A *Salmonella typhimurium* Endonuclease that Converts Native DNA to Fragments of about 8×10^5 daltons

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Crude extracts of *Salmonella typhimurium* were found to contain an endonuclease that degraded double-stranded linear DNA from bacteria and phages to fragments with a molecular weight of about 8×10^5 . The nuclease did not have an absolute requirement for Mg^{2+} . One discrete intermediate product had a molecular weight of 6.6×10^6 . Extracts from two different mutants were tested: one completely lacked the endonuclease activity (strain DB5575), and the other showed an absolute requirement for Mg^{2+} (strain 4543). No biological role has yet been found for this endonuclease of *S. typhimurium*.

INTRODUCTION

Escherichia coli contains several different endonucleases or enzymes with endonucleolytic activity. Endonuclease I catalyses the cleavage of DNA to oligonucleotides with an average chain length of seven nucleotides. In crude extracts, the enzyme is bound to and inhibited by tRNA (Lehman, Roussos & Pratt, 1962). Endonuclease II is characterized by its specificity for DNA alkylated with monofunctional alkylating agents (Friedberg, Hadi & Goldthwait, 1969; Kirtikar, Cathcart & Goldthwait, 1976). It produces single-strand breaks, or nicks, at or near apurinic sites (Hadi & Goldthwait, 1971). Other endonuclease activities act specifically on X-irradiated DNA (Strniste & Wallace, 1975), ultraviolet-irradiated DNA (Radman, 1976), or reside in the gene products of *uvrA* and *uvrB* (Braun & Grossman, 1974) and in the *recBC* enzyme (Goldmark & Linn, 1972). Restriction endonucleases represent another group of endonucleases; these are divided into two types. Type I enzymes are structurally complex having two or more different subunits per enzyme and are characterized by the requirement for adenosine 5'-triphosphate, S-adenosyl-L-methionine and Mg^{2+} (Arber, 1974). In contrast, type II enzymes are small and they require only Mg^{2+} for endonucleolytic activity (Boyer, 1974).

We report here the detection of an endonuclease in crude extracts of *Salmonella typhimurium* which degrades the double-stranded linear DNA of phages P22, L and λ to fragments of about 8×10^5 daltons. The enzyme does not have an absolute requirement for divalent cations.

METHODS

Bacterial and phage strains. The bacterial strains are listed in Table 1. The clear plaque mutants P22c2⁵ (Levine, 1957) and LcII² (Bezdek, Soska & Amati, 1970) were used to prepare phage DNA.

Preparation of labelled phage DNA. [methyl-³H]Thymidine or ³²P-labelled DNA of phages P22c2 and LcII was prepared as described by Schumann, Lindenblatt & Bade (1976). [methyl-³H]Thymidine or ³²P-labelled DNA of phage Muets62 was prepared as previously described (Bade, 1972; Schröder, Bade & Delius, 1974). Labelled DNA of phage λ cI857Sam7 was prepared as described by Wu *et al.* (1972).

Preparation of labelled bacterial DNA. [methyl-³H]Thymidine labelled *E. coli* DNA was prepared from

Table 1. *Bacterial strains*

Strain	Genotype	Source or reference
<i>Salmonella typhimurium</i>		
LT2	wild type	Levine (1957)
T4/2	<i>thy</i>	Schmieger (1970)
4543	<i>met trp str end</i>	C. Colson
4467	HfrK4 <i>serA13 hsd_{LT}85 hsd_{SA}41</i>	Colson & Van Pel (1974)
DB5575	<i>hisC cysA end-1 str</i>	Susskind & Botstein (1975)
<i>Escherichia coli</i>		
EB7566	<i>thy</i> (Mucts62)	Bade (1972)
EB417	<i>thy</i> (λ c1857Sam7)	Bade
W3110	<i>thy</i>	Schröder <i>et al.</i> (1974)

strain W3110 as described by Schröder *et al.* (1974). The DNA purified by CsCl-ethidium bromide density gradient centrifugation was extracted three times with phenol and dialysed extensively against 10 mM-Tris/HCl (pH 7.9) containing 1 mM-EDTA.

Preparation of crude extracts containing endonuclease activity. Bacteria were grown in nutrient broth (Difco) at 37 °C to mid-exponential phase, collected by centrifugation and stored at -20 °C. Crude extracts were prepared as described by Echols & Green (1971) and used without dialysis. The presence of detergent in the extracts did not influence the enzyme activity which, under our conditions, remained stable for at least one week when stored at 4 °C. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Determination of the endonucleolytic activity in crude extracts. The endonuclease activity was assayed by following the decrease in length of phage DNA as analysed by sucrose gradient centrifugation. Reactions were carried out for 20 min at 37 °C in a total volume of 0.2 ml containing 50 mM-Tris/HCl (pH 7.9), 10 mM-MgCl₂, 0.2 µg ³H-labelled DNA (P22, L or λ) and 1 to 5 µl of crude extract. Where indicated, 20 mM-EDTA was also included in the reaction mixture. The reactions were stopped by adding SDS to a final concentration of 1 % (w/v) and heating to 60 °C for 2 min. A 0.1 ml sample was then layered on to a 5 to 20 % (w/v) neutral sucrose gradient (Schröder *et al.*, 1974), together with ³²P-labelled marker DNA, and centrifuged in a Beckman SW 50.1 rotor at 40000 rev. min⁻¹ and 10 °C for 5 h. Recovery of radioactivity was between 70 and 90 %.

