. In 2007, the total number of people living with HIV was 33.2 million, newly infected with HIV was 2.5 million, and AIDS deaths was 2.1 million [1]. Every day, over 6800 persons become infected with HIV and over 5700 persons die from AIDS, mostly because of inadequate access to HIV prevention and treatment services [1]. The HIV pandemic remains the most serious of infectious disease challenges to public health. Sub-Saharan Africa has experienced the most severe epidemic. In many developing countries, HIV prevalence was above 1%, but in none of the developed countries HIV has prevalence ever crossed the 1% mark [2]. The fastest growth of HIV among women occurs in East Asia, here women living with HIV jumped by 56% in 2 years [3]. It was 1985 that the AIDS Surveillance Committee, Ministry of Health, Labor and Welfare (MHLW) announced the first AIDS case in Japan. Though, they knew that many of hemophiliacs were infected with HIV even before 1985. The number of people living with HIV (PLHIV) and AIDS patients in Japan has continued to increase over time and upward trend [4]. While the total figure for reported cases is low compared with other advanced countries, the increase in newly

reported AIDS cases is a phenomenon not seen in any other developed country. In those developed countries, the HIV infections have rapidly increased during the period between 1980s and the early 1990s, and a decline in the number of HIV/AIDS cases has been observed in the late 1990s. However, Japan did not experience such rapid increase but, instead, continued to increase slowly. The main route of transmission is sexual contacts, and enormously the sexual behavior of Japanese youth is drastically changing [5]. In recent years, HIV incidence rate has been rising dramatically, and the number of new HIV cases in Japan has been increasing year by year (Figures 1, 2). In 2004, the total number of new HIV/AIDS cases was over 1,000 and the cumulative reported number of HIV/AIDS cases was more than 10,000 [6]. The number of reported PLHIV has continued to increase since 1996, and the highest number of cases was reported in 2006, at 952 cases. The number consisted of 836 Japanese nationals and 116 foreign nationals. It is mentioned that 827 (86.8%) cases of infection were through sexual contact, of which 604 (63.4%) and 223 (23.4%) cases were between individuals of the same sex and of different sex respectively (Figure 3). The most significant increase in new HIV cases occurred among men who have sex with men (MSM), and 15 times more men than women reported a new HIV positive diagnosis in 2006 [4]. In addition, an increasing number of people ages 30 and older became HIV positive in 2006 compared with 2005, a nearly 10% increase in new HIV cases from 2005 to 2006, and a 6.3% increase for those who developed AIDS during the same time period [4]. A research group, funded by MHLW, predicts that the number of PLHIV will be approximately 30,000 by the end of 2006 in Japan. According to Hashimoto et. al. [7], there will be 50,000 HIV/AIDS cases by the year 2010 among Japanese nationals only. Several factors are believed to be conducive to large epidemics: high frequency of sexual intercourse outside marriage, multiple sexual partners, lack of condom use, lack of male circumcision, and infection with other sexually transmitted diseases (STD) [8]. The evidence for an important effect of genital ulcer disease and male circumcision practices is strong [9].

Currently, more than 100 million persons move voluntarily within or between nations each year and almost 40 million are either internally displaced or refugees outside their own countries. Many studies have revealed evidence of a potential association between human mobility and the epidemic [10,11], especially in the developing areas. The task of the study is to examine the hypothesis that the non-Japanese nationals' potential role in spreading the epidemics to the general population of Japan. The number of female migrant workers has been increasing, and many of them enter Japan and stay illegally and one avenue of illegal entry is through trafficking. Many of those women are forced into sex work as a means of survival, or have male partners who have had multiple sexual partners, resulting in exposure to high-risk sexual behavior [12]. The brothel-based commercial sex workers (CSW), and a huge number of freelance street-based CSW

