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*Rsa*I: a New Sequence-Specific Endonuclease Activity from *Rhodopseudomonas sphaeroides*

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A new type II sequence-specific endonuclease, *Rsa*I, has been identified from *Rhodopseudomonas sphaeroides* strain 28/5. An *Rsa*I purification scheme that yields enzyme which is free of contaminating exonuclease and phosphatase activities after a single column fractionation has been developed. The enzyme recognized the tetranucleotide sequence 5'-GTAC-3' and cleaved between the T and A, thereby generating flush ends. *Rsa*I should be extremely useful in deoxyribonucleic acid sequencing experiments.

Since their discovery over 10 years ago, more than 140 type II sequence-specific endonucleases have been identified (13, 14). The nucleotide sequences recognized by these endonucleases usually range from four to seven base pairs and contain a twofold axis of symmetry. Endonucleases which recognize tetranucleotide sequences cleave DNA frequently (on the average, every 256 base pairs) and are especially useful for DNA sequencing by the dideoxy chain terminating method of Sanger et al. (17) and the chemical modification method of Maxam and Gilbert (9). To date, enzymes which are specific for 8 of the 16 possible symmetrical tetranucleotide sequences have been identified (13, 14). In this report, we describe an endonuclease from *Rhodopseudomonas sphaeroides* strain 28/5, *Rsa*I, which recognizes the tetranucleotide sequence 5'-GTAC-3'.

MATERIALS AND METHODS

Strains and culture conditions. *R. sphaeroides* strain 28/5 was obtained from G. Drews, University of Freiburg, Freiburg, Germany. The conditions of growth and the media employed have been described previously (7). Cells (approximately 2×10^9 cells per ml) were harvested by centrifugation and stored as a cell paste at −20°C.

Preparation of *Rsa*I active fraction. Frozen cells were thawed and washed once in column buffer (10 mM potassium phosphate, pH 7.4; 10 mM β-mercaptoethanol; 0.1 mM EDTA; 10% [vol/vol] glycerol). All procedures were performed at 4°C. The washed cell pellet was suspended in column buffer (1:2, wt/vol) and disrupted by one passage through a French pressure cell at 20,000 lb/in^2. Phenylmethylsulfonyl fluoride was added to a final concentration of 50 μM, and the crude cell lysate was freed of particulate material by centrifugation at 150,000 × *g* for 2.0 h.

A freshly prepared 10% (wt/vol) streptomycin sulfate solution (0.35 volume) was slowly added with stirring to the above supernatant fluid. After an additional 30 min of stirring on ice, the precipitate was removed by centrifugation at 12,000 × *g* for 10 min. The remaining supernatant fluid was dialyzed overnight against column buffer.

The dialysate was again centrifuged at 12,000 × *g* for 10 min, and the supernatant fluid was loaded onto a Whatman DE-52 column (3 by 10 cm) equilibrated in column buffer. The column was washed with column buffer, and 2-ml fractions were collected. Those fractions exhibiting endonuclease activity were pooled and dialyzed against storage buffer (25 mM potassium phosphate, pH 7.4; 10 mM β-mercaptoethanol; 0.1 mM EDTA; 50% [vol/vol] glycerol). The preparation was stored at −20°C.

Enzyme assay conditions and electrophoresis. *Hae*III, *Hha*I, and *Taq*I were assayed by previously described procedures (11, 15, 18). Reaction mixtures (50 μl) for detecting *Rsa*I activity contained 2 μg of λ *c*I857 DNA, 10 mM Tris-hydrochloride (pH 7.9), 6 mM $MgCl_2$, and 0.5 mM dithiothreitol. Reactions were terminated after 1 h of incubation at 34°C by addition of 10 μl of electrophoresis sample buffer (1). Electrophoresis was performed on 5% polyacrylamide gels (1) or 1% (wt/vol) agarose gels (19).

