Molecular analysis of a synthetic tetracycline-binding riboswitch

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ABSTRACT

Riboswitches are newly discovered regulatory elements that consist solely of RNA, sense their ligand in a preformed binding pocket, and perform a conformational switch in response to ligand binding, resulting in altered gene expression. Regulation by a tetracycline (tc)-binding aptamer when inserted into the 5' untranslated region (UTR) of a reporter gene exhibits all characteristics of a riboswitch. Chemical and enzymatic probing reveals that the aptamer consists of two stems, P1 and P2, which are already present in the absence of tc and form the scaffold of the aptamer. They are separated by a bulge B1-2 and an opposing stem-loop (P3-L3). Tc-dependent changes in the probing pattern only appear in the upper part of the bulge B1-2 (nucleotides 9–13) and the loop L3. Saturating mutagenesis corroborates the involvement of these two regions in regulation. Structural probing of the mutant A55U, which contains a single-nucleotide exchange in loop L3 results in a changed probing pattern of the loop, but also of the opposing bulge B1-2. This denotes that both regions cooperate and form a composite binding pocket. Thus, our model for aptamer-mediated translational regulation is that the ligand-free aptamer has only marginal influence on translational initiation. Tc then leads to an intramolecular connection in a pseudoknot-like manner and turns the aptamer into its inhibitory form. This represents a new mechanism for riboswitch action clearly distinguished from currently known naturally occurring riboswitches, which function by sequestration of the ribosomal binding site, transcriptional attenuation, and ribozyme-mediated degradation.

Keywords: tetracycline; aptamer; riboswitch; structural probing; saturating mutagenesis

INTRODUCTION

The importance of RNA for gene regulation is essentially due to its conformational flexibility and functional versatility. Recently, novel regulatory elements controlling a wide set of basic metabolic pathways in prokaryotes have been reported (for review, see Winkler and Breaker 2003; Nudler and Mironov 2004; Soukup and Soukup 2004). These molecular switches, called riboswitches, consist solely of RNA, they sense their ligand in a preformed binding pocket, and undergo restructuring upon metabolite binding. This affects gene expression by either causing transcription attenuation (Mironov et al. 2002; Winkler and Breaker 2002), inhibition of translation initiation (Nahvi et al. 2002; Winkler et al. 2002b) or ribozyme-mediated mRNA degradation (Winkler et al. 2004). These riboswitches function in the absence of any sensor protein. Their novelty is that now RNA accom-

plishes both sensor and regulator functions and thereby integrates the tasks formerly performed by a protein and an RNA component.

Recent studies using specific RNA aptamers to design small molecule-dependent synthetic riboswitches have opened new perspectives in the field of translational control. Aptamers are RNA molecules selected by SELEX in vitro to bind specifically to their target molecules (Ellington and Szostak 1990; Tuerk and Gold 1990). They adopt a unique conformation upon ligand binding, wherein the ligand becomes an integral part of the complex (Patel et al. 1997; Hermann and Patel 2000). Naturally occurring riboswitches are therefore comparable to these in vitro-selected aptamers by both exploiting the remarkable structural and functional versatility of RNA and by exhibiting the outstanding binding affinity and specificity. Thus, in principle, RNA aptamers have the potential to act as synthetic riboswitches by having aptamer–ligand complex formation interfere with initial stages of translation when it is inserted into the 5'UTR of a reporter mRNA (Werstuck and Green 1998).

We have identified a tc-binding aptamer capable of controlling translation in *Saccharomyces cerevisiae* by direct

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RNA–ligand interaction (Suess et al. 2003). The aptamer leads to up to 15-fold reduction of reporter activity *in vivo* when inserted directly in front of the start codon (Hanson et al. 2003). The analysis of ribosomal distribution of *in vitro*-translated aptamer-containing RNA using sucrose gradients has shown that the tc-bound aptamer interferes with the formation of the 80S ribosome, probably by blocking scanning (Hanson et al. 2003). But, the aptamer is also active when placed behind the cap structure. Here, aptamer–tc complex formation prevents binding of the small ribosomal subunit to the cap structure.

Thus, the tc aptamer shows all characteristics of a riboswitch; it binds its ligand with high affinity and specificity by direct ligand–RNA interactions, and the resulting complex then effects gene expression. The aptamer is not dependent on the insertion site within the untranslated region. In addition, the aptamer is of suitable size and responds in a dose-dependent and reversible manner to a small molecule that is not a cellular metabolite (Suess et al. 2003). This makes the tc aptamer not just an excellent molecular switch for conditional gene expression, but also a suitable model system to elucidate molecular and structural mechanisms underlying these novel regulatory elements. Hence, we combined structural and enzymatic probing with saturation mutagenesis in the absence and presence of tc to define the secondary structure of the aptamer inserted within the 5'UTR directly in front of the start codon, and identified regions involved in ligand binding and occurring conformational changes. We ascertain that the aptamer forms a composite binding pocket. The ligand then connects the two distinct regions of the aptamer by a pseudoknot-like intramolecular linkage, which leads to the inhibition competent conformation. This reveals a novel mechanism for riboswitches differing from naturally occurring variants which are based on transcriptional attenuation, sequestration of the ribosomal binding site, and ribozyme-mediated degradation.

