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Structural requirement for the two-step dimerization of human immunodeficiency virus type 1 genome

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ABSTRACT

Generation of RNA dimeric form of the human immunodeficiency virus type 1 (HIV-1) genome is crucial for viral replication. The dimerization initiation site (DIS) has been identified as a primary sequence that can form a stem-loop structure with a self-complementary sequence in the loop and a bulge in the stem. It has been reported that HIV-1 RNA fragments containing the DIS form two types of dimers, loose dimers and tight dimers. The loose dimers are spontaneously generated at the physiological temperature and converted into tight dimers by the addition of nucleocapsid protein NCp7. To know the biochemical process in this two-step dimerization reaction, we chemically synthesized a 39-mer RNA covering the entire DIS sequence and also a 23-mer RNA covering the self-complementary loop and its flanking stem within the DIS. Electrophoretic dimerization assays demonstrated that the 39-mer RNA reproduced the two-step dimerization process, whereas the 23-mer RNA immediately formed the tight dimer. Furthermore, deletion of the bulge from the 39-mer RNA prevented the NCp7-assisted tight-dimer formation. Therefore, the whole DIS sequence is necessary and sufficient for the two-step dimerization. Our data suggested that the bulge region regulates the stability of the stem and guides the DIS to the two-step dimerization process.

Keywords: DIS; electrophoresis; HIV-1; kissing-loop; NCp7; nucleocapsid protein; RNA

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus identified as a cause of the acquired immunodeficiency syndrome (AIDS). As in all other retroviruses, the HIV-1 genome extracted from the virion consists of two identical genomic RNAs that are noncovalently linked near their 5′ ends by a dimer linkage structure, demonstrated by electron microscopy (Hoglund et al., 1997). This dimerization event during virion formation has been shown to be an essential step in influencing its infectivity (Paillart et al., 1996b; Clever & Parslow, 1997; Laughrea et al., 1997). The specific sequence required for spontaneous dimerization of HIV-1 5′ RNA fragments has been identified and named as the dimerization initiation site (DIS), which can form a stem-loop structure with a self-complementary sequence in the loop and a bulge in the stem (Laughrea & Jette, 1994; Skripkin et al., 1994). As a dimerization mechanism of the HIV-1 genomic RNA, it has been proposed that 6 nt in the DIS stem-loops first hybridize each other (a kissing-loop dimer) and then the intrastrand stems isomerize to form a stable interstrand duplex (an extended-duplex dimer), which is called the kissing-loop model (Laughrea & Jette, 1994; Skripkin et al., 1994).

Experimental results supporting this model have been accumulating (Laughrea & Jette, 1994, 1996a, 1996b; Paillart et al., 1994, 1996a, 1996b, 1997; Skripkin et al., 1994, 1996; Muriaux et al., 1995, 1996a, 1996b; Clever et al., 1996; Haddrick et al., 1996; Laughrea et al., 1997; Clever & Parslow, 1997). Among them, an especially important finding is that the dimerization is composed of a two-step process, as described below. In vivo experiments have demonstrated that the dimeric RNA in virion particles is converted to a heat-stable dimer as a viral protease-dependent maturation, resulting in the cleavage of the gag precursor to generate nucleocapsid protein, which has been shown initially for Moloney murine leukemia virus (Fu & Rein, 1993) and also for HIV-1 (Fu et al., 1994). In vitro experiments

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have also shown that HIV-1 RNAs containing the DIS each form two types of dimers with different stability, loose dimers and tight dimers. Loose dimers, but not tight dimers, are spontaneously formed at the physiological temperature after heat denaturation and then converted into tight dimers by 55 °C incubation (Laughrea & Jette, 1996b; Muriaux et al., 1996a) or by incubation at the physiological temperature with the nucleocapsid protein NCp7 (Muriaux et al., 1996b). These are results similar to those obtained for the Harvey sarcoma virus (Feng et al., 1995, 1996). It would be expected that loose dimers and tight dimers correspond to kissing-loop dimers and extended-duplex dimers, respectively.

Recently, structural analyses of the dimers of RNA molecules corresponding to partial sequences of the DIS have been reported, in which the dimers have been shown to be kissing-loop dimers (Dardel et al., 1998; Mujeeb et al., 1998). It is, however, not shown whether these RNAs have the character of two-step dimerization described above, including the effect of NCp7. To understand the mechanism of the two-step dimerization, the minimum structural requirement for the process should be elucidated.