Exonuclease and phosphatase assays. 5' Exonuclease activity was measured essentially as described by LeBon et al. (6). Restriction fragment DNA labeled with ^{32}P at the 5' end (approximately 10^6 cpm) was incubated for 24 h at 37°C in the standard *Rsa*I reaction mixture with 10 U of enzyme preparation per 0.01 μg of DNA substrate. Controls consisted of identical reaction mixtures lacking the *Rsa*I preparation. After incubation, the samples were subjected to electrophoresis on a 5% polyacrylamide gel, and the gel was autoradiographed. ^{32}P-containing gel slices were quantitated by Cerenkov radiation. 3' Exonuclease activity was not directly measured.

DNA sequencing. Labeling of DNA with [γ-^{32}P]-ATP by T4 polynucleotide kinase and sequencing were carried out by the procedure of Maxam and Gilbert (9).

Identification of 2'-deoxynucleoside 5'-phosphate after cleavage with *Rsa*I. Three independent *Rsa*I fragments were isolated and labeled on the 5' end with [γ-^{32}P]ATP by T4 polynucleotide kinase and reisolated from 5% polyacrylamide gels. These fragments were digested to completion with a combination

of pancreatic DNase I and snake venom phosphodiesterase and then chromatographed adjacent to standard 2'-deoxynucleoside 5'-phosphates as previously described (22).

Enzymes and chemicals. *Hha*I and *Taq*I were isolated by the procedures of Roberts et al. (15) and Sato et al. (18). T4 polynucleotide kinase was purchased from New England Bio-Labs. DE-52 was purchased from Whatman, Ltd. *Hae*III was purchased from Bethesda Research Laboratories. Plasmid DNA was isolated by the procedure of Hershfield et al. (5). Phage DNA was isolated as described previously (4). Simian virus 40 form I and φX174 replicative form were generous gifts from R. J. Hronek and K. Postle, respectively. 2'-Deoxynucleoside 5'-phosphates were purchased from P-L Biochemicals.

RESULTS

Isolation of *Rsa*I active fraction. The preparation of the *Rsa*I active fraction was relatively simple, involving a single column fractionation with no gradient elution. We routinely obtained 1,000 U of enzyme per g (fresh weight) of cells (1 U of activity will digest 1 μg of λ cI857 DNA in 1 h at 34°C). Optimal reaction conditions for *Rsa*I were found to be pH 7.9 and 34°C. NaCl up to 200 mM had no effect on the enzyme activity (data not shown). After extensive incubation with excess enzyme of DNA labeled with ^{32}P at the 5' end, there was less than 1% loss of radioactivity, indicating the absence of both 5' exonuclease and phosphatase activities (data not shown). There was no significant 3' exonuclease activity since digestion of a fragment labeled at the 5' end containing an *Rsa*I cleavage site generated a single band on DNA sequencing gels (see Fig. 2).

Specificity of *Rsa*I. The *Rsa*I cleavage sites were mapped relative to the known *Hae*III and *Taq*I sites of pBR322 (20; Fig. 1). Since the entire sequence of pBR322 is known (21), it should be possible to deduce the *Rsa*I recognition site by determining which *Taq*I or *Hae*III fragment(s) was cleaved by *Rsa*I and then searching the pBR322 sequence (21) for a common (or related) sequence at the suspected positions in each of the fragments. The occurrence of the sequence 5'-GTAC-3' coincided with the approximate position of the *Rsa*I sites in pBR322. In addition, the number of *Rsa*I cleavage sites in simian virus 40 and φX174 corresponded to the number of 5'-GTAC-3' sites in each DNA (Table 1). Other occurrences of *Rsa*I cleavage sites and the 5'-GTAC-3' sequence include a site in *trpO* DNA (R. Gunsalus and C. Yanofsky, personal communication) and three sites in the threonine (*thr*) operon regulatory DNA at positions −157, −76, and 24 (3).

