Functional Role of the Internal Guide Sequence in Splicing Activity of T4 Thymidylate Synthase Gene *in vivo*

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The structural and functional roles of IGS element of T4 *td* intron in thymidylate synthase activity *in vivo* were investigated. Site-directed mutagenesis was employed to create mutations of IGS element of T4 *td* intron. When a U·G pair was changed to a U·C pair in the 5′ splice site of P1 stem of *td* intron, the activity of thymidylate synthase was completely abolished whereas the wild type retained the normal activity of enzyme. When U at 12 position within IGS element was changed to C, the activity of thymidylate synthase was approximately 32% of that of the wild type. Comparison of enzyme activities suggests that IGS element within P1 structure is an essential requirement for splicing of *td* gene *in vivo*.

KEY WORDS □ IGS structure, T4 *td* intron, thymidylate synthase activity

The T4 thymidylate synthase gene (*td*), the first procaryotic split gene, possesses a group intron (10) which can undergo self-splicing *in vitro* in the absence of protein and exogenous energy (11, 12). Like the intron of *Tetrahymena thermophila* (8) rRNA gene, T4 phage *td* intron is spliced from the precursor RNA by two-step transesterification (6). The first step involves the nucleophilic attack at the 5′ exon-intron junction (5′ splice site) by guanosine, releasing a cleaved 5′ exon and guanosine added to the 5′ end of the intron-3′ exon intermediate. The second step involves the formation of the ligated 5′-3′ exons and linear intron. The third transesterification joins the intron's 3′ terminal guanosine to a nucleotide near the 5′ end of intron to yield a circular intron, releasing an oligonucleotide containing the added guanosine.

The T4 *td* intron contains a core structure common to most group introns for self-splicing activity. The basic core structure is comprised of nine paired segments (P1-P9), some of which are stem-loop structures such as P1, P2, P4, P5, P6, P8 and P9. In addition, the presence of base-paired consensus segments has been established by data obtained from site-directed mutagenesis studies of *Tetrahymena* rRNA intron (4). In the latter case, secondary structural elements P3, P4, P6, P7 and P8 are shown to be essential in the formation of the structural core of the ribozyme which may be involved in the splicing activity intrinsic to group I intron RNAs (2, 5, 7). One or more additional stem-loop structure is present in P2 and P3, P7 and P3[3′], and/or after P9 in a subgroup 1A. The T4 phage *td* intron belongs to this subgroup 1A.

Another essential feature of these introns is the requirement for an IGS (internal guide sequence) at a position proximal to the 5′-end of the intron and is postulated to form the base-pairing with a 3′-end of the upstream exon sequence very close to it, forming P1, which is believed to facilitate the accurate exon ligation (26). Furthermore, the IGS region is the least conserved among group I introns, because it forms the base-pairing with a complementary region in the upstream exon sequence, each of which possesses its own structural identity. The IGS has also been suggested to form P10 by base-pairing with the 5′-end of the downstream exon so that the ends of the exons are brought to within the distance of a single phosphodiester bond.

The mutational analysis of group I introns would suggest that the intact maintenance of P1 structure is crucial for splicing of the *Saccharomyces cerevisiae* mt COB4 intron *in vivo* (14, 22) and for self-splicing of the *T. thermophila* nuclear LSU rRNA intron *in vitro* (3, 27).

The expression and regulation of T4 thymidylate synthase gene *in vivo* has been suggested to be closely associated with the splicing activity (13). Therefore, in the present study, we attempted to define the functional importance of IGS structure in T4 thymidylate synthase activity

in vivo.

MATERIALS AND METHODS

Bacterial strains, phages and plasmids

Escherichia coli strain TG1, obtained from Amersham, was employed for the propagation of M13mp8 recombinant phage. M13mp8 phage was from Bethesda Research Laboratories. The 2.85 kb *Eco*RI fragment containing *td* gene was subcloned into M13 vector (Promega Biotec) to generate M13 *td* (11).

