A *Tetrahymena* intron nucleotide connected to the GTP/arginine site

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ABSTRACT

We have substituted all nucleotides at intron nucleotide 260 (N²⁶⁰) in transcripts related to the self-splicing *Tetrahymena* rRNA. Substitution slightly affects the binding and reaction of GTP with this group I catalytic center; k_{cat}/K_m varies over a three-fold range. The base of N²⁶⁰ therefore communicates with the rG site, but is unlikely to bond directly to GTP. Different nucleotides at this position also alter the binding of L-arginine to the intron, measured by inhibition of the reaction with GTP. Effects of similar small magnitude on interaction of RNA with both GTP and L-arginine support the previous argument from kinetic and structural comparison (Yarus, M. (1988) *Science* **240**, 1751) that placed the two ligands of the RNA in the same binding site. G²⁶⁰ RNA shows the greatest affinity for both D- and L-arginine, but uniquely lacks stereoselectivity for the amino acid. Therefore G²⁶⁰ alters spatial relations within the G site, otherwise conserved in C²⁶⁰, U²⁶⁰, and A²⁶⁰ RNA's. Guanyl urea was used as a probe for the G/guanidino H-bonding part of the rG/arginine site. G²⁶⁰ RNA's dissociation constant for guanyl urea is similar to that of the other RNA's, suggesting that G²⁶⁰ RNA is unaltered at the G/guanidino end of the rG/arginine binding site. To account for all observations, we suggest that the G²⁶⁰ substitution alters the relative location of the RNA backbone near the 5' exon-intron junction, making this location more flexible and closer to the $\alpha\text{-NH}_3^+$'s of L- and D-arginine.

INTRODUCTION

Specific inhibition of self-splicing of the *Tetrahymena* pre-rRNA by arginine (1) suggests that a binding site selective for an amino acid side chain, and stereoselective for the L-amino acid, can be folded from RNA alone. The location and structure of this site is of interest. Because arginine competitively inhibits the RNA's reaction with GTP, it appeared that the amino acid could occupy a subsite within the catalytic guanosine binding site. In fact, the free energy of binding for arginine has been attributed to groups which also contact bound GTP (2).

C²⁶⁰ is the only conserved C in the intron with no known Watson-Crick pairing partner. Therefore C²⁶⁰ could potentially base-pair with the exogenous GTP molecule which initiates the self-splicing reactions. In fact, when Williamson *et al.* (3) simultaneously substituted the flanking nucleotides N²⁵⁹ and N²⁶¹, they observed an effect on the K_m for GTP. This is consistent with a role for C²⁶⁰ in GTP binding. Thus whether or not N²⁶⁰ directly contacts GTP, N²⁶⁰ substitution was likely to reveal some properties of the rG site at which self-splicing is initiated, and by hypothesis, the arginine binding site also.

We now find that N²⁶⁰ mutant RNAs have altered saturation with GTP, as well as

Figure 1—Structure of the *Tetrahymena* catalytic center.

The 5' exon-intron junction is at the left, and the symbol for guanosine (with an arrow) shows the point of addition of the G-derivative which initiates the splicing cascade. The active catalytic configuration is probably generated by folding P1 against the group of explicit sequences (P3, P4, P6, P7...) at the right of the drawing (eg, 9).

altered L-arginine inhibition of the GTP reaction. This simultaneous perturbation of the GTP and arginine interactions unexpectedly helps to define the origin of the RNA's stereoselection for L-arginine.

MATERIALS AND METHODS

Site-directed alterations in the intron

Plasmid BGST7 was altered by oligonucleotide-directed mutagenesis as described (4). DNA sequence analysis covering the entire *Tetrahymena* insert confirmed the N²⁶⁰ mutations, and the absence of any other change.

Transcription

The plasmids were transcribed with T7 RNA polymerase and the transcripts prepared as previously described (2), except that 2 mM spermidine was added to limit splicing during the transcription reaction.

Splicing reactions

Transcripts were reactivated just before splicing reactions by heating in Mg⁺⁺ solutions as previously described (2). Reactions were all at 37°, and in 5 to 7.5 µl of 50 mM Hepes, pH 8.0, 30 mM NaCl, 10 mM MgCl₂, 0.2 mM EDTA. Reactions were stopped using EDTA-urea solutions and quickly resolved by electrophoresis on 5% polyacrylamide-8M urea gels, as previously described (1). Products were measured using Cerenkov scintillation from individual excised bands.