In the present study, we examined whether the isolated DIS itself and/or a part of the DIS reproduce(s) the two-step dimerization reaction and found that the whole DIS sequence is necessary and sufficient for the two-step dimerization.

RESULTS

The RNA sequences used in this study are shown in Figure 1. DIS39 is a 39-mer RNA corresponding to the whole DIS with two additional base pairs at the end of the stem. DIS23, a 23-mer RNA, is a part of the DIS, consisting of the self-complementary loop and its flanking stem.

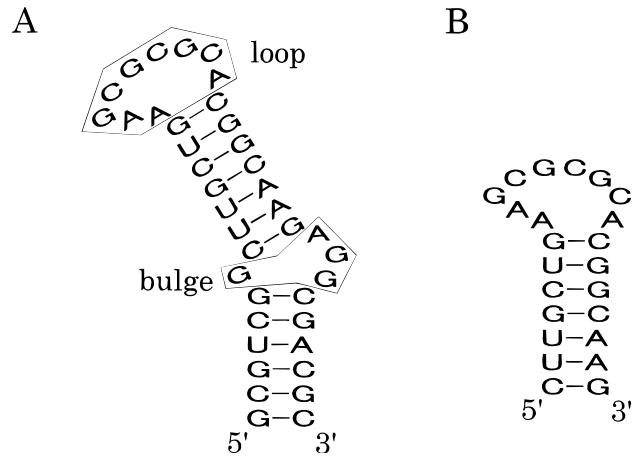

FIGURE 1. RNA sequences and possible secondary structures of DIS39 (**A**) and DIS23 (**B**).

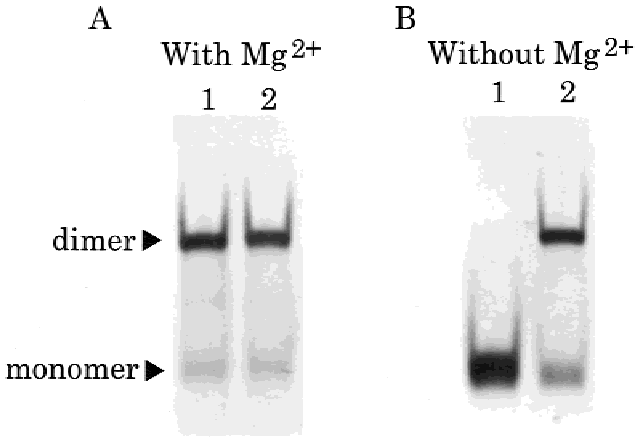

FIGURE 2. Two different dimeric states of DIS39. Electrophoreses were carried out at room temperature with (**A**) or without (**B**) Mg^{2+}. Lanes 1 and 2, DIS39 treated by incubation at 37 and 55 °C, respectively, after heat denaturation.

Electrophoretic characterization of DIS39

RNA samples were incubated at either 37 °C or 55 °C for 30 min after heat denaturation. Figure 2 shows that, depending on the incubation temperature, DIS39 can adopt two different dimeric states that are discriminated by polyacrylamide-gel electrophoresis. DIS39 treated by the 37 °C incubation behaved as a dimer in electrophoresis with Mg^{2+}, but as a monomer without Mg^{2+}. In contrast, DIS39 prepared by the 55 °C incubation behaved as a dimer under both electrophoretic conditions. According to the definition by Laughrea and Jette (1996b), we call the former state a loose dimer, and the latter state a tight dimer. The loose dimer was stable at 37 °C, the physiological condition, and converted into the tight dimer upon incubation at 55 °C.

Instead of incubation at 55 °C, an equimolar supplement of the NCp7 with respect to the RNA at 37 °C induced conversion of the loose dimer into the tight dimer (Fig. 3). For this activity, NCp7 did not require Zn^{2+} despite possessing two zinc-finger motifs (Fig. 3). Following the phenol/chloroform treatment to eliminate the NCp7 no difference appeared as the result of electrophoresis (data not shown), indicating that during the electrophoresis without Mg^{2+}, the dimer is not stabilized by the binding to the NCp7. Thus, it was concluded that the two-step dimerization reaction was able to be reproduced by using DIS39 and NCp7.

