Kinetic analysis of the 5′ splice junction hydrolysis of a group II intron promoted by domain 5

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ABSTRACT

The 5′ splice junction (5′SJ) of Group II Intron transcripts is subject to a specific hydrolysis reaction (SJH). This reaction occurs either within a single transcript containing Intron sequences through domain 5 (D5) or by cooperation of two separate transcripts, one bearing the 5′SJ and another contributing D5 (1). In this report we describe the latter reaction in terms of its kinetic parameters. A minimal D5 RNA of 36 nts (aD5) was sufficient to promote SJH of a second transcript containing the 5′ exon plus Intron domains 1, 2, and 3 (E1:123). Equimolar production of two RNAs, the 5′ exon (E1) and an Intron fragment containing domains 1, 2, and 3 (123) was observed. The kinetic coefficients were evaluated by an excess GGDD5 approach. The apparent $K_m$ was complex, varying with GGDD5 concentration. This behavior indicates heterogeneity in E1:123 with respect to GGDD5 binding. The binding heterogeneity may result from formation of E1:123 dimers or from nicks in some molecules of each E1:123 preparation. The heterogeneity was always evident, but to a variable degree, regardless of the procedure by which E1:123 was isolated. The system may be described in terms of parameters analogous to $k_{cat}$ and $K_m$. At infinite dilution of GGDD5, the characterizing values were: $k_{cat} = 0.0055$ min$^{-1}$ and $K_m = 0.22$ nM. In the limit of GGDD5 saturation, the values were: $k_{cat} = 0.012$ min$^{-1}$ and $K_m = 4.5$ nM. A natural variant D5, representing the sequence from Intron 1 of the yeast cytochrome-b gene, was also functional in SJH. This ggD5$^{EI}$ was governed by similar $K_m$ and $K_m$ values, but was only one-third as active over the entire D5 concentration range. A different D5 isomer was entirely ineffective for SJH.

INTRODUCTION

Group II introns are found in the genomes of chloroplasts, green plant mitochondria, and fungal mitochondria (2). Several members of the group are capable of self-splicing in vitro (3–8). The characteristic secondary structure of these introns is usually represented as six folded regions ('domains') arranged and numbered in order from 5′ to 3′ (Structure I; reviewed in ref. 2).

The mechanism of Group II intron splicing is similar in certain respects to that of the introns found in the nuclei of eukaryotic cells (9, a recent review). This similarity extends to the formation of lariat intermediates and excised intron lariats. The first step of the reaction for both intron families is release of the 5′ exon (E1) by attack of the 2′OH from the adenosine at the branch site. Group II introns can carry out a similar reaction, hydrolysis at the 5′SJ (SJH), under certain conditions of high salt (10) or if the branch site is absent from the transcript (11). For example, it has been shown that SJH proceeds efficiently for a transcript extending through D5 but lacking downstream sequences (12). Further analysis of deletion derivatives lacking multiple domains has shown that D5 probably interacts with D1 to activate the 5′SJ, since a transcript lacking D2, D3, D4, and D6, but retaining D1 and D5 was capable SJH (13).

SJH and other reactions of Group II introns can also use pairs of non-overlapping partial transcripts; such systems have been referred to as trans-splicing (1,14). This approach has been useful...
for examining the requirements of each step of splicing for individual elements of the structure. Specifically, the SJH reaction occurs efficiently in a system containing two transcripts, the first composed of the 5' exon plus domains 1, 2, and 3 (E1:123) while the second contains D5 (1).

We have undertaken an analysis of the properties of this trans-reaction system in order to compare the functional potencies of variant forms of GGDS and E1:123. The trans-reaction system allows us to partition the kinetic effects into apparent affinity, K_m, and reactivity, k_s, components. We also prepared three minimal D5 RNAs (Structure II) and tested them with E1:123 derived from the a5y intron of the Saccharomyces cerevisiae mitochondrial gene for subunit-1 of cytochrome oxidase.