Figure 2—Response of A²⁶⁰, C²⁶⁰, G²⁶⁰, and U²⁶⁰ precursor RNA's to GTP concentration.

The top panel is a radioautogram of a 5% polyacrylamide 8 M urea gel showing the GTP-labeled products after 10 second reactions with 0.2, 0.6, 1.2, 4.1, and 12.3 μ M GTP.

The lower panel is a Hanes plot of the velocity vs [GTP] data from the gel above. Velocities are expressed as fmol/min. The extrapolate to the abscissa indicates the K_m implied by the least squares line fit to the data. Circles—C²⁶⁰ RNA; x's—A²⁶⁰ RNA; triangles—U²⁶⁰ RNA; squares—G²⁶⁰ RNA, as indicated in the figure.

For measurement of K_i, incubations were 2 min, using 0.022 to 0.033 μ M [α ³²P]GTP, 3000 Ci/mmol.

For measurement of kinetics, incubations were 10 sec, using GTP diluted to 300 Ci/mmol. Short incubations are essential to measure a true initial velocity during the linear phase of these reactions, especially at high GTP concentrations near the V_{max}. This is particularly true of the self-splicing precursor RNA reaction because the precursor is consumed. We have measured initial velocities rather than full kinetic courses because

Table I Kinetic Constants for GTP Reaction with N²⁶⁰ RNA's

RNA	K_m , μM	V_{max} , fmol/min	V_{max}/K_m	k_{cat}/K_m	k_{cat} , m^{-1}
C ²⁶⁰	4.9 ± 0.3	420 ± 15	85 ± 5	0.40 ± 0.02	2.0 ± 0.07
U ²⁶⁰	5.0 ± 0.5	190 ± 13	38 ± 5	0.27 ± 0.04	1.4 ± 0.09
G ²⁶⁰	7.7 ± 0.7	180 ± 16	24 ± 3	0.13 ± 0.02	1.0 ± 0.09
A ²⁶⁰	5.7 ± 0.2	360 ± 7	64 ± 2	0.30 ± 0.01	1.7 ± 0.03

Each quantity is quoted with its standard error, from the fitted curve in Figure 2. k_{cat} 's are calculated from V_{max} , using the limiting fmol of GTP reactivity reached in long reactions otherwise like the kinetic assays. C²⁶⁰ reactions contained 212 fmol of active precursor RNA; U²⁶⁰, 142 fmol; G²⁶⁰, 179 fmol; and A²⁶⁰, 213 fmol.

the theoretical meaning of an initial velocity is clearer than an overall rate constant. In addition, if there is heterogeneity in reactivity, we wish to contemplate the most reactive molecules. After these short incubations, almost all radioactivity is in IVS-3' exon and L-IVS, because the 5'-15nt oligomer product of intron circularization is produced only after a lag. These two large [5' ³²P]RNAs from gels were counted together. Rate constants therefore represent the full velocity of reaction with GTP. The relative reactivities of the N²⁶⁰ RNAs were reproduced many times, though only a single experiment is presented.

For measurement of maximal GTP reaction to determine the amount of active precursor, incubations were performed in the presence of 0.8 μM GTP, 150 Ci/mmol, with samples at intervals to 90 min. These samples were resolved by electrophoresis on 10% polyacrylamide, 8M urea gels to sufficiently separate the [α^{32} P] 15nt oligomer product from residual GTP. In the experiment in Figure 2, 33 to 39% of the absorbance could be accounted for as active precursor. The rest appears to be discrete splicing products formed during transcription and isolated with precursor RNA (data not shown).

Analysis

We have used the Hanes form of the linearized Michaelis-Menten equation to analyze kinetic data because it automatically weights the measurements according to their reliability (5):

$$[GTP]/\text{velocity} = K_m/V_{max} + [GTP]/V_{max}$$

To determine K_i , we use (2):

$$1/f = 1 + [I]/K_i$$

This equation is valid for competitive inhibition when $[GTP] \ll K_m$. $[I]$ is the inhibitor concentration, and f is the fraction of the uninhibited splicing velocity.

All equilibrium and kinetic quantities are objective, in that they are calculated from the best least squares line fitted to the data. Standard errors are calculated directly from the variance of the data from the fitted lines, propagated to derived quantities K_i , K_m , and V_{max} , by standard techniques (6). Such errors are useful in deciding which values are experimentally distinguishable. To minimize random variation, data for each figure were obtained simultaneously for all precursors, or in successive experiments, using the same reagents.

