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NMR analysis of the hydrogen bonding interactions of the RNA-binding domains of the *Drosophila* Sex-lethal protein with target RNA fragments with site-specific [3-^{15}N]uridine substitutions

Insil Kim, Yutaka Muto, Makoto Inoue, Satoru Watanabe, Aya Kitamura, Shigeyuki Yokoyama*, Kazumi Hosono[1], Hiroshi Takaku[1], Akira Ono[2], Masatsune Kainosho[2], Hiroshi Sakamoto[3] and Yoshiro Shimura[4,5]

Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan, [1]Department of Industrial Chemistry, Chiba Institute of Technology, Narashino, Chiba 275, Japan, [2]Department of Chemistry, Faculty of Science, Tokyo Metropolitan University, Minami-ohsawa, Hachioji, Tokyo 192-03, Japan, [3]Department of Biology, Faculty of Science, Kobe University, Rokkodai, Nada-ku, Kobe 657, Japan, [4]Department of Biophysics, Faculty of Science, Kyoto University, Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto 606, Japan and [5]Biomolecular Engineering Research Institute, Furuedai, Suita, Osaka 565, Japan

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ABSTRACT

It has been reported that a 183 residue fragment, consisting of the two RNA-binding domains (RBD1–RBD2) of the *Drosophila melanogster* Sex-lethal (Sxl) protein, strongly binds an oligonucleotide of the target RNA sequence (5′-GUUUUUUUUC-3′) that regulates alternative splicing, and forms four or five hydrogen bonds with the imino groups of the RNA. In the present study, we used site-directed mutagenesis to improve the solubility of the didomain fragment of Sxl, and confirmed that this mutant fragment forms hydrogen bonds with the target RNA in the same manner as that of the wild-type fragment. The mutant fragment was shown to bind the cognate RNA sequences GUUUUUUUUC and AUUUUUUUUC more tightly than UUUUUUUUC. By using a [3-^{15}N]uridine phosphoramidite, we synthesized a series of ^{15}N-labeled target RNAs, in which one of the uridine residues was specifically replaced by [3-^{15}N]uridine. By observing the imino ^{1}H–^{15}N coupling of the labeled uridine residue, we assigned all four of the hydrogen-bonded imino protons to U1, U2, U5 and U6, respectively, of the target RNA. The imino protons of U2 and U6 exhibited nuclear Overhauser effects with aliphatic protons of the protein. All these results indicate that the A/G, U1, U2, U5 and U6 residues in the target sequence of (G/A)UUUUUUUU are specifically recognized by the two RNA-binding domains of the Sxl protein.

INTRODUCTION

Sexual differentiation of somatic and germline cells, as well as dosage compensation in *Drosophila melanogaster*, is controlled by the *Sex-lethal* gene (1). The Sex-lethal (Sxl) protein plays a key role in female-specific alternative splicing of the *transformer* (*tra*) pre-mRNA in somatic cells (2–4). This alternative splicing is regulated by direct binding of the Sxl protein to a characteristic uridine-rich polypyrimidine tract (PPT) prior to the regulated 3′ splice site of the *tra* pre-mRNA (5). In this process, Sxl reduces the splicing at the 3′ splice site by directly blocking the binding of the essential splicing factor, U2 snRNP auxiliary factor (U2AF^{65}), to the PPT (6). Sequences similar to the PPT sequence of the *tra* pre-mRNA are also found in the *Sxl* pre-mRNA, to which Sxl binds for autoregulation (7). Consensus sequences for Sxl binding have been proposed by *in vitro* selection and/or gel shift assays (8–10).

The Sxl protein contains two RNA-binding domains (RBDs), RBD1 and RBD2, in tandem (11). A 183 amino acid residue fragment consisting of RBD1–RBD2 retains the ability to bind a 10 nt RNA fragment, GUUUUUUUUC, derived from the *tra* PPT (12). A nuclear magnetic resonance (NMR) analysis indicated that four or five of the imino protons of GUUUUUUUUC are involved in hydrogen bonding with RBD1–RBD2 (12).

