The EMBO Journal vol.7 no.11 pp.3531 – 3537, 1988

Two guanosine binding sites exist in group I self-splicing IVS RNAs

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Communicated by M.Eiger

A shortened form of the self-splicing rRNA intervening sequence (IVS) of *Tetrahymena thermophila* can catalyze a transesterification reaction, termed G-exchange, between a monomeric guanosine derivative such as GTP and the substrate GpN (where N is A, C, G or U). The reaction is specific to the two guanosines involved, providing evidence that two guanosine binding sites exist in this group I IVS RNA. One binding site accommodates a guanosine which initiates self-splicing and the other recognizes the guanosine preceding the 3′ splice site. Previously, only one guanosine binding site was thought to be involved in the mechanism of self-splicing. Based on the two functionally distinguishable guanosine binding sites, a new model is proposed to explain how the two independent transesterification reactions required for self-splicing might proceed in a concerted manner.

Key words: group I intron/*Tetrahymena*/IVS/self-splicing/RNA catalyst

Introduction

Splicing of RNA is a widespread phenomenon in the nuclear and organelle RNAs of higher eukaryotes. RNA splicing reactions have been divided into three classes based on separate mechanisms (Cech, 1983). (i) Precursor tRNAs are spliced by two protein enzymes (Peebles *et al.*, 1983; Greer *et al.*, 1983). (ii) Two types of precursor RNAs are spliced through reactions mediated by their own intervening sequence (IVS or intron) RNAs. These group I and group II IVS RNAs (Davies *et al.*, 1982; Michel *et al.*, 1982) catalyze splicing either in the absence of protein (Kruger *et al.*, 1982; Garriga and Lambowitz, 1984; Chu *et al.*, 1986; Peebles *et al.*, 1986; Van der Veen *et al.*, 1986; Schmelzer and Schweyen, 1986) or assisted by protein components (Akins and Lambowitz, 1987). Most of these RNAs reside in the mitochondria or chloroplast. (iii) Nuclear precursor RNAs are spliced by a large apparatus consisting of ribonucleoprotein complexes (Sharp, 1987; Maniatis and Reed, 1987). It has been suggested that nuclear precursor RNA splicing has evolved from RNA-mediated splicing reactions similar to those of the group I and group II IVS RNAs (Sharp, 1985).

A group I IVS exists in the precursor rRNA of *Tetrahymena*. This IVS directs its own excision *in vitro* in the presence of a monomeric guanosine derivative and divalent magnesium ions. The splicing reaction proceeds via two transesterification reactions (Cech, 1987) (Figure 1a). In the first step, a guanosine derivative specifically attacks a phosphodiester bond at the 5′ splice site, resulting in the liberation of the 5′ exon and the creation of a 3′-5′ phosphodiester bond between the guanosine and the 5′ end of the IVS. In the second step, the 5′ exon attacks the 3′ splice site, generating a 3′-5′ phosphodiester bond between the exons.

We have investigated the mechanism of this self-splicing reaction and discovered that a large fragment of the *Tetrahymena* IVS RNA can mediate intermolecular transesterification reactions between two dinucleotides or between a mononucleotide and a trinucleotide (Kay and Inoue, 1987a). These transesterification reactions correlate

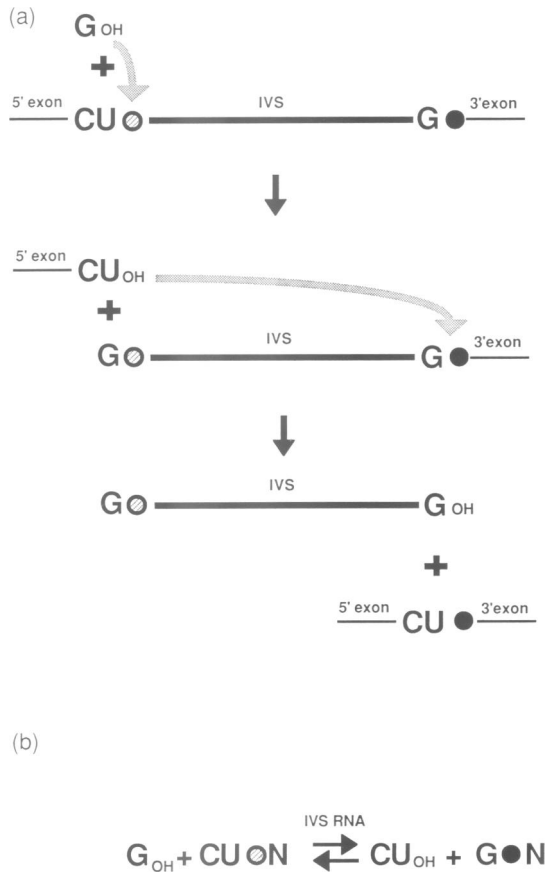

Fig. 1. The *Tetrahymena* self-splicing reaction. **(a)** A schematic representation of the two steps of self-splicing. G_{OH} represents a monomeric guanosine derivative which attacks the 5′ splice site. **(b)** A simplified transesterification reaction between short nucleotides catalyzed by the IVS RNA. The forward and reverse transesterification reactions represent the first and second step of self-splicing respectively (Kay and Inoue, 1987a). The reacted bonds are represented as circles.

