ISOLATION OF 5S RIBONUCLEIC ACID CISTRONS AND tRNA CISTRONS FROM ESCHERICHIA COLI

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ABSTRACT

5S ribonucleic acid genes were isolated from Escherichia coli concomitantly with the genes for transfer ribonucleic acid. The 5S cistrons constitute approximately 0.008% of the E. coli genome; enough DNA to specify between 5 and 6 copies of 5S ribonucleic acid.

Enrichment and isolation of the cistrons that code for bacterial ribonucleic acids (RNA) became feasible as RNA fractionation procedures, hybridization techniques and physical methods of separating nucleic acids with sufficient sensitivity were developed. Ribosomal RNA cistrons (rDNA), comprising about 0.3% of the bacterial genome (Yankofsky and Spiegelman, 1962; Doi and Igarashi, 1965) have been isolated from Escherichia coli and Proteus mirabilis (Kohne, 1968), and possibly from Salmonella typhimurium (Udvardy and Venetianer, 1971). Additionally, partially purified rRNA cistrons have been obtained from E. coli (Davison, 1966), Bacillus subtilis (Sgaramella et al., 1968; Colli and Oishi, (a) 1969, (b) 1970; Takahashi, 1969), and Mycoplasma sp. (Kid) (Ryan and Morowitz, 1969). Estimates of the amount of DNA complementary to transfer RNA (tRNA) in E. coli and B. subtilis vary from 0.02% to 0.07% (Giacomoni and Spiegelman, 1962; Goodman and Rich, 1962;

Zahavi-Willner and Comb, 1966; Baguley and Ralph, 1966; Morell et al, 1967; Daniel et al, 1969). We recently reported the isolation of tRNA cistrons (tDNA) from E. coli by repeated cycles of free solution hybridization of DNA with rate-limiting amounts of tRNA and subsequent chromatography on hydroxyapatite (HA) (Brenner et al, 1970). In these experiments the average value obtained for tDNA was 0.05% of the total E. coli genome. An alternative method of isolation (Marks and Spencer, 1970) indicated 0.02% tDNA in E. coli. This method was also used to isolate E. coli tyrosine tDNA (Marks et al, 1971). Enrichment of tDNA has been reported from Mycoplasma sp. Kid (Ryan and Morowitz, 1969), and the complete synthesis of E. coli alanine tDNA has been carried out de novo (Agarwal et al, 1970).

5S RNA cistrons (5S DNA) are complementary to only between 0.005 and 0.015% of the genomes of E. coli and B. subtilis (Zehavi-Willner and Comb, 1966; Morell et al, 1967; Pace and Pace, 1971). The isolation of 5S DNA from bacteria has not been reported.

We now report the isolation of 5S DNA from E. coli. Due to the small amount of 5S DNA, it was practical to isolate the 5S DNA concomitantly with tDNA. The isolation procedures (Brenner et al, 1970) were therefore modified so that tDNA and 5S DNA are jointly partially purified, separated from each other and then further purified. The isolation procedures are described below.

Transfer RNA and 5S RNA were purified from bulk E. coli RNA and separated from one another by repeated chromatography on Sephadex G-100 columns (Schleich and Goldstein, 1966) until both RNA species formed homogeneous peaks on Sephadex G-100, and after electrophoresis on acrylamide gels (Peacock and Dingman, 1967). $^{32}PO_4$-labeled DNA from E. coli (75 μg/ml, 350,000 Cerenkov cpm/μg, suspended in 0.14 M phosphate buffer ((PB), pH 6.8) was sheared to an average single-stranded fragment size of 1.2×10^5 daltons (Brenner et al, 1970) and denatured in a boiling water bath for four minutes. The DNA was quickly cooled to 60°C and passed through an HA column

equilibrated in 0.14 M PB + 0.4% sodium dodecyl sulfate (SDS) and held at 60°C. Single-stranded DNA passes through HA under these conditions. Material bound to HA, mainly double-stranded DNA that is cross-linked, (Alberts and Doty, 1968) was discarded. This step was repeated twice during the purification of 5S DNA and tDNA to insure removal of all cross-linked DNA and any non-DNA material binding to HA.