RESULTS AND DISCUSSION

Probing secondary structure of the tc aptamer in its mRNA context

We probed the secondary structure of the tc-binding aptamer with structure-

specific nucleases. RNase V1 cleaves double-stranded RNA or stacked nucleotides, while S1 cleaves single-stranded RNA (Ehresmann et al. 1987; Knapp 1989). We used a 150-nucleotide long RNA fragment carrying the tc aptamer in its mRNA context directly in front of the start codon (complete 5'UTR and the first 48 nucleotides of the GFP-encoding reading frame). ³²P-labeled RNA was treated with the respective nucleases, and the resulting fragments were sized by electrophoresis on a denaturing polyacrylamide gel. The fragmentation pattern is shown in Figure 1A. The results of the enzymatic cleavage are summarized schematically onto the secondary structure of the tc aptamer calculated by the mfold Web server (version 3.1, <http://www.bioinfo.rpi.edu/applications/mfold>) (Zuker 2003; Fig. 2A).

Double-strand specific cuts at the positions G1, C3–A6, U64–G66, and C69 suggest that the stem P1 is formed in solution but extended to include the base pair A6–U64. The

FIGURE 1. Enzymatic and chemical probing of the tc-binding aptamer. (A) Limited digestion was performed using a 150-nucleotide-long RNA containing the aptamer in its mRNA context. Probing was carried out using RNase T1 (0.5 and 0.25 U), RNase V1 (0.005 and 0.001 U), and S1 nuclease (1 and 0.5 U) in the absence (–) and presence (+) of 10 μM tc. The two left-hand lanes of each nuclease probing correspond to the respective higher enzyme concentration. Alkaline hydrolysis of the RNA is denoted by H. G residues probed with RNase T1 are marked at left, and stem regions proposed by secondary structure prediction are denoted at the right side of the plot with open bars. (B) Chemical modifications were performed in the absence and presence of tc and monitored by primer extension reaction. Untreated RNA is marked with an 0. The incubation time (T) and the concentration of tc is given above the figure. (C, U, A, G) Sequencing lanes. (S) DMS modification carried out under semidenaturing conditions in the presence of EDTA. Nucleotide positions with occurring tc-dependent changes in the probing pattern are denoted and marked with arrowheads at the left side of the plot.

FIGURE 2. Summary of the secondary structure analyses and tc-dependent changes of the tc-binding aptamer. (A) RNA secondary structure prediction was conducted at the mfold Web server (version 3.1; Zuker 2003). The AUG start codon of the *gfp* reading frame is underlined. Nucleotide positions with double-strand specific V1 cuts are highlighted with filled circles, the intensity of the filling correlates with the strongness of the V1 signal. Positions amenable to S1-mediated single-strand cuts are highlighted with open circles. Positions that were modified by chemical probes are marked with symbols (DMS, circle; DEPC, arrowhead; kethoxal, square; CMCT, diamond). The size of the symbols correlates with the intensity of the signal; the largest symbols correspond to the highest level of modification. (B) Positions with tc-dependent changes of the chemical modification pattern are marked with symbols (DMS, circle; DEPC, arrowhead; kethoxal, square; CMCT, diamond) onto the improved secondary structure derived from the structural probing analysis. The largest symbols correspond to the highest tc-dependent protection (a scaling in terms of degree reduction of signal intensity is given *top, right* of the blot). Open and closed symbols indicate an increase and decrease, respectively, in signal strength. Nucleotides highlighted with circles were saturating mutagenized. Resulting mutants were analyzed in vivo for their ability for tc-dependent regulation of gene expression when inserted into the 5'UTR of a reporter mRNA. Positions highlighted in black are completely intolerant toward mutation, gray circles indicate positions at which mutations lead to reduced regulatory activity, and open circles indicate positions at which all nucleotide exchanges do not influence regulation.

lower part of P1 is less susceptible to RNase V1. V1 cuts at positions C15-A18 and C38-G40 agree with the existence of the lower part of stem 2. However, the lack of V1 cuts, combined with extended single-stranded regions between the nucleotides (nt) C25 and U37 indicate that the predicted P2' stem-loop structure is not reliably formed. V1 cuts at G46-G48 and C62 support formation of P3. Further S1 cleavages are consistent with all predicted single-strand regions as follows: the bulge B1-2 (A7-C14), the joining region J2-3 (G43), and the loop L3 (A50, G51, A53-C56).