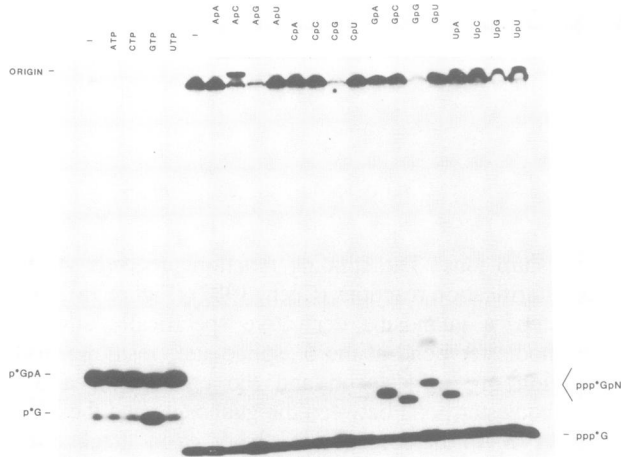

Fig. 2. The G-exchange reaction catalyzed by L-21 *Sca*I IVS RNA. **Lanes 1–5**: 5′-end-labeled pGpA (20 μM) was incubated in the presence of L-21 *Sca*I IVS RNA (0.8 μM) with no co-factor, or with 100 μM of ATP, CTP, GTP or UTP at 42°C for 1.5 h. **Lanes 6–22**: [α-^{32}P]GTP (80 nM) was incubated in the presence of L-21 *Sca*I IVS RNA (2.0 μM) with no co-factor or each of the 16 possible dinucleotides (20 μM) at 42°C for 1.5 h. The bands appearing near the origin of the gel are the products of the transesterification at and near the 5′ end of L-21 *Sca*I IVS RNA. An extra band appearing above pppGpG is presumably pppGpGpG due to the reaction between pppGpG and GpG. Note that the asterisk indicates the labeled phosphate.

to the first and second steps of group I self-splicing (Figure 1b).

There are several reports describing an unusual transesterification reaction mediated by the IVS RNAs of *T. thermophila* (Been and Cech, 1985; Price *et al.*, 1987), *Neurospora crassa* (Garriga and Lambowitz, 1984) and yeast (van der Horst and Tabak, 1987). This reaction involves the nucleophilic attack by the 3′ hydroxyl group of a guanosine derivative at a phosphodiester bond preceded by another guanosine. The reaction has been termed the 'G-exchange reaction', since one guanosine displaces another via a transesterification mechanism. It was, however, not shown whether the reaction is specific to two guanosine derivatives.

We have investigated the guanosine exchange reaction mediated by a large fragment of self-splicing *Tetrahymena* IVS RNA, utilizing monomeric nucleotides and the 16 possible dinucleotides combinations. Our results show that the exchange reaction is indeed specific to two guanosines, one as a nucleophile and the other as a nucleotide preceding an electrophilic phosphodiester bond. The kinetic study of the G-exchange reaction strongly suggests that two functionally different guanosine binding sites exist in the IVS. It was also found that this reaction is analogous to the transesterification by a nucleophilic guanosine derivative at the 3′ splice site in the precursor RNA. The existence of two guanosine binding sites in the IVS implies a more