Enzymes and chemicals

Restriction enzymes *Eco*RI and *Hin*dIII were obtained from New England Biolabs. *Exo*III nuclease, *Nci*I, lysozyme, DNA ligase, and DNA polymerase were from Bethesda Research Laboratories. [γ-^{32}P]ATP and [α-^{35}S]dATP were from Amersham.

Site-directed mutagenesis

Oligonucleotide-directed mutagenesis using single-stranded recombinant M13 template was performed using a single mutagenic primer oligonucleotide (25). The mutagenic oligonucleotide was annealed to the single-stranded M 13 DNA containing *td* gene and extended by DNA polymerase in the presence of dCTPαS and ligated by T4 DNA ligase. The unwanted single-stranded template was effectively removed by passing through a nitrocellulose filter. Single-strand nicks were generated by the treatment of restriction enzyme *Nci*I. *E. coli* exonuclease III was used to digest away all or part of the non-mutant sequence from the site of mutation. The mutant DNA strand is then employed as a template to reconstruct the double-stranded closed circular molecule by DNA polymerase I and T4 DNA ligase, thus creating a homoduplex mutant DNA.

Transformation

Two m*l* of overnight culture was inoculated into TBYE medium (10g Bacto tryptone, 5g yeast extract and 5g NaCl per 1 *l*) in 250 m*l* flask and grown until OD_{550} reached about 0.3. These log phase cells were resuspended in 1/2 volume of sterilized 50 mM $CaCl_2$ pre-chilled on ice and left on ice for 20 min. After centrifugation at 3000 $\times$g for 2 min, cells were resuspended in 1/10 volume of cold 50 mM $CaCl_2$. Twenty μl of ligated mutant DNA was mixed with 300 μl of competent TG1 cells and left on ice for 40 min. Heat shock was done on cells at 42°C for 3 min and cells were placed on ice for 5 min before plating out. Transformants were poured onto the lower agar plate and incubated at 37°C overnight.

Preparation of single-stranded DNA

Mutant plaques were transferred into 1 m*l* TBYE medium along with 3 drops of TG1 cells (0.25 OD_{650}/m*l*) and grown at 37°C for 5 h. Then cells were harvested and supernatants and pellets were saved for DNA preparation and thymidylate synthase activity assay, respectively. The phage supernatant was mixed with 0.2 volume of 20% polyethylene glycol 6000 and left at 40°C for 1 h. The phage pellet obtained by centrifugation was resuspended in TE buffer. Equal volume of phenol/chloroform was added and vortexed for 20 s and allowed to stand for 15 min at room temperature. After vortexing for 15 s, the solution was centrifuged for 5 min and the upper aqueous phase was mixed with 1/20 volume of 3 M sodium acetate and 2 volume of ethanol. The sample tube was placed in a dry ice and ethanol bath for 20 min and DNA pellet was obtained by centrifugation for 15 min. After washing the pellet with cold ethanol, it was dissolved in small volume of distilled water.

Dot blot hybridization analysis

DNA samples (0.3 μg) were electrophoresed in a 0.8% agarose gel in TBE buffer [100 mM Tris-HCl (pH 8.3), 100 mM boric acid, and 2 mM EDTA] and then blotted onto a nitrocellulose filter by the procedure of Southern (1975). The oligodeoxynucleotide was labeled with ^{32}P at the 5′ end in the presence of [γ-^{32}P]ATP and T4 polynucleotide kinase. DNA was spotted on Hybond nylon membrane in 1 μl volume. Pre-hybridization was carried out at 48°C for 5 h in the presence of salmon sperm DNA (0.1 mg/m*l*) and hybridization at 48°C for 16 h in the presence of ^{32}P-labeled probe (10^6 cpm/m*l*). The membranes were subjected to autoradiography after exhaustive washing in buffer.

