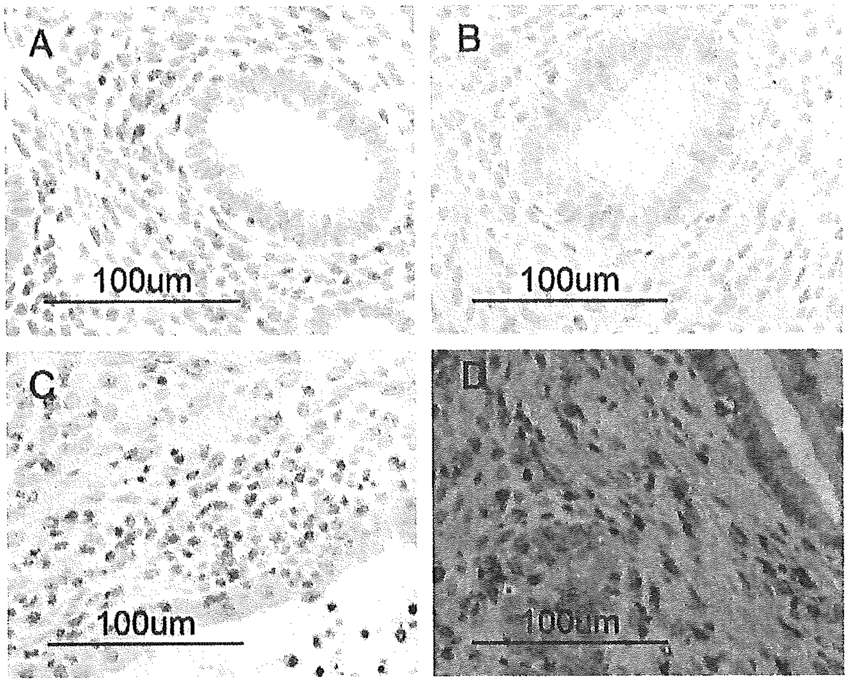

Figure 2. Immunohistochemical staining of human CD56. **(A)** Pretransplantation endometrial tissue: human CD56-positive cells are present in small numbers (magnification ×400). **(B)** Day 14 after transplantation: human CD56-positive cells have completely disappeared from the stroma (magnification ×400). **(C)** Day 21 after transplantation: human CD56-positive cells are present in small numbers (magnification ×400). **(D)** Day 28 after transplantation: human CD56-positive cells have significantly increased in number in the stroma (magnification ×400).

After 28 days hormone treatment was stopped, the decidual change ceased and tissue destruction accompanied by bleeding was observed in the stroma (Figure 1F). These findings suggest the occurrence of menstruation. By day 31, the mean serum concentrations of E_2 and progesterone were 0.9 pg/ml and 0.5 ng/ml, respectively. However, by day 35 the tissues contained many small narrow glands with columnar cells and pseudostratification of the nuclei was detected (Figure 1G). The stromal cells were dense, and mitotic figures were detected in both the glandular and stromal cells, suggesting the return of the proliferative phase.

Immunohistochemical analyses

A number of nuclei in the glandular and stromal cells were stained with Ki-67 on day 35 after transplantation (Figure 1H). Human CD56-positive cells were detected in small numbers before transplantation (Figure 2A); however, these had disappeared from the stroma by day 14 (Figure 2B). A few human CD56-positive cells were again observed in the stroma at day 21 (Figure 2C) and their numbers had significantly increased by day 28 (Figure 2D). Moderate numbers of human CD3-positive cells were detected before transplantation and at day 14; however, they had increased by days 21 and 28. Small numbers of human CD16-positive cells were detected in all specimens throughout the menstrual cycle; in contrast, human CD79a-positive cells were barely detected at any stage. Moreover, CD56-positive cells (Figure 3A) did not stain for CD16 (Figure 3B), and CD56-positive cells hardly stained for CD3 (Figure 3C) by single immunohistochemical staining of paired mirror-image sections for CD56 and CD16 and double staining for CD56 and CD3.

Discussion

We have demonstrated that human endometrial tissue transplanted into the subcutaneous space of NOG mice responded to sex-hormone treatment by showing the normal cyclical changes observed in the human endometrium. The recovery rate of the fragments was 94%, and the failures were probably caused by the transplants being too small to detect at day 35 (one contained only mouse tissue and the other only stromal tissue of human endometrium).

Several previous workers have transplanted human endometrial tissue into immunodeficient mice (Zamah *et al.*, 1984; Bergqvist *et al.*, 1985; Zaino *et al.*, 1985; Aoki *et al.*, 1994; Awwad *et al.*, 1999; Tabibzadeh *et al.*, 1999; Nisolle *et al.*, 2000a; b; Grummer *et al.*, 2001; Beliard *et al.*, 2002; Hull *et al.*, 2003). These experiments were highly variable

plus progesterone for 14 days, and then no hormones for the remaining 7 days. The glands are small and narrow with tall columnar cells. Evidence of pseudostratification of the nuclei is present. The stromal cells are dense (magnification ×400). **(H)** Endometrial tissue 35 days after transplantation: a number of nuclei have been immunohistochemically stained with Ki-67, showing proliferation of glandular and stromal cells (magnification ×400).

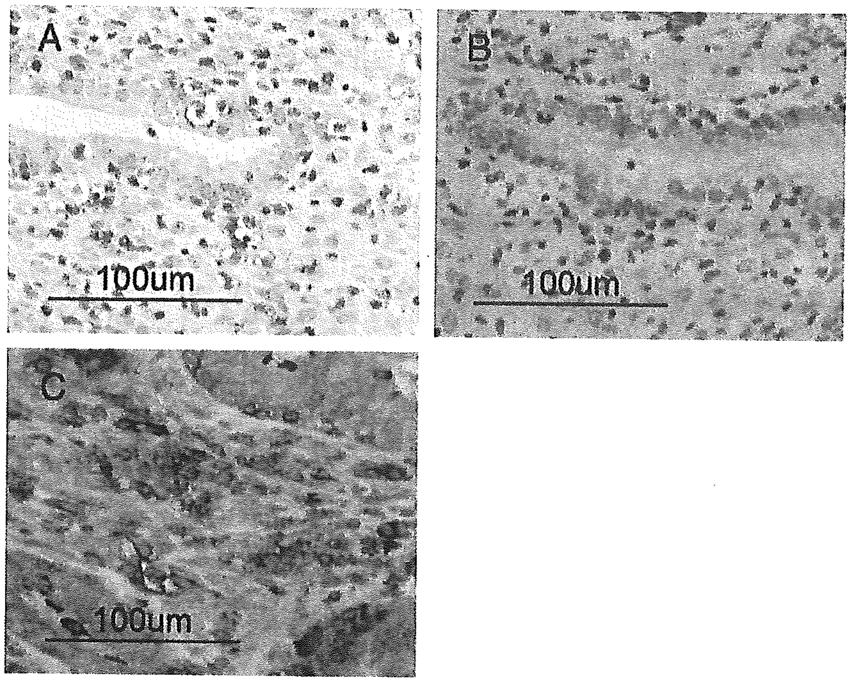

Figure 3. Paired mirror-image sections for CD56 (**A**) and CD16 (**B**), and double immunohistochemical staining section for CD56 and CD3 (**C**) at day 28: There are many CD56-positive cells in the stroma; however, they are not stained with CD16. There are many CD56-positive cells and also many CD3-positive cells in the stroma (brown deposit shows CD56-positive cells and the blue CD3-positive cells); there was minimal overlap in labelling for CD56 and CD3 (magnification ×400).

with respect to the strain of mouse used, the phase of the menstrual cycle of the transplanted tissue, the site of transplantation, whether or not ovariectomy was performed, whether or not the endometrium was pretreated and the method by which sex hormones were administered. However, despite these differences, most previous studies concluded that this system was a suitable model of endometriosis.

Zaino and colleagues transplanted human endometrial tissue into the subcutaneous space of ovariectomized athymic mice in four treatment groups (E_2, E_2 and progesterone, progesterone, and no exogenous hormone) and suggested that their model was useful for examining the histological response of normal endometrial tissue to sex hormones (Zaino *et al.*, 1985). The novelty of the approach described in the present report involves the simultaneous administration of sex steroids similar to those involved in the human menstrual cycle. Moreover, our model utilizes NOG mice that lack both T and B lymphocytes and NK-cell activity.

We observed many small narrow glands with columnar cells and pseudostratification of the nuclei in the grafted tissue 14 days after transplantation. Treatment with E_2 plus progesterone resulted in the following sequence of secretory changes in the endometrial tissue: subnuclear vacuolation and glandular secretion, followed by notable dilation of the glands, the presence of cuboidal glandular cells and the gradual decidualization of the stromal cells. This corresponds to the normal secretory phase of the endometrium. Furthermore, lymphocytes were observed during E_2 plus progesterone treatment in the mid-to-late secretory phase. After the sex-hormone treatment had ceased, tissue destruction and bleeding were observed in the stroma. We subsequently observed the reconstruction of the endometrium, the presence of small glands with columnar cells, pseudostratification of the nuclei, and mitoses in both the glandular and stromal cells. The proliferative activity was confirmed by immunohistochemistry. Ferenczy (1976) demonstrated that reconstruction of the eutopic endometrium during the early stage of the menstrual cycle is independent of ovarian hormone. Our findings suggest that the transplanted human endometrial tissue in our model returned to the proliferative phase, despite the low concentration of E_2.

The endometrial tissue in our system contained numerous lymphocytes at 21 and 28 days after transplantation. The number of large granular lymphocytes in human endometrial tissue has been reported to increase during the mid to late secretory phase, and CD56-positive, CD16-negative uterine NK cells have been identified (King *et al.*, 1989; Bulmer *et al.*, 1991; Klentzeris *et al.*, 1992) We detected small numbers of CD56-positive and CD16-negative cells on day 21, and their numbers had increased by day 28. These cells showed very little CD3 staining. It was also reported that CD3-positive cells increased from 4–7 days after the LH surge, and remained unchanged in number after that (Klentzeris *et al.*, 1992). In this model, moderate numbers of CD3-positive lymphocytes were detected at day 14; they increased on day 21 and similar numbers of these cells were detected on day 28. From these points of view, our model successfully maintained the human endometrial structure and reproduced normal menstrual changes. It is therefore suitable for studying the human endometrium both from a histological perspective and from the appearance of lymphocytes, such as CD56-positive NK cells and CD3-positive cells.