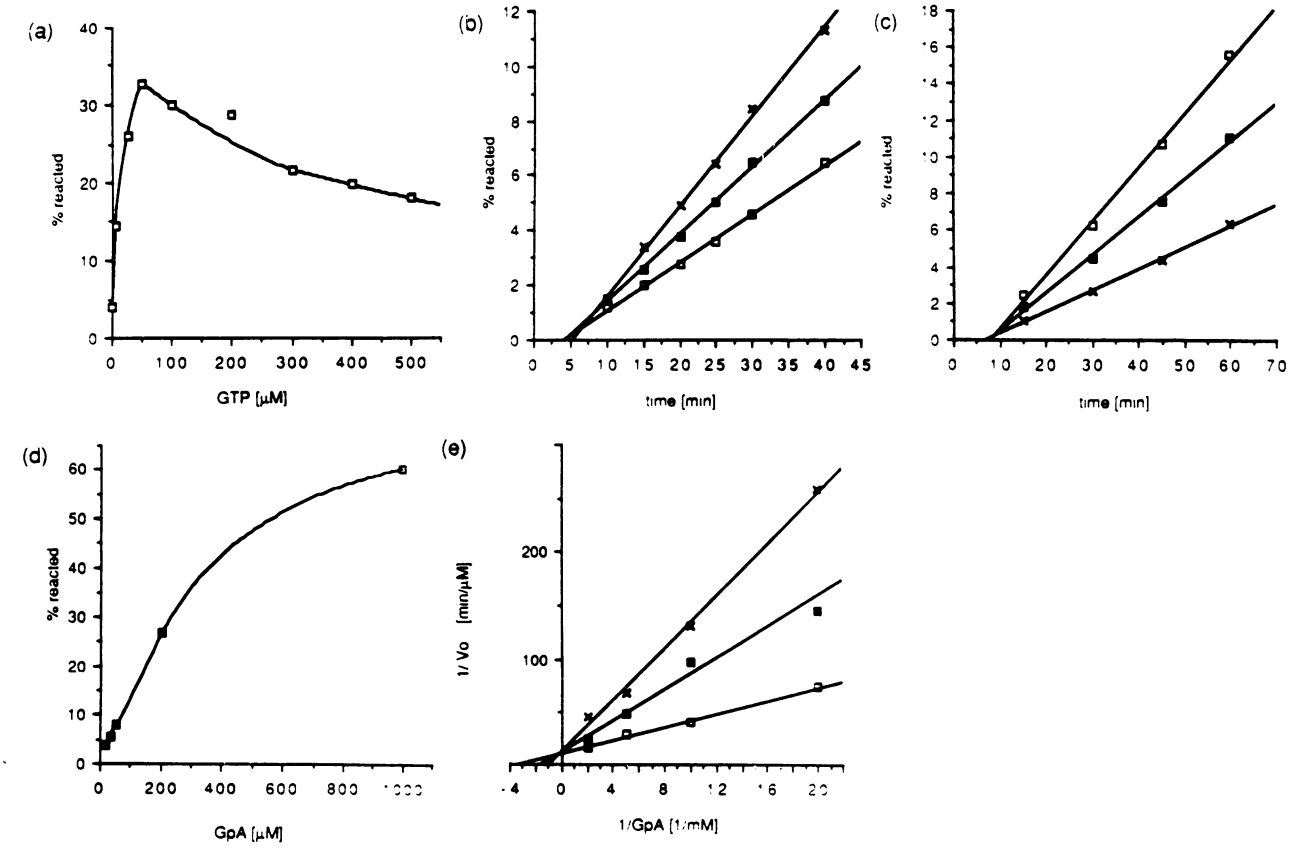

Fig. 3. Kinetic analysis of the G-exchange reaction. (**a**) [5′-^{32}P]pGpA (2.5 μM) was incubated in the presence of L-21 *Sca*I IVS RNA (2.0 μM) with various concentrations of GTP at 42°C for 1.0 h. (**b**) The time course of the reaction in the presence of L-21 *Sca*I IVS RNA (2.0 μM) with [5′-^{32}P]pGpA (20 μM) at low GTP concentrations: □, 2.5 μM; ■, 5.0 μM; ×, 10.0 μM. (**c**) Time course of the reaction in the presence of L-21 *Sca*I IVS RNA (2.0 μM) with [5′-^{32}P]pGpA (20 μM) at high GTP concentrations: □, 200 μM; ■, 500 μM; ×, 1000 μM. (**d**) [α-^{32}P]GTP (10 μM) was incubated in the presence of L-21 *Sca*I IVS RNA (2.0 μM) with various concentrations of GpA at 42°C for 0.5 h. (**e**) Competitive inhibition by dGTP. Time course data were obtained from the reactions of GTP (10 μM) with various concentrations of GpA by employing L-21 *Sca*I IVS RNA (2.0 μM) as a catalyst in the presence or absence of dGTP at 42°C. Lineweaver–Burk plots were obtained from three sets of time course data: □, no co-factor; ■, dGTP (200 μM); ×, dGTP (500 μM).

sophisticated self-splicing mechanism than was previously thought.

Results

Reaction of GTP with dinucleotides

A truncated form of the IVS RNA termed as L-21 *Sca*I IVS RNA (lacking the first 21 nucleotides and last five nucleotides of the full length IVS released by self-splicing) (Kay and Inoue, 1987a) was used as a catalyst in all our experiments, unless otherwise stated. We have reported that in the presence of high concentrations of GTP (5 mM), L-21 *Sca*I IVS RNA is not able to catalyze transesterification reactions by GTP at the phosphodiester bond of any dinucleotide. Recently, however, we found that GTP can perform a transesterification reaction at the phosphodiester bond contained in the dinucleotide GpN (N = A, C, G or U), if the concentration of GTP is relatively low (<1 mM).

The specificity of the reaction was examined by incubating either each of all four mononucleotides with [5′-^{32}P]pGpA or each of all 16 possible dinucleotides with [α-^{32}P]GTP, in the presence of L-21 *Sca*I IVS RNA. The resulting reaction mixtures were electrophoresed on a 20% polyacrylamide denaturing gel (Figure 2). In the reactions of NTP (N = A, C, G or U), with [5′-^{32}P]pGpA, it was found that only GTP can serve as a nucleophile. When GTP is used as the nucleophile with each of the 16 dinucleotides, only four dinucleotides, GpNs, are found to be suitable substrates. These results demonstrate that the reaction is in fact a G-exchange reaction and requires two guanosines: one as a nucleophile and the other as a nucleoside preceding a phosphodiester bond which is reacted. Thus at least two guanosine binding sites must exist in the group I IVS. When the newly formed [5′-α-^{32}P]pppGpN product was digested with RNase T1, the reaction yielded [5′-α-^{32}P]pppGp (data not shown). Thus the G-exchange reaction produces a normal 3′-5′ phosphodiester linkage as a result of transesterification.

Inhibition of G-exchange by GTP

Since the G-exchange reaction does not proceed in the presence of high concentrations of GTP, it was of interest to study the kinetics of the reaction. Experiments were conducted by varying the concentration of GTP. Under our standard reaction conditions, GTP (1 μM−1 mM) was incubated with 2.5 μM of [5′-^{32}P]pGpA for 1 h (Figure 3a). The conversion of [5′-^{32}P]pGpA to [^{32}P]pG increased dramatically as the GTP concentration increased and reached its optimum at 50 μM, after which the yield decreased proportionally to the increase in GTP concentration.