RESULTS

The site studied

The first figure shows the location of the nucleotide substituted in this work, relative to other sequences typical of the group I catalytic center. Nucleotide 260 (N²⁶⁰) is a part

Figure 3—Inhibition by L-arginine of the reaction between N^{260} precursor RNA's and GTP.

$1/f$ (the ordinate) is the reciprocal fraction of the initial velocity of GTP reaction at the indicated L-arginine concentration (the abscissa). The slope of the curve is the inhibitor binding constant; the reciprocal slope is K_i , the dissociation constant of the inhibitor. Symbols are as in Figure 2.

of an invariant structure, a short bridge or joining sequence (called J6/7, by convention (7)) between two conserved paired regions, P6 & P7. P7 and J6/7 are formed from the highly conserved 1° sequence elements R and S (8; Figure 1). N^{260} itself is always a pyrimidine nucleotide; 56/66 group I sequences have C^{260} (85%, ref 8) and the remainder have U^{260} . This conserved C is the 5' neighbor of A^{261} , an even more highly conserved nucleotide (8). The role of N^{260} was probed by site-directed mutagenesis of the intron on pBGST7 (4).

The resulting kinetics are distinguishable

Using the four synthetic precursor RNAs synthesized by T7 RNA polymerase, the initial rate of reaction with $[\alpha^{32}P]GTP$ was measured. Figure 2A shows the $[\alpha^{32}P]$ splicing intermediates which result after brief incubation at 37° in the presence of 0.21 to 12.3 μM GTP. Because the precursor RNAs were at similar concentrations, the conclusions

Table II Dissociation Constants of L-arginine

RNA	K_i , mM
C^{260}	2.9 ± 0.2
U^{260}	3.7 ± 0.2
G^{260}	1.6 ± 0.1
A^{260}	4.0 ± 0.3

The K_i 's shown are averages of two consistent experiments for each precursor RNA, one of which is shown in Figure 3. The average K_i 's and standard errors were calculated by weighting each individual determination, from a least squares line fit to data as in Figure 3, by the reciprocal of its variance.

Figure 4—Inhibition by *D*-arginine of the reaction between N^{260} precursor RNA's and GTP. Axes and symbols are the same as Figure 3.

given in quantitative detail in Figures 2B and Table I can be confirmed by inspection of the radioautogram. At low GTP concentrations, radioactivity will be proportional to $V_{\max}/K_m$, proportionate to a lower bound for the second order rate constant for the reaction of free RNA and GTP (10). It can be seen by comparing the leftmost lanes for each RNA in Figure 2 that A^{260} and U^{260} RNAs are somewhat less reactive than the wild type C^{260} RNA, and that G^{260} RNA is substantially less reactive.

The lower part of Figure 2 plots a linearized form of the Michaelis-Menten equation for all the RNAs, so that they may be closely compared. The Figure makes it quantitatively apparent that the RNAs are different. Firstly, there is a consistent difference in reactivity between the four intron sequences at all GTP concentrations, seen by comparing columns of points. Secondly, the slopes of the lines reflect $1/V_{\max}$ and the extrapolate on the abscissa at the left is the K_m . Both kinetic parameters evidently differ among the N^{260} RNAs. Thus the nature of N^{260} influences catalysis of the GTP reaction with the *Tetrahymena* intron.

Kinetic differences are quantified in Table I, which shows the K_m and $V_{\max}$ implied by the data of Figure 2. K_m 's for GTP vary slightly; G^{260} RNA has a higher K_m than does the wild type C^{260} sequence. The variation in the rate constant k_{cat} is more significant. The wild type is fastest, and the G^{260} RNA is again the most affected; G^{260} RNA turns over at 1/2 the normal rate. The combination of these two factors produces the order of reactivities also observable without computation in the radioautogram: $V_{\max}/K_m$ and k_{cat}/K_m are greatest for wild type C^{260} RNA, less for A^{260} RNA and U^{260} RNA, and slowest for G^{260} RNA, which is 3-fold less reactive than the unsubstituted intron.

Table III Dissociation Constants of D-arginine

RNA	K_i , mM	Stereoselectivity $K_{i,D\text{-arg}}/K_{i,L\text{-arg}}$
C ²⁶⁰	7.0 ± 0.5^2	2.4 ± 0.2
U ²⁶⁰	7.3 ± 0.6^2	2.0 ± 0.2
G ²⁶⁰	1.7 ± 0.1^1	1.1 ± 0.1
A ²⁶⁰	9.4 ± 0.8^2	2.3 ± 0.3

¹ Average of three experiments, one shown in Figure 4.