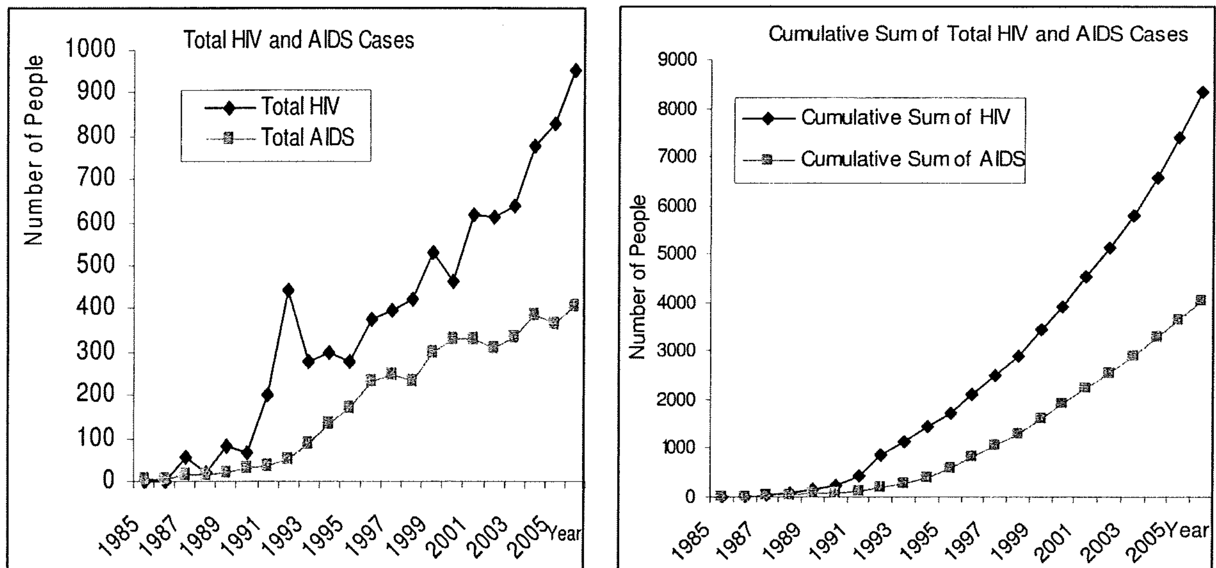

Figure 1
Number of people living with HIV and AIDS Cases and their Cumulative Sum in Japan up to 2006 [4].

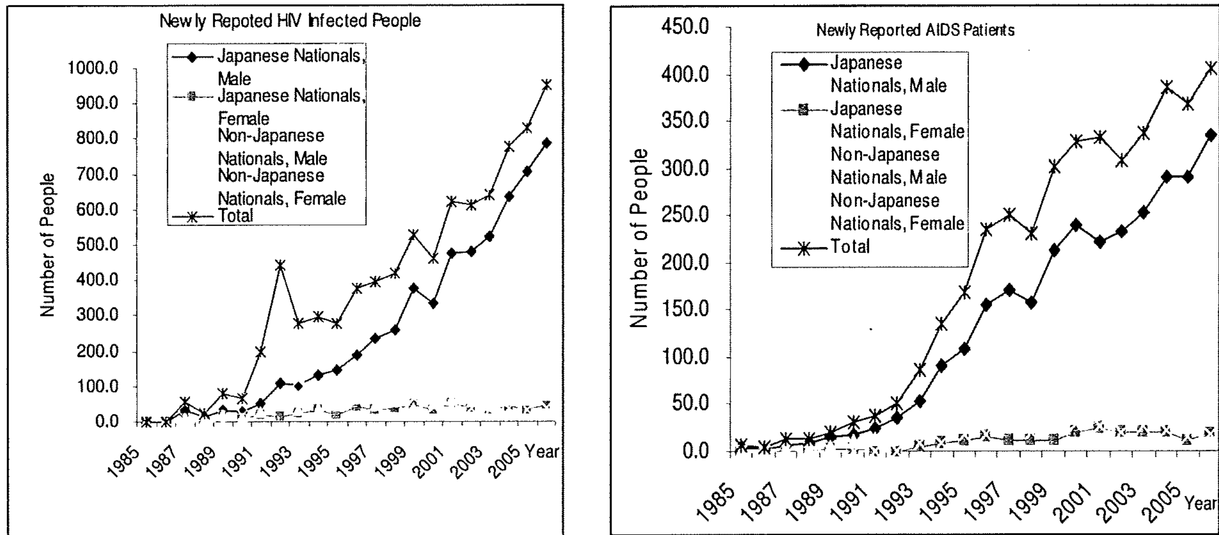

Figure 2
Trends in the number of newly reported HIV infected people and AIDS Patients, 1985–2006 [4].

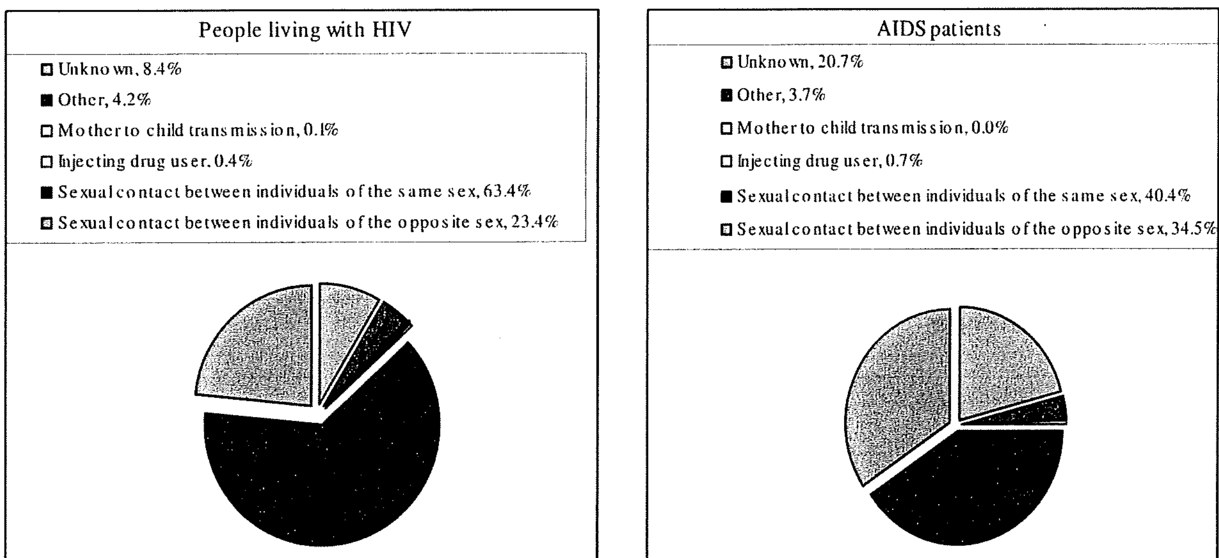

Figure 3
Breakdown of infectious routes of PLHIV and AIDS patients reported in 2006 [4].