The time course of the G-exchange reaction was examined to see whether the reaction rate is kinetically limited or determined by GTP concentration. The reaction proceeds linearly relative to time under all the conditions employed. At lower GTP concentrations, the reaction rate increases relative to the increase of GTP (Figure 3b). In the higher range, however, the reaction rate decreases in relation to increased concentrations of GTP (Figure 3c). The reaction was not inhibited by the presence of high concentrations of ATP, CTP or UTP (data not shown). This demonstrates that the inhibition is specific to guanosine.

The effect of GpA concentration was also investigated over the range of 20 μM−1 mM in the presence of 10 μM of

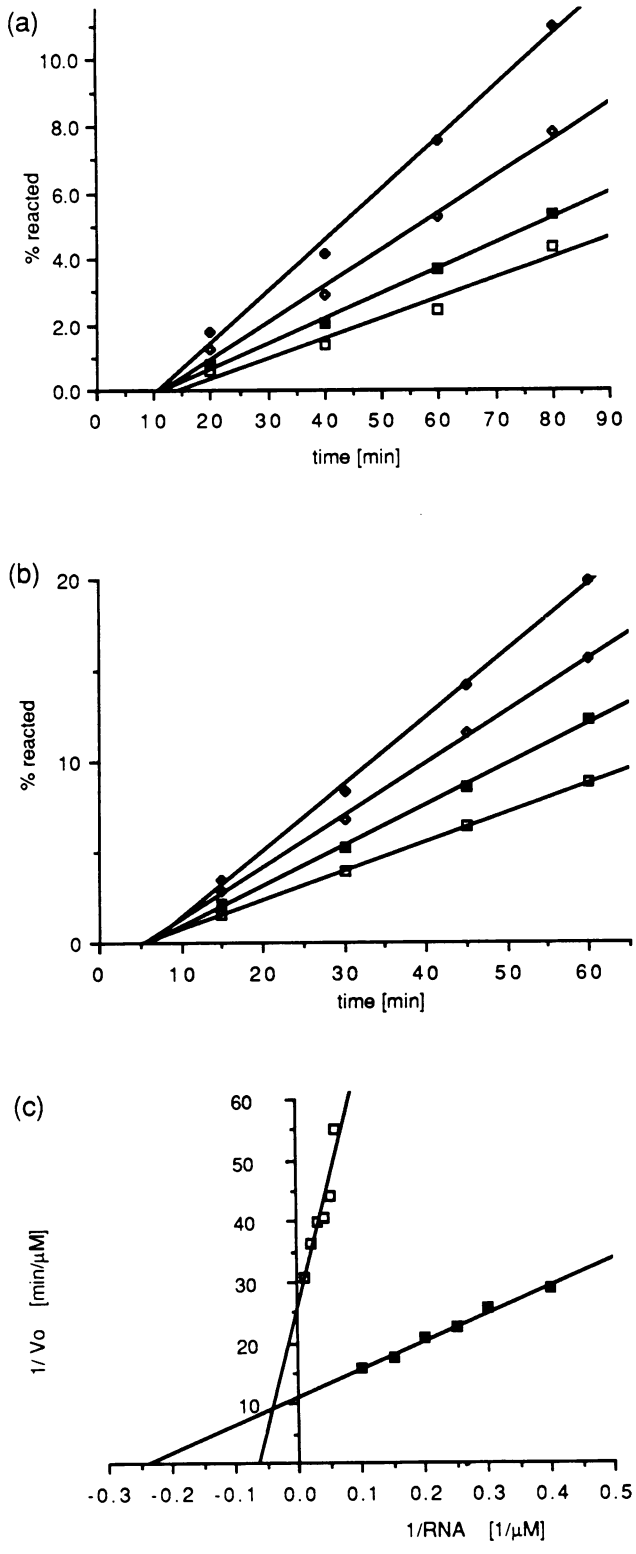

Fig. 4. The relative reactivity of GTP to CpU in reaction with [5′-^{32}P]pGpA. (**a**) The time course of the reaction of CpU (1 mM) with [5′-^{32}P]pGpA (2.5 μM) in the presence of L-21 *Sca*I IVS RNA (2.0 μM) at 42°C. (**b**) The time course of the reaction of GTP (10 μM) with [5′-^{32}P]pGpA (2.0 μM) in the presence of L-21 *Sca*I IVS RNA (2.0 μM) at 42°C. For both (**a**) and (**b**), pHs were: □, 6.0; ■, 6.5; ◇, 7.0; ◆, 7.5. (**c**) Kinetics of the two reactions as a function of nucleophile concentration in the presence of L-21 *Sca*I IVS RNA (2.0 μM) with [5′-^{32}P]pGpA (20.0 μM) at 42°C: □, with GTP; ■, with CpU.

[α-^{32}P]GTP under our standard conditions (Figure 3d). The yield of pppGpA increased linearly relative to the increase of GpA concentration in both the high and low ranges. Since no significant inhibitory effect was observed in this case, it appears that GpA binds specifically to only one site in the IVS and therefore cannot interfere with the binding of GTP.

The inhibitory effect of GTP can be explained in two ways: (i) GTP displaces pGpA competitively at one binding site or (ii) GTP accommodated by a third guanosine binding site deactivates the catalytic function of the IVS. In order to distinguish between these two possibilities, we conducted the reaction in the presence of dGTP, which contains a guanine base but is known as an inefficient inhibitor of self-splicing (Bass and Cech, 1984, 1986). It was of interest to see whether dGTP could competitively inhibit the binding of GpA. The Lineweaver–Burk plots of our data (Figure 3e) show that dGTP does inhibit the reaction in a competitive manner. This strongly suggests that only two guanosine binding sites are involved in the G-exchange reaction.