In the present study, we improved the solubility of RBD1–RBD2 on the basis of our recent finding that the solubility of the RBD1 fragment was remarkably increased by site-directed mutagenesis (13). We also developed a site-specific ^{15}N-labeling method: a series of RNAs were chemically synthesized with a [3-^{15}N]uridine phosphoramidite. Thus, we succeeded in the

*To whom correspondence should be addressed. Tel: +81 3 3812 1805; Fax: +81 3 5689 5609; Email: yokoyama@y-sun.biochem.s.u-tokyo.ac.jp

assignment of the resonances of the four hydrogen-bonded imino protons for the complex of GU_8C and Sxl RBD1–RBD2.

MATERIALS AND METHODS

Preparation of the RNA binding domains (RBD1–RBD2) of the Sxl protein

The gene encoding RBD1–RBD2 of the Sxl protein was cloned by PCR methods, and site-directed mutagenesis of Phe166 to Tyr was performed as described (13). *Escherichia coli* strain BL21 (DE3), transformed with a T7 RNA polymerase expression vector containing the gene for RBD1–RBD2 (pK7-RBD1-RBD2) (13), was pre-cultured in 20 ml LB medium to stationary phase. This pre-culture was added to 1 l of culture medium, and the cells were cultured, induced with IPTG, and harvested; 4 g of wet cells were collected from 1 l of 2× M9 medium with 1 g/l NH_4Cl, 240 mg/l $MgSO_4$, 15 mg/l $CaCl_2$, 20 mg/l thiamine and 4 g/l glucose. Chromatographic purification of the mutant RBD1–RBD2 protein was performed on DEAE Sephacel, CM-Toyopearl and FPLC Mono S columns. About 10–20 mg of RBD1–RBD2 was obtained from 4 g well cells. Yields of the RBD1–RBD2 protein were estimated by specific absorbance, A_{280} = 0.56 cm/1 mg × 1 ml.

[3-^{15}N]Uridine phosphoramidite

We synthesized the [3-^{15}N]uridine as described by Ariza *et al.* (14). In preparing the 3′-phosphoramidite of [3-^{15}N]uridine, first the 5′ position was protected with the dimethoxytrityl (DMTr) group, and then the 2′ position was protected with the *tert*-butyldimethylsilyl (tBDMS) group. 5′-O-dimethoxytritylation was accomplished with 4,4′-dimethoxytrityl chloride in a pyridine solution (15). Then, silylation was performed with *tert*-butyldimethylsilyl-chloride in the presence of silver nitrate ($AgNO_3$) in THF (16). Phosphitylation of the 5′, 2′-protected [3-^{15}N]uridine was performed by the use of 2-cyanoethyl-*N*,*N*-diisopropylamino-chlorophosphine as the phosphitylation agent, in the presence of diisopropylethylamine (17–19). All building blocks were satisfactorily characterized by ^{1}H- and ^{31}P-NMR.

Preparation of RNAs

Chemical syntheses of RNAs (GUUUUUUUUC, AUUUUUUUUC and UUUUUUUUC) were performed on a DNA/RNA synthesizer using 1 µmol of protected nucleoside grafted onto a long chain alkylamine CPG support. The final DMTr-group was removed. A freshly prepared 3:1 saturated solution of 28% ammonia in ethanol (2.0 ml) was added with a syringe, and the mixture was heated overnight at 55°C and then evaporated. To remove the 2′ protection groups, 400 µl of 1 M TBAF in THF was added to a solid pellet. The solution was mixed well and incubated overnight at room temperature. For desalting, 100 µl of 2 M TBAF and 1.5 ml of distilled H_2O were added to this solution, which was then loaded on a Sep-Pak cartridge (Waters) equilibrated with 2 M TEAA (pH 7.0). The column was washed with 10 ml of 0.1 M TEAA and distilled H_2O, and was then eluted with 40% acetonitrile. The eluted fractions were evaporated and then quantified by UV spectroscopy. RNA oligomers were purified by 20% PAGE. After PAGE, the band located by UV shadowing was cut out and eluted with distilled H_2O at 50°C for 2 days. The purified RNA sample was desalted on a Sep-Pak cartridge, which was washed with 50 ml of 0.1 M TEAA and distilled H_2O, and then eluted with 40% acetonitrile. The fractions were evaporated and then checked by UV spectroscopy.