 MATERIALS AND METHODS
Preparation, purification, and maintenance of RNA
D5s were made by transcription with T7 RNA polymerase using synthetic DNA templates (15). The top strand and various bottom strand templates were constructed at the University of Pittsburgh DNA Synthesis Center. To improve transcription yields, the bottom strands were designed to generate transcripts having a S'-GG leader followed by 34 nts corresponding either to the D5 sequence found in the a5y intron (GGDS^5y) or the b1 intron (GGDS^b1). GU and UU precede D5 in the natural a5y and b1 introns, respectively. Reactivity was introduced by adding [α-32P]-UTP to the transcription reactions (ICN). Transcriptions for GGDSs were carried out for 24 hours. Transcriptions to make RNAs bearing E1 and differing lengths of intron a5y were carried out for 1 hour from HindIII digested plasmids [pJD3'-673 for E1:123 (1), pJD3'-851 for E1:12345 (12), and pJD2Δa5 for E1:1234_6_E2 (13)]. After incubation at 37°C, the reaction mixtures were applied directly to a 20% (for GGDS) or to a 4% (for all larger transcripts) polyacrylamide denaturing gel (5% crosslinked, containing 8 M urea, 0.1% SDS, and TBE buffer). After electrophoresis and either UV-shadowing or autoradiography, the RNA was eluted into TBE buffer with 0.1 % tank buffers being 10 mM MgCl_2, 0.1 mM EDTA, and 0.1 M Tris-Heps, pH 7.5 (17). To approximate the condition of the SJH reaction, the sample preparation, gel loading, and quantitation of products was achieved with the AMBIS Radioanalytic Image Analyzer. The distribution of background radioactivity due to non-specific hydrolysis of phosphodiester bonds appeared to follow the progress function described by the random scission model (16). The rate of loss of E1:123 by non-specific hydrolysis varied somewhat from preparation to preparation but could be characterized by an apparent decay constant of only 0.005–0.012 min^{-1} (equal to 5–12×10^{-6} min^{-1} on a phosphodiester bond basis). Since reaction rates were measured from product formation progress curves during the first twenty minutes, the loss of reactant due to random hydrolysis had negligible influence on the measured rates. In practice, the radioactivity envelope representing nonspecific hydrolysis was subtracted using the multiple level background approach was tried. This method involved phenol extraction followed by gel-exclusion chromatography through a column (0.8 by 29 cm) of Sephacyr-S200 (Pharmacia) equilibrated with 0.5 M KCl and 0.02 M Tris, pH 7.5. The RNA concentration of the void volume fraction was determined by spectrophotometry. Aliquots of this fraction were immediately used in SJH reactions by mixing with equal volumes of GGDS dissolved in 0.5 M KCl, 0.2 M MgCl_2, and 0.02 M Tris, pH 7.5.

 Splice junction hydrolysis reactions
The standard reaction temperature for SJH was 45°C, and the buffer was 0.5 M KCl, 0.1 M MgCl_2, 0.02 M Tris (pH 7.5, Cl^- counterion) (10). Either 4 or 10 μl of the reaction buffer was added directly to the tubes containing the dried reactants. For the smaller volume reactions, 4 μl of mineral oil was added to retard evaporation. Reactions were initiated by chilling the tubes and adding 0.75 volumes of 0.25 M EDTA. The samples were then heated for 1.5 minutes in a boiling water bath, chilled on ice, and applied to a 4% polyacrylamide denaturing gel. Quantitation of products was achieved with the AMBIS Radioanalytic Image Analyzer. The distribution of background radioactivity due to non-specific hydrolysis of phosphodiester bonds appeared to follow the progress function described by the random scission model (16). The rate of loss of E1:123 by non-specific hydrolysis varied somewhat from preparation to preparation but could be characterized by an apparent decay constant of only 0.005–0.012 min^{-1} (equal to 5–12×10^{-6} min^{-1} on a phosphodiester bond basis). Since reaction rates were measured from product formation progress curves during the first twenty minutes, the loss of reactant due to random hydrolysis had negligible influence on the measured rates. In practice, the radioactivity envelope representing nonspecific hydrolysis was subtracted using the multiple level background mode of the AMBIS software (Fig. 1B). Practical specific radioactivity was defined as the quotient of the total analyzer count rate for the entire length of a given gel lane and the amount of RNA applied to the lane based on spectrophotometry. The specific radioactivities of the products of the reaction were based on their relative contents of uridylicate.