as well as illegal immigrant CSW in Japan who rarely visit STD clinics for routine screening purposed increased the risk of HIV transmission to the general population [13]. Kimoto et. al. [13] work on the CSW situation in Osaka has been well recognized, including the CSW's vulnerability and the situations relationships with the entry and spread of HIV. The present study also examines the hypothesis that late average age at marriage is another factor contributing to the spread of HIV because late marriage may lead to a long period of premarital sexual activity. Throughout the developing world marriage is the central social institution that regulates and sanctions sexual behavior. This suggests that age at marriage and sexual behavior before and after marriage could play a vital role in spreading HIV [14,15]. Bongaarts [16] has closely examined the relationship of age at marriage and HIV status. Ecological data indicate that the countries in southern Africa with very late age at marriage also have large epidemics. Thus, much has been known about what determines the spread as well as prevention of the HIV/AIDS epidemics. From best of our knowledge no study has been concentrated on the associations of mean age at marriage, and immigration with HIV and AIDS epidemics in Japan. So, it differs from other studies in several important aspects and it will use a broader range of explanatory variables based on more current and relevant data to offer a more comprehensive view of HIV and AIDS implications. By using best known statistical tools, it will be examined carefully how strongly mean age at marriage, and migration influence the sizes of HIV and AIDS epidemics in general and the scale of these diseases among non-Japanese nationals in particular. Hopefully, the study will focus on HIV and AIDS pandemics in Japan, and this can offer to the policy makers in socioeconomic options to combating both the diseases and its routes of infection.

Study design and data

A dataset was created by compiling data from two different sources. The total number of HIV and AIDS and the number HIV and AIDS of non-Japanese nationals, and mean age at marriage were collected from MHLW [4] and the data on migration was collected from the Ministry of Justice [17] for the period 1985 to 2007. The ecological panel data has been used for bivariate and multivariate linear regression analysis. Keeping with the purpose of the study, two dependent variables were chosen for the multivariate linear regression analysis: (i) sizes of HIV and AIDS epidemics in a year as measured by total HIV and AIDS prevalence and (ii) share of non-Japanese nationals in the epidemics as measured by the proportion of population living with HIV and AIDS. Explanatory variables were (i) time, t measured in years; (ii) mean age at marriage as measured by years of the average age at first marriage of men, and (iii) migration as measured by the number of non-Japanese nationals living in Japan.

Results

Bivariate and multivariate approaches have been applied in the analysis. Roles of time, mean age at marriage, and migration are examined here in turn.

Bivariate Analysis

Correlation coefficients (r) were derived to examine direction, strength and significance of linear relationships between the variables included in the study (Table 1).

Table 1: Correlation between the variables that were examined

	Time	Total HIV Prevalence	Non-Japanese HIV Prevalence	Total AIDS Prevalence	Non-Japanese AIDS Prevalence	Age at Marriage	Migration
Time	1.00						
Total HIV Prevalence	.967**	1.00					
Non-Japanese HIV Prevalence	.341*	.436*	1.00				
Total AIDS Prevalence	.976**	.931**	.244	1.00			
Non-Japanese AIDS Prevalence	.880**	.797**	.316	.926**	1.00		
Age at Marriage	.976**	.964**	.211*	.955**	.795**	1.00	
Migration	.991**	.973**	.380*	.960**	.857**	.971**	1.00

** Significant at $p < 0.01$ level, * Significant at $p < .05$ level.

The significant similar relationships were found between time and the sizes of the HIV and AIDS epidemics for both the cases. The correlation was considerably lower for the size of the HIV epidemic among non-Japanese nationals (r = .341) than the overall HIV prevalence (r = .967). Again for the case of AIDS epidemic, the correlation was slightly lower for the size of AIDS epidemic among non-Japanese nationals (r = .880) than the overall AIDS prevalence (r = .976).

As expected, age at marriage is likely associated with the overall HIV prevalence (r = .964) and the non-Japanese nationals' share of the epidemic (r = .211) is very lower compared to the former. Again for the case of AIDS epidemic, the correlation was slightly lower for size of the AIDS epidemic among non-Japanese nationals (r = .795) than the overall AIDS prevalence (r = .955).

Migration is the most important issue in HIV and AIDS prevalence perspective. Correlation coefficients show that the higher migration, the greater HIV and AIDS epidemics. However, among the four explanatory variables considered, migration score appeared to have a significant, and the strongest, relationship with the overall HIV and AIDS prevalence (r = .973 and r = .960 respectively). But its correlation was significant and slightly lower with the proportion of HIV and AIDS prevalence who are non-Japanese nationals (r = .380 and r = .857 respectively).

Multivariate Analysis

Four sets of multivariate linear regressions were conducted (Tables 2, 3). In the first two sets, the dependent variables were total HIV and non-Japanese nationals HIV prevalence, and in the second two sets were total AIDS prevalence and non-Japanese nationals AIDS prevalence.

