Siebenlist, U. (1979) *Nature (London) 279*, 651-652.
Siebenlist, U., Simpson, R. B., & Gilbert, W. (1980) *Cell (Cambridge, Mass.) 20*, 269-281.
Stevens, A. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M. J., Eds.) pp 617-627, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Stüber, D., & Bujard, H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 167-171.
Travers, A. A., & Burgess, R. R. (1969) *Nature (London) 222*, 537-540.
Vogt, V. (1969) *Nature (London) 223*, 854-855.
von Hippel, P. H., Bear, D. G., Winter, R. B., & Berg, O. G. (1982) in *Promoters: Structure and Function* (Rodriguez, R. L., & Chamberlin, M. J., Eds.) pp 3-33, Praeger, New York.
Wang, J. C., Jacobsen, J. H., & Saucier, J. M. (1977) *Nucleic Acids Res. 4*, 1225-1241.
West, R. W., Jr., & Rodriguez, R. L. (1980) *Gene 9*, 175-193.
Wu, F. Y.-H., Yarbrough, L. R., & Wu, C.-W. (1976) *Biochemistry 15*, 3254-3258.

Guanosine Binding Required for Cyclization of the Self-Splicing Intervening Sequence Ribonucleic Acid from *Tetrahymena thermophila*†

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ABSTRACT: We have converted the intramolecular cyclization reaction of the self-splicing intervening sequence (IVS) ribonucleic acid (RNA) from *Tetrahymena thermophila* into an intermolecular guanosine addition reaction. This was accomplished by selectively removing the 3′-terminal nucleotide by oxidation and β-elimination; the β-eliminated IVS thereby is no longer capable of reacting with itself. However, under cyclization conditions, a free guanosine molecule can make a nucleophilic attack at the normal cyclization site. We have used this guanosine addition reaction as a model system for a Michaelis–Menten kinetic analysis of the guanosine binding site involved in cyclization. The results indicate that functional groups on the guanine that are used in a G-C Watson–Crick base pair are important for the cyclization reaction. This is the same result that was obtained for the guanosine binding site involved in splicing [Bass, B. L., & Cech, T. R. (1984) *Nature (London) 308*, 820–826]. Unlike splicing, however, certain additional nucleotides 5′ to the guanosine moiety make significant binding contributions. We conclude that the guanosine binding site in cyclization is similar to, but not identical with, the guanosine binding site in splicing. The same binding interactions used in cyclization could help align the 3′ splice site of the rRNA precursor for exon ligation. We also report that the phosphodiester bond at the cyclization site is susceptible to a pH-dependent hydrolysis reaction; the phosphodiester bond is somehow activated toward attack by the 3′-hydroxyl of a guanosine molecule or by a hydroxyl ion.

The 26S rRNA precursor of the ciliated protozoan *Tetrahymena thermophila* contains an intervening sequence (IVS)[1] that has intramolecular catalytic activity. It is capable of excising itself from the precursor RNA, in vitro, in the absence of any protein or external energy source (Kruger et al., 1982). The reaction is initiated by the nucleophilic attack of the 3′-hydroxyl of a guanosine cofactor at the phosphodiester bond at the 5′ splice site. The 5′ exon is released, and the guanosine cofactor is covalently attached to the 5′ end of the IVS (Figure 1, step a). The free 3′-hydroxyl of the 5′ exon can then make a nucleophilic attack at the phosphodiester bond at the 3′ splice site to release the IVS and ligate together the exons (Figure 1, step a′). It is now known that this reaction is not unique to the *Tetrahymena* pre-rRNA; group I introns from other organisms are capable of a similar, self-catalyzed splicing reaction (Garriga & Lambowitz, 1984; Van der Horst & Tabak, 1985; Chu et al., 1986).

The released IVS RNA retains catalytic activity. The 3′-hydroxyl of the guanosine nucleotide at the 3′ end of the IVS can make a nucleophilic attack at the phosphodiester bond between nucleotides 15 and 16, release a 15-nucleotide fragment (15-mer) containing the nonencoded guanosine, and form a covalently closed circle (Figure 1, step b). A minor product is formed by nucleophilic attack between nucleotides 19 and 20 to form a slightly smaller circle and release a 19-nucleotide fragment (19-mer). These cyclization reactions share many features with splicing. All are transesterification reactions where the total number of phosphodiester bonds is conserved

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[1] Abbreviations: RNA, ribonucleic acid; IVS, intervening sequence; pre-rRNA, ribosomal RNA precursor; L - 14 IVS, linear IVS RNA with the first 15 nucleotides replaced by guanosine or by a guanosine analogue; L - 15 IVS, linear IVS RNA produced from site-specific hydrolysis at the cyclization site of either the circular IVS or the linear IVS; EDTA, ethylenediaminetetraacetic acid; G, guanosine; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; 15-mer, an RNA fragment released during the cyclization reaction that contains the first 15 nucleotides of the linear IVS; 19-mer, a 19-nucleotide RNA fragment released during cyclization at a minor site; L IVS_β, linear IVS RNA with its 3′-terminal guanosine removed by periodate oxidation and β-elimination; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

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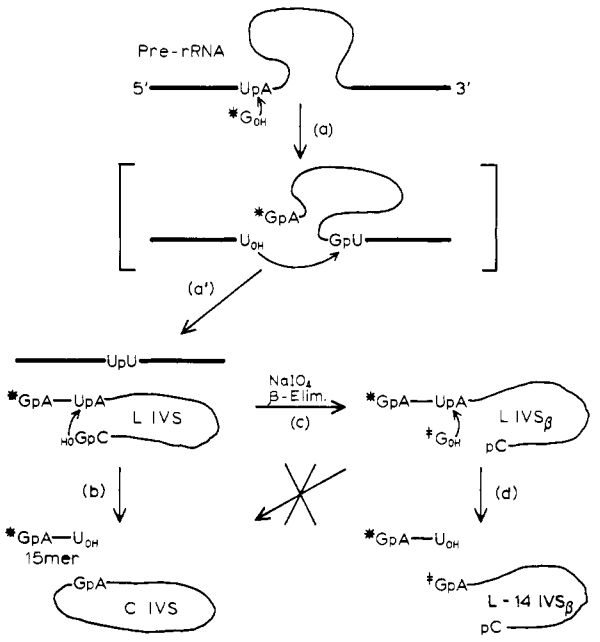

FIGURE 1: RNA self-splicing, cyclization, and guanosine addition to L IVS$_\beta$ RNA. (a and a') Splicing pathway (Kruger et al., 1982). (b) Cyclization reaction (Zaug et al., 1983). (c) Reactions involved in removing the 3'-terminal nucleotide of the IVS. (d) Reaction where a free guanosine attacks the phosphodiester bond at the site of cyclization. Asterisks denote the nonencoded guanosine that becomes covalently attached during the splicing reaction. Daggers indicate the nonencoded guanosine that becomes covalently attached during the guanosine addition reaction. The bold lines represent the exons and the light lines the IVS. The figure is not drawn to scale.

through the reactions. In each of these reactions, the 3'-hydroxyl of a guanosine molecule makes a nucleophilic attack at a specific phosphodiester bond that follows a stretch of pyrimidines (Sullivan & Cech, 1985; Inoue et al., 1986). Finally, in each case, the guanosine molecule must be precisely positioned in the catalytic site so that the reaction can proceed efficiently. The difference between the reactions is that splicing is an intermolecular reaction where a free guanosine molecule attacks the IVS, while cyclization is an intramolecular reaction where the 3'-terminal guanosine of the IVS attacks itself.

The guanosine binding site in splicing has been extensively analyzed by Michaelis–Menten kinetics (Bass & Cech, 1984, 1986). The results showed that functional groups on the guanosine molecule that are used in a G-C Watson–Crick base pair are important for the splicing reaction. This was consistent with, but not conclusive for, hydrogen bonds from the RNA to O6 and N1 of guanosine. Two additional hydrogen bonds seemed to be made with the C2 amino group. No reaction was seen when all three functional groups were absent. In addition, the 3'- and 2'-hydroxyls were absolutely required. Modifications of N7 on the base and of the 5'-hydroxyl on the sugar had no effect on binding or splicing.

We wished to determine if the same guanosine binding site was involved in cyclization, but this intramolecular reaction does not lend itself to Michaelis–Menten kinetic analysis. We have now circumvented this problem by converting the intramolecular cyclization reaction into an intermolecular reaction. This is accomplished by oxidation and β-elimination of the 3'-terminal guanosine nucleotide (Figure 1, step c). The 3' terminus that is left is no longer capable of reacting. However, the 3'-hydroxyl of a free guanosine molecule can substitute for the missing terminal guanosine of L IVS$_\beta$. It can make a nucleophilic attack at the phosphodiester bond between nucleotides 15 and 16, release a 15-nucleotide fragment, and become covalently attached to a linear molecule, which is now 14 nucleotides shorter (L - 14 IVS$_\beta$; Figure 1, step d).

This guanosine addition reaction was used as a model system for a Michaelis–Menten study of the cyclization reaction. The results indicate that functional groups on guanosine that are used in a Watson–Crick G-C base pair are also important for the cyclization reaction, as was observed for splicing. However, unlike splicing, the addition of two or more specific nucleotides to the 5'-position of guanosine significantly improves the reaction efficiency. This finding results in a model for cyclization that involves a guanosine binding site that is similar to, but not identical with, the guanosine binding site for splicing. In addition, on the basis of these results, we propose a model for how the 3' splice site of the precursor is correctly aligned during the splicing reaction.