The accessibility of RNA toward modifications by base-specific chemical reagents allows conclusions about the involvement of specific nucleotides in base pairing or tertiary interactions (Ehresmann et al. 1987). DMS (dimethylsulphate) methylates position N-1 of A, CMCT (1-cyclohexyl-3-[2-morpholinoethyl] carbodiimidemetho-p-toluenesulfonate) modifies position N-3 of U and kethoxal (β -ethoxy- α -ketobutyraldehyde) reacts with G, generating a cyclic adduct between N-1 and N-2. Position N-7 of A involved in Hoogsteen or reverse-Hoogsteen interactions are probed by

DEPC. The RNA fragments were treated with DMS, CMCT, kethoxal, and DEPC and subjected to a primer extension reaction using a 32 P-labeled primer. A control of unmodified RNA was run in parallel to discriminate between stops of the reverse transcriptase specifically induced by modification and pausing due to secondary structures or spontaneous cleavages. The modification pattern is shown in Figure 1B. Bases accessible to the respective chemicals are marked with symbols in the secondary structure in Figure 2A.

None of the proposed stem regions show signals caused by base modification. The failure of DMS modification at position A6 and a signal at position A44 caused by DMS methylation support the formation of the base pair A6-U64 of stem P1 instead of A44-U46 of stem P3. Strong signals at G21, G28, C29, and G30 indicate that the upper part of P2 does not form, as it was already suggested by nuclease digestion. A refined secondary structure, which includes all new insights from structural probing, is shown in Figure 2B.

Nearly all bases located in the proposed single-stranded regions B1-2, J2-3, and L3 are accessible to chemical modification, however, to different degrees. The DMS and CMCT signals at positions 11, 12, and 13 are less intense than signals of the adenines 7, 8, and 9

in the lower half of bulge B1-2. This is in agreement with the gradual decrease in signal strength for the S1 cuts in this bulge. The reduced accessibility indicates an involvement of the upper part of the bulge in tertiary interaction. Single V1 cuts at the positions 10 and 12 confirm these tertiary interactions. A similar effect is observed for loop L3. We observe a better accessibility for nt 51–55 than for 49, 50, 57, or 58 (see Fig. 1A). These tertiary interactions occur in the presence of Mg^{2+} . Whereas all stem regions are already formed under semidenaturing conditions (Fig. 1B, second lane [S] of the DMS probing), the differences in the accessibility within the single-stranded region B1-2 and L3 first appear in the presence of Mg^{2+} (compare the second and third lane of the DMS probing). Interestingly, we observe a signal caused by kethoxal for the adenine at position 55. This may be due to conformational constraints leading to a local pKa shift of the adenine, which would then allow a cyclic addition of kethoxal.

Taken together, the results of the structural probing analyses are nearly consistent with the proposed secondary

structure of the aptamer located in its mRNA context. All stem regions are already formed in the absence of the ligand. This explains the partial decrease of translation efficiency *in vivo* when the aptamer is inserted in the non-translated region of a reporter gene (Hanson et al. 2003; Suess et al. 2003). Parts of bulge B1-2 and loop L3 are single-stranded, but become involved in tertiary interaction after the addition of Mg^{2+} .

Bulge B1-2 and Loop L3 are involved in tc binding

Our next concern was to assess regions involved in tc binding. Therefore, we repeated the structural analyses in the presence of the ligand tc. RNA was incubated with 0.1–10 μM tc and subsequently subjected to enzymatic and chemical modification analyses as described above. The probing patterns are shown in Figure 1. Positions exhibiting tc-dependent changes in the probing pattern were quantified. The data are summarized in Table 1 and marked with symbols upon an improved illustration of the secondary structure incorporating the structural probing data (Fig. 2B). Thereby, the size of the symbols corresponds to the degree of signal change.

The addition of tc does not effect the overall secondary structure, because nearly all signals observed by enzymatic and chemical probing exist also in the presence of tc. We observe a strong decrease of the probing signal at nucleotide positions A9, U12, and A13 located in the upper half of bulge B1-2, for which an involvement in tertiary interaction had already been proposed. Furthermore, a drastic decrease is observed for nearly all nucleotides of the loop L3 (Table 1; Fig. 2B). The exceptions are A52 and A53 with only marginal signal reduction. In addition, tc-dependent protection of A50, G51, U54, A55, and C56 from S1 digestion supports the importance of the loop for ligand binding.

Tc binding to the aptamer was further investigated by hydroxyl radical probing. This method makes use of the ability of Fe^{2+} to replace the Mg^{2+} -ion complexed with tc. After addition of hydrogen peroxide, Fe^{2+} generates short-lived, highly reactive hydroxyl radicals that can cleave proteins and nucleotides in proximity of bound tc (Ettner et al. 1993; McMurry et al. 2002; Bauer et al. 2004). In contrast to other probing methods, Fe^{2+} -mediated hydroxyl radicals cleave nucleic acids with little or no sequence specificity (Tullius et al. 1987; Balasubramanian et al. 1998), and a significant secondary structure preference has not been observed in radical-induced cleavage of single- and double-stranded forms of RNA and DNA (Celander and Cech 1990).