In the fist two sets of regression models, age at marriage and migration were included, and all these variables were significant predictors of HIV prevalence. For the case of non-Japanese nationals' HIV prevalence, the migration was stronger than that of age at marriage and better explained the model as indicated by the higher values of R^2 (Models 1–3). In the set 2 of regression models showed that migration and age at marriage were the more significant predictors compared to non-Japanese nationals' share in the total HIV epidemic (Models 4–7). When non-Japanese nationals' prevalence was included in the model, migration was no longer significant (Model 7), although it was significant independently (Model 5). Finally, age at marriage and non-Japanese nationals HIV prevalence are the most significant predictors for the total prevalence of HIV epidemic in Japan.

Similarly, in the second two sets of regression models, age at marriage and migration were included, and all these variables were significant predictors of AIDS prevalence. For the case of non-Japanese nationals' AIDS prevalence, the migration was stronger than that of age at marriage

Table 2: Multivariate linear regression models explaining the HIV epidemic

Explanatory Variables	Dependent Variables and Standardized Coefficients			
Set 1	**Non-Japanese Nationals HIV Prevalence**			
	Model 1	Model 2		Model 3
Age at Marriage	.211			-2800**
Migration		.380*		3.100**
Adjusted R^2	-.003	.102		.544
Set 2	**Total HIV Prevalence**			
	Model 4	Model 5	Model 6	Model 7
Age at Marriage	.964**			1.150**
Migration		.973**		-.254*
Non-Japanese nationals HIV Prevalence			.435*	.290**
Adjusted R^2	.926	.944	.150	.986

** Significant at $p < 0.01$ level, * Significant at $p < 0.05$ level.

Table 3: Multivariate linear regression models explaining the AIDS epidemic

Explanatory Variables	Dependent Variables and Standardized Coefficients			
Set 3	**Non-Japanese Nationals AIDS Prevalence**			
	Model 8	Model 9		Model 10
Age at Marriage	.795**			-.661
Migration		.857**		1.499**
Adjusted R^2	.614	.721		.734
Set 4	**Total AIDS Prevalence**			
	Model 11	Model 12	Model 13	Model 14
Age at Marriage	.955**			.716**
Migration		.960**		.154
Non-Japanese Nationals AIDS Prevalence			.926**	.488**
Adjusted R^2	.908	.918	.850	.986

** Significant at $p < 0.01$ level, * Significant at $p < 0.05$ level.

and better explained the models independently as indicated by higher values of R^2 (Models 8–9). But, when mean age at marriage and migration were included in the models, mean age at marriage was no longer significant (Model 10), although this was significant independently (Model 8). Thus, migration is the more significant for the non-Japanese nationals' AIDS prevalence. The set 4 of regression models showed that migration, and mean age at marriage were the two significant predictors compared with non-Japanese nationals share in the total AIDS epidemic (Models 4–7). When non-Japanese nationals' prevalence was included in the model, migration was no longer significant (Model 14), although this was significant independently (Model 12). As a final point, age at marriage and non-Japanese nationals' AIDS prevalence are the most significant predictors for the total prevalence of AIDS epidemic in Japan.

Discussion

Good health is an important component of human wellbeing and the improvements in health and life expectancy are likely to contribute to greater economic growth and human resource development. HIV/AIDS affects not only the infected person, but also his or her family, community, and country. At the household level, people have loss of companionship and income. At the community and national levels, they experience loss of productivity because of absenteeism and deaths. The prevalence of HIV and AIDS are still low and little public attention is given to the epidemic as a serious issue confronting Japanese society. Experts point out that the epidemic may be spreading much more quickly than available figures indicate (Figures 1, 2, 3). Various underlying factors may be cited for the trend, the most prominent being changes in the sexual behavior of young people which fuel to the late marriage, greater migration across national borders, and delays in the identification of the infection.

Comparing other countries, Japan has three particular characteristics in HIV/AIDS situation: (i) a majority of reported HIV cases is infected through male-to-male sexual conducts, (ii) the higher prevalence rate among the non-Japanese nationals, and (iii) very few reported HIV cases is infected through intravenous drug use (IDU). The people considered being high risk and vulnerable to the epidemics are MSM, migrant workers, CSW and their clients. In the recent years, more than half of new HIV reported cases are infected through male-to-male sexual conducts. The main route of infection was sexual contact, in particular, MSM, which accounted for 63.4% of all PLHIV (Figure 3). Commonly cited statistics estimate the MSM population in Japan at approximately 1–2% of the total male population [18]. Many MSM reside in downtown Tokyo and Osaka, which are said to have the largest

gay communities in Asia. As this number grows, there is an increasing need to improve the means of detection and the provision of swift treatment. The primary reason for this is that HIV infection is actually increasing among gay communities and MSM populations and thus gay communities in Japan have been exposed to the high risk of HIV infection. Heterosexual contact was the second most common mode of transmission (23.4%) and the rates of infection through IDU and mother to child transmission (MTCT) are both very low (<1%) (Figure 3). On the other hand, the key reason for few HIV infections through IDU is that the population of injecting drug users (IDUs) is comparatively small and they are isolated. Furthermore, in Japan, social stigma against drug users is very strong and drug controls is also very strict, thus the people with HIV may not report it, even if they were infected through drug injection. The proportions of foreign nationals who live in Japan are only 2%, but among all HIV/AIDS reported cases since 1985, the proportion of foreigners with HIV is 28.8% and the proportion of foreigners with AIDS is 24.1% and in 2004, among all HIV/AIDS reported cases, the proportion of foreigners with HIV was 13% and the proportion of foreigners with AIDS was 19.7% [4]. In terms of gender, Japanese men account for 89% of all cumulative HIV cases since 1985, as compared with Japanese women (11%). In contrast, foreign men account for 38% of all cumulative HIV cases, as compared with foreign women (62%). The possible reason for this difference is that the major route of HIV transmission among Japanese HIV/AIDS cases is male-to-male sexual conducts, where as foreigners are infected with HIV primary through sexual conducts between the opposite sexes. In terms of age group, those aged 30 - 39 years represented the highest number of cases of infection (41.0%), followed by those aged 15 - 29 years (29.6%).