Preparation of NMR samples

For NMR measurements, 4 mg of the Sxl RBD1–RBD2 in 50 mM ammonium formate buffer (pH 6.5) was concentrated by ultrafiltration using either Centricon-3 or Centriprep-3 units (Amicon). 99.85% ^2H_2O (Isotec. Inc) was added to a concentration of 10% for lock stabilization. The final samples used for NMR measurements had 0.2 ml sample solution volumes containing 40 or 80 nmol protein. To prepare the sample of the RNA–protein complex, the protein solution was added to the evaporated RNA samples. After NMR measurements, the pH values of the samples were checked.

NMR measurements

All of the ^{1}H NMR experiments were performed on a Bruker AMX-600 spectrometer at a probe temperature of 25°C. ^{1}H chemical shifts were determined relative to internal DSS. Solvent suppression was achieved using the jump–return method (20). The two-dimensional (2D) nuclear Overhauser effect (NOE) spectrum was acquired with a mixing time of 150 ms and by the method of time-propotional phase incrementation (TPPI; 21).

RESULTS AND DISCUSSION

Improvement of the solubility of the Sxl RBD1–RBD2 by mutagenesis

We have already succeeded in increasing the solubility of a single-domain fragment of the Sxl RBD1 by ~10-fold, without affecting the RNA-binding properties, through the mutation of Phe166 to Tyr (13). Accordingly, in the present study, we introduced the Phe166→Tyr mutation into the didomain fragment, Sxl RBD1–RBD2 (12). Actually, the mutant didomain fragment exhibited a much higher solubility than the wild type, as judged from the degree of aggregation at high concentrations. Furthermore, the mutant RBD1–RBD2 protein was shown to bind the *tra*-PPT (GUUUUUUUUCUAGUG) as well as the wild-type RBD1–RBD2, by the UV-cross linking method (data not shown), similar to the case of the single-domain fragment (13).

Imino proton resonances of GUUUUUUUUC bound to the mutant Sxl RBD1–RBD2

In this study, we examined the interaction of the *tra*-derived decamer (5′-GUUUUUUUUC-3′ or GU_8C) with the highly soluble mutant of the Sxl RBD1–RBD2. In the imino proton region (10–13 p.p.m.) of the 600 MHz proton NMR spectrum of the RNA–protein complex (Fig. 1A), five resonances were observed, at 10.34, 11.37, 11.42, 11.85 and 12.58 p.p.m. The resonances at 11.37, 11.85 and 12.58 p.p.m. exhibit line widths of ~60 Hz, which are much narrower than those of the other two. The broad resonance at 11.42 p.p.m. is partly overlapped with the signal at 11.37 p.p.m. It has been established that hydrogen bonded imino proton resonances of nucleic acids characteristically appear in a low field region of 10–15 p.p.m. (22). The effects of hydrogen bonding include not only the significant decrease in the exchange rate of the imino proton with the solvent water, but also the deshielding due to the adjacent electronegative atom of the hydrogen bond (23–25). In this context, it has been reported that