Comparison of GTP to CU in reactions with GpA

As we have previously reported, CpU reacts with GpN to perform a mini-exon ligation reaction (Kay and Inoue, 1987a). To help characterize the G-exchange reaction, the reactivity of GTP and CpU were compared side by side under a variety of conditions. First, pH dependence of the transesterification reaction of GTP or CpU with GpA were examined (Figure 4a and b). In the range of 6.5–7.5, both reactions were accelerated relative to the increase in pH. These results indicate that the G-exchange reaction and the reaction of CpU with GpA are conducted by the IVS RNA in a similar fashion.

The time courses of both reactions were investigated by employing a variety of nucleophile concentrations. Reactions of both GTP and CpU proceeded linearly relative to time and to the concentration of the nucleophiles, under the conditions employed. The reaction rates were determined and subsequently converted into Lineweaver–Burk plots (Figure 4c). The *K*m for CpU is 16.0 μM, while the *K*m for GTP is 4.1 μM. The V_{max} of the reaction with GTP is ~2.4 times that of CpU. Although the G-exchange reaction proceeds more efficiently than the mini-exon ligation reaction of CpU with GpN, these similar values support the idea that the G-exchange reaction proceeds via the same reaction mechanism that conducts the two steps of self-splicing.

The G-exchange reaction at the 3′ splice site

Since the G-exchange reaction seems related to the mini-exon ligation of CU with GpA, further investigation was undertaken to elucidate the relationship between the two. The IVS-catalyzed nucleophilic attack of CpU at the phosphodiester bond of the dinucleotide GpN has been shown to be equivalent to the second step of self-splicing (Kay and Inoue, 1987a). In this reaction, CpU and the phosphodiester bond of GpN represent the 5′ exon and the 3′ splice site respectively (Figure 1). GTP has also been reported to attack at the 3′ splice site of some precursor RNAs which contain group I IVSs (Price *et al.*, 1987; van der Horst and Tabak, 1987). To investigate whether the phosphodiester bonds of GpNs are also equivalent to the 3′ splice site in the G-exchange reaction, two experiments involving the

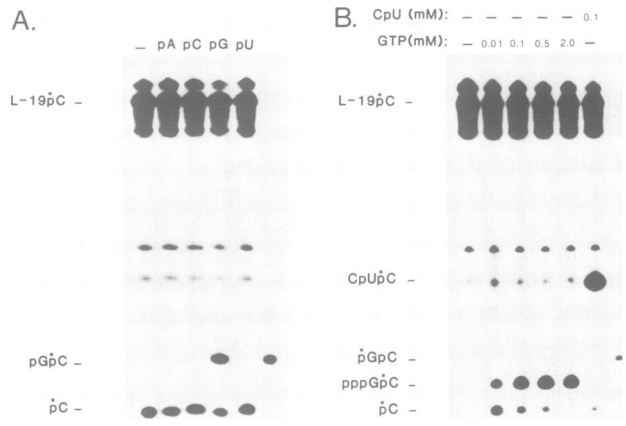

Fig. 5. The G-exchange reaction between a monomeric guanosine derivative and the 3′ splice site. (**a**) L-19 IVS RNA with [5′-^{32}P]pC covalently attached at its 3′ end was incubated with no co-factor, or 2 mM of pA, pC, pG or pU at 42°C for 2 h. [5′-^{32}P]pC shown on the film is a product due to the specific hydrolysis at the artificially created 3′ splice site of the starting material (Inoue *et al.*, 1986). (**b**) L-19 IVS RNA with [5′-^{32}P]pC covalently attached at its 3′ end was incubated in the presence of various concentrations of GTP or 0.1 mM of CpU at 42°C for 2 h. The first lane on the left shows the starting material without incubation. Note that the asterisk indicates the labeled phosphate.

guanosine located at the 3′ end of the IVS RNA were attempted.

L-19 IVS RNA, a linear form of the IVS RNA that lacks the first 19 nucleotides at the 5′ end of the full-length IVS RNA released by self-splicing, was ligated with [5′-^{32}P]pCp and then treated with alkaline phosphatase to create an artificial 3′ splice site. The resulting L-19 IVS p*C (where p* is a ^{32}P-labeled phosphoester bond) was purified and reacted with pA, pC, pG or pU to see whether any mononucleotide could attack at the 3′ splice site (Figure 5a). The result shows that only pG can attack specifically at the artificially created 3′ splice site to produce [^{32}P]pG p*C. The reaction was further examined by varying the concentration of GTP (Figure 5b). Contrary to the G-exchange reaction with GpA, the reaction at the 3′ splice site was not significantly inhibited even in the presence of 2 mM of GTP. This indicates that GTP does not efficiently compete with the guanosine preceding the 3′ splice site, which is presumably predisposed to associate with a guanosine binding site due to its location in the tertiary structure of the IVS.

