The distal end of the ribosomal RNA operon *rrnD* of *Escherichia coli* contains a *tRNA*^{thr} gene, two 5S rRNA genes and a transcription terminator

Gregg L. Duester and Walter M. Holmes

Medical College of Virginia, Virginia Commonwealth University, Box 678-MCV Station, Richmond, VA 23298, USA

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ABSTRACT

The distal region of the *Escherichia coli* ribosomal RNA operon *rrnD* has been sequenced to identify transfer RNA genes which might be contained within this transcriptional unit, and to define a possible transcription termination site. A nucleotide sequence corresponding to the gene for *tRNA*₁^{thr} is located between two genes for 5S rRNA. The spacer region between the proximal 5S rRNA gene and the *tRNA*₁^{thr} gene is 12 base pairs long, whereas the spacer region between the distal 5S rRNA gene and the *tRNA*₁^{thr} gene is 37 base pairs. Possible sites for RNA processing are discussed, and the sequence of the *tRNA*₁^{thr} gene is found to possess the same anticodon as the *tRNA*₃^{thr} gene. A putative transcription termination site for *rrnD* was³ located about 25 base pairs distal to the 3' end of the second 5S rRNA gene. The *rrnD* and *rrnC* terminators are compared with each other and with other prokaryotic terminators. Located at exactly the same site on the complementary strand is another putative transcription termination site for a gene being transcribed towards *rrnD*. The possibility of overlapping terminators is discussed.

INTRODUCTION

The regulation of bacterial stable RNA genes has been studied extensively, but the molecular mechanisms involved are for the most part unknown. Most, if not all, stable RNA genes are controlled by the stringent response, a mechanism which probably operates at the level of transcription initiation(1,2). The *tRNA*₁^{tyr} gene of *Escherichia coli* was the first stable RNA gene for which a transcription promoter sequence was determined(3). Recently, the promoter regions of five of the seven rRNA (*rrn*) operons in *E. coli* have been sequenced (4,5,6). Less information is available on transcription termination sites for stable RNA genes. The terminator for the *tRNA*₁^{tyr} gene(7) and the terminator for the rRNA operon *rrnC*(8) were recently sequenced.

Each of the rRNA operons of *E. coli* has been shown to contain tRNA genes, thus exemplifying the coordinate control of these two classes of genes. All seven rRNA operons have tRNA genes located in the spacer region between the 16S and 23S rRNA genes, and at least four have tRNA genes located distal to the 5S rRNA gene(9). The spacer tRNA genes are all co-transcribed with rRNA(2), but the distal tRNA genes present a different picture. The genes for tRNA^{asp}₁ and tRNA^{trp} located at the distal end of rrnC are co-transcribed with rRNA(9). The genes for tRNA^{thr}₄, tRNA^{tyr}₂, tRNA^{gly}₂, and tRNA^{thr}₃ located at the distal end of rrnB are evidently not co-transcribed with rRNA (10). Ikemura and Nomura(11) indicated that a gene for tRNA^{thr} was probably located near the distal end of rrnD which maps near minute 71 on the *E. coli* chromosome, but it was not determined whether or not this gene is co-transcribed with rRNA. We have examined the nucleotide sequence of the distal region of rrnD, and confirm that a gene for tRNA^{thr}₁ is located in this operon. Only 12 base pairs separate the gene for tRNA^{thr}₁ from the 5S rRNA gene which suggests that this tRNA is most likely co-transcribed with rRNA. We also have located a site at the end of rrnD which resembles many known prokaryotic transcription terminators (12).

EXPERIMENTAL PROCEDURES

Materials

[γ -³²P] ATP was purchased from New England Nuclear (specific activity 2000-3000 Ci/mmol). T4 polynucleotide kinase and calf intestine alkaline phosphatase were obtained from Boehringer Mannheim. The restriction endonucleases EcoRI, SaI, and BglII were purchased from Bethesda Research Laboratories. AvaI and TaqI were obtained from New England Biolabs. Acrylamide, bis-acrylamide, ammonium persulfate, xylene cyanol, bromphenol blue, and urea were from Biorad. Agarose and a 40x30 cm sequencing gel apparatus were from Bethesda Research Laboratories. Yeast bulk tRNA and bovine serum albumin were obtained from Sigma Chemical Co.