² Average of two experiments, one shown in Figure 4.

Each determination, from a least squares fit to data as in Figure 4, was weighted by its reciprocal variance to calculate the average.

The K_m for GTP of C²⁶⁰ RNA is similar, but significantly larger than the value we previously measured for a wild type *Tetrahymena* intron under the same conditions: $2.1 \pm 0.4 \mu M$ (1). One possibly significant variation is that the earlier precursor has the same intron sequence as C²⁶⁰ RNA, but differs in the exons. Differential interaction with exon sequences could make small alterations in catalysis by the intron.

Inhibition by L-arginine is also distinguishable

Figure 3 and Table II show that the binding of L-arginine also distinguishes precursor sequences that differ at N²⁶⁰. At GTP concentrations which are small with respect to K_m (as in these experiments), the slope of the lines is the equilibrium binding constant for the inhibitor (or the reciprocal of K_i , the dissociation constant, ref 1).

G²⁶⁰ RNA binds L-arginine most strongly, wild type C²⁶⁰ RNA is intermediate, and U²⁶⁰ RNA and A²⁶⁰ RNA are least inhibited, and indistinguishable (Figure 3). The extremes differ by 2.5-fold (Table II).

Binding of L-arginine to C²⁶⁰ RNA in these experiments is in very good agreement with prior measurements on slightly differing *Tetrahymena* precursor RNAs; previous measurements gave $K_i = 3.2 \pm 0.3 \text{ mM}$ (2), and $4.0 \pm 0.3 \text{ mM}$ (1).

G²⁶⁰ RNA has Altered Stereoselection

One of the engaging properties of the L-arginine site on the intron is its ability to select the L-stereoisomer of the amino acid (1). If RNA sites like these were the evolutionary agents that linked specific amino acids to specific RNA sequences (that is, during the origin of the genetic code), then this finding suggests that a preexisting asymmetric RNA might have initiated the asymmetric choice of L- instead of D-amino acids found in modern proteins. The N²⁶⁰ variants give new information about stereoselection.

Figure 4 shows an experiment that determines binding of D-arginine to the N²⁶⁰ RNA precursors. G²⁶⁰ RNA is most sensitive to D-arginine inhibition, and comparison with Figure 3 shows that G²⁶⁰ RNA alone is nearly indifferent to the stereochemistry of the amino acid. The other three precursors definitely bind L-arginine more strongly than D-arginine. One result is that D-arginine is the ligand which distinguishes most effectively among N²⁶⁰ RNAs, which have K_i 's that vary 6-fold (Table III, Figure 4). This data is interpreted with the aid of Table III, by calculating an index for stereoselectivity, $K_{i,D\text{-arg}}/K_{i,L\text{-arg}}$, which will be larger the greater the preference for the L-amino acid. The stereoselectivities of C²⁶⁰ RNA, U²⁶⁰ RNA, and A²⁶⁰ RNA are indistinguishable from each other, and all in good agreement with previous measurements on an intron of normal sequence ($K_{i,D\text{-arg}}/K_{i,L\text{-arg}} = 2.1 \pm 10.2$; ref 1). In contrast, the stereoselectivity of G²⁶⁰

Figure 5—Inhibition by guanyl urea of the reaction between N^{260} precursor RNA's and GTP. The top panel compares the structures of the substrate guanosine, and the competitive inhibitors arginine and guanyl urea. Guanine positions discussed in the text are numbered. The lower panel shows the inhibition of GTP reaction with the four precursor RNA's by guanyl urea.

RNA does not differ significantly from 1.0, implying no selection at all. G^{260} RNA is plainly not denatured by its substitution. It is catalytically quite competent (Figure 2, Table I), and in fact exhibits the highest affinity for both D- and L-arginine (Figures 3 and 4, Tables II and III). Thus stereoselection is a reproducible attribute of the normal *Tetrahymena* catalytic center, but it may also be lost by single mutation with relatively minor effect on transesterification and the RNA structure. Notably, the other transversion substitution, in A^{260} RNA, probably perturbs the amino acid site only slightly, but in a contrary direction, giving the lowest measured affinities for arginine (Table II and III). However, A^{260} is without effect on stereoselection (Table III). *Guanyl urea gives information about the 'location' of N^{260}* In previous experiments (2), arginine binding appeared to rely on interaction with separate

Table IV Dissociation Constants of Guanyl Urea

RNA	K_i , mM
C^{260}	18 ± 3
U^{260}	17 ± 3
G^{260}	15 ± 1
A^{260}	39 ± 5

Each value is from a least squares line fit to the data in Figure 5.