Thymidylate synthase assay

Thymidylate synthase activity was measured by the ^{3}H release method of Robert (23). Cell pellets were resuspended in 70 μl of TE buffer along with addition of 1 μl of 1 M dithiothreitol and 3 μl of lysozyme (10 mg/m*l*). After freezing and thawing the resuspended cells twice, the final cell lysates were obtained from the addition of 4 μl of 1 M $MgCl_2$ and DNase. Aliquots of the sample were incubated with substrate mixture containing 100 mM Tris, pH 7.1, 10 mM formaldehyde, 1 mM L-tetrahydrofolate, 200 mM mercaptoethanol, 20 mM sodium ascorbate, 50 mM NaF, 100 mM $MgCl_2$ and 0.1 mM [5-^{3}H]dUMP (1×10^5 cpm/nmol). After 30 min at 37°C, the reaction was terminated by addition of Norit A in 2% trichloroacetic acid. Following centrifugation in a microfuge for 2 min, radioactivity of 100 μl of supernatant was counted in 4 m*l* of Aquasol (New England Nuclear).

Protein determination

Protein concentration was estimated by the method of Lowry *et al.* (18) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Thymidylate synthase (EC 2.1.1.45) is a key

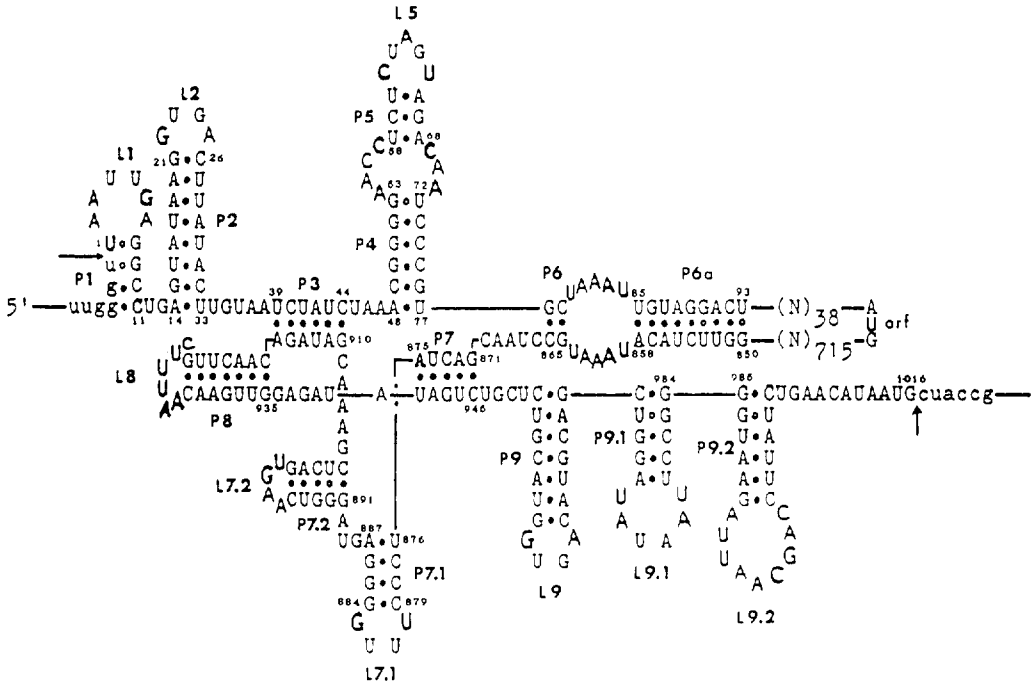

Fig. 1. *Proposed secondary structure of phage T4 td intron RNA.*
Arrows indicate the 5′ end and 3′ end splice sites. Numerical values are numbered from 5′ end of intron. Intron bases are in upper case letters and exon bases in lower case letters.

enzyme in DNA synthesis, responsible for formation of dTMP from dUMP in the *de novo* biosynthetic pathway (13). The enzyme is essential for survival of proliferating cells that are not provided with alternate sources of thymidine. Intron containing genes have been extensively reviewed in eucaryotes, and the expression of these genes involves the processes of intron cleavage and exon ligation, usually known as RNA splicing which can be either small nuclear ribonucleoprotein particle (snRNP) dependent (19) or autocatalytic (15).

