Characterization of the restriction site of a prokaryotic intron-encoded endonuclease

(T4 bacteriophage/td intron open reading frame/hybridization/dideoxynucleotide sequencing/gene conversion)

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ABSTRACT The 1016-base-pair (bp) intron in the T4 bacteriophage thymidylate synthase gene (td) contains a 735-bp open reading frame that encodes a protein product with endonucleolytic activity. The endonuclease shows specificity for the intronless form of the td gene. Highly purified endonuclease cleaves the DNA of the intronless form of the td gene in vitro at 24 bp upstream of the exon 1–exon 2 junction, generating a 2-base staggered cut with 3' hydroxyl overhangs. Although the endonuclease cleaves in exon 1, it requires some exon 2 sequence for recognition. The maximum recognition sequence lies in an 87-bp stretch, from 52 bp upstream to 35 bp downstream of the cleavage site, ending at 11 bp into exon 2. The td intron endonuclease appears involved in the conversion of the intronless form of td to intron-containing td gene in the T-Even phases. A role for intron mobility is discussed.

Introns are ubiquitous in the eukaryote kingdom, but only a few have been found in prokaryotic genes. Apart from the presence of small introns in some tRNA genes in archaeabacteria (1), three introns of prokaryotic origin have been found in the T-Even phases (2–8), and one such intron has been found in the DNA polymerase gene of the Bacillus subtilis phage SP01 (9). Three genes in the T4 phase—td, nrdB (5, 6), and sunY (7, 8)—have been shown to be interrupted by a group I intron that contains an open reading frame, in addition to specific sequence determinants characteristic of group I introns (10, 11), described in Tetrahymena thermophila and later in the mitochondria of many yeasts and fungi (for review, see ref. 12).

Group I introns, including those in the T4 phase, are able to self-excite in vitro concomitant with exon ligation in the presence of a guanosine cofactor and Mg++ (13, 14). Unlike some yeast and fungal introns, the reading frames in these phase introns do not encode mRNA maturases (15–17) that facilitate intron excision at the RNA level. However, similar to other yeast and fungal introns, the phase td intron open reading frame (iORF) encodes an endonuclease (18) that has been genetically implicated in promoting intron mobility among the T-Even phase td genes (19, 20).

The best characterized intron endonuclease to date is that derived from the group I intron (ω+I) of the mitochondrial 21S rRNA gene of Saccharomyces cerevisiae (21, 22), where the product of a 235-codon-long iORF, termed α transposase, was shown to effect a double-strand break in the intronless (ω−I) copy of the same gene (21). This cut site consists of a 4-base staggered cut with 3' hydroxyl overhangs and occurs at the junction between two exons where the intron is eventually inserted. The recognition sequence for this enzyme is shown to extend over 18 base pairs (bp), centered around the cleavage site. For the T4 phase td gene, the protein produced from a 735-bp open reading frame (3) in its 1016-bp intron (2) also possesses double-stranded endonucleolytic activity specific for the intronless copy of the td gene (tdΔ1) (18, 20). With highly purified preparations of the td intron endonuclease, we have determined its cleavage site to be upstream of the exon 1–exon 2 junction. In this paper, we show that the endonuclease effects a cleavage that is centered at 24 bp upstream of the exon fusion site in the tdΔ1 DNA, creating a staggered cut with a 2-base hydroxyl overhang at the 3' end of each DNA strand; the endonuclease recognition sequence spans an 87-bp region, from 52 bp upstream to 35 bp downstream of the cleavage site in the tdΔ1 gene.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Enzymes, and Chemicals. Escherichia coli strains DH5α (19), JM103, and TB-1 were from Bethesda Research Laboratories. Plasmid pET3c, CE6A phage, and their respective hosts, HMS174 and ED8739, were supplied by F. W. Studier (Brookhaven National Laboratory, Upton, NY) (23, 24). Recombinant plasmids pKtdΔ1 and pUCΔΔ1, containing tdΔ1, have been described (see refs. 25 and 18, respectively). The following enzymes were obtained from United States Biochemical and used according to directions: T4 DNA ligase, T4 polynucleotide kinase, and T7 DNA polymerase. Restriction enzymes were purchased from several suppliers and used according to the suppliers' instructions. [γ-32P]ATP (>5000 Ci/mmol; 1 Ci = 37 GBq) and deoxyadenosine-5'-triphosphate (>4000 Ci/mmol) were from Amersham. The oligodeoxynucleotides were chemically synthesized by using an Applied Biosystems model 381A DNA synthesizer.

