Binding Enhancement by Tertiary Interactions and Suicide Inhibition of a *Candida albicans* Group I Intron by Phosphoramidate and 2′-*O*-Methyl Hexanucleotides†

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ABSTRACT: *Candida albicans* is one of many infectious pathogens that are evolving resistance to current treatments. RNAs provide a large class of targets for new therapeutics for fighting these organisms. One strategy for targeting RNAs uses short oligonucleotides that exhibit binding enhancement by tertiary interactions in addition to Watson−Crick pairing. A potential RNA target in *C. albicans* is the self-splicing group I intron in the LSU rRNA precursor. The recognition elements that align the 5′ exon splice site for a ribozyme derived from this precursor are complex [Disney, M. D., Haidaris, C. G., and Turner, D. H. (2001) *Biochemistry 40,* 6507−6519]. These recognition elements have been used to guide design of hexanucleotide mimics of the 5′ exon that have backbones modified for nuclease stability. These hexanucleotides bind as much as 100000-fold more tightly to a ribozyme derived from the intron than to a hexanucleotide mimic of the intron's internal guide sequence, r(GGAGGC). Several of these oligonucleotides inhibit precursor self-splicing via a suicide inhibition mechanism. The most promising suicide inhibitor is the ribophosphoramidate rn(G**C**CUC)rU, which forms more trans-spliced than cis-spliced product at oligonucleotide concentrations of >100 nM at 1 mM Mg^{2+}. The results indicate that short oligonucleotides modified for nuclease stability can target catalytic RNAs when the elements of tertiary interactions are complex.

Fungal pathogens such as *Candida albicans* are acquiring resistance to current treatments and are emerging as a serious threat to humans (*1*). Functionally important RNAs in these pathogens provide a large class of targets for therapeutic development. Self-splicing group I introns in fungi (*2*−*7*) are one type of potential target. Group I introns are attractive drug targets in *C. albicans* for several reasons: ∼40% of the strains contain a group I intron in their LSU[1] rRNA precursor (*3*), strains containing a group I intron are more susceptible to growth inhibition by pentamidine, which is known to inhibit self-splicing (*2*), self-splicing is a necessary step in the maturation of RNAs that contain group I introns (*8*), and no group I introns have been identified in humans.

Two strategies have recently been used to target group I introns: binding enhancement by tertiary interactions (BETI) and suicide inhibition. BETI incorporates tertiary structure elements into antisense design principles (*6*, *7*, *9*−*12*). The result is that short oligonucleotides (e.g., a hexamer) bind to a target with an affinity much higher than expected from base pairing alone. The advantages of this approach are selective binding to target, reasonable cost of synthesis, and the potential of short oligonucleotides to enter cells at higher concentrations than their larger counterparts (*13*).

Suicide inhibition takes advantage of the catalytic potential of RNA. In suicide inhibition applied to group I introns, a short oligonucleotide with a sequence that mimics the 3′ end of a group I intron's 5′ exon is spliced to the intron's 3′ exon, producing a dead-end product (*6*). If this reaction occurred in vivo, the organism's growth would be reduced because it would not form mature rRNA (*8*).

Previous investigations into these strategies used the *Pneumocystis carinii* group I intron as a model system (*6*, *7*, *9*−*12*). *P. carinii*, however, cannot be easily grown in cell culture (*14*). To provide a foundation for studies in cell culture, this paper describes in vitro targeting of a group I intron from *C. albicans* (*3*), an organism that can easily be grown in cell culture. Results are presented for oligonucleotides that are relatively stable to degradation by nucleases because they have the following backbones: deoxyphosphoramidate (*15*), ribophosphoramidate (*16*), thiophosphoramidate (*17*), and 2′-*O*-methyl (*18*). The work demonstrates the generality of the BETI and suicide inhibition strategies for targeting group I introns with oligonucleotides that can potentially serve as therapeutics.

MATERIALS AND METHODS

General Protocols. The splicing and gel binding assay buffer is termed H*X*Mg and contains 50 mM Hepes (25 mM

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[1] Abbreviations: BETI, binding enhancement by tertiary interactions; C-10/1x, *C. albicans* ribozyme; C-h, *C. albicans* truncated LSU rRNA precursor; dn, deoxyphosphoramidate; dns, deoxythiophosphoramidate; HPLC, high-performance liquid chromatography; IGS, internal guide sequence; LSU, large subunit; m, 2′-*O*-methyl sugar; pG, guanosine 5′-monophosphate; T_m, melting temperature in degrees Celsius; T_M, melting temperature in kelvin; rn, ribophosphoramidate; rRNA, ribosomal RNA.

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Table 1: Thermodynamics for Binding to r(GGAGGC) in H10Mg Buffer[a]