DNA

The chimeric plasmid pGD4 was constructed by ligating pBR322 and an EcoRI restriction endonuclease fragment containing the distal end of rrnD from *E. coli*. This plasmid was identified by screening

a clone bank of bacteria containing most of the EcoRI restriction endonuclease fragments of E. coli K12-C600 chromosomal DNA. Radio-labelled bulk tRNA and tRNA^{thr}₁ were used as hybridization probes to screen cloned plasmid DNA (Duester et al., manuscript in preparation). Plasmid DNA was prepared in milligram quantities as previously described (13).

Blotting and DNA/RNA Hybridization

Chromosomal DNA from E. coli K12-C600 was digested, blotted to nitrocellulose, and hybridized to ³²P-labeled tRNA^{thr}₁ using procedures which will be outlined elsewhere (Campen et al., manuscript submitted to J. Bacteriol.).

Mapping Restriction Endonuclease Sites

Single or double digestions of pGD4 DNA with the restriction endonucleases mentioned above were performed to generate a restriction endonuclease map sufficient for DNA sequencing. One µg of DNA was digested in 50 µl of 6mM Tris-HCl, pH 7.4, 6mM MgCl₂, 6mM dithiothreitol for one hour at 37C (or at 65C for TaqI), using one unit of enzyme per µg of DNA. Cleavage products were subjected to electrophoresis at 100V in 1.0% agarose or 5% acrylamide gels. All agarose and acrylamide gels contained 90 mM Tris-borate, pH 8.3, 2.5 mM EDTA, and this served as the electrophoresis buffer. DNA bands were visualized by ethidium bromide staining, and molecular sizes in base pairs(bp) were determined using digests of lambda and pBR322 DNA as standards.

Purification and Labeling of DNA Fragments

DNA fragments generated from complete digestion of pGD4 DNA with AvaI or TaqI were separated by electrophoresis in 5% acrylamide gels. Ethidium bromide stained DNA fragments were visualized by 330 nm ultraviolet light, and were cut out of the gel with a razor. Gel slices were placed in dialysis tubing (Union Carbide, 2cm width, pre-treated by rinsing with 1.0 mg/ml bovine serum albumin) with 1.0 ml of electrophoresis buffer. The dialysis tubing was submerged in buffer and oriented perpendicular to the current in a horizontal gel electrophoresis apparatus. After electrophoresis at 300V for one hour, the polarity was reversed for one minute to move the DNA away from the side of the tubing. The dialysate was removed and concentrated by ethanol precipitation as described by Maxam and Gilbert(14). This method is very rapid and

generally resulted in a greater than 50% yield.

DNA fragments for sequence analysis were treated with calf intestine alkaline phosphatase to remove 5' phosphates; the conditions were 100 mM Glycine-NaOH, pH 9.5, 10 mM MgCl₂, 1.0 mM ZnCl₂, 0.1 µg enzyme per 1.0 µg DNA, 37C for 30 min. DNA was 5'-labeled with [γ -³²P] ATP as described by Maxam and Gilbert(14). Complementary DNA strands were separated as described(14), and single-stranded 5'-labeled DNA fragments were electrophoretically eluted from acrylamide as described above except that the dialysis tubing was rinsed with 1.0 mg/ml tRNA instead of bovine serum albumin.

DNA Sequencing

DNA fragments were sequenced using a modification of the chemical method of Maxam and Gilbert(14). Four base-specific reactions were employed to give cleavage at G, G+A, C+T, or C. The G, C+T, and C reactions were performed as described(14), and the G+A reaction was carried out as follows: 5.0 µl DNA, 3.0 µl pyridinium formate (1.0 M formate-pyridine, pH 2.0), 2.0 µl carrier DNA (10 mg/ml), and 15 µl H₂O were mixed; heated at 37C for 35 min (less than 100 bp) or 15 min (greater than 100 bp); then treated with piperidine as for the G reaction(14). Cleavage products were fractionated on 20% or 8% acrylamide-50% urea gels (40 cm long). A constant voltage of 1200-2000V was employed such that the wattage never exceeded 60W, and electrophoresis times ranged from 3-8 hours. The gel was subjected to autoradiography at -70C for 1-6 days using Kodak X-RP film.

RESULTS

Mapping Restriction Endonuclease Sites

The restriction endonuclease map shown in Fig. 1 was generated by the following experiments. Digestion of pGD4 with EcoRI produced two fragments, the 4400 bp pBR322 vector and the 3900 bp cloned fragment containing the distal end of rrnD. Fig. 2(a) shows that this 3900 bp fragment hybridizes to tRNA^{thr}₁; pGD4 also hybridizes to 23S and 5S rRNA (data not shown). That this segment of DNA is really derived from rrnD was determined from three lines of evidence. First, Ikemura and Nomura indicated that the rrnD operon possessed a tRNA^{thr} gene very near its distal end(11). Second, Boros et al. recently published a detailed restriction endonuclease map of all seven rRNA

Fig. 1. Physical map of the recombinant plasmid pGD4.