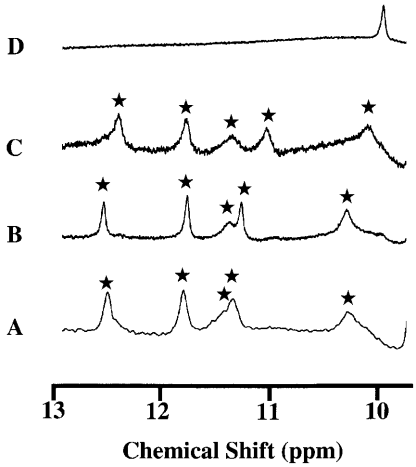

Figure 1. The imino proton regions of the 600 MHz proton NMR spectra of (**A**) GUUUUUUUUC, (**B**) AUUUUUUUUC and (**C**) UUUUUUUUC in the complex with the soluble mutant of the Sxl RBD1-RBD2, and (**D**) the protein in the RNA-free state.

imino protons in RNA internal loops exhibit the resonances in the characteristic low field region only when they are involved in hydrogen bonds (26). Accordingly, in the complex of GU_8C with the mutant Sxl RBD1–RBD2, the imino groups exhibiting the downfield shifted imino proton resonances are likely to be involved in hydrogen bonding.

All of such properties as the chemical shifts, the linewidths, and the overlapping pattern of the low-field resonances of the protein-bound GU_8C, are the same between the mutant RBD1–RBD2 (this study) and the wild-type RBD1–RBD2 (12). We conclude, therefore, that these hydrogen bonds are formed in the same manner in the two GU_8C complexes of the mutant and wild-type proteins. The four resonances between 11.37 and 12.58 p.p.m. are ascribed to the imino proton resonances of the RNA fragment, on the basis of their chemical shifts, while that at 10.34 p.p.m. has been proposed to be another imino proton resonance (12).

Imino proton resonances of AUUUUUUUUC bound with the mutant Sxl RBD1–RBD2

In addition to the *tra*-derived decamer sequence (GU_8C), the Sxl protein is known to bind to other uridine-stretches of the *Sxl* pre-mRNA, and is also suggested to bind to those of the *msl*-2 (5′) transcript, where the nucleotide residue just prior to the uridine stretch is usually an adenosine residue, in contrast to the guanosine residue of the *tra* pre-mRNA (7,9,27–30). Correspondingly, Sakashita and Sakamoto (8) selected *in vitro* the Sxl-binding RNAs of a consensus sequence with A prior to a uridine stretch. Therefore, in the present study, we also prepared another target RNA, AUUUUUUUUC (AU_8C), and measured the 600 MHz proton NMR spectrum of its complex with the mutant Sxl RBD1–RBD2. In the imino proton region (Fig. 1B), five resonances were observed, in much the same manner as that of GU_8C (Fig. 1A), except that the highest-field peak of the three sharp imino proton resonances was observed at 11.32 p.p.m. for AU_8C, but was at 11.37 p.p.m. for GU_8C. Therefore, the hydrogen-bond formation involving the imino protons is essentially the same between the complexes of AU_8C and GU_8C with the mutant RBD1–RBD2.

