Inefficient Translation of T7 Late mRNA by *Bacillus subtilis* Ribosomes

IMPLICATIONS FOR SPECIES-SPECIFIC TRANSLATION*

Paul W. Hager and Jesse C. Rabinowitz

From the Department of Biochemistry, University of California, Berkeley, California 94720

(Received for publication, March 21, 1985)

*Bacillus subtilis* 30 S subunits inefficiently recognize initiation sites in mRNAs from Gram-negative bacteria, but they are able to efficiently recognize initiation sites in mRNA derived from Gram-positive bacteria. McLaughlin et al. (McLaughlin, J. R., Murray, C. L., and Rabinowitz, J. C. (1981) J. Biol. Chem. 256, 11283-11291) have suggested that *B. subtilis* ribosomes require a strong Shine-Dalgarno sequence for translation initiation. To test whether this criterion is sufficient to explain the translational specificity of *B. subtilis* ribosomes, T7 late mRNA, which contains strong Shine-Dalgarno sequences before many of the late genes (Dunn, J. J., and Studier, F. W. (1983) J. Mol. Biol. 166, 477-535), was translated in vitro with both *Escherichia coli* and *B. subtilis* ribosomes. The identification of several of the in vitro products upon gel electrophoresis indicated that *B. subtilis* ribosomes recognize correct translation initiation sites in late T7 mRNA, but they do not translate these products efficiently. Competition experiments demonstrated that late T7 mRNA does not inhibit *B. subtilis* ribosomal translation of *B. subtilis* derived mRNA (from the bacteriophage φ29). It is concluded that strong Shine-Dalgarno sequences may be necessary in *B. subtilis* translation initiation sites; however, additional determinants of initiation which differ from those found in the translation initiation sites of *E. coli* mRNAs must exist.

The initiation of protein synthesis by procaryotic ribosomes involves the selection of an appropriate site on the mRNA by the 30 S subunit of the ribosome (1-3). In general, ribosomes derived from Gram-positive bacteria translate homologous mRNAs (i.e., Gram-positive derived mRNAs) efficiently, but translate mRNAs derived from Gram-negative bacteria inefficiently (4-12). This species-specific translation implies that Gram-positive derived ribosomes and mRNAs are functionally distinct from those of *Escherichia coli*. Since *E. coli* ribosomes translate mRNA derived from Gram-positive bacteria, it has been assumed that such mRNA contains, at a minimum, determinants of translation initiation which are similar to those found in mRNA from *E. coli* (13). One of the features of mRNA important for its recognition by *E. coli* ribosomes is found in the “ribosome binding site” which includes an initiation codon, a Shine-Dalgarno sequence, and an appropriate spacing (“window”) between these two (1-3, 14, 15). The Shine-Dalgarno sequence consists of a polyurine stretch of variable length which is located 5' to the initiation codon and is capable of base pairing to the 3' end of the 16 S rRNA (16).

McLaughlin et al. (13) have suggested that the Shine-Dalgarno complementarity required by *Bacillus subtilis* and other Gram-positive ribosomes is significantly greater than that required by *E. coli* and other Gram-negative ribosomes, and is required for species-specific translation. The sequence information for over 40 Gram-positive derived translation initiation sites that has appeared since its formulation supports this hypothesis since all of the sites contain “strong” Shine-Dalgarno sequences (17). The “strength” of the Shine-Dalgarno sequence was estimated by determining the free energy of formation of the most stable double helical complex between the 3' end of the 16 S rRNA and the Shine-Dalgarno sequence according to the rules of Tinoco et al. (18). The Gram-positive derived translation initiation sites have Shine-Dalgarno sequences with free energies of binding with an average value of −16.7 kcal/mol (standard deviation of 2.3), as compared to *E. coli* translation initiation sites which have an average of −10.9 kcal/mol (standard deviation of 3.4).

