A HYBRIDASE FROM ESCHERICHIA COLI

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SUMMARY

An endonuclease has been isolated and purified from Escherichia coli which degrades RNA hydrogen bonded to DNA and no other polynucleotide substrates, including double stranded RNA, single stranded RNA, double stranded DNA or single stranded DNA.

INTRODUCTION

We wish to report the isolation and characterization of an enzyme from Escherichia coli which degrades specifically an RNA chain hydrogen bonded to DNA, i.e., the RNA portion of an RNA-DNA hybrid. We propose the term "hybridase" for this enzyme. Enzymes of similar specificity have been previously described for mammalian systems (1).

MATERIALS AND METHODS

Bacteria. Apparently identical enzymes were isolated from an E. coli K12 strain (H560 pol A^- endo I^-) and from an E. coli C strain (pol A^+).

Nucleic acid substrates. The substrates were prepared

essentially according to the references given: ^{14}C-RNA-bacteriophage fd-DNA hybrid or ^{3}H-RNA-fd-DNA hybrid (with γ-^{32}P-GTP) (1); "melted" fd hybrid (1); double stranded phage fr RNA (2); phage T7-^{3}H-mRNA (3); T7-^{32}P-DNA (4); ^{3}H poly d(AT) (5); ^{14}C-bacteriophage ϕX 174-RF I (6).

Enzyme assays. Hybridase assays were performed in either 50 mM tris-HCl pH 8.1, 5 mM $MgCl_2$ or 50 mM potassium phosphate pH 7.5, 5 mM $MgCl_2$. One unit of hybridase is defined as the activity which solubilizes 1 pmole ^{14}C-RNA from RNA-fd-DNA hybrids in 30 minutes at 25^{o}C. DNA polymerase I assays were performed as in (7). Assays using the 5'→3' exonuclease of pol I ("small fragment") were performed according to (8).

All assays were carried out at 25^{o}C for 30 minutes unless otherwise noted. Assays were terminated by the addition of 1 ml of 10% trichloroacetic acid (TCA). Then 5 ml of 5% TCA, 0.01 M in sodium pyrophosphate was added, the solution was filtered through nitrocellulose filters and washed once with 5 ml 5% TCA-pyrophosphate. The filters were dried, 5 ml toluene scintillation fluid added, and the samples counted in a scintillation counter.

RESULTS AND DISCUSSION

Isolation of Hybridase. About 100 g frozen E.coli cells from an exponentially growing culture were broken in an X press. After stirring at 4^{o}C in 300 ml standard buffer (50 mM tris-HCl pH 7.6; 1 mM EDTA; 10 mM mercapto-

ethanol), the suspension was centrifuged at 16,000 x g for 50 minutes. The pellet was then extracted with 310 ml standard buffer, 4 M in NaCl, and centrifuged as above. The supernatant was dialyzed against standard buffer diluted by two and applied to a DEAE-cellulose column (2 cm x 18 cm), previously equilibrated with the same buffer. The column was eluted with a linear gradient (1000 ml total volume) from 0.0 M to 0.6 M NaCl. Hybridase activity appeared at a salt concentration of about 0.16 M NaCl. The corresponding fractions were combined, dialyzed against standard buffer and applied to a DNA agarose column (2 cm x 15 cm) (9). The column was washed with standard buffer and eluted with a linear gradient (500 ml total volume) from 0.0 M to 1.0 M NaCl. The fractions were assayed for hybridase and polymerase activities (Fig. 1). Table I summarizes the purification procedure which resulted in approximately a 200 fold purification.

Biochemical characteristics. The hybridase was equally active in tris-HCl (50 mM) buffer and in potassium phosphate buffer (50 mM) between pH 7 and 8. The presence of magnesium ions is required for activity. Magnesium ions cannot be replaced by manganese or calcium. Optimal magnesium concentration is between 5 and 10 mM. Monovalent cations do not influence the reaction at concentrations below 0.1 M. NaCl or NH_4Cl concentrations above 0.2 M were found to be inhibitory.