The increase of PLHIV amongst Japanese males was most prominent; the number reported in 2006 (604 cases) greatly exceeded the previous year's figure (529 cases), and represented a record high. Also, the number of Japanese female PLHIV increased from 32 cases in 2005 to 49 cases in 2006. In terms of Japanese male PLHIV, the number of cases resulting from MSM (571 cases) had increased from the previous year (514 cases), representing the highest reported to date. Moreover, there were 173 cases of Japanese males infected through sexual contact with individuals of the opposite sex, up from 161 cases in the previous year. The number of Japanese female PLHIV infected through sexual contact with individuals of the opposite sex increased yearly until 1999, after which the numbers appeared to have stabilized. Figures (Figures 1, 2) reported in 2006, however, showed an increase in new cases, from 32 cases in 2005 to 49 cases in 2006. Looking at a gender breakdown by age groups of Japanese PLHIV infected through sexual contact with individuals of the opposite sex, females made up the majority in the 15–19 years and 20–24 years groups, which was in contrast with other age groups. The total number of AIDS patients reported in 2006 was 406, showing a continued increase from previous years, and representing the highest recorded level to date. Of this total, 355 (87.4%) were Japanese nationals, which reached the highest ever, and the number of foreign national AIDS patients decreased from 65 in 2005 to 51 in 2006. Out of AIDS patients reported in 2006, 74.9% of the patients were infected through sexual transmission, with 140 (34.5% of all cases) infected through sexual contact with individuals of the opposite sex and 164 (40.4% of all cases) with individuals of the same sex. Cases with unknown infection routes totaled 84 (20.7%). The assumed location of infection was within Japan for 315 cases (77.6%). The number of Japanese male AIDS patients was 335 (82.5%), increasing from the previous year (291). Out of these, 110 (32.8%) were infected through sexual contact with individuals of the opposite sex, 156 (46.6%) with individuals of the same sex, and 54 (16.1%) through unknown infection routes.

The trend of foreign nationals reported as PLHIV or affected by AIDS has flattened out. In 2006, there were 116 cases (12.2%) of foreign national PLHIV and 51 AIDS patients (12.6%) in Japan. These PLHIV were, in order of those nationalities most frequently reported, from Latin America, Southeast Asia, and the East Asia and Pacific Area excluding Japan. Among foreign AIDS patients, those from Southeast Asia were most frequently reported, followed by Sub-Saharan Africa, and the East Asia and Pacific Area excluding Japan. Looking at regional trends, Tokyo and the Kanto Koshinetu area (excluding Tokyo) remained areas with high levels of infection, representing 528 (55.2%) PLHIV and 211 (52.0%) AIDS patients in 2006. The number of PLHIV increased in Hokkaido, the Tohoku region, the Kanto Koshinetu area, Tokyo, and the Tokai and Kinki regions.

The average age at marriage has risen in most industrial countries, especially in Japan (28 year for female and 32 year for male). Even countries with less development, or who have come lately to industrialization show a rise in the average age at marriage for women. These statistics may reflect a greater feminist stance in most countries, with more women working and completing college. As well they reflect a trend away from marriage. Which results the considerably change the sexual behaviors among the youth and they participate in more sex with causal friends or multiple partners. In Japan, a network of youth having unprotected sex is rapidly expanding, leading to sharp increases in sexually transmitted infections (STI) and terminations of unplanned pregnancies [19]. As a result the abortion rate of teenagers has doubled and the number of Chlamydia and Gonorrhea cases among youth

has also increased since 1995. Homosexual infection is expanding almost exponentially among Japanese males in age group of 20's and 30's, that is, before their marriages. HIV infection through homosexual route is increasing so acutely because of the changes occurring in the society of Japanese younger generations. Importantly, in the last several years, there are increasing incidents caused by "two-shot dials", "telephone clubs", "encounter web sites", and "legal drug" markets on webs, all related to the development of electronic webs [20]. Changing the sexual behaviors of Japanese youth is the key to reduce the expected HIV epidemic in Japan.