The known *E. coli* translation initiation sites (19) include a number which have strong Shine-Dalgarno sequences with calculated free energies of binding that overlap those of *B. subtilis* (17). The *E. coli* phage T7 contains an unusually large number of these strong ribosome binding sites (20). To determine whether a ribosome binding site composed of a strong Shine-Dalgarno sequence with an appropriately placed initiation codon is a sufficient determinant for translation by *B. subtilis* ribosomes, we tested mRNA prepared from T7 DNA for activity with an *in vitro* *B. subtilis* translation system. We decided to test the late region of T7 for this purpose because of its complete characterization and abundance of strong ribosome binding sites and the ease of identification of protein products using mutants and because there are few polar effects in T7 gene expression (20). We find that *B. subtilis* ribosomes do not translate authentic T7 late proteins, although at a markedly reduced level compared to *E. coli* ribosomes. There is some correlation between the strength of the Shine-Dalgarno sequence and the relative expression of the protein by *B. subtilis* ribosomes; however, there must be other features of a Gram-positive translation initiation site which are important for efficient translation which remain to be elucidated.
Translation of T7 Late mRNA by B. subtilis Ribosomes

The relative amounts of each protein made by E. coli and B. subtilis ribosomes were quantitated by scanning fluorograms with a densitometer (Table 1). B. subtilis ribosomes show greater relative translation of proteins that have strong Shine-Dalgarno interactions (note 3.5, 11, and 14) and less relative translation of those which have weaker Shine-Dalgarno interactions, as compared to E. coli ribosomes. The exception to this is seen with protein 9, which does not have a strong Shine-Dalgarno interaction but is translated by B. subtilis ribosomes at an increased relative amount as compared to E. coli ribosomes. These results indicate that while B. subtilis ribosomes do not efficiently translate T7 mRNA they may prefer initiation sites that have strong Shine-Dalgarno sequences.

**DISCUSSION**

T7 late mRNA is inefficiently translated by B. subtilis ribosomes despite the strong Shine-Dalgarno sequences in many of these mRNAs. Thus, although strong Shine-Dalgarno sequences appear to be necessary for translation of mRNA by B. subtilis ribosomes (23), such sequences are not sufficient to allow the efficient translation of E. coli phage mRNA by a system containing B. subtilis ribosomes. The products of the low levels of translation include the same proteins made by E. coli ribosomes, so B. subtilis ribosomes appear to recognize the correct initiation sites on T7 late mRNA; however, B. subtilis ribosomes prefer different initiation sites than E. coli ribosomes. The translation of T7 late mRNA by B. subtilis ribosomes could be inhibited at any stage, but the ability of B. subtilis ribosomes to translate φ29 mRNA following preexposure to T7 late mRNA (Fig. 4) suggests that B. subtilis ribosomes inefficiently bind to initiation sites on T7 mRNA. Poor translation from what is otherwise a good initiation site is analogous to some recent examples of translational regulation (23–26). It is possible that there is a factor which inhibits the translation of heterologous mRNA by B. subtilis ribosomes. Unlike gene 32 protein (24), or the ribosomal proteins (25) which inhibit translation of specific mRNAs, such a factor would have to be quite nonspecific. In addition it would have to block translation for B. subtilis ribosomes but not E. coli ribosomes, since the components of the translation systems are interchangeable (11, 12).

The inducible resistance genes derived from Gram-positive organisms appear to use a different mechanism for translational regulation (26). The mRNAs are capable of at least two mutually exclusive conformations. In the repressed state the Shine-Dalgarno is sequestered in a stem structure and would

---

**Table I**

| Protein product | E. coli ribosomes | φ29 to E. coli | B. subtilis ribosomes | φ29 to B. subtilis |
|-----------------|------------------|----------------|-----------------------|--------------------|
| 2.5             | 0.09             | -12.8          | 0.05                  | -14.0              |
| 3.5             | 0.08             | -18.8          | 0.18                  | -21.2              |
| 5.5             | 0.30             | -11.6          | 0.12                  | -13.8              |
| 9               | 0.02             | -14.6          | 0.16                  | -12.8              |
| 10              | 0.10             | -12.8          | 0.04                  | -14.2              |
| 11              | <0.02            | -17.6          | 0.04                  | -12.2              |
| 14              | <0.02            | -17.8          | 0.02                  | -14.2              |

---

Portions of this paper (including "Experimental Procedures," portions of "Results," and Figs. 2–4) are presented in miniprint at the end of this paper. The abbreviations used are: DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-863, cite the authors, and include a check or money order for $4.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
be less accessible to the ribosome. A second conformation, stabilized when a ribosome stalls while translating the leader peptide, exposes the Shine-Dalgarno and the initiation codon facilitating translation of the mRNA. T7 translation initiation sites might be blocked in secondary structures, hence poorly translated by *B. subtilis* ribosomes. However, our computer analysis of T7 translation initiation sites shows them to be generally free of stable interactions which would mask the Shine-Dalgarno and initiation codon. In the functional sense, these sites are accessible since *E. coli* ribosomes do translate these mRNAs. It is possible that *E. coli* but not *B. subtilis* ribosomes are able to read through particular secondary structures. Such a function could be performed by the *E. coli* ribosomal protein S1, which has no homologue in *B. subtilis* (33).