Dallenbach-Hellweg (1971) suggested that endometrial granulated lymphocytes develop from undifferentiated endometrial stromal cells. In this study, the human CD56-positive cells that were present in the stroma before transplantation completely disappeared by day 14, reappeared at day 21 and had significantly increased in number by day 28. As the mice used in this study lacked B and T lymphocytes and NK cells, these cells could not have been derived from the peripheral blood of the mice. Therefore, there are two possibilities of the origin of CD56-positive NK cells: one is that they differentiated from the transplanted endometrial stromal cells, the other is precursor cells were located within the transplanted endometrial tissue and they arose and differentiated gradually. It could be possible to investigate the origin of the uterine NK cells by using this model; however, further studies will be necessary to clarify this issue.

We noted some differences between the transplanted tissue in our model and the normal human endometrium. Spiral arteries were not observed in the transplants after either 21 or 28 days; this was probably due to the fragmentary nature of the endometrial tissues. Normally, the uterine arteries penetrate the uterine wall and advance into the middle layer of the myometrium, where they ramify into arcuate arteries. These divide into radial arteries, which, in turn, divide into basal arteries near the myoendometrial junction. The basal arteries ascend through the stratum functionale to the endometrial surface, where they become spiral arteries. However, the transplants comprised endometrial tissue only, and lacked arcuate and radial arteries. This resulted in a different pattern of vascularization to that of normal endometrial tissues, with an absence of spiral arteries. Menstruation has long been recognized as an ischaemic necrosis of the endometrium caused by contraction of the spiral arteries due to a decrease in the levels of the sex steroid hormones (Markee, 1940; Bartelmez, 1957). However, Hoopwood and Levison (1976) reported the presence of apoptotic bodies in the human endometrium, and recent reports have also pointed to the involvement of apoptosis in the endometrium during the menstrual cycle (Tabibzadeh, 1995; Kokawa *et al.*, 1996). Igarashi *et al.*, (2001) confirmed the increase of CD56-positive cells in the late secretory phase and suggested that CD56-positive NK cells induce the apoptosis of endometrial glandular cells associated with the onset of menstruation. Marbaix *et al.*, (1996) also demonstrated *in vitro* that matrix metalloproteinases were involved in the initiation of menstruation. In the present study, bleeding and tissue destruction were observed in the stroma 31 days after transplantation. These findings suggest that our model is suitable for investigating the mechanism of menstruation in the absence of spiral arteries.

We were careful to use only natural sex steroid hormones in our model, and the appropriate periods of administration and doses were determined in a preliminary study using one piece of endometrial tissue. Interestingly, 7 days of pretreatment with E_2 followed by 14 days of E_2 plus progesterone treatment did not result in decidualization of the stromal cells, despite the administration of high E_2 and progesterone concentrations. Furthermore, after treatment with E_2 for 28 days, the transplants largely comprised dilated glands that resembled endometrial hyperplasia. Therefore, 14 days was selected as the appropriate period of E_2 administration in order to reproduce the proliferative and late secretory phases of the normal endometrium. These findings indicate that our animal model might also be useful for studying endometrial dysfunction or hyperstimulation due to under- or overexposure to estrogen.

In conclusion, we have developed a mouse model of the human menstrual hormone cycle and the recurring changes that take place in the endometrium. This novel approach may be useful for identifying the mechanism of action of steroid hormones, the origin of uterine NK cells and the mechanism of initiation of menstruation, all of which have yet to be clarified.

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Sequence Note

Phylogenetic Heterogeneity of new HTLV Type 1 Isolates from Southern India in Subgroup A

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ABSTRACT

Seven isolates of human T cell leukemia virus type 1 (HTLV-1) were taken in southern India and phylogenetically analyzed to gain new insights into the origin and dissemination of HTLV-1 in the subcontinent. The new Indian HTLV-1s were found to be members of subgroup A (Transcontinental subgroup) of the Cosmopolitan group. They formed three different clusters (South African/Caribbean, Middle Eastern, and East Asian clusters). These results demonstrate that Indian HTLV-1s are genetically heterogeneous and include the most divergent strain of subgroup A. On the basis of these results, we speculate that subgroup A HTLV-1s may have been present for thousands of years in India.

HUMAN T CELL LEUKEMIA VIRUS type 1 (HTLV-1) is the causative agent of adult T cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[1–3] HTLV-1 has unique geographic and ethnologic distribution patterns. It is endemic mainly in Melanesia, the Caribbean basin, sub-Saharan Africa, and southwestern Japan and is highly prevalent among certain ethnic populations including Jews in Iran, the Ainu in Japan, Aboriginals in Australia, and Native Americans. These sporadic infections are thought to be vestiges of various migrations of HTLV-1 carriers from endemic areas, although how this distribution was established is not clear.

Seroepidemiological surveys have shown sporadic HTLV-1 infections in India. In a previous phylogenetic study,[4] Indian HTLV-1s were found to belong to the phylogenetic group that includes the Middle Eastern HTLV-1 strains, suggesting a possible link between these two areas. However, from that work it was unclear whether Indian HTLV-1 strains are indeed the closest to Middle Eastern strains, because clear phylogenetic separation of HTLV-1 strains could not be accomplished on the basis of the protein-encoding regions (*gag*, *pol*, *env*, and *pX* genes) analyzed in the previous work. In contrast to analyses based on these regions, analysis of the long terminal repeats (LTR) region permits finer separation of taxa within the genotype that includes Indian and Middle Eastern HTLV-1s. Thus, to gain new insights into the origin and dissemination of HTLV-1 in India, we phylogenetically analyzed seven new HTLV-1s from southern India on the basis of the LTR region.

The prevalence of HTLV-1 in southern India was examined by serological assays. Sera were collected in three districts of southern India (Kerala, Andhra Pradesh, and Tamil Nadu), and were tested by a particle agglutination (PA) test (Serodia HTLV-1; Fujirebio, Tokyo, Japan). Reactive sera in the PA test were further confirmed by an immunofluorescence assay as described previously.[2] Three serum samples were collected from Dravidian speakers living in Andhra Pradesh in southern India (Table 1) and phylogenetically analyzed. In addition, we analyzed HTLV-1s of four reported seropositives (two from the

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†We are deeply saddened to report that Palla-George Babu died in a traffic accident.

Table 1. Seven HTLV-1s Isolated from Southern India in This Study

HTLV-1 isolate	Place	Age (years)	Sex	Disease	Subject			Notes
					PA[a]	*Western blot*	*IFA*	
AP15	Andhra Pradesh	60s	M	Nervous disease	NI	NI	NI	Inhabits inland near the Tamil Nadu district
IND001	Andhra Pradesh	NI	F	NI	+	NI	NI	
IND002	Andhra Pradesh	11	NI	NI	NI	NI	NI	Blood sample on filter paper
IN2	Kerala	47	F	NI	NI	NI	NI	Wife of the subject from whom IN3 was isolated
IN3	Kerala	52	M	ATL	+(16)	+	+	A migrant worker in the Middle East
Ma1	Tamil Nadu	20	F	ARC	+(8192)	Not done	+	Also positive for HIV-1
TNA	Tamil Nadu	43	F	NI	+(512)	NI	NI	Also positive for HIV-1

Abbreviations: ARC, AIDS-related complex; ATL, adult T cell leukemia; IFA, immunofluorescence assay; NI, no information available; PA, particle agglutination test.
[a]Figures in parentheses represent the titer of HTLV-1 antibodies.

Tamil Nadu district[5] and the other two from the Kerala district[6]) (Table 1). The two seropositives from Kerala were also Dravidian speakers. Two of the three Andhra Pradesh subjects (from whom IND001 and IND002 were isolated) said that they had had blood transfusions. It is unknown whether the other five subjects had received blood transfusions. Of the two Tamil Nadu subjects, the one from whom TNA was isolated was a commercial sexual worker and the one from whom Ma1 was isolated had sexual contacts with prostitutes.

For all but one of the subjects, chromosomal DNA (containing HTLV-1 proviral DNA) was extracted from either cultured or uncultured peripheral blood mononuclear cells (PBMCs) by a standard procedure with proteinase K. For the subject from whom IND002 was isolated, chromosomal DNA was extracted from whole blood that was blotted onto filter paper.[4] Randomly selected regions (approximately 1 cm^2) of the filter paper were cut into more than 100 pieces with sterile scissors. The pieces were put into 3 ml of 0.85% NaCl solution and shaken at room temperature for 1 hr. Supernatants were centrifuged for 1 min to precipitate free PBMCs, which were utilized for the subsequent extraction of DNA.

The extracted DNA was subjected to nested polymerase chain reaction (PCR) to amplify a part of the LTR region that corresponds to nucleotide positions 99 to 685 of ATK, a prototypic Japanese HTLV-1 strain, with special care to avoid cross-contamination of the amplified products as described previously.[7] Throughout this study, all negative controls gave negative signals. Nucleotide sequences of a part of the amplified LTR fragments that correspond to positions 122 to 628 of ATK were determined in both directions, using an automated DNA sequencer (Applied Biosystems, Foster City, CA). Nucleotide sequences were aligned with the computer software CLUSTAL W with minor manual modifications. Phylogenetic trees were constructed by the neighbor-joining (NJ) method. For construction of NJ trees, bootstrapping was done to generate 1000 resamplings of the original sequence alignments, and pairwise genetic distances were estimated on each resampling by the Kimura two-parameter method. Phylogenetic trees were then constructed with CLUSTAL W and the trees were visualized by TREEVIEW. Detailed procedures and references for construction of the trees are described elsewhere.[7] The GenBank accession numbers for the new strains are AY607576 to AY607582.

We amplified a part of the LTR region of seven new HTLV-1s from India (Table 1). HTLV-1 isolates are phylogenetically separated into three major genotypes. Most HTLV-1 isolates from the world form a large group called the Cosmopolitan group or HTLV-1a. HTLV-1 isolates from Central Africa and those from Melanesia have diverged from those of the Cosmopolitan group, and are thus collectively called the Central African (HTLV-1b and -1d) and Melanesian (HTLV-1c) groups, respectively. The LTR sequences of the newly isolated HTLV-1s exhibited higher genetic similarities to HTLV-1 strains of the Cosmopolitan group (97.1%) than to those of the Central African (94.4%) and Melanesian groups (91.3%). The phylogenetic analysis also placed the newly isolated HTLV-1s into the Cosmopolitan group (Fig. 1). This group has been divided into five subgroups (A to E) on the basis of their LTR sequences. Each of the five subgroups closely correlates with the geographic origins of its HTLV-1 isolates. According to our data (Fig. 1), all of the new HTLV-1 strains belong to subgroup A (Transcontinental subgroup). This was confirmed by their restriction fragment length polymorphism (RFLP) profiles, which are consistent with those of subgroup A in our classification. Namely, the analyzed LTR fragments of the new HTLV-1s have *Nde*I and *Sac*I sites, but not *Dra*I and *Mae*III sites, which is consistent with the RFLP patterns of subgroup A strains. All of these data demonstrate that the prevalent genotype of HTLV-1 in southern India is subgroup A of the Cosmopolitan group.