Preparation of iORF Protein. Induced cells [E. coli strain HMS174 harboring the pETdiORF (pETtdlfr) plasmid] were processed, as described by West et al. (18). The endonuclease released from the inclusion bodies was renatured according to the procedure of P. C. Babbitt (University of California at San Francisco; personal communication). This entailed solubilization of the isolated inclusion bodies in 6 M guanidine hydrochloride, followed by resolution on a Bio-Gel column in 4 M guanidine hydrochloride and refolding by dialysis against 20 mM potassium phosphate, pH 7.5/0.5 M NaCl/20 mM 2-mercaptoethanol. Purification to near homogeneity was obtained by chromatography on a column of phenyl-Sepharose. A detailed description of the purification procedure will be presented elsewhere.

Analysis for Endonuclease Activity. The reaction buffer contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 100 mM

Abbreviations: iORF, intron open reading frame (iORF protein and endonuclease are used interchangeably in this work); tdΔ1, intronless copy of the td gene.

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NaCl, and 10 mM dithiothreitol. The reaction was performed in a volume of 10 µl containing 0.1 pmol of DNA and 2 µg of iORF protein at 23°C for 5–10 min, stopped by the addition of 1 µl of 0.4 M Na₂EDTA (pH 7.5) and then chilled on ice. The products were electrophoresed in 0.8% agarose slab gel in TBE buffer (0.1 M Tris base/0.1 M boric acid/2 mM Na₂EDTA) and visualized by ethidium bromide staining.

**Blotting and Hybridization.** After electrophoresis, the DNA in the agarose slab gel was denatured in solution A (1.5 M NaCl/0.5 M NaOH) at 23°C for 30 min, neutralized in solution B (0.5 M Tris-HCl, pH 7.2/1.5 M NaCl/0.01 M Na₂EDTA) at 23°C for 30 min, and then transferred onto Hybond-N membrane (Amersham) by capillary blotting at 23°C for 16 hr. The membrane was then rinsed briefly in distilled water and allowed to air dry. The transferred DNA was fixed to the membrane by UV irradiation for 5 min. Hybridization with ³²P-labeled oligodeoxynucleotides (1 × 10⁵ cpm/ml of hybridization mixture) was carried out in solution C (0.05 M sodium phosphate, pH 7.7/0.9 M NaCl/0.5 mM Na₂EDTA/5 × Denhardt’s solution/0.5% SDS) (1 × Denhardt’s solution is 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin) at 60°C for 2 hr and then cooled to room temperature. The membrane was washed at 23°C, twice with solution D (solution C without Denhardt’s solution and SDS) and once with 0.2 × solution D, each time for 20 min. The membrane was then air dried and subjected to autoradiography.

**DNA Sequencing.** The dideoxynucleotide chain-termination method (26) was used for sequencing of double-stranded DNA templates. The reagents for DNA sequencing with Sequenase were purchased from United States Biochemical and used according to the supplier’s protocol. Where unlabeled primer was used, deoxyadenosine [α-³²P]thiotriphosphate was employed. Where ³²P-labeled (5') primer was used, deoxyadenosine [α-³²P]thiotriphosphate was replaced by dATP.

