CLUSTERING OF tRNA CISTRONS IN ESCHERICHIA COLI DNA

Maurille J. Fournier*, William L. Miller* and B. P. Doctor

Division of Biochemistry
Walter Reed Army Institute of Research
Washington, D. C. 20012

Received August 27, 1974

SUMMARY: Characterization of tRNA:DNA hybrids reveals that many, perhaps most, of the tRNA genes in E. coli DNA are clustered. Density and double-isotope measurements show that 3-4 molecules of tRNA can hybridize with DNA fragments that are only 4-5 times larger than a mature tRNA. Treatment of the hybrids with a single-strand-specific endonuclease results in the solubilization of 30-35% of the DNA and the formation of monocistronic hybrids.

While it is clear that there are from 40-60 genes for tRNA in E. coli DNA (1) little is known about the arrangement of these cistrons in the chromosome and the mechanism by which their expression is coordinated. The eleven tRNA genes that have been mapped thus far are scattered around the chromosome (1). These results rule out the possibility that the entire complement of cistrons is organized into one or even a few polycistronic operons. Recent evidence however, reveals that certain of the tRNA cistrons are clustered (2-5) and may be transcribed in a polycistronic manner (6). In an earlier report we described the isolation of total E. coli tDNA (the DNA complementary to tRNA) and presented results suggesting that there is extensive clustering of the tRNA cistrons in this organism (7). This conclusion was based on: 1) the finding that the yield of tDNA corresponded well to the content of tDNA in the chromosome although the tDNA fragments were 4-5 times larger than a tRNA and 2) the finding that the density of tRNA:tDNA hybrids was the same as that reported for hybrids in which the ratio of RNA:DNA is close to one. The results reported here extend the earlier findings and establish conclusively that clusters of at least 3 or 4 tRNA genes occur.

*Present Address: Department of Biochemistry, University of Massachusetts, Amherst, Mass. 01002 (MJF) and Department of Biochemistry, University of Wisconsin, Madison, Wisc. 53706 (WLM).

MATERIALS AND METHODS: [^{32}P]-phosphoric acid, [^{35}S]-sulfuric acid and Liquifluor were purchased from the New England Nuclear Corp. Cesium sulfate was obtained from Schwarz/Mann.

All DNA and tRNA was derived from E. coli B and purified as described earlier (7). [^{32}P]-tDNA was isolated by hybridization of fragments of [^{32}P]-DNA with tRNA and purification of the RNA:DNA hybrids by chromatography on hydroxyapatite (7). Three or four cycles of hybridization and chromatography were required to remove all DNA fragments which were not complementary to tRNA. The molecular weight of the tDNA fragments was about 125,000 daltons. [^{35}S]-tRNA was prepared according to the method of Peterkofsky and Lipsett (8) and purified by Sephadex G-100 chromatography.

Neurospora crassa endonuclease was isolated by the method of Rabin and Fraser (9). The ratio of single-stranded to double-stranded activity with E. coli DNA was 150:1.

Radioactivity was measured by liquid scintillation spectrometry by: a) collecting samples precipitated in 5% trichloroacetic acid (TCA) on Millipore filters (cellulose nitrate) and counting the dried filters in Liquifluor-toluene or b) counting aliquots directly in Liquifluor-toluene-Triton X-100 (1:12:6). Samples with less than 100 cpm were counted for 20 minutes. All others were counted for 10 minutes. Instrument background activity was about 20 cpm.

RESULTS: If the tRNA cistrons in E. coli are clustered to the extent that some tDNA fragments contain two or more cistrons, the density of tRNA:tDNA hybrids should reflect this situation. Figure 1 shows the density profile obtained when a preparation of [^{32}P]-tDNA hybridized with tRNA is fractionated in a cesium sulfate density gradient. The bulk of the material bands at a density of 1.505 g cm^{-3} which is the density of authentic RNA:DNA hybrids as reported by others (10). Under these conditions RNA bands at a density of about 1.650 and single-stranded E. coli DNA at ρ = 1.450 (11). The hybrid band is broad and skewed toward the region of lower density indicating hetero-

