Mapping the *lacZ* Ribosome Binding Site by RNA Footprinting†

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ABSTRACT: The ribosome binding site of the *Escherichia coli lacZ* mRNA has been characterized by using an RNA footprinting technique. Purified *E. coli* 70S ribosomes and fMet-tRNA were incubated with mRNA, and the complex was treated with RNA-reactive reagents or RNases as probes. The protected sites on the mRNA were then mapped by extending a radioactive primer with reverse transcriptase. Dimethyl sulfate, diethyl pyrocarbonate, and 1,10-phenanthroline–copper ion oxidative complex were used as reagent probes; they detected interaction sites *within* the ribosome binding site. A region of approximately 35 nucleotides was protected by the ribosome, specifically across the Shine–Dalgarno region, around the fMet initiation codon, and at a region 7–12 nucleotides distal to the fMet codon. In addition, an enhanced reaction occurred between the fMet codon and the distal site. These results imply an internally selective interaction between the ribosome and the mRNA sequence. The enhanced reactivity of a site distal to the initiation site—flanked by the AUG codon and a site previously identified as conserved in a study of initiation sequences—may indicate a region where the mRNA is specifically exposed.

The translational initiation complex is a relatively stable complex of ribosomes and mRNA that is competent to begin polypeptide elongation (Kozak, 1983; Hershey, 1987; Shinedling et al., 1987). It is formed as the result of a multistep pathway that involves ribosomes, messenger, fMet-tRNA, three initiation factors, and GTP. Experiments with purified components suggest that the initiation factors, bound to ribosomes, serve in the dissociation and association of the ribosomal subunits and binding of the fMet-tRNA and that the pathway includes a preinitiation complex of 30S subunits, mRNA, factors, and fMet-tRNA as an intermediate. It is at the stage of formation of the preinitiation complex that recognition of the initiation codon and its associated Shine and Dalgarno (S–D)[1] sequence occurs (Shine & Dalgarno, 1974; Gold, 1981; Hershey, 1987).

The messenger sequences involved in initiation were originally identified as ribosome-protected fragments derived by treating initiation complexes with various ribonucleases (Steitz, 1980). Such studies employed single-stranded phage RNAs as messenger as well as a few bacterial mRNAs. They revealed that the ribosome has the capacity to discriminate initiation AUG codons from internal AUG's and delineated a protected region around the initiation codon of approximately 35 residues. Nonetheless, this approach had limitations. Primary among these was that the protected fragments included both those larger and those smaller than the binding site (Kang & Cantor, 1985). Thus, analysis of the data was enhanced by comparison of several ribosome binding sites experimentally and, in time, the assembly of a database of initiation sequences (Gold et al., 1981; Stern et al., 1979).

Additional information on the initiation site has come from the alignment of many more initiation sequences deduced from genetic sequences (Gold et al., 1981; Schneider et al., 1986). These strengthen the identification of the S–D sequence and additionally show that the sequences 1–4 and 8–11 bases beyond the initiation codon are also nonrandom, as with a moderately conserved A residue at −3 and a low abundance of purine residues in the region between the S–D sequence and the initiation codon. It remains to be seen what role, if any, these latter sites play in ribosome binding or initiation (Childs et al., 1985). Such studies also show that in addition to AUG, GUG directs incorporation of fMet-tRNA in about 10% of *Escherichia coli* genes and UUG is also utilized but less frequently.

The rate of translational initiation is affected by a variety of mechanisms. These include features of the environment of the initiation site such as the local mRNA secondary structure, upstream sequences, or the proximity of a preceding cistron's stop codon, which may facilitate translational coupling (Looman et al., 1987; Noll et al., 1973; Shinedling et al., 1987; Stanssens et al., 1985). However, of primary importance is the sequence of the ribosome binding site itself: the choice of the initiation codon, the degree of homology between the S–D sequence and the 16S rRNA, and distance of the S–D sequence from the initiation codon (Childs et al., 1985; de Boer et al., 1983; Hui & de Boer, 1987; Looman et al., 1987; Munson et al., 1984; Reddy et al., 1985). Their role in initiation has been established by genetic alteration of the binding region and measurement of the effect on protein synthesis in vitro and in vivo. Unfortunately, little else is known regarding the interaction of the ribosome with its initiation site. Such studies have been limited by the methods available. Specifically, understanding of the interaction of the binding of RNA in complexes with different macromolecules is less advanced than similar studies with DNA, which has rapidly progressed with the development of DNA footprinting (Galas & Schmitz, 1978). However, recent developments in DNA footprinting and in mapping the sites on RNA molecules exposed to chemical probes suggested to us that a procedure similar to DNA footprinting might be used successfully in mapping the ribosome binding site, or another site, on an RNA (Gralla, 1985; Inoue & Cech, 1985; Stern et al., 1988). Thus, we

