Volume 280, number 1, 171–174 FEBS 09483 March 1991

ADONIS 001457939100192B

Mutations in a semiconserved region of the *Tetrahymena* intron

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Received 3 January 1991

The A-rich bulge in paired region P5a of the *Tetrahymena* intron is a structural feature that is conserved in the sub-group Ib self-splicing introns. We have constructed a series of substitution and deletion mutations in this region of the intron. Kinetic analysis has shown that some of the mutants have a reduced maximal extent of splicing, while others have a reduced V_{max}. These mutations could be reactivated to a great extent by spermidine and high Mg^{2+} concentrations. These data are consistent with the hypothesis that the A-rich bulge of P5a has a role in stabilizing the higher-level structure of the ribozyme.

Group I self-splicing intron; Ribozyme; P5 extension

1. INTRODUCTION

The group I introns are defined on the basis of conserved primary sequence elements and a conserved secondary structure [1]. In addition to these universally conserved features, partially conserved elements define two subclasses of group I introns. Group Ia is defined by the presence of additional stems and loops between stems P7 and P3, while group Ib is defined by the presence of a large extension of the paired stem P5, called the L5 element or large P5 extension [2]. These two subgroups are not mutually exclusive, since at least one group I intron has both a large P5 extension and the extra P7 stems and loops [3]. Collins [3] analyzed the large P5 extension in group Ib introns and concluded that there is a high degree of conservation of some structural elements. Two stems P5a1 and P5a2 are formed by the conserved sequence elements J/M and L/K respectively, and an adenine-rich bulge is always present in P5a (Fig. 1). Joyce et al. [4] have shown that deletions of some of these sequences inactive self-splicing in vitro. Latham and Cech [5] have found that the A-rich bulge of P5a is protected from cleavage by the reagent Fe(II) EDTA, implying that it may interact with other portions of the intron.

We have constructed a number of mutations in the A-rich bulge of P5a in the *Tetrahymena* intron. We characterized the effects of these mutations on the rate and extent of self-splicing. Our results suggest that the A-rich bulge helps stabilizing the active conformation of the ribozyme.

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2. MATERIALS AND METHODS

2.1. *Materials*

All chemicals were purchased from Sigma. All nucleotides were from Pharmacia. Ribonucleotides for RNA synthesis were extracted twice with phenol and washed with ether. [α-^{32}P]GTP was purchased from Amersham. Restriction enzymes and T7 RNA polymerase were purchased from New England Biolabs and RNAsin from Promega.

2.2. *Mutagenesis*

Cassette mutagenesis was performed on plasmid pSZ241 [6], which contains the entire sequence of the *Tetrahymena* intron, with some 5′ and 3′ exon sequences, downstream of a T7 RNA polymerase promoter. The A-rich bulge is included in a 64-bp *Bgl*II/*Eco*NI restriction fragment. This fragment was replaced with synthetic oligonucleotides (synthesized in our lab on a Biosearch model 8750 DNA synthesizer by J.A. Doudna and D.R. Green), containing the desired mutation. Every mutant plasmid was verified by DNA sequencing.

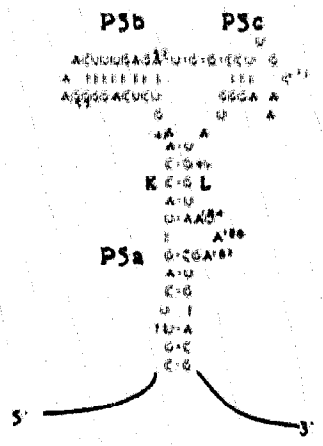
2.3. *Preparation of the RNA*

Internally labelled RNA was prepared by transcribing *Bam*H1-linearized plasmid at 37°C overnight in 100 μl of 40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 2.5 mM each ATP, UTP, CTP and [α-^{32}P]GTP (15 μCi), 10 mM dithiothreitol, 60 U RNAsin and 100 U T7 RNA polymerase. The RNA transcripts were isolated from 4% polyacrylamide/8 M urea gels by cutting out bands visualized by UV shadowing. The RNAs were eluted overnight into 0.8 ml of 0.2 M Tris-HCl, pH 7.5, 25 mM EDTA, 0.3 M NaCl and 0.2% SDS, followed by phenol/chloroform extraction. After ethanol precipitation the RNAs were resuspended in water and stored at −70°C.