The DNA, adjusted to 0.10 M PB + 0.4% SDS, at 50 μg/ml, plus 25 μg/ml each of 5S RNA and tRNA was heat denatured, quickly cooled to 60°C and incubated at 60°C for 15 minutes. At RNA concentrations of 25 μg/ml, the hybridization reactions between DNA and both 5S RNA and tRNA are complete after 15 minutes, whereas the DNA self-reaction is very limited (Brenner et al, 1970; Britten and Kohne, 1966; Kohne, 1968). The hybridization mixture was then passed through an HA column (2.8 g HA/mg nucleic acid) equilibrated in 0.10 M PB + 0.4% SDS at 60°C. The column was washed several times with 0.10 M PB + 0.4% SDS to remove single-stranded DNA. 5S RNA/DNA and tRNA/DNA hybrids, as well reassociated DNA duplexes, and unreacted 5S and tRNA all bind to HA in 0.1 M PB. About 6.1% of the input DNA was bound to HA as the result of both hybridization reactions and DNA self-reaction. This back peak material was eluted from HA in several 10 ml portions of 0.3 M PB, and the fractions were then pooled and diluted to 0.1 M PB. A sufficient amount of 2 N sodium hydroxide was added to this solution to obtain 0.2 N sodium hydroxide concentration and the material was incubated at 60°C for two hours to degrade both free and hybridized 5S RNA and tRNA. At this stage the sample contained reassociated duplexes, dehybridized 5S DNA and tDNA and degraded 5S RNA and tRNA. The preparation was neutralized with hydrochloric acid and extensively dialysed to remove the ribonucleotides. The material was concentrated to a desired volume (usually between 10 and 15 ml) and equilibrated agains 0.10 M PB + 0.4% SDS by dialysis. In control experiments DNA that did not bind to HA in the first cycle was reincubated in the presence and absence of tRNA and 5S RNA. In the absence of RNA about

1.5% bound to HA. In the presence of either RNA, binding to HA was 1.5-2.0%, indicating that essentially all 5S DNA and tDNA was hybridized in the first cycle.

Three additional DNA/RNA hybridization cycles using both 5S RNA and tRNA were carried out. In each cycle the concentration of 5S RNA and tRNA were kept at 25 μg/ml and the incubation temperature, volume and time were held constant. The DNA concentration decreased with each cycle, thereby making DNA self-reaction progressively less probable. The DNA remaining after the third cycle was again denatured and immediately passed through HA to eliminate remaining cross-linked DNA that binds to the column without any incubation at 60°C. The results are shown in Table 1. After four cycles of hybridization 0.17% of the input readioactivity bound to HA. These bound counts were present either in DNA hybridized to 5S RNA or tRNA, in reassociated DNA duplexes or in nonspecific material that had not been removed in the background elimination procedures.

TABLE 1

Concomitant Purification of 5S DNA and tDNA from Escherichia coli.

Cycle	Hybridization Reaction*	% of Original DNA Bound to HA
1	Total *E. coli* DNA + 5S RNA + tRNA	6.1
2	Back peak DNA from Cycle 1 + 5S RNA + tRNA	1.3
3	Back peak DNA from Cycle 2 + 5S RNA + tRNA	0.75
Unincubated background elimination	Back peak DNA from Cycle 3	0.56
4	Front peak DNA from background elimination + 5S RNA + tRNA	0.17
5	Back peak DNA from Cycle 4 + 5S RNA	0.07% Back peak

*The hybridization reaction was carried out initially with 3 mg of labeled DNA at 50μg/ml + 25μg/ml each of 5S RNA and tRNA. The nucleic acids were denatured at 100°C for 4 minutes, incubated at 60°C for 15 minutes and then passed through HA as described in the text. After each cycle the fractions eluted from HA in 0.3 M PB were diluted to 0.1 M PB and hydrolysed with 0.2M NaOH for 2 hours at 60°C to degrade RNA. After the solution was neutralized by the addition of HCl the ribonucleotides were removed by dialysis, the solution was then concentrated and again dialysed against 0.1 M PB before the next hybridization cycle was carried out. DNA concentration decreased in each cycle, whereas the concentration of RNA, incubation temperature and duration were identical in all cycles.

At this point 5S DNA and tDNA were purified separately. DNA bound to HA in the fourth cycle was dehybridized and incubated in a fifth cycle with 5S RNA. As shown in Table 2, the fraction that passed through HA in 0.1 M PB + 0.4% SDS was enriched for tDNA (0.10% of the input) whereas the fraction eluted in 0.3 M PB was enriched for 5S DNA. The front peak, tDNA enriched fraction, was treated with 0.2 N NaOH to remove any 5S RNA and was then subjected to two further cycles of hybridization and HA chromatography (Table 2). At the end of the regimen the tDNA fraction accounted for 0.05% of the total DNA. This amount of tDNA is similar to the amount isolated from E. coli in previous experiments (Brenner et al, 1970).