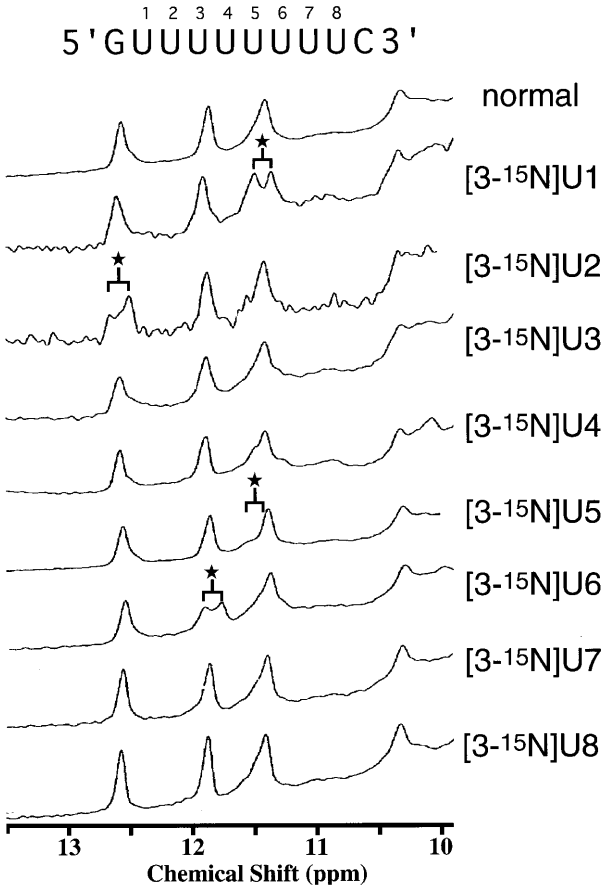

Figure 2. The imino proton regions of the 600 MHz proton NMR spectra of a series of GUUUUUUUUC samples, with and without a site-specific substitution of [3-^{15}N]uridine in the complex, with the mutant Sxl RBD1–RBD2. The observed doublet signals are indicated with asterisks.

Imino proton resonances of UUUUUUUUC in interaction with the mutant Sxl RBD1–RBD2

As a control, we also prepared an RNA fragment lacking the 5′ purine residue, UUUUUUUUC (U_8C). The imino proton spectrum of the complex with the mutant RBD1–RBD2 has five resonances (Fig. 1C). The imino proton resonances at 11.42 and 11.85 p.p.m. were observed for all three of the RNAs bound with RBD1–RBD2 (Fig. 1). In contrast, the resonances at 11.37 and 12.6 p.p.m. in the GU_8C complex appeared to be shifted up to 11.10 and 12.46 p.p.m., respectively, in the U_8C complex (Fig. 1A and C). Furthermore, the five hydrogen-bonded proton resonances of U_8C are much broader than those of GU_8C or AU_8C (Fig. 1). This is probably because U_8C binds to the Sxl RBD1–RBD2 more weakly than the other two. This is in good agreement with the previous reports that the adenosine residue immediately 5′ to the poly(U) tract is important for high affinity binding to Sxl (8,9).

Assignment of the hydrogen-bonded imino proton resonances by site-specific [3-^{15}N]uridine substitution of GUUUUUUUUC

We assigned the imino proton resonances from the repetitive uridine residues by replacing each specific residue with [^{15}N]uridine by the ribonucleotide phosphoramidite method. For GUUUUUU-UUC, we constructed a complete set of decamers, in which

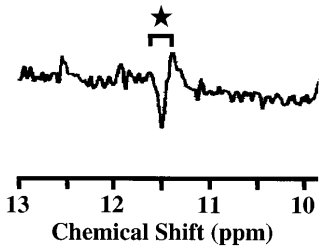

Figure 3. The imino protein of the difference between the spectra, with and without ^{15}N-decoupling, for the GUUUUUUUUC sample with the fifth uridine residue (U6) replaced by [3-^{15}N]uridine in the complex with the mutant Sx1 RBD1–RBD2.