**Restriction of cDNA Products.** To determine the restriction of cDNA products by the endonuclease, half of the sequencing reaction (=3 µl) was heated at 65°C for 10 min to inactivate the Sequenase and then slow-cooled (=30 min) to room temperature to allow renaturation of DNA complementary strands. After 10-sec centrifugation in a microcentrifuge to collect moisture, 2 µg of the endonuclease was added, followed by 5-min incubation at 23°C. A one-tenth volume of 0.4 M Na₂EDTA (pH 7.5) was added, and the incubated mixture was heated at 80°C for 3 min and then subjected to electrophoretic analysis in 8% polyacrylamide/6 M urea gel.

**RESULTS**

**Localization of the Restriction Site of td Intron-Encoded Endonuclease.** The 735-bp iORF of the T₄ phase td gene has been shown (3) to encode a protein endonuclease that effects a specific double-strand break at or near the exon junction in tdAI (18, 20). To define the limits of the break, we have synthesized several oligodeoxynucleotides and labeled their 5' ends with ³²P. The radioactive oligodeoxynucleotides, complementary in sequence to regions progressively upstream and downstream of the exon 1–exon 2 junction (see Table 1 for their size, designation, and location), were used as hybridization probes for the endonuclease-generated products of tdAI DNA. In the endonuclease-cuts the EcoRI-linearized tdAI DNA (4.26-kilobase-pair (kb) fragment A) at the junction of exon 1 and exon 2, the resulting products, consisting of a 3.5-kb exon 2-containing fragment B and a 0.76-kb exon 1-containing fragment C (Fig. 1 A and B), should not hybridize to the exon 1–exon 2 junction sequence represented by probe 1. To our surprise, fragment B, and not C, hybridized strongly to probe 1 (Fig. 1C), indicating the presence of some exon 1 3'-end sequence in the larger exon 2-containing fragment. This presence was confirmed by the observed hybridization of fragment B to probe 5, representing the last 21 bases in exon 1 and corroborated by hybridization data showing that the 3' end of exon 1 (at least 21 bp in length) is absent in the smaller exon 1-containing fragment C. On the other hand, fragment B contains, in addition to the 3' end of exon 1, the entire exon 2, as determined by its hybridization to probes 6, 7, and other probes.

**Table 1. Synthetic oligodeoxynucleotides**

<table>
<thead>
<tr>
<th>Probe designation</th>
<th>Exon location</th>
<th>Size (bp)</th>
<th>Nucleotide position</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Exon 1-2</td>
<td>18</td>
<td>541–558</td>
</tr>
<tr>
<td>2*</td>
<td>Exon 1</td>
<td>18</td>
<td>453–470</td>
</tr>
<tr>
<td>2+</td>
<td>Exon 1</td>
<td>13</td>
<td>490–502</td>
</tr>
<tr>
<td>3</td>
<td>Exon 1</td>
<td>21</td>
<td>516–536</td>
</tr>
<tr>
<td>4</td>
<td>Exon 1</td>
<td>21</td>
<td>529–549</td>
</tr>
<tr>
<td>5</td>
<td>Exon 2</td>
<td>13</td>
<td>555–567</td>
</tr>
<tr>
<td>6</td>
<td>Exon 2</td>
<td>16</td>
<td>591–606</td>
</tr>
</tbody>
</table>

*Nucleotides are numbered from the initiation codon (ATG) in tdAI, which is 858 bp in length (2, 25).

1Exon 1 and 2 designation pertain to the exon topography in the intron-containing td gene, in which exon 1 runs from nucleotide 1 through 549, exon 2 runs from nucleotide 550 through 858, and the exons are separated by a 1016-bp intron (2, 27).

2This oligodeoxynucleotide contains the exon mRNA sequence, in contrast to the others, which all contain anticodon (anti-mRNA) sequences.