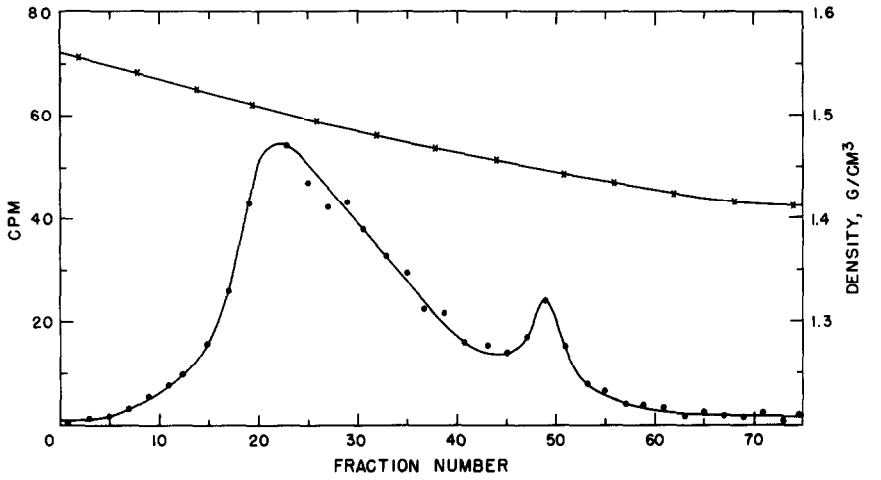

Fig. 1. Cesium sulfate density gradient centrifugation of tRNA:tDNA hybrids. tRNA:[^{32}P]-tDNA hybrids were formed by incubation of heat denatured [^{32}P]-tDNA (4 µg, 2750 cpm) with an excess of tRNA (50 µg) in 1 ml 2x SSC^{+} buffer, pH 7. (72°C). After incubation for one hour the sample was diluted to 4.5 ml and the density adjusted to 1.4699 g cm^{-3} with Cs_2SO_4. Final concentration of SSC was 0.5X. Centrifugation was in a No. 40 rotor (Beckman Instruments) at 33,000 rpm for 72 hours at 24°C. Fractions (6 drops) were obtained by puncturing the bottom of the tube and the resulting radioactivity and density profiles determined. [^{32}P]-activity was measured by the filter method described in Methods. Density values were determined from refractive index measurements. (•——•) [^{32}P]-DNA; (x——x) density.

$^{+}$SSC = 0.015 M sodium citrate, 0.15 M sodium chloride.

geneity of the duplexes. The minor band at density 1.449 g cm^{-3} corresponds to single-stranded DNA and is presumed to consist of fragments which cannot hybridize with tRNA. In this particular preparation of tDNA, the single-stranded material accounts for about 5% of the DNA.

The density profile for the hybrids indicates the occurrence of a distribution of hybrids which vary in RNA content. The pattern suggests a mix of hybrids containing from one to three or four tRNAs. Hybrids which contain multiple tRNA molecules per DNA fragment would be expected to band in the region of higher density and those containing a single cistron and thus, a lower ratio of RNA:DNA will band at densities closer to that of single-stranded DNA. In this experiment the majority of the hybrids banded at ρ = 1.500 and probably consist of tDNA fragments hybridized with 3 or 4 tRNAs. Because the density of tRNA in Cs_2SO_4 has not been firmly established, it is

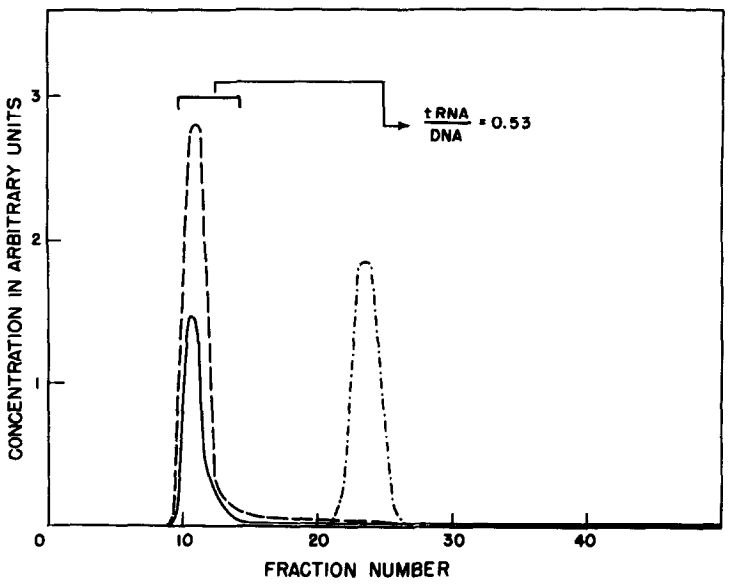

Fig. 2. Fractionation of [^{35}S]-tRNA:[^{32}P]-tDNA hybrids and non-hybridized tRNA by Sephadex G-100 column chromatography. [^{32}P]-tDNA (3.5 µg @ 3700 cpm/µg, 75-80% pure) was hybridized with an excess of [^{35}S]-tRNA (40 µg @ 4535 cpm/µg) in one ml 2x SSC, pH 7.5 (60°C). The hybrids were separated from tRNA by 2 successive cycles of chromatography on a column of Sephadex G-100 (1 x 150 cm) developed with 1 M NaCl at ambient temperature. 3 ml fractions were collected and the hybrid-containing fractions from the first column pooled and re-chromatographed. The profile shown is from the second column. (— —) [^{32}P]-DNA; (●——●) [^{35}S]-tRNA; (●——●) marker tRNA. The [^{32}P]- and [^{35}S]-activity profiles were determined by the TCA-filter method and have been normalized to a common specific activity. The results (expressed in arbitrary units) indicate the relative amount of RNA and DNA on a wt/wt basis.