[3-^{15}N]uridine was substituted for non-labeled uridine at one of the eight positions. First, we measured two-dimensional HSQC or HMQC spectra of the complex of these ^{15}N-labeled RNAs with the mutant Sxl RBD1–RBD2. However, we could not observe any ^{1}H–^{15}N cross peak (data not shown), as the labeled imino ^{1}H–^{15}N resonances are too broad probably because of some aggregation and/or exchange process. Therefore, even with the remarkable improvement of the solubility by the F166Y mutation of the Sxl RBD1–RBD2, the sample of the RNA•protein complex is not good enough for the multi-dimensional NMR measurements. Then, the one-dimensional ^{1}H NMR spectra of the eight ^{15}N-labeled species of GU_8C bound to the mutant RBD1–RBD2 were measured, the imino proton regions of which are shown in Figure 2. Actually, in four of the eight ^{15}N-labeled samples, one of the five imino proton resonances was observed as a doublet, rather than a singlet, because of the ^{1}H–^{15}N coupling (~90 Hz) in the labeled uridine. In fact, the ^{1}H–^{15}N splittings of these four imino proton resonances were not observed when the ^{15}N nuclei were decoupled (data not shown). First, for the decamer in which the first uridine residue (U1) was replaced by [3-^{15}N]uridine, G(^{15}N-U)UUUUUUUC, the imino proton resonance at 11.37 p.p.m. was observed as a doublet, and therefore was assigned to the imino proton of U1. Similarly, the resonances at 12.58 and 11.85 p.p.m. were split in the spectra of GU(^{15}N-U)-UUUUUUC and GUUUUU(^{15}N-U)UUC, respectively, and were unambiguously assigned to the imino protons of U2 and U6, respectively. When U5 was ^{15}N-labeled, the broad resonance at 11.42 p.p.m. was a doublet, which was confirmed by taking the difference spectrum between the non-decoupled and decoupled spectra (Fig. 3). The ^{15}N-labeled imino proton resonances appeared as asymmetric doublet signals with the lower-field line broader than the higher-field line, as in the cases of ^{15}N-labeled transfer RNAs (31–33). In contrast, no imino proton resonance exhibited ^{1}H–^{15}N coupling, when either U3, U4, U7 or U8 of GUUUUUUUUC was ^{15}N-labeled. This clearly shows that none of the imino protons of these four uridine residues is involved in the putative hydrogen bonds.

Finally, the broad resonances observed at 10.34 p.p.m. for GU_8C and AU_8C and at 10.16 p.p.m. for U_8C in their complexes with RBD1–RBD2 remained to be assigned. It is possible that this resonance is due to an OH group of the RNA backbone (the Sxl protein much prefers RNA to DNA). On the other hand, it should be noted here that the spectrum of the free protein of the mutant RBD1–RBD2 exhibited a resonance at 10.01 p.p.m., which has been assigned to an amide proton (Y.M. and S.Y., unpublished results).

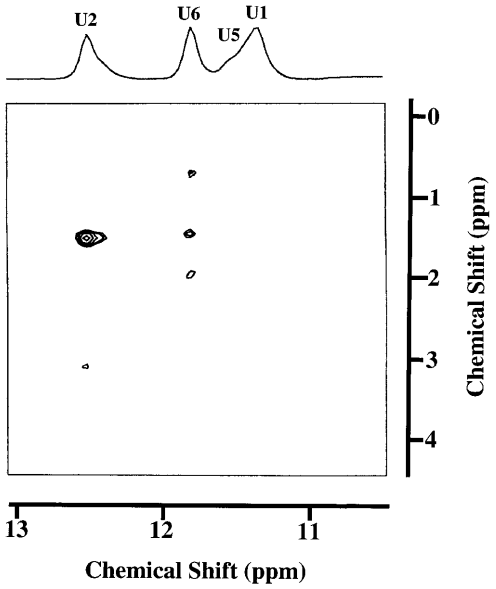

Figure 4. A region of the NOESY spectrum of a complex of GUUUUUUUUC with the mutant Sx1 RBD1–RBD2 in 10 mM ammonium acetate buffer (pH 6.0)

Thus, the assignments have been established for the hydrogen-bonded imino proton resonances of the first, second, fifth and sixth uridine residues in the uridine stretch of GU_8C in the complex with the Sxl RBD1–RBD2. On the basis of these unambiguous assignments, each of the four imino proton resonances observed for both AU_8C and U_8C may also be assigned by comparison. Even in the spectra of AU_8C and U_8C in the protein-bound states (Fig. 1B and C), two resonances were observed at exactly the same positions as the imino proton resonances of the fifth and sixth uridine residues of GU_8C. Accordingly, the 3′ halves, at least, of the three RNA fragments are properly bound with RBD1–RBD2, even though the binding is stronger for GU_8C and AU_8C, and weaker for U_8C. On the other hand, the resonance of the first uridine residue appears to be slightly shifted between GU_8C and AU_8C (Fig. 1A and B), probably reflecting the difference of the 5′-neighboring base. Furthermore, in the absence of the 5′ purine residue (i.e., the case of U_8C), the resonances of the first and second uridine residues are shifted appreciably upfield, to larger and smaller degrees, respectively, again reflecting the modification of the RNA sequence near these two residues. In addition, the removal of the 5′ G/A residue resulted in significant broadening of all four imino proton resonances, probably because of the weaker binding of the U_8C fragment to the protein as compared with GU_8C and AU_8C. The recognition of the 5′ pair of uridine residues seems to be coupled with that of the 5′ G/A, and occurs independently of the recognition of the downstream pair of the hydrogen-bonded uridine residues. In summary, the present analysis demonstrates that the Sxl RBDs recognize the uridine stretch, through hydrogen bonding, in a precise, site-specific manner, together with the 5′ G or A.