Because all of the new Indian isolates belong to subgroup A (Fig. 1), their nucleotide alignments were compared with those of subgroup A strains. The new strains formed three different clusters (Fig. 2). One cluster contained two of the new strains (IND001 and IND002) together with South African and Caribbean HTLV-1s (Caribbean/South African/Indian cluster). Another cluster contained two other Indian HTLV-1s (AP15 and TNA) together with mostly Middle Eastern HTLV-1s (Middle Eastern cluster). The third cluster contained the other three Indian isolates (IN2, IN3, and Ma1) together with Japanese HTLV-1 strains. We tentatively refer to this cluster as the East Asian cluster. These clusters had the following characteristic nucleotide substitutions: HTLV-1s of the Caribbean/South African/Indian cluster have a G-to-A substitution at nucleotide position 246 in ATK, a C-to-T substitution at position 386, and an A-to-G substitution at position 575. Two of the Indian isolates (AP5 and TNA), like strains of the Middle Eastern cluster, have G rather than A at 240. It is noteworthy that within subgroup A, AP15 and TNA were phylogenetically located at the nodes of the Middle Eastern cluster, while Ma1 was branched off at the node of the East Asian cluster (Fig. 2). Collectively, these results indicate that subgroup A HTLV-1s circulating in India are polyphyletic.

The finding that HTLV-1s in southern India formed three different clusters indicates that the HTLV-1s of southern India are highly heterogeneous. This heterogeneity is in sharp contrast to the homogeneity of subgroup A HTLV-1s from other geographic areas. For example, HTLV-1s of South America and the Middle East form respective clusters (the Latin American and Middle Eastern clusters) within subgroup A. In addition, as shown in Fig. 1, India has the most divergent strain of subgroup A (SG).[8,9] The present analysis further showed that within subgroup A, some of the new Indian HTLV-1s are phylogenetically located at or branched off the node of the Middle Eastern and East Asian clusters. As a result, subgroup A HTLV-1s in southern India are clearly distinguishable from those of other geographic areas in that southern India has both the most divergent HTLV-1 and highly heterogeneous HTLV-1s.

These findings have an interesting implication with respect to the origin of HTLV-1 in India. Earlier studies hypothesized that HTLV-1 was introduced into India by recently migrated peoples such as African slaves several hundreds years ago, or by Jewish populations that migrated from the Middle East about 1000 to 1300 years ago.[4] However, our results indicate that Indian HTLV-1 is much more heterogeneous and includes more divergent strains than subgroup A HTLV-1 of other geographic origins such as Africa and the Middle East. This implies that HTLV-1 has been present in India for much longer than 1300 years.

If this is correct, how did HTLV-1 get to India? HTLV-1 isolates analyzed in the present study were isolated from the Dravidians, a population that migrated to India several thousand years ago. The high heterogeneity and divergence of HTLV-1 in southern India may be explained by the genetic vari-

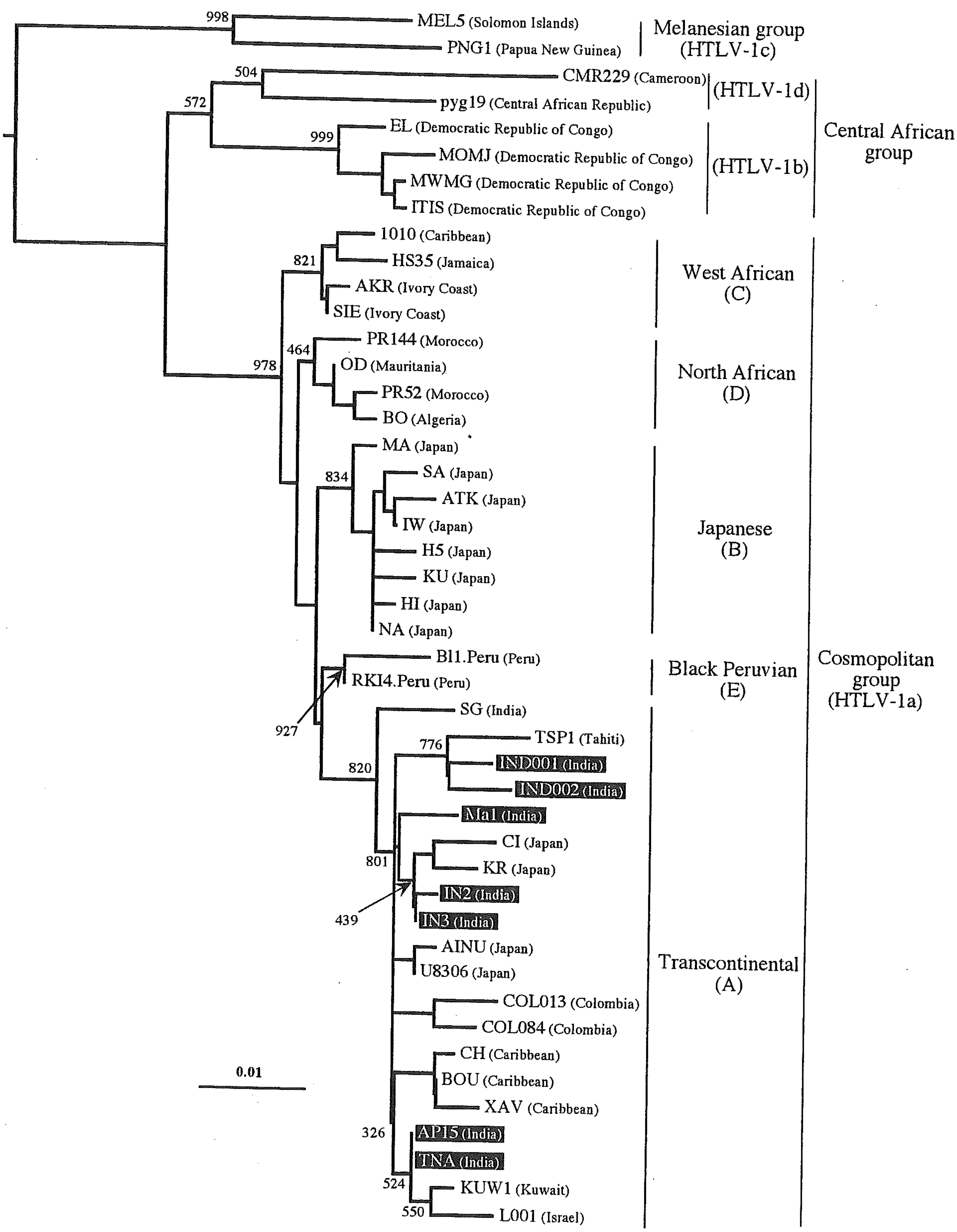

FIG. 1. Phylogenetic tree of HTLV-1 isolates based on a part of the LTR region (nucleotide positions 122–628 in ATK), showing the evolutionary relationships between the new isolates from India and isolates previously reported. Newly isolated HTLV-1s from India are highlighted. The tree was constructed by using the neighbor-joining (NJ) method. The scale at the bottom of the tree indicates the number of nucleotide substitutions per site. The horizontal branch lengths are proportional to the genetic distance. Numbers at nodes are bootstrap values. The tree was rooted with a prototype isolate of HTLV-2, MoT. The other DNA sequences used for construction of the phylogenetic tree have been described previously.[7]

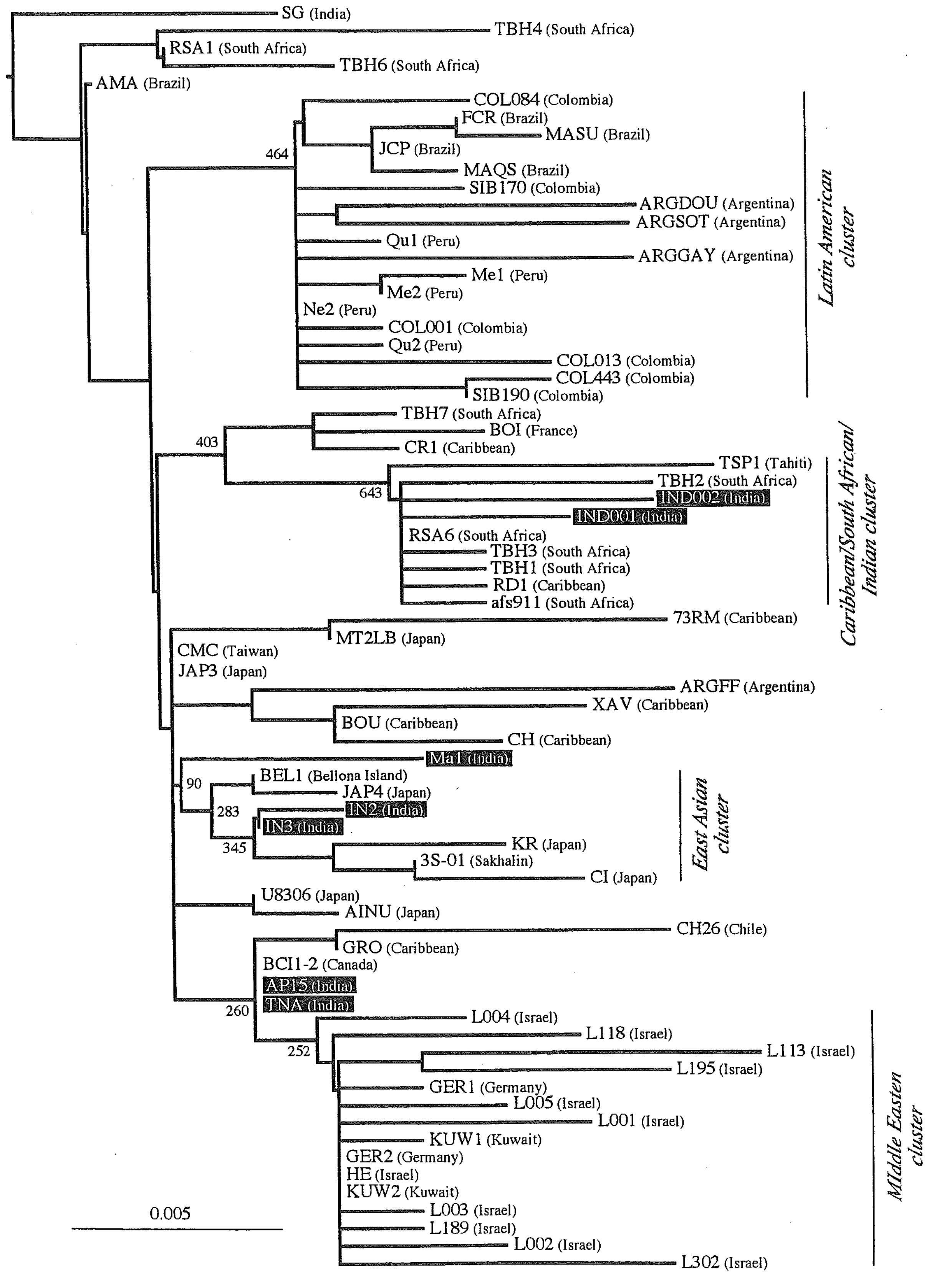

FIG. 2. Phylogenetic tree of HTLV-1 isolates that belong to subgroup A. The new isolates from India are boxed in black. The tree was constructed on the basis of a part of the LTR region (nucleotide positions 122–628 in ATK) by using the NJ method. The tree was rooted with strains of the Central African and Melanesian groups. For details, see the legend to Fig. 1.

ability of populations in the southern parts of India, where the Dravidians are the major inhabitants and HTLV-1 is highly prevalent.[10–12] The genetic diversity of the southern Indian populations was estimated to be as high as that of Africans and even higher than that of Europeans and other Asians based on sequence data of mitochondrial DNA and the allele frequency of several genetic loci.[13] Taken together, these findings imply that different lineages of the Dravidians carried different genotypes of subgroup A HTLV-1 when they reached India.