Experimental Procedures

Preparation of L IVS and Precursor RNA. IVS RNA labeled at its 5' end with ^{32}P was prepared essentially as described by Tanner and Cech (1985). Transcriptions were carried out either with SP6 polymerase and plasmid pJK43-SP6 (Price et al., 1985) or with T7 polymerase and plasmid pT7TT1A3 (Zaug et al., 1986). Both plasmids were first linearized with *Eco*RI; 2 mM spermidine was used in the transcription reactions, instead of the 4 mM spermidine used previously, to minimize the possibility of the RNA precipitating during transcription. However, no difference in yield was noted between transcriptions using the two different spermidine concentrations. The RNA transcript was spliced in the presence of [α-^{32}P]GTP as described previously (Tanner & Cech, 1985).

Precursor RNA that was body labeled with ^{32}P was transcribed exclusively from *Eco*RI-cut plasmid pT7TT1A3. This yielded a 537-nucleotide trancript containing 42 nucleotides of the 5' exon, the entire 413 nucleotides of the IVS, and 82 nucleotides of the 3' exon. The transcription conditions were the same as described above except 100 μCi of [α-^{32}P]CTP or [α-^{32}P]GTP (800 Ci/mmol, NEN) was added, and the [^3H]UTP was omitted.

Preparation of L IVS$_\beta$ RNA. The 3'-terminal nucleotide of the IVS was oxidized by incubating the RNA ($\sim$50 μg) in 40 μL of 20 mM sodium periodate for 30 min at 25 °C in the dark. Excess periodate was removed by making the solution 20–25 mM in rhamnose and incubating for an additional 30 min at 25 °C. Finally, the RNA was β-eliminated by incubating the oxidized IVS for 30 min at 45 °C in the presence of 250 mM aniline and 50 mM sodium acetate, pH 5.0. This procedure is similar to that described by Winter and Brownlee (1978). The reaction gave complete or nearly complete removal of the 3'-terminal nucleotide as indicated by the inability of body-labeled IVS to form a circle. Little degradation occurred. The periodate oxidation reaction requires a *cis*-diol, so only the 3'-terminal nucleotide is removed from the IVS RNA.

The resulting L IVS$_\beta$ RNA was ethanol precipitated and purified by polyacrylamide gel electrophoresis (Tanner & Cech, 1985). Material isolated from the gel was further purified by chromatography on a Sephadex G50-150 column (in 10 mM Tris, pH 7.5, 250 mM sodium acetate, and 1 mM EDTA). The RNA was then ethanol precipitated, dissolved in water, and stored at −20 °C.

The precursor RNA was also β-eliminated, so all the RNA received the same treatment. No difference was noted in the extent of splicing between β-eliminated and non-β-eliminated precursor RNA. The precursor was purified and stored as described above.

Substrate Preparation. G, 5′GMP, 5′UMP, 5′AMP, 5′CMP, 3′-*O*-methyl-G, ApG, CpG, UpG, and purine ribonucleoside were obtained from PL Pharmacia; GpG, inosine, dG, 7-methyl-G, and xanthosine were obtained from Sigma; and 2-aminopurine β-D-ribofuranoside (2-aminopurine ribonucleoside) was obtained from VEGA Biochemicals. ApGpUpApCpUpCpG (octanucleotide) was kindly synthesized by R. Kierzek and M. Caruthers by phosphoramidite procedures. All compounds were dissolved in water and stored at −20 °C. Concentrations were determined by using an HP 8451A diode array spectrophotometer.

The nucleotides, nucleosides, and dinucleotides that were active in the substrate addition reaction were checked for purity on a Beckman HPLC with an Altex Ultrasphere–ODS, 5-μm reverse-phase column (4.6 mm × 250 mm). Nucleosides and 5′GMP were separated with a 5-min flow of 10 mM ammonium acetate, pH 5.0, and then a linear gradient of 0–5% acetonitrile in this buffer over a period of 50 min. The flow rate was 1.2 mL/min, and elution was monitored with a Beckman UV_{254} detector. All the nucleosides and GMP were well resolved except 7-methyl-G and dG, which comigrated. Dinucleotides were well resolved with a 5-min flow of 10 mM ammonium acetate, pH 5.0, and a linear gradient of 0–7% acetonitrile in this buffer over 50 min. All the compounds were rerun with 1% G and GMP added to serve as markers and as a reference to the amount of impurity present. The compounds had negligible contamination, as judged by this assay, and were used without further purification. 5′-Nucleotides were often very poorly resolved, even with a variety of elution conditions, and were generally not used in the reactions.

The identity of the reactive compounds was verified by performing an end analysis of L - 14 IVS_β RNA produced by incubation with each nucleoside analogue. L IVS_β RNA that was body labeled with ^3H was incubated with the various compounds. The RNA was then 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and the resulting end-labeled L - 14 IVS_β RNA was purified by polyacrylamide gel electrophoresis. This material was completely digested with nuclease P1, to yield nucleoside 5′-monophosphates, and spotted on a polyethylenimine thin-layer chromatography (TLC) plate (Macherey–Nagel) with authentic nucleoside 5′-monophosphates as markers. The TLC plate was eluted with 1 M LiCl and then subjected to autoradiography. The resulting spots were matched with the markers to identify the products. In all cases, except for dG (see Results), most of the ^{32}P label was on the same compound as had been added to the substrate addition reaction.

In some reactions, 20% or more of the product was [^{32}P]-AMP. This was also the major product of L IVS_β RNA that was incubated in the absence of any substrate. Evidently, the 5′-terminal A, produced from site-specific hydrolysis at the cyclization site, becomes labeled by the polynucleotide kinase phosphate exchange reaction, even though conditions were chosen to minimize this reaction. The other three nucleotides (GMP, UMP, and CMP) were also present in all the reactions, apparently because degradation products were not sufficiently resolved from the L - 14 IVS_β RNA. However, these nucleotides together constituted less than 10% of the total products. Thus, greater than 90% of the 5′-terminal nucleotide produced by the substrate addition reaction, excluding the site-specific hydrolysis product, contained the expected nucleotide analogue.

G Addition Reaction Conditions. The standard reaction solution contained 20 mM $MgCl_2$, 10 mM HEPPS, pH 7.5, and 1–2 nM L IVS_β RNA labeled at its 5′ end with ^{32}P. [The L IVS_β RNA concentration was based on the specific activity of the ^{32}P incorporated and therefore concerns the ^{32}P-labeled molecules only. Since some splicing occurs during transcription and before end labeling, the total L IVS_β RNA concentration (^{32}P labeled and unlabeled) was higher. The L IVS_β RNA concentration based on the specific activity of the ^3H incorporated during transcription was routinely 10-fold higher. However, it contained unreactive RNA and consequently overestimated the L IVS_β RNA concentration. The total L IVS_β RNA concentration was estimated to be between 1.5 and 15 nM.] Reaction mixtures (10 μL) were prepared on ice in siliconized Eppendorf tubes (Tanner & Cech, 1985) and then incubated in a 42 °C water bath. Reactions were stopped by plunging the tubes in a dry ice/ethanol bath and fixed by adding 10 μL of urea loading buffer (Tanner & Cech, 1985) containing 40 mM EDTA. The products of the reaction were separated by electrophoresis on a 0.5 mm thick, 20% polyacrylamide/7 M urea gel.

The gel was subjected to autoradiography, the ^{32}P-bands were cut out, and each gel slice was extracted overnight with 400 μL of Protosol (NEN), containing 10% water, at 37 °C. Each sample was neutralized with 50 μL of glacial acetic acid, 4 mL of toluene-based fluor was added (Tanner & Cech, 1985), and the sample was counted in a Beckman LS7000 scintillation counter. The IVS and 15-mer bands were always counted. The 19-mer, 7-mer, and GTP bands were counted only for the longest time point of each substrate concentration; combined, they constituted less than 10% of the total reaction.

Splicing Reaction Conditions. The splicing reaction conditions were similar to the guanosine addition reaction conditions. Splicing was carried out in a solution containing 10 mM H-EPPS, pH 7.5, and ~50 nM ^{32}P-body-labeled precursor. The salt concentration either was 20 mM $MgCl_2$ or was 5 mM $MgCl_2$/100 mM $(NH_4)_2SO_4$. The reaction products were separated by electrophoresis on a 1.5 mm thick, 4% polyacrylamide/7 M urea gel [with 19:1 acrylamide:bis-(acrylamide)]. The gel was soaked in water to remove the urea, dried, and subjected to autoradiography. Bands corresponding to the precursor, IVS–3′ exon, 5′ exon–IVS, IVS, L - 15, circle, and large circle (when present) were cut out and counted directly with 4 mL of toluene-based fluor.

RESULTS

G Addition to β-Eliminated IVS Mimics Cyclization. Oxidation and β-elimination of the 3′-terminal nucleotide destroy the ability of the IVS RNA to react with itself: it is no longer capable of forming a circle (Figure 2). However, in the presence of a guanosine cofactor, a linear molecule that is 14 nucleotides shorter than the starting material is formed. This is consistent with the free guanosine attacking at the normal site of cyclization. Over 90% of the L IVS_β RNA can be converted to the L - 14 IVS_β RNA under these conditions.

The reaction is not specific for L IVS_β RNA. Intact L IVS RNA, when incubated with sufficiently high guanosine concentrations, will also form L - 14 molecules. There is 65% more reaction of intact L IVS at pH 7.5 in the presence of 1 mM guanosine than in the absence (Figure 2, lanes 2 and 3). Almost all of this increase is in the form of the band migrating at L - 14/L - 15. On the basis of such experiments, we estimate that the effective concentration of the 3′-terminal guanosine of the intact L IVS, with respect to the proposed binding site, is equivalent to approximately 1 mM free guanosine.