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[1] Abbreviations: S–D, Shine and Dalgarno; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; DMS, dimethyl sulfate; DEP, diethyl pyrocarbonate; Cu–OP, 1,10-phenanthroline–copper ion complex.

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report here on the interaction of the *E. coli* ribosome with the *lacZ* translation initiation site.

Materials and Methods

Construction of Transcription Vector and Preparation of lac Transcript. A 97-nucleotide *Msp*I–*Eco*RI fragment spanning residues −19 to +63 of *lac* and containing the ribosome binding site of the *lacZ* gene was joined to the T7 promoter in plasmid pGEM2 (Promega Biotec). The starting material for the construction was a plasmid (Stefano & Gralla, 1980) that carried a 203 base pair *Hae*III fragment of the *lac* operon, containing the promoter and a 5′ region of the *lacZ* gene, to which *Eco*RI ends had been added. The purified *Eco*RI fragment was digested with *Msp*I, and the products were inserted into the bacterial alkaline phosphatase treated, *Eco*RI/*Acc*I cloning site of the vector pGEM2. The resulting plasmid, pGM820, was identified by screening alkaline-lysed cell extracts (Maniatas et al., 1982) and its sequence verified directly (Figure 1). To transcribe the RNA template, the plasmid was digested with *Sph*I and treated with DNA polymerase I (Klenow fragment; BRL, Bethesda, MD) to remove the 3′ overhang remaining after restriction cleavage. Transcription was performed using T7 RNA polymerase as described by Davanloo et al. (1984). This produces a single 554-base transcript: as numbered in the figures, residues 1–63 are *lac* mRNA sequences, −1 to −19 are derived from the *lacOP* region, and sequences −20 to −42 and 64–512 are derived from the pGEM vector.

Preparation of Ribosomes. Ribosomes were prepared as described by Henderson et al. (1983). Frozen cells (5 g) of *E. coli* strain Q13 ($RNase^-$, $PNPase^-$) were resuspended in 10 mL of buffer (10 mM Tris-HCl, pH 7.5, 60 mM NH_4Cl, 10 mM magnesium acetate, 0.5 mM ethylenediaminetetraacetate, and 1.0 mM DTT), and the cells were disrupted by two passages through a French press (10000 psi). The extracts were then DNase I treated (3.0 μg/mL) and centrifuged 30 min at 30000*g*. Small portions of the supernatant were quick-frozen for experiments using S-30 fractions. The balance of the supernatant was layered over a 1.1 M sucrose pad in the same buffer. After centrifugation for 16 h at 100000*g* in a Beckman ultracentrifuge with a Ti50 rotor, the ribosome-containing pellet was resuspended in buffer and stored frozen at −70 °C. Alternatively, the supernatant was layered on a linear 15–45% sucrose gradient in buffer A (Noll et al., 1973) and the 70S ribosome peak collected after centrifugation. Ribosomes were activated at 37 °C for 5 min prior to use. The $tRNA_f^{Met}$ (Boehringer Mannheim) was charged and formylated as described by Kaempfer and Jay (1979).

Binding Experiments. RNA was incubated for 15 min at 37 °C in 25 μL of ribosome binding buffer (50 mM Tris-HCl, pH 7.5, 50 mM NH_4Cl, 10 mM magnesium acetate, 1.0 mM DTT, and 200 μM GTP; for reactions with Cu–OP, DTT was replaced with 2.0 mM β-mercaptoethanol) containing 0.1 pmol of *lac* RNA, 5 pmol of fMet-tRNA, and 70S ribosomes (as indicated). The optimal amount of ribosomes was determined by titration with each batch of ribosomes; protection was concentration dependent up to a point where ribonucleases associated with the ribosomes seriously interfered (see Results). At this time, samples were treated with DMS (20 mM; Inoue & Cech, 1985), DEP (diluted 30-fold; Inoue & Cech, 1985), or Cu–OP (167 μM 1,10-phenanthroline, 5 mM 3-mercaptopropionic acid, and 37.5 μM $CuSO_4$; Sigman et al., 1985), and the reaction was allowed to proceed 5 min longer. The reactions were terminated with 15 μL of stop mixture (Sigman et al., 1985), and the nucleic acids were phenol extracted and precipitated with ethanol (Maniatas et al., 1982). Cu–OP reactions received 2.5 μL of quench solution (Sigman et al., 1985) prior to addition of stop mixture.