The size of the P22 DNA fragments produced by the endonucleolytic activity was estimated by sucrose gradient centrifugation using ³²P-labelled fragments produced by *Eco*RI restriction endonuclease digestion of phage Mu DNA as internal markers. Isolation of endodeoxyribonuclease *Eco*RI and digestion of Mu DNA were performed as described by Green *et al.* (1974). The 5 to 20 % (w/v) neutral sucrose gradients were centrifuged in a Beckman SW41 rotor at 26000 rev. min⁻¹ and 10 °C for 12 h.

RESULTS

Crude extracts of *Salmonella typhimurium* degraded double-stranded linear DNA from phage P22 to a homogeneous population of high molecular weight fragments (Fig. 1*a*). The molecular weight of the end-product fragments was not altered within the range of the protein concentrations tested (0.3 to 30 µg protein per incubation mixture; data not shown). When the incubation mixture also contained 20 mM-EDTA, fragments of the same size were produced only at high protein concentrations (30 µg; Fig. 1*a*). At protein concentrations below 20 µg per incubation mixture only larger fragments were produced (Fig. 1*a*). With 1 µg or less of extract protein, the phage DNA sedimented uncleaved in the presence of EDTA (Fig. 1*a*).

Incubation of P22 DNA with extracts (30 µg protein per incubation mixture) for 2 h in the presence of Mg²⁺ resulted in a complete, unspecific degradation of the DNA (Fig. 1*b*). When the same incubation was performed in the absence of Mg²⁺ (20 mM-EDTA present), no degradation beyond the fragment size obtained after 20 min incubation occurred (Fig. 1*b*). The more extensive degradation after prolonged incubation of the DNA in the presence of Mg²⁺ was most likely due to exonuclease action, which is completely inhibited by EDTA.

Fig. 1. Detection of endonuclease activity in crude extracts of *S. typhimurium* wild-type strain LT2. Extracts and assay conditions are described in Methods. Centrifugation was for 5 h at 130000 *g*. The direction of sedimentation was from right to left. The arrow indicates the position of ^{32}P -labelled DNA from phage Mu which has a molecular weight of about 25×10^6 (Martuscelli *et al.*, 1971) and which sedimented in the same position as untreated P22 DNA.

(a) ^3H -labelled P22 DNA treated with various amounts of extract protein in the presence or absence of EDTA: ●, 30 μg protein, no EDTA; ○, 30 μg protein, 20 mM-EDTA; ▲, 5 μg protein, 20 mM-EDTA; △, 1 μg protein, 20 mM-EDTA. (b) ^3H -labelled P22 DNA treated for 2 h with 30 μg extract protein: ●, in the absence of EDTA; ○, in the presence of 20 mM-EDTA. (c) ^3H -labelled L DNA (●) and ^3H -labelled λ DNA (○) treated with 30 μg extract protein in the absence of EDTA. (d) ^3H -labelled *E. coli* DNA: ●, untreated; ○, treated with 30 μg extract protein in the absence of EDTA.

Fig. 2. Detection of endonuclease activity in crude extracts of three different mutants of *S. typhimurium*. The conditions were as described in the legend to Fig. 1.

(a) ${}^3\text{H}$ -labelled P22 DNA treated with $30\ \mu\text{g}$ extract protein of strain 4543: ●, in the absence of EDTA; ○, in the presence of 20 mM-EDTA. (b) ${}^3\text{H}$ -labelled P22 DNA treated with $20\ \mu\text{g}$ extract protein of strain DB5575: ●, in the absence of EDTA; ○, in the presence of 20 mM-EDTA. (c) ${}^3\text{H}$ -labelled P22 DNA treated with a mixture of $60\ \mu\text{g}$ extract protein (total) of strains 4543 and DB5575 in the presence of 20 mM-EDTA. (d) ${}^3\text{H}$ -labelled P22 DNA treated with $30\ \mu\text{g}$ extract protein of strain 4467: ●, in the absence of EDTA; ○, in the presence of 20 mM-EDTA.

The endonucleolytic activity is not P22-specific, since it degraded DNA from *Salmonella* phage L (Fig. 1c) and *E. coli* phage λ (Fig. 1c) as well as *E. coli* DNA (Fig. 1d).

Strain 4543 was isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment. Colonies of this strain remain fluorescent on plates containing ethidium bromide after toluene treatment (C. Colson, personal communication). Strain DB5575 was obtained after mutagenesis by ethyl methanesulphonate and screening with methyl green (Susskind & Botstein, 1975). Both strains are therefore assumed to lack a major endonuclease activity. Incubation of P22 DNA with crude extracts of strain 4543 (Fig. 2a) resulted in fragments of the same size as those obtained with extracts of wild-type LT2 (see Fig. 1a). Addition of 20 mM-EDTA to the reaction mixture completely prevented the cleavage of the phage DNA (Fig. 2a). Crude extracts of DB5575 did not cleave the phage DNA in the presence of Mg^{2+} nor with added EDTA (Fig. 2b).