Guanosine addition has a high (20 mM) $MgCl_2$ concentration requirement. Very little reaction occurs with 5 mM $MgCl_2$/200 mM NaCl at 42 or 55 °C, even after a 2-h in-

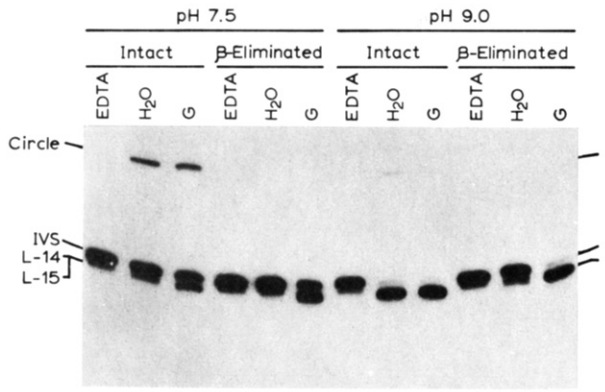

FIGURE 2: Reactions of IVS RNA. IVS RNA, body labeled with ^3H, either was left unmodified (intact) or the 3′-terminal nucleotide was removed (β-eliminated). The RNA was incubated at 42 °C for 10 min in 10 mM HEPPS, pH 7.5, 20 mM $MgCl_2$ with 1 μL of 400 mM EDTA, H_2O, or 10 mM guanosine added as indicated to bring the reaction solution to 10 μL final volume. The RNA was electrophoresed on a 1.5 mm thick, 4% polyacrylamide/8 M urea gel. The gel was then soaked in 1 M sodium salicylate and subjected to autoradiography at −70 °C with Kodak XAR 5 film. No circle was seen with the L IVS_β RNA even with a much longer exposure. The circular form of the IVS RNA undergoes a site-specific hydrolysis reaction at the site of cyclization (Zaug et al., 1985). This reaction is greatly accelerated at higher pHs, so in 10 min at pH 9.0, most of the IVS has cyclized and then autoreopened. A small amount of circle, however, was obvious on the original autoradiogram. Slower migrating species were also apparent in the lanes with the intact IVS in the original autoradiogram. These probably arose from an oligomerization reaction described previously (Zaug & Cech, 1985). L - 14 and the L - 15 are not resolved on this gel. Gel slices corresponding to the circle, IVS, and L - 14/L - 15 were isolated and quantified. Only background counts were present in the gel slices corresponding to the circle in the reaction of L IVS_β RNA.

cubation in a sealed glass pipet (data not shown). This is in contrast to the cyclization reaction where 80–90% reaction occurs after 20 min at 42 °C with 5 mM $MgCl_2$/200 mM NaCl (Tanner & Cech, 1985). Monovalent salts have little or no effect on the guanosine addition reaction. We have not demonstrated why there is such a high $MgCl_2$ concentration requirement; perhaps, it is needed to stabilize the guanosine molecule in the binding site.

The guanosine cofactor becomes covalently attached to the L - 14 product. This is shown in Figure 3 where unlabeled L IVS and L IVS_β RNAs become ^{32}P labeled when incubated with [α-^{32}P]GTP. A 5′ end-labeled octaribonucleotide ([^{32}P]pApGpUpApCpUpCpG), which has a sequence corresponding to the last eight nucleotides of the IVS, produced more labeling than GTP. It formed a product that was seven nucleotides shorter than the starting material. More efficient reaction was also obtained at 55 °C, but 42 °C was routinely used for more consistent results.

RNA similar to that shown in Figure 3, obtained following reaction of both intact and β-eliminated IVS, was gel purified and sequenced by the enzymatic method (Donis-Keller et al., 1977). That is, it was subjected to partial digestion under denaturing conditions with either G-specific RNase T1, A-specific RNase U2, U- and A-specific RNase PhyM, or nonspecific alkaline hydrolysis. The cleavage products were separated by sequencing gel electrophoresis and localized by autoradiography (data not shown). The most prominent sequence was that expected if the substrate (GTP or the entire octanucleotide) had become covalently attached to A^{16} of the IVS through a 3′–5′-phosphodiester bond. In addition, some minor sites of addition were apparent. One corresponded to addition at U^{20}; this is known as a minor site of cyclization as well (Zaug et al., 1985; Tanner & Cech, 1985). Another apparent minor addition site mapped to A^2. This guanosine exchange reaction has also been noted previously (Zaug & Cech, 1985).

During the cyclization reaction, an oligonucleotide (15-mer) is released; it contains the nonencoded guanosine, which was covalently attached during splicing. The 15-mer is also the major product formed when 5′ end-labeled L IVS_β RNA is incubated with guanosine (Figure 4). This provides further evidence that intermolecular guanosine addition involves the same reaction mechanism used in cyclization. One of the minor products, the 19-mer, is formed in approximately the same amounts as in cyclization (1–2% of the total reaction; Tanner & Cech, 1985). The other minor products are less well characterized. The production of [^{32}P]GTP is consistent with a guanosine exchange reaction, but the amount produced

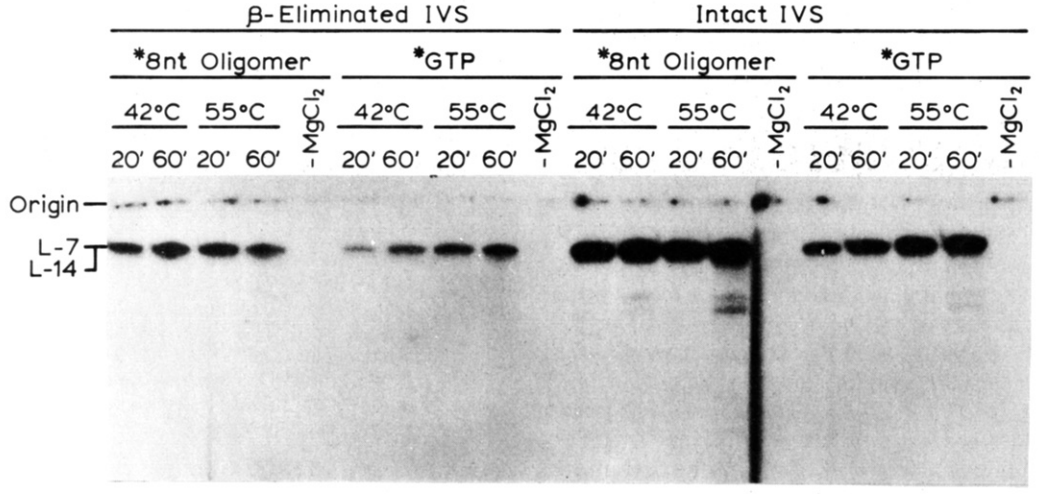

FIGURE 3: Covalent addition of GTP and of an octanucleotide to IVS RNA. L IVS_β RNA (β-eliminated IVS) or L IVS RNA (intact IVS) was incubated for the indicated times, in minutes, and at the indicated temperatures with ^{32}P-labeled octanucleotide or GTP. The same specific activity and concentration of the octanucleotide and GTP were used for each reaction. The $MgCl_2$ concentration was 20 mM, and the pH was 7.5. In the absence of $MgCl_2$, no ^{32}P was incorporated into the IVS. L - 14 forms when guanosine replaces the 5′-terminal 15 nucleotides. L - 7 forms when the octanucleotide is exchanged for the terminal 15 nucleotides. These two products are not resolved on this gel. The material was separated by electrophoresis on a 0.5 mm thick, 20% polyacrylamide/7 M urea gel and subjected to autoradiography with a DuPont Cronex intensifying screen and X-ray film at −70 °C. The lower intensity in the reaction product of β-eliminated RNA was due to less RNA being loaded onto the gel rather than to a difference in the level of incorporation between the β-eliminated and the intact IVS. However, approximately the same amount of RNA was loaded on the gel within the β-eliminated RNA lanes and within the intact RNA lanes.