not possible to accurately calculate the ratio of RNA:DNA based on density shifts of non-hybridized and hybridized tDNA.

To better quantitate the degree of clustering, double-labeled tRNA:tDNA hybrids were constructed. In this experiment, [^{35}S]-tRNA[a] was hybridized with a preparation of [^{32}P]-tDNA judged to be 75-80% pure with regard to content of DNA fragments hybridizable with tRNA. (Purity was estimated by hydroxyapatite-ref. 7 and cesium sulfate gradient analyses.) The hybrids were separated from non-hybridized tRNA by two cycles of chromatography on a column of Sephadex G-100. The resulting profile is shown in Fig. 2. The ratio of tRNA:DNA on a wt/wt basis is 0.53. When this value is corrected for

[a]On the average there is one sulfur-containing nucleoside per molecule of tRNA--ref. 12.

the content of non-hybridizable fragments, it is estimated that 60-70% of the nucleotides of the tDNA are hybridized with tRNA. This amounts to, on the average 3 to 4 tRNA cistrons per fragment of tDNA.

An independent means of determining the proportion of the tDNA fragments that is complementary to tRNA is to treat the tRNA:tDNA hybrids with a single strand-specific nuclease and then determine the extent of DNA degradation. In such an experiment we treated [^{35}S]-tRNA:[^{32}P]-tDNA hybrids with a single strand-specific endonuclease prepared from N. crassa and analyzed the digestion products by Sephadex column chromatography. The fractionation pattern obtained is shown in Figure 3. Total and acid-soluble radioactivity profiles are shown. The acid-soluble [^{32}P]-activity results from hydrolysis of the single-stranded DNA unprotected by tRNA. About 60% of the [^{32}P]-activity was

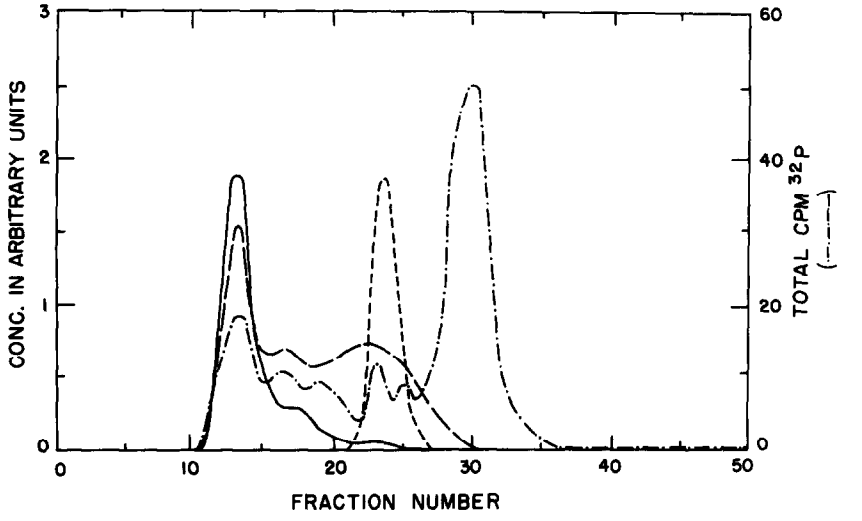

Fig. 3. Sephadex G-100 column chromatography of N. crassa endonuclease digest of [^{35}S]-tRNA:[^{32}P]-tDNA hybrids. A preparation of [^{35}S]-tRNA:[^{32}P]-tDNA hybrids was constructed from a sample of tDNA estimated to be 50% pure by hydroxyapatite analysis (7) and separated from non-hybridized tRNA by Sephadex G-100 chromatography as described in Fig. 2. 2 µg hybrid (5750 cpm ^{35}S, 5670 cpm ^{32}P)was incubated overnight with 0.3 units of N. crassa nuclease under the conditions described in ref. 9. The reaction mixture (0.5 ml) was then applied to a column of Sephadex G-100 and fractionated. The column dimensions and fractionation conditions are described in Figure 2. (———) [^{32}P]-DNA insoluble in 5% trichloroacetic acid (TCA); (— —) TCA insoluble [^{35}S]-tRNA; (— — — —) marker tRNA; (— · —) total [^{32}P]-activity (TCA soluble plus insoluble). The acid insoluble ^{32}P and ^{35}S profiles have been normalized to a common specific activity and the results expressed in arbitrary units.