An aliquot of hybridase was sedimented through a sucrose gradient (20% to 5% sucrose, buffered by standard buffer made 1 M in NaCl; SW 41 rotor of the Spinco centrifuge; 36 hours at 38,000 rpm and 2°C). Compared to horse hemo-

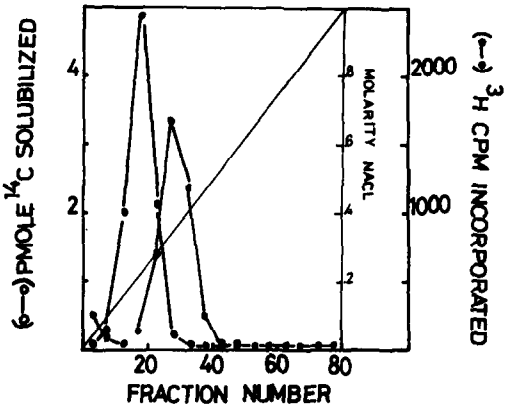

Fig. 1.

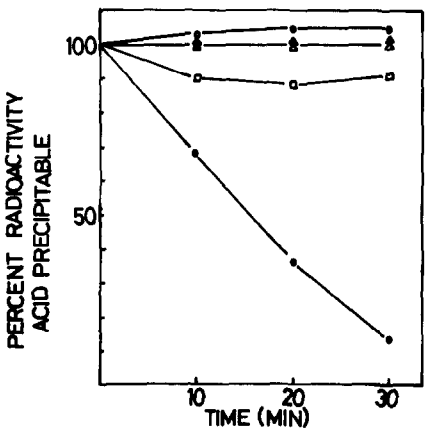

Fig. 2.

Figure 1. DNA agarose chromatography. The hybridase activity was determined in 0.2 ml buffer, composed of 50 mM tris-HCl pH 8.1, 5 mM $MgCl_2$, 9 pmole ^{14}C-RNA fd-DNA hybrid, and 4 µl from each fraction. DNA polymerase was assayed at 37°C in 0.2 ml containing 50 mM KCl, 50 mM tris-HCl pH 7.6, 8 mM $MgSO_4$, 5 mM mercaptoethanol, 16 µg calf thymus DNA, 2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 0.2 µCi dTTP, and 4 µl from each fraction.

Figure 2. Hybridase digestion of various nucleic acid substrates. All assays contained in 1.0 ml: 50 mM tris-HCl pH 8.1, 5 mM $MgCl_2$, 25 units hybridase and appropriate substrate. (●--●) 3500 cpm ^{14}C-RNA-fd-DNA hybrid; (o--o) 3500 cpm "melted" ^{14}C-RNA-fd-DNA hybrid; (▲--▲) 1600 cpm ^{3}H double stranded fr RNA; (Δ--Δ) 7000 cpm ^{3}H-T7-mRNA; (□--□) 2100 cpm ^{32}P-T7-DNA. At the times indicated, 0.2 ml was withdrawn from the incubation solution, the reaction terminated with the addition of TCA and radioactivity determined as described in Methods.

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globin (S^{w}_{20} = 4.25), the sedimentation coefficient of the hybridase was determined to be S = 2.6 - 2.8.

The digestion by hybridase of various radioactively labeled nucleic acid substrates is shown in Fig. 2. It can be seen that the enzyme degrades only the RNA portion of the RNA-fd-DNA hybrid and none of the other polynucleotides tested. Since it has been reported that the 5'→3' exonuclease from DNA polymerase I can excise pieces of

Table 1

Purification of E.coli Hybridase

	Fraction	Protein (mg/ml)	Specific Activity
1.	Crude extract	14.0	285
2.	4 M NaCl Supernatant	1.4	1320
3.	DEAE-cellulose peak	0.3	3500
4.	DNA agarose peak	< 0.02	> 53000