Based on this and previous experiments, the mechanism of 3′ splice site specification in self-splicing can be summarized as follows. The binding site for the guanosine preceding the 3′ splice site is essential for recognition of the 3′ splice site. The location of the 3′ splice site relative to this guanosine binding site in the tertiary structure of the precursor RNA substantially helps to select this guanosine over others near the 3′ splice site (Kay and Inoue, 1987a; Barfod and Cech, 1988). In addition, recent experiments based on site-directed mutagenesis at and around the 3′ splice site indicate that the sequence around the 3′ splice site is recognized by the IVS RNA and considerably enhances the accuracy of splicing (J.V.Price and T.R.Cech, submitted). Therefore, not all the phosphodiester bonds preceded by

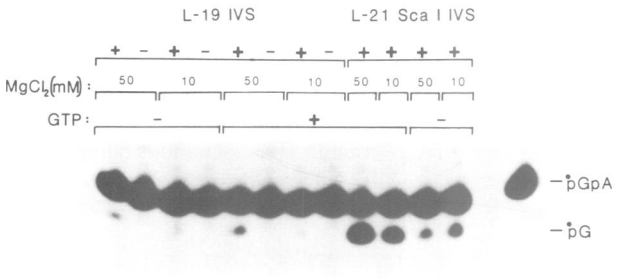

Fig. 6. The G-exchange reaction with L-19 IVS RNA. [5′-^{32}P]pGpA (40 nM) was incubated in the presence of either L-19 IVS RNA (1.0 μM) or L-21 *Sca*I IVS RNA (1.0 μM) with no co-factor or GTP (10 μM) in the presence of either 10 or 50 mM $MgCl_2$ at 42°C for 1.5 h.

guanosine in the precursor RNA are eligible to serve as 3′ splice site equivalents in self-splicing.

The G-exchange reaction employing the 3′-terminal guanosine of the IVS as a nucleophile

Alternatively, we attempted to use the 3′-terminal guanosine of L-19 IVS RNA as a nucleophile in the reaction with [5′-^{32}P]pGpA. Since the 3′-terminal guanosine of the IVS is known as an efficient nucleophile in other IVS-catalyzed reactions, such as cyclization (Zaug *et al.*, 1983), it was of interest to determine whether the 3′-terminal guanosine would be equivalent to GTP in the G-exchange reaction.

The reactions were conducted alongside the standard reaction catalyzed by L-21 *Sca*I IVS RNA (Figure 6). The 3′ end guanosine of L-19 IVS RNA reacted only as a very weak nucleophile with GpA. Furthermore, L-19 IVS RNA catalyzes the G-exchange reaction of GTP with [5′-^{32}P] pGpA in a much less efficient manner than L-21 *Sca*I IVS RNA. The results indicate that the 3′-terminal guanosine of the IVS may compete with pGpA for the same binding site and thereby inhibit the reaction.

In conjunction with our results, it has been reported that GTP and the 3′-terminal guanosine of the IVS do not compete in the transesterification reaction catalyzed by L-19 IVS RNA (Zaug *et al.*, 1986). In this case, the transesterification reaction by GTP, which is equivalent to the first step of self-splicing, proceeds at the same rate whether or not the terminal guanosine has been removed from L-19 IVS RNA. Therefore we suggest that the transesterification reactions proceeding by the nucleophilic attack of the 3′-terminal guanosine, such as cyclization, are equivalent to the reverse of the second step of self-splicing rather than the first step, as was formerly suggested.

Discussion

Two guanosine binding sites in Tetrahymena group I IVS

As demonstrated by our comparative kinetic study, the G-exchange reaction belongs to the same class of IVS-mediated transesterification reactions that accomplish self-splicing. The reaction is not unique to the activity of the *Tetrahymena* IVS. It has also been reported in the catalysis of the mitochondrial group I IVS RNAs of *N. crassa* (Garriga and Lambowitz, 1984) and yeast (van der Horst and Tabak, 1987).

It has been generally considered that only one guanosine binding site in group I IVS RNAs facilitates self-splicing and extensive study to characterize that binding site has been done (Bass and Cech, 1984, 1986). Since it appears that the G-exchange reaction requires two guanosine derivatives, a guanosine serving as a nucleophile and the other guanosine preceding an electrophilic phosphodiester bond, we conclude that two guanosine binding sites exist in group I IVS RNAs. Though at present we do not know whether the two sites are physically separate or share certain component(s) in the tertiary structure of the IVS, it is apparent from our inhibition experiments that they have two functionally distinguishable roles in the transesterification reactions. One binding site which we refer to as the α-G binding site recognizes a guanosine derivative that serves as a nucleophile. The other binding site which we refer to as the β-G binding site recognizes the guanosine preceding the phosphodiester bond which serves as an electrophile.

During the course of our investigation, we examined the G-exchange reaction over a wide range of GTP and GpA concentrations (Figure 3). GTP, acting as a nucleophilic guanosine derivative, was found to serve as a highly efficient inhibitor of the reaction. This inhibition was verified by experiments employing dGTP, which was shown to inhibit the reaction in a competitive manner (Figure 3e). In contrast, GpA, a dinucleotide with a guanosine at its 5′ end, was not able to serve as a critical inhibitor. This difference can be interpreted by hypothesizing that the β-G binding site interacts with only the guanine base of a guanosine derivative. The α-G binding site, we propose, is engaged to another binding site for a ribose moiety and must accommodate both a guanine base and its ribose moiety with a free 3′ OH group. In this way GTP can compete effectively with GpA at the β-G binding site, but GpA cannot significantly inhibit GTP at the α-G binding site.