rendered soluble. Inasmuch as approximately 50% of the DNA fragments in this preparation of tDNA did not hybridize with tRNA, about 30% of the DNA in the tRNA:tDNA duplexes was digested by DNase. This estimate of the proportion of non-hybridized DNA per tDNA fragment agrees with the value determined in the previous experiment. The ratio of RNA:DNA in the hybrid peak (fractions 11 to 16) has increased from about 0.6-0.7 to nearly one indicating that there is little non-hybridized DNA in the nuclease-treated hybrids.

Also of interest is the finding that the hybrids are not in the void volume but, rather, are included and elute 3 or 4 fractions later than did the non-nuclease treated hybrids shown in Fig. 2. This behavior suggests that nuclease treatment resulted in the formation of hybrids containing a single tRNA and cistron. Such would be the case if the hybridized segments were separated by single-stranded gaps. Indeed, the molecular weight of these hybrids was estimated to be about 60,000 daltons when chromatographed on a Bio-Gel-5m column with suitable marker compounds (results not shown). Inasmuch as the endonuclease is believed to be unable to cleave nicked duplex structures in which only a phosphodiester bond is broken (13), the results of this experiment appear to rule out the possibility that the cistrons are truly contiguous with no single-stranded spacer sequences--however small.

DISCUSSION: The results presented here establish that there is extensive clustering of the tRNA cistrons in the *E. coli* chromosome. The ratio of RNA:DNA in the hybrids, determined by density and isotopic measurements, was found to be about 0.6-0.7 for hybrids constructed from tRNA fragments of 125,000 daltons. Thus, these fragments which are 4-5x larger than a mature tRNA molecule contain, on the average, 3-4 tRNA cistrons.

Treatment of the hybrids with *N. crassa* endonuclease resulted in the production of monocistronic hybrids indicating that single stranded regions occur between adjacent cistrons. If spacer sequences do occur between tRNA cistrons and the cistrons are distributed evenly along the tDNA, it is possible to estimate the size of the spacer regions from our results.

Approximately 30-35% of the 125,000 dalton tDNA fragment is not complementary to native tRNA. This corresponds to 120-140 unpaired nucleotides. If each tDNA fragment contains 3-4 tRNA cistrons, then the average 'spacer' region is approximately 30-45 nucleotides in length. In view of the possibility that some tDNA fragments may also contain other cistrons, this estimate could be somewhat high. It is interesting, however, that the estimated number of bases which could serve as a spacer corresponds to the number of 'extra' nucleotides in a precursor to E. coli tyrosine tRNA (14).

REFERENCES:

1. Smith, J. D., Ann. Rev. of Genetics 6, 235 (1972).
2. Russell, R. L., Abelson, J. N., Landy, A., Gefter, M. L., Brenner, S., and Smith, J. D., J. Mol. Biol. 47, 1 (1970).
3. Ohlsson, B. M., Strigini, P. F., and Beckwith, J. R., J. Mol. Biol. 36, 209 (1958).
4. Miller, R. C., Besmer, P., Khorana, H. G., Fiandt, M., and Szybalski, W., J. Mol. Biol. 56, 363 (1971).
5. Squires, C., Konrad, B., Kirschbaum, J., and Carbon, J., Proc. Natl. Acad. Sci. USA. 70, 438 (1973).
6. Schedl, P. and Primakoff, P., Proc. Natl. Acad. Sci. USA. 70, 2091 (1973).
7. Brenner, D. J., Fournier, M. J., and Doctor, B. P., Nature 227, 448 (1970).
8. Peterkofsky, A. and Lipsett, M. N., Biochem. Biophys. Res. Commun. 20, 780 (1965).
9. Rabin, E. Z. and Fraser, M. J., Can. J. Biochem. 48, 389 (1970).
10. Chamberlin, M. and Berg, P., J. Mol. Biol. 8, 297 (1964).
11. Szybalski, W. in Methods in Enzymology, Grossman, L. and Moldave, K., editors, Academic Press, New York 12B, 330 (1968).
12. Hall, R. H., The Modified Nucleosides in Nucleic Acids, Columbia University Press, New York (1971).
13. Kato, A. C., Bartok, K., Fraser, M. J. and Denhardt, D. T., Biochim. Biophys. Acta 308, 68 (1973).
14. Altman, S., Nature 229, 19 (1971).