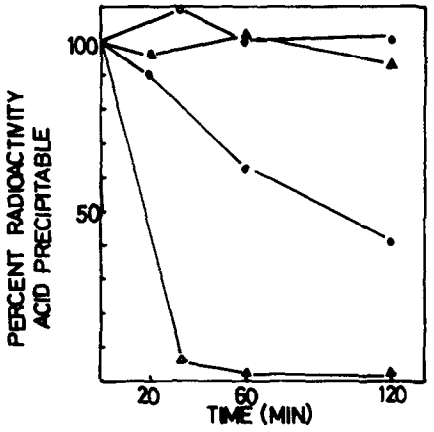

Figure 3. Hybridase and "small fragment" activities compared. Assays were performed at 30°C and contained in 0.8 ml: 10 mM potassium phosphate pH 7.6, 6.5 mM $MgCl_2$, 1.3 mM mercaptoethanol, 0.1 mg/ml BSA, 500 cpm ^3H poly d(AT) (filled symbols) or 3500 cpm ^{14}C-RNA-fd-DNA hybrid (open symbols) with 0.2 µg "small fragment" (circles) or 25 units hybridase (triangles). At the times indicated, 0.2 ml samples were withdrawn, the reaction terminated with TCA and the radioactivity determined as described in Methods.

RNA (10), we compared the action of the "small fragment" (a generous gift of H. Klenow), which retains the 5'→3' exonuclease activity of DNA polymerase I (8), with hybridase (Fig. 3). Under our conditions, the "small fragment" degrades ^3H poly d(AT), but not the ^{14}C-RNA portion of the RNA-fd-DNA hybrid. It was also found that under the same conditions DNA polymerase I would not degrade the RNA in the RNA-fd-DNA hybrid. Fig. 3 also shows that under

these conditions, hybridase does not attack ^{3}H poly d(AT). It seems unlikely, therefore, that the hybridase activity observed is due to the exonuclease activity associated with DNA polymerase I.

An experiment (data not shown) to investigate the mode of action of hybridase followed with time the hybridase digestion of RNA-fd-DNA hybrid labeled internally with ^{3}H-AMP and at the 5'-termini with γ-^{32}P-GTP. It was found that the solubilization of ^{3}H and ^{32}P increases at approximately the same rate. This suggested that the hybridase was acting as an endonuclease, as opposed to an exonuclease in which case the ^{32}P-label would be expected to be solubilized at a rate different from ^{3}H label. In order to determine definitely the mode of hybridase action, the digestion products were characterized.

Labeled RNA-fd-DNA hybrid was digested with hybridase at 25°C until 5-20% of the hybrid remained acid precipitable. An equal volume of 8 M urea was added to terminate the digestion. Pyrimidine oligonucleotides (11) were added to the sample as internal standards before applying to a DEAE Sephadex A 25 column. Fig. 4 shows the results of a typical experiment. It was determined that 5.2% of the counts were recovered as monophosphates and that 75.2% of the degradation products were probably oligomers larger than pentamers.

In order to more conclusively show that the hybridase was cleaving in an endonucleolytic manner, Col E_1 DNA (a gift of W. Goebel) was treated with hybridase, then sedimented through a sucrose gradient. It has been shown that