Determination of the *Rsa*I cleavage site. The *Rsa*I cleavage site was determined by a variation of the method of Brown and Smith (2) as described by McConnell et al. (10), using DNA carrying the *thr* operon regulatory region (3). The *Hha*I site at −183 was 5' end labeled

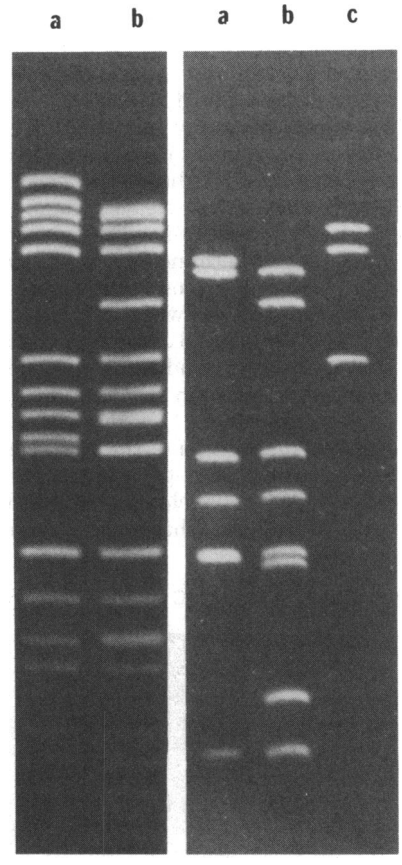

FIG. 1. *Restriction endonuclease mapping of RsaI sites in pBR322 on 5% polyacrylamide gels. Left: (a) HaeIII; (b) HaeIII + RsaI. Right: (a) TaqI; (b) TaqI + RsaI; (c) RsaI. Digestion conditions are described in the text.*

TABLE 1. *Frequency of RsaI cleavage sites in known DNA sequences*

DNA substrate	No. of *Rsa*I sites observed	No. of *Rsa*I sites predicted from DNA sequences
φX174 replicative form	11	11[a]
Simian virus 40 form I	10	10[b]
pBR322	3	3[c]
λ cI857	>40	—[d]

[a] As determined by Sanger et al. (16).
[b] As determined by Reddy et al. (12).
[c] As determined by Sutcliffe (21).
[d] Sequence data not available.

with [γ-^{32}P]ATP, using T4 polynucleotide kinase (9), and redigested with *Taq*I. The mixture was subjected to electrophoresis on a 5% polyacrylamide gel, and the labeled fragments were visualized by autoradiography and eluted from the gel. The labeled fragment carrying an *Rsa*I site was then sequenced by the method of Maxam and Gilbert (9). The *Rsa*I-cleaved fragment was subjected to electrophoresis adjacent to the sequencing lanes to determine the site of cleavage. As shown in Fig. 2, the mobility of the *Rsa*I-cleaved fragment lay between the A and C of the sequence 5′-GTAC-3′. According to McConnell et al. (10), this indicates that the *Rsa*I cleavage site lies between the T and A of the sequence 5′-GTAC-3′.

To confirm the position of the *Rsa*I cleavage site, three different *Rsa*I-generated restriction fragments were 5′ end labeled and digested to 2′-deoxynucleoside 5′-phosphates with a combination of pancreatic DNase I and snake venom phosphodiesterase as described above. In a representative experiment (Table 2), 93% of the radioactivity appeared in dAMP, indicating that the *Rsa*I cleavage site lies between the T and A of the sequence 5′-GTAC-3′.

