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Biochemical characterization of the kink-turn RNA motif

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ABSTRACT

RNA, which acts as a medium for transmitting genetic information, plays a variety of roles in a cell. As with proteins, elucidation of the three-dimensional (3D) structures of RNAs is important for understanding their various roles. Determination of the atomic structures of crystallized ribosome has enabled the identification of previously unknown RNA structural motifs. The kink-turn (K-turn or GA) motif, which causes a sharp bend in an RNA double helix, was identified as one of these structural motifs. To biochemically characterize the K-turn, the motif was inserted into a hinge region of P4-P6 RNA, which is the most extensively studied self-folding RNA, and its properties were investigated. The stability and metal ion requirement of the constructs containing three different K-turn motifs were analyzed using native PAGE and dimethyl sulfate (DMS) modification. The formation of the sharp bending structure depends on the presence of divalent cation like Mg^{2+} or Ca^{2+}, although its required concentration is different for each motif.

INTRODUCTION

The determination of the atomic structure of the ribosome has considerably increased the size of the database of known three-dimensional (3D) RNA structures (1–5). This in turn has led to the identification of new RNA structural motifs (5,6). The kink-turn (K-turn or GA) motif is one such motif originally identified in the crystal structure of the 50S ribosomal subunit of *Haloarcula marismortui* (7,8). It contains two short helices that are connected via an asymmetric internal loop composed of a consensus sequence (Fig. 1A). Six and two K-turns were found in the crystal structure of *H.marismortui* 50S large ribosomal subunit and *Thermus thermophilus* 30S small ribosomal subunit, respectively. In addition, a K-turn was also found in the crystal structure of human U4 snRNA (9). A sharp (~120°) bend was observed in all of them. Five out of the six K-turns in *H.marismortui* 23S rRNA are associated with at least one ribosomal protein, indicating that the motif acts as a protein recognition motif.

High resolution 3D RNA structures have enabled the design and construction of artificial RNAs based on the structural units of naturally occurring RNAs (10,11). The K-turn motif with sharp bent structure is also a candidate for RNA design and construction. To incorporate a structural motif successfully, it is critical to understand its biochemical properties, such as structural stability and the requirements for structure formation. However, no biochemical property was investigated prior to the present study so that the requirements for forming the bent structure were unknown.

In this study, the biochemical properties of the K-turn motif were investigated. To evaluate the stability of its higher order structure, a flexible internal loop of P4-P6 RNA, which is the most extensively studied self-folding RNA, was replaced with one of three naturally occurring K-turns, named U4, KT-38 and KT-42 (Fig. 1). The structural stability and metal ion requirement of the constructs were analyzed using native PAGE and dimethyl sulfate (DMS) modification.

MATERIALS AND METHODS

Design and construction of the derivatives of the P4-P6 RNA containing a K-turn motif

Molecular modeling of the RNA was performed using Insight II (Molecular Simulation Inc.) on a graphics workstation Octane (Silicon Graphics Inc.). Molecular models of the RNAs were constructed from the coordinates of the crystal structure of the P4-P6 domain of the *Tetrahymena* ribozyme (Protein Data Bank i.d. 1GID) and the respective K-turn motif, whose 3D structure was extracted from the crystal structure of the *H.marismortui* 50S subunit (1JJ2). Plasmids encoding the derivatives of the P4-P6 RNA were prepared from pTZIVSU using PCR and verified by sequencing.

Preparation of RNAs

Template DNAs for transcribing P4-P6, J5/5a, U4, KT-38, KT-42 and BP5/5a were generated by performing 25 cycles of PCR using *Ex Taq* DNA polymerase (Takara Shuzo, Japan). All RNAs employed in this study were prepared by *in vitro* transcription with T7 RNA polymerase followed by purification on 5% denaturing polyacrylamide gels. For preparation of RNAs used in native gel analyses, the transcriptions were performed in the presence of [α-^{32}P]GTP.

Native polyacrylamide gel electrophoresis (PAGE)

Native PAGE was performed as described (12) with the following modifications: 5% polyacrylamide gels (39:1 acrylamide:bisacrylamide), buffer containing 50 mM Tris–OAc (pH 7.5) and appropriate concentrations of $Mg(OAc)_2$.