	thermodynamics from $1/T_M$ vs $\ln(C_T/4)$ plots				thermodynamics from the average of curve fits			
oligonucleotide	$-\Delta G°_{37}$ (kcal/mol)	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (eu)	T_m (°C)	$-\Delta G°_{37}$ (kcal/mol)	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (eu)	T_m (°C)
r(GACUCU)[b]	4.2	–	–	–	–	–	–	–
r(G**C**CUCU)[c]	8.51 ± 0.07	59.29 ± 2.14	163.63 ± 6.67	47.7	8.79 ± 0.49	66.35 ± 12.14	185.60 ± 37.62	47.9
r(G**U**CUCU)[c]	4.89 ± 0.16	52.14 ± 3.57	152.33 ± 11.97	27.6	4.86 ± 0.32	54.01 ± 6.56	158.47 ± 22.05	27.7
dn(GACTC)rU	5.45 ± 0.46	85.92 ± 19.48	259.46 ± 63.24	33.1	5.56 ± 0.45	74.19 ± 44.81	221.28 ± 145.89	33.0
dn(G**C**CTC)rU	10.99 ± 0.43	67.93 ± 6.49	183.60 ± 19.58	58.8	10.14 ± 0.52	54.59 ± 7.18	143.32 ± 21.61	59.0
dn(G**T**CTC)rU	7.76 ± 0.14	88.25 ± 9.03	259.53 ± 27.01	41.4	7.95 ± 0.38	95.53 ± 42.05	282.39 ± 134.36	41.7
dns(G**C**CTC)rU	9.18 ± 0.46	47.56 ± 7.13	123.72 ± 21.77	55.3	10.73 ± 1.43	74.10 ± 19.54	204.32 ± 58.65	55.6
rn(GA**C**UC)rU	5.21 ± 0.68	56.25 ± 14.78	164.58 ± 58.62	29.9	5.36 ± 0.14	49.58 ± 7.82	142.57 ± 24.86	29.8
rn(G**C**CUC)rU	10.93 ± 0.39	61.63 ± 6.21	163.45 ± 18.82	60.9	11.31 ± 1.07	68.62 ± 20.00	184.79 ± 61.10	60.2
rn(G**C**CUCU)	11.76 ± 2.35	68.10 ± 22.46	181.66 ± 66.10	62.8	12.32 ± 1.19	74.83 ± 10.93	201.56 ± 31.50	63.0
m(GACUCU)	(5.24 ± 0.57)	(27.33 ± 6.40)	(71.20 ± 21.58)	(23.0)	(4.94 ± 0.78)	(37.07 ± 10.83)	(103.61 ± 35.85)	(24.2)
GmAmCmUmCm(rU)	(5.39 ± 0.16)	(38.01 ± 4.50)	(105.15 ± 14.92)	(28.0)	(5.17 ± 0.38)	(69.28 ± 48.58)	(206.72 ± 15.73)	(31.0)
GmAmCm(rU)Cm(rU)	5.06 ± 0.84	37.76 ± 12.90	105.42 ± 42.98	25.4	5.27 ± 0.25	31.29 ± 3.95	83.87 ± 12.50	25.0
Gm**C**mCmUmCm(rU)	8.99 ± 0.32	51.73 ± 6.04	137.81 ± 18.64	52.5	9.28 ± 0.44	57.78 ± 8.65	156.35 ± 26.67	52.5
Gm**C**mCm(rU)Cm(rU)	8.75 ± 0.42	48.62 ± 8.46	128.52 ± 26.35	51.9	9.01 ± 0.26	56.56 ± 9.87	153.30 ± 31.08	51.2
Gm**U**mCmUmCm(rU)	5.85 ± 0.35	55.72 ± 8.06	160.81 ± 26.47	33.2	5.72 ± 0.23	63.62 ± 9.82	186.69 ± 31.61	33.1
Gm**U**mCm(rU)Cm(rU)	5.84 ± 0.44	64.82 ± 11.15	190.17 ± 36.52	33.7	5.76 ± 0.35	76.06 ± 17.97	226.69 ± 58.55	33.9

[a] The T_m is reported for a total strand concentration of 100 μM. Differences in the $\Delta H°$ values of duplex formation of >15% may indicate non-two-state melting of these duplexes; these duplexes are in parentheses. [b] Thermodynamics for r(GACUCU) were predicted as described in the preceding paper (*19*). [c] Values from ref *19*.

NaHepes), 135 mM KCl, and *X* mM Mg^{2+} at pH 7.5. General protocols, including RNA synthesis, optical melting experiments, and gel binding assays, were completed as described in the preceding paper (*19*).

Modified Oligonucleotide Synthesis. The 2′-*O*-methyl RNA was synthesized with a 6 min coupling time and deprotected as described for DNA (*19*). Deoxyphosphoramidate (*20*), thiophosphoramidate (*17*), and ribophosphoramidate (*16*) oligonucleotides were synthesized as described previously. The identity of each of these oligonucleotides was confirmed by electrospray mass spectrometry on a Hewlett-Packard Series 1100 LC/MS Chemstation.

Suicide Inhibition of Precursor Self-Splicing. A solution containing internally labeled truncated precursor rRNA was reannealed in H*X*Mg buffer by heating at 55 °C for 5 min, cooled for 2 min at room temperature, and then incubated at 37 °C. Another solution containing 2 mM pG and/or 60 μM 5′ exon mimic in H*X*Mg buffer was incubated at 37 °C for 5 min. Reactions were initiated by addition of an equal volume of the precursor solution to the solution containing pG and/or the 5′ exon mimic, then the mixtures incubated at 37 °C for 1 h, and the reactions stopped by addition of $^2/_3$ volume of stop buffer. The 5′ exon−3′ exon and 5′ exon mimic−3′ exon products were used to monitor cis splicing and trans splicing, respectively. Reaction products were separated on a 5% polyacrylamide, 8 M urea gel. Radioactivity was quantified with a Molecular Dynamics Phosphorimager and normalized for the number of adenines in each product.

The dependence of self-splicing inhibition on oligonucleotide concentration was tested with internally labeled precursor in either H1Mg or H2Mg buffer as described above, except that a solution of serially diluted 5′ exon mimic and 2 mM pG in H1Mg or H2Mg was added to an equal volume of the solution containing the internally labeled precursor.

RESULTS

Binding to r(GGAGGC). The thermodynamics for base pairing of various 5′ exon mimics to r(GGAGGC) to form

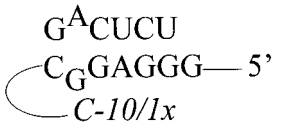

FIGURE 1: *C. albicans* P1 helix. The internal guide sequence (IGS) is on the bottom, and the 5′ exon mimic is on the top.