In summary, *B. subtilis* translation initiation sites differ from *E. coli* sites, both with respect to the strength of the Shine-Dalgarno and possibly to sequence preferences within the ribosome binding site (31, 32); for example, these sites tend to be A-U rich. The Gram-positive derived initiation sites accentuate this characteristic. A collection of *B. subtilis* and other Gram-positive derived mRNAs contain 42% A residues for a 50-base region around the initiation site (33).

In summary, *B. subtilis* translation initiation sites differ from *E. coli* sites, both with respect to the strength of the Shine-Dalgarno and possibly to sequence preferences within the translation initiation site. A strong Shine-Dalgarno sequence is not a sufficient signal for efficient translation of a mRNA by *B. subtilis* ribosomes, but is probably a necessary one. Additional features of a strong *B. subtilis* translation initiation site remain to be elucidated.

REFERENCES

1. Steitz, J. A. (1979) in *Biological Regulation and Development* (Goldberger, R. F., ed) Vol. 1, pp. 349–399, Plenum Press, New York
2. Grunberg-Manago, M. (1980) in *Ribosomes: Structure, Function, and Genetics* (Chambless, G., Craven, G. R., Davies, J., and Davis, K., eds) pp. 445–477, University Park Press, Baltimore
3. Gold, L., Prinbrow, D., Schneider, T., Slindling, S., Singer, B. S., and Stornoo, G. (1981) *Annu. Rev. Microbiol.* 35, 368–403
4. Lodish, H. F. (1970) *Nature* 226, 705–707
5. Stallcup, M. R., and Rabinowitz, J. C. (1973) *J. Biol. Chem.* 248, 3208–3215
6. Stallcup, M. R., and Rabinowitz, J. C. (1973) *J. Biol. Chem.* 248, 3216–3219
7. Stallcup, M. R., Sharrock, W. J., and Rabinowitz, J. C. (1976) *J. Biol. Chem.* 251, 2499–2501
8. Leffler, S., and Szer, W. (1974) *J. Biol. Chem.* 249, 1465–1468
9. Lewenthal, J. M., and Chambless, O. H. (1979) *Biochim. Biophys. Acta* 564, 162–171
10. McLaughlin, J. R., Murray, C. L., and Rabinowitz, J. C. (1981) *Proc. Natl. Acad. Sci. U. S. A.* 78, 4912–4916
11. McLaughlin, J. R., Murray, C. L., and Rabinowitz, J. C. (1981) *J. Biol. Chem.* 256, 11273–11282
12. McLaughlin, J. R., Murray, C. L., and Rabinowitz, J. C. (1981) *J. Biol. Chem.* 256, 11283–11291
13. Steitz, J. A. (1980) in *Ribosomes: Structure, Function, and Genetics* (Chambless, G., Craven, G. R., Davies, J., and Davis, K., eds) pp. 479–495, University Park Press, Baltimore
14. Taniguchi, T., and Weissman, C. (1978) *J. Mol. Biol.* 118, 533–565
15. Shire, J., and Dalgarno, L. (1974) *Proc. Natl. Acad. Sci. U. S. A.* 71, 1342–1346
16. Hager, P. W., and Rabinowitz, J. C. (1985) in *The Molecular Biology of the Bacilli* (Dubnau, D., ed) Vol. II, pp. 1–29, Academic Press, New York
17. Tinoco, I., Borer, P. N., Dengler, B., Levine, M. D., Uhlenbeck, O. C., Crothers, D. M., and Gralla, J. (1973) *Nature New Biol.* 246, 40–41
18. Gren, E. J. (1984) *Biochimie* (Paris) 66, 1–29
19. Dunn, J. D., and Studier, F. W. (1983) *J. Mol. Biol.* 166, 477–555
20. Murray, C. L., and Rabinowitz, J. C. (1982) *J. Biol. Chem.* 257, 1063–1062
21. Yoshikawa, H., and Ito, J. (1982) *Gene* (Amst.) 17, 323–335
22. Band, L., and Henner, D. J. (1984) *DNA* (N. Y.) 3, 17–21
23. Lemaire, G., Gold, L., and Yaras, M. (1978) *J. Mol. Biol.* 126, 73–90
24. Nomura, M., Gourse, R., and Baughman, G. (1984) *Annu. Rev. Biochem.* 53, 75–117
25. Dubnau, D. (1984) *CRC Crit. Rev. Biochem.* 16, 103–132
26. Isono, K., and Isono, S. (1976) *Proc. Natl. Acad. Sci. U. S. A.* 73, 767–770
27. Isono, K., Isono, S., Stoffler, G., Visentin, L. P., Yaguchi, M., and Matheson, A. T. (1973) *Mol. Gen. Genet.* 127, 191–135
28. Higo, K., Otaka, E., and Osawa, S. (1982) *Mol. Gen. Genet.* 185, 239–244
29. Isono, S., and Isono, K. (1975) *Eur. J. Biochem.* 56, 15–22
30. Stormo, G. D., Schneider, T. D., and Gold, L. M. (1982) *Nucleic Acids Res.* 10, 2971–2996
31. Scherer, G. F. E., Walkinsaw, M. D., Arnott, S., and Morre, D. J. (1980) *Nucleic Acids Res.* 8, 3595–3597
32. Hager, P. W. (1984) Ph.D. thesis, University of California, Berkeley
33. Studier, F. W. (1989) *Virology* 39, 562–574
34. Studier, F. W. (1981) *J. Mol. Biol.* 153, 493–502
35. Studier, F. W. (1972) *Science* 176, 367–376
36. Davison, B. L., Leighton, T., and Rabinowitz, J. C. (1979) *J. Biol. Chem.* 254, 9231–9238
37. Kasavetis, G. A., and Chamberlin, M. J. (1977) *J. Virol.* 29, 196–208
38. Sharrock, W. J., and Rabinowitz, J. C. (1979) *J. Mol. Biol.* 135, 611–629
39. Lasmil, U. K. (1970) *Nature* 227, 680–685
40. Chamberlain, J. P. (1979) *Anal. Biochem.* 89, 132–135
41. Laskey, R. A., and Mills, A. D. (1975) *Eur. J. Biochem.* 56, 334–341
42. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254
43. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
44. Sharrock, W. J., Gold, B. M., and Rabinowitz, J. C. (1979) *J. Mol. Biol.* 135, 627–638