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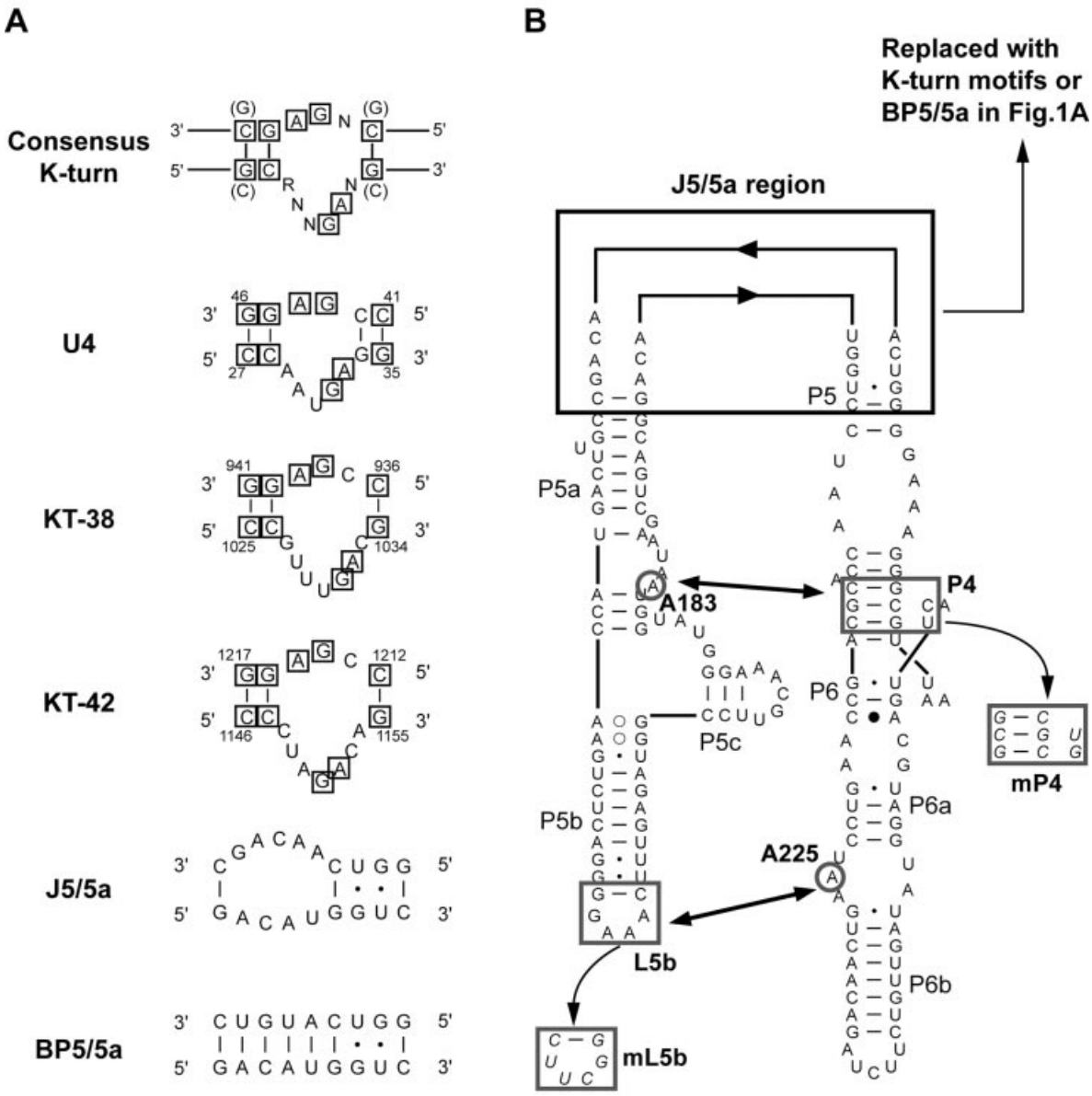

Figure 1. Secondary structures of the RNAs. (**A**) Secondary structures of K-turn consensus sequence, three K-turn motifs and the control RNAs employed in this study. Boxes indicate highly conserved residues in the K-turn (7,8). (**B**) Secondary structure of the wild-type P4-P6 RNA. A black box indicates the J5/5a region replaced with K-turn motifs or BP5/5a. Red circles indicate A183 and A225 residues participating in intramolecular RNA–RNA tertiary interactions in the wild-type P4-P6 RNA (14,17). Red boxes indicate the L5b loop and P4 helix and their substitutions mL5b and mP4 for disrupting RNA–RNA tertiary interactions.

Uniformly [α-^{32}P]-labeled RNAs were incubated in distilled water at 80°C for 3 min. Aliquots of 10× folding buffer [50 mM Tris–OAc, pH 7.5, and appropriate concentrations of $Mg(OAc)_2$] were added to the RNA solution and incubated for 10 min at 30°C. Then 5% glycerol and 0.25% bromophenol blue (BPB) were added to the samples and loaded on the gels containing $Mg(OAc)_2$ at the same concentration as that in the mixture for preincubation. Gels were run at constant voltage for 4–6 h at 30°C.

DMS modification

Aliquots of 200 µl of a solution containing 50 pmol RNA, 50 mM Tris–HCl (pH 7.5) and 0, 2.5, 5, 10 or 20 mM $MgCl_2$ were incubated at 80°C for 3 min. Mixtures were cooled and then incubated for 10 min at 30°C. Then 0.4 µl of 10% DMS (Sigma) in ethanol was added, followed by incubation for 5 min at 37°C. Reactions were stopped with 50 µl of 1 M 2-mercaptoethanol and RNAs were precipitated by adding 50 µl of 1.5 M sodium acetate, 1 µl of glycogen (Roche) and 990 µl of ethanol.