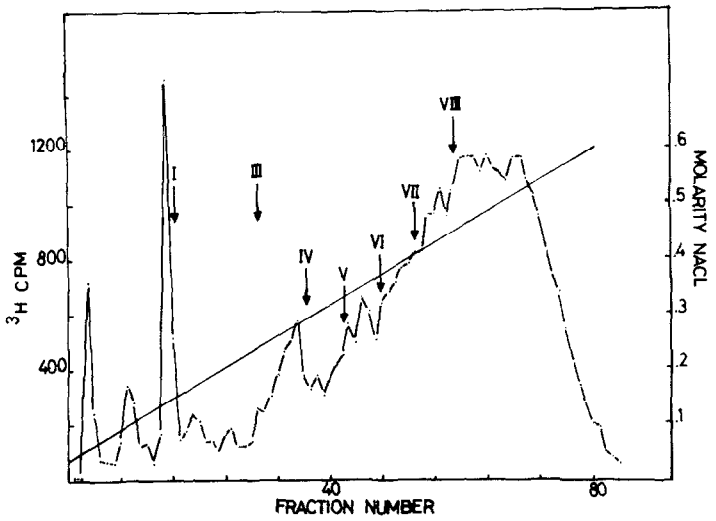

Figure 4. DEAE-Sephadex A25 chromatography of hybridase digestion products. The column was equilibrated at 25°C with 50 mM tris-HCl pH 8.1. The enzyme treated ^{3}H-RNA-fd-DNA hybrid was washed into the column with 1-2 ml buffer, then a linear salt gradient was applied (600 ml total volume, from 0.05 M to 0.6 M NaCl in 50 mM tris-HCl pH 8.1). 0.5 ml aliquots from the 8 ml fractions were used to determine radioactivity. Vertical arrows indicate the positions of marker mono- and oligo nucleotides as determined by ultraviolet light absorption at 260 nm.

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supercoiled Col E_1DNA isolated from cells grown in chloramphenicol contain a small section of ribonucleotides integrated into DNA (12). Such structures should, therefore, be susceptible to hybridase if it acts as an endonuclease. Fig. 5 shows that after incubation with hybridase, more than 50% of the supercoiled Col E_1DNA (23 S) has been shifted to the position of nicked "relaxed" Col E_1DNA (17 S). The control demonstrated the presence of only supercoiled Col E_1DNA (23 S). As a second control it was also determined that hybridase did not have the same effect on supercoiled bacteriophage ϕX RF I, which does not contain sections of ribonucleotides. Thus it appears that hybridase acts as an endonuclease specific for RNA regions hydrogen

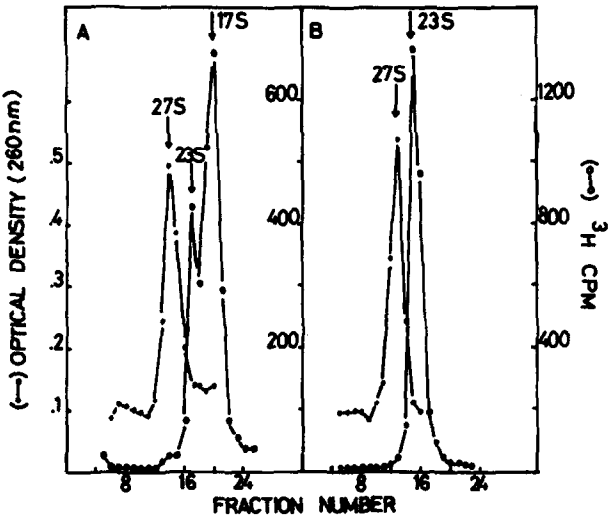

Figure 5. Sucrose gradient sedimentation of Col E_1 DNA.

(A) After incubation with hybridase. The incubation mixture contained in 0.2 ml: 50 mM tris-HCl, 5 mM $MgCl_2$, 2000 cpm ^{3}H-Col E_1 DNA, and 50 units in 50 µl of hybridase. After incubation at 30°C for 60 minutes, EDTA was added to 30 mM and fd DNA (27 S) added as internal marker. The solution was sedimented through a linear sucrose gradient (20% to 5% sucrose, buffered with standard buffer in 1 M NaCl) in the SW 27 rotor of the Spinco centrifuge at 25,000 rpm for 14 hours at 5°C. Fractions were collected through a hole punched in the bottom of the centrifuge tube. The optical density was read at 260 nm to determine the position of the marker and then amount of radioactivity determined for each fraction.

(B) Control. The conditions and procedures were exactly as in (a) but without hybridase.

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bonded to DNA. These results are in agreement with those found by Keller (13) for ribonuclease H from chick embryos.

Conclusion. An endonucleolytic enzyme has been isolated from E. coli with a striking specificity: it degrades only an RNA chain hydrogen bonded to DNA. No direct information concerning the in vivo function of the hybridase is presently available. A number of recent reports indicate that RNA may serve as a primer for DNA synthesis in DNA replication (14, 10, 15). Such an RNA primer must be excised

after the DNA chain has been sufficiently elongated. Because of its high specificity, the hybridase would be a likely candidate to perform this excision.

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