A 71-nucleotide-long RNA fragment containing the tc-binding aptamer was probed with 250 μM Fe^{2+} , 2.5 mM sodium ascorbate, and 2.5 mM H_2O_2 in the presence of 1–100 μM tc, resulting in one tc-dependent signal at position A55 (Fig. 3). Beside this signal, no additional cleavage sites were obtained. The Mg^{2+} -competition experiment shows decreasing signal intensities for increasing amounts

TABLE 1. Quantification of tc-dependent changes of the chemical probing analysis

Aptamer position	Chemical probe	Wild-type aptamer	Mutant aptamer A55U
		Normalized intensity/%	Normalized intensity/%
A9	DMS	36.5 $\pm$ 4.9	131 $\pm$ 16.0
A13	DMS	38.2 $\pm$ 3.2	68.1 $\pm$ 11.2
A49	DMS	94.9 $\pm$ 0.5	72.3 $\pm$ 9.2
A50	DMS	36.8 $\pm$ 2.8	26.5 $\pm$ 3.3
A53	DMS	62.7 $\pm$ 3.0	79.4 $\pm$ 10.5
A55	DMS	60.5 $\pm$ 6.2	
A58	DMS	38.0 $\pm$ 6.5	38.4 $\pm$ 6.0
A8	DEPC	83.2 $\pm$ 2.5	90.4 $\pm$ 5.7
A9	DEPC	77.6 $\pm$ 1.4	110 $\pm$ 5.8
A13	DEPC	56.3 $\pm$ 2.2	84.3 $\pm$ 3.6
A49	DEPC	28.2 $\pm$ 2.1	40.3 $\pm$ 3.2
A50	DEPC	31.6 $\pm$ 2.5	42.4 $\pm$ 2.3
A52	DEPC	65.2 $\pm$ 3.3	63.7 $\pm$ 2.3
A53	DEPC	91.7 $\pm$ 5.9	115 $\pm$ 7.1
A55	DEPC	68.5 $\pm$ 4.1	
A58	DEPC	73.5 $\pm$ 3.3	63.4 $\pm$ 3.1
U12	CMCT	46.0 $\pm$ 2.8	75.4 $\pm$ 3.0
U32	CMCT	192 $\pm$ 3.4	120 $\pm$ 5.7
U33	CMCT	196 $\pm$ 4.9	123 $\pm$ 5.7
U34	CMCT	187 $\pm$ 6.3	115 $\pm$ 7.8
U54	CMCT	18.9 $\pm$ 3.1	95.5 $\pm$ 7.7
U55	CMCT		105 $\pm$ 5.2
G43	kethoxal	47.6 $\pm$ 5.2	39.9 $\pm$ 4.6
G51	kethoxal	13.6 $\pm$ 2.3	30.5 $\pm$ 3.3
G57	kethoxal	19.8 $\pm$ 2.1	17.9 $\pm$ 1.5

Quantification of tc-dependent changes in the chemical probing pattern. Chemical modifications of the tc-binding aptamer and the mutant variant A55U were carried out for 20 min (DMS) and 30 min (CMCT, kethoxal, DEPC) in the absence and presence of 1 μM tc. The signals were quantified in a phosphorimager, and intensities were normalized after correction for background. The signals in the absence of tc were taken as 100%.

of Mg^{2+} , indicating that Fe^{2+} and Mg^{2+} might interact with overlapping sites. As the aptamer alone shows no conformational changes upon addition of increasing amounts of Mg^{2+} (C. Berens, pers. comm.), this indicates a direct competition between Fe^{2+} and Mg^{2+} . Thus, the pronounced cleavage signal at position A55 provides additional evidence for the importance of loop L3 in tc binding and is also in nice agreement with lead cleavage data that indicated an involvement of bulge B1-2 and the loop L3 in tc binding (Berens et al. 2001).

The observed tc-dependent changes are restricted mainly to bulge B1-2 and loop L3 of the aptamer as summarized in Figure 2B. The stems P1 and P2 do not interact with tc and may only be of structural importance. This conclusion is supported by *in vivo* data obtained previously. Both stems have been varied in length and sequence without loss of regulation (Hanson et al. 2003; Suess et al. 2003). Hence, we propose that P1 and P2 may be responsible only for maintaining the scaffold of the aptamer structure, whereas bulge

FIGURE 3. Fe^{2+} -mediated hydroxyl radical cleavage reaction of the tc-binding aptamer. Cleavage was performed using the 71-nucleotide-long aptamer RNA. Probing was carried out with increasing amounts of tc in the presence of Fe^{2+} and $\text{NaOAsc}/\text{H}_2\text{O}_2$. For Mg^{2+} competition, the RNA was treated with $\text{Fe}^{2+}/\text{H}_2\text{O}_2$ and increasing amounts of Mg^{2+} in the presence of 100 μM tc. (H) Alkaline hydrolysis; (T1) RNase T1 sequencing ladder.

B1-2 and loop L3 are responsible for the formation of the tc-binding pocket.

Investigating structure-function relationships by saturating mutagenesis

Saturating mutagenesis was performed on the basis of chemical and enzymatic protection data to identify base

positions important for the regulatory activity in vivo. Aptamers were mutagenized at the nt 8–13 and 49–58 by PCR mutagenesis and inserted directly upstream of the start codon of a constitutively expressed *gfp* gene (Suess et al. 2003). The resulting plasmids were transformed into *S. cerevisiae*, and GFP fluorescence was determined in the absence and presence of 100 μM tc. The data are summarized in Table 2 and displayed as highlighted positions in Figure 2B.