2.4. *Self-splicing conditions*

Self-splicing reactions were carried out according to Price and Cech [7]. These conditions reduce circularization of the excised intron, facilitating quantitation of the results.

Each reaction contained 10 000 to 30 000 cpm of internally labelled RNA in 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 200 mM NH_4 acetate and 0.02% SDS. The RNA was incubated at 80°C for 2.5 min, transferred to a 30°C water bath and incubated for at least 20 min. This step was performed in order to allow the RNA to refold to the optimal conformation. The self-splicing reaction was started by ad-



Mutant number	Mutation	Extent of splicing in low Mg (% of WT)
1	Del A181-G182	6
2	Del A181-A183	16
3	A184U	12 ± 1
4	A187G	42 ± 12
5	A187C	69 ± 5
6	A187U	82 ± 16
7	A186G	91 ± 13
8	A186C	15 ± 5
9	A186U	76 ± 26
10	A184G	72 ± 19
11	A184C	9 ± 1
12	A184U	76 ± 2

Fig. 1. Numbering and description of the mutants. The structure diagram and numbering of the bases are according to Burke et al. [2].

ding GTP and stopped by adding one Vol of 2X loading dye followed by heating at 95°C for 3 min. The reaction temperature was 30°. The experiments shown in Fig. 2a were performed in the presence of 2 mM GTP (final concentration) and run for 3 min while those of Fig. 2b (high Mg and spermidine) contained in addition 2 mM spermidine and 15 mM $MgCl_2$. Times and GTP concentrations were varied in order to measure kinetic parameters: for the experiment of Fig. 3a large volume reactions were run (GTP concentration 2 mM) and aliquots of the reactions were mixed with an equal volume of 2X loading dye at the indicated times. For the experiment of Fig. 3b GTP concentrations were varied from 3 to 300 μM, and the reaction time was 1 min.

2.5. *Quantitation of the results*

The reaction products were separated on a 4% denaturing polyacrylamide gel and the bands corresponding to the various products were quantitated on a Betascope two dimensional array detector (Betagen, Waltham, MA). The extent of self-splicing was calculated as the percentage of the linear intron released (for the low Mg^{2+} experiments) or as 100 minus the percentage of the precursor remaining (for the spermidine)/high Mg^{2+} experiments). In order to compare the results, the data were normalized to the wild-type values.

3. RESULTS

Twelve mutations in the A-rich bulge of stem P5a (Fig. 1) were constructed by cassette mutagenesis of plasmid pSZ241. Runoff T7 transcription of *Bam*H1-digested pSZ241 yields a 511 nt RNA; this transcript contains the entire wild-type *Tetrahymena* intron and portions of the flanking exons. It has previously been shown to undergo correct self-splicing and exon ligation reactions [6]. Similar runoff transcripts were prepared from each of the 12 mutant plasmids, and their self-splicing properties were examined under two sets of reaction conditions (Fig. 2). The first set of conditions (Fig. 2a) are the low Mg^{2+} conditions of Price and Cech [7], which minimize cyclization and circle reopening. The second set of conditions (2 mM spermidine and 15 mM Mg^{2+}, Fig. 2b) increase cyclization and circle-re-opening, but have been shown by Joyce et al. [4] to partially revert the effect of large deletions of the P5 region. We found that two deletions of 5