The idea that a ribose binding moiety exists in conjunction with the guanine base binding site in the IVS RNA has been proposed based on the results obtained from previous studies (Bass and Cech, 1984, 1986). Furthermore, as one of our earlier studies indicated, the nucleophilicity of a monomeric guanosine derivative in self-splicing is greatly affected by modifications at its ribose moiety (Kay and Inoue, 1987b). In that study we concluded that the presence of a 2′ OH group, or its equivalent, is absolutely necessary to make the 3′ OH group, or its equivalent, available as a nucleophile. We therefore suggested that the ribose moiety of a guanosine derivative might play a significant role in the mechanism of group I self-splicing.

A model for the reaction mechanism of self-splicing based on two guanosine binding sites

In all group I IVS RNAs, the guanosine which initiates the splicing, the uridine at the 3′ end of the 5′ exon and the guanosine at the 3′ end of the IVS are conserved features. There are also three characteristic recognition sites in the IVS; (i) the α-G binding site, in conjunction with the ribose binding site, for the nucleophilic guanosine derivative, (ii) the internal guide sequence (IGS) (Waring *et al.*, 1986; Been and Cech, 1986) for the 3′ end of the 5′ exon and (iii) the β-G binding site for the guanosine at the 3′ end of the IVS. It is reasonable to assume that these three binding sites aid in conducting the first and second steps of self-splicing

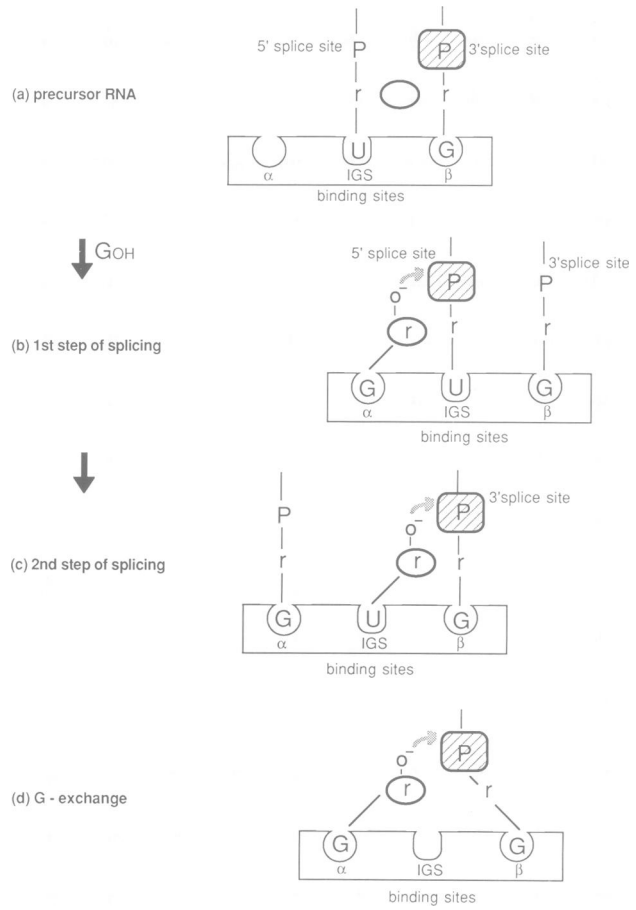

Fig. 7. A model for the two-step self-splicing mechanism and the G-exchange reaction based on the IGS, two guanosine binding sites, a ribose binding site and a single phosphoester bond cleavage site. (**a**) A schematic representation of the local conformation at the active site of precursor RNA. The shaded square-shaped element and the ellipsoidal element represent the P-cleavage site and the ribose binding site respectively. The α-G binding site, the IGS (which accommodates the U preceding the 5′ splice site), and the β-G binding site (which accommodates the G preceding the 3′ splice site) are shown in the element entitled 'binding sites'. G, guanine; U, uridine; r, ribose; P, phosphoester bond. At this stage, the 3′ splice site resides in the P-cleavage site. (**b**) The first step of self-splicing. A nucleophilic guanosine derivative binds to the α-G and ribose binding sites. The local conformation at the active site is changed by the relocation of the three binding sites (α, IGS and β) relative to the P-cleavage and ribose binding sites. This change orients the attack of the 3′ OH group (represented by O^-) of G_{OH} at the 5′ splice site. An arrow indicates the site of the nucleophilic attack. (**c**) The second step of self-splicing. With guanosine now covalently attached to the 5′ end of the IVS, the local conformation at the active site returns to its original form as depicted in (**a**). This allows the newly exposed ribose at the 3′ end of the 5′ exon to occupy the ribose binding site and also positions the 3′ splice site at the P-cleavage site in preparation for exon ligation. (**d**) The G-exchange reaction. A guanosine derivative at the α-G binding site attacks a phosphodiester bond preceded by a guanine that is accommodated by the β-G binding site. Note that the relative position of the reaction site to the binding sites shown here is only one of several possible schemes and the distances between the nucleotides and the bond angles are not necessarily proportional to the actual values.

reaction in a concerted manner. By summarizing the results presented in this report, we now propose a new model for the reaction mechanism of group I self-splicing RNAs which we hope will introduce a new perspective.