When P22 DNA was incubated with a mixture of crude extracts of both mutant strains in the presence of 20 mM-EDTA, no degradation occurred (Fig. 2c), indicating that under these conditions the extracts did not complement each other.

In order to test whether or not *S. typhimurium* restriction enzymes are involved in the degradation process, P22 DNA was incubated with crude extracts of strain 4467. This strain is mutant in the two restriction and modification systems LT and SA (Colson & Van Pel, 1974). Extracts of the restriction-deficient strain cleaved P22 DNA to give products of similar size to those obtained with extracts of wild-type *S. typhimurium*, both in the absence and in the presence of EDTA (Fig. 2d).

The molecular weights of the P22 DNA fragments found as apparent end-product and as one intermediate (Fig. 1a) of digestion were determined by sucrose gradient centrifugation using *Eco*RI-generated fragments of ^{32}P -labelled Mu DNA as internal markers (Fig. 3). Based on the molecular weight of the three Mu fragments (fragment A = 11.6×10^6 ; B = 9.4×10^6 ; C = 3.2×10^6 ; Bade, Delius & Allet, 1977), the molecular weight of the end-product fragments was estimated to be 8×10^5 , and that of the intermediate to be 6.6×10^6 .

DISCUSSION

Incubation of phage P22 DNA with crude extracts of *S. typhimurium* wild-type strain LT2 resulted in breakdown of the linear DNA to fragments of about 8×10^5 daltons, representing 1/32 of the length of the phage DNA (27×10^6 daltons; Rhoades, MacHattie & Thomas, 1968; 28×10^6 daltons; Tye, Chan & Botstein, 1974). This reaction was not completely dependent on the presence of Mg^{2+} cations provided that large amounts of extract were used (Fig. 1a). At a protein concentration of 5 μ g per incubation mixture and in the absence of Mg^{2+} , a homogeneous population of larger fragments was produced (Fig. 1a); the molecular weight of these was estimated to be about 6.6×10^6 . This corresponds to approximately one-quarter of the molecular weight of the P22 chromosome.

Two different *Salmonella* mutants, originally isolated by C. Colson and D. Botstein as endonuclease-deficient by bacteriological criteria, were tested for the presence of detectable endonuclease activity using the gradient centrifugation technique. Incubation of P22 DNA with crude extracts of strain 4543 revealed that this strain has retained the nuclease activity in the presence of Mg^{2+} but the extracts were completely inactive with added EDTA (Fig. 2a). The second mutant tested (strain DB5575) did not show any endonuclease activity (Fig. 2b). Therefore, in both strains the mutational event affected a gene coding for an endonucleolytic activity. It is not possible to determine from the existing data whether or not the same gene is mutant in both strains.

From our results we conclude that the endonuclease activities do not correspond to a type I restriction endonuclease because DNA of phage P22 grown in wild-type LT2 was well degraded. Furthermore, P22 DNA was also cleaved by crude extracts prepared from

Fig. 3. Sedimentation of the end-product and of one intermediate in the digestion of P22 DNA with LT2 crude extract. Centrifugation was for 12 h at 90000 *g*. The direction of sedimentation was from right to left. ●, ^{32}P -labelled Mu DNA digested with *Eco*RI; △, ^3H -labelled P22 DNA treated with 30 μg extract protein of LT2; ▲, ^3H -labelled P22 DNA treated with 5 μg extract protein of LT2 in the presence of 20 mM-EDTA. A, B and C indicate the three *Eco*RI fragments of Mu DNA. The figure is a composite of two identical gradients with respect to the *Eco*RI-digested Mu DNA, one containing the P22 end-product and the other, the P22 intermediate.

strain 4467 (Fig. 2*d*) which carries mutations affecting the two restriction and modification systems LT and SA^v (Colson & Van Pel, 1974).

How does the endonuclease work? The fact that the different types of DNA so far investigated are cleaved to end-products of about the same size raises the question of how the enzyme can 'measure' this size. From our results we propose that the endonuclease degrades DNA by attaching initially to both ends of the molecule and then migrating along the DNA and cleaving it at or close to the collision point approximately equidistant from either end. The resulting DNA fragments of approximately equal length are then sectioned again according to a similar mechanism until the fragment size reaches the size class of about 8×10^5 daltons. This model is supported by the finding of an intermediate size class of DNA fragments of about 6.6×10^6 daltons in the cleavage of P22 DNA. Experiments are in progress to obtain purified enzyme preparations to find out the exact role of Mg^{2+} in the breakdown process, and to identify other intermediates that may verify the cutting model proposed here.

A nuclease apparently similar to the activity described here has been identified and purified from extracts of *Bacillus subtilis* (Scher & Dubnau, 1973, 1976).

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