According to this scenario, some of the HTLV-1s in India could have been taken to other HTLV-1-prevalent areas such as the Middle East, South Africa, and the Caribbean basin. The genetic similarities between two of the new Indian isolates (AP15 and TNA) and the Middle Eastern isolates (Fig. 2), as well as the geographic proximity of India and the Middle East, strongly suggest movements of HTLV-1 carriers between these two areas, which is consistent with previous reports.[4] If this is the case, HTLV-1 might have been brought from India to the Middle East on the basis of the higher diversity among HTLV-1s in southern India than among Middle Eastern HTLV-1s (Fig. 2). Our results also show that two of the new isolates (IND001 and IND002) were phylogenetically related to South African and Caribbean HTLV-1s. This raises the possibility that some Indian HTLV-1s were introduced to South Africa and the Caribbean basin, as was previously proposed.[14] This possibility is consistent with the facts that more than a half million Indians migrated to the Caribbean basin as indentured laborers after the abolition of the trans-Atlantic slave trade in the early nineteenth century, and that South Africa served as a waystation during the migration.

After the putative introduction of Indian HTLV-1 to South Africa, some of the migrants may have gone back to India, possibly carrying human immunodeficiency virus type 1 (HIV-1). This is because the seropositivity against HTLV-1 among HIV-1 seropositives was significantly higher than that among HIV seronegatives in southern India.[11] This suggests that HTLV-1 was sexually transmitted among some HIV-1 seropositives in southern India. As Indian HIV likely originated from South Africa, some Indian HTLV-1s may have originated from the same place.

In summary, we speculate that Dravidian speakers originally carried HTLV-1 to southern India. This is supported by the following two points. First, the seven HTLV-1s isolated in southern India in this study are all in subgroup A and are highly heterogeneous. Second, India has the most divergent strain of subgroup A[9] (Fig. 1). Nonetheless, it is unclear how Dravidian speakers originally acquired HTLV-1. With respect to the origin of HTLV-1 of the Dravidians, it is interesting that the sickle cell gene haplotypes in southern India are the same as those in Africa,[15] and that the Dravidian languages have some similarities to those spoken in the Sahel Belt of Africa (from Sudan to Senegal).[16] It also remains unclear why HTLV-1s of Japan and South America are phylogenetically related to those of India. Future phylogenetic analyses of HTLV-1s in the Sahel Belt and South and Central Asia will help to identify the origin of Indian HTLV-1 and elucidate how it was disseminated in Asia.

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Solution RNA Structures of the HIV-1 Dimerization Initiation Site in the Kissing-Loop and Extended-Duplex Dimers

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Dimer formation of HIV-1 genomic RNA through its dimerization initiation site (DIS) is crucial to maintaining infectivity. Two types of dimers, the initially generated kissing-loop dimer and the subsequent product of the extended-duplex dimer, are formed in the stem-bulge-stem region with a loop including a self-complementary sequence. NMR chemical shift analysis of a 39mer RNA corresponding to DIS, DIS39, in the kissing-loop and extended-duplex dimers revealed that the three dimensional structures of the stem-bulge-stem region are extremely similar between the two types of dimers. Therefore, we designed two shorter RNA molecules, loop25 and bulge34, corresponding to the loop-stem region and the stem-bulge-stem region of DIS39, respectively. Based upon the chemical shift analysis, the conformation of the loop region of loop25 is identical to that of DIS39 for each of the two types of dimers. The conformation of bulge34 was also found to be the same as that of the corresponding region of DIS39. Thus, we determined the solution structures of loop25 in each of the two types of dimers as well as that of bulge34. Finally, the solution structures of DIS39 in the kissing-loop and extended-duplex dimers were determined by combining the parts of the structures. The solution structures of the two types of dimers were similar to each other in general with a difference found only in the A16 residue. The elucidation of the structures of DIS39 is important to understanding the molecular mechanism of the conformational dynamics of viral RNA molecules.

Key words: DIS, HIV-1, NMR, RNA, structure.

Abbreviations: DIS, dimerization initiation site; HIV-1, human immunodeficiency virus type 1.

Two molecules of viral genomic RNA are packaged in a dimeric state in the virion of human immunodeficiency virus type 1 (HIV-1), and this dimer formation is crucial to maintaining their infectivity (*1–4*). Accumulating evidence from both *in vivo* and *in vitro* experiments has shown that the specific sequence, the dimerization initiation site (DIS) located close to the 5′ terminus of the genomic RNA, is required for spontaneous dimerization of HIV-1 RNA. DIS can form a stem-loop structure with a self-complementary sequence in the loop and a bulge in the stem (*5, 6*). The dimerization of DIS forms the kissing-loop dimer as the first step; then, their intramolecular stems are converted into intermolecular stems, generating the extended-duplex dimer (*7, 8*). This two step dimerization process is called the kissing-loop mechanism. The kissing-loop dimer is converted into the extended-duplex dimer by incubation at 55°C (*9, 10*) or by incubation at physiological temperature with the HIV-1 nucleocapsid protein, NCp7, which includes two basic regions and two zinc-fingers (*11*). A number of experiments have been performed to gain an understanding of the role of the zinc-fingers as well as the basic regions (*12–16*). Our previous results show that, for the two step dimerization from the kissing-loop dimer to the extended-duplex dimer, the two basic regions surrounding the N-terminal zinc finger of NCp7 have RNA chaperone activity by themselves, and the zinc fingers increase the efficiency of the activity (*17, 18*).

A number of three dimensional structural analyses using NMR and X-ray methods have been performed to determine the conformation of each region of DIS, the loop region in the kissing-loop (*19, 20*) or extended-duplex dimers (*21–24*), as well as the bulge-out region (*25–27*). However, our previous studies suggested that the 39mer RNA sequence, DIS39, which covers the entire bulge and loop regions, is necessary and sufficient for the two step dimerization (*28, 29*). Thus, it is still relevant to determine the structures of the kissing-loop and extended-duplex dimers for DIS39 with the same sequence and conditions.

In the present study, we designed two shorter RNA molecules, loop25 and bulge34; loop25 includes the loop-stem region of DIS39, and bulge34 includes the stem-bulge-stem region (Fig. 1), respectively we then determined the solution structures of loop25 in each of the kissing-loop and extended-duplex dimers as well as bulge34. By combining the structure parts, the solution structures of DIS39 in the kissing-loop and extended-duplex dimers were able to be determined.

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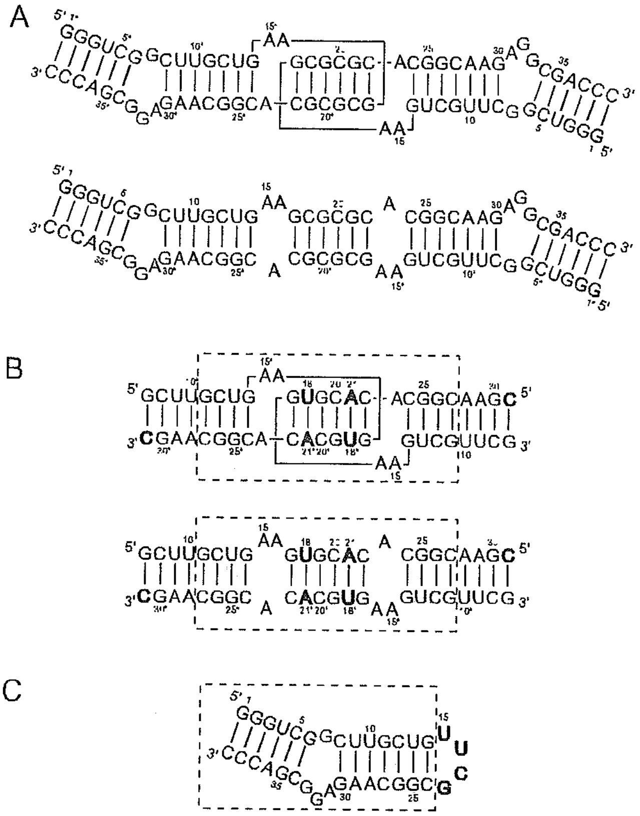

Fig. 1. **Secondary structure of a 39mer RNA corresponding to the dimerization initiation site (DIS39) and its fragments used in this study.** (A) The kissing-loop and extended-duplex dimers of DIS39. (B) The kissing-loop and extended-duplex dimers of loop25, which is composed of the loop and stem of DIS39. Modified residues are indicated by open characters. The sequence of the self-complementary loop was modified to increase the dispersion of NMR signals, and a base pair was added to the stem. The broken box indicates the part to be used for structure calculation. (C) Bulge34 consists of the stem-bulge-stem region of DIS39 and the connecting UUCG loop. The broken box indicates the part to be used for structure calculation. Gray shading indicates the two base pairs, C12–G26 and U13–G25, that are superimposed to combine the structures of the kissing-loop or extended-duplex dimer region and the stem-bulge-stem region. Asterisks indicate residues in the other strand.

MATERIALS AND METHODS

RNA Synthesis, Purification and Preparation—Non-labeled loop25 was synthesized chemically by the phosphoramidite method with an automatic DNA/RNA synthesizer, Expedite model 8909 (PerSeptive Biosystems Inc., MA, USA). The protection groups were removed with ammonia and tetra-*n*-butylammonium fluoride. Non-labeled DIS39 and bulge34 were synthesized enzymatically by the *in vitro* transcription reaction method with AmpliScribe T7 transcription kits (Epicentre Technologies Co., WI, USA). Purification for each RNA sample was performed by PAGE using 30 cm × 40 cm glass plates (Nihon Eido Co. Ltd., Tokyo, Japan) under denaturing conditions, and extensive desalting by ultrafiltration (Centricon YM3, Amicon Inc., MA, USA) was carried out. For stable isotopic labeling by the *in vitro* transcription with ^{13}C- and ^{15}N-labeled NTPs (Nippon Sanso, Tokyo, Japan), we used DIS39 rather than shorter loop25 and bulge34 because the efficiency of *in vitro* transcription is better for larger RNA.