Continued on next page.
Translation of T7 Late mRNA by B. subtilis Ribosomes

Supplementary Material to

In vitro mRNA-directed Protein Synthesis—Protein synthesis reactions were carried out similarly to a previous description (5) in a 30 ul reaction volume containing 67 mM Tris-cl, pH 8.0, 0.1 mM MgCl2, 50 mM KCl, 0.83 mg/ml, 12mM ATP, 0.5 mM CTP, 2 mM GTP, 12 mM NADH, 10 mM KCl, 1.6M thiocetic acid (pH 7.0, initiation factos were supplied from salt wash protein of E. coli ribosomes (22 pg) or E. coli ribosomes (17 pg), 0.6 nM ATP and E. coli ribosomes (2 micrograms). The reaction were incubated at 37 °C for 2 h. Reactions were stopped by the addition of 50 ul of 25% trichloroacetic acid, and the samples were counted in a 30 ml scintillation vial. For analysis by SDS-PAGE, p-mercaptoethanol was added to the sample and heated to 95 °C prior to loading on the gel.

Gel Electrophoresis—Polyacrylamide gel electrophoresis using SDS and a discontinuous buffer system (40) was used. A 25% acrylamide/8.8% acrylamide ratio of 3:1 was performed in a slab electrophoresis cell. Gradient gels containing 10-20% acrylamide with a starting gel of 4% were used. For fluorography the gels were fixed in 10% acetic acid 25% isopropanol and then stained with 0.1 M sodium salicylate (41) before baking. Sample volumes loaded were adjusted to give reasonable exposure overnight at -80 °C using Kodak XAR films. Film which was used for densitometric scanning was pre-flashed to an F1.0/2.0 to give a linear response. Gel images were band in Kodak X-Radiography film. Gel images were scanned using the ScanJet 4c scanner and analyzed using NIH Image 1.55 software. The intensities were normalized to 100% and the results were plotted on log-log paper.