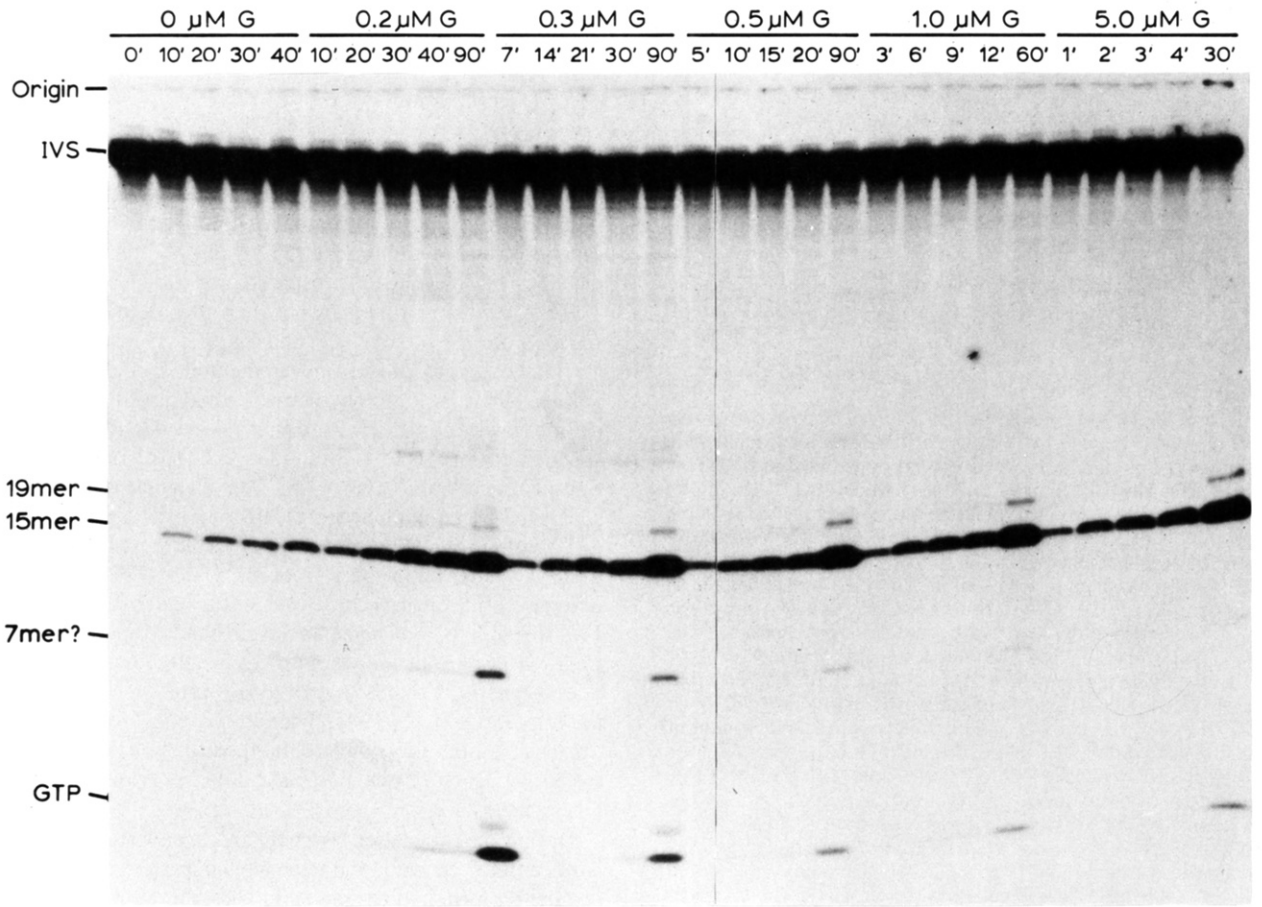

FIGURE 4: Kinetics of addition of guanosine to L IVS$_\beta$ RNA assayed by release of the first 15 nucleotides of IVS. L IVS$_\beta$ RNA, labeled at its 5′ end with ^{32}P, was incubated for various times (in minutes) and with various guanosine concentrations at 42 °C in 20 mM $MgCl_2$, pH 7.5. The RNA was separated on a 0.5 mm thick, 20% polyacrylamide/7 M urea gel and subjected to autoradiography. The extent of reaction was intentionally kept low for determination of initial velocities.

in relation to the 15-mer can be somewhat variable. Generally, it constitutes 5–8% of the total reaction. The other minor product, the 7-mer, runs as an eight-nucleotide fragment in relation to an alkaline hydrolysis ladder. However, if it has a 3′-hydroxyl, like the other products, then it is migrating one nucleotide slower than the corresponding hydrolysis fragments, which have 3′-phosphates. This would be consistent with addition after nucleotide G[7]. Addition after G[7] was not obvious from the RNA sequence analysis because the sequence was partially superimposed on the sequences of the other addition products. The amount of 7-mer produced was generally 1–2% of the total reaction.

The guanosine addition reaction also appears to be reversible. Unlabeled GTP or octanucleotide will displace [^{32}P]GTP from the 5′ end of gel-purified L - 14. The product was analyzed by polyethylenimine TLC and shown to be pppG, indicating that it was not produced simply by alkaline hydrolysis (data not shown). The reverse reaction, however, was very inefficient under the conditions used for these reactions.

Activation of Specific Phosphodiester Bonds. The phosphodiester bond at the cyclization site is susceptible to a slow hydrolysis reaction (Figure 4, 0 μM G). On the basis of its comigration with the 15-mer released by cyclization or guanosine addition, the 15-mer that is released by hydrolysis contains a 3′-hydroxyl. This is in contrast to random alkaline hydrolysis which leaves a 3′-phosphate. The half-life for this site-specific hydrolysis is 980 ± 20 min (mean ± standard deviation) at pH 7.5. Increasing the pH from 7.5 to 8.5–9.0 increased the site-specific hydrolysis rate 11-fold (Figure 5). There is also a 3–5-fold increase in hydrolysis when the temperature is raised to 55 °C at either pH 7.5 or pH 9.0 (data not shown). Over 70% of the L IVS$_\beta$ RNA underwent site-specific hydrolysis when it was incubated for 40 min at pH 9.0, 55 °C. Thus, most of the IVS RNA is susceptible to this reaction.

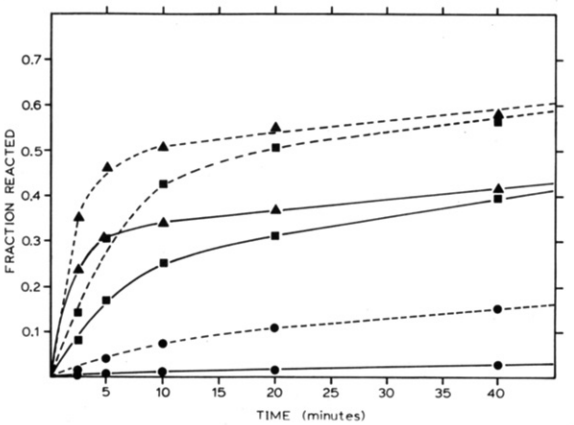

FIGURE 5: Fraction of total IVS RNA reacted (15-mer/total RNA) as a function of time. Conditions were 20 mM $MgCl_2$, 42 °C, at pH 7.5 (solid lines) and pH 8.5–9.0 (dashed lines). (●) Site-specific hydrolysis reaction of L IVS$_\beta$ RNA; (■) L IVS$_\beta$ RNA incubated with 5 μM GTP; (▲) intact IVS RNA in the absence of GTP. Note that these reactions show an initial fast reaction velocity and then a secondary, slower reaction velocity.

Both intact L IVS and L IVS$_\beta$ RNA incubated with guanosine show an increased velocity or level of reaction at pH 9 relative to pH 7.5 (Figure 5). Some of this is simply due to the increased rate of hydrolysis. The rest of it may be due

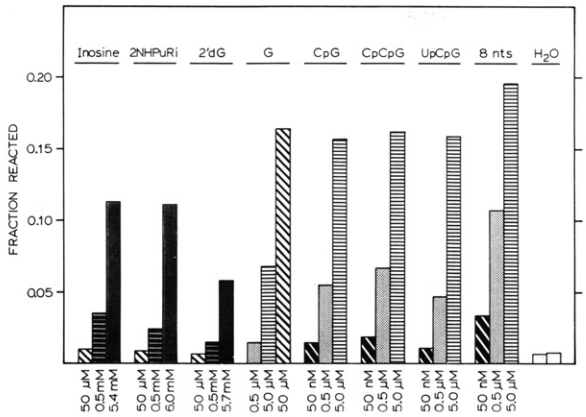

FIGURE 6: Comparison of guanosine analogues in the substrate addition reaction. Values are plotted as the reaction extent of 5′ end-labeled, L IVS_β RNA (15-mer/total RNA). Reaction conditions were 42 °C, 20 mM $MgCl_2$, pH 7.5, and 10-min incubation. The extent of reaction was intentionally kept low so differences between the compounds were maximized. The concentrations were carefully chosen so the extent of reaction would be similar in each case. Bars with the same stippling or shading have similar substrate concentrations. Abbreviations: 2NHPuRi, 2-aminopurine ribonucleoside; 8 nts, octanucleotide AGUACUCG. The small amount of reaction with dG was entirely due to less than 0.1% contaminating G (see text).

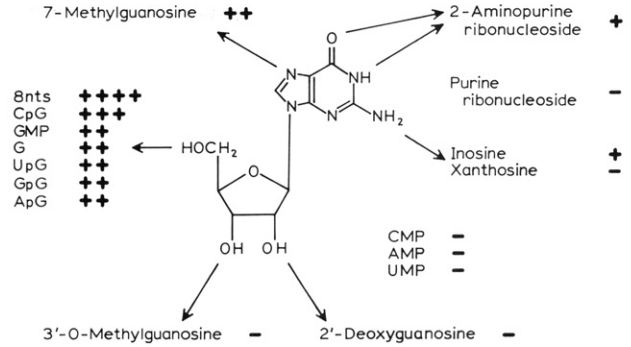

FIGURE 7: Summary of the activity of the guanosine analogues used in the substrate addition reaction. Compounds with a minus sign (−) were inactive in the reaction. Compounds with two plus signs (++) were effective at the same concentrations as guanosine in the reaction, compounds with one plus sign were less effective, and compounds with more than two plus signs were more effective than guanosine.

to an increase in the number of molecules in an activated conformation (Zaug et al., 1985).

Other Nucleosides Can Substitute for Guanosine. A number of different substrates were tested for addition to L IVS_β RNA. A representative sample is shown in Figure 6. Substrate concentrations were carefully chosen to give 20% or less reaction. This is because at high $MgCl_2$ concentrations we obtained biphasic reaction velocities with an initial fast rate followed by a secondary slower reaction rate (see Figure 5). High $MgCl_2$ concentrations may inhibit conformational changes that are necessary for the IVS to become reactive (Tanner & Cech, 1985). Typically, 25% or more of the molecules show the fast reaction velocity.