For the preparation of the kissing-loop dimer, DIS39 or loop25 in water was incubated at 368 K for 5 min and chilled on ice for 5 min. Then, the solvent was adjusted to 1× PN-buffer [10 mM sodium phosphate (pH 7.0) and 50 mM NaCl] by adding concentrated buffer. For the preparation of the extended-duplex dimer, DIS39 or loop25 in 1× PN-buffer was incubated at 368 K for 5 min and slowly cooled to room temperature. Bulge34 was annealed by heating at 363 K for 5 min and snap-cooling on ice. To confirm the formation of the hairpin structure, the samples were subjected to a native PAGE experiment. For NMR measurements, RNA samples were dissolved in 10 mM sodium phosphate buffer (pH 7.0) containing 50 mM NaCl. The final concentration of chemically synthesized loop25 was 1.8 mM. The concentrations of DIS39 and bulge34 (transcripts) were 1.0 and 0.5 mM, respectively. The concentration of the kissing-loop and extended-duplex dimers of [G-$^{13}C/^{15}N$] and [A-$^{13}C/^{15}N$] DIS39 were 0.4, 0.3, 0.2 and 0.1 mM, respectively.

NMR Measurements—NMR spectra were recorded on Bruker DRX-500 and DRX-600 spectrometers. Spectra were recorded at probe temperatures of 283 to 303 K and NMR data at 298 K were used for structure calculation. The imino proton signal of the G and U residues in H_2O were distinguished from each other by the HSQC selected and HSQC filtered 1D spectra measured with ^{13}C and ^{15}N-labeled DIS39. Exchangeable proton NOEs were determined by 2D NOESY in H_2O with a mixing time of 150 ms using the jump-and-return scheme and gradient pulses for water suppression. For resonance assignments, well-established procedures were used (*30*). The H2 protons of adenosine were assigned based on a 2D HSQC experiment with natural abundance ^{13}C. NOE distance restraints from non-exchangeable protons were obtained from 2D NOESY experiments (mixing times of 50, 100, 200, and 400 ms) in D_2O. The intensities of the NOEs between exchangeable protons were interpreted as distances of 2.1–5.0 Å. For loop25, distances were estimated by analyzing the initial slope of NOE intensities for mixing times of 25, 50, 100, 200 ms. Judgment of intermolecular NOE is described in the result section. Two restraints (>5 Å) were added to the distance restraints based on the absence of NOE cross peaks in the case of the kissing-loop dimer. For bulge34, the intensities of NOEs due to nonexchangeable protons were interpreted as distances with a margin of −1.5 to +1.5 Å for the 100 ms 2D NOESY and −1.0 to +2.0 Å for the 200 ms 2D NOESY. Two restraints (>5 Å) were added to the distance restraints based on the absence of NOE cross peaks. The formation of hydrogen binding of G:C, A:U or G:U base pairs is interpreted as distance constraints as 1.8–2.1 Å for hydrogen and acceptor atoms and 2.8–3.2 Å for donor and acceptor atoms; G11:C27 to G14:C24, G11*:C27* to G14*:C24* and G17:C22* to C22:G17* for loop25 in the kissing-loop dimer, G11:C27* to G14:C24*, G11*:C27 to G14*:C24 and G17:C22* to C22:G17* for loop25 in the extended-duplex dimer, and G1:C39 to C5:G35 and U9:A29 to G14:C24 for bulge34. Dihedral restraints were obtained as described below. The absence of crosspeaks between H1′–H2′ in the 2D TOCSY and DQF-COSY experiments was interpreted as the residue being in the C3′-*endo* conformation. On the other hand, the presence of strong crosspeaks between H1′–H2′ in the 2D TOCSY and DQF-COSY experiments was interpreted as the residue being in the C2′-*endo* conformation. The correction of sugar puckering is interpreted as dihedral restraints for ν_2 as 40.00 ± 20.00° (C3′-*endo*) or −35.00 ± 20.00° (C2′-*endo*). Based on the sequential connectivity of the Watson-Crick and G-U base pairs, the RNA-A conformation was assumed for the stem region and dihedral restraints were introduced for backbone torsion angles (α, β, γ, δ, ε and ζ) as the ideal conformation with a margin of ±10.00°. For loop25 in the kissing-loop dimer, information about the C3′-*endo* conformation (G11–G14, G17–C27), the C2′-*endo* conformation (A16) and RNA-A conformation in the stem region (G11–U13, U18–A21, G25–C27) was used as the dihedral restraints. For loop25 of the extended-duplex dimer, information about the C3′-*endo* conformation (G11–G14, G17-C27) and RNA-A conformation in the stem region (G11–U13, U18–A21, G25–C27) was used as the dihedral restraints. For bulge34, the information about the C3′-*endo* conformation (G1–G14, C24–A31, C34–C39) and RNA-A conformation in the stem region (G1–C5, G11–G14, C24–C27, G35–C39) was used as the dihedral restraints.

Structure Calculation—A set of 100 structures was calculated using the simulated annealing protocol described below with the InsightII/Discover package, and the amber force field was used. The force constants were 100 kcal mol^{-1} Å^{-2} for distance restraints and 100 kcal mol^{-1} rad^{-2} for dihedral restraints. The starting coordinates were randomized, and the randomized structures were heated to 2,000 K in 5 ps, and the temperature was kept to 2,000 K for another 5 ps. After that, all restraints were increased to full values in 20 ps, then, decreased to 1/10 of full values in 5 ps at 2,000 K. Van der Waals radii were increased from 0.1 to 0.825 in 20 ps at 2,000 K. All restraints were increased to full value again in 10 ps at 2,000 K. Scalings for non-bond interactions were increased to full value in the next 20 ps at 2,000 K, and the temperature was kept to 2,000 K for another 5 ps. Then, the temperature was gradually scaled to 300 K in 10 ps. After that, the structure was heated from 300 to 1,000 K in 5 ps, and the van der Waals radii were increased from 0.825 to 1 at 1,000 K, and then decreased from 1 to 0.825 at 1,000 K. An additional 5 ps of dynamics was performed at 1,000 K, and the temperature was gradually scaled to 300 K for 10 ps. A final minimization step was performed, which included a Lennard-Jones potential and electrostatic terms with a dielectric constant of 7. The ten final structures with the lowest total energies were chosen.

RESULTS AND DISCUSSION

Analysis of the NMR Spectra of DIS39, Loop25 and Bulge34—Our previous NMR study revealed that the two types of dimers of DIS39 prepared as described in "MATERIALS AND METHODS" correspond to the kissing-loop and extended-duplex dimers (*31*). NMR spectra of DIS39 in each of the kissing-loop and extended-duplex dimers were measured in D_2O, and the signals due to H1′, H6/H8 were assigned by the sequential assignment method (Fig. 2). Figure 3A shows the difference in the chemical shift of H1′, H6/H8 between the two types of dimers. It was found that the difference is concentrated in the loop region. Interestingly, structures of the stem-bulge-stem region of the kissing-loop and extended-duplex dimers were extremely similar, even though the stems are formed by intra and inter molecules. This was also shown by analysis of the TOCSY spectrum; differences are located in the loop regions. Most residues were adapted to the C3′-*endo* conformation except for G32, G33 in the bulge-out region of both forms, A16 in the kissing-loop dimer and A15, A16 in the extended-duplex dimer, which might be a mixture of the C2′-*endo* and C3′-*endo* conformations.

To reveal further authentic structure, two RNA molecules were designed; loop25 includes the loop region and bulge34 includes the stem-bulge-stem region (Fig. 1, B and C). Loop25 was constructed to determine the authentic structure of the loop region. In order to increase the dispersion of the NMR signals, the sequence of the loop was modified from GCGCGC to GUGCAC. One base pair was added by replacing A31 by C31 in the stem to increase the stability of the kissing-loop dimer. It is noted that the loop sequences of GCGCGC and GUGCAC correspond to those of HIV-1 subtypes B and F (*32*), respectively, and both sequences have dimerization activity (*6, 9, 10*). The chemical shifts of loop25 were compared with those of DIS39 in each of the kissing-loop and extended-duplex dimers (Fig. 3, B and C). For both conformations, the chemical shifts for most of the stem region and A15, A16 and A23 were strikingly similar between the loop25 and DIS39. Due to the base alterations, the chemical shifts of the self complement loop were slightly different for both dimers. The chemical shift of H8 was shifted more than 0.2 ppm due to the addition of the terminal base pair. It is noteworthy that the chemical shift difference in loop25 between the kissing-loop and extended-duplex dimers (Fig. 3D) was almost identical to that of DIS39 (Fig. 3A). These results indicate that the structures of loop25 in the kissing-loop and extended-duplex dimers are essentially identical to those of DIS39. Upon analysis of the TOCSY spectrum, it was found that most of the residues were adapted to the C3′-*endo* conformation except A15 and A16 for the extended duplex dimer and A16 for the kissing loop dimer, and these results also agree with the results for DIS39.

Bulge34 was constructed to determine the authentic structure of the stem-bulge-stem region. Bulge34 consists of the stem-bulge-stem region of DIS39 and the connecting UUCG loop. The NMR signals of bulge34 were assigned by the sequential assignment technique. The chemical shift of H1′, H6/H8 of bulge34 were compared to those of DIS39 in the kissing-loop dimer (Fig. 3E). The chemical shifts for the stem-bulge-stem regions of bulge34 and DIS39 were identical, although the chemical shifts of the residues adjacent to the loop were slightly different by reflecting the difference in the closing loop sequences. Upon analysis of the TOCSY spectrum, it was found that most residues were adapted to the C3′-*endo* conformation except for G32, G33 in the bulge-out region and C in the UUCG loop, and that the conformation in the stem-bulge-stem region also agreed with that of DIS39. These results indicate that the structure of the stem-bulge-stem region of bulge34 is identical to that of DIS39.

Thus, the structures of DIS39 for two types of dimers can be determined by determining the structures of loop25 and bulge34, and combining them.