There has been a dramatic change in Japan's sex industry since the 1980's, especially in urban areas, where the traditional CSW is being marginalized by new type who offers manual stimulation, cunnilingus, and fellatia, but not vaginal intercourse. All types CSW are vulnerable population at the higher risk of HIV infection as well as transmission to their partners. The actual reported number of HIV/AIDS cases that involve sex workers is unknown. Japanese youth are actively involved in paid sex. Prostitution has been prohibited in Japan since the enforcement of the Anti-Prostitution Law in 1957. However, the sex industry has never disappeared and has continued to diversify since the 1980s as certain adult entertainment businesses were legitimized under a law regulating their establishments and services. More than 20,000 shops are registered throughout the country and it is said that many more operate illegally [21]. Among these businesses, it appears that the insistence on the use of condoms has not become a general practice and the risk of HIV infection is therefore high. Recently, informal sex work through the Internet is said to have dramatically increased among the younger generation, which is another serious cause for concern. However, the non-Japanese female sex workers are particularly vulnerable to infection as an ever widening segregation between them and native-born Japanese sex workers has led to tendency for the foreign nationals to engage in higher-risk practices.

Japan is simultaneously facing crises of exclusion and inclusion. By adopting more stringent laws to exclude foreign workers, it faces tremendous difficulties in coming to terms with its demographic future diminishing population and labor force. Yet because these laws invariably become less effective in stemming the entrance of foreign workers as both Japanese enterprises and recruitment networks find new ways to recruit them, it also perpetuates a system of illegal migration. While perhaps convenient to enterprises wishing to pay very low wages, this risks an equally problematic crisis of inclusion of foreign workers into everyday life accompanying the advent to Japan as a multicultural society. The non-Japanese nationals, most are believed to be migrant workers. Although a smaller portion, migrant workers, especially unregistered non-Japanese nationals without legal status, are similarly vulnerable and seriously at risk of HIV infection. A particularly high number of cases were reported in 1992, but since then the number has leveled off, meaning that this group cannot be used to explain the increasing number of HIV cases in Japan. In this period many women from Asian countries come to Japan as CSW and most of them were infected outside Japan [22]. There are two main factors that make migrant workers in Japan more vulnerable to HIV infection. One is the language barrier, which results in a lack of information on prevention and limited access to proper testing, treatment, and care. In many cases, they do not seek treatment until they are in the advanced stages of AIDS or need to be admitted to the emergency room. The second factor is treatment costs. Many migrant workers either do not have valid legal status or they possess visas that make them ineligible for public health insurance. The reluctance of such people to undergo testing or receive treatment because they cannot afford the costly medical bills, and of some hospitals to refuse such patients, has been a serious issue. Given this situation, many such non-Japanese have never been tested for HIV and tend not to visit medical institutions until they are in the advanced stages of AIDS or carried in he near death which is so much pathetic as human being. Everybody is at risk of contracting HIVAIDS regardless of nationality, race, ethnicity, gender, or age. One solution to the difficulties these populations find themselves in would be for the Infectious Disease Prevention Law to remove the condition of nationality and residency status from eligibility for health insurance.

In Japan, social awareness and public perception about HIV/AIDS is extremely low and few people voluntarily attempt to have HIV testing, except for a part of gay communities in the large cities. So, it is reasonably difficult to know the actual number of people with HIV, HIV infection rates, and the trends of HIV/AIDS at the early stages based only on the reported cases of HIV/AIDS. In reality, there is a big difference between actual number of HIV/AIDS and the trend, and the number of HIV/AIDS and the trend, which are represented by the Committee of AIDS Trends (CAT) and MHLW, etc. Thus, HIV/AIDS cases in Japan seem to continue increasing slowly but the increase rates are becoming faster and it seems to be just an iceberg. In Japan, HIV testing system is not strong and totally lacks counseling and the motivation for HIV testing is also weak. It is impossible to know the latest trend in the actual number of HIV/AIDS cases, based on the present surveillance systems, which depends on only reported HIV/AIDS cases. Using the present surveillance system, it is also impossible to know which population groups and communities are at the risk of increasing HIV infections. The government did not recognize the threat posed by the

pandemic and only declared HIV/AIDS a national disaster. This is the major limitation to understand the current situation of HIV/AIDS in Japan. If the prevalence rates of HIV and AIDS epidemics rise tremendously, Japan must face the most critical situation, which is not expected.

Conclusion

Among most developed countries, the number of newly reported HIV positive and AIDS patients has been decreasing, but it is increasing steadily in Japan. Based on the analyses of the present surveillance data, the HIV infections through sexual conducts tend to increase tremendously in the next few years. The major limitation to understand the current situation of HIV and AIDS is the present surveillance systems, which only depends on the reported HIV and AIDS cases. Added to this is a belief that only a low percentage of people testing to HIV in Japan actually report as required, suggesting that the number of HIV positive people in Japan is much higher than MHLW survey states. The continuous and simultaneous increase in both STD and HIV infection trigger the current situation and lead to a full-blown HIV epidemic, if no immediate action is taken. However, Japan is now in the low level of HIV and AIDS epidemics, it is considered as shifting to "concentrated epidemics". The study results suggest that the urgent need for introducing prevention measure for HIV and STD among youth which are currently appropriate for the target population. The findings support the hypothesis that a high average age at marriage in a population contributes to the spread of HIV because a higher age at marriage is strongly associated with a longer period of premarital exposure to the risk of infection. So, it is burning need to find out why the youth are disheartened to get marriage in proper time. Japan's anti-prostitution law needs to amend in the context of HIV/STD control because strict police security drives the problem underground and discourages street girls, especially those living in the country illegally, from seeking proper health services for HIV/STD. Consequently, HIV infection is rising enormously among non-Japanese females. Group-based interventions, better access to health care and a comprehensive public approach should be applied to the non-Japanese nationals. In fine, an AIDS center for prevention should be established to acquire knowledge and expertise and to demolish the epidemics.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NIM performed statistical analysis and drafted the manuscript. YO developed the analytical approach and econometric methods. HT contributed to the drafting of the manuscript. All authors read and approved the final manuscript