The small amount of reaction obtained with dG, shown in Figure 6, was entirely due to less than 0.1% contaminating G. Nuclease P1 analysis of L - 14 IVS_β RNA, that was generated by reaction with dG and then 5′ end labeled with polynucleotide kinase, gave GMP as the only labeled product. In addition, when the end-labeled L - 14 was completely digested with G-specific RNase T1, all the label was released as a mononucleotide. This indicated that the dG had not reacted.

UMP, AMP, and CMP were inactive in the substrate addition reaction (data not shown). Purine nucleosides with functional groups on the pyrimidine ring different from those of guanosine were much less reactive. In contrast, substrates with additional nucleotides 5′ to the guanosine, having the same sequence as that found on the 3′ end of the IVS, were much more reactive. These results are summarized in Figure 7. Xanthosine was not very soluble and was not tested at the millimolar concentrations used for inosine and 2-aminopurine ribonucleoside. However, it gave no indication of reactivity. 7-Methylguanosine, the only compound tested with a modified imidazole ring, worked as well as guanosine in the reaction. Compounds with modifications at the 2′- or 3′-position of the sugar were totally inactive even at millimolar substrate concentrations.

Michaelis–Menten Kinetics. To further compare the analogues, they were analyzed with Michaelis–Menten kinetics. L IVS_β RNA labeled at its 5′ end with ^{32}P was incubated with different substrate concentrations for various times (see Figure 4). The end-labeled 15-mer that was released provided a direct measure of the reaction extent. Reaction times were selected to give approximately the same extent of reaction for each substrate concentration. Even at the lowest concentration used, the substrate was in great excess over the L IVS_β RNA, thereby maintaining pseudo-first-order kinetics. The bands corresponding to the L IVS_β and 15-mer were isolated and quantified as described under Experimental Procedures. The 19-mer, 7-mer, and GTP were isolated and quantified only for the longest time points. These minor products combined constituted less than 10% of the total reaction products and were generally ignored in the subsequent calculations. The pattern shown in Figure 4 is typical of that obtained with all the analogues.

The velocity of the guanosine addition reaction, V, at each substrate concentration is given by

$$V = -\mathrm{d}[\mathrm{IVS}]/\mathrm{d}t = k[\mathrm{IVS}]_t \quad (1)$$

where k is a pseudo-first-order rate constant and $[\mathrm{IVS}]_t$ is the L IVS_β RNA concentration at some time t. The integrated form is

$$\ln ([\mathrm{IVS}]_t/[\mathrm{IVS}]_0) = -kt \quad (2)$$

where $[\mathrm{IVS}]_0$ is the initial L IVS_β RNA concentration. A plot of ln (1 − fraction reacted), which is equal to ln ($[\mathrm{IVS}]_t/[\mathrm{IVS}]_0$), against time has a slope equal to $-k$. An example is shown plotted, in log base ten, in Figure 8A. The initial reaction velocity, V_0, for each substrate concentration is equal to $k[\mathrm{IVS}]_0$. V_0 was corrected for the hydrolysis reaction and analyzed by using a Lineweaver–Burk plot (Figure 8B) to obtain values for the Michaelis–Menten constant, K_m, and the maximum reaction velocity, V_{max}. Since [IVS] is changing during the reaction, V_{max} occurs only at initial time. This differs from the situation with an enzyme, where the total enzyme concentration does not change during the reaction (Bass & Cech, 1984).

Since the experimental procedure and method of analysis were somewhat different from those used previously (Bass & Cech, 1984), kinetic measurements were also made for guanosine and CpG in the splicing reaction. Reactions were carried out at 42 °C, pH 7.5, either with 20 mM $MgCl_2$ (same conditions used for guanosine addition) or with 5 mM $MgCl_2$/100 mM $(NH_4)_2SO_4$ [conditions of Bass and Cech (1984)]. Like the reaction where guanosine is added to L IVS_β RNA, the splicing reaction had multiphasic velocities: an initial fast velocity constituting approximately 25% of the starting material and a slower secondary velocity. Up to 70%

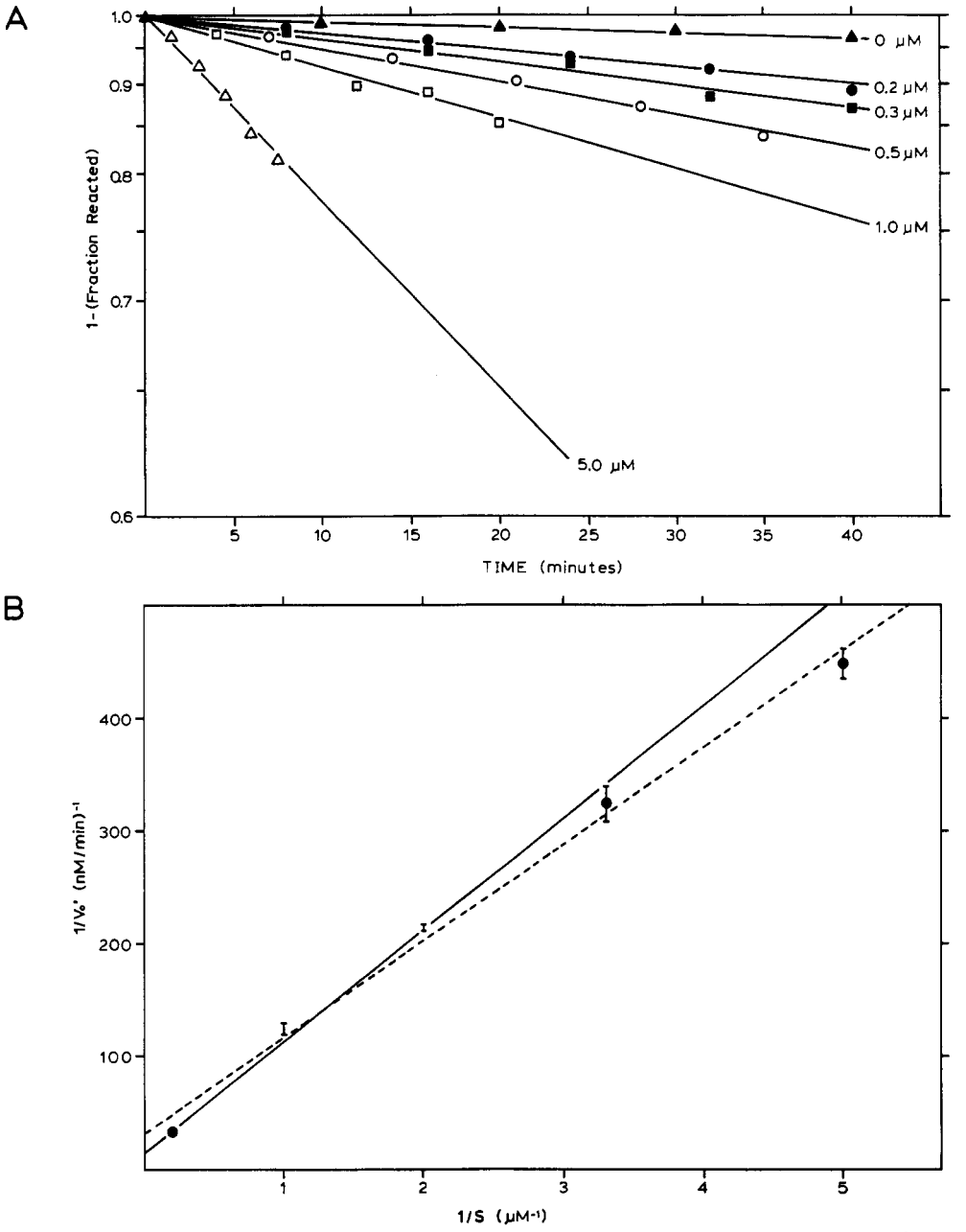

FIGURE 8: (A) Quantification of kinetic data similar to those shown in Figure 4. Data were plotted as the log (1 − fraction reacted) or log (1 − 15-mer/total RNA) against time. Rate constants and initial velocities of the reaction were determined for each substrate concentration from a least-squares linear regression fit to the equation ln (1 − fraction reacted) = $-kt + b$ where t is time, k is the rate constant, and b is generally equal to 0. Time points representing greater than 20% reaction or longer than 40-min reaction time were not used. The velocity of the hydrolysis reaction (0 μM) was normally fairly constant, but it was always determined for each experiment. (B) Lineweaver–Burk plot for the velocities determined in (A). The velocity of the hydrolysis reaction was subtracted from the reaction velocity at each substrate concentration to give the initial reaction velocity, V_0', actually due to substrate addition. Data were fit to the equation $1/V_0' = (K_m/V_{max})(1/S) + 1/V_{max}$ where K_m is the Michaelis–Menten constant, S is the substrate concentration, and V_{max} is the maximum velocity, using a weighted linear regression (solid line; Wilson, 1952; Neter et al., 1983). The weighted linear regression gave more consistent and reproducible results than an unweighted regression (dashed line). The error bars represent the standard deviations associated with each datum point; these were determined from the linear least-squares fit shown in (A). The datum point was deleted from the error bar in some cases for clarity. Data were also analyzed by an unweighted least-squares fit to the Eadie–Hofstee equation $V_0' = -K_m(V_0'/S) + V_{max}$. As expected, the fit, as determined from the correlation coefficient (Neter et al., 1983), was not as good. However, the values obtained were generally well within the calculated uncertainty of those obtained from the Lineweaver–Burk plot.

of the precursor had reacted after 100 min, with the products still increasing at that time. No correction was made for unreactive material.