![Fig. 1](image-url)  
**Fig. 1.** Mapping of the cleavage site: hybridization analysis. (A) Schematic diagram showing the generation of linear double-stranded DNA substrate (pUCtdAI) for the td intron-encoded endonuclease. Short arrow at exon junction indicates the anticipated endonuclease lytic site; ex: exon; int: intron; amp: ampicillin resistance gene. (B) Products of cleavage by the endonuclease. Lane 1 shows the EcoRI-linearized pUCtdAI DNA substrate (fragment A), and lane 2 shows the resulting products of the endonuclease (fragments B and C). (C) Oligonucleotide hybridization analysis of the cleavage site. Six sets of substrate and products shown in B were resolved by electrophoresis in an 0.8% agarose horizontal slab gel. They were then blotted onto Hybond-N membrane (Amersham), and each subset was subjected to hybridization analysis by using a specific oligodeoxynucleotide labeled with ³²P in its 5' end as probe. The size of tdAI DNA fragments was estimated from a marker lane containing HindIII-restricted A DNA fragments. Ex1 and Ex2, exon 1 and 2, respectively.

*See Table 1 for description of oligodeoxynucleotide probes.

1+, Hybridizing; -, nonhybridizing.
exon 2 probes not shown. Analysis of the 3' limit of fragment C and 5' limit of fragment B by hybridization to exon 1 probes (probes 2 and 3 in Table 1) corresponding to nucleotide sequences further upstream of the exon junction suggests the site of endonucleolytic cleavage to fall between bp 502 (present in fragment C) and 529 (present in fragment B), which specify codons 168-176 in the tdAI gene. Another recombinant plasmid pKtdAI (25), constructed by cloning the tdAI gene fragment into pUC8 (28), exhibits identical probe-specific hybridization characteristics in its products after treatment with the td intron endonuclease (data not shown).

The td Intron Endonuclease Generates a 2-Base Staggered Cut with 3'-Hydroxyl Overhangs Centered at 24 bp Upstream of the Exon Junction. To determine the precise point of cleavage by the td intron endonuclease, we synthesized 5'-end-labeled double-stranded substrates in vitro by priming the double-stranded DNA from recombinant plasmid pUCtdAI with 5'-end-labeled oligodeoxynucleotide (probes 2 and 7 in Table 1). The cDNA was synthesized in the presence of the 4 dNTPs (mixture of dATP, dCTP, dGTP, and dTTP) with and without the individual dideoxynucleotide chain terminators. Probe 2, which contains exon 1 sequence located at 32 bases upstream of bp 502, uses the coding strand as template and is elongated in the exon 2 direction, whereas probe 7 containing exon 2 sequence at 62 bases downstream of bp 529 copies the noncoding strand towards the exon 1 region. In each case, the double-stranded DNA product, consisting of an unlabeled template strand and a 32P-labeled cDNA strand produced in the absence of dideoxynucleotide chain terminators, was used as substrate for the endonuclease. The products were denatured and electrophoresed in an 8% polyacrylamide/6 M urea gel alongside the respective sequence ladder (the four dideoxynucleotide-containing cDNA reactions), which was used also as a size marker. Fig. 2A shows the endonucleolytic cleavage site on the noncoding (mRNA-like) DNA strand to be right of bp 526, yielding a 74-base product; Fig. 2B reveals this site to be left of bp 525 on the coding (mRNA-complementary) strand, giving an 82-base product. Taken together, the sequencing results allow us to conclude that the endonuclease cleaves each strand at a specific position, two bases apart, to produce a 3' overhang (Fig. 2C). The cut also results in hydroxyl groups at the 3' protruding ends and phosphoryl groups at the 5' recessed ends, as deduced from the observed ligation of the cut ends by T4 DNA ligase (data not shown). Two additional lines of experimental evidence confirm the location of the endonucleolytic cut in the tdAI gene. (i) By using pUCtdAI DNA precut by the endonuclease as template and primed with the same exon-1 and exon-2 probes, runoff synthesis resulted, in each case, in a cDNA fragment that was 2 nucleotides shorter than that in the previous experiment: 72 nucleotides long instead of 74 when primed with probe 2 on the coding strand (Fig. 3A); and 80 nucleotides instead of 82 when primed with probe 7 on the noncoding strand (Fig. 3B). This result is to be expected when a 2-base 3' overhang exists at the cleavage site. (ii) A 21-mer oligodeoxynucleotide (Table 1 probe 4) that spans the nucleotide sequence centered at the observed endonucleolytic cut in exon 1 failed to hybridize to pUCtdAI DNA pretreated with the endonuclease (Table 2). In this instance, the truncation in the tdAI sequence greatly destabilized its interaction with probe 4, leading to loss of the probe during stringent washing. The data in Table 2 also show that the double-stranded pUCtdAI DNA, which contains the 1016-bp intron juxtaposed between exon 1 and exon 2, and the single-stranded M13tdAI DNA, containing the noncoding tdAI sequence, are not substrates for the endonuclease. These results reemphasize both the sequence and double-strand specificities of the enzyme. Determination of the Recognition Sequence of the td Intron Endonuclease. Although the pUCtd DNA contains the endo-
the recognition sequence lying upstream and downstream of the cleavage site, an extension of the experiments described in Fig. 2 was executed. The cDNA products in the dioxygenucleotide-containing lanes, which served as a sequence ladder in Fig. 2, actually contain a nested set of truncated molecules differing in length by one nucleotide. Thus, the double-stranded hybrid molecules, each made up of a $^{32}$P-labeled cDNA of different length base-paired to its unlabeled template strand, could be used as substrates for theendonuclease. By following the number of bases that must be converted to a double-stranded form before the endonuclease will cut, an upper limit for the recognition sequence can be determined. The boundary of recognition can be identified by the marked reduction or even disappearance of cDNA bands beyond a specific point when the endonuclease-treated cDNA products are resolved on a sequencing gel. The result of this experiment is depicted in Fig. 4, which shows the sequence profile both downstream (A) and upstream (B) of the endonuclease cut. Overexposure of the x-ray film to the radioactive sequencing gel was necessary to reveal the cDNA bands proximal to the boundaries of the recognition sequence. Based on the respective sequence profiles, we conclude that the maximum endonuclease recognition sequence is 87 bp long, running from 52 bp upstream of the cleavage site in exon 1 to 35 bp downstream of it (C), which includes the first 11 bp of exon 2.