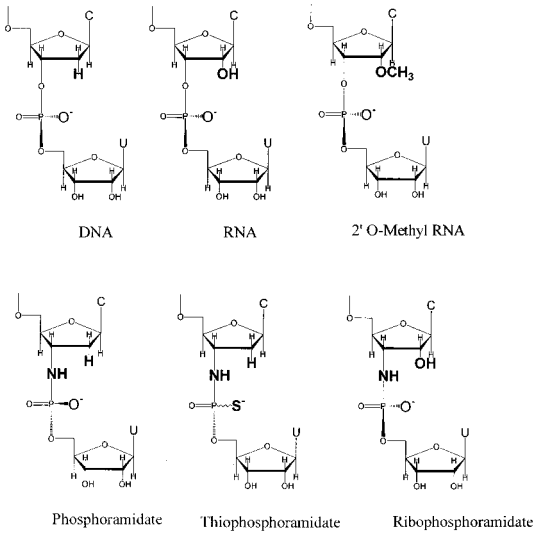

FIGURE 2: The 3′ ends of the oligonucleotides used in this study. Groups that are varied are in bold.

a mimic of the ribozyme's P1 helix (see Figure 1) were measured via optical melting experiments in H10Mg buffer (Table 1). The backbone was deoxyphosphoramidate, ribophosphoramidate, thiophosphoramidate, or 2′-*O*-methyl RNA (Figure 2). For most of these modifications, different base compositions were tested to allow formation of a G·A, G·U, G·T, or G-C pair at the −5 position when bound to r(GGAGGC).

The deoxyphosphoramidates, dn(GACTC)rU, dn(G**T**CTC)rU, and dn(G**C**CTC)rU, base pair to r(GGAGGC) with $\Delta G°_{37}$ values of −5.45, −7.76, and −10.99 kcal/mol, respectively (Table 1). The ribophosphoramidates, rn-

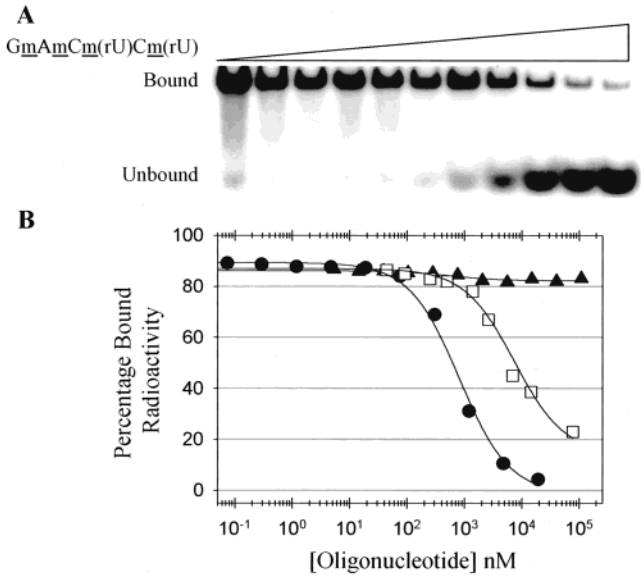

FIGURE 3: Competitive gel binding assay for binding to C-10/1x in H10Mg buffer. (A) An autoradiogram of a typical assay with GmAmCm(rU)Cm(rU). (B) Data and curve fit for GmAmCm(rU)-Cm(rU) (●), GmAmCmUmCm(rU) (□), and m(GACUCU) (▲).

(GACUC)rU and rn(G**C**CUC)rU, base pair with stabilities of −5.21 and −10.93 kcal/mol, respectively (Table 1). These duplexes are approximately 1−2 kcal/mol more stable than the corresponding all-RNA duplexes (Table 1 and ref *19*).

Oligonucleotides with four 2′-*O*-methyl substitutions, GmAmCm(rU)Cm(rU), Gm**U**mCm(rU)Cm(rU), and Gm**C**mCm(rU)Cm(rU), base pair with free energies of −5.06, −5.84, and −8.75 kcal/mol at 37 °C, respectively. Oligonucleotides with five 2′-*O*-methyl substitutions, GmAmCmUmCm(rU), Gm**U**mCmUmCm(rU), and Gm**C**mCmUmCm(rU), base pair with similar free energies of −5.39, −5.85, and −8.99 kcal/mol at 37 °C, respectively.

Binding to C-10/1x. Binding of 5′ exon mimics to the C-10/1x ribozyme was assessed via a competitive gel binding assay in H10Mg buffer (Figure 3 and Table 2). The contributions of tertiary interactions, i.e., binding enhancement by tertiary interactions (BETI), were calculated by dividing the dissociation constant, K_d, for binding to the IGS mimic, r(GGAGGC), by the K_d for binding to the ribozyme (Table 2) (*7*, *9*). BETI gives an estimate of the oligonucleotide's specificity for binding to the target sequence embedded within the intron rather than within a bystander RNA (*7*, *9*).

For the deoxyphosphoramidates, the oligonucleotide that forms a G·A pair when bound to the ribozyme, dn(GACTC)-rU, binds too weakly to afford accurate measurement of its binding affinity (K_d > 8000 nM) (Table 2). Replacing the G·A pair with a G-C or G·T pair, however, increases the level of binding more than 1000-fold to yield K_d values of 2.4 and 7.4 nM for dn(G**C**CTC)rU and dn(G**T**CTC)rU, respectively (Table 2). The BETIs for dn(G**C**CTC)rU and dn(G**T**CTC)rU are 10- and 430-fold, respectively.