Because of the multitude of products formed, the splicing reaction with 20 mM $MgCl_2$ was much more complicated than the guanosine addition reaction. The L IVS and circular IVS are produced as outlined in Figure 1. The other products are described elsewhere (Inoue et al., 1986). The large circle (when present), circular IVS, precursor, IVS–3′ exon, 5′ exon–IVS, L IVS, and L - 15 IVS bands were all isolated from the polyacrylamide gel and quantified as described above. The exons were too small to be retained on the gel. Only the L IVS, circular IVS, L - 15 IVS, and IVS–3′ exon bands were attributed to splicing. The other products were included with the precursor. The rate constants and reaction velocities for each substrate concentration were determined as described above. The mean K_m value for guanosine in the splicing reaction with 20 mM $MgCl_2$ is 11 ± 2 μM. The K_m value with 5 mM $MgCl_2$/100 mM $(NH_4)_2SO_4$ is 19 ± 6 μM, which is well within the experimental uncertainty of the value deter-

Table I: Values of K_m, k_{cat}, and k_{cat}/K_m[a]

substrate	K_m (μM)	k_{cat} (min^{-1} × 10^2)	k_{cat}/K_m (M^{-1} min^{-1} × 10^{-3})
Substrate Addition to L IVS_β RNA with 20 mM $MgCl_2$, 42 °C, pH 7.5			
GMP	3.0 ± 0.3	3.6 ± 0.4	12 ± 1
	5.6 ± 0.6	8.1 ± 0.8	15 ± 2
G	4.4 ± 0.8	2.8 ± 0.5	6.4 ± 1.2
	7.5 ± 0.8	6.9 ± 0.7	9.1 ± 0.9
7-methyl-G	5.1 ± 0.5	5.6 ± 0.6	11 ± 1
	16 ± 3	13 ± 3	8.3 ± 1.7
ApG	5.6 ± 0.6	2.9 ± 0.3	5.2 ± 0.5
GpG	7.2 ± 0.7	6.3 ± 0.6	8.8 ± 0.9
UpG	15 ± 5	5.4 ± 0.5	3.7 ± 1.2
	28 ± 5	11 ± 2	3.7 ± 0.7
CpG	1.4 ± 0.1	6.0 ± 0.6	43 ± 4
	2.5 ± 0.3	12 ± 1	48 ± 5
octanuc	0.74 ± 0.07	9.0 ± 0.9	120 ± 10
inosine	1300 ± 130	3.7 ± 0.4	0.029 ± 0.006
	1700 ± 170	6.4 ± 0.6	0.038 ± 0.004
2NHPuRi	4700 ± 600	5.3 ± 0.7	0.011 ± 0.002
	6100 ± 610	8.7 ± 0.9	0.014 ± 0.001
Splicing of Pre-rRNA with 20 mM $MgCl_2$, 42 °C, pH 7.5			
G	9.4 ± 0.9	6.5 ± 0.7	6.9 ± 0.7
	12 ± 1	7.2 ± 0.7	6.2 ± 0.6
CpG	14 ± 2	7.0 ± 1.0	5.0 ± 0.8
	12 ± 3	4.7 ± 0.8	3.8 ± 0.8
Splicing with 5 mM $MgCl_2$/100 mM $(NH_4)_2SO_4$, 42 °C, pH 7.5			
G	23 ± 8	16 ± 5	7.0 ± 2.1
	14 ± 4	11 ± 2	7.5 ± 2.0
CpG	30 ± 4	8.8 ± 1.1	3.0 ± 0.4

[a] A minimum of 10% uncertainty was assigned to each value. The larger uncertainties represent the standard deviations determined from least-squares linear regression. The values shown for the substrate addition reaction were determined from a weighted linear regression fit to the Lineweaver–Burk equation $1/V_0' = S/V_{max} + K_m/V_{max}$. The Lineweaver–Burk equation also gave values for splicing similar to those shown in the table. The standard deviations shown for splicing were determined from the Eadie–Hofstee equation. Abbreviations: 2NHPuRi, 2-aminopurine ribonucleoside; octanuc, the octanucleotide AGUACUCG. Duplicate values are independent experiments with different RNA preparations.

Table II: Association Constants, K_a (Molar), for Substrate Binding and the Free Energy of Binding, $\Delta G°$ (kcal/mol), for the Values from Table I[a]

substrate	K_a ($=1/K_m$ × 10^{-5})	$\Delta G°$ ($=-RT$ ln K_a)	$\Delta\Delta G°$[b]
Substrate Addition Reaction			
GMP	3.3 ± 0.3	−8.0 ± 0.8	
	1.8 ± 0.2	−7.6 ± 0.8	0.0 ± 0.3
G	2.3 ± 0.2	−7.7 ± 0.7	
	1.3 ± 0.1	−7.4 ± 0.7	
7-methyl-G	2.0 ± 0.2	−7.6 ± 0.8	
	0.64 ± 0.13	−6.9 ± 1.4	0.5 ± 0.4
ApG	1.8 ± 0.2	−7.6 ± 0.8	0.1
GpG	1.4 ± 0.1	−7.4 ± 0.7	0.3
UpG	0.68 ± 0.22	−7.0 ± 2.3	
	0.35 ± 0.06	−6.6 ± 1.2	0.9 ± 0.2
CpG	7.2 ± 0.7	−8.5 ± 0.9	
	4.1 ± 0.4	−8.1 ± 0.8	−0.6 ± 0.2
octanuc	14 ± 1	−8.9 ± 0.9	−1.2
inosine	0.0078 ± 0.0008	−4.2 ± 0.4	
	0.0059 ± 0.0006	−4.0 ± 0.4	3.6 ± 0.1
2NHPuRi	0.0022 ± 0.0003	−3.4 ± 0.5	
	0.0017 ± 0.0002	−3.2 ± 0.3	4.4 ± 0.1
Splicing Reaction with High [$MgCl_2$]			
G	1.1 ± 0.1	−7.3 ± 0.7	
	0.83 ± 0.08	−7.1 ± 0.7	0.5 ± 0.1
CpG	0.71 ± 0.11	−7.0 ± 1.1	
	0.83 ± 0.17	−7.1 ± 1.5	0.7 ± 0.1
Splicing Reaction with Low [$MgCl_2$]			
G	0.43 ± 0.16	−6.7 ± 2.5	
	0.71 ± 0.19	−7.0 ± 1.9	0.9 ± 0.2
CpG	0.33 ± 0.05	−6.5 ± 1.0	1.2

[a] R is 1.99 (calories per degrees kelvin per mole), and T is 315 K. [b] $\Delta\Delta G° = \Delta G° - \Delta G°$(average), where $\Delta G°$(average) = −7.7 (the average value of $\Delta G°$ for GMP and G binding to the cyclization site). The uncertainty shown for $\Delta\Delta G°$ indicates the range of values obtained. Negative values for $\Delta\Delta G°$ indicate substrates that bind better than G and GMP by the value shown, while positive values indicate substrates that bind less well. Duplicate values are the same as in Table I.

mined previously (Bass & Cech, 1984). The difference between the values determined in low and high $MgCl_2$ concentrations probably reflects Mg(II)-dependent stabilization of guanosine binding. The K_m values for CpG are the same as the values determined for guanosine.

The 5′ and 3′ splice sites undergo site-specific hydrolysis, in the absence of substrate, to form the L IVS, L - 15 IVS, circular IVS, and the IVS–3′ exon. The half-life for the formation of these products is 360 ± 80 min in the presence of 20 mM $MgCl_2$, pH 7.5, and 2000 ± 90 min in the presence of 5 mM $MgCl_2$/100 mM $(NH_4)_2SO_4$, pH 7.5. [The 5′ exon–IVS and the large circle are formed only by site-specific hydrolysis of the 3′ splice site (Inoue et al., 1986); these products are distinct from the products formed by the splicing reaction.] The individual rates of hydrolysis at the 5′ and 3′ splice sites could not be accurately determined because both reactions produce many of the same products [see Inoue et al. (1986)]. However, we estimate the half-life for site-specific hydrolysis to be 500–1000 min at the 5′ splice site and 150–300 min at the 3′ splice site at the high $MgCl_2$ concentration.

There is a slight tendency for guanosine to increase the rate of hydrolysis at the 3′ splice site, in the presence of 20 mM $MgCl_2$ (data not shown). However, the amount of 5′ exon–IVS produced seems to level off fairly early in the reaction, probably because these molecules undergo further reaction.

Table I summarizes the values K_m, k_{cat}, and k_{cat}/K_m determined for each substrate in the substrate addition reaction of the L IVS_β RNA and in the splicing reaction. The data for the substrate addition reaction can be interpreted in terms of a reaction scheme:

$$\text{IVS} + \text{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{IVS·S} \xrightarrow{k_2} \text{L - 14 IVS} + \text{15-mer}$$

where the substrate (S) is guanosine or an analogue thereof and k_2 is equivalent to k_{cat}. If k_{-1} is much greater than k_2, then

$$K_m = (k_{-1} + k_2)/k_1 \approx k_{-1}/k_1 = K_d$$

where K_d is the dissociation constant of the IVS–substrate complex. Thus, K_m is approximately inversely proportional to the substrate binding constant. From these binding constants, the free energy of binding, $\Delta G°$, was calculated for each substrate (Table II).