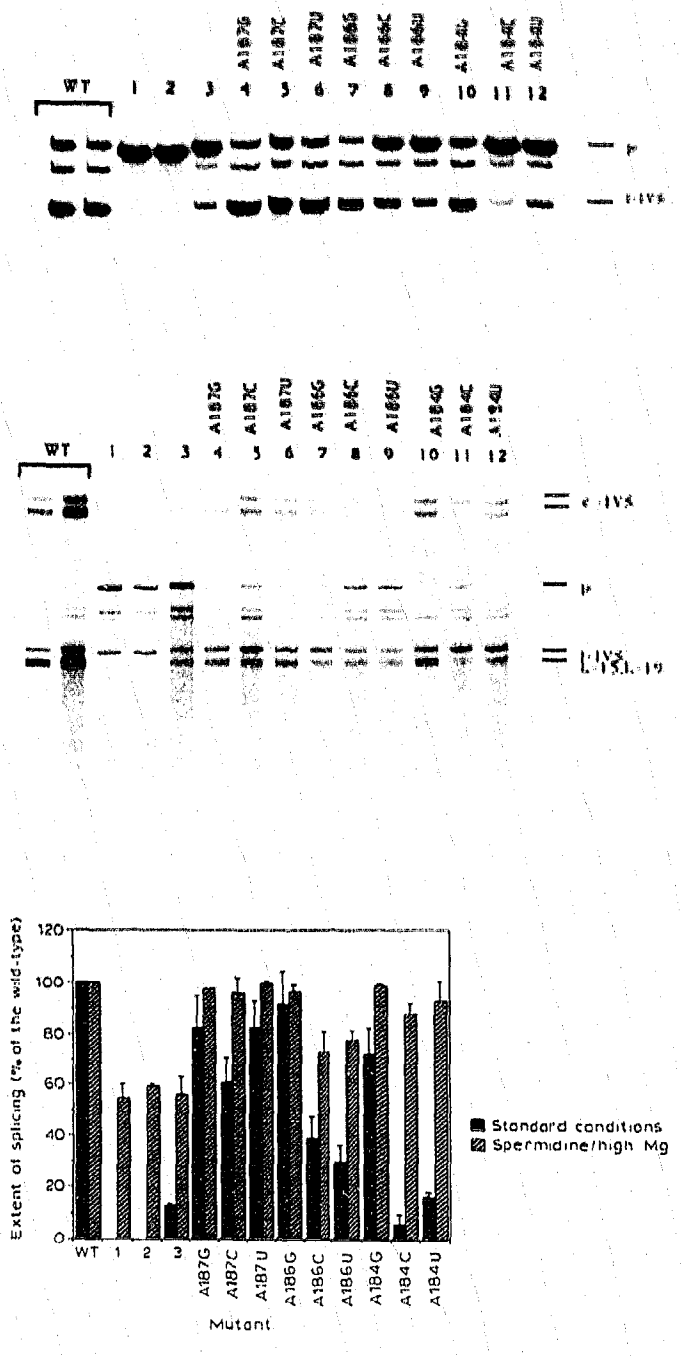


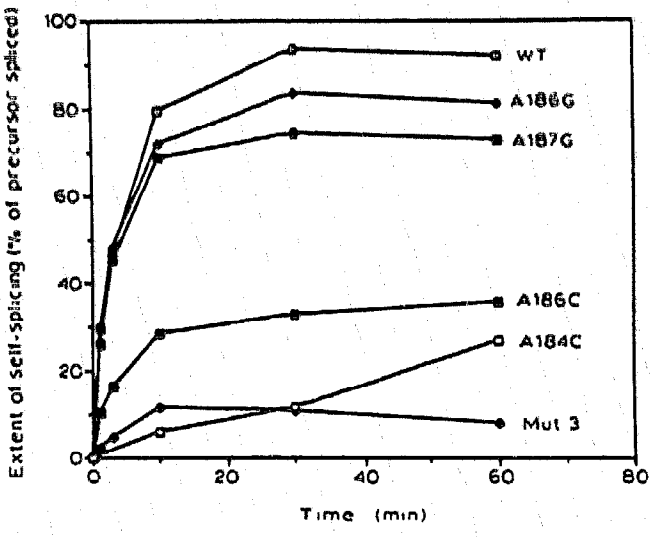
Fig. 2. Self-splicing of the wild-type and mutant introns. (a) Reactions performed in low Mg^{2+} conditions. WT, wild-type; the mutant introns are numbered as in Fig. 1; P, precursor RNA; 1-IVS, linear intron, the major product of the self splicing reaction. The intermediate band corresponds to traces of 5′-exons-IVS, generated by hydrolysis of the precursor at the 3′ splice site. The band corresponding to ligated exons ran near the bottom of the gel and is not shown in this figure. (b) Reaction performed in the presence of spermidine and 15 mM Mg^{2+}. Circular (c-IVS) and shortened forms (L-15, L-19) of the intron are visible in all lanes. Other symbols are as above. (c) Quantitation of extents of splicing (see section 2 for details). Data express the average ± SD of 6 experiments (low Mg^{2+} conditions, 3 different RNA preps) and 3 experiments (spermidine/high Mg^{2+}, 2 different RNA preps).