Mutations at the positions 9–11 and 13 in B1 and 49, 50, and 56–58 in L3 result in a complete loss of regulation, irrespective of the nucleotide exchange introduced. This agrees nicely with the in vitro results that show strong tc-dependent changes and an involvement of these positions in binding-pocket formation. Mutations at positions 8, 12, 51, 54, and 55 are tolerated, but lead to reduced regulation. Thus, these positions participate in ligand binding, maybe not by direct interaction, but by an involvement in binding-pocket formation, and are therefore significant for tight binding of tc. All nucleotide exchanges in the probing pattern at positions 52 and 53, which were proposed to be not involved in tertiary interactions or tc binding, show regulatory properties like wild type. Mutations U10, G13, A51, U50, G55, U56, and U57 are not amenable to analysis due to strong reduction of GFP expression already in the ab-

TABLE 2. Regulatory properties of aptamer mutants in vivo

Position	Relative fluorescence in %			Relative fluorescence in %			Relative fluorescence in %			Relative fluorescence in %		
	–tc	100 μM tc	F	–tc	100 μM tc	F	–tc	100 μM tc	F	–tc	100 μM tc	F
Exchange to	$x = A$			$x = C$			$x = G$			$x = U$		
wild type	100 $\pm$ 0	17 $\pm$ 4.1	5.8									
A8x				128 $\pm$ 9.2	45 $\pm$ 5.1	2.8	119 $\pm$ 11	39 $\pm$ 1.9	3.1	129 $\pm$ 9.6	62 $\pm$ 9.0	2.1
A9x				128 $\pm$ 12.8	114 $\pm$ 4.6	1.1	132 $\pm$ 12	134 $\pm$ 5.4	1.0	147 $\pm$ 9.1	149 $\pm$ 6.3	1.0
C10x	99 $\pm$ 9.3	104 $\pm$ 11	0.9				144 $\pm$ 10	145 $\pm$ 6.8	1.0	3.1 $\pm$ 2.5	3.5 $\pm$ 2.9	0.9
A11x				103 $\pm$ 8.2	123 $\pm$ 5.7	0.9	106 $\pm$ 9.8	107 $\pm$ 11	1.0	145 $\pm$ 6.9	137 $\pm$ 6.5	1.1
U12x	87 $\pm$ 6.6	33 $\pm$ 6.0	2.6	92 $\pm$ 14.4	102 $\pm$ 7.3	0.9	125 $\pm$ 12	66 $\pm$ 5.0	1.9			
A13x				90 $\pm$ 14.2	103 $\pm$ 9.2	0.9	6.5 $\pm$ 1.4	7.5 $\pm$ 2.6	0.9^a	90 $\pm$ 7.0	97 $\pm$ 8.0	0.9
A49x				98 $\pm$ 9.1	96 $\pm$ 7.0	1.0	54 $\pm$ 6.6	56 $\pm$ 16	1.0	104 $\pm$ 8.5	92 $\pm$ 11	1.1
A50x				83 $\pm$ 1.1	79 $\pm$ 7.0	1.0	79 $\pm$ 0.1	84 $\pm$ 13	0.9	8.9 $\pm$ 0.6	9.7 $\pm$ 1.1	0.9^a
G51x	7 $\pm$ 3.3	5.5 $\pm$ 3.5	1.3	114 $\pm$ 8.7	37 $\pm$ 0.7	3.1				94 $\pm$ 8.1	31 $\pm$ 7.9	3.0
A52x				76 $\pm$ 0.6	15 $\pm$ 1.5	5.1	80 $\pm$ 14	14 $\pm$ 6.4	5.7	105 $\pm$ 11	17 $\pm$ 5.9	6.0
A53x				97 $\pm$ 8.7	15 $\pm$ 4.7	6.5	100 $\pm$ 13	24 $\pm$ 3.8	4.1	88 $\pm$ 11	13 $\pm$ 4.5	6.4
U54x	101 $\pm$ 11	18 $\pm$ 3.9	5.4	92 $\pm$ 3.0	23 $\pm$ 2.3	4.0	81 $\pm$ 11	24 $\pm$ 7.4	3.3			
A55x				63 $\pm$ 3.5	19 $\pm$ 0.1	3.3	79 $\pm$ 10	26 $\pm$ 3.9	2.9^b	88 $\pm$ 7.1	34 $\pm$ 11	2.6
C56x	111 $\pm$ 12	101 $\pm$ 12	1.1				120 $\pm$ 14	128 $\pm$ 8.4	0.9	40 $\pm$ 2.9	36 $\pm$ 3.9	1.1^a
G57x	115 $\pm$ 16	118 $\pm$ 8.6	1.0	81 $\pm$ 9.9	80 $\pm$ 7.3	1.0				1.8 $\pm$ 2.4	1.7 $\pm$ 2.1	1.1
A58x				73 $\pm$ 8.4	77 $\pm$ 0.2	1.0	85 $\pm$ 12	85 $\pm$ 9.4	1.0	97 $\pm$ 3.8	101 $\pm$ 11	1.0
mutant A55U												
A53x				33 $\pm$ 2.3	27 $\pm$ 1.8	1.2	62 $\pm$ 3.3	53 $\pm$ 8.2	1.2	62 $\pm$ 4.4	42 $\pm$ 2.1	1.4