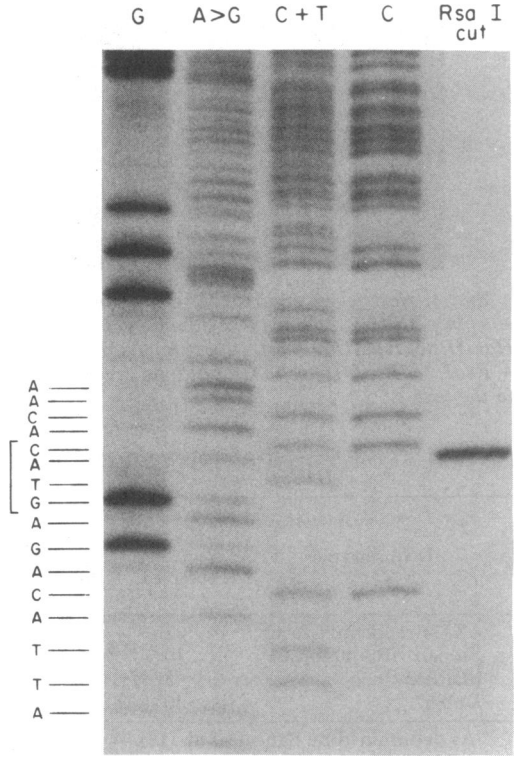

FIG. 2. *Determination of the RsaI cleavage site. The HhaI ^{32}P-labeled fragment described in the text was sequenced by the method of Maxam and Gilbert (9). The mobility of the RsaI-cleaved fragment (between the A and C of the sequence 5′-GTAC-3′) indicates that the site of cleavage lies between the T and A of that sequence (10). Only one band is present in the RsaI-cut lane, indicating that there is no significant 3′ exonuclease activity present.*

TABLE 2. *Identification of 5′-terminal 2′-deoxynucleoside 5′-phosphate (dNMP) generated after cleavage with RsaI*

dNMP[a]	cpm (%)
dAMP	93.0
dCMP	2.5
dGMP	1.8
dTMP	2.4

[a] Determination of the 5′-terminal 2′-deoxynucleoside 5′-phosphate after digestion with pancreatic DNase I and snake venom phosphodiesterase was as described by Wells et al. (22).

DISCUSSION

The procedure employed for the preparation of the *Rsa*I active fraction represents a modification of the previously described procedure for the identification of another site-specific endonuclease (*Rsh*I) from *R. sphaeroides* strain 2.4.1 (8). The activity, virtually free of exonuclease, was recovered in the void volume of a single DE-52 column after fractionation of the streptomycin sulfate-treated crude extract. Applying a 0.0 to 1.0 M KCl gradient after recovery of the majority of the endonuclease activity (>90%) resulted in elution of a small amount of additional *Rsa*I activity at 0.15 M KCl.

The reason(s) for the second peak of activity is unclear, although secondary interactions with other macromolecules, probably nucleic acids, are implicated. Fractionation of the soluble extract before DE-52 chromatography on either Sephadex G-100 or phosphocellulose did not alter its elution from the anion-exchange resin. Omission of the streptomycin sulfate precipitation step decreased the amount of activity eluting in the void volume and increased the amount of activity displaced in the gradient. All activity derived by gradient elution of the DE-52 column was contaminated with exonuclease or phosphatase or both.

We have shown that *Rsa*I recognizes and cleaves the sequence 5′-GTAC-3′. Only one site has been sequenced directly, but the frequency of this sequence, the numbers and sizes of fragments generated by cleavage of known DNA sequences (simian virus 40, ϕX174, and pBR322), the DNA sequencing, and the 5′ end analysis are convincing. This core sequence (GTAC) appears to be all that is necessary for recognition and cleavage since 14 of the 16 pos-

sible combinations of nucleotides at the 5′ and 3′ ends of the site can be found in the aforementioned sequences. *Rsa*I should be included in the growing list of site-specific endonucleases used for DNA sequencing studies because of its ease of isolation, tetranucleotide recognition sequence, and freedom from objectional contaminating activities.

We have identified five other *R. sphaeroides* strains producing isoschizomers of *Rsa*I. The origin of these strains is quite dispersed, coming from three continents. No further work has been done with these strains to determine their site(s) of cleavage.

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