The 4400 bp fragment corresponds to the pBR322 vector. The 3900 bp fragment corresponds to the cloned insert. The hatched regions in the inset correspond to non-coding DNA sequences.

operons of *E. coli*, including the positions of *EcoRI* sites. An inspection of this map shows that a 3900 bp *EcoRI* fragment carrying 23S and 5S rRNA genes could only be derived from the distal end of *rrnD*. Third, it was found that the *SalI* and *BglII* sites predicted by Boros *et al.* (15) for the distal region of *rrnD* were present in exactly the right positions in pGD4.

AvaI and *TaqI* sites were located to complete the map. The plasmid pGD4 was cut into five fragments by *AvaI* as shown in Fig. 2(C). The three *AvaI* sites in the 23S rRNA gene (Fig. 1) were predicted by inspection of the recently published sequence of this gene(16). Also, pBR322 was found to be cleaved only once by *AvaI*, thus necessitating the presence of one additional *AvaI* site distal to the 5S rRNA gene located 650 bp away from an *AvaI* site in the 23S rRNA gene. The 650 bp *AvaI* fragment was found to hybridize to $\text{tRNA}^{\text{thr}}_1$ as shown in Fig. 2(C) and to 23S and 5S rRNA (data not shown), and was therefore purified in large quantities for DNA sequencing.

Because the gene for $\text{tRNA}^{\text{thr}}_1$ was on this 650 bp fragment, one additional restriction endonuclease site could be predicted. An inspection of the RNA sequence for $\text{tRNA}^{\text{thr}}_3$, and for most tRNAs, indi-

Fig. 2

DNA was digested, electrophoresed in agarose, stained with ethidium bromide (lanes 1,2,3), blotted to nitrocellulose, hybridized to 32 P-tRNA^{Met}, and autoradiographed (lanes 4,5). A: lane 1 is λ -HindIII; lanes 2 and 4 are pGD4-EcoRI; lanes 3 and 5 are *E. coli* chromosomal DNA-EcoRI. B: lane 1 is λ -HindIII; lanes 2 and 4 are pGD4-EcoRI; lanes 3 and 5 are pRC24-EcoRI. C: lane 1 is pBR322-AluI; lanes 2 and 4 are pGD4-AvaI; lanes 3 and 5 are pRC24-AvaI. The numbers are in base pairs.

cated the presence of a TaqI site in the cognate gene at a position corresponding to the T Ψ CGA loop(17). The 650 bp AvaI fragment was indeed found to be cleaved by TaqI, and was conveniently cut into just two fragments (400 and 250 bp) which were isolated for DNA sequence analysis (data not shown).

Organization of Distal End of *rrnD* in the Chromosome

When *E. coli* K12-C600 chromosomal DNA is digested with *Eco*RI and hybridized to ³²P-labeled tRNA^{thr}₁, a band is seen which corresponds in size to a similar digest of pGD4 (Fig. 2A). This experiment shows that the distal *rrnD* region cloned on pBR322 is the same as that in the chromosome with respect to molecular size. Also, we have isolated a plasmid, pRC24 (Duester *et al.*, manuscript in preparation), which was obtained from an entirely separate cloning experiment than that for pGD4, but which has a restriction endonuclease pattern identical to pGD4. An *Eco*RI digest of both plasmids shows that each has a 3900 bp fragment which hybridizes to tRNA^{thr}₁ (Fig. 2B); an *Ava*I digest shows that each has a 650 bp fragment which hybridizes to tRNA^{thr}₁ (Fig. 2C). Since it would be highly improbable for these two independently isolated plasmids to be rearranged identically, we believe that no rearrangements have occurred during the cloning of this region.

DNA Sequencing of the Distal Region of *rrnD*

Both strands of the 400 and 250 bp *Ava*I-*Taq*I fragments were sequenced using the Maxam and Gilbert chemical technique(14). A representative DNA sequence gel is shown in Fig. 3. The entire DNA sequence of the 650 bp *Ava*I fragment is shown in Fig. 4. The sequence as read in the direction of transcription contains the last 133 bp of the 23S rRNA gene, a spacer region of 92 bp, the entire 5S rRNA gene, a spacer region of 12 bp, the tRNA^{thr}₁ gene, a spacer region of 37 bp, another 5S rRNA gene, and an extensive region of dyad symmetry (consisting of two perfectly homologous 9 bp inverted repeat sequences separated by 4 bp) with an axis of rotation located about 25 bp distal to the 3' end of the second 5S rRNA gene.