nucleotides within the A-rich bulge are completely inactive in low Mg^{2+} and splice very poorly even in spermidine and high Mg^{2+}. A mutation in which all three A's of the A-rich bulge were changed to G's spliced to abot 10% of the level of the wild-type transcript in low Mg^{2+}, and to 50-60% of the wild-type level in high Mg^{2+}.

We also examined the effect of changing each of the three A's of the A-rich bulge to each of the three other possible nucleotides (Figs. 1 and 2). A187 is the least conserved position in the bulge [3]. Mutations at this position had only small effects, which were fully reversed by spermidine and high Mg^{2+}. In contrast, A186 and A184 are highly conserved (always A, see [3]), and mutations at these positions have stronger effects. In both cases, pyrimidine substitution has a much stronger effect than the A to G substitution. In all cases, the mutants are largely suppressed by spermidine and high Mg^{2+}, suggesting that the mutations interfere with structure and do not directly affect catalysis.

To determine the maximal extent of self-splicing, we examined time courses of reaction for some of the mutants. Most of the mutants examined splice at a rate that is similar to wild-type, but have a lower final extent of reaction (Fig. 3a), suggesting that a fraction of the RNA molecules are trapped in inactive conformations. The A184C mutation appeared to cause much slower splicing, so that the final extent of splicing could not be determined.

The K_m for GTP and V_{max} of the wild-type and selected mutants were also measured (Fig. 3b, Table I). The values for the wild-type RNA are similar to previously published values [8,9], and most of the mutants examined had only minor changes in these kinetic parameters, suggesting that the fraction of molecules that were correctly folded spliced normally. Again, the exceptional mutant was A184C, with a 5-fold increase in K_m and a 10-fold decrease in V_{max} (Table I).