Experiments using the ribonucleases A (Sigma), *Bacillus cereus* (Pharmacia), PhyM (Pharmacia), and T_1 (Pharmacia) were performed in the same way. Amounts used were determined by titration.

Primer Extension Assays. RNA samples were annealed with a radioactive oligonucleotide primer complementary to the transcript at residues 78–94, and the primer was extended by using AMV reverse transcriptase (2.0 units; Bio-Rad) as described by Inoue and Cech (1985). After completion of the incubations, the samples were subjected to electrophoresis on 8.0% sequencing gels containing 7.0 M urea.

Filter Binding Assays. Filter binding was used to assess the specificity of ribosome complex formation (Dubnoff & Maitra, 1971). The reactions were identical with those used in the footprinting reactions except that a radioactive *lac* transcript was used. Addition of fMet-tRNA stimulated binding severalfold to a maximum of 50% of the mRNA retained. Higher concentrations of ribosomes increased this value but were not used in footprinting experiments due to interference by ribonuclease associated with the ribosomes (above).

Results

RNA Footprinting. Samples of RNA, with or without bound ribosomes, were treated with chemical reagents or ribonucleases, and the footprint of the ribosome binding site was determined by comparing the patterns of modified or cleaved sites obtained on electrophoresis. Our method is based on the technique of Inoue and Cech, who used chemical reagents to probe the secondary structure of RNA molecules, and is similar to a method developed independently by Noller to study the binding of ribosomal proteins to rRNA (Inoue & Cech, 1985; Stern et al., 1986). An important feature of the method is that reactive residues are identified by primer extension. Since this requires that a 3′ site for annealing the primer be intact, RNA molecules that have been partially degraded by 3′-exonucleases are excluded.

With this method, we have analyzed the ribosome binding site on the *lacZ* messenger RNA of *E. coli*. The RNA used was synthesized in vitro by transcription with T7 RNA polymerase of a fragment of the *lac* operon cloned in the vector pGEM2 (see Materials and Methods). Its sequence was confirmed by dideoxy sequencing as shown in Figure 1. In this and the subsequent figures, we have numbered the sequence relative to that of the *lac* mRNA. In this numbering, the *lacZ* AUG initiation codon begins at +39 and the S–D sequence at +28.

Probing the Ribosome Binding Site with Chemical Reagents. We have used three reagents, dimethyl sulfate (DMS), diethyl pyrocarbonate (DEP), and 1,10-phenanthroline–copper ion complex (Cu–OP), as chemical probes. DMS and DEP have been used by Inoue and Cech as well as others (Ehresmann et al., 1987; Inoue & Cech, 1985; Stern et al., 1986). They modify base residues of nucleic acids although their reaction is suppressed in base-paired regions. Cu–OP is a reagent, originally described by Sigman and his associates, that cleaves DNA (Sigman, 1986). With DNA, the reagent forms a coordination complex in the minor groove. Subsequent oxidation by hydrogen peroxide leads to a hydroxyl radical that results in the oxidation of a nearby backbone deoxyribose. The reagent cleaves double-stranded DNA faster than single-stranded DNA, but we have found that in reactions with double-stranded RNA the reaction is strongly inhibited (Murakawa et al., 1989).