DISCUSSION

The nucleotide sequence of the distal region of the rRNA operon *rrnD* in *E. coli* has been determined. A gene for 5S rRNA is located 92 bp from the 3' end of the 23S rRNA gene. This 92 bp spacer region was recently sequenced in *rrnD* and *rrnX*(18), and our sequence exactly matches the one for *rrnD*. The distal 133 bp of the 23S rRNA gene which we sequenced in *rrnD* is identical to the published sequence for this gene in *rrnB*(16). The sequence of this 5S rRNA gene in *rrnD* is identical to that obtained by direct RNA sequencing(19), and identical to the 5S rRNA gene in *rrnC*(8). Very surprisingly, we have identified a second

Fig. 3. DNA sequence of the putative rrnD transcription terminator. The region shown is from nucleotide 579-647 of the 667 bp *Ava*I fragment. The sequence is from 3' (579) to 5' (647) on the bottom strand (see Fig. 4).

5S rRNA gene located 37 bp distal to the $tRNA^{thr}_1$ gene. There are three bp differences between this 5S and the other 5S rRNA gene (located at positions 473, 482, and 587 in Fig. 4). However, these correspond to positions which have previously been shown to be variable by RNA sequencing(19). Of the four rRNA operons in which 5S rRNA sequences have been determined, only rrnD has been shown to have two 5S rRNA genes.

Fig. 4. DNA sequence of the *rrnD* distal region.

The entire DNA sequence of the 667 bp *Ava*I fragment in Fig. 1 is shown. Nucleotide sequences corresponding to mature tRNA and rRNA are underlined. The region of dyad symmetry corresponding to the stem and loop of the putative terminator is overlined. Possible RNA processing sites are indicated by asterisks for 5S rRNA and crosses for tRNA.

We have discovered that a gene for tRNA^{thr}₁ resides near the end of *rrnD* 12 bp distal to the first 5S rRNA gene. Thus, earlier indications of the location of this gene have been confirmed(11). To the best of our knowledge this is the first published sequence of tRNA^{thr}₁ from either the DNA or RNA. There are four classes of threonine tRNAs designated 1-4. The reasons for designating this the gene for tRNA^{thr}₁ are two-fold. First, the sequence for tRNA^{thr}₁ is clearly different than the published sequences for tRNA^{thr}₃ (20) and tRNA^{thr}₄ (21). Second, pGD4 hybridized very strongly to tRNA^{thr}₁, but hybridized only very weakly to tRNA^{thr}₂ (pure tRNA^{thr}₁ and tRNA^{thr}₂ available from Plenum Scientific; data not shown). We propose to call this gene *thrV*, in keeping with the established terminology for tRNA^{thr} genes.

A tRNA cloverleaf structure(22) can be drawn from the *thrV* sequence, and Fig. 5 compares the structures of tRNA^{thr}₁ and tRNA^{thr}₃. It can be seen that the structures are very similar, and interestingly they share the same anticodon 5'-GGU-3' which is homologous to the threonine codons 5'-ACC-3' and 5'-ACU-3' by the rules of the "wobble hypothesis" of Crick(23). Of the 76 nucleotides present in each tRNA,

fully resolve the issue. Likewise, we believe the second 5S rRNA gene located distal to thrV is co-transcribed with rrnD.

An additional feature of the thrV sequence is the presence of a seven bp inverted repeat flanking its 5' (5'-AATTTAG-3') and 3' (5'-TTAGGTT-3') ends. It is possible that these sequences could be involved in RNA processing to form the mature tRNA, and could be the site of recognition by tRNA processing enzymes such as RNase P (3). Similar structures have been observed flanking the 16S-23S rRNA spacer tRNA genes of rrnD and rrnX(25), and the tRNA^{thr}₄ gene, thrU(21).

Other RNA processing sites which reside in this region are those for 5S rRNA. An examination of the sequences flanking the 5S rRNA genes in rrnD and rrnC uncovers conserved sequences on the 5' (5'-GAATT-3') and 3' (5'-CAAAT-3') sides. Following the model for the secondary structure of 5S rRNA proposed by Madison(26) which assumes that the 5' and 3' ends are base paired, it is possible that some of the bases in the flanking sequences could pair if a three base bulge loop is allowed. Perhaps these conserved sites function in the RNase E processing event which has been shown to produce mature 5S rRNA from a 9S precursor(27).