Fig. 1 shows the proposed secondary structure of *td* RNA intron and flanking exons constructed on the basis of predicted models of group I introns (4). The basic core structures are P1 to P9.2 and L1 to L9.2 which represent stems and loops, respectively. The finding that the intron excision and exon ligation for thymidylate synthase gene expression *in vitro* occurs in an autocatalytic fashion indicates that the information for the RNA processing is contained within the nucleotide sequence of the primary transcript. To determine if the self-splicing reaction could be correlated with secondary structures intrinsic to self-splicing RNA, we looked for the functional importance of splicing in the alignment of 5′ splice site by an internal guide sequence consisting of 9 nucleotides (5′-AGGCCUGAG-3′), which are 6 nucleotides downstream of the 5′ splice site.

Thus the mutagenesis, outlined in Fig. 2, was such that the point mutation was achieved mainly by substitution such as G8C and U12C within IGS element. G8C and U12C indicate a G to C and U to C mutation in the intron at positions 8 and 12, respectively. These mutations were created using mutagenic oligodeoxynucleotides which were designed so that regions to be mutated were situated in the middle of oligomers (Table 1).

Agarose gel elctrophoresis of wild type and mutant DNAs revealed a discrete band. Each sample was simultaneously run on 0.8% agarose gel and the substitution did not affect the mobility to any extent (data not shown). Figure 3 shows the hybridization results of wild type and IGS mutant DNAs with their respective oligonucleotide probes. Radioactivity-labeled oligonucleotides were employed as probes to screen for corresponding sequences. All mutagenic oligonucleotides hybridized to their respective mutant DNAs but failed to hybridize to wild type DNA. This suggests the presence of significant base pairing between mutant DNAs and their respective probes.

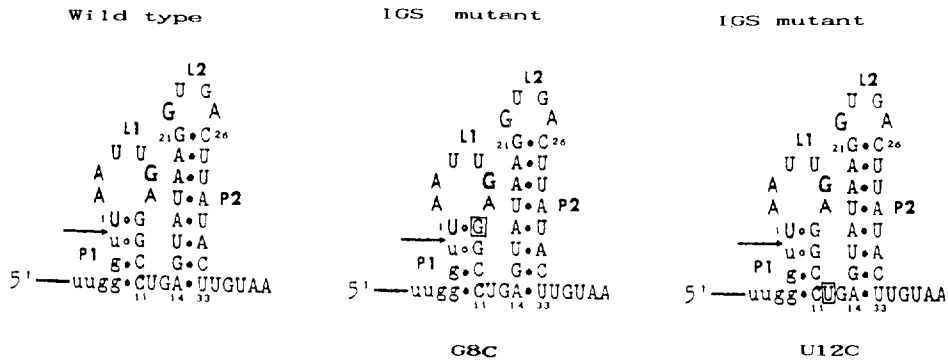

Fig. 2. *Constructed mutants of IGS structure within td intron RNA.*
Boxes indicate base substitutions. G8C represents the change of G to C and U12C the change of U to C.

Table 1. *Site-specific mutations of IGS structure in T4 td intron.*
The nucleotide point mutations (underscored) are situated in the middle of mutagenic oligodeoxynucleotide.

Oligodeoxynucleotide	Target sequence
5′-CTTATACTCAGGOGTCAATTAACC-3′ (24mer)	G8C ($G^{8}\rightarrow C$)
5′-CTTATACTCGGGCCTCAATTAACC-3′ (24mer)	U12C ($U^{12}\rightarrow C$)

Table 2. *Effect of IGS mutation of td intron gene on thymidylate synthase activity in vivo*

Intron	Corrected[a] ^{3}H dpm	Corrected ^{3}H dpm/min/μl	^{3}H dpm/min mg protein	TS[b] activity nmol/mg protein
Wild type	19,690	2,422	4,844	3.62
IGS mutant (G8C)	none	none	none	none
IGS mutant (U12C)	5,120	631	1,295	1.16

[a]Correction was obtained subtracting the background count 3,994 dpm.
[b]Thymidylate synthase.