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RNA interference targeted to the conserved dimerization initiation site (DIS) of HIV-1 restricts virus escape mutation

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Short hairpin RNAs (shRNA) targeting viral or cellular genes can effectively inhibit human immunodeficiency virus type 1 (HIV-1) replication. This inhibition, however, may induce mutations in the targeted gene, leading to rapid escape from the shRNA-induced inhibition. We generated a lymphoid cell line that stably expressed a 19-bp shRNA targeting a well-conserved dimerization initiation site (DIS) of HIV-1, which strongly inhibited viral replication, thereby delaying virus escape. Furthermore, treatment of HIV-1 infection with DIS- and vif-shRNA combination therapy resulted in superior anti-viral responses compared to vif-shRNA monotherapy. Continuous challenge with HIV-1, however, generated virus mutants that could overcome the RNA interference restriction. Such anti-genes may be promising tools for HIV-1 gene therapy for HIV/acquired immunodeficiency syndrome.

Key words: combination therapy, lentiviral vector, HIV-1, RNA interference, shRNA, well-conserved dimerization initiation site (DIS), virus escape mutation.

Abbreviations: HIV-1, human immunodeficiency virus type1; DIS, dimerization initiation site; vif, HIV-1 virion infectivity factor; RNAi, RNA interference; shRNA, short hairpin RNA; EGFP, enhanced green fluorescent protein.

RNA interference (RNAi) is a natural biological phenomenon mediated by small interfering RNA (siRNA) molecules that target specific mRNA for degradation by cellular enzymes. RNAi has become a popular method for studying gene function, especially in mammalian systems. With proof-of-concept studies already presented against a diverse range of human pathogens and several innovative methods described for delivering siRNA to a wide variety of primary cells, there is great potential for siRNA as a therapeutic strategy (*1–8*). Many groups have reported the general use of siRNAs to specifically inhibit HIV-1 replication by targeting viral or cellular genes (*9–17*), suggesting a role for RNAi in the therapeutic treatment of HIV-1 infection.

One disadvantage of targeting viral genes with RNAi, however, is that viral mutations can lead to a loss of sensitivity to RNAi. This is particularly problematic in the case of RNA viruses, which accumulate point mutations up to 107 times more rapidly than DNA viruses (*18*). RNAi-resistant mutants have been shown to arise in cell culture models while targeting HIV (*19–22*), poliovirus (*23*, *24*), hepatitis C virus (*25*) and hepatitis B virus (*26*). Although most of these mutants achieved resistance through a point mutation or deletion of the target sequence, one HIV mutant escaped RNAi suppression by accumulating mutations outside of the target sequence, thereby creating a new local RNA secondary structure that presumably excluded the RNA-induced silencing complex (*21*). These findings demonstrate that any effective RNAi-based anti-viral therapy must compensate for the evolutionary potential of the pathogen. Importantly, work with HIV-1 also indicates that tolerance to target sequence mismatches may depend on the particular sequence of the siRNA tested (*27*). Sabariegos *et al.* (*22*) reported that optimal HIV-1 gene silencing by siRNA requires complete homology within the central region of the target sequence, and that substitutions at only a few positions at the 5′ and 3′ ends are partially tolerated. Thus, targeting single sequences in the viral genome as a therapeutic approach for RNA viruses has limited efficacy.

In the present study, we found that siRNAs that specifically target the well-conserved dimerization initiation site (DIS) (*28–30*) of HIV-1, compared to those targeting non-conserved regions, drive RNAi-resistant mutants to emerge at a slower rate. We selected the HIV-1 vif region as a target site and constructed a lentiviral vector expressing the shRNA. The HIV-1 *vif* gene encodes a highly basic, 23,000-M_r phosphoprotein that collapses intermediate filaments, localizes in the cytoplasm of its infected target cells, and acts during virus assembly by an unknown mechanism to enhance viral infectivity (*31–33*). The siRNAs targeting the DIS drive the accumulation of point mutations at 77 days, almost 2 months slower than those targeting the vif region. Furthermore, coexpression of DIS- and vif-siRNAs

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following lentiviral-mediated transduction of SupT1 cells provides enhanced, longer-term inhibition of HIV-1 infection relative to single expression. The data support the utility of combining different anti-viral modalities in a gene therapy setting to effectively suppress HIV-1 replication.

