The Anti-Shine–Dalgarno Region in *Escherichia coli* 16S Ribosomal RNA Is Not Essential for the Correct Selection of Translational Starts†

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ABSTRACT: Plasmid pPM114, which contains the *Escherichia coli* 16S rRNA gene under control of a T7 promoter, was linearized upstream of the 3′ end of the gene and used in an in vitro transcription assay to yield a 16S rRNA lacking about 30 nucleotides at its 3′ end. This truncated 16S rRNA was assembled into 30S subunits which contain the full complement of 30S proteins, including S21, but were impaired in their capacity to associate to the 50S subunits. This impairment was paralleled by a decrease in their protein synthesis activity under the direction of natural or artificial messengers. However, although the anti-Shine–Dalgarno sequence was missing, the initiation step was not specifically affected, and the mutated ribosomes could initiate translation at the correct start sites. This supports previous suggestions that the translational efficiency and the selection of translational starts are not solely controlled by the Shine–Dalgarno interaction. A novel interpretation of the role of protein S21 is also proposed which is independent of the activation by this protein of the base-pairing potential of the anti-Shine–Dalgarno sequence of 16S rRNA.

The Shine–Dalgarno sequence is a polypurine stretch of variable length, which is located 5′ to the initiation codon of prokaryotic messenger RNAs and can base pair to a complementary pyrimidine-rich sequence, the anti-Shine–Dalgarno sequence, at the 3′ end of the 16S rRNA. It has been suggested that it plays a crucial role in the selection of the translation initiation starts in prokaryotes and the control of translational efficiency (Shine & Dalgarno, 1974). Considerable evidence supports this suggestion [reviewed by Steitz (1980), Gold et al. (1981), Kozak (1983), and Gren (1984); see also Dahlberg (1989)], but a survey of the literature indicates that other regions in the messenger RNAs are also involved in the control of translation efficiency (Dreyfus, 1988; Gold, 1988).

We have previously developed a system which functions entirely in vitro and enables the study of mutations in the 16S rRNA (Melançon et al., 1987; Gravel et al., 1989). A similar system was developed independently by Krzyzosiak et al. (1987). In these systems, *Escherichia coli* 16S rRNA gene has been introduced into a pUC vector, directly downstream of a T7 promoter. We have shown that in vitro transcription of the linearized plasmid yields a 16S rRNA transcript which can be assembled into 30S subunits. These subunits are active in the translation of artificial and natural messengers, although their 16S rRNA lacks the 10 methylations which occur posttranscriptionally in natural 16S rRNA. We have previously constructed a deletion mutant of the 16S rRNA by linearizing the plasmid at a *Bst*EII site upstream of the 3′ end of the gene (Figure 1). The truncated 16S rRNA which lacked the 1510–1542 sequence at the 3′ end could be efficiently assembled into 30S subunits which comigrated with native 30S subunits in a sucrose gradient and could translate poly(uridylic acid) [poly(U)],[1] albeit with a reduced efficiency (Melançon et al., 1987). In the present study, we have investigated whether the deletion in the 16S rRNA could affect the association between ribosomal subunits, the binding of S21, a protein thought to interact with the 3′-end region of the 16S rRNA, and the capacity of the mutated ribosomes to translate natural messengers. Our results indicate that the deletion in the 16S rRNA did not prevent S21 binding but impaired subunit association. The translation of natural messenger RNAs was decreased in parallel with this impairment, but the correct recognition of translation start sites was not affected.

Materials and Methods

Bacterial Strains and Plasmids. *E. coli* K12A19, RNaseI⁻, was the source of 30S and 50S ribosomal subunits and enzymes for cell-free protein synthesis assays. Plasmid pPM114 contains the entire sequence of 16S rRNA gene under the control of a synthetic T7 promoter. When transcribed in vitro with T7 polymerase after linearization at an appropriate *Mst*II site, it yields a full-length 16S rRNA identical with natural 16S rRNA, except that it lacks the 10 posttranscriptional methylations and contains 2 additional residues (G and C) at its 5′ end (Gravel et al., 1989). More recently, *Mst*II was replaced by an isoschizomer, *Bsu*361, following a suggestion of Denman et al. (1989a).