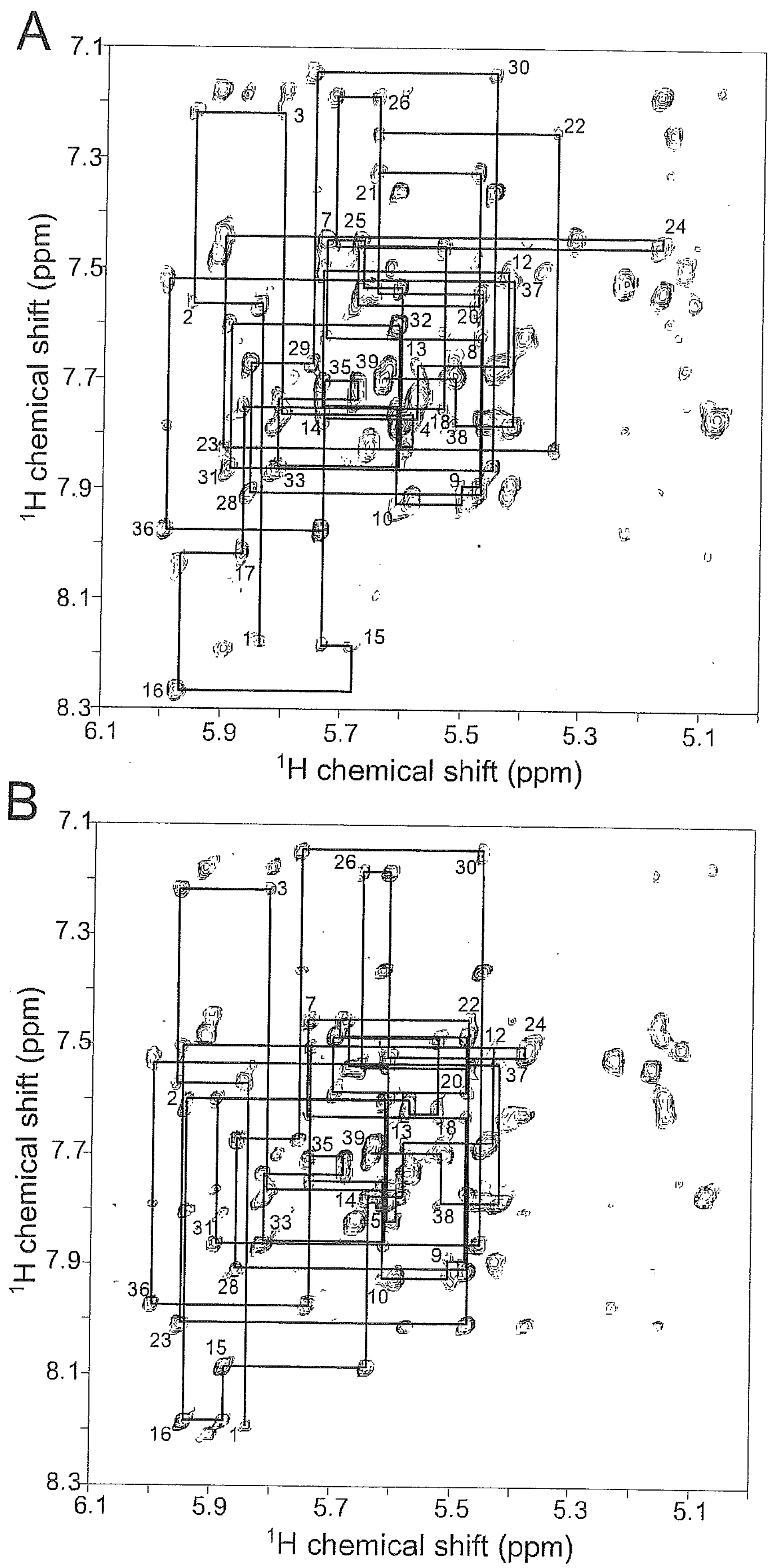

Fig. 2. **2D NOESY spectra of the (A) kissing-loop and (B) extended-duplex dimers of DIS39 measured in D_2O at 25°C with a mixing time of 200 ms.** Cross-peaks between aromatic H6/H8 protons and ribose H1′ protons are shown, and the sequential NOE connectivity is indicated.

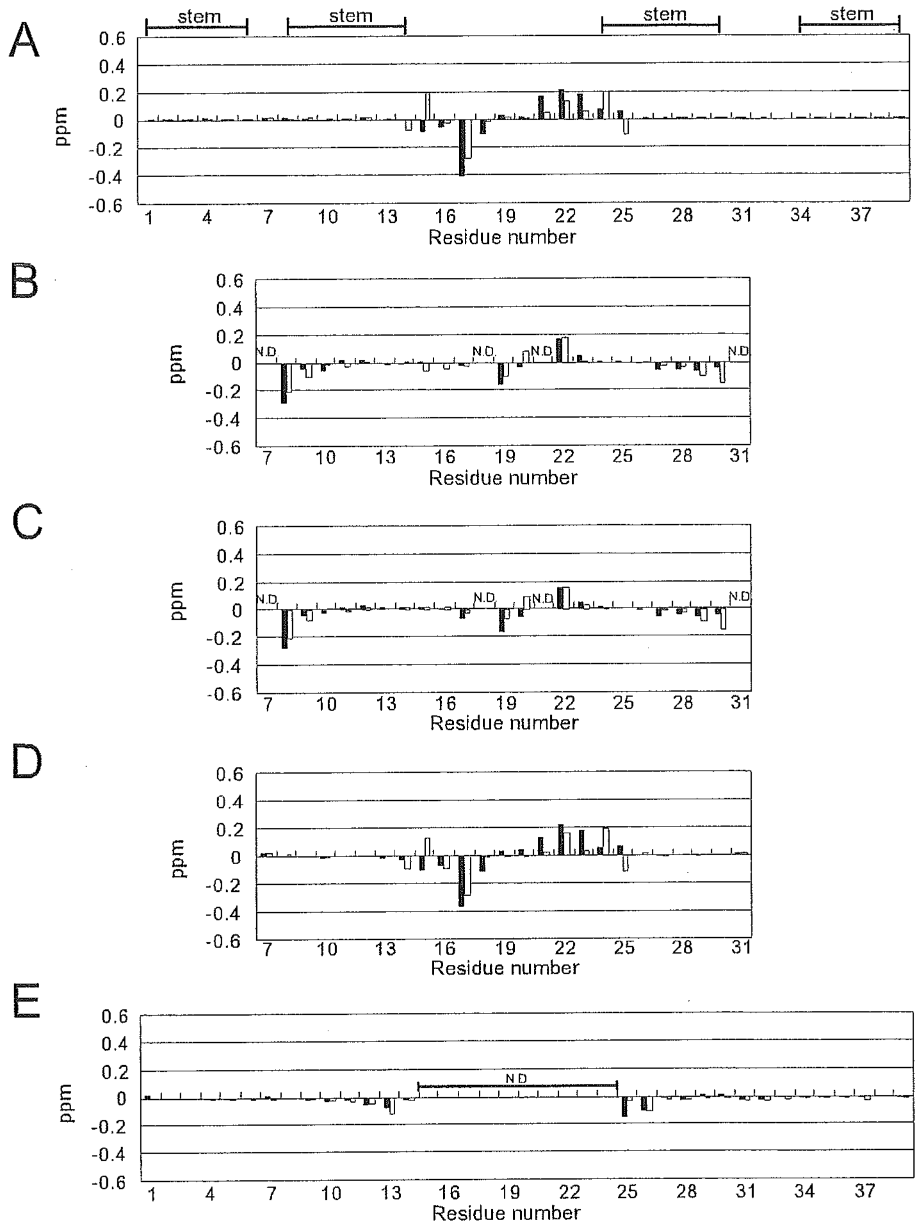

Fig. 3. **Chemical shift differences for H6/H8 and H1′.** Filled and open bars indicate H6/H8 and H1′, respectively. (A) Chemical shift differences between the kissing-loop and extended-duplex dimers of DIS39. Lines above the graph indicate the stem regions. (B) Chemical shift differences between DIS39 and loop25 in the kissing-loop dimer (data for replaced residues 7, 18, 21 and 31 are not shown). (C) Chemical shift differences between DIS39 and loop25 in the extended-duplex dimer (data for replaced residues 7, 18, 21 and 31 are not shown). (D) Chemical shift differences between the kissing-loop and extended-duplex dimers of loop25. (E) Chemical shift differences between DIS39 and bulge34 in the kissing-loop dimer (data for residues 15–24 are not shown).

Structure Determination—The loop region of loop25 in the kissing-loop dimer: To determine the structure of the loop region of DIS39 in both the kissing-loop and extended-duplex dimers, the NMR signals of loop25 were further analyzed and structural information was collected. The structure of the loop region consisting of the nine nucleotide loop and the stem with four base pairs was determined as shown by the broken box in Fig. 1B. A total of 286 distance restraints, 76 hydrogen bonding distance restraints, 140 dihedral restraints (Table 1), and 136 chiral restraints were used for the structural calculation. Three NOEs in the loop region, H2(A21)–H1′(U18), H2(A21)–H1′(G19) and H2(A21)–H8(G19), were judged to be intermolecular by analysis of the imino proton spectra. Four NOEs in the stem-loop linking region were considered to be intermolecular or intramolecular based on the results of the isotope filter NMR measurement (data not shown), and it was concluded that two NOEs, H2(A23)–H1′ (G17), H2(A16)–H1′ (G16), are intermolecular and three NOE, H8(A16)–H1′ (A16), H8(A16)–H2′ (A16), are intramolecular. One NOE in the stem-loop linking region was considered to be intermolecular or intramolecular in the structure calculation, and it was concluded that this NOE, H2(A23)–H2(A15), is intramolecular. Each restraint is used twice for two molecules. The structures were calculated by the restrained molecular dynamic calculation with the simulated annealing method. The structure was defined with a heavy atom r.m.s.d. of 2.14 Å for the ten converged structures (Fig. 4A, left panel), and the minimized average structure is shown in Fig. 4A (right panel). Although the overall convergence was not very good, the self-complementary region was well defined with 0.76 Å, and the stem-loop linking region was defined with 1.86 Å. The structural statistics are summarized in Table 1.