To investigate whether the loose and tight dimers of DIS39 correspond to the kissing-loop and extended-duplex dimers, respectively, we synthesized two pairs of mutants: one of the pairs of mutants, DIS39K1 and DIS39K2, will form only a kissing-loop dimer, whereas the other pair of mutants, DIS39E1 and DIS39E2, will form only an extended-duplex dimer. DIS39K1 and DIS39K2, by themselves, cannot form homodimers because the self-complementarity in the loop has been

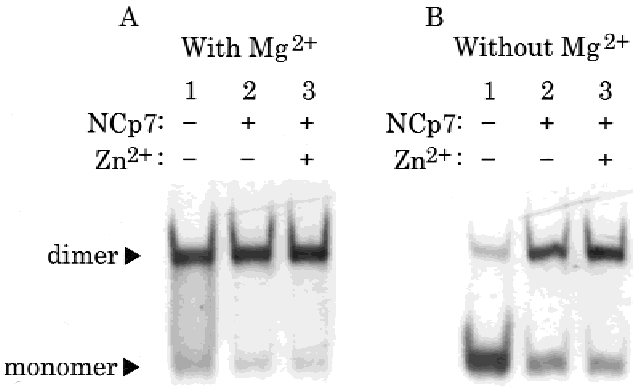

FIGURE 3. Effect of NCp7 on dimerization of DIS39. Electrophoreses were carried out at room temperature with (**A**) or without (**B**) Mg^{2+}. DIS39 was incubated at 37 °C in the absence (lane 1) or presence (lanes 2 and 3) of NCp7 prior to electrophoresis. Four equivalent molar concentrations of Zn^{2+} relative to NCp7 were also mixed during the incubation for lane 3.

lost by 1-nt substitutions, but will form a heterodimer because of the complementarity in the loop sequences. Any heterodimer thus formed cannot be an extended-duplex dimer because the stem sequence of DIS39K2 is modified not to be complementary to that of DIS39K1. Thus, the heterodimer of DIS39K1 and DIS39K2 must be a kissing-loop dimer. On the other hand, DIS39E1 and DIS39E2 were designed such that the intramolecular complementarity in the stem sequence was disrupted; instead, an intermolecular complementarity was created between the two mutants. Accordingly, the heterodimer of DIS39E1 and DIS39E2 is nothing other than an extended-duplex dimer.

In fact, electrophoretic assay confirmed the binding character of these mutants. DIS39K1 did not form a homodimer (Fig. 4B, lane 1); neither did the other three mutants. The pair of DIS39K1 and DIS39K2, a model of a kissing-loop dimer, behaved as a dimer in electrophoresis with Mg^{2+}, but as a monomer in electrophoresis without Mg^{2+} (Fig. 4B, lane 4). This electrophoretic pattern is almost identical to that of the DIS39 loose dimer (Fig. 4B, lane 2). The pair of DIS39E1 and DIS39E2, a model of an extended-duplex dimer, behaved as a dimer under both electrophoretic conditions (Fig. 4B, lane 5). This electrophoretic pattern is similar to that of the DIS39 tight dimer (Fig. 4B, lane 3), which supports the assertion that the loose and tight dimer of DIS39 correspond to a kissing-loop dimer and an extended-duplex dimer, respectively.

Electrophoretic characterization of DIS23

In the same way as for DIS39, we carried out an electrophoretic dimerization assay of DIS23. We also designed 23-mer RNA mutants similar to the 39-mer RNA mutants: two pairs of mutants that only form heterodimers with secondary structure of either a kissing-loop