 Native gel electrophoresis
Electrophoresis under non-denaturing conditions was performed in 5% polyacrylamide gels (5% crosslinked) with both the gel and tank buffers being 10 mM MgCl_2, 0.1 mM EDTA, and 0.1 M Tris-Heps, pH 7.5 (17). To approximate the condition of the SJH reaction, the sample preparation, gel loading, and
electrophoretic separation were performed at 42°C. The sizes of the RNAs in the electrophoretically resolved bands were estimated with reference to the mobility of several RNA size markers.

**Thermal denaturation profiles**

Melting curves were obtained with the Aviv 14DS UV-Vis Spectrophotometer. Heating was controlled by the master computer operating an external bath. The heating ramp advanced by 1°C steps over a total time of 3 hours. The criterion for equilibration at each temperature was the maintenance of a probe temperature change rate of less than 0.03°C min⁻¹ for 0.5 minutes. The probe was inserted in the heated brass cuvet holder, 3 mm from the body of the cuvet. At the end of each run the RNA was analyzed to determine the extent of any degradation.

**Data analysis**

The experiments used to evaluate kinetic parameters were performed with GGD5 in excess. The initial rate data were analyzed by established excess enzyme methods (18, 19). We chose to use the excess enzyme technique for several technical reasons. Since E1:123 (1003 nts) is almost thirty times larger than GGD5 (36 nts) the mass ratio of E1:123 to GGD5 is almost thirty times larger than the molar ratio in any solution of the two RNAs. Therefore, reactions with excess E1:123 would require the preparation of a much larger mass of E1:123 than of GGD5, particularly in the concentration range where virtual saturation of GGD5 might be achieved. In addition, the high mass concentrations of E1:123 needed to achieve saturation might exceed the limit of RNA monodispersity, if not the limit of RNA solubility, under the standard SJH assay conditions. Also, the background radioactivity envelope becomes larger with increasing E1:123 concentration. In contrast, the background can be minimized by keeping the E1:123 concentration low and using GGD5 in excess, with its concentration as the experimental variable. Finally, from the point of view of a formalism in which GGD5 and E1:123 are identified respectively as enzyme and substrate, if kₙₑᵣᵢᵣ is very small, the system may not progress to the steady-state during the period of assay. Since this small kᵣᵢᵣ situation is relevant to the current study, the steady-state condition cannot be legitimately invoked to analyze rate data obtained using substrate in excess over enzyme.

According to the theoretical analysis of Kasserra and Laidler for enzyme in excess of substrate (the establishment of the steady-state thus being precluded), the initial rate of reaction is given by equation 1 (18, 19).

\[ v = v \cdot [S₀] \]

The reduced reaction velocity, v, is a function of the enzyme concentration according to equation 2.

\[ v = k₂ \cdot [E_{act}] / ([E_{act}] + Kₘ) \]

Equation 2 applies to Scheme A

\[ S + E \xrightarrow{k₁} E \cdot S \xrightarrow{k₋₁} E + P \]

where \( Kₘ \) is defined as usual as \( (k₋₁ + k₂) / k₁ \).

Reduced velocities for a set of reactions representing a range of GGD5 concentrations and a common E1:123 concentration were obtained from the initial slopes of progress curves. Single time point measurements were used to obtain velocities for data set B of Figure 3. Separately, it was determined for the E1:123 corresponding to data set B that the velocities determined by single and multiple time point measurements were within experimental error of each other at both the low and high extremes of the GGD5 concentration range. Rearrangement of equation 2 according to the Eadie-Hofstee format allows the values of \( Kₘ \) and \( k₂ \) to be obtained from the slope and y-intercept of the corresponding plot. However, as described in Results, complexities in the behavior of this system required a more elaborate formulation of the analysis.