To confirm that these hydrogen-bonded imino protons are really in close proximity to the protein, we obtained the NOESY spectrum of the complex of GU_8C and the mutant Sxl RBD1–RBD2. Among the four hydrogen-bonded imino protons, those assigned to U2 and U6 exhibited intense and well resolved NOE cross peaks in the region of methyl/methylene resonances

(Fig. 4). This clearly shows that these two imino protons, at least, of GU_8C are involved in direct interaction with the protein. Intriguingly, the 5′- and 3′-halves of the U_8 stretch show the interaction modes quite similar to each other; the first and second U residues of the 5′- and 3′-U_4 sequences (U1–U2 and U5–U6, respectively) are involved in hydrogen bonding interaction, and the second one (U2 and U6) are in close proximity to aliphatic amino acid residues of the protein. This repetitive nature of the RNA–protein interaction may suggest that the 5′ half of GU_8C binds to one of the two RBDs while the 3′ half binds to the other. A remarkably strong NOE was observed between the imino proton of U2 and some aliphatic protons resonating at 1.55 p.p.m. (Fig. 4). Note that RBD1, but not RBD2, of the Sxl protein characteristically has an aliphatic residue (Ile128) in place of the widely-conserved, functionally important aromatic residue in the center of the RNP2 consensus sequence (13,34).

The importance of the uridine stretch in the binding of the Sxl protein to the *tra* pre-mRNA has been suggested by biochemical experiments (5,9,12). Both of the consensus sequences of the Sxl-binding RNAs selected *in vitro*, UUUUU(U/G)UU(U/G)-UUUUUUUU (10) and AU_nN_nAGU (8), have a GU_8 or AU_8 sequence, which appears to be necessary for hydrogen-bond formation with the Sxl RBD1–RBD2, as found in the present study. The Sxl protein has been reported to not bind the mutant *tra* pre-mRNA having the GUUCUCUCUC sequence in place of GUUUUUUUUC (4,5,12). It should be noted that one of the three positions of the cytidine substitution corresponds to the fifth uridine residue, which was revealed to be involved in the hydrogen bonding. It also has been reported that Sxl cannot bind to the stretch of cytidine residues that substituted for the uridine residues in the *Sxl* pre-mRNA (7). These effects of the replacement of a uridine residue by a cytidine are likely to be due to the loss of the hydrogen bonding involving the imino proton at the 3-position of the uracil base. On the other hand, it has been reported that the Sxl protein can bind to a uridine stretch as short as U_6 (the Sxl binding is naturally weaker for U_6 than the longer *tra* and *Sxl* PPTs), but not to shorter uridine stretches (9). This may be consistent with the present result that the hydrogen bonded uridine residues span over the U_6 portion of the GU_8C sequence.

The preference of $U2AF^{65}$ for PPTs is similar to, but not exactly the same as, that of Sxl, so Sxl has been suggested to block $U2AF^{65}$ binding to the PPTs for negative regulation of sex-specific splicing (6). Actually, most of the reported $U2AF^{65}$-binding sequences have cytidine residues in the middle (10,35). Therefore, it will be interesting to examine the differences in the RNA recognition mechanism between Sxl and $U2AF^{65}$.

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