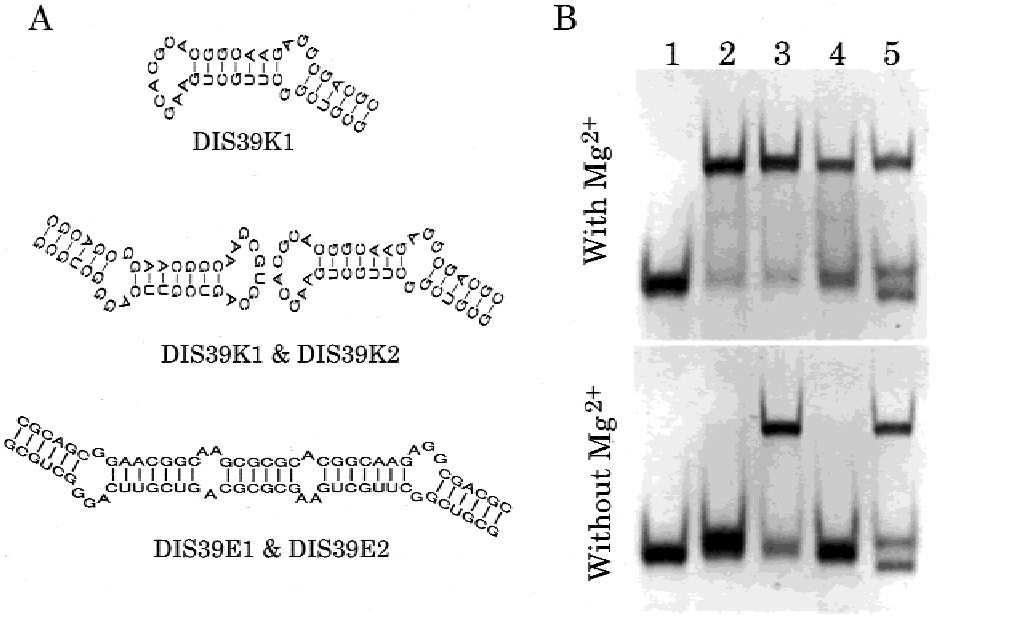

FIGURE 4. Model RNAs of a kissing-loop dimer and extended-duplex dimer (**A**) and their electrophoretic patterns (**B**). A pair of 39-mer RNA mutants DIS39K1 and DIS39K2 (DIS39E1 and DIS39E2) was designed to form only a heterodimer with secondary structure of a kissing-loop dimer (an extended-duplex dimer) (see text). Lane 1: DIS39K1; lanes 2 and 3: the loose and tight dimers of DIS39, respectively, which were generated by incubation at 37 and 55 °C, respectively, after heat denaturation; lane 4: mixture of DIS39K1 and DIS39K2; lane 5: mixture of DIS39E1 and DIS39E2. Electrophoreses were carried out at room temperature with or without Mg^{2+}. Probably because of the difference in GC content for DIS39E1 and DIS39E2, two weak bands for monomers were observed in lane 5.

dimer (DIS23K1 and DIS23K2) or an extended-duplex dimer (DIS23E1 and DIS23E2) (Fig. 5A). Like the case of the 39-mer RNA mutants, the former pair of mutants behaved as a loose dimer, whereas the latter behaved as a tight dimer in the electrophoretic analysis (Fig. 5B, lanes 4 and 5). However, DIS23 almost exclusively formed a tight dimer, regardless of the incubation temperature (Fig. 5B, lanes 2 and 3). Thus, DIS23 did not show the character of two-step dimerization.

To clarify the origin of the different behaviors of DIS23 and DIS39, we designed DIS25 and DIS35Δ: the former consists of DIS23 and one additional G-C base pair at the end of the stem to increase stability of the stem; the latter corresponds to DIS39 without the four-base bulge (Fig. 6A). Although the kissing-loop dimer was not detectable in DIS23, the additional base pair(s) in DIS25 and DIS35Δ resulted in the kissing-loop dimer formation (Fig. 6B). The kissing-loop dimer of DIS25 was then converted to the extended-duplex dimer by the treatment with NCp7 (Fig. 6C). However, NCp7 was unable to convert DIS35Δ from the kissing-loop dimer to the extended-duplex dimer (Fig. 6C). These results indicate that well-adjusted stability of the stem is critical for the exhibition of the two-step dimerization, and the bulge regulates the stability of the long stem of DIS39.