Comparison of the binding affinities of ribophosphoramidates and deoxyphosphoramidates suggests that the ribose sugar imparts stronger binding affinity and higher BETIs than sugars that lack 2′-hydroxyls. The oligonucleotide rn-(GACUC)rU binds to the ribozyme with a K_d of 6.4 nM, which is at least 1000-fold tighter than dn(GACTC)rU. Furthermore, rn(GACUC)rU exhibits a BETI of 34000-fold, which is much larger than the upper limit of 17-fold for dn-(GACTC)rU (Table 2). The oligonucleotide rn(G**C**CUC)rU binds to the intron with a K_d of 1.0 nM, which is only a 2-fold increase in binding affinity relative to that for dn-(G**C**CTC)rU. The larger effect of adding 2′-hydroxyl groups to dn(GACTC)rU than dn(G**C**CTC)rU is consistent with the suggestion that the free energy of tertiary interactions mediated by 2′-hydroxyls is sequence-dependent (*19*).

The implications of changing the ribose sugar to a 2′-*O*-methyl were also studied. The effects of these substitutions are dependent on position because the 2′-methoxy group cannot act as a hydrogen bond donor in a tertiary interaction (Figure 2). As expected, the fully substituted oligonucleotide m(GACUCU) binds very weakly to the ribozyme (Table 2 and Figure 3). Replacement of a 2′-*O*-methyl sugar with ribose in positions where there is evidence for a tertiary interaction to a 2′-hydroxyl group (*19*) increases the overall level of binding; e.g., GmAmCmUmCm(rU) and GmAmCm-(rU)Cm(rU) have K_d values of 366 and 15 nM, respectively (Figure 3 and Table 2). Binding was also studied for oligonucleotides forming either a G-C or G·U pair instead of the natural G·A pair. In general, substitution of a 2′-*O*-methyl group with ribose at position −3 increases the overall binding affinity by 3−20-fold relative to oligonucleotides that only have ribose at position −1 (Table 2). The largest BETI of 102000-fold is measured for Gm**U**mCm(rU)Cm-(rU), which has a K_d of 0.8 nM.

Suicide Inhibition as a Function of Mg^{2+} Concentration. Various oligonucleotide 5′ exon mimics were tested for their ability to suicide inhibit self-splicing of the C-h precursor as a function of Mg^{2+} concentration in H*X*Mg buffer at pH 7.5 (Figure 4). Each assay contained 30 μM 5′ exon mimic and 1 mM pG, so cis splicing was allowed to compete with trans splicing. An oligonucleotide is considered an inhibitor if the level of cis splicing decreases in the presence of oligomer.

For the deoxyphosphoramidate oligonucleotides, inhibition is observed from 1 to 2 mM Mg^{2+} with dn(G**T**CTC)rU and dn(G**C**CTC)rU, but no inhibition is observed with dn-(GACTC)rU. This lack of inhibition at 30 μM for dn-(GACTC)rU could be due to its weak binding affinity for the intron (Table 2).

The ribophosphoramidate oligonucleotides, rn(GACUC)-rU and rn(G**C**CUC)rU, also inhibit self-splicing at both 1 and 2 mM Mg^{2+}. Thus, addition of 2′-hydroxyl groups to the deoxyphosphoramidate, dn(GACTC)rU, imparts both a large increase in binding affinity (Table 2) and the ability to inhibit self-splicing.

The 2′-*O*-methyl oligonucleotides that retained two rU groups were also analyzed for inhibition, since they bind tightly to the ribozyme (Table 2 and Figure 4). Little inhibition is observed for GmAmCm(rU)Cm(rU). Replacing the G·A pair with a G-C or G·U pair, however, results in inhibition.

Suicide Inhibition as a Function of Oligonucleotide Concentration at 1 and 2 mM Mg^{2+}. The dependence of trans splicing on oligonucleotide concentration was measured at 1 and 2 mM Mg^{2+} (Figure 5). The concentration dependence was measured at both Mg^{2+} concentrations because significantly more cis-spliced product is formed at 2 mM Mg^{2+}

Table 2: Binding of 5′ Exon Mimics in H10Mg Buffer at 37 °C[a]

	binding to the ribozyme		binding to r(GGAGGC)		tertiary interactions	
oligonucleotide	$K_{d,total}$ (nM)	$-\Delta G°_{37,total}$[b] (kcal/mol)	$K_{d,BP}$ (mM)	$\Delta G°_{37,BP}$[b] (kcal/mol)	$\Delta G°_{37,BETI}$[c] (kcal/mol)	K_2[d] (BETI)
r(GACUCU)[e]	6.9 ± 0.9	11.6	(1.1)	(4.2)	7.4	159000
r(G**C**CUCU)[e]	0.7 ± 0.1	13.0	0.001	8.5	4.5	1500
r(G**U**CUCU)[e]	0.6 ± 0.2	13.1	0.4	4.9	8.2	670000
dn(GACTC)rU	>8000	–	0.14	5.5	–	<17
dn(G**C**CTC)rU	2.4 ± 0.4	12.2	1.8×10^{-5}	11.0	1.2	10
dn(G**T**CTC)rU	7.4 ± 0.7	11.5	0.003	7.8	3.7	430
dns(G**C**CTC)rU	1.9 ± 0.1	12.4	3.3×10^{-4}	9.2	3.2	170
rn(GACUC)rU	6.4 ± 0.3	11.6	0.22	5.2	6.4	34000
rn(G**C**CUC)rU	1.0 ± 0.3	12.8	2.1×10^{-5}	10.9	1.9	21
rn(G**C**CUCU)	0.8 ± 0.2	12.9	4.8×10^{-6}	11.8	1.1	6
m(GACUCU)	>8000	–	0.24	5.2	–	–
GmAmCmUmCm(rU)	366 ± 69	9.1	0.16	5.4	3.7	430
GmAmCm(rU)Cm(rU)	15 ± 2	11.1	0.25	5.1	6.0	17000
Gm**C**mCmUmCm(rU)	2.6 ± 1	12.2	4.5×10^{-4}	9.0	3.2	170
Gm**C**mCm(rU)Cm(rU)	0.7 ± 0.2	13.0	6.2×10^{-4}	8.8	4.2	890
Gm**U**mCmUmCm(rU)	16 ± 4	11.1	0.07	5.9	5.2	4300
Gm**U**mCm(rU)Cm(rU)	0.8 ± 0.1	12.9	0.08	5.8	7.1	102000