The loop region of loop25 in the extended-duplex dimer: The loop region of loop25 in the extended-duplex dimer was

Table 1. **NMR restraints and structural statistics.**

	Number of restraints		
	loop25 in the kissing-loop dimer (17 mer × 2)	loop25 in the extended-duplex dimer (17 mer × 2)	bulge34 (30 mer)
Distance restraints	286	384	345
imino-imino	12	12	10
intra residue	154	182	163
intra molecule	106	174	170
inter molecule	12	16	–
>5 Å	2	0	2
Hydrogen bonding distance restraints	76	76	58
Dihedral restraints	140	138	126
3′-*endo*	30	30	28
2′-*endo*	2	0	0
RNA-A stems	108	108	98
r.m.s.d. from the idealized geometry (Å)			
Bonds (Å)	0.00897 ± 0.00004	0.00803 ± 0.00020	0.00775 ± 0.00015
Angle (°)	2.43 ± 0.23	2.33 ± 0.05	2.24 ± 0.07
Impropers (°)	1.57 ± 0.10	1.82 ± 0.64	1.53 ± 0.21
Heavy-atoms r.m.s.d. (Å)[a]			
All	2.14	1.45	1.98
Stem-loop linking region[b]	1.86	1.31	
Bulge region[c]			1.90

[a]Averaged r.m.s.d. between an average structure and the 10 converged structures were calculated. The converged structures did not contain experimental distance violations >0.2 Å or dihedral violations >5°, [b]The stem-loop linking region consists of residues 14 to 17, 22 to 24, 14* to 17* and 22* to 24*, [c]The bulge region consists of residues 6 to 10 and 28 to 34. Asterisks indicate residues in the other molecule.

determined (broken box in Fig. 1B). A total of 384 distance restraints, 76 hydrogen bonding distance restraints, 138 dihedral restraints (Table 1) and 136 chiral restraints were used for the structure calculation. For the stem-loop linking region, H2 of A23 was connected by intermolecular NOEs to H1′ and H2 of A15, H2 of A16 and H1′ of G17. The structures were calculated by the restrained molecular dynamic calculation with the simulated annealing method described above. The structure was well defined with a heavy atom r.m.s.d. of 1.45 for the ten converged structures (Fig. 4B, left panel), and the minimized average structure is shown in Fig. 4B (right panel). The stem-loop linking region was defined with 1.31 Å. The structural statistics are summarized in Table 1.

The stem-bulge-stem region of bulge34: A structural determination was performed for bulge34 except for the UUCG loop (broken box in Fig. 1C). A total of 345 distance restraints, 58 hydrogen bonding distance restraints, 126 dihedral restraints (Table 1) and 120 chiral restraints were used for the structure calculation. Two NOE restraints (>5 Å), H2(A31)–H1′(U9) and H1′(A31)–H1′(U9), were added to the distance restraints based on the absence of NOE cross peaks. The structures were calculated by the restrained molecular dynamic calculation with a simulated annealing protocol. The structure was defined with a heavy atom r.m.s.d. of 1.98 for the ten converged structures (Fig. 4C, left panel), and the minimized average structure is shown in Fig. 4C (right panel). Although the overall convergence is not very good, the stem regions are well defined with 0.83 or 0.78 Å, respectively. The bulge region was defined with 1.90 Å. The structural statistics are summarized in Table 1.

The two types of dimers of DIS39: Solution structures of DIS39 were then constructed by combining the structure parts. The structures of the kissing-loop or extended-duplex dimer region and stem-bulge-stem region were combined by superimposing two base pairs, C12:G26 and U13:G25 (Fig. 1, gray area). The left panels of Fig. 5 show the ten structures prepared by using the minimized average structure of the stem-bulge-stem region (Fig. 4C, right) and each of the ten lowest energy structures of the loop region (Fig. 4, A or B, left) superimposed by the loop region. The right panels of Fig. 5 show the structures prepared using the minimized average structure of the stem-bulge-stem region (Fig. 4C, right) and the loop region (Fig. 4, A or B, right). The relative angles between the stem-bulge-stem regions differ between the kissing-loop and extended-duplex dimers as shown in the right panels of Fig. 5. However, the fluctuations of the relative angles are rather large and the ranges overlap between the two dimers as shown in the left panels of Fig. 5. In fact, the values of the residual dipolar coupling for the stem-bulge-stem region are similar between the kissing-loop and extended-duplex dimers (to be published). A preliminary normal mode analysis suggested the existence of hinge motion, and, in order to reveal the dynamic properties of the dimers, a molecular dynamics analysis, as well as the thermodynamics analysis (*33*), is required. The most obvious local difference was observed for A16; for the kissing-loop dimer, A16 was close to the same residue in the other molecule (Fig. 6A, left) and did not stack above A15 of the same molecule nor G17 of the other molecule (Fig. 6A, right), whereas for the extended-duplex dimer, A16 was apart from the same residue of the other molecule (Fig. 6B, left) and stacked between A15 and G17 (Fig. 6B, right).

Structural Comparison with Related Structures— Ennifar *et al.* (*20*) determined the crystal structure of

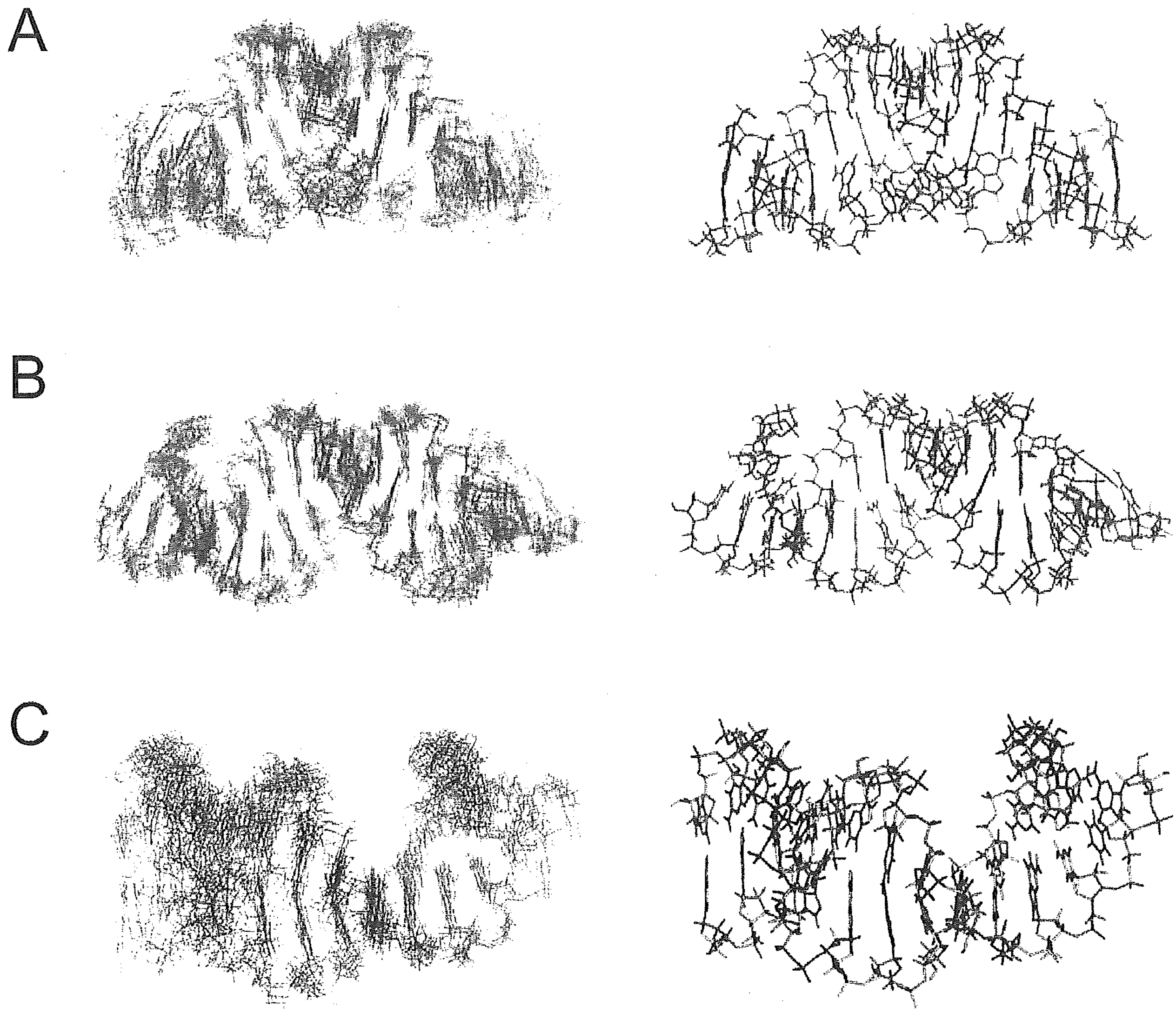

Fig. 4. **Solution structures of each part of DIS39.** Left panels show the superimposition of the 10 lowest energy structures and the right panels show the minimized average structures. (A) The loop region of loop25, as shown by the broken box in Fig. 1b, in the kissing-loop dimer. Each strand is colored in red or blue. (B) The loop region of loop25 in the extended-duplex dimer. (C) The stem-bulge-stem region of bulge34.

the kissing-loop dimer. The present structure is similar to the crystal structures in general, except for A15 and A16. In the present structure, A15 stacks on G14 and A16 interacts with the same residue in the other molecule (Fig. 6A, right). On the other hand, in the crystal structure, A15 and G16 are flipped out (*20*). It is noted that the numbering system of DIS39 is used for other structures for convenience, and position 16 is occupied by A or G depending on the strain. A15 and A16 (or G16) might be flexible and can be flipped out even in solution. Mujeeb *et al.* (*19*) determined the solution structure of the kissing-loop dimer. In this structure, A16 interacts with A15 and C24 in the other molecule, and, as a result, the distance between the two stems is relatively short. Thus, this restricted interaction makes the global structure different from the present structure and the crystal structure. However, the location of A15 is similar in the two solution structures. The difference in the conformation of A16 between the two solution structures may reflect the difference in the sequence of the stem adjacent to the loop and/or in the sample condition, including the salt concentration. The NOE connectivity determined in the present study agrees in general with those of Dardel *et al.* who analyzed the structure of the stem-loop region in the kissing-loop dimer by NMR (*34*).

Girard *et al.* (*21*) and Mujeeb *et al.* (*22*) determined the solution structures of extended-duplex dimers. In these two structures, as well as in the present structure, A15, A16 and A23 form a zipper like structure (Fig. 6B, right). On the other hand, in the case of the crystal structure of the extended-duplex dimer, G16 forms a G:A base pair and A15 is flipped out, and it was assumed that this in-out bulge transconformation is magnesium-dependent (*23*).