DISCUSSION

HIV-1 5′ RNA fragments dimerize in vitro by a two-step mechanism involving NCp7 (Muriaux et al., 1996b), which is suggested to be related to the virion maturation in vivo: the viral genome dimer is stabilized according to the cleavage of the gag precursor p55 to NCp7 by viral protease after releasing the virus from a host cell (Fu et al., 1994). To understand the molecular mechanism of the two-step dimerization reaction of HIV-1 genomic RNA, we determined the minimum-requirement region for this reaction. As described in the Results section, a part of the DIS, consisting of the self-complementary loop and its flanking stem (DIS23), was insufficient to accomplish the two-step dimerization; the bulge and the other stem were needed in addition, that is, the whole DIS sequence (DIS39) was required and sufficient. This is the minimum set of secondary structural elements (loop-stem-bulge-stem). If high conservation of the bulge is the evolutionary result for having kept the capability of the two-step dimerization reaction, this reaction should occur in vivo and possess biological significance. The recent report showing that the disruption of the shorter stem of the DIS in HIV-1 genome heavily reduces yield of loose dimer, but not tight dimer, and inhibits the viral replication (Laughrea et al., 1999) is also consistent with our conclusion. Using this DIS39, we investigated the molecular nature of the two types of dimers, the loose dimer and the tight dimer. Our data supported the assertion that the loose dimer and the tight dimer correspond to a kissing-loop dimer and an extended-duplex dimer, respectively. It should be noted that the mechanism of conversion between the two types of dimers by NCp7 can be investigated in detail by using this DIS39 system.

The reason why DIS23 did not show the character of two-step dimerization seems to be the low stability of the kissing-loop dimer. In the case of DIS39, the kissing-loop dimer is formed and maintained at 37 °C, and then incubation at 55 °C converts it into the extended-duplex dimer. This indicates that the kissing-loop dimer is less

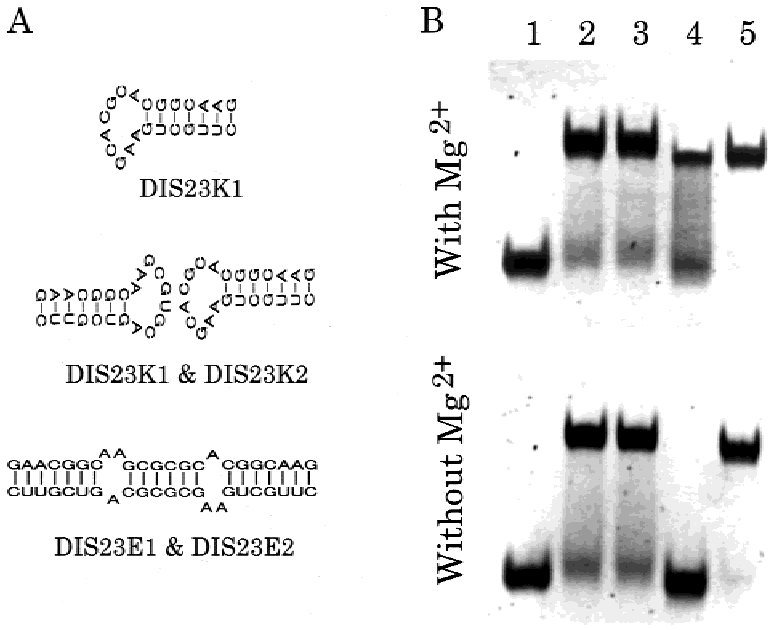

FIGURE 5. Dimerization assay of DIS23 and its mutants. **A**: A pair of 23-mer RNA mutants DIS23K1 and DIS23K2 (DIS23E1 and DIS23E2) was designed to form only a heterodimer with secondary structure of a kissing-loop dimer (an extended-duplex dimer) in a similar way to those of the 39-mer RNA mutants. **B**: Lane 1: DIS23K1; lanes 2 and 3: DIS23 generated by incubation at 37 and 55 °C, respectively, after heat denaturation; lane 4: mixture of DIS23K1 and DIS23K2; lane 5: mixture of DIS23E1 and DIS23E2. Electrophoreses were carried out at room temperature with or without Mg^{2+}.