MATERIAL AND METHODS

Construction of U6 Expression Plasmids and Lentiviral-based Vectors—A short hairpin RNA (shRNA) was designed to target the DIS and vif sequences (Fig. 1A). The shRNA sequences were chemically synthesized as two complementary DNA oligonucleotides: (DIS sense: 5′-CGGCTTGCTGAAGCGCGCACGGTTCAAGAG ACCGTGCGCGCTTCAGCAAGCCTTTTTTCTAGAG-3′; DIS antisense: 5′-GATCCTCTAGAAAAAAAGGCTTGCT GAAGCGCGCACGGTCTCTTGAACCGTGCGCGCTTCA GCAAGCCGGTAC-3′; vif sense: 5′-CCAGATGGCAGGTG ATGATTGTTTCAAGAGAACAATCATCACCTGCCATCT GTTTTTTTCTAGAG-3′; vif antisense: 5′-GATCCTCTAG AAAAAAACAGATGGCAGGTGATGATTGTTCTCTTGAA ACAATCATCACCTGCCATCTGGGTAC-3′; LacZ sense: 5′-CGTGACCAGCGAATACCTGTTCTTCAAGAGAGAAC AGGTATTCGCTGGTCACTTTTTTTCTAGAG-3′; LacZ antisense: 5′-GATCCTCTAGAAAAAAAGTGACCAGCGA ATACCTGTTCTCTCTTGAAGAACAGGTATTCGCTGGT CACGGTAC-3′). The oligonucleotides were mixed in equimolar amounts, heated for 5 min at 95°C, and then gradually cooled to room temperature in annealing buffer (10 mM Tris–HCl, 100 mM NaCl). The resultant duplex was ethanol-precipitated and ligated into *Kpn*I and *Bam*HI cloning sites upstream of the U6 promoter of pSV2neo (TAKARA, Otsu, Japan). The U6 DIS-shRNA vector encoded HIV-1 DIS, the U6 LacZ shRNA vector encoded LacZ and the U6 vif-shRNA vector encoded vif.

To construct the lentiviral vectors, the *Eco*RI fragment of the U6 vectors listed above containing the U6 promoter and the siRNA duplex was cloned into the *Eco*RI site of the lentiviral transfer vector (pCS-CDF-CG-PRE), generating the CS-DIS-shRNA, CS-vif-shRNA, and control the transfer vectors CS-LacZ-shRNA and CS-U6-ter.

Cell Culture—Sup-T1 cells were grown in either RPMI 1640 (Sigma, St Louis, MO, USA) or Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). All cultures were maintained at 37°C under a 5% CO_2 atmosphere.

Lentivirus Preparation—293T cells were co-transfected with 15 μg transfer vector construct, 15 μg helper constructs coding for Gag-Pol (pMDLg/p.RRE), 5 μg Rev-expressing construct pRSV-Rev and 5 μg VSV-G-expressing construct pMD.G, using the calcium phosphate precipitation method (*34*). Supernatants were harvested 72 h post-transfection, filtered through a 0.45 μm filter disc and concentrated 100-fold by centrifugation at 6,000*g* overnight. The resultant viral pellet was resuspended in serum- and antibiotic-free RPMI medium and stored at –80°C until use. To determine the viral titre, 5×10^5 293T cells were transduced with the prepared viral stock, and the number of EGFP-positive cells was counted after a 72 h culture using flow cytometric analysis (*35*).

Transduction of SupT1 Cells—SupT1 cells (3×10^5) were seeded in 12-well plates in 1 ml culture medium. Cells were transduced with the CS-U6-shRNAs and control lentiviral vectors at a multiplicity of infection (MOI) of 20 in the presence of 4 μg/ml polybrene. After incubation at 37°C for 4 h, the cells were washed three times with phosphate buffered saline (PBS) and resuspended in growth medium.

HIV-1 Challenge and Long-term Culture Assay—After transduction, SupT1 cells expressing the transgenes were challenged with HIV-$1_{NL4\text{-}3}$ at an MOI of 0.1. Following infection, the cells were washed three times with PBS and resuspended in growth medium. Mock infection was performed under the same conditions except that the supernatants were generated from control/vector-transduced cells. Half of the culture volume was harvested and replaced with an equal volume of fresh culture medium at regular intervals. The harvested

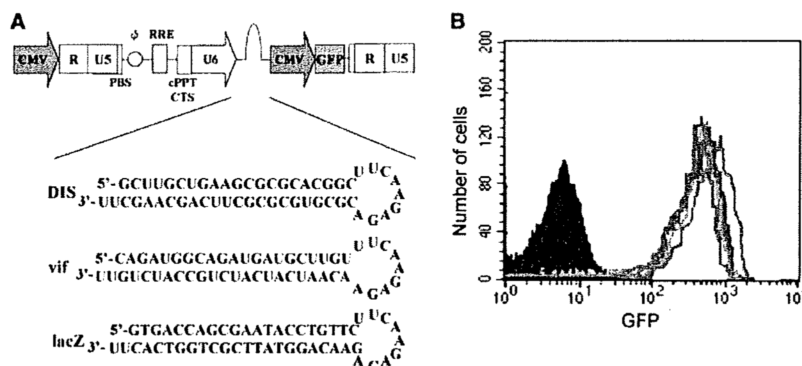

Fig. 1. **RNAi targets in the HIV-1 genome and the shRNA vector.** (A) Lentiviral vectors were designed to express shRNA against the following regions of the HIV-1 sequence: 246–266 nt of the DIS sequence, containing a stem–loop structure with six self-complementary nucleotides at the top; and 5049–5069 nt of the vif sequence. Expression of the shRNA was driven by the pol III. (B) The transgenic EGFP expression in SupT1 cells expressing vector transgenes was examined by FACS analysis using *CELLQUEST* software. Purple, (SupT1); green, CS-U6-DIS-shRNA; red, Cs-U6-vif-shRNA; blue, CS-U6-lacZ-shRNA; orange, CS-U6-ter.