Reverse transcription

To detect the DMS-modified adenines and cytosines, one-tenth of the modified RNAs (5.0 pmol) was subjected to reverse transcription with ReverTra Ace (Toyobo, Japan) with a 5′-^{32}P-labeled DNA primer complementary to the last 28 nt

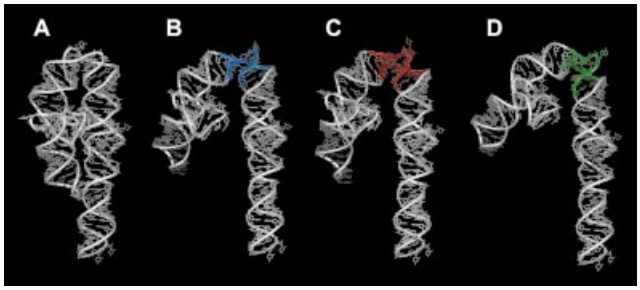

Figure 2. 3D models of the designed variants of P4-P6 RNA. (**A**) Wild-type P4-P6 and the constructs with (**B**) U4, (**C**) KT-38 and (**D**) KT-42, respectively. Blue, red and green regions indicate the K-turn motifs that have a similar bending angle.

of the respective RNA. Products were electrophoresed on 6% denaturing polyacrylamide gels and quantitated with a Bio Imaging Analyzer BAS 2500 (Fuji Film, Japan).

RESULTS

Design of the variants of P4–P6 RNA containing the K-turn motif

To investigate the biochemical properties of the K-turn motif, we constructed new RNA molecules by inserting the motif between two distinctive stem regions of a self-folding RNA called P4-P6 RNA. P4-P6 RNA derived from the *Tetrahymena* group I intron was used because its 3D structure (13–15) and biochemical properties (16–21) have been characterized in detail. A structurally flexible internal loop called J5/5a was used as the insertion site because it also forms a sharp bend (~180°) (Fig. 2A). The derivatives of P4-P6 with the K-turn were designed using a molecular modeling program (see Materials and Methods) in order to minimize unnecessary structural perturbation due to the replacement.

The J5/5a loop plays a passive role in forming the hairpin shaped structure of the P4-P6 RNA (18), which is clamped by two sets of RNA–RNA tertiary interactions: one interaction is located between the GAAA tetraloop in the L5b region and the 11 nt receptor in P6a/b and the other is between the A-rich bulge and the P4 helix (Fig. 1B) (14,17). Previous analyses demonstrated that disruption of one or both of the interactions significantly reduces the folding ability so that the resulting mutant RNA is in a rapid equilibrium between the bent and extended conformation due to the flexibility of the J5/5a hinge (18).

To investigate the stability of the K-turn motifs in the constructs without the two interactions, they were disrupted in the construct containing a K-turn called U4, KT-38 or KT-42 (Fig. 1B): U4, KT-38 and KT-42 are derived from human U4 snRNA (9) and helix 38 and helix 42 of *H.marismortui* 23S rRNA (1,7), respectively. The three motifs share a similar bending angle in the native RNA–protein complex in the crystal structure (see model 3D structures). However, they have different structural features. U4 possesses the K-turn consensus primary and secondary structure whereas the corresponding structures of KT-38 and KT-42 are considerably different from that of U4 (Fig. 1A). Interestingly, proteins are associated with U4 and KT-42 but not with KT-38 (7).