**RESULTS**

**Rate of SJH under excess GGD5 conditions**

Previous studies of trans SJH promoted by D5 RNA have used D5 transcripts comprising the bona fide D5 RNA flanked by 5' and 3' sequences derived from the original intron and vector polylinker (1). The activity of the D5 used here, consisting of the dinucleotide GG plus the 34 nt stem and loop, demonstrates that the flanking sequences play no required functional role. The autoradiogram in Figure 1A qualitatively shows the progress of a typical SJH reaction carried out with GGD5 in excess over E1:123 to yield products E1 and 123. The center panel of this figure shows radioanalytic image analyzer tracings of selected lanes. A multilevel background was generated manually with the instrument software and is included to illustrate how we compensated for the background resulting primarily from nonspecific hydrolysis of the substrate. Except in the region near E1:123, the observed background was a monotonically declining function of migration distance. This pattern is qualitatively like that predicted from the theory of random scission of a polymer (16). We did not construct any background envelope under E1:123 because the background there was a more complicated function, rising from zero to a maximum at the position of those random scission products slightly smaller than the intact substrate. Also, we could not unambiguously account for the E1:123 consumed either by random scission, by SJH, or by other specific but D5-independent cleavages. This uncertainty of how to apportion the radioactivity near the E1:123 region of the gel precluded quantitative and unbiased measurement of E1:123 decay along any one of several paths. On the other hand, the yield of the principal products 123 and E1 could be readily computed from the accumulation of net radioactivity in these bands (Fig. 1B).

The minor product 12' may arise from a slow, secondary cleavage in D2, 567 nts from the 5'SJ. There the sequence AUUUUC, resembling IBS1 at the 3' end of E1, precedes the scissile bond (10). A D5-dependent band, putatively the other fragment '23 (108 nts), was detected lower in the gel. A very faint band is seen near the position of intron fragment '23 in the zero time and zero GGD5 lanes (Fig. 1A); it was probably produced during E1:123 preparation by cleavage of the transcript.

The kinetic progress curve of the reaction is presented in Figure 1C. The initial slope of this progress curve divided by the initial concentration of E1:123 yielded the reduced reaction velocity. The equivalent yields of the primary reaction products 123 (708 nts) and E1 (295 nts) at early times substantiate the reliability of the measurements. Only the production of E1 was monitored in many experiments, since the background correction for 123 was much greater than for E1, and since the longer 123 was more susceptible to degradation by random cleavage and was...
Figure 1. Splice junction hydrolysis of E1:123 with excess GGD5. The initial concentrations of E1:123 and GGD5 were 400 nM and 30 μM respectively in a reaction volume of 4 μl. A.) Autoradiogram of denaturing gel electrophoretic separation of SJH reaction components. The gel lanes represent reactions quenched after the indicated times of incubation at 45°C. B.) Radioanalytic image analyzer tracings of lanes 0', 5', 15', and 25'. Incubation time increases from the top scan (0 min) to the bottom one (25 min). Each extends from the top of a lane to beyond the E1 band. The hatched areas represent the multilevel background generated with the AMBIS software. The background was subtracted to give the net radioactivity in the product bands. C.) Progress curve of the reaction. The reaction products are symbolized as follows: E1, O; 123, △; 12', □. The initial rate for the SJH reaction was 3.7 nM min⁻¹ of E1 (or 123), and the reduced reaction velocity was 9.2×10⁻³ min⁻¹. The solid curve represents the predicted yield of E1 with concomitant loss of E1:123 by non-specific hydrolysis occurring with a decay constant of 0.012 min⁻¹ (equivalent to 1.2×10⁻³ min⁻¹ per phosphodiester bond available). The dashed curve represents the predicted yield of either product for SJH occurring without non-specific hydrolysis.

SJH of E1:123 by GGD5 proceeds beyond a single turnover

Although it is more convenient to measure reaction rates in this system using GGD5 in excess, it was necessary to use excess E1:123 to determine whether one molecule of GGD5 could promote the SJH of more than one E1:123 molecule. It appears that in the seven hour time period GGD5 was not used more than once, the limit yield of product being achieved in three hours (Fig. 2A, solid circles). Three hours is about the time required for two potential turnovers (see k₁ values below). However, since the supply of E1:123 was almost exhausted at the end of three hours, the limit on the production of E1 might simply reflect depletion of E1:123. In three other experiments (Fig. 2B, open symbols) fresh transcript was added after three or four hours of incubation. The amount of infused E1:123 was equal to that present at the start of the reaction but was sufficiently concentrated to dilute the GGD5 by no more than 10%. In one of these experiments, two fresh additions of E1:123 were made. Each addition of fresh E1:123 led to an increase in E1 formation. The final yield of E1 became significantly greater than the amount of GGD5 present.