Enzymes and Chemicals. T7 RNA polymerase was isolated from *E. coli* BL21/pAR1219, a gift from Dr. F. W. Studier, and purified as described by Ikeda and Richardson (1987). Restriction enzymes were purchased from Pharmacia, except *Bsu*361 which was from New England Biolabs. The RNase inhibitor (RNA guard) and the T4 RNA ligase were from Pharmacia. Aprotinin was from Boehringer-Mannheim. [^{3}H]Valine (26 Ci/mmol), [^{3}H]leucine (45 Ci/mmol), [^{3}H]phenylalanine (28 Ci/mmol), [^{35}S]methionine (1330 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were from Amersham. [5′-^{32}P]pCp was synthesized according to the method of England et al. (1980).

In Vitro Transcription of 16S rRNA and Reassembly of 30S Subunits. In vitro transcription of pPM114, linearized either with *MstII* (or *Bsu*361) or with *Bst*EII, which yields full-length or truncated 16S rRNA, respectively, was performed with T7 RNA polymerase, as described previously (Gravel et al., 1989). The 30S subunits were reassembled by

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[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; poly(U), poly(uridylic acid); RNase, ribonuclease.

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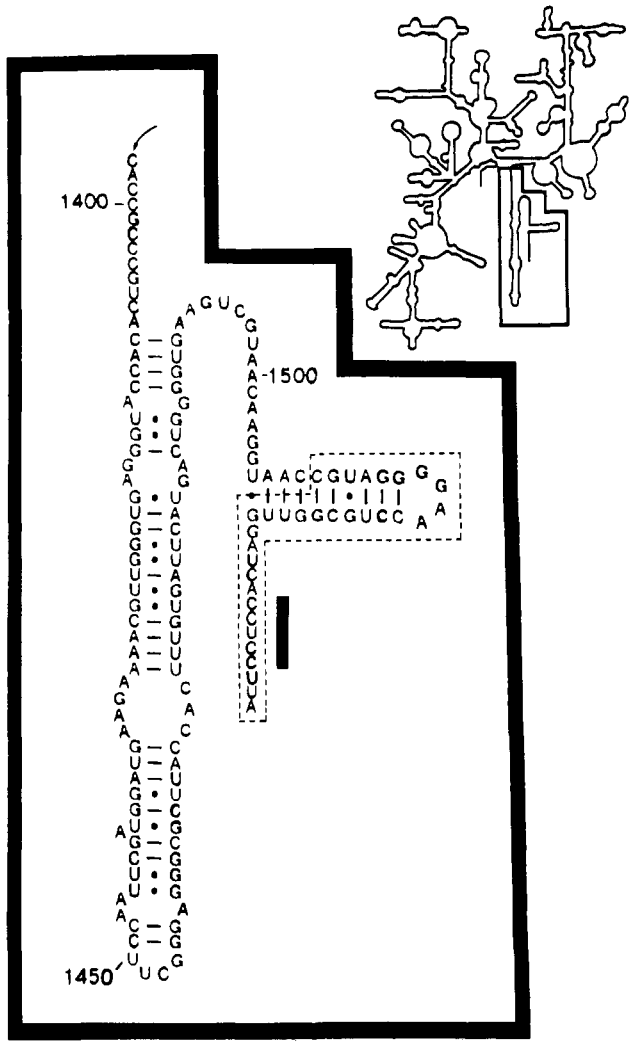

FIGURE 1: Sequence and secondary structure of the 16S rRNA 3′ minor domain enlarged from the model of Stern et al. (1988b). The dashed line delineates the 1510–1542 sequence which is lacking in the truncated 16S rRNA. This RNA is generated by in vitro transcription of a plasmid, pPM114, which harbors the 16S rRNA gene under control of a T7 promoter and which had been linearized at a *Bst*EII site, upstream of the 3′ end of the 16S rRNA gene (see the text). The vertical bar indicates the anti-Shine–Dalgarno sequence.

incubation at 43 °C of LiCl–urea-extracted ribosomal proteins and in vitro synthesized or natural 16S rRNA in reconstitution buffer (30 mM Hepes–KOH,[1] pH 7.4, 26 mM $MgCl_2$, 292 mM KCl, 3 mM spermidine, and 6 mM 2-mercaptoethanol). The procedure was as described (Melançon et al., 1987) with some modifications (Gravel et al., 1989). Reassembled 30S subunits were purified by centrifugation through a 5–20% linear sucrose gradient in reconstitution buffer, at 44 000 rpm for 150 min in a Beckman SW50.1 rotor. The purified subunits were either pelleted by centrifugation overnight or precipitated with poly(ethylene glycol) (Expert-Bezançon et al., 1974).