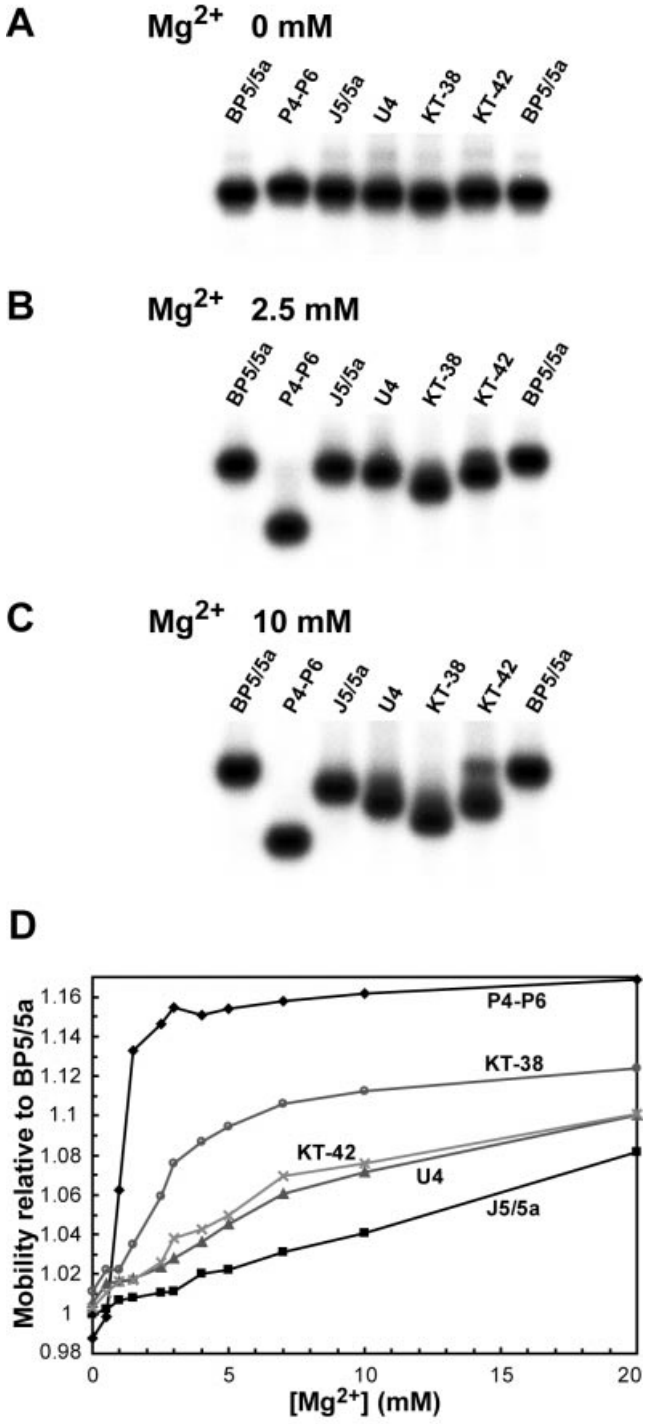

Figure 3. Native PAGE of the RNAs. (A–C) Autoradiograms of the native PAGE of the P4-P6 derivatives (see Fig. 1) without Mg^{2+} (**A**), with 2.5 (**B**) and with 10 mM Mg^{2+} (**C**), respectively. (**D**) Relative mobilities of the P4-P6 derivatives were plotted as a function of Mg^{2+} concentration. Mobilities were normalized by employing BP5/5a as the standard (20). Closed diamonds, squares, triangles, open circles and crosses indicate the relative mobilities of P4-P6, J5/5a, U4, KT-38 and KT-42, respectively.

Native PAGE

The constructs containing K-turns were electrophoresed on native polyacrylamide gels to determine whether their