The curve drawn in parts A and B of Figure 2 is that generated from the model described by Scheme B.

Scheme B: 

\[ S + E \xrightleftharpoons[k_{-1}]{k_1} E \cdot S \xrightarrow{k_2} E + P \]

nicked S
Figure 2. Progress of the SJH reaction with excess El:123. A.) Reaction between 2.27 uM El:123 and 0.16 uM GGD5, solid circles. Progress curve according to Scheme B of the text, solid curve. The values of the adjustable parameters k_{2} and k_{\alpha} were obtained by minimizing the sum of the squares of the deviations between the observed product yield and that calculated using the Simultaneous Ordinary Differential Equation Solver from the International Mathematical and Statistical Library (IMSL). The error index was minimized over the k_{2} by k_{\alpha} surface using the Minimization Routine also from the IMSL. The ratio k_{2}/k_{\alpha} was fixed at 0.2 \muM, with k_{-1} and k_{1} chosen such that S and E were in virtual equilibrium with E:S throughout the reaction. The resulting values of k_{2} and k_{\alpha} were 0.01 min^{-1} and 0.03 min^{-1}, respectively. B.) Data symbols for reactions summarized in this panel are identified according to the following tabulation: Symbol El:123 Addn, hr [El:123], mM [GGD5], mM

<table>
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<th>Symbol</th>
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<th>[GGD5], mM</th>
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<tr>
<td></td>
<td>3</td>
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<td>3.55</td>
<td>0.16</td>
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</tbody>
</table>

The curve is a duplicate of that in part A.

where k_{\alpha} defines the rate of loss of functional El:123 by random scission. The minimization procedure gave k_{2} and k_{\alpha} values of 0.01 min^{-1} and 0.03 min^{-1}, respectively. The k_{2} value is within the error of that obtained from excess enzyme experiments (see Fig. 3 and related text), and the k_{\alpha} value is similar to that obtained from estimates of the decay of the El:123 gel band (see legend to Fig. 1C). The behavior of the system in excess El:123 mode is therefore consistent with that of the system in excess GGD5 mode.

To determine whether GGD5 subjected to one round of SJH promotion could act again when isolated from the reaction mixture, we separated the contents of a three hour reaction mixture (E_{1} formed/GGD5 added = 0.9) in a 20% polyacrylamide denaturing gel, recovered the GGD5 by electroelution (20% recovery), and reacted it with fresh excess El:123. In three hours the recovered GGD5 produced about half as much El as fresh GGD5. Thus it seems that GGD5 is not irreversibly inactivated by participation in SJH.

The saturation of the SJH transcript by GGD5 is non-hyperbolic

Reaction velocities were determined over a 700-fold range of GGD5 concentration. The data were cast in Eadie-Hoffstee form; an unexpected non-linear response was regularly observed (Fig. 3). By comparing the two data sets of this figure, one can see that upward concavity appeared in the plot regardless of the method of El:123 preparation (exposure or non-exposure to RNA denaturing conditions). The open circles display the behavior of El:123 purified on a denaturing gel. The closed triangles show the behavior of phenol extracted El:123 purified by exclusion chromatography through a column equilibrated with SJH reaction buffer (without Mg^{2+}), after which the El:123...
was mixed with GGD5 plus Mg^{++} and assayed directly. Although the characterizing parameters of these two E1:123 preparations were quantitatively distinct, both behaved qualitatively as heterogeneous systems. Using the ratio of the slopes of the limiting lines of Figure 3, K_m^-/?K_m^+, as an index of heterogeneity, Method A and Method B produced E1:123s with index values of 20 and 4, respectively. Two other Method B preparations of E1:123s had corresponding heterogeneity indices of 10 and 12. In all cases the corresponding limiting K_m's and k_2's were of similar magnitude. The response to GGD5 therefore was apparently not significantly determined by the conditions and treatments to which the E1:123 had been subjected during purification and preparation for assay. In another experiment, we tried to melt and anneal E1:123 to produce a single, more reactive form. We preheated the reaction mixtures (without Mg^{++}) at 65°C for 10 minutes, shifted to 45°C for 10 minutes, and initiated reactions by addition of Mg^{++}. The initial reaction rates were unaffected by this particular treatment (data not shown). Pre-incubation of E1:123 and/or GGD5 for various times and temperatures in the presence of Mg^{++} (20) was performed but gave no enhancement of the SJH rate. Indeed, preheating at 60°C with Mg^{++} present led to extensive transcript degradation. Preheating at 100°C in the absence of Mg^{++}, followed by cooling in the presence of a buffer containing Mg^{++} (21) also resulted in no increase in the SJH reaction rate. Thus the annealing conditions which provide marked enhancements of Group I intron activities have little effect on this Group II system.