RESULTS

To identify some of the major protein products of T7 late mRNA the DNA of T7 mutants were transcribed with T7 RNA polymerase and then translated with B. subtilis ribosomes. In general, the amber mutants of T7 are those of genes 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, 10.5, 11.5, and 17.1. The protein is missing in the deletion, and other proteins are missing due to their amber mutations. Gene 10 produces 2 protein products identified as 10A and 10B. Dunn and Studier have concluded that 10A and 10B. Protein 6, from strain 6-147, should be identified from its normal molecular weight of 39,000 by 24 amino acids (30). There is an altered band at a molecular weight of about 45,000 which would indicate that this band represents protein E. However, this band corresponds to both positions and is located at about 108 kDa. The mobility of 10B is slightly altered in lane m of Fig. 3. This isolate of 6-147 has probably picked up a second mutation which has altered the mobility of both 10A and 10B, therefore protein 6 is not identified on these fluorograms.
Translation of T7 Late mRNA by B. subtilis Ribosomes

T7 late mRNA was prepared from wild type and mutant T7 DNA as described under Experimental Procedures and added to translation reactions as indicated. Lanes 1 and 2 contained transcription reactions minus DNA. Translation assays containing E. coli ribosomes were performed as described under Experimental Procedures. Labeled proteins were separated on 10-20% polyacrylamide gradient gels and visualized by fluorography.

Some of the products of the translation of T7 by B. subtilis ribosomes are identified in Fig. 3. The proteins which are most easily identified include 1.3, 2.5, 9, 10, 11, and 14. In the cases of 3.5 and 5.5, the protein band at the expected location is missing, but there appear to be other changes in the pattern of translation products as well. There are two prominent protein bands from the B. subtilis in vitro translations which have not been identified (found between 3.5 and 5.5 in Fig. 3). When equal amounts of acid-precipitable counts are compared, B. subtilis and E. coli ribosomes prefer to translate different products (see Fig. 3, lanes b and j).

It is not clear why B. subtilis ribosomes translate T7 mRNA poorly. Since the B. subtilis ribosomes do translate several of the T7 proteins, they recognize the correct initiation sites for these proteins. This inability to efficiently translate could be due to poor initiation of translation (i.e., weak binding to the initiation region). Alternatively, initiation complex formation could proceed normally followed by a block at a later point in the translation such as suggested by Sharrock et al. (45). If B. subtilis ribosomes bind to T7 mRNA in unproductive complexes, these complexes might prevent the ribosomes from translating a mRNA presented at a later time. Such a competition experiment was performed by preincubating B. subtilis ribosomes with T7 late mRNA and then adding T7 mRNA as shown in Fig. 4. T7 late mRNA (the transcription reaction) does not inhibit translation of 29 since B. subtilis ribosomes with T7 late mRNA or with 29 plus T7 late mRNA give the same incorporation. B. subtilis ribosomes are not inactivated by preexposure to T7 late mRNA, since addition of 29 mRNA at 5 min results in immediate incorporation at a rate similar to the initial rate using 29 mRNA alone. The absolute level of incorporation for this competition assay is lower than for 29 mRNA alone, but this is probably a consequence of the loss of activity these ribosomes display after 15 min (33). Thus, the relatively inefficient translation of T7 late mRNA cannot be explained as B. subtilis ribosomes binding to T7 late mRNA in nonproductive complexes.

FIGURE 1
Electrophoretic analysis of proteins translated by B. subtilis ribosomes using T7 late mRNA.

FIGURE 4
Translation of T7 late mRNA and 29 mRNA.

Amino acid incorporation by B. subtilis ribosomes with T7 late mRNA and 29 mRNA in a competition assay. The translation reaction was initiated using ribosomes, ribosomal salt wash, and 5-15% from B. subtilis and T7 late mRNA. For reactions which contained 29 mRNA, 3% of 29 transcription reaction was added directly.