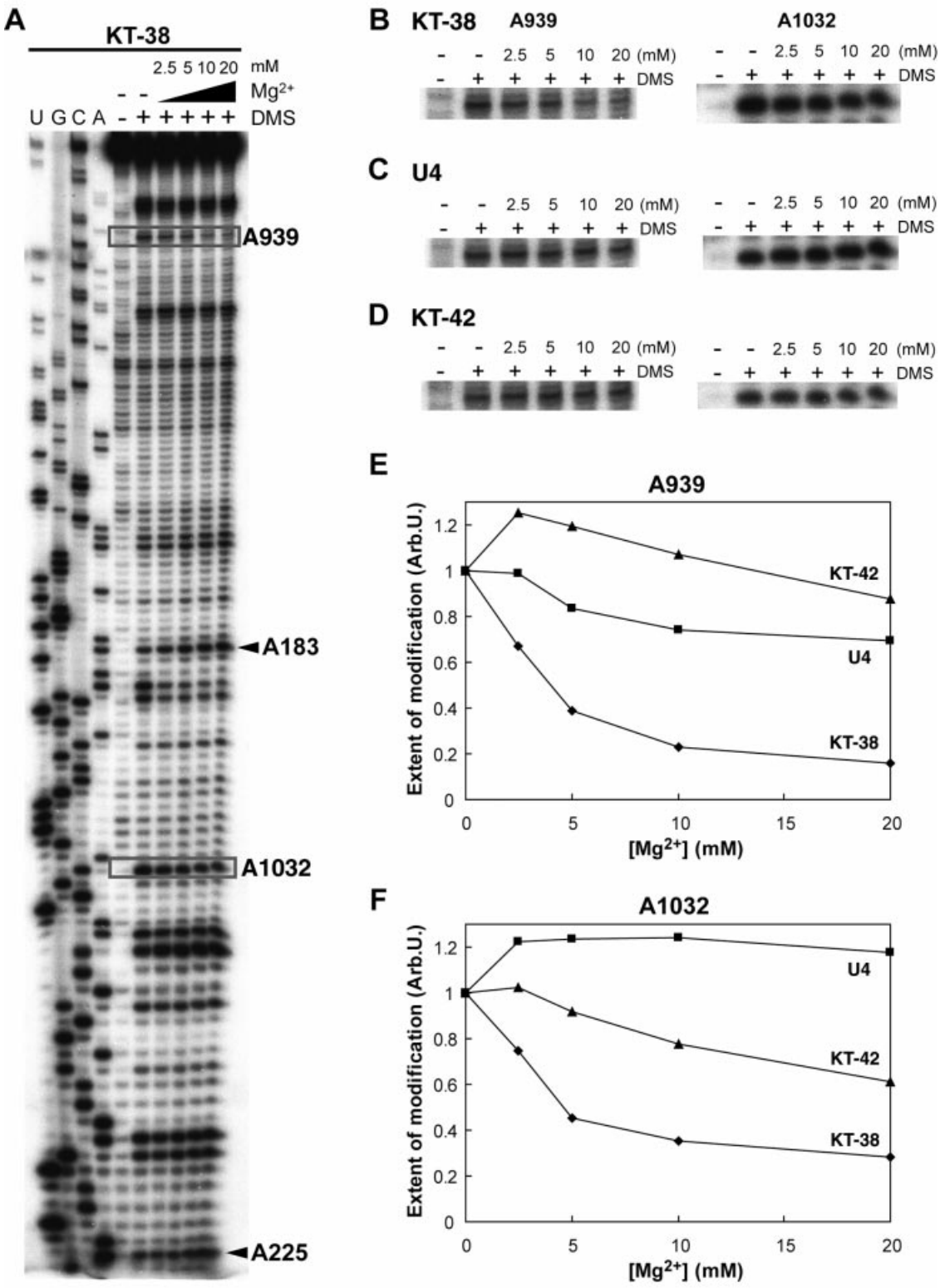

Figure 4. Chemical probing of the RNAs with U4, KT-38 and KT-42 motifs by DMS modification. (**A**) Autoradiogram of the DMS modification of RNA with KT-38. Red boxes with solid lines indicate A939 and A1032. Arrowheads indicate A183 and A225 participating in intramolecular RNA–RNA tertiary interactions in the wild-type P4-P6 RNA. (**B**–**D**) Autoradiograms of the DMS modification at A939 and A1032 in the KT-38 motif (B) and the adenines corresponding to A939 and A1032 in the U4 (C) and KT-42 motifs (D). (**E** and **F**) The extent of the modification of A939 and A1032 (KT-38) and the adenines corresponding to A939 and A1032 (U4 and KT-42) were plotted as a function of Mg^{2+} concentration. The extent of the modification was normalized by taking account of the background observed in the absence of Mg^{2+}. Closed squares, diamonds and triangles indicate the extent of the modification of the adenines in U4, KT-38 and KT-42, respectively.

conformation depended on the dihedral angles between the two helices or reflected the equilibria between a bent and extended form. The wild-type P4-P6 RNA and its two derivatives (BP5/5a and J5/5a) were used as the controls for native PAGE. The P4-P6 RNA with the compactly folded structure and the BP5/5a variant in which J5/5a was replaced with consecutive base pairs resulting in an extended form were anticipated to exhibit fastest and slowest mobility, respectively (Fig. 1A). The J5/5a variant missing the two tertiary interactions that clamp the P5abc subdomain and the P4-P6 helices, used as a control reflecting the relative stability of the J5/5a loop, was presumed to exhibit intermediate mobility because of rapid kinetic equilibrium between its folded and extended forms (Fig. 1A).

The relative mobility at several Mg^{2+} concentrations and dependence on Mg^{2+} were examined by employing the three constructs and the control RNAs. The three K-turn motifs migrated in a similar manner without magnesium ions (Fig. 3A), indicating that they all require a divalent cation to form the bent structure.

The crystal structures predicted that the RNAs containing K-turns should migrate faster than BP5/5a but slower than the native P4-P6 because a K-turn motif causes an ~120° bend (Fig. 2). Accordingly, the three K-turn RNAs migrated faster than BP5/5a but slower than the native P4-P6 (Fig. 3B and C), indicating that Mg^{2+} ions induce conformational change.

The three constructs with the K-turns migrate differently (Fig. 3D). KT-38 exhibited the highest mobility. U4 and KT-42 migrated more slowly than KT-38 and had similar mobilities. The mobility of the three K-turns, which is dependent on Mg^{2+} concentration, was faster than that of J5/5a, whose folding depends on the flexible J5/5a loop. Thus it can be concluded that Mg^{2+} induces the bent conformations of the three K-turns and that the conformation of KT-38 is stable compared with that of U4 or KT-42.

Chemical probing using DMS modification

Chemical modification experiments were performed in the presence of magnesium ions. DMS, which methylates the N1 position of adenine and N3 position of cytosine, was used for the modification. First, modification of the construct with KT-38 was compared with that of the native P4-P6 or BP5/5a. Two adenines (positions 183 and 225) of the A-rich bulge and 11 nt receptor were protected from modification in the native P4-P6 RNA because the adenines participate in tertiary interactions between the P5abc subdomain and P4-P6 helices (22). As anticipated, they were accessible in BP5/5a lacking the interactions. The two adenines of the construct with KT-38 were modified at low and high concentrations of Mg^{2+} (Fig. 4A) as the two interactions were disrupted, indicating that the conformational change due to Mg^{2+} is independent of the interactions between the two structural subdomains of P4-P6 RNA.

Two adenines (A939 and A1032) in KT-38 were strongly protected from modification when 10 mM or higher concentrations of Mg^{2+} were present. The degree of protection depended on Mg^{2+} concentration. It is thus conceivable that KT-38 forms a bent structure by employing Mg^{2+}. This is consistent with the fact that N1 of A939 and of A1032 form hydrogen bonds with neighboring nucleotides in the crystal structure of KT-38 (Fig. 5). The same modification was performed for the constructs with U4 and KT-42. They possess adenines corresponding to A939 and A1032 of KT-38 in their K-turns. The adenines were not or were hardly protected in the presence of 20 mM Mg^{2+}, indicating that the corresponding hydrogen bonds are either transient or not formed in the absence of the binding proteins (Fig. 4C and D). The data indicate that the U4 and KT-42 constructs fold less compactly (or less stably) than the KT-38 construct.