One consequence of the biphasic behavior shown in Figure 3 is that it is not possible to assign unique values of k_2 and K_m to the system. The system could be considered to be heterogeneous, having any number of E1:123 forms, each of which binds GGD5 (the component in excess) with its own affinity. We have chosen to characterize the system in terms of the two limiting concentration regions spanned by the plot of Figure 3. The definition of the identity of the limiting parameters for such a heterogeneous system follows the theoretical analysis of ligand binding by a macromolecule bearing multiple classes of nonidentical noninteracting sites (22).

To ascertain whether or not E1:123 was monodisperse under SJH reaction conditions, native gel electrophoresis was performed at 42°C with 10 mM Mg^{++} present in the gel (17). Although it would have been desirable to employ SJH buffer in the gel, the high conductance of SJH buffer precluded its use. A sample of E1:123 was dissolved in SJH reaction buffer, however, and loaded on the gel. Two bands were resolved for that part of the sample which entered the gel (data not shown). The faster band contained about two-thirds of the applied RNA and migrated with a mobility consistent with that of a dimer of E1:123. The remainder of the RNA was entrapped at the top of the lane. E1:123 dissolved in water and loaded on the native gel produced only a monomer band of slightly greater mobility than the monomer band seen after loading under SJH conditions.

**Splice junction hydrolysis with variant forms of D5 and with alternative substrates**

Replacing GGD5^{55} with GGD5^{51} (see Structure II) led to a three-fold reduction in the reduced velocity over the entire range of GGD5 concentrations corresponding to those of Figure 3. Hence E1:123 had the same distribution of affinities for GGD5^{51} as it had for GGD5^{55}, but the phosphodiester linkage at its splice junction was hydrolyzed with a three-fold smaller k_2. The other variant D5 we tested, isoGGD5^{55}, was not only completely ineffective for SJH, but also failed to inhibit SJH promoted by GGD5^{55} when present at great molar excess (29 nM E1:123, 0.25 mM GGGD5^{55}, 40 µM isoGGD5^{55}). SJH for the system E1:1234_6:E2 and GGD5 was also measured, but only over the dilute solution range of GGD5 concentration corresponding to binding to the high affinity substrate form(s). From the slope of the Eadie-Hofstee plot in this range (data not shown) we deduce that the K_m^{iso} of the high affinity population was 0.27 mM, and the corresponding v^o value was 0.007 min^{-1}. Thus the inclusion of D4, D6, and E2 only mildly affected the capability of the 5'SJ-bearing transcript to interact functionally with D5. Deletion of D4 previously had been shown to have no discernable effect on the SJH or self-splicing activities of this GII intron (1).

Next, the effect of internalizing D5 was examined by assaying the first order cleavage of the splice junction of E1:12345 under SJH conditions and by comparing these results with SJH of E1:123 promoted by GGD5. The value of the first order rate constant for the self-cleavage of E1:12345 was 0.032 min^{-1}. The secondary cleavage in D2 that produced intron fragment I2 during the SJH reaction between E1:123 and GGD5 was also observed to occur at about the same rate in the self-cleavage of E1:12345.