Figure 6—An interpretation of the G^{260} effect.

Arginine and guanosine are shown in the same site, aligned by their common hydrogen bonds, shown as dash-and-dotted lines at the right (2). The cross-hatched disks at the left are a section from the negatively-charged RNA precursor backbone at the 5' exon-intron junction. In the normal (C^{260}) precursor, the junction is fixed near the 3'-OH of guanosine with which it must react. G^{260} substitution introduces flexibility in the relative position of the precursor backbone, which may now approach the α -amino of L-arginine (or D-arginine). This same flexibility interferes with the reactive alignment at the 3'-OH of guanosine (see discussion).

regions of the RNA, at opposite ends of the binding site. In principle, we can ask which end of the GTP/arginine site is most closely coupled to N^{260} by using a probe specific to one end of the site, guanyl urea (2).

The top panel of Figure 5 illustrates the structural rationale for this experiment. Guanyl urea closely resembles the Watson-Crick H-bonding face of guanine. It can participate in the same set of H-bonds, including the equivalent of the O^6 of guanine, which is missing in arginine. Guanyl urea inhibits the GTP reaction with a K_i greater than, but the same order as, L-arginine (2). However, guanyl urea is not large enough to reach the ribose/ α -carbon end of the site (Figure 5, top). Thus if N^{260} substitution were to alter arginine binding, but not guanyl urea binding, we would say that N^{260} is uncoupled to the guanine/guanidino end of the site.

Such measurements have implications for, but differ from determination of, true relative spatial proximity. The argument makes an (undemanding) assumption about the propagation of the effect of a nucleotide substitution through the RNA structure. However, such structure will likely be anisotropic, and occasionally subject to cooperative change. Nevertheless, because the energy required to distort bonds increases approximately as the square of the displacement, a small displacement due to a site-directed substitution will spread over many bonds in order to minimize the energy of the system. More precisely, the energy of accommodation to the distortion introduced by mutation is reduced by $1/N$ when distortions

are spread over N bonds instead of concentrated in a single bond. The consequent linear displacement will normally be quickly attenuated as it spreads through the rapidly (with approximately the cube of distance from the site) increasing number of primary and secondary bonds in an increasing volume. Therefore displacements due to small perturbations are expected to be localized. The operation of this mechanism is clearly visualized in numerous crystallographic studies which show only localized change after single substitution in a macromolecule (for example, 11–15). Therefore the use of a half-site probe like guanyl urea to determine the locus of the G²⁶⁰ effect should usually supply useful information.

The data are shown in the lower panel of Figure 5 and Table IV. C²⁶⁰ RNA behaves as determined earlier ($K_i = 11 \pm 1$ mM, ref 2) for guanyl urea inhibition of a wild-type intron. In general appearance, inhibition resembles that by arginine (Figures 3 and 4), but the magnitude of the effects of N²⁶⁰ substitution on guanyl urea binding are smaller. The critical point is that G²⁶⁰ RNA cannot be distinguished from C²⁶⁰ RNA and U²⁶⁰ RNA by use of guanyl urea (Table IV and Figure 5; contrast Table II). Thus on the basis of this experiment, the G²⁶⁰ perturbation is either most closely linked to the ribose/ α -carbon end of the site, or shifts the ends of the GTP/arginine site relative to each other.

DISCUSSION

Physical evidence for a single, active-site-bound arginine

In these experiments, we have examined the rG binding site within the self-splicing intron from the *Tetrahymena* macronuclear rRNA gene. This site is normally used for a derivative of guanosine, the exogenous splicing substrate (16). Because K_m , k_{cat} , and k_{cat}/K_m for reaction with GTP are changed by N²⁶⁰ substitution, we conclude that the base moiety of N²⁶⁰ communicates with the rG site.