[a] Ribozyme binding K_d values were determined via competitive gel binding assays as described in ref *19*. Bold and underlined nucleotides are different from the natural sequence, r(GACUCU). dn is a phosphoramidate linkage, dns a thiophosphoramidate linkage, rn a ribophosphoramidate linkage, m a 2′-*O*-methyl linkage, and rU uridine. The thermodynamics for binding r(GGAGGC) were obtained from $1/T_M$ vs $\log(C_T/4)$ plots. The reported K_d values are the average of at least two independent measurements, and the reported errors are the standard deviations. [b] $\Delta G°_{37}$ is determined from the equation $\Delta G°_{37} = RT \ln(K_d)$, where $R = 0.001987$ kcal mol^{-1} K^{-1} and $T = 310$ K. [c] The free energy change of tertiary interactions is determined as the difference between the free energy of binding to the ribozyme and of binding to r(GGAGGC). [d] K_2 is a measure of the binding enhancement by tertiary interactions (BETI) and is determined by dividing the K_d for base pairing by the K_d for ribozyme binding. [e] From ref *19*.

than at 1 mM Mg^{2+}. At 1 mM Mg^{2+}, the deoxyphosphoramidates and thiophosphoramidate, dn(G**C**CTC)rU, dn(G**T**CTC)rU, and dns(G**C**CTC)rU, form more trans- than cis-spliced products at concentrations of >100, >1000, and >500 nM, respectively (Figure 5). At 2 mM Mg^{2+}, all three hexamers require more than 1000 nM to form more trans- than cis-spliced product (Figure 5). In contrast, the ribophosphoramidate, rn(G**C**CUC)rU, forms more trans- than cis-spliced product at concentrations of >50 and >100 nM at 1 and 2 mM Mg^{2+}, respectively (Figure 5).

For the 2′-*O*-methyl oligonucleotides, only oligonucleotides that form a G-C or G•U pair at the −5 position induce more trans- than cis-spliced products. At 1 mM Mg^{2+}, the concentrations of oligonucleotide needed for the level of trans splicing to be greater than that of cis splicing are 4000 nM for Gm**U**mCm(rU)Cm(rU) and 300 nM for Gm**C**mCm(rU)Cm(rU) (Figure 5). At 2 mM Mg^{2+}, only Gm**C**mCm(rU)Cm(rU) forms more trans- than cis-spliced product, and this requires an oligonucleotide concentration of 4000 nM (Figure 5).

DISCUSSION

C. albicans is one of many pathogens that are becoming prevalent because the number of both humans with compromised immune systems and pathogens with resistance to known treatments are increasing (*1*). To aid the development of new potential therapeutics for such pathogens, strategies for targeting RNA are being tested (*2*−*4*, *6*, *7*, *9*−*12*, *21*, *22*). *C. albicans* provides a good model system because it is easily grown in cell culture and contains a self-splicing group I intron, whose activity can be monitored in vitro.

Group I introns provide an attractive target because self-splicing is a necessary step in the maturation of RNAs that contain them and several pathogens harbor group I introns (*3*, *5*, *23*), but none have been found in the human genome. The results presented here show that both the BETI and suicide inhibition approaches can be used as design strategies for targeting the group I intron from *C. albicans*, although the functional group requirements are considerably more complex than those encountered in *P. carinii* (*9*, *11*, *12*, *19*).

Binding of Modified Oligonucleotides to r(GGAGGC). The deoxyphosphoramidate, thiophosphoramidate, ribophosphoramidate, and 2′-*O*-methyl oligonucleotides all base pair more tightly than r(GACUCU) to r(GGAGGC) (Table 1). Previous studies of the stabilities of such duplexes indicate that adding deoxyphosphoramidate (*15*), deoxythiophosphoramidate (*17*), and ribophosphoramidate (*16*) modifications increase the T_m of the duplex by ∼2 °C/modification relative to a DNA oligonucleotide binding to an RNA target. The 2′-*O*-methyl linkages have been shown to increase the T_m values of duplexes by ∼0.4 °C/modification, relative to DNA oligonucleotide binding to an RNA target (*24*). The results presented here are in qualitative agreement with these increases, but further quantify the thermodynamic consequences of these modifications. For canonically paired duplexes, substitution of the RNA backbone with a deoxyphosphoramidate or ribophosphoramidate increases duplex stability by ∼0.5 kcal/mol at 37 °C for each linkage, corresponding to an ∼2-fold more favorable K_d per substitution (Table 2). A 2′-*O*-methyl linkage has a modest effect on duplex stability of ∼0.1 kcal/mol at 37 °C per modification (Table 1).