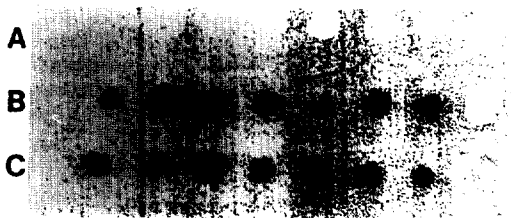

Fig. 3. *Hybridization analysis of mutant IGS DNAs of T4 td gene with mutagenic deoxyribonucleotide probes.*
A, wild type; B, IGS mutant (G8C); C, IGS mutant (U12C).

The alteration of IGS structure of *td* intron and its consequent effect on thymidylate synthase activity *in vivo* are presented in Table 2. As a preliminary screening method for splicing defective mutants, we assayed thymidylate synthase activity from phage-infected cells. As expected, the wild type showed the normal enzyme activity (3.62 nmol/mg protein). When a U · G pair was changed to U · C pair in the 5′ splice site of P1 stem structure of *td* intron, the activity of thymidylate synthase *in vivo* was completely abolished. The alteration of G to C would result in a partial disruption of base pairing at U · C position. The 5′ splice site of

a primary transcript of all known group I introns is usually preceded by a U that is base-paired to a G in the IGS. This conserved U · G pair is almost invariant among group I introns examined, forming part of P1 element (17). Thus it appears likely that the maintenance of the intact U · G pair is crucial for 5′ splice site activity. Using site-directed mutagenesis and analysis of the rate and accuracy of splicing activity of *T. thermophila* group I intron, it was found that both the U and the G of the U · G base pair are critical for the first step of self-splicing *in vitro* which occurs by the nucleophilic attack of GTP at the 5′ splice site (1).

However, the activity of thymidylate synthase was approximately 32% of that of the wild type when U at 12 position within IGS element was changed to C. Even if U is not directly involved in the formation of base-pairing of both P1 and P2 structures, it appears that this particular base bridging between P1 and P2 structures may exert some influence on splicing activity *in vivo*. Thymidylate synthase activity results suggest that P1 pairing may exist *in vivo* and is essential for splicing of *td* intron RNA. The loss or reduction of enzyme activity could be associated with changes in the formation of the active core structure in *td* intron (26).

This supports the tentative suggestion that the precise nucleotide sequence as well as the intact base pairing of IGS can be a crucial element for the proper splicing activity *in vivo*. Furthermore, mutations in other regions such as P2 and P6a of the intron also resulted in either loss or reduction of thymidylate synthase activity (20, 21). In the case of non-directed mutagenesis, thirteen of the 100 *td* mutants were mainly localized to 5′ splice site and gave rise to the functionally inactive thymidylate synthase production, which is indicative of splicing defectiveness (9).

Taken altogether, it can be speculated that the intact IGS is a very important structural element for the accurate cleavage at 5′ splice site. Thus, the functional analysis of splice products generated from mutations will shed light on the better understanding about the involvement of IGS in splicing mechanism.

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초 록: T4 티미딜산 생성효소 유전자의 Splicing 활성에 있어 Internal Guide Sequence 구조의 기능적 역할

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T4 *td*(티미딜산 생성효소를 발현하는 유전자) intron의 IGS 구조가 *in vivo* 티미딜산 생성효소 활성에 미치는 영향에 관하여 조사하였다. 자리-지정 돌연변이를 이용하여 IGS 구조내 뉴클레오티드를 치환 돌연변이 시켰다. 5′ Splice site 근접한 부위에 있는 P1 구조내 U · G pair를 U · C pair로 돌연변이 시켰을 때 효소의 활성은 완전히 상실되었다. IGS 구조내 위치하면서 P1과 P2 구조 사이에 있는 U(intron의 5′ end로 부터 12번째 위치)를 C로 돌연변이 시켰을 때 효소의 활성은 야생형에 비하여 32%를 나타냈다. 이는 IGS의 온전한 구조가 *in vivo*에서 *td* 유전자 발현에 필수적인 요인임을 의미한다.