The k_{cat}/K_m for GTP varies 3-fold among the N²⁶⁰ RNAs, and the dissociation constant for L-arginine varies 2.5-fold. Thus N²⁶⁰ substitutions produce effects of similar magnitude on GTP and L-arginine, though the changes appear mainly in the binding of the transition state for GTP. A substitution like these, which makes a limited perturbation, is unlikely to produce similar effects at two or more distant sites. Thus these experiments add physical evidence to the previous kinetic argument (arginine is competitive with GTP, ref 1), and the previous stereochemical argument (arginine's isostery with G is essential to inhibition, ref 2) for inhibition of the GTP reaction by a single, site-bound arginine.

Both the K_m 's for GTP, and the K_i 's for arginine in the C²⁶⁰, U²⁶⁰, and A²⁶⁰ RNAs behave similarly, with A²⁶⁰ RNA slightly affected in all assays (Tables I, II, and III). However, G²⁶⁰ RNA, while the most significantly altered RNA to both GTP and arginine, has the least apparent affinity for GTP (Table I) but the greatest affinity for arginine (Tables II and III). The contrary direction of these effects presumably results from the differences in the RNA contacts of the two molecules. While GTP and arginine can bind via partially overlapping H-bond patterns (Figure 6), arginine can not utilize the putative H-bond to O⁶ of rG (17), nor can it form precisely equivalent bonds to either of the H-bonds inferred for ribose (18). We will return to the reason for the differential effects of G²⁶⁰ after developing a more complete model for its role in the structure (see Figure 6).

N²⁶⁰ is not in direct contact with GTP and arginine

Because the reaction of GTP is so slow (≤ 2 min⁻¹, Table I), it seems reasonable to expect equilibration of the RNA with free GTP during GTP binding. Such slow chemical conversion would not disturb the normally more rapid binding steps. Thus we believe the

K_m will reflect changes in the dissociation constant for GTP. This idea has strong empirical support from previous work which shows that alteration of H-bonding groups on both guanine and ribose produces changes of the expected direction and magnitude in K_m for GTP (17,18).

The equilibrium constant for L-arginine binding has been measured directly, as K_i . Therefore N²⁶⁰ substitution alters the apparent equilibrium constants for GTP and L-arginine binding by 1.6 to 2.5-fold (Tables I and II), implying a free energy difference for binding of 0.27 to 0.55 kcal/mol. These effects on GTP or L-arginine binding are inconsistent with changes implying free energies of ≥ 1.4 kcal/mol per H-bond, observed when several of the exocyclic H-bonding groups of substrate guanosine are deleted (17). Therefore the base of N²⁶⁰ is unlikely to H-bond to GTP.

The same conclusion follows from a qualitative, rather than quantitative argument. C²⁶⁰, U²⁶⁰, and A²⁶⁰ RNAs are similar in many respects, for example, with regard to their K_m 's for GTP (Figure 2, Table I). The observed numerical similarity of K_m 's implies that were there to be (for example) an H-bond between the base of N²⁶⁰ and GTP, it would have to be formed *equivalently* to C, U, and A. It is difficult to devise any plausible bond to these specifications. Certainly one otherwise attractive possibility, that C²⁶⁰ might form a standard base pair with GTP, is eliminated by the results of substitution.

Instead, the N²⁶⁰ base is probably close enough to move the groups which do directly participate in the GTP/arginine site. The size of the consequent effect is due to the spreading, over many bonds, of the deformation due to nucleotide substitution. C²⁶⁰ is partially accessible to dimethylsulfate alkylation, though it does not appear to be as reactive as are single-stranded nucleotides (19). It is positioned (Figure 1) in a joining segment of conserved sequence and length. Therefore it seems likely that C²⁶⁰ plays a role in positioning the helices attached to it within the group I catalytic core, with consequent effects on the GTP/arginine site.

Changes of similar magnitude to these occurred when both the nucleotides flanking C²⁶⁰ were simultaneously changed, U²⁵⁹ to A, and A²⁶¹ to C (3). Williamson, *et al.* measured a 4-fold alteration in the K_m for GTP, with little or no effect on k_{cat} , despite substitution for the completely conserved A²⁶¹ (3). Changes in the sequence of bases in J6/7 can apparently displace groups in the G site, but none are likely to make vital contacts with GTP. *The altered GTP/arginine site in G²⁶⁰ precursor RNA*

The molecular nature of the change in the GTP site produced by G²⁶⁰ is strongly constrained by three results: 1) G²⁶⁰ removes stereoselectivity for L-arginine, 2) G²⁶⁰ simultaneously yields the highest affinity for both D- and L-arginine (Figures 3 and 4, Tables II and III), and 3) the G²⁶⁰ change is not perceived when guanyl urea is bound instead of arginine (Figure 5). We now consider these in order (compare Figure 6).