The truncated 16S rRNA transcript was extracted with phenol from reconstituted 30S subunits and was labeled at its 3′ end with T4 RNA ligase and [5′-^{32}P]pCp, according to the method of England et al. (1980). It was subjected to a complete digestion with RNase T1, in order to analyze the homogeneity of the 3′-terminal oligonucleotide. Control natural 16S rRNA was subjected to the same treatment. The ladder was made by heating in a 50 mM sodium bicarbonate–1 mM EDTA buffer (pH 9) at 90 °C for 10 min a fragment generated by partial digestion with RNase T1 of the [^{32}P]pCp-labeled 16S rRNA transcript. Electrophoresis was on 0.4-mm 20% polyacrylamide gels in a Tris–borate–EDTA buffer [89 mM Tris base, 89 mM boric acid (pH 8.3), and 2.5 mM EDTA] containing 7 M urea.

The protein content of the reconstituted 30S subunits was assessed by one-dimensional and two-dimensional gel electrophoresis. Proteins were extracted from the 30S subunits by the acetic acid procedure (Hardy et al., 1969) and precipitated with acetone (Barritault et al., 1976). Electrophoresis was performed according to Knopf et al. (1975), using mini Protean II cells (Bio-Rad).

Protein Synthesis Assays. Polypeptide synthesis programmed with natural mRNAs (phage MS2 RNA or phage T7 late mRNAs) was performed as described previously (Gravel et al., 1989), with the difference that equimolar amounts of 30S and 50S subunits were used and not an excess of 30S subunits over 50S subunits, except when mentioned. The incorporation mixture (100 μL), supplemented with 5 units of RNA guard and 0.1 μg of aprotinin, contained, in addition to the other components, 0.16 A_{260} unit of 30S subunits, 0.33 A_{260} unit of 50S subunits, 20 μg of MS2 RNA or T7 late mRNAs, and 25 μM each of [^{3}H]leucine (1.9 Ci/mmol) and [^{3}H]valine (1.8 Ci/mmol). MS2 RNA was obtained by a standard procedure (Goldman & Hatfield, 1979). The preparation of T7 phage was obtained according to Studier (1969), and T7 DNA was extracted according to Minkley and Pribnow (1973). T7 late mRNAs were obtained by in vitro transcription with T7 RNA polymerase, using the same procedure as for the transcription of 16S rRNA. Poly(U)-directed incorporation of phenylalanine was as described in standard procedures (Grisé-Miron et al., 1981; Melançon et al., 1987). The incorporation mixture (100 μL) contained 0.16 A_{260} unit of 30S subunits, 0.33 A_{260} unit of 50S subunits, and 10 μM [^{3}H]phenylalanine (3.4 Ci/mmol). With either the artificial or the natural mRNAs, incubation was for 60 min, unless when specified. Translational products of MS2 RNA and T7 late mRNAs were analyzed by gel electrophoresis. For this analysis, [^{35}S]methionine (243 Ci/mmol) at 3 μM was used instead of [^{3}H]leucine and [^{3}H]valine. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed as described (Laemmli, 1970) using 15% and 20% acrylamide for MS2 RNA and T7 late mRNAs, respectively. After electrophoresis, gels were processed for fluorography and exposed to Fuji RX X-ray films at −70 °C.

Formation of 70S Ribosomes from 30S and 50S Subunits. Association of 30S subunits to 50S subunits was assessed by sucrose gradient centrifugation: 0.16 A_{260} unit of 30S subunits and 0.33 A_{260} unit of 50S subunits were incubated for 15 min at 37 °C in 20 μL of reassociation buffer (10 mM magnesium acetate, 60 mM NH_4Cl, 30 mM Hepes–KOH, pH 7.4, and 6 mM 2-mercaptoethanol) and centrifuged through a 5–20% linear sucrose gradient in a Beckman SW50.1 rotor at 44 000 rpm for 135 min.