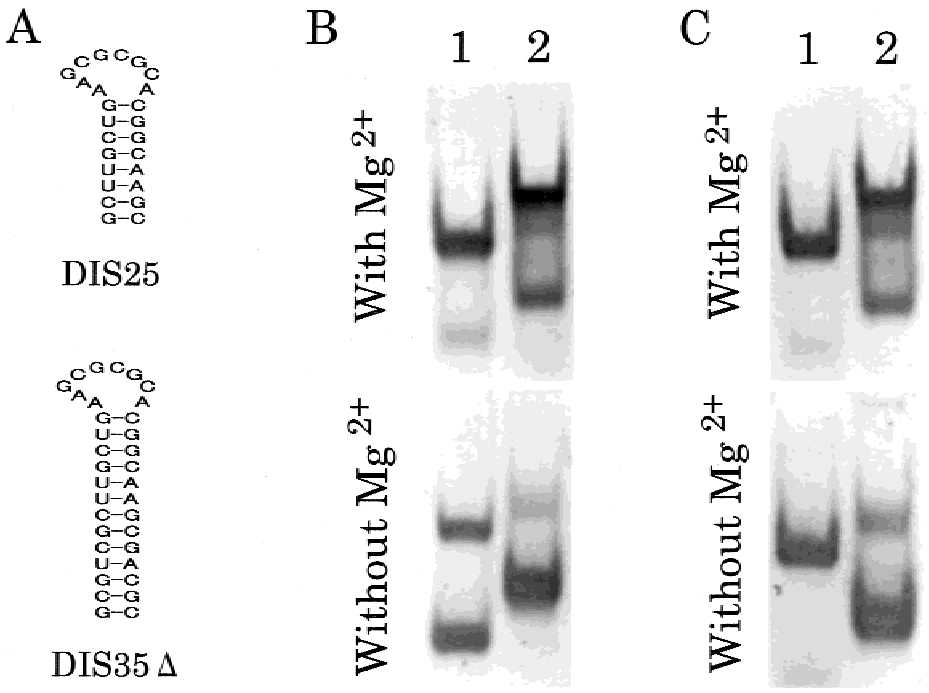

FIGURE 6. Sequences (**A**) and dimerization assay of DIS25 (lane 1) and DIS35Δ (lane 2) in the absence (**B**) and presence (**C**) of NCp7. Each of the RNAs was treated by incubation at 37 °C with and without NCp7 after heat denaturation. Electrophoreses were carried out at room temperature with or without Mg^{2+}.

stable than the extended-duplex dimer, but is kinetically trapped at 37 °C because of the high-energy barrier between the two conformational states, which may correspond to the energy required to unfold the stems. On the other hand, DIS23 is in the state of the extended-duplex dimer at 37 °C, and the kissing-loop dimer is hardly detected. Thus, it is suggested that the stability of the stems in the kissing-loop dimer is so low that conformational conversion can easily occur. In fact, when the stability of the stem was raised by the addition of base pairs to the end of the stem (DIS25 and DIS35Δ), the kissing-loop dimer was formed.

Recently, structural analyses of 23-mer and 19-mer RNAs corresponding to partial sequences of the DIS have been reported, whose homodimers have been demonstrated to be in the states of kissing-loop dimers at 25 °C and 17 °C, respectively (Dardel et al., 1998; Mujeeb et al., 1998). The stem of the 23-mer RNA (named SL1Δ) in Mujeeb et al. (1998) has base substitutions. The stem of the 19-mer RNA (named DIS19) in Dardel et al. (1998) is also different from that of our DIS23 because of the difference in HIV-1 strain and the replacement of the 3 bp at the end of the wild-type stem with one G-C base pair. It is possible that these differences in sequence raise the stability of the stem in the kissing-loop dimers. To support this possibility, we found that SL1Δ and DIS19 are theoretically more stable than DIS23 by 2.8 and 3.8 kcal/mol, respectively, when we calculated free energy in the unfolding of the RNA stem-loop structures using empirical thermodynamic parameters (Tinoco et al., 1973).

Although low stability of the stem makes the first step of the two-step dimerization undetectable, excessive stability inhibits the appearance of the second step of the reaction, that of NCp7-assisted conformational conversion. DIS25 possessing 8 bp in its stem was converted from the kissing-dimer to the extended-duplex dimer by NCp7, but the kissing-loop dimer of DIS35Δ, which has a stem of 13 continuous base pairs due to deletion of the four-base bulge from DIS39, was not converted at all by NCp7. This is because of the higher stability of the stem in the kissing-loop dimer of DIS35Δ. In fact, the melting temperature of the stem in DIS35Δ (84 °C) was higher by 18 °C than that in DIS39 (66 °C; K.-I. Takahashi & G. Kawai, to be published). The presence of the bulge in DIS39, therefore, is important in adjusting the stability of the long stem to make the conformational conversion possible. Because the bulge of the DIS has been shown to be one of the binding sites of NC proteins (Damgaard et al., 1998), the bulge may directly play a role in NCp7-assisted conformational conversion of the DIS, and may also be involved in other processes associated with NCp7.