Discussion

We have shown that the removal of the 3′-terminal guanosine residue incapacitates the IVS RNA for cyclization but leaves it competent for an intermolecular guanosine addition reaction. Analysis of the products of guanosine addition for both intact and β-eliminated IVS indicates that this reaction occurs with the same fidelity as the cyclization reaction. The β-elimination reaction does not affect the catalytic site. Furthermore, the reaction shows high specificity for guanosine relative to the other three ribonucleotides. The fact that intermolecular guanosine addition to the cyclization site occurs

with efficiency and high fidelity indicates that a guanosine binding site is involved in IVS cyclization. Furthermore, the properties of the intermolecular guanosine addition reaction indicate that it is a good model system for studying this guanosine binding site. High $MgCl_2$ concentrations, however, seem to be necessary to promote this intermolecular reaction. This reaction is similar to the IVS RNA oligomerization reaction noted previously, where the 3′-terminal nucleotide of one IVS molecule makes a nucleophilic attack at the cyclization site of another IVS molecule to form a linear dimer molecule (Zaug & Cech, 1985). The heating/cooling step required for that reaction seems to be necessary to make the ends accessible.

Site-specific hydrolysis has been noted previously for the circular IVS (Zaug et al., 1985) and for the precursor at the 5′ and 3′ splice sites (Inoue et al., 1986). We now report that site-specific hydrolysis occurs at the cyclization site of L IVS_β RNA as well. The reaction is the same as that shown in the diagram of Figure 1d except that OH^- replaces G_{OH} and the larger product is therefore L - 15 IVS_β RNA. Hydrolysis at the cyclization site had not been previously noticed with the intact IVS, probably because the cyclization reaction is much faster than site-specific hydrolysis and because the final products are the same as those generated by cyclization followed by site-specific hydrolysis of the circle.

Like the products of splicing, cyclization, and guanosine addition to L IVS_β RNA, the 5′ fragments produced by site-specific hydrolysis contain 3′-hydroxyls instead of the 3′-phosphates expected from random alkaline hydrolysis. Our interpretation is that the phosphodiester bond between nucleotides 15 and 16 of the IVS is activated for nucleophilic attack by the 3′-hydroxyl of a guanosine molecule (the cyclization reaction) and that in the absence of guanosine it is subject to attack by a hydroxyl ion from the aqueous solvent. The half-life for hydrolysis at the cyclization site at pH 7.5 is 980 ± 20 min. The half-lives for site-specific hydrolysis at the 5′ and 3′ splice sites are estimated to be 500–1000 and 150–300 min, respectively, under the same conditions used for guanosine addition. Thus, the cyclization site and the 5′ splice site, both of which follow a stretch of pyrimidines, undergo hydrolysis at about the same rate.

By comparing the reaction kinetics of cyclization of intact IVS RNA to guanosine addition to L IVS_β RNA, we estimate that the effective concentration of the 3′-terminal guanosine of the IVS with respect to the cyclization site is equivalent to ~1 mM free guanosine. This is at least 4 orders of magnitude less than the effective concentrations often calculated for intramolecular reactions (Fersht, 1977; Jencks, 1969). We interpret this to mean that the 3′-terminal guanosine and preceding nucleotides of the intact IVS are not rigidly held in place but must search for the cyclization binding site in a manner analogous to the binding required for intermolecular guanosine addition. This is consistent with the conclusions of Price et al. (1985).

Michaelis–Menten kinetics certainly do not prove the existence of a single substrate binding site for cyclization. However, we feel the data are strongly indicative of such a site. The results of the substrate analogue comparison indicate that functional groups on the guanosine molecule that are normally involved in a Watson–Crick G-C base pair are important for substrate binding. Thus, compounds that have functional groups on the 1-, 2-, or 6-position of the purine ring that are different from those of guanosine are much less reactive in substrate addition. Likewise, a modification on the N7 position of the purine ring has no apparent effect on the reaction. These results are the same as those obtained for the guanosine binding site in splicing (Bass & Cech, 1984). Furthermore, the K_m values for G or GMP addition to L IVS_β RNA (5 ± 2 μM) and for splicing under the same conditions (11 ± 2 μM) are in reasonable agreement.

The K_m values and calculated free energies are consistent with inosine and 2-aminopurine ribonucleoside making two fewer hydrogen bonds than guanosine. These contribute 1.7–2.2 kcal/mol per hydrogen bond. This is similar to the 1.5 kcal/mol calculated previously (Bass & Cech, 1984). While 2.2 kcal/mol is somewhat higher than expected, the free energy contribution for each hydrogen bond is within the experimentally determined range for hydrogen bonds to RNA (Freier et al., 1986; Fersht et al., 1985). Hydrogen bonding is only one of several possible binding modes; van der Waals forces, solvent interactions, stacking interactions, etc. may also be important in substrate binding.

The hydroxyls on the 2′- and 3′-positions of the ribose sugar are absolutely required for the addition reaction. The 3′-hydroxyl is important because it acts as the reactive nucleophile. The importance of the 2′-hydroxyl is less obvious. Bass and Cech (1986) have shown, from competitive binding studies, that the 2′- and 3′-hydroxyls contribute significant binding energy for guanosine binding in splicing. However, dG is a better binder than inosine and 2-aminopurine ribonucleoside, which do work in the splicing reaction, so the 2′-hydroxyl appears to have importance for facilitating the splicing reaction beyond its contribution to binding. It is likely that this is also the situation for the guanosine binding site involved in cyclization.

The lower K_m values for CpG and the octanucleotide are considered to be significant, despite the fact that our calculations show the additional nucleotide(s) contribute(s) only ~1 kcal/mol binding energy. The results are very striking and reproducible (see Figure 6). We feel the calculated K_m values are actually underestimating the contributions made by the nucleotides preceding the guanosine. This is because only the 3′-terminal nucleotide is removed from the IVS; the other nucleotides near the 3′ end are still free to bind. The effective concentration of these 3′-terminal nucleotides, with respect to the binding site, is probably equivalent to ~1 mM free dimer or octanucleotide. This is 1000 times more than the highest dimer or octanucleotide concentration used in the Michaelis–Menten kinetic analysis. Thus, the 3′-terminal bases present in the L IVS_β RNA are likely to compete effectively with the nucleotide(s) preceding guanosine in the substrate for binding to the IVS RNA. The K_m values measured for the dimer and octanucleotide would represent the combination of the full contribution from binding of G plus a partial contribution from binding of the preceding nucleotides. The deletion of additional nucleotides from the 3′ end of the IVS would enable a test of this theory.

ApG and GpG have about the same K_m values as G alone, so an A or G preceding the terminal G is probably not able to make significant binding interactions. UpG, in contrast, does not bind as well as guanosine. Perhaps the poor stacking ability of uridine interferes with binding of the guanosine moiety, or perhaps the uridine causes the dimer to bind in a nonproductive fashion. In summary, we conclude that two or more specific nucleotides preceding the guanosine residue are important for substrate binding in the intermolecular addition reaction and presumably for binding the 3′ end of the IVS RNA for the normal cyclization reaction.

This is in contrast to the splicing reaction, where CpG binds no better than guanosine (Table I). We conclude that the

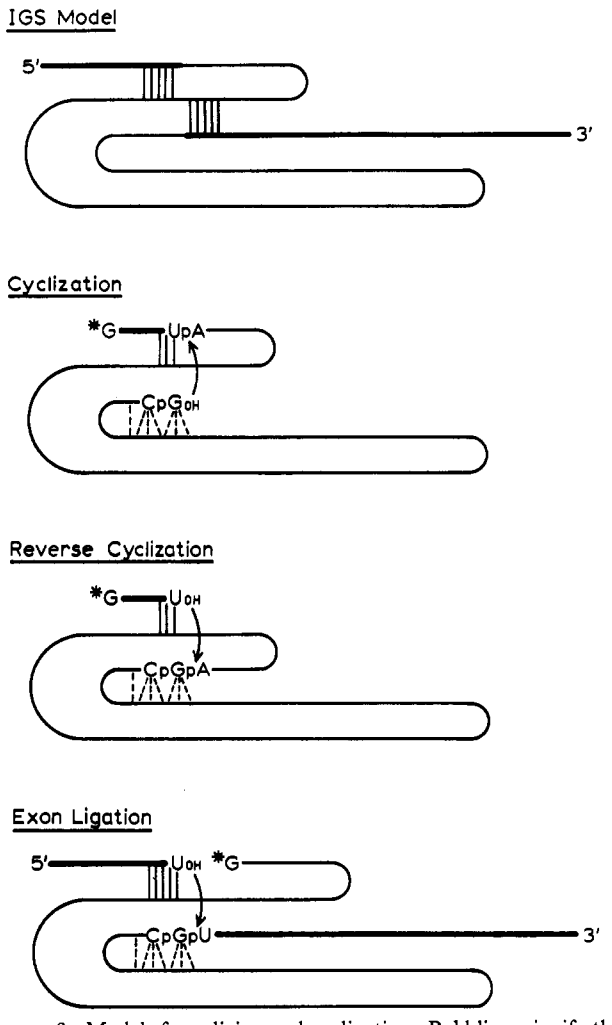

FIGURE 9: Models for splicing and cyclization. Bold lines signify the exons, and the thin line signifies the IVS. For the purpose of these models, the 15-mer released during cyclization can be thought of as a mini 5′ exon. The solid vertical lines connecting the strands designate Watson–Crick base pairing. Dashed lines signify binding interactions that have not yet been definitively determined. Arrows indicate where new phosphodiester bonds are formed. See the text for a description of the models.

guanosine binding site for cyclization is similar to, but not identical with, the guanosine binding site for splicing. This could mean that there are two independent binding sites within the IVS with similar properties or that there is a single binding site that is somehow modified during the various reactions. The latter possibility is not unreasonable, because the exons of the precursor RNA make significant interactions with the IVS (Waring et al., 1986; Been & Cech, 1986).