The DNA sequence in Fig. 4 was searched for regions of dyad symmetry using the computer program of Korn *et al.*(28). The results were quite surprising. A huge inverted repeat was found which begins at position 572 and extends for 74 bp to position 661. Fig. 6 (top) shows how the inverted repeat in the complementary RNA transcript could possibly fold to form a stem and loop structure. Out of 35 bp in the stem there are only three mismatches, giving rise to a very stable structure with a ΔG of -33.5 kcal/mole using the base-stacking rules of Gralla and Crothers(29). The obvious implication is that this could be a possible transcription termination site since most prokaryotic terminators sequenced so far have been shown to possess an inverted repeat just prior to the 3' end of the transcript (12).

Two general classes of terminators have been recognized; those dependent on the *E. coli* termination protein, rho, and those which are rho-independent(12). The rho-independent terminators all have an A-T rich region immediately following the inverted repeat, and the transcript usually terminates in a run of uridine residues. In contrast, the two well characterized rho-dependent terminators (t_{R1} and

Fig. 6

Fig. 7

Fig. 6 shows the RNA stem and loop structures which could possibly be formed at the terminus of the *rrnD* transcript. Fig. 7 shows the RNA stem and loop structures which might form at the terminal regions of a gene being transcribed towards *rrnD*. No definite 3' end is indicated for any of these structures.

tRNA^{tyr}) have the common sequence 5'-CAATCAA-3' immediately following an inverted repeat, and termination occurs at an A or T in, or near, this sequence. Such a sequence was not found anywhere in the distal region of *rrnD*, but this does not necessarily rule out the presence of a rho-dependent terminator.

The terminator for *rrnC* was recently determined(8) and found to be rho-independent by *in vitro* transcription studies. The *rrnC* terminator was found to have an inverted repeat consisting of a nine base pair stem with a four base loop, followed by a string of eight thymine residues. A remarkably similar structure can be observed within the large inverted repeat at the distal end of *rrnD*. Fig. 6 (bottom) shows the possible RNA structure which could form a nine bp stem and four base loop (positions 600-639 in Fig. 4). This inverted repeat is followed by a string of eight thymine residues separated by one guanine. The stability of this structure is somewhat better than the *rrnC* terminator; $\Delta G = -12.5$ kcal/mole for *rrnD* compared to $\Delta G = -11.1$ kcal/mole for *rrnC*(8). Since the *rrnC* terminator has been found to efficiently terminate transcription *in vitro*, we feel that the nine bp structure in *rrnD* will be equally effective as a terminator. Detailed studies are in progress in our laboratory to define the termination site of *rrnD* *in vitro* and *in vivo*.

Whether or not the entire 35 bp stem ever would be formed *in vivo* is unclear. If the nine bp putative terminator stem is efficient, then the 35 bp stem would never have a chance to form. In addition, part of this stem and loop lies inside the structural gene for the second 5S rRNA gene, thus making its formation unlikely. Possibly this structure is a stop-gap for those few RNA polymerase molecules which manage to find their way past the initial termination signal. In this manner, as more and more RNA is transcribed, a more and more stable stem is formed which further enhances termination.

Another extremely interesting facet of the sequence in Fig. 4 is the presence of an inverted repeat followed by a string of thymine residues on the bottom strand (proceeding 5' to 3' on the bottom strand from position 647-578). Fig. 7 (top) shows a 23 bp stem and loop which could be formed in the RNA. Like the structure in Fig. 6 (top) it is very stable since it contains only one mismatch; $\Delta G = -25.1$ kcal/mole(29). A nine bp structure resembling the putative *rrnD* terminator can also be observed (from positions 631-592) as

shown in Fig. 7 (bottom). Due to the one mismatch this structure has a $\Delta G = -7.3$ kcal/mole(29) which is significantly lower than the value for rrnD. However, it is still stable enough to be a possible terminator since an inspection of 20 terminators(12) shows that the ΔG values for the stem and loop structures range from -5.2 kcal/mole for tRNA^{tyr}₁ to -31.4 kcal/mole for the his attenuator(29). So, it is entirely possible that this is a terminator for a gene being transcribed towards rrnD. If so, it will be the first case to the best of our knowledge of overlapping terminators of transcription. The functionality of the putative terminators in either orientation is presently being pursued in our laboratory. An initial in vitro transcription experiment using the 667 bp AvaI fragment has indicated that termination occurs to give two major RNA transcripts which are about 620 and 70 nucleotides in length (W.M. Holmes, unpublished results).

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