Regulatory properties of aptamer mutants with nucleotide exchanges at positions proposed to be involved in tc binding. Fluorescence was measured after 48 h incubation in the absence (column 2, 5, 8, and 11, –tc) or presence (column 3, 6, 9, and 12) of 100 μM tc. The fluorescence of the constitutively expressed GFP gene with the wild-type aptamer in the absence of tc was set to 100%. Columns 4, 7, 10, and 13 (marked with F) show the efficiency of regulation given as the ratios of relative fluorescence values without and with tc.

^aMutations lead to the introduction of a premature start codon in the 5'UTR.

^bMeasured as double-mutant C53G55 to avoid the formation of a premature start codon.

sence of tc. For G13, U50, G55, and U56, this is caused by the introduction of a premature start codon in the 5'UTR.

Thus, the *in vivo* results show that several positions of both the bulge B1-2 and the loop L3 are not at all exchangeable, and thereby support the assumption that both regions participate in the formation of the ligand-binding pocket.

Aptamer mutant A55U suppose the formation of a composite binding site

Structural probing and mutational analysis revealed that the binding pocket is composed of two parts. We now ask whether each part can bind one single molecule tc, or do both regions that are separated in the secondary structure, and contribute to interaction by forming a composite binding pocket. To elucidate this, we analyzed an aptamer mutant, A55U, which is regulatory active, but with reduced efficiency (Suess et al. 2003). We performed enzymatic and chemical probing assays as described above for the wild type. The fragmentation and modification patterns were similar to that of the wild-type aptamer (data not shown), indicating that there are no overall changes in the secondary structure. We have quantified all tc-dependent changes. The data are included in Table 1. Segments of the gels with prominent changes in the probing pattern are shown in Figure 4. Astonishingly, the nucleotide exchange at position 55, which is located in loop L3, affects the probing pattern of bulge B1-2. We observe an increase of the DMS signal at A9 and a slight reduction at the U12 and A13, whereas the wild-type aptamer shows a strong reduction at all three positions (Table 1; Fig. 4). These data support the hypothesis that bulge B1-2 and loop L3 interact with each other and participate in formation of a composite binding pocket for tc.

FIGURE 4. Comparative analyses of the DMS modification pattern of tc-binding aptamer and the aptamer mutant A55U. DMS modification of a 150-nucleotide-long RNA fragment containing the aptamer (wild-type or A55U mutant) in its mRNA context were performed in the absence and presence of tc and monitored by primer extension reaction. Untreated RNA is marked with 0. The concentration of tc is given above the plot. (S) DMS modification carried out under semi-denaturing conditions in the presence of EDTA. Positions with tc-dependent changes in the probing pattern are denoted at left.

A55 participates in tc binding

The comparison of wild-type with the A55U mutant shows that the strong tc-dependent decrease at the wild-type adenine of position 55 is now shifted to the adenine at position 53, a position which is completely uninfluenced in the wild-type aptamer. This presumes that adenine at position 55 may be directly participating in tc binding. If the adenine is then mutated to a uridine, as in the case of A55U, the adenine at position 53 appears to take on the task of position 55. To test this, we mutagenized position 53 of the mutant. The data are included in Table 2. Whereas in the wild-type aptamer, position 53 is not sensitive to mutations, in the mutant A55U context, a complete loss of regulation is observed, irrespective of the nucleotide exchange introduced. This shows that the adenine at position 53 is essential when the wild-type adenine at position 55 is mutated and is able to functionally suppress the defect caused by mutation of A55.

Intramolecular linkage—the mechanistic basis for tc activity?

The crystal structure of the small ribosomal subunit of *Thermus thermophilus* shows two main binding sites for the antibiotic tc (Brodersen et al. 2000; Pioletti et al. 2001). Both sites are formed by at least two separate parts of the 16S rRNA, both are composed of an irregular part of a helix that forms a pocket-like structure and a second part that shapes a lid to close the pocket. The primary tc-binding site is formed by a distorted minor groove of helix h34. The stacked nt 1196–1200 and 1056–1053 form a clamp that holds the tc molecule by hydrophobic interactions and hydrogen bonding. Residues 965 and 966 from the h31 stem-loop make contacts to tc from the opposite side and close the binding pocket. We find a similar assembly for the secondary binding site (Brodersen 2000, site 2; Pioletti 2001, site 5), which is composed of h27 and nt of h11 and h20. Thereby, the nt 894 and 895, as well as 891–894 of h27, form a rather tight binding pocket mainly through stacking interactions. U244, C245 of h27, and G761 of h20 shape the lid. The sequence similarities between the primary binding pockets of the *Escherichia coli* 16S rRNA sequence and loop L3 of the aptamer are noticeable. The sequence of the ribosome (site 1: AAG [1196–1198] UACG [1056–1053]; site 2/5 AA [894,895] UACG [891–894]) fits well to the nucleotides AAG (49–51) and UACG (54–57) of loop L3 that we have determined to be important for tc binding in this study.