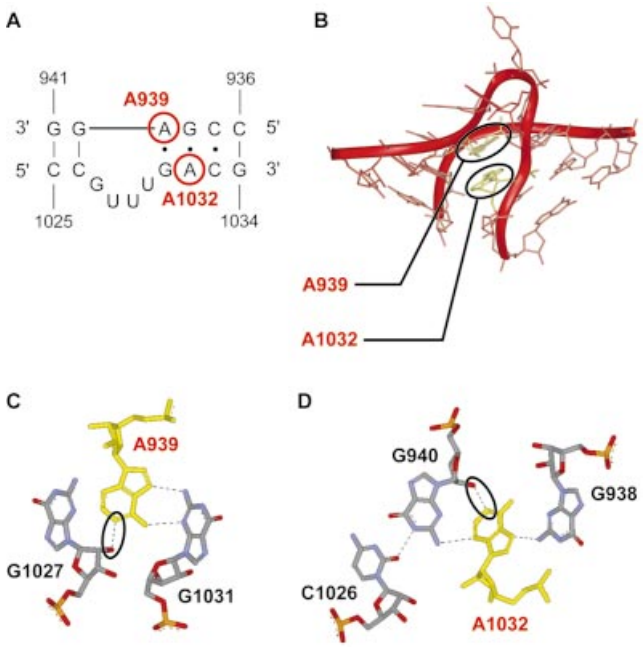

Figure 5. Base pair formation in the structure of KT-38 motif in the crystal structure of the ribosome (1,7). (**A**) Base pair formation in the KT-38 motif. Red circles indicate A939 and A1032. (**B**) 3D structure of the KT-38 motif in the ribosome. A939 and A1032 are indicated (yellow residues). (**C** and **D**) Atomic details of the hydrogen bonds of A939 and A1032 (yellow residues) with respective neighboring nucleotides.

DMS modification and native PAGE with cations other than Mg^{2+}

In most cases, magnesium ions participate in the folding of naturally existing functional RNAs and RNA–protein complexes such as ribosomes (23,24). They are also required for translation by the bacterial ribosome (25,26). However, they can be substituted by other metal ions such as Ca^{2+} or Mn^{2+} (27,28). Eight K-turn motifs in the 50S and 30S ribosomal subunits seem to play roles in forming the ribosomal 3D structure. Thus it was of interest to see whether other metal ions could substitute Mg^{2+} in the bent structure formation of the K-turn motifs.

To investigate the cation specificity, the modification of the KT-38 construct was performed in the presence of a variety of cations. The adenine residues, A939 and A1032 in KT-38, were protected in the presence of Ca^{2+} and Mn^{2+} but not in the presence of Na^{+} and K^{+} (Fig. 6), indicating that the bent structure is formed using divalent cations. Native PAGE analysis of the KT-38 construct was also performed in the presence of 10 mM Ca^{2+}. Its mobility relative to the derivatives of P4-P6 RNA was the same as that in the presence of Mg^{2+} (Fig. 7). The results suggest that Ca^{2+} (and