RESULTS

Analysis of the 3′-Terminal Region of the Truncated 16S rRNA Transcript. We have previously shown by nuclease mapping that linearization of plasmid pPM114 at the *Bst*EII site, upstream of the 3′ end of the 16S rRNA gene, yields a truncated 16S rRNA lacking the sequence 1510–1542 (Melançon et al., 1987). In this study, the 3′-end region of the truncated transcript was verified after its extraction from the reconstituted 30S subunits. After complete digestion of the transcript with RNase T1, a single band was detected for the 3′-terminal oligonucleotide. It was sized to four nucleotides by comparison with the dodecanucleotide (1531–1542) ori-

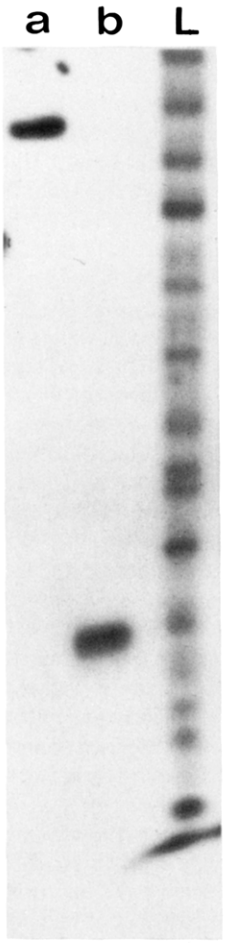

FIGURE 2: Analysis of the 3′-terminal oligonucleotide of the truncated 16S rRNA generated by transcription of *Bst*EII-linearized plasmid pPM114. The transcript was extracted from reconstituted 30S subunits, 3′ end-labeled, and subjected to complete digestion with RNase T1. (a) Control natural 16S rRNA; (b) truncated 16S rRNA; (L) ladder.

ginating from the complete digestion with RNase T1 of the control natural 16S rRNA (Figure 2). This is the expected size, corresponding to a cleavage after G-1505. The homogeneity of the 3′-terminal oligonucleotide of the truncated RNA confirms that the mutant 30S subunits do not contain a significant amount of natural 16S rRNA, which could have resulted from a contamination of the preparation of ribosomal proteins. In some experiments, a faint amount of contamination by natural 16S rRNA could be detected in the reconstituted subunits, but it was always inferior to 5% of the mutant 16S rRNA transcript (data not shown).

Protein Content of the Reconstituted Subunits. We have shown (Melançon et al., 1987) that the truncated synthetic 16S rRNA generated by transcription of *Bst*EII-linearized pPM114 could assemble with ribosomal proteins into 30S subunits which comigrated with native 30S subunits in a sucrose gradient. However, protein S21 cannot assemble into 30S subunits reconstituted with 16S rRNA lacking about 50 nucleotides at its 3′ end, after cleavage with colicin E3 (Bowman et al., 1971). The absence of S21 would not affect the sedimentation coefficient of reassembled 30S subunits. In the present study, we have examined the protein content of 30S subunits reassembled with the truncated 16S rRNA lacking bases 1510–1542. Analysis by one-dimensional and two-dimensional gel electrophoresis indicates that the mutated 30S subunits contain the full complement of 30S ribosomal proteins, including S21 (Figure 3).

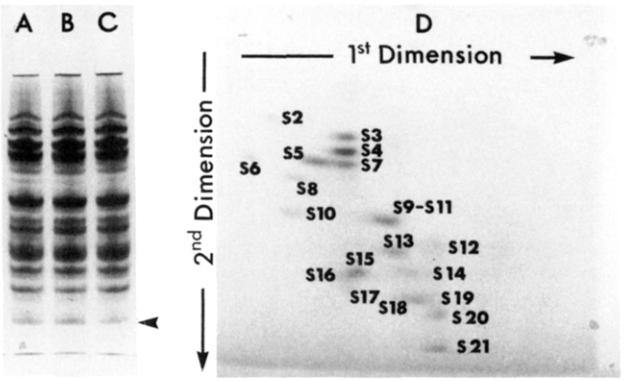

FIGURE 3: Analysis by gel electrophoresis of the protein content of reassembled 30S subunits. Electrophoresis was performed according to Knopf et al. (1975): one-dimensional 18% polyacrylamide/urea acidic gel electrophoresis of the proteins extracted from 30S subunits reassembled with natural 16S rRNA (A), with full-length synthetic 16S rRNA (B), and with truncated synthetic 16S rRNA (C). Proteins were stained with Coomassie blue. The arrowhead points to protein S21; two-dimensional polyacrylamide/urea acidic gel electrophoresis of the proteins extracted from 30S subunits reassembled with the truncated 16S rRNA (D). Identical patterns were obtained with proteins extracted from 30S subunits reassembled with natural or full-length synthetic 16S rRNA.