Binding Enhancement by Tertiary Interactions (BETI). BETI is a design strategy that takes advantage of the higher-order folding of RNAs. In this strategy, tertiary contacts to the target are employed to allow short antisense agents, which base pair only weakly to their target, to obtain high affinity and specificity (*7*, *9*). The use of short oligonucleotides is beneficial because they enter cells more easily than their

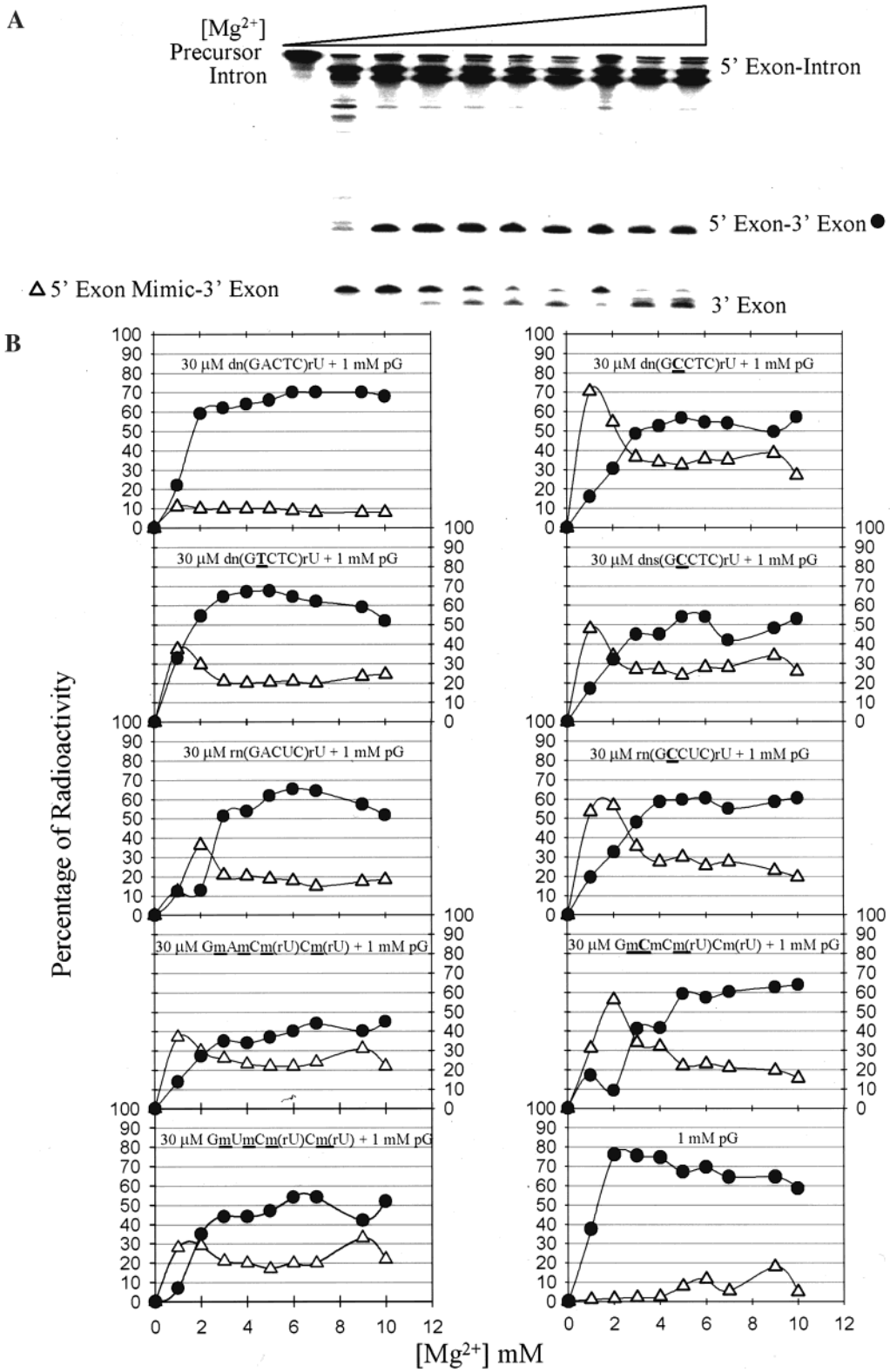

FIGURE 4: Trans (△) and cis (●) splicing as a function of Mg^{2+} concentration as measured with the internally labeled precursor in H*X*Mg buffer. The reactions were carried out with 1 mM pG and the indicated oligonucleotide (30 μM). (A) An autoradiogram of a typical assay with GmCmCm(rU)Cm(rU). (B) The data obtained from the experiments.

larger counterparts (*13*), are less expensive to synthesize, and have potential for fewer side effects because binding affinity is derived from the three-dimensional shape of the target, not simply from duplex stability.

The results listed in Table 2 show that oligonucleotides modified to enhance nuclease stability can still exhibit BETI. The largest BETIs are exhibited by oligonucleotides containing functional groups and/or sequences that have previously been shown to accommodate tertiary interactions (*19*). The smallest BETIs are exhibited by oligonucleotides that form a G-C base pair at the −5 position when bound to the intron (Figure 1), and the largest by oligonucleotides that form either a G·A or G·U pair at the −5 position (Table 2). Furthermore, selective inclusion of 2′-hydroxyl groups