Thus, we propose that the loop L3 is responsible for the formation of a tc-binding pocket for tc generated by the nucleotides AAG (49–51) and UACGA (54–58). The tc molecule is probably buried deeply in this pocket and makes contact with multiple residues, explaining the strong chemical protection of nearly all nucleotides of the loop in

the presence of tc and the strong sensitivity against nucleotide exchanges *in vivo*. The upper part of the bulge B1-2 would then form a lid, thereby trapping tc in the binding pocket.

This leads to our model of tc-aptamer-mediated translational regulation. The aptamer forms a scaffold by the stems P1 and P2 already in the absence of the ligand, which leads to a somewhat reduced reporter gene expression. The addition of tc then leads to the formation of the binding pocket. Multiple contacts of the tc to both the bulge B1-2 and loop L3 region mediate an intramolecular linkage within the aptamer in a pseudoknot-like manner. This complex is then able to efficiently interfere with the ribosome, thereby inhibiting translational initiation.

This ligand-mediated intramolecular linkage forms the mechanistic basis of the synthetic tc riboswitch. Thus, it cannot be classified among the currently known natural riboswitches that are based on transcriptional attenuation, sequestration of the ribosomal binding site, or mRNA degradation, and consequently, represents a new class of riboswitches.

MATERIALS AND METHODS

Preparation of RNA

PCR amplification using pWHE601-AN32sh (wild-type aptamer) and -AN28sh (A55U mutant) (Suess et al. 2003) as template and the primers T7 in (5'-TCTAATACGACTCACTATAGGAGCATA CAATCAACTCC, the T7 recognition sequence is underlined) and GFP_rev (5'-CAAGAATTGGGACA ACTCC) was performed to obtain a 170-bp-long PCR product carrying the tc-binding aptamer in its mRNA context behind a T7 promoter. RNA transcription was carried out in 40 mM Tris-HCl (pH 7.5), 26 mM MgCl₂, 3 mM Spermidine, 20 mM DTT, 2.5 mM NTPs, 1 U RNase inhibitor with 200 U T7 RNA polymerase for 16 h at 37°C, followed by DNase I treatment for 1 h at 37°C. The RNA was purified on a 5% denaturing polyacrylamide gel (20:1) and eluted from gel slices by shaking for 6 h at room temperature in 10 mM Tris-HCl (pH 7.5), 0.1% SDS, 2 mM EDTA, and 250 mM sodium acetate. After precipitation, the RNA was resuspended in chromatography grade water.

RNA dephosphorylation and 5'end labeling

Dephosphorylation of 100 pmol RNA was carried out in 0.1 M Hepes-KOH (pH 6.7) with 10 U Calf Intestinal Phosphatase in a total volume of 20 μ L for 1 h at 37°C. After phenolchloroform isoamylalcohol extraction and precipitation, 10 pmol of RNA were 5'end labeled with 30 μ Ci [γ -³²P]ATP and 10 U T4 polynucleotide kinase in a total volume of 10 μ L for 30 min at 37°C, then precipitated and gel purified.

RNase protection assay

A total of 100,000 of cpm 5'end-labeled RNA was incubated in 50 mM sodium acetate (pH 4.5), 280 mM NaCl, 4.5 mM ZnSO₄ (S1),

or 50 mM Tris-Cl (pH 7.5) (T1 and V1) in the presence of 5 ng of unlabeled yeast tRNAs for 2 min at 56°C and for 10 min at 37°C. Afterward, 5 mM MgCl₂ was added and the RNA was incubated at room temperature for 5 min without or with 1 or 10 μ M tc. A total of 1 or 0.5 U of S1 nuclease (Promega), 0.25 or 0.5 U of RNase T1 (Roche), or 0.001 or 0.005 U of RNase V1 (Ambion) was then added and incubated for 5 min at room temperature. Alkaline hydrolysis was carried out in 50 mM sodium carbonate buffer (pH 9.0) for 3 min at 90°C. All reactions were stopped by the addition of the equal volume of sample loading buffer (95% formamid, 0.1% bromophenol blue, 20 mM EDTA) and immediately putting on ice. Samples then were separated through 10% denaturing polyacrylamide gels at 40 W for 2 h and visualized by phosphorimager.

Chemical modification assays

Modification with DMS

A total of 5 pmol of RNA was incubated in 80 mM cacodylate buffer (pH 7.5) for 2 min at 56°C and for 10 min at 37°C. Afterward, 5 mM MgCl₂ was added, and the RNA was incubated for 5 min at room temperature without or with 1 or 10 μ M tc. Methylation was performed by incubation with 1 μ L DMS (1:8 dilution in 96% ethanol) for 20 min at room temperature. Reactions were stopped by addition of 1 μ L β -mercaptoethanol (1:5 dilution in water). After precipitation, 5'end-labeled primer was annealed and primer extension performed.