The k_{cat} values in Table I show a fair amount of variability. This may reflect differences in IVS RNA preparations or experimental inaccuracies. However, the mean value of k_{cat}, 0.07 ± 0.03 min^{-1}, is basically the same for all the substrates used in the addition reaction. It is also within the mean value obtained for splicing in the presence of 20 mM $MgCl_2$ (0.06 ± 0.01 min^{-1}). Therefore, the guanosine analogues that work in the reaction only have altered binding properties in the catalytic site; they do not affect the maximum velocity of the reaction. The k_{cat} value tends to be larger (0.12 ± 0.04 min^{-1}) for splicing at 5 mM $MgCl_2$/100 mM $(NH_4)_2SO_4$, suggesting that high $MgCl_2$ concentrations inhibit the reaction somewhat. High $MgCl_2$ concentrations may stabilize the IVS RNA structure and thereby inhibit conformational changes that are necessary for reaction (Burke et al., 1986; Tanner & Cech, 1985). The mean k_{cat} value for splicing at 42 °C is 5 times less than that determined previously at 30 °C, under the same ionic conditions (Bass & Cech, 1984). This is probably because of the different experimental procedures and method of analysis used.

The two nucleotides preceding the terminal guanosine show some conservation among group I IVS's. A subclass of group I IVS's that includes four mitochondrial large rRNA IVS's, *Neurospora crassa* cytochrome *b* IVS 2, and yeast cytochrome *b* IVS 5 has been recognized on the basis of unique features of its core structure (Waring & Davies, 1984). We note that in this subclass the IVS's terminate with the sequence UUG, with one example of CUG. The other group I IVS's have a terminal consensus sequence of $A^A_U G$. The implication is that one or more of the features that distinguishes the subclass—for example, the dinucleotide AC instead of GA preceding the paired region of sequence element 9L—is responsible for binding the terminal nucleotides and forming part of the active site for cyclization. Such binding could involve non-Watson–Crick interactions. An alternative model that seems plausible for the *Tetrahymena* IVS involves pairing of U^{412} and C^{413} with a portion of the internal guide sequence, i.e., with A^{24} and G^{23} or with A^{28} and G^{27}. The possibility of such an interaction is not conserved among group I IVS's and therefore seems less attractive than the first model described above.

The implication that C^{413} and other nucleotides in the terminal eight nucleotides of the IVS interact with some internal site may have important implications for understanding exon ligation during splicing. Figure 9 shows the internal guide sequence (IGS) model first proposed by Davies et al. (1982). In this model, the 5′ and 3′ exons are held in precise alignment for splicing by Watson–Crick base pairing with sequences within the IVS. A great deal of evidence in support of the interaction of the 5′ exon with the IGS has been obtained (Been & Cech, 1986; Waring et al., 1986). However, the proposed IGS–3′-exon interaction can be completely eliminated without an apparent effect on splicing efficiency or accuracy (Been & Cech, 1985). How then is the 3′ exon held in place?

For the cyclization reaction, we have argued that the 3′ end of the IVS is held in precise position by three or more sequence-specific interactions with other residues within the IVS. These same interactions could also be used for the reverse cyclization reaction (Sullivan & Cech, 1985). We propose that the same interactions that are important for cyclization are also used to hold the 3′ exon in position for exon ligation. Watson–Crick base pair interactions between the IVS and the 3′ exon could still take place, but they are evidently not necessary.

ACKNOWLEDGMENTS

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Registry No. G, 118-00-3; 7-methyl-G, 20244-86-4; GMP, 85-32-5; ApG, 3352-23-6; GpG, 3353-33-1; UpG, 3474-04-2; CpG, 2382-65-2; ApGpUpApCpUpCpG, 107940-96-5; 2NHPuRi, 4546-54-7; OH^-, 14280-30-9; inosine, 58-63-9.

REFERENCES

Bass, B. L., & Cech, T. R. (1984) *Nature (London) 308*, 820–826.

Bass, B. L., & Cech, T. R. (1986) *Biochemistry 25*, 4473–4477.

Been, M. D., & Cech, T. R. (1985) *Nucleic Acids Res. 13*, 8389–8407.

Been, M. D., & Cech, T. R. (1986) *Cell (Cambridge, Mass.)* *47*, 207-216.
Burke, J. M., Irvine, K. D., Kaneko, K. J., Kerker, B. J., Oettgen, A. B., Tierney, W. M., Williamson, C. L., Zaug, A. J., & Cech, T. R. (1986) *Cell (Cambridge, Mass.)* *45*, 167-176.
Chu, F. K., Maley, G. F., West, D. K., Belfort, M., & Maley, F. (1986) *Cell (Cambridge, Mass.)* *45*, 157-166.
Davies, R. W., Waring, R. B., Ray, J. A., Brown, T. A., & Scazzocchio, C. (1982) *Nature (London)* *300*, 719-724.
Donis-Keller, H., Maxam, A. M., & Gilbert, W. (1977) *Nucleic Acids Res.* *4*, 2527-2538.
Fersht, A. (1977) *Enzyme Structure and Mechanism*, W. H. Freeman, San Francisco.
Fersht, A. R., Shi, J.-P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, D., Cartor, P., Waye, M. M. Y., & Winter, G. (1985) *Nature (London)* *314*, 235-238.
Freier, S. M., Sugimoto, N., Sinclair, A., Alkema, D., Neilson, T., Kierzek, R., Caruthers, M. H., & Turner, D. H. (1986) *Biochemistry* *25*, 3214-3219.
Garriga, G., & Lambowitz, A. M. (1984) *Cell (Cambridge, Mass.)* *39*, 631-641.
Inoue, T., Sullivan, F. X., & Cech, T. R. (1986) *J. Mol. Biol.* *189*, 145-165.
Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, McGraw-Hill, New York.
Kruger, K., Grabowski, P. J., Zaug, A. J., Sands, J., Gottschling, D. E., & Cech, T. R. (1982) *Cell (Cambridge, Mass.)* *31*, 147-157.
Neter, J., Wasserman, W., & Kutner, M. H. (1983) *Applied Linear Regression Models*, Richard D. Irwin, Inc., Homewood, IL.
Price, J. V., Kieft, G. L., Kent, J. R., Sievers, E. L., & Cech, T. R. (1985) *Nucleic Acids Res.* *13*, 1871-1889.
Sullivan, F. X., & Cech, T. R. (1985) *Cell (Cambridge, Mass.)* *42*, 639-648.
Tanner, N. K., & Cech, T. R. (1985) *Nucleic Acids Res.* *13*, 7741-7758.
Van der Horst, G., & Tabak, H. F. (1985) *Cell (Cambridge, Mass.)* *40*, 759-766.
Waring, R. B., & Davies, R. W. (1984) *Gene* *28*, 277-291.
Waring, R. B., Towner, P., Minter, S. J., & Davies, R. W. (1986) *Nature (London)* *321*, 133-139.
Wilson, E. B. (1952) *An Introduction to Scientific Research*, McGraw-Hill, New York.
Winter, G., & Brownlee, G. G. (1978) *Nucleic Acids Res.* *5*, 3129-3139.
Zaug, A. J., & Cech, T. R. (1985) *Science (Washington, D.C.)* *229*, 1060-1064.
Zaug, A. J., Grabowski, P. J., & Cech, T. R. (1983) *Nature (London)* *301*, 578-583.
Zaug, A. J., Kent, J. R., & Cech, T. R. (1985) *Biochemistry* *24*, 6211-6218.
Zaug, A. J., Been, M. D., & Cech, T. R. (1986) *Nature (London)* *324*, 349-353.

Yeast and Horse Liver Alcohol Dehydrogenases: Potential Problems in Target Size Analysis and Evidence for a Monomer Active Unit†

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ABSTRACT: Yeast and horse alcohol dehydrogenases are commonly used as standards for radiation inactivation analysis of proteins, usually assuming that the minimal functional unit corresponds to the physical size in solution, a tetramer (M_r = 148 000) and a dimer (M_r = 80 000), respectively. Results described in this paper demonstrate that molecular weight overestimates may be obtained for the yeast protein as a result of its unusual sensitivity to secondary radiation products. Irradiation in the presence of sulfhydryl reagents results in a smaller functional size estimate (67 000 ± 3000) than that obtained in their absence (128 000 ± 5000), indicating that some sulfhydryl groups in the enzyme may be particularly susceptible to attack by radiolytic species. Analysis of the horse liver enzyme reveals that although it has structural and functional similarities to the yeast protein, it is not as prone to secondary radiation damage and gives a minimal functional size estimate (33 000 ± 1000) that most closely corresponds to a monomer. Quantitation of disappearance of the protein from a sodium dodecyl sulfate gel as a function of radiation dose also gives a target size (48 000 ± 3000) in reasonable agreement with the monomer molecular weight. These results indicate that the individual subunits of horse liver alcohol dehydrogenase have independent catalytic capacity and imply that the same may be true for the yeast enzyme.

Radiation inactivation or target size analysis is a technique that has been widely used to determine the functional molecular weight of proteins in pure and impure states. The functional size determined by this method often corresponds well with the actual physical molecular weight measured by amino acid sequence analysis or by hydrodynamic methods (Kempner & Schlegel, 1979; Shikita & Hatano-Sato, 1972; Lowe & Kempner, 1982; Kempner & Haigler, 1982; Haigler et al., 1985; Suarez, 1986; Kempner, 1987). In the case of multisubunit enzymes, information may be obtained regarding the catalytic capacity of individual subunits within an ag-

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