**Effect of temperature on the SJH reaction and on the structures of E1:123 and GGD5**

In an effort to understand the functional form of GGD5 as it interacts with E1:123, we examined the temperature dependence of the SJH activity of GGD5 acting on E1:123 and the melting curves of each of these reactants as monitored by UV-hyperchromicity measurements (Fig. 4). SJH activity increased along with the melting of secondary structure up to the optimum...
reaction temperature of 45°C. Additional heating from 45°C to 50°C induced further structure disruption and was accompanied by a precipitous activity loss. GGDS showed little change in absorbance across the temperature range where SJH is active. Instead, GGDS displayed a relatively sharp melting transition at about 75°C (Fig. 4). These melting experiments were performed in the absence of added Mg²⁺ and with 0.5 mM EDTA present. Electrophoretic analysis of the RNA carried through the melting process showed little or no degradation of either El:123 or GGDS. In the presence of excess Mg²⁺, extensive degradation of the RNA occurred during the melting experiment cycle, especially above 55°C, thus obscuring hyperchromicity due specifically to melting of secondary structure. The melting of GGDS¹ followed essentially the same profile as GGDS⁰. On the other hand, isoGGDS yielded a melting profile of the same shape but shifted toward higher temperatures by 5°C.

DISCUSSION

The early time course of cleavage at the 5'SJ of El:123 produced equal amounts of El and the intron fragment 123 (Fig. 1), and the initial rates measured with GGDS in excess were directly proportional to the El:123 concentration. These experiments used the 36 nt GGDS, demonstrating that this D5 with only a 2 nt leader is sufficient for D5 function. Furthermore, preliminary work with a sample of chemically synthesized 34 nt D5 indicates that the minimal stem-loop structure per se is functional. A second specific D5-dependent cleavage occurred later within D2 of the intron fragment (Fig. 1). Others have ascribed this particular cleavage to the presence of a nucleotide sequence within D2 similar to IBS1, the 3' end of El (10). Other minor gel bands also appeared, but these were not D5-independent and formed at smaller rates than the canonical SJH reaction products.

To establish whether D5 in trans might be analogous to an enzyme, experiments were performed to determine whether more than one El:123 cleavage was performed per D5 molecule (Fig. 2). In our earliest experiments the presence of excess El:123 led to the appearance of SJH product El and 123 only in molar amounts equal to the amount of GGDS present (data for 123 not shown). However, the limit on the amount of product formed was probably not due to irreversible GGDS⁺ product complex formation or covalent modification of GGDS, since subsequent addition of fresh El:123 caused the accumulation of El in amount greater than the amount of GGDS present. Also, the ability to isolate active GGDS from a reaction mixture that had already gone through one round of SJH demonstrates that recycling of GGDS is feasible. The apparent 'single-turnover' progress curve of Figure 2A with no late additions of El:123 was probably due to the substantial breakdown of El:123 by random scission during the slow specific cleavage at the 5'SJ. The parameter values of the theoretical curve of Figure 2A are compatible with such a model. We therefore conclude that GGDS turns over, albeit slowly.

In principle, the observed upward concavity of the response curve of Figure 3 could result from a dual-reaction-site model, one site being the 5'SJ and the other being the phosphodiester link located an equal distance from the 3' end. This model can be readily discarded since the gels show no D5-dependent fragment corresponding to the length of El:123 less two El-length fragments (413 nts). Moreover, there is no AUUUUC sequence near 295 nts from the 3' end. The observed curve is not consistent with an effector-site/reaction-site model either. If the effector-site were antagonistic to reaction at the primary reaction site, 'enzyme inhibition' should have occurred; i.e., the addition of more and more GGDS should have led to lower and lower SJH reaction rates. On the other hand, if the effector-site stimulated reaction-site activity, positive cooperativity should have been observed.

The results shown in Figure 3 would be seen if El:123 were dimerized and if one of the two SJH reaction sites in each dimer held D5 more avidly than the other. Native gel electrophoresis results suggest that multimeric forms of El:123 might be present under SJH conditions. This could account for the heterogeneity in El binding site affinity suggested by Figure 3.