Table I: Association of Reassembled 30S Subunits to 50S Subunits[a]

origin of the 16S rRNA	% of associated 30S subunits
natural	81 ● 3 (103)
full-length synthetic	79 ● 3 (100)
truncated synthetic	44 ● 4 (56)

[a] The association of the 30S subunits to the 50S subunits was assessed by velocity sedimentation through a 5–20% sucrose gradient as described under Materials and Methods. Results are the means ± standard deviation of three experiments with independent preparations of 30S subunits. Numbers in parentheses represent the data as percent of the results obtained with 30S subunits reassembled with full-length synthetic 16S rRNA.

Subunit Association. The deletion of about 30 nucleotides at the 3′ end of 16S rRNA could interfere with ribosomal subunit association and, concomitantly, would decrease the protein synthesis activity of the mutated ribosomes. Mutated or unmutated 30S subunits were incubated with 50S subunits in a buffer of ionic strength and composition similar to that used for protein synthesis assays. The association of the ribosomal subunits was then assessed by velocity centrifugation through a sucrose gradient, as indicated under Materials and Methods. The deletion at the 3′ end of 16S rRNA resulted in a decrease in association to about 56% of that of 30S subunits containing the full-length synthetic 16S rRNA (Table I). Although this assay is performed under nonequilibrium conditions and does not perfectly reproduce the conditions of protein synthesis, it nevertheless clearly demonstrates that subunit association is impaired by the removal of the 1510–1542 sequence. The association capacity of the 30S subunits reconstituted with the full-length synthetic 16S rRNA was the same as that of 30S subunits reconstituted with the natural 16S rRNA, under the conditions used in this assay.

Protein Synthesis Activity. The impairment of subunit association might be the sole factor affecting the protein synthesis activity of the mutated 30S subunits or, in addition, the mutation might also perturb the activity of the 30S subunits associated to the 50S subunits. In Table II, we have compared the translational activity of 30S subunits reconstructed with the natural phenol-extracted 16S rRNA ("natural 30S subunits") with the full-length synthetic 16S rRNA ("full-length 30S subunits") and with the truncated

Table II: Protein Synthesis Activity of Reassembled 30S Subunits[a]

origin of the 16S rRNA	activity (cpm) with MS2 RNA	with T7 late mRNAs	with poly(U)
natural	11958 (130)	17356 (135)	280696 (196)
full-length synthetic	9209 (100)	12810 (100)	143154 (100)
truncated synthetic	6814 (74)	9266 (72)	89184 (62)

[a] The protein synthetic activity of the reassembled 30S subunits was assessed by measuring the MS2 RNA or T7 late mRNAs directed incorporation of [^{3}H]leucine and [^{3}H]valine into trichloroacetic acid insoluble material. Each sample (100 μL) contained 0.16 A_{260} unit of 30S subunits and 0.33 A_{260} unit of 50S subunits. Incubation was for 60 min. Results are the means of four assays with independent preparations of 30S subunits. Blanks with no added 30S subunits were subtracted. Standard deviation on the mean was inferior or equal to 15%. Numbers in parentheses correspond to protein synthesis activity expressed as the percent of that of 30S subunits reassembled with full-length synthetic 16S rRNA.