Structures of the stem-bulge-stem region were shown by Lawrence *et al.* (*26*) and Yuan *et al.* (*27*). In the solution structure determined by Lawrence *et al.* (*26*), continuous stackings were formed for each strand, G6-C8 and

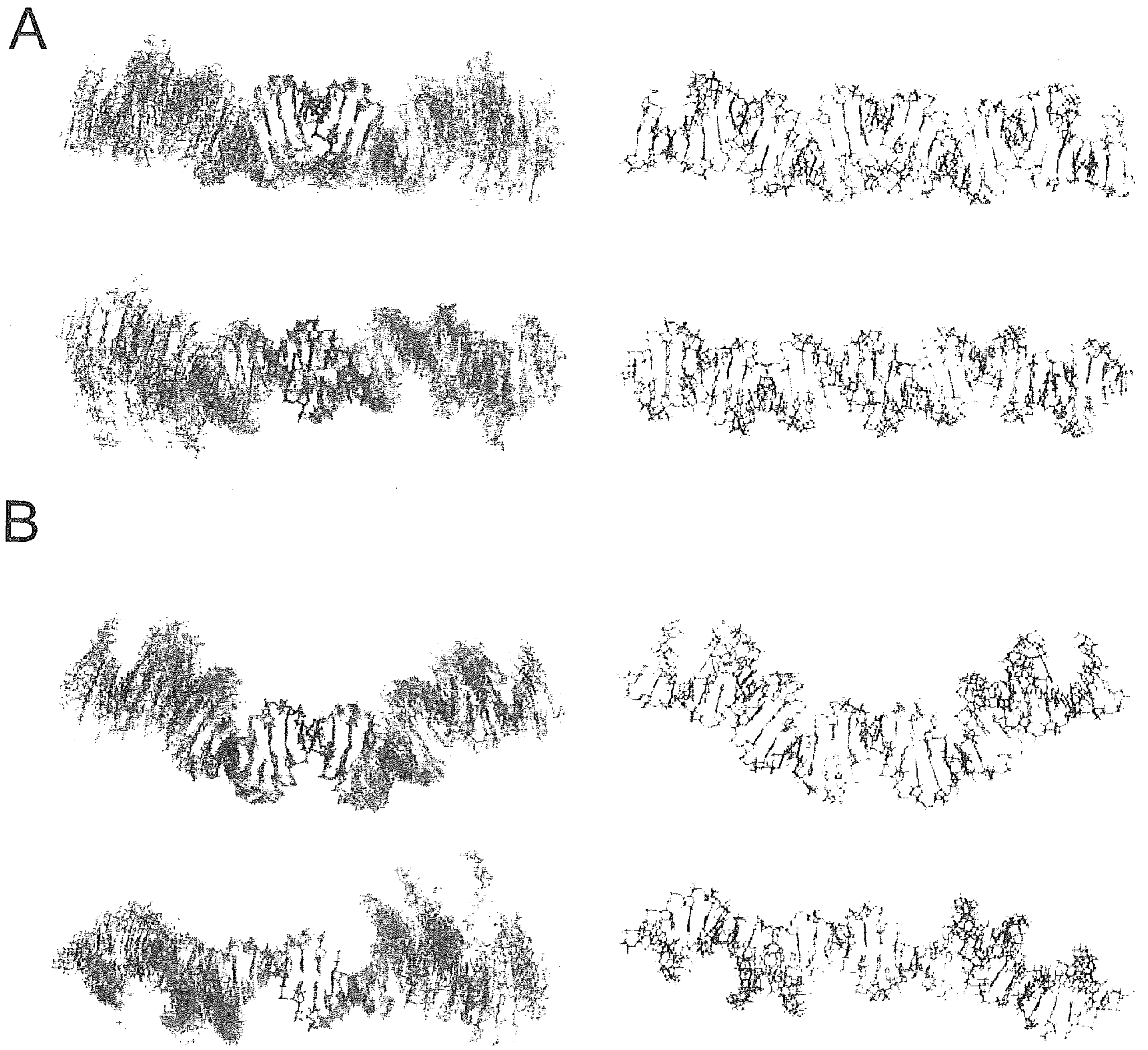

Fig. 5. **Solution structures of the (A) kissing-loop and (B) extended-duplex dimers of DIS39.** Left panels show the structures constructed by combining the structures of the loop (the 10 lowest energy structures of the kissing-loop or extended-duplex dimers) and the stem-bulge-stem (minimized average structure) regions. Right panels show the structures constructed by combining the minimized average structures of the loop and stem-bulge-stem regions. The two regions were combined by superimposing two base pairs, C12–G26 and U13–G25 (Fig. 1, gray area). Each strand is colored in red or blue and views from two different directions are shown.

G30-C34. Yuan *et al.* (*27*) showed that G7 and A31 form a base pair, and that G33 is not always stacked on G32 or C34, and, in general, the present structure is identical to the latter structure. Greatorex *et al.* (*25*) showed that the bulge region is too flexible to determine the conformation. These conformational differences may be caused by differences in the stability of the terminal stem. Lawrence *et al.* (*26*) adopted a stable 7 base-pair stem, and their structure forms an ordered conformation in the bulge region. In contrast, Greatorex *et al.* (*25*) adopted an unstable 4 base-pair stem and the bulge region is flexible. Yuan *et al.* (*27*) adopted a 4 base-pair stem and a flanking adenosine residue at the 3′ terminal that must stabilize the stem. In the present study, a 6 base-pair stem was used.

Mechanism of the Two Stem Dimerization—Between the kissing-loop and extended-duplex dimers, A16 shows the most drastic change in interaction with other residues, suggesting that A16 is the key residue in the two step dimerization reaction. The difference in the A16 conformation among structures with different sequences and determined under different conditions as described above, also suggests the importance of this residue. Mujeeb *et al.* (*19*, *22*) also pointed out the flexibility around the junction of the loop and the stem of DIS in the kissing-loop and

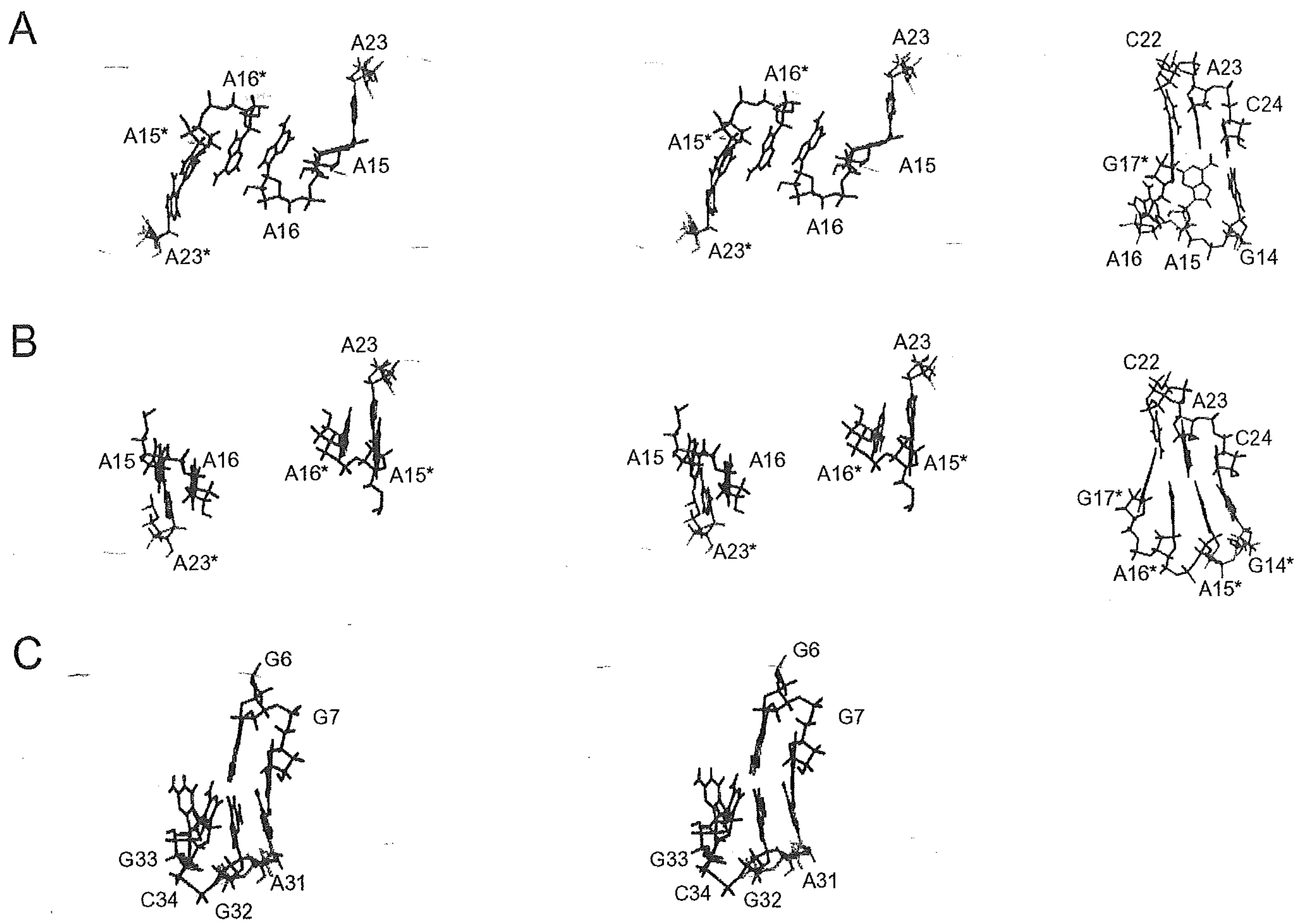

Fig. 6. **Structures of the linking regions.** (A) Regions linking the stem and loop in the kissing-loop dimer. The left panels show the positions of A15, A16, and A23 in the entire structure in a stereo view, and the right panels show residues linking the stem and loop. Asterisks indicate residues in the other strand. (B) Regions linking the stem and loop in the extended-duplex dimer. (C) The bulge region linking the two stems.

extended-duplex dimers. Imino proton signals due to U9:A29 and U10:A28 are much broader than other signals in the stem region, and no imino proton signal due to C8:G30 was observed. Thus, the stem between the loop and bulge is destabilized by the bulge region. Our previous experiments also showed that the bulge region is required for the two-step dimerization to adjust the thermal stability of DIS, and Greatorex *et al.* (*25*) also indicated that the flexibility of the bulge region is critical based on the fact that mutations in the bulge region strongly affect the melting temperature, as well as the fact that none of the wild-type sequences in the bulge region that increase the melting temperature is ever found in wild-type viruses. Thus, the conformational conversion from the kissing-loop dimer to the extended-duplex dimer might require two factors, the movement of A16 and the modest stability of the stem caused by the presence of the bulge region.

In the present study, a set of structures corresponding to the initial and final structures of the two-step dimerization of DIS are provided; these structures will promote studies to elucidate the molecular mechanism of the conformational change in the two-step dimerization, including an analysis of the interaction between DIS and NCp7, in addition to the molecular dynamics approach.

Coordinates: The structure has been deposited in the Protein Data Bank (accession code 2D17: the stem-bulge-stem region of bulge34, 2D18: the extended-duplex dimer of loop25, 2D19: the kissing-loop dimer of loop25, 2D1A: the extended-duplex dimer of DIS39 and 2D1B: the kissing-loop dimer of DIS39).

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