Even freshly prepared El:123 samples contain some nicked molecules whose fragments presumably remain assembled under SJH conditions. The nicked fraction ranges from 10% to as much as 25% of a transcript preparation. Though nicked, many such molecules may retain both D5-binding and SJH activities but with altered kₚ and Kₘ parameters. The presence of nicked molecules thus provides another possible explanation for the observed El:123 heterogeneity with respect to D5 binding.

There are five tertiary pairings involving D1 and adjacent El sequences that have been established experimentally or proposed on the basis of phylogenetic comparisons (2). Mutational alterations that prevent base pairing between these elements impair self-splicing. For example, substitution of nucleotides in either EBS1 or IBS1 abolished self-splicing, but subsequent compensating substitution in the partner region restored splicing (23). Similarly, the intra-D1 α-α' interaction (Structure I) has been shown to contribute to the function of the intron at 45°C (24). We believe that most of these interactions must persist at 45°C, even though heating to this temperature results in melting one-third of the hypochromicity-producing structure. In particular, the seven basepair interaction between the EBS1 sequence of intron D1 and the partner IBS1 sequence of El must remain intact at 45°C in a functional transcript.

We have addressed the question of whether D5 complexed to an intron fragment lacking D5 is as effective for promoting SJH as resident D5 by comparing the kinetic data for the inter-molecular reaction between El:123 and D5 with those for the intra-molecular reaction of El:12345. Since El:12345 preparations contain about the same fraction of nicked molecules as El:123 preparations, the observed rate constant for SJH is probably a mole-fraction average first order rate constant, k. The observed k value for El:12345, 0.032 min⁻¹, was less than three times larger than the corresponding mole-fraction average rate constant, kₚ, for El:123 + GGDS. Consider too that the secondary cleavage in D2 to make intron fragment 12' occurred in both the intra- and inter-molecular SJH reaction. Thus, binding of D5 by El:123 produced a binary complex which performed SJH almost as well as covalently integrated D5. The complex was also capable of promoting the second site cleavage. Analysis of the SJH reaction between GGDS and El:12345: El:12345:6: El:123 showed that the inclusion of D4, D6, and E2 did not have great effect on the parameters kₚ and Kₘ.
The fact that these base pair substitutions did not change the melting behavior of D5 suggests that the GAAA tetraloop or another feature may stabilize the stem structure in a manner that overrules the effect of a small number of basepair exchanges. One other modification of D5, represented in isoGGDS, resulted in complete loss of SJH activity. Since isoGGDS also failed to inhibit D5 promoted SJH, it probably could not bind at all to E1:123.

As measured by hyperchromicity, the secondary structure of D5 is essentially unperturbed throughout the temperature range where SJH is observed and over which E1:123 melts significantly (Fig. 4). Therefore, whether D5 acts in cis or in trans, functional D5 is probably a compact, folded form (Structure 2) with additional internal stacking of three of the four bases in the GAAA loop (25). The binding of D5 to a specific site to promote reaction at the 5'SJ evidently does not require extensive Watson/Crick basepairing between D5 and a complementary sequence on E1:123. The challenge ahead is to identify the site(s) of D5 binding more precisely within D1 and to elucidate the nature of the interactions responsible for activation of the splice junction.

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ABBREVIATIONS

(Abbreviations for RNA molecules are set in boldface type.): intron domains D1, D2, D3, D4, D5, and D6; E1:123, a transcript with the 5' exon and intron a5y domains 1, 2, and 3; E1, the 5' exon reaction product; I23, a product containing intron domains 1, 2, and 3; E3, the 3' exon; EBS1, exon-binding-site-1, a 7 nt sequence in D1 that base pairs to a complementary sequence at the 3' end of the 5' exon; IBS1, intron-binding-site-1, the 5' exon complement of EBS1; EDTA, ethylenediaminetetraacetic acid; GgD5, intron domain 5 preceded by the dinucleotide GG; nts, nucleotide units; SDS, sodium dodecylsulfate; 5'SJ, 5' splice junction (5' exon to intron linkage); SJH, splice junction hydrolysis; Tris, tris(hydroxymethyl)aminomethane-HCl buffer; TBE, 89 mM Tris-Borate, 2.5 mM EDTA, pH 8.3.

REFERENCES