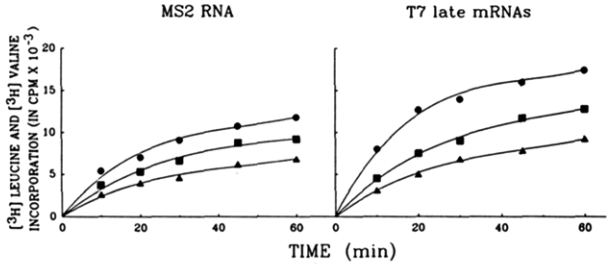

FIGURE 4: Time course of [^{3}H]leucine and [^{3}H]valine incorporation under the direction of MS2 RNA or T7 late mRNAs with "natural 30S subunits" (●), with "full-length 30S subunits" (■), and with "truncated 30S subunits" (▲). Experimental points are the means of three experiments with independent preparations of 30S subunits. Standard deviation on the means is inferior or equal to 17%.

synthetic 16S rRNA ("truncated 30S subunits"). The activity of the "truncated 30S subunits" was decreased to about 70% of that of "full-length 30S subunits", when assayed under the direction of either MS2 RNA or T7 late mRNAs. When poly(U)-directed assays were performed with the same preparations of 30S subunits, the deletion in 16S rRNA reduced the activity of the 30S subunits to about 60%. The activity of the "full-length 30S subunits" was reduced to about 75% of that of "natural 30S subunits", in the MS2 RNA or T7 late mRNAs directed assays, and to about 50% in the poly(U)-directed assay. These latter effects are probably related to the absence of methyl groups in the synthetic 16S rRNA.

Kinetic studies were also performed to determine whether the difference in activity between mutated and unmutated 30S subunits is influenced by the time course of the reaction. Figure 4 presents a time course study comparing the protein synthesis activity of "natural", "full-length", and "truncated 30S subunits" under the direction of either MS2 RNA or T7 late mRNAs. This shows that the mutation in 16S rRNA similarly affects the initial rate of the reaction and the maximum incorporation, whatever the origin of the mRNA.

The decrease in protein synthesis activity of the mutated ribosome does not exceed the impairment in subunit association, which makes it likely that this impairment primarily accounts for the change in activity. In some experiments, the standard amount of 50S subunits was used with a 4-fold excess of 30S subunits under the direction of MS2 RNA or T7 late mRNAs. Under these conditions, which favor the formation of 70S ribosomes, we observed a decrease of only about 10–15% when comparing the activity of "truncated 30S subunits" to "full-length 30S subunits" (data not shown). This confirms that the absence of the 1510–1542 region has no major direct effect on the translational activity of the ribosomes, apart from impairing subunit association.

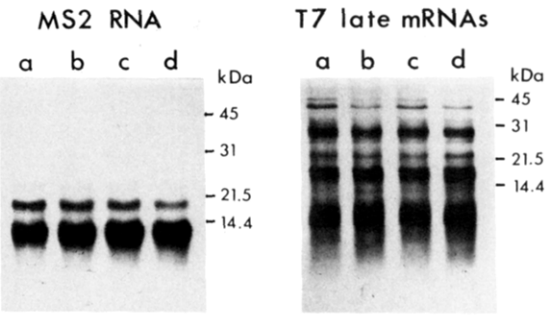

FIGURE 5: Fluorograms of the [^{35}S]methionine-labeled cell-free translational products of MS2 RNA and T7 late mRNAs obtained with native 30S subunits (a), with "natural 30S subunits" (b), with "full-length 30S subunits" (c), and with "truncated 30S subunits" (d). For comparison, equal amounts of radioactivity (about 10000 cpm) were loaded in each lane.

Analysis of the Translational Patterns. However, the pattern of translation products could be altered without an observable change in the overall activity of the mutated ribosomes. Figure 5 compares the patterns of translation products obtained under the direction of MS2 RNA or T7 late mRNAs with mutated or unmutated 30S subunits. In vitro synthesized proteins labeled with [^{35}S]methionine were resolved by sodium dodecyl sulfate gel electrophoresis and fluorography. With MS2 RNA, one major product, corresponding to the coat protein, was detected whereas a more complex pattern was detected with T7 late mRNAs. In all cases, identical patterns of translation were observed, whether the 16S rRNA was natural or synthetic, full-length, or truncated.