Stereoselection must result from differential interactions by groups on the arginine α -carbon. Therefore a change in stereoselectivity implies a change in the position of groups around the α -carbon relative to the rest of the site. A more explicit hypothesis can be given in terms of the previous binding model (2); the relative positions of the H-bonding acceptors for the sidechain guanidino group and the 5'exon-intron junction phosphodiester region are changed in G²⁶⁰ RNA.

To account for the increase in affinity for arginine on substitution of G²⁶⁰ in the RNA, either the guanidino H-bonding, or the strongly ionic (2) interaction of α -NH₃⁺ with the exon-intron junction must improve (or both). Because these possibilities invoke the two ends of the site, the ambiguity is resolved by the experiment with the half-site probe, guanyl urea.

Figure 5 and Table IV show that the unique affinity of the G²⁶⁰ RNA binding site for arginine is undetectable with guanyl urea. The only feature which guanyl urea lacks, which is possessed by arginine, is the extension (leftward in Figure 5) which carries the α -carbon. Thus the simplest interpretation is that the site on the G²⁶⁰ RNA has been changed by movement of the ribose/ α -carbon region, without relative change in the H-bonding groups at the guanine/guanidino end. To expand this conclusion in terms of the binding model (2), the RNA backbone at the 5' intron-exon junction has shifted within the G²⁶⁰ RNA site.

The movement at the ribose/ α -carbon end of the site in G²⁶⁰ RNA must increase affinity for both D- and L-arginine. G²⁶⁰ substitution has probably allowed the α -carbon nearer the negative phosphate charges, and thereby improved the previously inferred interaction with the arginine α -NH₃⁺ (2). However, this improvement must be unaffected by the interchange of α -COO⁻ and α -NH₃⁺. Therefore the RNA backbone is probably not static, which would favor either D- or L-arginine. While it might at first appear that the plane of symmetry between the α -COO⁻ and α -NH₃⁺ is a tenable position, this is inconsistent with the generally improved interaction of G²⁶⁰ RNA with arginine, which rules out a stronger interaction with the α -COO⁻. Thus the position of the intron-exon junction within the site is probably more flexible in G²⁶⁰ RNA, so that inversion at the bound arginine's α -carbon can be accommodated.

Though this interpretation makes no strong specific prediction about the GTP reaction, it is consistent with the data on the effects of substitution (Table I). If the ribose 3'-OH of GTP and the α -NH₃⁺ of arginine are accommodated at contiguous, but not identical positions (2), then the proposed flexibility could aid arginine binding, but slightly obstruct GTP binding and the precise alignment of groups required for catalysis, as is observed. These ideas are illustrated in Figure 6, which shows the effect of the G²⁶⁰ mutation at the ribose/ α -carbon end of the rG site.

For completeness' sake, note that there are two variants of this structural interpretation, equally consistent with the data. In the less obvious variant, movement at the ribose/ α -carbon end of the site is actually produced by oscillation of the entire guanine/guanidino end of the site as a rigid unit.

Evolution of L-stereoselection

The sidechain-specific and stereoselective interaction of arginine with this intron is the best available experimental model for the sort of RNA-amino acid association which, arguably, produced the genetic code (1). Therefore the molecular origin for L-stereoselection is of interest. Were a preference for L-arginine due to a stable, and therefore reproducible feature of RNA structure, it would be fair to say that preexistence of RNA having an approximately modern structure could have determined the choice of L-amino acids for use in coding and protein biosynthesis. As an example of such strong structural constraint, the L-arginine site might lie entirely within the reproducible confines of a helical groove.

The properties of the N²⁶⁰ substitutions argue that this is not the case for the *Tetrahymena* site. A single mutation near the G site, in a nucleotide which has no apparent stable partner, removes stereoselection without dramatic effects on catalysis. It would be of interest to see if the *Tetrahymena* arginine site can be made D-selective by other site-directed mutations. But on the basis of the *Tetrahymena* model, L-stereoselectivity is not implied because of an extremely stable structure intrinsic to α -D-ribose RNA. Instead, L-selection might necessarily arise in the *Tetrahymena* intron as a consequence of a

requirement for optimum catalysis (2), or might even be fortuitous. These possibilities may be distinguished by examining other group I introns.

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