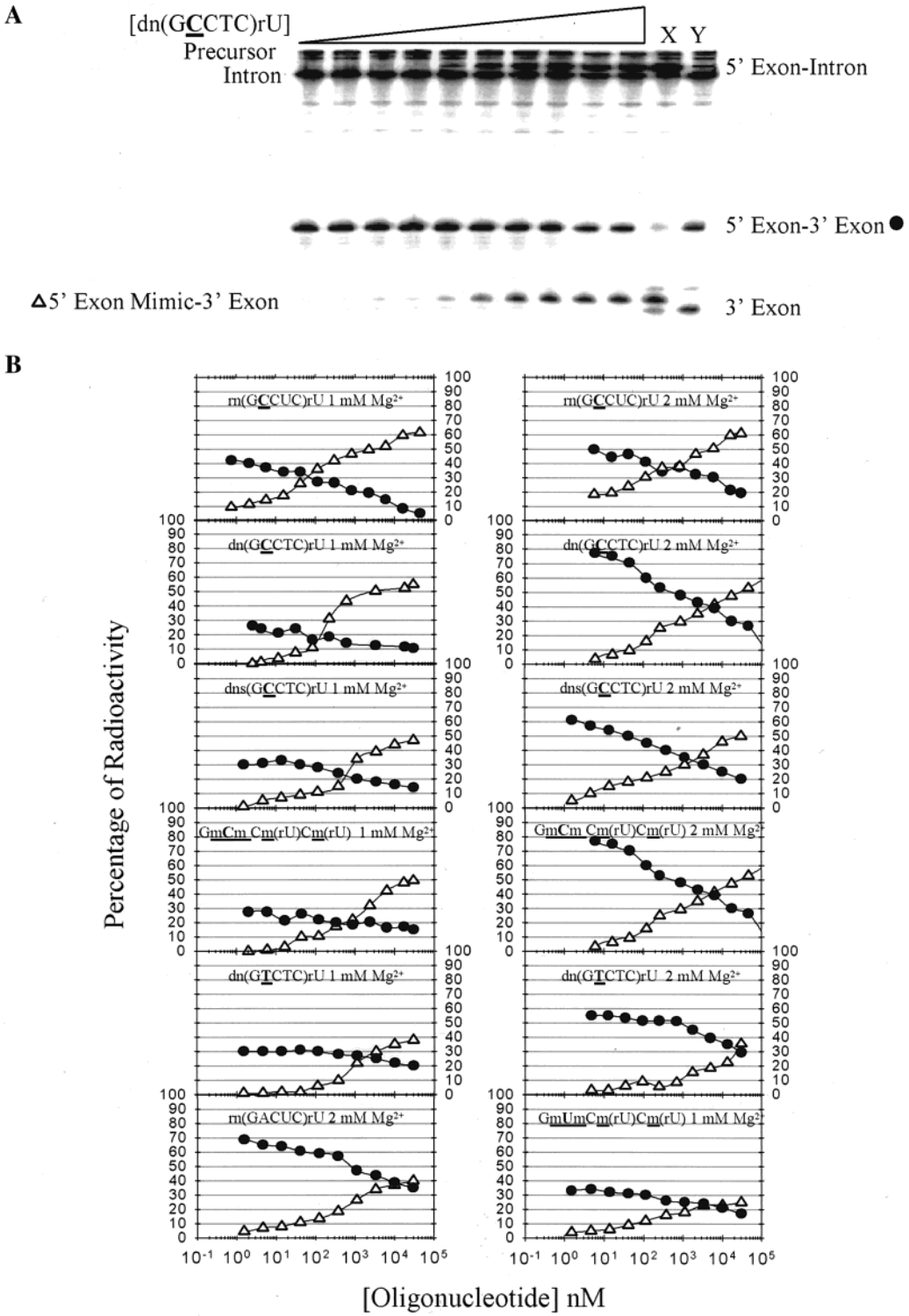

FIGURE 5: Dependence of trans (△) and cis (●) splicing on oligonucleotide concentration in either H1Mg or H2Mg buffer. All reactions were carried out with 1 mM pG present to allow for the formation of the cis-splicing, 5′ exon−3′ exon product. (A) An autoradiogram of a gel from a typical experiment with dn(G**C**CTC)rU in H1Mg buffer. Lane X contained the trans-spliced product. Lane Y contained the 3′ exon or hydrolysis product. (B) The data obtained from averaging two experiments. The error is about ±6% for each point.

increases BETI; e.g., BETIs for GmAmCm(rU)Cm(rU) and GmAmCmUmCm(rU) are 17 000 and 430, respectively (Table 2). Thus, short oligonucleotides can be substituted for nuclease stability without destroying potential tertiary contacts; this allows specificity in binding to a target.

Suicide Inhibition. The results in Figures 4 and 5 show that short, modified oligonucleotides can act as inhibitors of precursor self-splicing. Formation of more trans- than cis-spliced products only occurs between 1 and 2 mM Mg^{2+}, however. Inhibition at these low Mg^{2+} concentrations may indicate that the intron is more compactly folded at higher Mg^{2+} concentrations, thus rendering the active site of the intron inaccessible for binding exogenous substrates. Alternatively, inhibition at low Mg^{2+} concentrations may be due to participation of metal ions in the first step of precursor self-splicing (*25*−*27*). At the concentration of Mg^{2+} where

inhibition is observed, these binding sites may not be sufficiently filled to allow for efficient reaction. Since the inhibitor oligonucleotides bypass the first step of self-splicing, they may have an advantage over the endogenous substrate for reaction with the 3′ exon at low Mg^{2+} concentrations. On average, the results in Figure 5 suggest that trans splicing, which mimics the second step of self-splicing, is less sensitive to Mg^{2+} concentration than cis splicing, which requires the first step of splicing.

The available results suggest that there are several opportunities for the oligonucleotide to bind to the precursor target and subsequently inhibit self-splicing in vivo. For example, the oligonucleotide could bind to precursor while it is being transcribed, since base pairing of these oligonucleotides is strong (Tables 1 and 2). In addition, the inhibitor may be able to bind tightly to the IGS when the intron is not in a catalytically active structure and inhibit self-splicing once the intron has folded into an active structure. In some cases, proteins are known to bind to group I introns and organize their structures for catalysis (*28*−*32*). If a protein is required for self-splicing in vivo, then the inhibitor would also have an opportunity to bind to the IGS before the protein. Thus, there are several opportunities for inhibitor binding before splicing.