Modification with DEPC, CMCT, and kethoxal

A total of 5 pmol of RNA was incubated in 80 mM cacodylate buffer (pH 7.0) DEPC (Sigma), 50 mM potassium borate buffer (pH 8.0) CMCT (Sigma), or 50 mM potassium borate buffer (pH 7.0) kethoxal (ICN) at 56°C for 2 min and at 37°C for 10 min. Afterward, 5 mM MgCl₂ was added, and the RNA was incubated for 5 min at room temperature without or with 1 or 10 μ M tc. A total of 10 μ L of DEPC, 10 μ L of CMCT (42 mg mL⁻¹ stock in 50 mM potassium borate buffer at pH 8.0) or 5 μ L of kethoxal (37 mg mL⁻¹ in 20% ethanol) were added. The DEPC-containing reaction mixtures were incubated at 37°C for 30 and 60 min with intermittent shaking. CMCT and kethoxal modifications were carried for 30 and 60 min at room temperature. Reactions were stopped by precipitation. 5'end-labeled primer was annealed and primer extension performed.

Primer extension

Hybridization of modified RNA (2.5 μ L) and 1 μ L of 5'end-labeled primer was carried out in 50 mM Hepes-KOH (pH 7.0) and 100 mM KCl by heating the mixture for 1 min at 96°C, followed by 15 min incubation at room temperature. Extension reaction was started in 50 mM Tris-HCl (pH 7.5), 75 mM KCl, 3 mM MgCl₂, 0.25 mM dNTPs, 10 mM DTT with 10 U SuperscriptII RNaseH⁻ Reverse Transcriptase (Invitrogen) at 42°C for 50 min. The reactions were stopped by alkaline hydrolysis of the RNA template. After precipitation, the DNA fragments were separated through 10% denaturing polyacrylamide gels at 40 W for 2 h and visualized by phosphorimager. The sequencing reactions were carried out

using the PCR templates with the T7 Sequencing kit (USB Corporation).

Fe²⁺-mediated hydroxyl radical cleavage reactions

A 71-nucleotide-long RNA fragment containing the tc-binding aptamer was generated by in vitro transcription using plasmid pSP64B_{mono} as a template. pSP64B_{mono} encodes the wild-type aptamer stabilized by an additional G-C pair introduced in stem 1 under control of the T7-promotor. Homogenous 3' ends were obtained by introduction of a hammerhead ribozyme sequence. RNA dephosphorylation and 5' end labeling was performed as depicted above.

The hydroxyl radical cleavage was carried out similar as described before (Berens et al. 1998). A total of 1 μ L RNA (20 pmol cold RNA, spiked with ~50,000 cpm of 5' [³²P]-labeled RNA) was added to 1 μ L of 5 $\times$ cleavage buffer (5 $\times$ CB: 125 mM MOPS-KOH (pH 7.0); 25 mM MgCl₂; 500 mM NaCl; the 5 $\times$ CB additionally included 5–500 μ M tc for samples, in which the RNA was cleaved in the presence of tc) and incubated for 1 min at 60°C, followed by 2 min incubation at room temperature. A total of 1 μ L of 1.25 mM FeCl₂ was added to the reaction tube, mixed by centrifugation, and incubated for 1 min before adding 1 μ L of 12.5 mM sodium ascorbate. After 1 min, 1 μ L of 12.5 mM hydrogen peroxide was added to initiate the reaction and rapidly mixed. The final concentrations were 250 μ M for Fe²⁺ and 2.5 mM for both sodium ascorbate and hydrogen peroxide. The cleavage reaction was stopped after 1 min by the addition of 1 μ L 1M thio-urea, 1 μ L glycogen (10 μ g/ μ L), and 60 μ L ethanol. The RNA was precipitated, resuspended in gel-loading buffer (0.3% each of bromophenol blue and xylene cyanol; 10 mM EDTA (pH 7.5); 97.5% deionized formamide) and electrophoresed in denaturing 15% polyacrylamide sequencing gels.

Mg²⁺-competition of Fe²⁺-cleavage

In the Mg²⁺-competition experiment, the cleavage reaction was carried out in the presence of increasing amounts of MgCl₂ (5–80 mM) leading to Fe²⁺:Mg²⁺ ratios ranging from 1:20 to 1:320, since Fe²⁺ shows a 100-fold higher affinity to tc than Mg²⁺ (Ettner et al. 1995). MgCl₂ was added as a 5 $\times$ stock solution of the final Mg²⁺ concentration to the 1.25 mM FeCl₂ solution. The Fe²⁺/Mg²⁺ mixture was then pipetted into the reaction tube, and the cleavage reaction continued as above.

Site-directed mutagenesis

Mutations were introduced into pWHE601-AN32sh (wild-type aptamer) by directed PCR mutagenesis with the three primer method (Landt et al. 1990) using oligonucleotides carrying the respective nucleotide exchanges.

GFP measurements

Fluorescence measurements were done as previously described (Suess et al. 2003).

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