To convert the kissing-loop dimer into the extended-duplex dimer, NCp7 did not require Zn^{2+}, which is required for the folding of the two zinc-finger motifs of NCp7. It has been reported that the deletion of the two zinc-fingers did not affect the annealing activity of NCp7, such as the annealing of tRNA to the primer binding site of the HIV-1 genomic RNA (De Rocquigny et al., 1992) and the annealing of complementary DNA strands to the region R of the HIV-1 genome during strand

transfer (Lapadat-Tapolsky et al., 1995). However, there is a recent, contradictory report that point mutations in the zinc-fingers impaired the annealing of tRNA to the primer-binding site (Remy et al., 1998). On the other hand, the zinc-fingers have been shown to be involved in binding to HIV-1 RNAs (Dannull et al., 1994; De Guzman et al., 1998). The activity of NCp7 in the two-step dimerization of HIV-1 RNAs (the unfolding of two intrastrand stems and annealing of them into an interstrand duplex) may also tolerate the deletion of the zinc-fingers because no Zn^{2+} is required.

The kissing-loop dimer of DIS39 behaved as a dimer in electrophoresis with Mg^{2+}, but as a monomer without Mg^{2+}. At the submillimolar concentration of DIS39, the NMR signals corresponding to the dimer were observed without Mg^{2+} (data not shown). These results indicate that the kissing-loop dimer of DIS39 is unstable without Mg^{2+} at lower RNA concentration, and adding Mg^{2+} or increasing the concentration of DIS39 stabilizes the kissing-loop dimer. On the other hand, the extended-duplex dimer of DIS39 behaved as a dimer in electrophoresis even without Mg^{2+}. The extended-duplex dimer has 32 bp between the two monomers, whereas the kissing-loop dimer has only 6 bp. This large number of interstrand base pairs should contribute to the dimer stability.

In conclusion, the stem-bulge-stem-loop motif is required and sufficient to exhibit the two-step dimerization process, including NCp7. In other words, DIS39 should be the smallest system to reproduce the two-step dimerization of HIV-1 genomic RNAs. This system would help us to understand the molecular mechanism of the initial step in the virion maturation, including conformational change of the genomic RNA and the formation of nucleocapsid structure. Structural study of this system is in progress in our laboratory.

MATERIALS AND METHODS

All RNA oligonucleotides were chemically synthesized by a phosphoramidite method, using an automatic DNA/RNA synthesizer, Expedite Model 8909 (PerSeptive Biosystem, Inc., Massachusetts, USA). Deprotection with ammonia and tetra-*n*-butylammonium fluoride, purification with polyacrylamide gel electrophoresis (Nihon Eido Co. Ltd., Tokyo, Japan) under denaturing conditions, and extensive desalting by ultrafiltration (Centricon YM-3, Amicon Inc., Massachusetts, USA) were carried out. A lyophilized sample of synthetic nucleocapsid protein NCp7 (LAV strain 72-aa sequence) was purchased from Peptide Institute Inc. (Osaka, Japan).

Dimerization assay of 39-mer (or 23-mer) RNA was performed in the following way. First, 12 (or 20) μM of RNA in 4 μL of water were heated at 95 °C for 5 min and chilled on ice for 5 min. Second, in experiments without NCp7, 4 μL of 2 $\times$ PN-buffer (1 $\times$ PN-buffer contains 10 mM sodium phosphate [pH 7.0] and 50 mM NaCl) were added and the solutions were incubated at 37 or 55 °C for 30 min. In the case of experiments with NCp7, 4 μL of NCp7 solution (12 μM in 2 $\times$ PN-buffer) in the presence or absence of four equivalent molar concentrations of $ZnCl_2$ were added, and the mixture solutions were incubated at 37 °C for 30 min. After the incubation, the phenol/chloroform treatment either was or was not carried out. Third, after the addition of 8 μL of loading buffer containing glycerol, the solution was divided into two aliquots, which were separately analyzed by electrophoreses through nondenaturing 10% (or 15%) polyacrylamide gels in TBM-buffer (89 mM Tris, 89 mM borate, 0.1 mM $MgCl_2$) or TBE-buffer (89 mM Tris, 89 mM borate, 2 mM EDTA), respectively, at room temperature. After electrophoresis the gels were stained with ethidium bromide.

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