Discussion

The deletion of a sequence at the 3′ end of 16S rRNA, which comprises the anti-Shine–Dalgarno region and a large part of the final helix, did not affect the binding of the ribosomal proteins. Bowman et al. (1971) have shown that natural 16S rRNA deprived of 49 nucleotides at its 3′ end, after cleavage between residues 1493 and 1494 with colicin E3, cannot bind protein S21. This result taken together with our present observations, that the 16S rRNA lacking the 1510–1542 segment can bind S21, suggests that the region around residue 1500 must be important for S21 binding. In agreement with this conclusion, Stern et al. (1988a) have studied the effect of the assembly of protein S21 into 30S subunits on the reactivity of 16S rRNA to chemical agents. They probed the 16S rRNA up to residue 1500 and observed that S21 had a small effect on very few bases, which led them to suggest that the incorporation of S21 into 30S subunits probably involves protein–protein interactions and/or interactions in the 3′-terminal region of 16S rRNA, downstream from residue 1500.

We found that the deletion of the 1510–1542 region impairs subunit association. This observation is in accord with previous reports, based on changes in the reactivity of specific residues of 16S rRNA, which suggested that the final helix of 16S rRNA is involved in subunit association (Herr et al., 1979; Meier & Wagner, 1985). However, although the deleted sequence contains the anti-Shine–Dalgarno sequence, its absence did not specifically interfere with the initiation step. Indeed, the mutation caused a decrease in protein synthesis activity which was very similar whether translation was carried out under the direction of natural messenger RNAs or of poly(U) which bypasses the initiation step. Since this decrease did not exceed the impairment in subunit association, we suggest that this impairment is the major cause for the change

in activity of the mutated ribosomes.

The ribosome binding sites of T7 late mRNAs are known to contain a high proportion of strong Shine–Dalgarno sequences (Dunn & Studier, 1983), whereas, in MS2 RNA, the ribosome binding site of the cistron coding for the coat protein, which is predominantly translated, contains a weak Shine–Dalgarno sequence (Gren, 1984). However, as seen in Table II and Figure 4, the absence of the Shine–Dalgarno interaction did not differentially affect the translational efficiency of the different sets of mRNAs. However, it could occur that the overall activity of the mutated ribosomes is not affected but that they yield an aberrant pattern of translational products. We have therefore analyzed the patterns generated by the mutated and unmutated ribosomes under the direction of MS2 RNA or T7 late mRNAs, with the hypothesis that the absence of the Shine–Dalgarno interaction could favor random initiation. In contrast to this hypothesis, as illustrated in Figure 5, the patterns of translational products were identical, whether the 30S subunits were mutated or not. These results indicate that the absence of the Shine–Dalgarno interaction did not prevent the mutated ribosomes from correctly selecting the translational starts. Recently, in perfect agreement with our findings, Calogero et al. (1988) compared the in vitro translation of two synthetic messengers, identical except for the presence or the absence of a Shine–Dalgarno region. They found that the absence of the Shine–Dalgarno sequence affected neither the efficiency of translation of the messenger nor the selection of the correct reading frame.

As mentioned in the introduction, a large body of evidence, among which the effect of various mutations in the Shine–Dalgarno sequence of different messengers, supports the existence of the Shine–Dalgarno interaction. More recently, mutations were also introduced in the anti-Shine–Dalgarno region of 16S rRNA (Jacob et al., 1987; Hui & de Boer, 1987). When these mutations were expressed in vivo, they were found to drastically reduce the translation of natural messengers, except those for which the complementarity between the mutated anti-Shine–Dalgarno region and the Shine–Dalgarno sequence was restored. This apparently contrasts with our results as well as with those of Calogero et al. The translation efficiency of cell-free systems is much lower than that of in vivo systems (Armstrong-Major & Champney, 1985), and this probably accounts for the failure of in vitro assays to detect effects related to the absence of the Shine–Dalgarno interaction. Nevertheless, the in vitro assays clearly indicate that the Shine–Dalgarno interaction is dispensable for the correct selection of translation starts. Several results (Gold, 1988; Dreyfus, 1988) stress the importance of various determinants in mRNAs located upstream and downstream of the Shine–Dalgarno sequence including the coding region, but the ribosomal determinants which could interact with these mRNA determinants are not yet identified. Ribosomal proteins as well as rRNA are involved in these interactions. In vitro protein synthesis assays have shown that S21 is essential for the initiation of translation, and it was assumed that it exerts its effect by unfolding the 3′ terminus of the 16S rRNA and thus promoting the Shine–Dalgarno interaction (Backendorf et al., 1981; Van Duin & Wijnands, 1981). Our results showing that initiation can occur without the Shine–Dalgarno interaction suggest that S21 can exert its role independently of its effect on the anti-Shine–Dalgarno region. As explained above, we have inferred that S21 interacts with 16S rRNA around the 1500 region. Thomas et al. (1988) have shown that the 1500 region interacts with the 1400 region, since replacement of G1505 with A, C, or U can suppress lethal mutations in the 1400 region. Denmann et al. (1989b), with a system similar to ours, have introduced a series of deletions and insertions around residue 1400 and demonstrated that the 1400 area is crucial for the initiation of translation. Our results are consistent with the suggestion that S21 could exert its role in initiation by affecting the interaction between the 1400 and the 1500 regions.