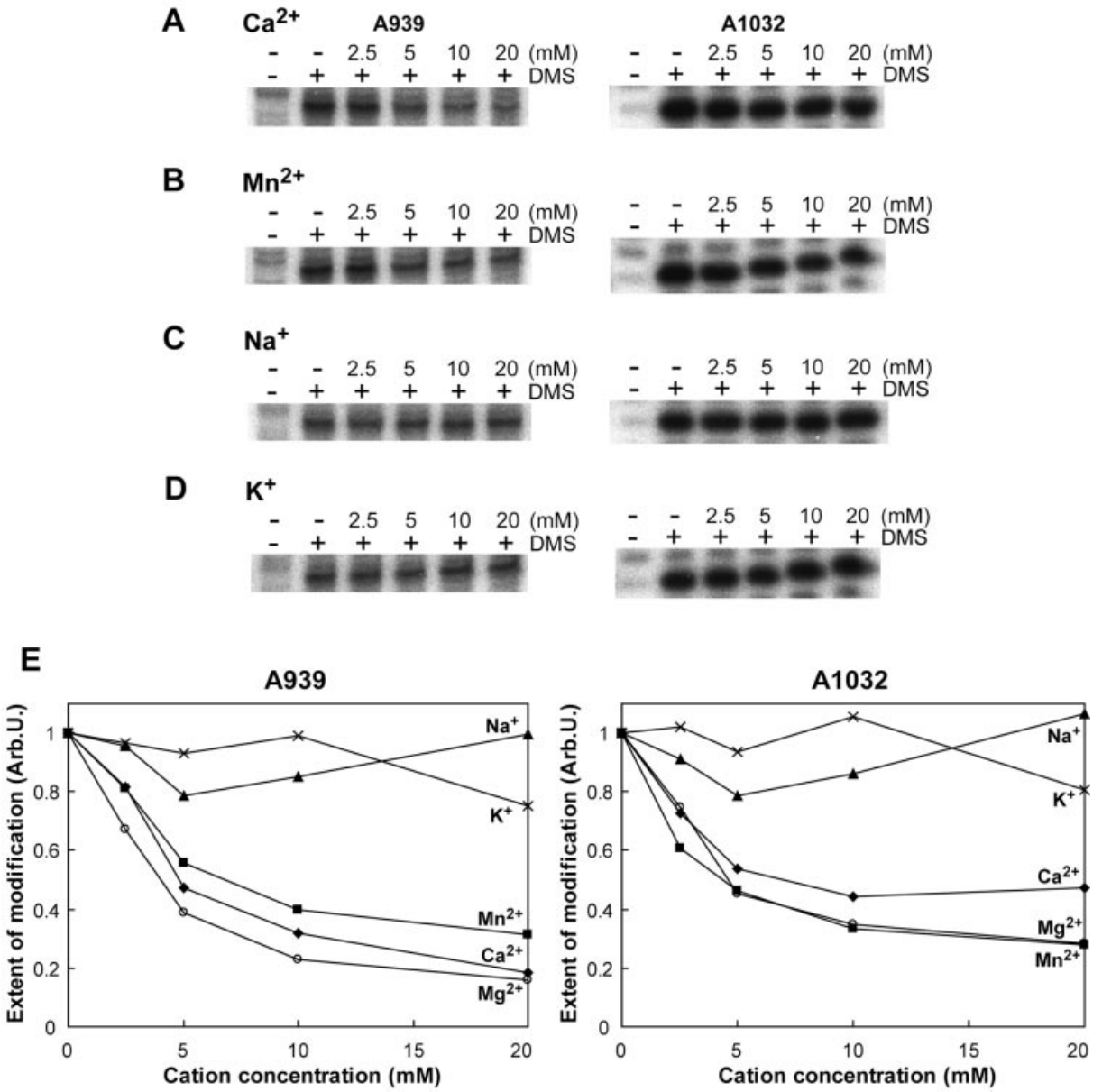

Figure 6. Chemical probing of the RNA with KT-38 by DMS modification with cations other than Mg^{2+}. (**A**–**D**) Autoradiograms of the DMS modification at A939 and A1032 in KT-38 with Ca^{2+} (A), Mn^{2+} (B), Na^{+} (C) and K^{+} (D), respectively. (**E**) The extent of the modifications at A939 and A1032 were plotted as a function of the cation concentration. The extent of the modification was normalized by taking account of the background observed in the absence of metal ions. Open circles, closed diamonds, squares, triangles and crosses indicate the extent of the modification of the adenines in the presence of Mg^{2+}, Ca^{2+}, Mn^{2+}, Na^{+} and K^{+}, respectively.

probably Mn^{2+}; however, native PAGE is not practical with Mn^{2+}) can substitute for Mg^{2+} ions in folding of the K-turn motif in KT-38.

DISCUSSION

General properties of the K-turn

The properties of three K-turns, U4, KT-38 and KT-42, were characterized biochemically and it was found that they are capable of forming a bent structure depending on the concentration of Mg^{2+}. This indicates that the K-turn is a self-folding RNA structural motif. It was also found that the stabilities of the three structures and their folding abilities are distinctly different. The native PAGE and chemical modification assays indicate that KT-38 forms a stable bend at 10 mM Mg^{2+}, whereas U4 and KT-42 are incapable of forming a bend at less than 20 mM Mg^{2+}.

In the crystal structure, U4 and KT-42 are associated with a particular protein whereas KT-38 is not (1,7,9), suggesting that U4 and KT-42 form a bend depending on association with the protein *in vivo* whereas KT-38 bends by itself. This is consistent with the observation that a 15.5 kDa protein induces and stabilizes the sharp bend of the U4 K-turn in the crystal

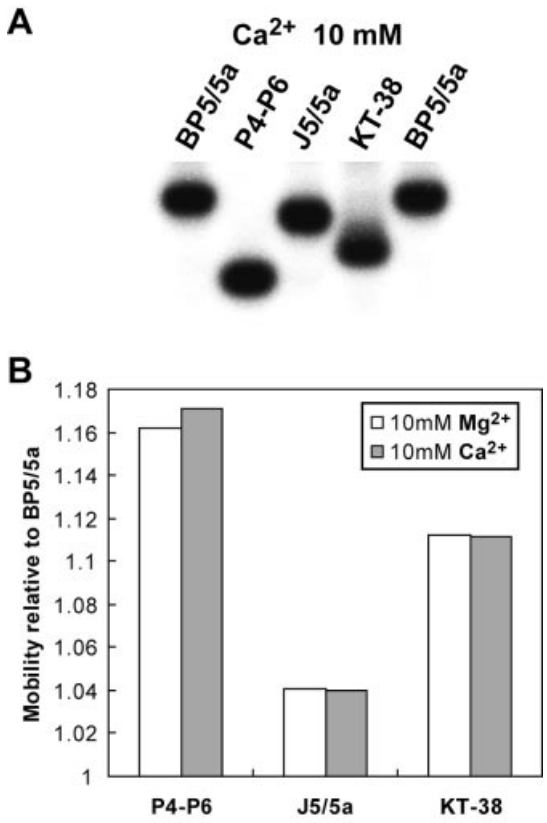

Figure 7. Native PAGE in the presence of Ca^{2+}. (**A**) Autoradiogram of native PAGE of the RNAs, P4-P6, J5/5a, KT-38 and BP5/5a at 10 mM Ca^{2+}. (**B**) Relative mobilities of the RNAs, P4-P6, J5/5a and KT-38 at 10 mM Mg^{2+} or Ca^{2+}. The mobilities were normalized by employing BP5/5a as the standard. Open and shaded bars indicate the mobilities at 10 mM Mg^{2+} and Ca^{2+}, respectively.