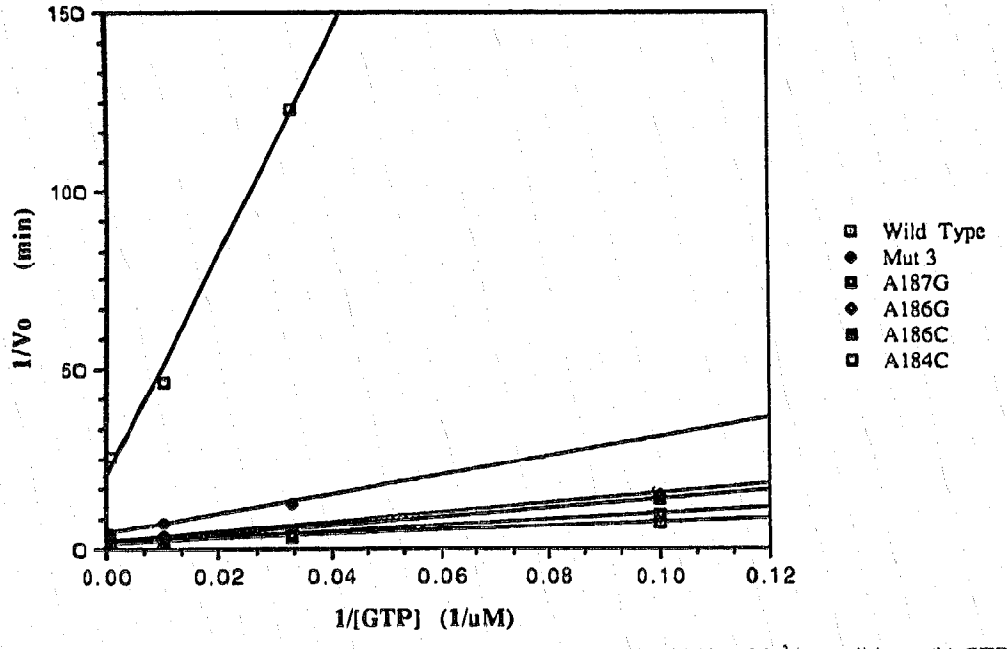


Fig. 3. (a) Time course of splicing reaction for wild-type and selected mutants, determined in low Mg^{2+} conditions. (b) GTP dependence of the activity of wild-type and selected mutants, plotted as double reciprocal plot. The values for V_o were the percentage of precursor converted to 1-IVS per minute, normalized to the maximal level of self-splicing, obtained from the plateau of Fig. 3a. K_m and V_{max} values have been calculated from this plot and are shown in Table I.

Table 1

K_m for GTP and V_{max} for the wild-type and some of the mutants. K_m and V_{max} were calculated from the double reciprocal plot shown in Fig. 3b. In mutant Mut3 all 3 A's in the A-rich bulge are changed to G's.

RNA	K_m (μM)	V_{max} (min^{-1})
WT	32	0.59
Mut3	67	0.25
A187G	22	0.40
A186G	65	0.50
A186C	103	0.83
A184C	158	0.051

4. DISCUSSION

The A-rich bulge of the P5 extension is a feature of the self-splicing *Tetrahymena* intron that is conserved only in a subset of group I introns [3]. Nevertheless, this structural feature appears to have taken on an essential role in the *Tetrahymena* intron. Our results suggest that changes in the A-rich bulge may lead to the appearance of mis-folded and inactive molecules, possibly as a result of the loss of a stabilizing effect of the A-rich bulge on the structure of the folded intron. The role of the A-rich bulge may be filled by other structural interactions in the subclass Ia introns, which lack this structure.

Our results with deletion mutations are consistent with the findings of Joyce et al. [4] that show that removal of the P5 region affects self-splicing in low Mg^{2+} conditions. Our analysis of substitution mutations within the A-rich bulge has shown that the sensitivity of each position parallels the degree of conservation of the various A's. A187 can be replaced with any other nucleotide without strongly inhibiting self-splicing, but that the more highly conserved positions A186 and A184 can only be substituted by G without substantial loss of activity. Spermidine and high Mg^{2+} largely suppress the effects of all of the mutations we examined, suggesting that the mutations destabilize the active intron in a way that can be compensated for by additional salt bridges or charge neutralization. Joyce et al. [4] have also shown that spermidine and high Mg^{2+} partially suppress the effects of extensive deletions spanning most of the P5 extension. The hypothesis that the A-rich bulge acts to stabilize the active form of the intron is supported by our kinetic analysis of the mutants. The most striking difference between the wild-type and the mutants is the lower maximal extent of self-splicing of the mutants, suggesting that a fraction of the molecules become trapped in an inactive conformation. Changes in K_m and V_{max} were small except for mutant A184C, which has a K_m for GTP 5-fold higher than the wild-type and a V_{max} 10-fold lower. These effects could reflect either a less active folded enzyme, or a slow equilibration between an inactive mis-folded form and an active form.

The relatively severe effect of the A184C mutation could be caused by a negative interaction such as the formation of a G:C base pair with G134. This would displace the bulge and move it one or two (if U185 pairs with A133) nucleotides down the P5a stem.

It is unclear how the P5 extension helps stabilizing the catalytic core. Our results suggest that the A-rich bulge may have an important role, since its removal is as effective as the removal of all the P5 extension [4]. Our recent finding that thiophosphate substitution in the A-rich bulge interferes with self-splicing (U.P. and J.W.S., unpublished) is consistent with such a model, as are the observations of Latham and Cech [5] that the backbone of the A-rich bulge is protected from hydroxyl radical attack in the folded intron.

Acknowledgements: We wish to thank Drs Andy Ellington and Jennifer Doudna for comments on the manuscript. U.P. is a recipient of a Chaim Weizmann Postdoctoral Fellowship. This work has been supported by a grant from Hoechst AG.

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