Finally, Petersen et al. (1988) recently hypothesized, after a screening of 251 genes from *E. coli*, that a region comprising the first 16 nucleotides at the 5′ end of 16S rRNA is a likely candidate for an interaction with the coding region of messenger RNAs and could be involved in the selection of translational starts. Our in vitro system for studying the consequences of mutations in 16S rRNA provides a convenient tool to further investigate this hypothesis.

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Drug Binding by Branched DNA: Selective Interaction of the Dye Stains-All with an Immobile Junction†

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ABSTRACT: The thiacarbocyanine dye Stains-All (4,5:4′,5′-dibenzo-3,3′-diethyl-9-methylthiacarbocyanine bromide) is one of a large number of cyanine dyes introduced as photosensitizers in the photographic industry. Stains-All is used in histology as a stain for nucleic acids, proteins, polysaccharides, and lipids. We report here that the dye colors branched DNA molecules differently from linear duplexes and use footprinting experiments with methidiumpropyl-EDTA·Fe(II) [MPE·Fe(II)] and bis(*o*-phenanthroline)copper(I) [$(O\text{-}P)_2Cu(I)$] to show that Stains-All interacts preferentially at the branch point of a four-arm DNA structure. A titration experiment allows us to estimate that the interaction of the dye with the branch has a dissociation constant below 45 nM, tighter than that of ethidium or methidium by over 2 orders of magnitude. Probing the interaction with the purine-specific reagent diethyl pyrocarbonate (DEPC) implies that the dye induces an asymmetric distortion near the branch in the major grooves of double helix in the junction.

The properties of unstable Holliday recombination intermediates have been modeled by stable synthetic branched DNAs formed from oligonucleotides in which the sequence symmetry needed for branch migration is eliminated (Seeman, 1982; Kallenbach et al., 1983; Kallenbach & Seeman, 1986). Experiments in solution (Churchill et al., 1988; Chen et al., 1988) show that the dominant stable conformation of the tetramer consists of a pair of adjacent duplexes, each consisting of two neighboring arms stacked (or nearly so) over each other. This structure is determined by the sequence of bases flanking the branch (Chen et al., 1988), as well as by the nature and concentration of cations in the solution (Seeman et al., 1985). In the presence of Mg^{2+}, the immobile junction formed from four 16-mers, referred to as J1, favors the conformation in which the two noncrossover strands lie antiparallel, as illustrated in Figure 2 (Cooper & Hagerman, 1987, 1989; Churchill et al., 1988; Seeman et al., 1989), and this appears to hold for immobile junctions of other sequences as well (Duckett et al., 1988). Base pairs flanking the branch site in J1 appear to remain hydrogen bonded (Wemmer et al., 1985).

In early experiments with J1, we observed that the dye Stains-All (see Figure 1 for structure) (Hamer, 1975) colors dilute solutions of J1 differently from any of the duplex arms alone. This dye is used as a differential stain for DNA, RNA, polysaccharides, and membrane phospholipids (Green, 1975). It has the eponymous property of staining each of these components a distinct color. We report here that J1 is stained differently by the dye from 16-mer DNA duplexes with the same sequences as the arms in J1 and use different footprinting agents for precise characterization of the Stains-All binding site in the four-arm junction, J1. We estimate the dissociation constant for the dye–J1 interaction to be about 45 nM.

Materials and Methods

Nucleic Acids. All DNA strands used in these experiments are synthesized on an ABI 380B automated synthesizer, using standard phosphoramidite chemistry (Caruthers, 1982). Strands are purified following deprotection and removal from

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