(9). According to the RNA World hypothesis, the functional RNAs were gradually converted into ribonucleoprotein (RNP) in the course of evolution (29), implying that the U4 and KT-42 K-turns were originally self-folding and might subsequently have acquired RNA-binding proteins during the course of evolution.

In native PAGE, U4 and KT-42 exhibited similar properties with regard to the relationship between the concentration of Mg^{2+} and structure formation. However, their nucleotide sequences are significantly different, suggesting that the differences in the sequences of the K-turns do not influence their folding abilities and that KT-38 with its prominent folding ability may be exceptional.

The internal loops of RNAs can be divided into two classes: 'structured loops' and 'flexible loops' (30). In solution, the former are structurally ordered and relatively rigid. The latter are disordered and flexible, but in many instances become structured upon binding with an external ligand. In previous studies, it has been demonstrated that asymmetric internal loops often exhibit the properties of 'flexible loops'. For example, the J5/5a loop in P4-P6 RNA (18) and the internal loop B in the bacterial signal recognition particle (SRP) (31) are disordered and flexible. Our study suggests that asymmetric K-turns such as U4 and KT-42 form unstable bends and can be regarded as flexible loops, whereas KT-38, which forms a stable bend in the presence of Mg^{2+}, should be regarded as a structured loop. Therefore, KT-38 would appear to be an exceptional motif among asymmetric internal loops.

Self-folding motif KT-38

KT-38 seems to form a stable bend in the presence of ~10 mM Mg^{2+} (Figs 3 and 4). In the modification experiment, the hydrogen bonds contributing to bend formation were identified in two adenine residues, A939 and A1032. In the crystal structure, N1 of A939 and A1032 in KT-38 form hydrogen bonds with the ribose 2′-OH group of the adjacent nucleotides (G1027 and G940) (1,7), suggesting that the two hydrogen bonds are essential to the formation of the bent structure (Fig. 5). A939 and A1032 were protected from the modification depending on Mg^{2+} concentration and the extent of modification was decreased relative to the increase in electrophoretic mobility on a native gel, indicating that they directly contribute to the bent structure formation (Fig. 4). Thus it is clear that the K-turn in KT-38 autonomously forms the bent structure equivalent to that in the ribosome.

Cations for folding of KT-38

Our results show that KT-38 is capable of forming a bend with Ca^{2+} or Mn^{2+} but not with Na^{+} or K^{+} at <20 mM. The relationship between the concentrations of Ca^{2+} or Mn^{2+} and the extent of modification at A939 and A1032 is closely related to that with Mg^{2+} (Fig. 6). In the presence of Ca^{2+}, the electrophoretic mobility on a native gel was almost equivalent to that with Mg^{2+} (Fig. 7). This suggests that KT-38 is stabilized by Mg^{2+}, Ca^{2+} and probably by Mn^{2+} in the same manner.

In chemical probing of the P4-P6 variant with KT-38, alterations of the modification pattern were observed only at the A-rich bulge (Fig. 4). They were consistent with the known secondary structure rearrangement and tertiary folding of the P5abc subdomain by itself. Modifications of A183 and A225, which are known to participate in the interactions between the P5abc and P6 subdomains, were observed (see Results), indicating that the interactions between these subdomains do not exist. Thus these indicate that self-folding of the KT-38 motif by itself causes the bending of the P4-P6 variant: the sharp bend structure is formed due to the divalent cations on the KT-38 motif that facilitate hydrogen bond formation between A939 and A1032 and neighboring nucleotides.

A model for the folding of KT-38

In native PAGE, the mobility of the construct with KT-38 increases relative to the increase in the concentration of Mg^{2+} up to 10 mM (Fig. 3). 'A bent molecule with an intermediary angle' or 'a molecule in rapid equilibrium between fully extended and folded states' is a possible form for KT-38 under these conditions. A939 and A1032 should be protected from DMS in order for KT-38 to form a fully bent structure, whereas they should be unprotected for it to form a bent molecule with an intermediate angle. The two adenines were protected from modification at 2.5 mM Mg^{2+}. The relative mobility was increased on the native gel by increasing the concentration of Mg^{2+}, suggesting that fully bent molecules exist under these conditions and that the population of bent molecules increases relative to an increase in the concentration of Mg^{2+} (Figs 4 and 6). Thus it seems that a molecule

exhibiting intermediate mobility on a native gel at less than 10 mM Mg^{2+} is in rapid equilibrium between extended and folded states, as is the case for the folding equilibrium of the P4-P6 RNA.

K-turn as a tool for constructing artificial RNAs

It was established that KT-38 is an independent structural motif that can be formed autonomously under certain physiological conditions. Thus it is likely to be a useful structural unit in constructing artificial RNA when a sharp turn is needed in the RNA design. It can also be utilized as a structural unit whose conformation can be controlled by the presence or absence of a divalent cation. The other K-turns can also be utilized when employed together with the binding proteins for switching the conformations of the motifs.

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