**DISCUSSION**

Group I introns, characterized by their ability to undergo self-splicing in vitro (for review, see ref. 29), are found in such diverse genetic sources as the *Tetrahymena* nucleus, yeast and fungal mitochondria, the *Physarum* nucleus, some chloroplasts, and the T-even bacteriophages. Although some group I introns, such as that in the *Tetrahymena* nuclear large rDNA, are relatively small and devoid of open reading frames, others contain extensive open reading frames that have become the subject of intense study. These group I iORFs can be generally divided into two categories: (i) those encoding maturases that facilitate splicing of the intron in which they are encoded at the RNA level and (ii) those that encode endonucleases that may be involved in unidirectional gene transfer at the DNA level, resulting in intron acquisition by homologous intronless genes. Some examples in the latter category include the 21S rDNA (21, 22) and *cox1* genes (30, 31) in yeast mitochondria, *Physarum* nuclear extrachromosomal rDNA (32), and T4 bacteriophage *td* (18, 20) and *sunY* (19) genes. Analysis of the substrate specificity of two yeast mitochondrial intron-encoded endonucleases in vitro has revealed that they produce 4-base-long 3'-hydroxyl overhangs, either at the intron-insertion site for the 21S rRNA gene (22) or 1 bp downstream of that site for the *cox1* *al4α* gene (30, 31).