TABLE 2

Isolation of 5S DNA and tDNA from E. coli.

tRNA Cycle	Hybridization Reaction*	% of Original DNA bound to HA
5	Front peak DNA from Cycle 5 + tRNA	0.061
6	Back peak DNA from Cycle 5 + tRNA	0.055
5S RNA Cycle		
6	Back peak DNA from Cycle 5 + 5S RNA	0.037
Elimination of unincubated background	Back peak DNA from Cycle 6	0.018
7	Front peak DNA from background elimination + 5S RNA	0.0081
8	Back peak DNA from Cycle 7 + 5S RNA	0.0077

*Hybridization reactions were carried out as described in Table 1.

The fraction enriched for 5S DNA was subjected to a sixth cycle of hybridization with 5S RNA, a third unincubated background elimination, and finally two further cycles of hybridization with 5S RNA. After the eighth cycle of hybridization the amount of DNA hybridizing with 5S RNA remained constant at about 0.0077%. Using 2.8×10^9 daltons as the molecular weight of DNA from E. coli B (Brenner et al, in press), and 39,000 daltons as the molecular weight of 5S RNA, this amount of 5S DNA is sufficient to specify between 5 and 6 copies of 5S DNA in E. coli. It

has been estimated the E. coli DNA contains 6 copies of both rDNA (Kohne, 1968; Pace and Pace, 1971) and 5S RNA (Pace and Pace, 1971).

The single-stranded DNA fragments have an average molecular weight of 1.2×10^5 daltons, some three times the size of 5S RNA. Therefore, if only one 5S molecule hybridized with each labeled DNA fragment the final yield of hybridized DNA would have been between 0.02 and 0.025% instead of the observed 0.0077%. This argument depends on the fact that 5S RNA cistrons are not preferentially lost during the 8 cycles of hybridization and chromatography on HA. The average DNA loss per cycle is between 2 and 3%. We have no reason to believe this technical loss is not random. The coincidence of this value with that obtained in RNA/DNA saturation studies (Morell et al, 1967; Pace and Pace, 1971) suggests that most of the hybrid molecules contain as many as three molecules of 5S RNA per DNA fragment. Thus the 6 copies of 5S DNA in E. coli may be clustered; possibly in 2 groups of three, or in one contiguous group of six.

Further evidence for purity of 5S DNA and tDNA was obtained in hybridization reactions with specific RNAs. 5S DNA was denatured, and aliquots were incubated without RNA and in the presence of rate-limiting amounts of 5S RNA, tRNA and rRNA from E. coli and tRNA from rat liver. Results from these experiments are shown in Table 3. In the absence of added RNA approximately 2% of 5S DNA bound to HA, while 91% of the 5S DNA hybridized with 5S RNA and bound to HA. There was no reaction of 5S DNA with rat liver tRNA, a slight reaction with tRNA from E. coli and, as expected, a significant reaction with bulk rRNA from which 5S RNA had not been removed. The reaction observed between the 5S cistrons and E. coli tRNA may indicate a small percentage of tRNA contamination in our 5S RNA preparation, or might represent tDNA located contiguously to 5S DNA. The second alternative cannot be ruled out, but the first possibly is strengthened by the fact that this tDNA preparation exhibited some 6% reaction with 5S RNA (Table 3). No reaction occurred between tDNA and either rRNA from E. coli or tRNA from rat liver.

TABLE 3

5S DNA and tDNA Hybridization Reactions

Source of RNA	% reaction of 5S DNA	% reaction of tDNA
---	2.0	1.5
5S RNA (50 μg/ml)	91	6
E. coli tRNA (50 μg/ml)	10	94
Rat liver tRNA (200 μg/ml)	1.5	1.0
rRNA (200 μg/ml)	50	2.0

0.001 μg DNA in 0.12 M PB, alone, or with RNA at the indicated concentration was denatured by heating at 100°C for 4 min., quickly cooled to 60°C and incubated for 15 minutes (E. coli tRNA and 5S RNA) or 2 hours (rat liver tRNA, rRNA, no RNA). The samples were then assayed at 60°C on HA equilibrated with 0.12 M PB + 0.4% SDS.

DNA/DNA reassociation studies to be reported elsewhere (Brenner et al, in preparation) indicated that DNA specifying both 5S RNA and tRNA are highly conserved within the Enterobacteriaceae.

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