In the absence of the 5′ splice site a nucleophilic guanosine derivative such as GTP can perform a transesterification reaction at the 3′ splice site (Figure 5). In contrast, regardless of the presence of the 3′ splice site, GTP specifically attacks at the 5′ splice site in the precursor RNA (Zaug *et al.*, 1986). This indicates an apparent mechanism that specifies the 5′ splice site over the 3′ splice site when a nucleophilic guanosine derivative is bound to the α-G and accompanying ribose binding sites. A logical explanation of this selection mechanism is that one unique reaction site in the IVS (Been and Cech, 1987) activates the phosphodiester bond to be attacked and is involved in all the reactions catalyzed by the IVS. When the phosphodiester bond either at the 5′ or 3′ splice site is to be reacted, that bond must be located at this particular site. We refer to this as the P-cleavage site (phosphoester bond cleavage site). Although numbers of complex models can be conceived, we suggest one simplified model for group I self-splicing based on the P-cleavage site, the IGS, the α-G binding site (accompanied by the ribose binding site) and the β-G binding site (Figure 7).

In the absence of a nucleophilic guanosine molecule the precursor RNA can be hydrolyzed specifically at the 3′ splice site, leaving a 3′ OH group at the 3′ end of the IVS and a 5′ phosphate at the 5′ end of the 3′ exon (Inoue *et al.*, 1986). This IVS-mediated hydrolysis reaction is mechanistically different from that of the alkalkine hydrolysis of RNA and is equivalent to the transesterification reaction in the second step of self-splicing. Thus we propose that the 3′ splice site in the precursor RNA must reside in the P-cleavage site in the absence of a nucleophilic guanosine derivative (Figure 7a).

In the first step of the splicing reaction, a nucleophilic guanosine derivative is bound at the α-G binding site with engagement of its ribose moiety. We postulate that this alters the local conformation at the active locus in the IVS. Guided by the IGS the 5′ splice site now resides at the P-cleavage site and the 3′ hydroxyl group of the guanosine residing in the ribose binding site is positioned to attack the activated phosphodiester bond (Figure 7b). Thus the 5′ splice site is precisely selected as the substrate for nucleophilic attack in the first step of splicing.

When the first step is completed, the α-G binding site is occupied by guanosine that is now covalently attached to the 5′ end of the IVS and no longer in association with the free ribose binding domain. This formation blocks access to the α-G binding site for other nucleophilic guanosine derivatives. In the absence of a free ribose moiety at the ribose binding site, the local conformation at the active locus returns to that of the precursor RNA. The 3′ splice site, directed by the β-G binding site, now occupies the P-cleavage site. Concurrently, the exposed free ribose at the 3′ end of the 5′ exon occupies the ribose binding site to orient its attack at the 3′ splice site (Figure 7c). The use of the same P-cleavage site allows the nucleophilic uridine at the 3′ end of the 5′ exon to perform the transesterification reaction accurately at the 3′ splice site. This model proposes that the two transesterification reactions proceed at a single P-cleavage site in the same manner. If this is the case, the IVS RNA is a sophisticated catalyst in that its mechanism prevents the reverse reaction of the first step, and guarantees that once initiated, splicing proceeds to completion.

Our work shows that GTP is able to perform the G-exchange reaction at the newly created 3′ splice site constructed by the ligation of pC to the 3′ end of L-19 IVS RNA (Figure 5a and b). This indicates that, in the G-

exchange reaction catalyzed by L-21 *Sca*I IVS, the phosphodiester bond of GpN can be regarded as equivalent to the one at the 3′ splice site. Our model can readily explain the mechanism of the reaction by locating the guanosine of GpA at the β-G binding site, where the guanosine preceding the 3′ splice site would primarily reside (Figure 7d). In this reaction system, there is no 5′ splice site or its equivalent, so that GTP bound at the α-G binding site can promote the G-exchange reaction in a highly efficient manner.

Materials and methods

Nucleotides, enzymes and plasmid

Mononucleotides and dinucleotides were purchased from Sigma. [γ-^{32}P]ATP and [α-^{32}P]GTP were obtained from ICN Radiochemicals. Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim. T4 polynucleotide kinase was purchased from US Biochemical Corp. T4 RNA ligase and endonuclease *Sca*I were purchased from New England Biolabs. [5′-^{32}P]pCp was prepared from cytidine 3′-monophosphate and [γ-^{32}P]ATP and utilized without further purification for ligation with L-19 IVS RNA, as described by England *et al.* (1980). [5′-^{32}P]pGpA was prepared as described by Kay and Inoue (1987a). T7 RNA polymerase, prepared and purified as described by Davanloo *et al.* (1984) was provided by G.F.Joyce. pT7TT1A3 (Zaug *et al.*, 1986) was provided by A.J.Zaug.

*Preparation of L-21 Sca*I *IVS RNA and L-19 IVS RNA*

L-21 *Sca*I IVS RNA was prepared from pT7TT1A3 as described by Kay and Inoue (1987a). L-19 IVS RNA was prepared as described by Zaug and Cech (1986).

G-exchange reaction

The standard reaction condition was 50 mM $MgCl_2$, 50 mM Hepes (pH 7.5) at 42°C. The reaction volume was 5–10 μl and 2.0 μM of L-21 *Sca*I IVS RNA was used unless noted. The reactions were stopped by addition of EDTA to 55 mM. The time course reactions were performed without pre-incubation. Aliquots (5 μl) were withdrawn at each time point, instantly frozen at −70°C and then mixed with EDTA.

Acknowledgements

We thank L.E.Orgel and G.F.Joyce for their comments on the manuscript. This work was supported by the National Institutes of Health and the Alfred Krupp von Bohlen und Halbach-Stiftung.

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Received on June 10, 1988; revised on July 20, 1988