To study the structure and function of the product encoded by the 735-bp *td* iORF, the iORF was cloned into a high-expression vector, and the resulting partially purified protein was shown to possess endonucleolytic activity specific for the *tdΔ1* gene (18). The data of Quirk et al. (19) obtained from genetic analyses showed that deleting a 21-bp stretch from 11 bp upstream to 10 bp downstream of the exon junction abolished endonuclease-facilitated *td* intron mobility. Our present work, which made use of a highly purified preparation of the cloned *td* intron endonuclease, has allowed us to determine unequivocally from a direct analysis of the endonucleolytic products that the cleavage site is centered at 24 bp upstream of the exon junction (Fig. 2) and outside of the 21-bp region. The cleavage is such that it forms a 2-base staggered cut, with 3'-hydroxyl protruding ends and 5'-phosphoryl recessed ends (Fig. 3 and F.K.C., unpublished results). That the *td* intron endonuclease cuts a site fairly removed from the exon junction and generates a 2-base 3'-hydroxyl overhang contrasts with the yeast mitochondrial intron endonucleases, which cut at or very close to the exon junction and produce 4-base overhangs (22, 30, 31). Regardless of the source, all intron-encoded endonucleases characterized thus far belong to a subclass of type 2 endonucleases that cleave nonsymmetrical sequences (33) and exhibit a very high degree of specificity.

Although the intron-containing *td* gene possesses the endonuclease cleavage sequence, it nevertheless is not a substrate (Table 2), indicating that some exon 2 sequence may be
required by the endonuclease for recognition. Delineation of the recognition sequence (Fig. 4) lends support to this notion. The td intron endonuclease readily cleaves tdΔ1 DNA containing a continuous 87-bp region, which stretches from 52 bp upstream to 35 bp downstream of the cleavage site. The downstream sequence ends 11 bp into exon 2. It should be pointed out that the cleavage is not all-or-none because cDNAs just inside the boundary of the recognition sequence showed a slight, but detectable, loss when treated with the endonuclease (Fig. 4A and B). Therefore, our experimental data define only the optimum recognition sequence. The absolute recognition sequence in the case of the td intron endonuclease may be much shorter than this, particularly in the exon 1 direction because the cox1Δ1α-encoded endonuclease in yeast mitochondria recognizes a fairly short sequence of 18 bp (22, 30). The extensive recognition sequence on either side of the tdΔ1 endonucleolytic cleavage site some distance from the exon junction may improve the probability of successful intron acquisition by the recipient tdΔ1 DNA, a phenomenon shown by genetic experiments (19).

What function does the td intron-encoded endonuclease serve? Intron mobility in the T-even bacteriophages was demonstrated for the td and sunY introns by a genetic approach that showed this process to absolutely depend on the integrity of the corresponding iORF (19). We have shown that the td iORF encodes a protein endonuclease specific for the double-stranded tdΔ1 DNA, which was subsequently confirmed by others (20). Because the intron transfer appears always to be in the direction of acquisition, it probably occurs by a mechanism similar to that postulated for the site-specific transfer of the 21S rDNA intron in yeast mitochondria (22), where Colleaux et al. invoked the double-strand break-repair model of Szostak et al. (34). However, they also suggested that the endonuclease-generated 3′ overhang can invade the corresponding intron-containing gene and act directly as a primer to elongate a copy of the intron sequence, using the appropriate intron-containing strand as template (22). Such a duplicative recombination mechanism will result in the conversion of flanking exon sequences.

If the td endonuclease facilitates intron mobility among T-even phages, what does intron spreading accomplish? Speculations abound for lack of definitive experimental data in this area. We prefer to view intron acquisition by the phage td gene and phage intron mobility, in general, as a means to expand and/or diversify the coding capacity of a given gene in an organism, such as bacteriophage (27), which contains a relatively limited amount of genetic material compared to the eukaryotes. Thus, precise intron acquisition by the tdΔ1 gene increases the number of potential reading frames within this gene from one to four (td, exon 1, exon 2, and iORF), thereby expanding its coding capacity. In this context, we had shown that the exon 1 peptide is expressed early in a temporal fashion from the intron-containing td gene both in vivo (35) and in an in vitro-coupled transcription-translation system (36). Finally, genetic evidence from studying T4 phage deletion mutants has implied that phage introns could provide target sequences for recombination during exon shuffling, resulting in new combinations of exons (37).

Note. Shortly after submission of this work, Perlman and Butow (38), citing a personal communication of M. Belfort, indicated that the T-even intron-encoded endonucleases cleave at a distance (up to 25 bp) from the intron-insertion site.

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