Inspection of the data reveals that inhibition can be optimized by several factors. Base composition and oligonucleotide modifications play critical roles. Qualitatively, the amount and concentration dependence of inhibition correlate with the stability of base pairing to the IGS in the following order: G•A < G•T or G•U < G-C (Figures 4 and 5 and Table 1). Thus, the non-natural base sequences are more effective than the natural sequence, r(GACUCU). The dependence on oligonucleotide backbone and sugar also suggests that base pairing is critical. For example, the order of base pairing stability with regard to oligonucleotide modifications is as follows: 2′-*O*-methyl < deoxyphosphoramidate ∼ ribophosphoramidate. The amount and concentration dependence of inhibition qualitatively follow this trend (Figures 4 and 5 and Table 1). These results suggest that for inhibition to occur with *C. albicans*, the exogenous 5′ exon mimic must base pair more tightly to the IGS than the endogenous 5′ exon. This is not true for every intron since an RNA tetramer can suicide inhibit self-splicing of the *P. carinii* group I intron at concentrations of <1 μM (*11*). Such differences may reflect different accessibilities of the IGS due to different foldings of the rRNA precursors.

Conclusions. Hexanucleotides that are stable to nuclease degradation bind tightly and specifically to the *C. albicans* ribozyme. Several of these oligonucleotides can inhibit self-splicing of the precursor in vitro at concentrations of <1 μM (Figures 4 and 5). These insights provide a foundation for testing BETI and suicide inhibition strategies in an easily grown human pathogen, and thus may lead to more effective antisense-based or small-molecule targeting of functionally important RNAs.

REFERENCES

1. Sternberg, S. (1994) *Science 266*, 1632−1634.
2. Miletti, K. E., and Leibowitz, M. J. (2000) *Antimicrob. Agents Chemother. 44*, 958−966.
3. Mercure, S., Montplaisir, S., and Lemay, G. (1993) *Nucleic Acids Res. 21*, 6020−6027.
4. Mei, H. Y., Cui, M., Lemrow, S. M., and Czarnik, A. W. (1997) *Bioorg. Med. Chem. 5*, 1185−1195.
5. Sogin, M. L., and Edman, J. C. (1989) *Nucleic Acids Res. 17*, 5349−5359.
6. Testa, S. M., Gryaznov, S. M., and Turner, D. H. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 2734−2739.
7. Testa, S. M., Gryaznov, S. M., and Turner, D. H. (1998) *Biochemistry 37*, 9379−9385.
8. Nikolcheva, T., and Woodson, S. A. (1997) *RNA 3*, 1016−1027.
9. Testa, S. M., Haidaris, C. G., Gigliotti, F., and Turner, D. H. (1997) *Biochemistry 36*, 15303−15014.
10. Testa, S. M., Disney, M. D., Turner, D. H., and Kierzek, R. (1999) *Biochemistry 38*, 16655−16662.
11. Disney, M. D., Gryaznov, S. M., and Turner, D. H. (2000) *Biochemistry 39*, 14269−14278.
12. Disney, M. D., Testa, S. M., and Turner, D. H. (2000) *Biochemistry 39*, 6991−7000.
13. Loke, S. L., Stein, C. A., Zhang, X. H., Mori, K., Nakanishi, M., Subasinghe, C., Cohen, J. S., and Neckers, L. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3474−3478.
14. Merali, S., Chin, K., Grady, R. W., and Clarkson, A. B., Jr. (1996) *Antimicrob. Agents Chemother. 40*, 1298−1300.
15. Gryaznov, S. M., and Chen, J.-K. (1994) *J. Am. Chem. Soc. 116* 3143−3144.
16. Matray, T. J., and Gryaznov, S. M. (1999) *Nucleic Acids Res. 27*, 3976−3985.
17. Pongracz, K., and Gryaznov, S. M. (1999) *Tetrahedron Lett. 40*, 7661−7664.
18. Sproat, B. S., Lamond, A. I., Beijer, B., Neuner, P., and Ryder, U. (1989) *Nucleic Acids Res. 17*, 3373−3386.
19. Disney, M., Haidaris, C. G., and Turner, D. H. (2001) *Biochemistry 40*, 6507−6519.
20. Gryaznov, S. M., Lloyd, D. H., Chen, J. K., Schultz, R. G., DeDionisio, L. A., Ratmeyer, L., and Wilson, W. D. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 5798−5802.
21. Liu, Y., and Leibowitz, M. J. (1994) *J. Eukaryotic Microbiol. 41*, 101S.
22. Liu, Y., and Leibowitz, M. J. (1995) *Nucleic Acids Res. 23*, 1284−1291.
23. Netzker, R., Kochel, H. G., Basak, N., and Kuntzel, H. (1982) *Nucleic Acids Res. 10*, 4783−4794.
24. Freier, S. M., and Altmann, K. H. (1997) *Nucleic Acids Res. 25*, 4429−4443.
25. Shan, S., Yoshida, A., Sun, S., Piccirilli, J. A., and Herschlag, D. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 12299−12304.
26. Piccirilli, J. A., Vyle, J. S., Caruthers, M. H., and Cech, T. R. (1993) *Nature 361*, 85−88.
27. Weinstein, L. B., Jones, B. C., Cosstick, R., and Cech, T. R. (1997) *Nature 388*, 805−808.
28. Weeks, K. M., and Cech, T. R. (1995) *Cell 82*, 221−230.
29. Weeks, K. M., and Cech, T. R. (1995) *Biochemistry 34*, 7728−7738.
30. Weeks, K. M., and Cech, T. R. (1996) *Science 271*, 345−348.
31. Ho, Y., Kim, S. J., and Waring, R. B. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 8994−8999.
32. Ho, Y., and Waring, R. B. (1999) *J. Mol. Biol. 292*, 987−1001.

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