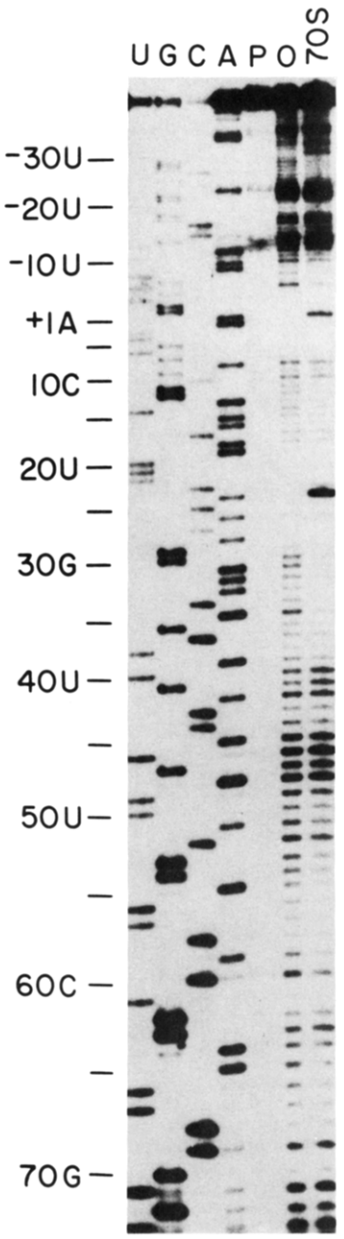

FIGURE 1: Footprinting with Cu–OP. Samples were treated with Cu–OP after incubation with and without 70S ribosomes and fMet-tRNA (lanes 70S and 0, respectively). Additional lanes show the RNA sequence obtained by dideoxy sequencing (U, G, C, A). Lane P (primer extension of untreated *lac* RNA) shows the bands that appear as "pause" sites; such sites are relatively refractory to protection. Numbers refer to positions determined on the sequence ladder with the *lac* operon transcriptional start site indicated as +1 (see Results and Materials and Methods).

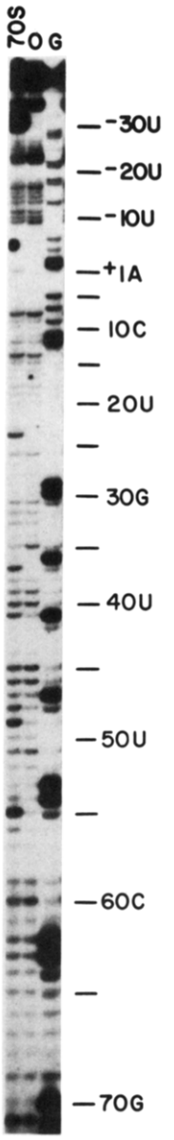

FIGURE 2: Footprinting with DEP. The DEP reaction probes for A and some G residues. Samples were incubated with and without 70S ribosomes and treated with DEP (lanes 0 and 70S). Lane G is the dideoxy C ladder generated.

The results of experiments with the three reagents are shown in Figures 1, 2, and 3. In interpreting the results, one has to remember that the different reagents lead to a band after primer extension that is one residue short of the site of reaction because the residue suffering the modification or cleavage can no longer serve as a template.

Figure 1 shows the results obtained with Cu–OP. Cleavage sites in the Cu–OP-treated control sample are somewhat uniformly distributed along the RNA except in the region of −7 to 6 and 16–28, where the reaction of the nucleotides base paired in a stem–loop structure that is located there is suppressed. Sites of protection can be distinguished when the control sample is compared to one that received 5 pmol of 70S ribosomes (about 50 ribosomes/mRNA). Protection is seen generally at the nucleotides between residues 27–38 and 48–59, and particularly at 29–34, 50, and 59. Positions −35, −33, −1, and +23 appear to be sites of enhanced reactivity relative to the control lane but are actually sites of RNA cleavage due to an endonuclease associated with the ribosome preparation. This was demonstrated in reaction mixtures which received ribosomes but no Cu–OP treatment (data not shown). However, residues 45–47 are slightly enhanced, and no similar endonuclease cleavage was seen to occur here. This indicates that the binding of ribosomes to the *lac* RNA facilitates the reaction with Cu–OP within this region. The results of this experiment are summarized in Figure 4A and discussed below. The region protected from Cu–OP extends generally from nucleotide 27 to 59; it includes the S–D sequence and nucleotides immediately surrounding it, and the nucleotides around the initiation codon but not the AUG itself.

Figure 2 shows the pattern of protection by the ribosome on the *lac* RNA when the probe is DEP. Chemical cleavage of the RNA does not occur in these experiments with either DEP or DMS. However, the modified nucleotides produced prevent replication by reverse transcriptase. Although DEP reveals the least information regarding mRNA–ribosome interaction, a strongly protected site is seen at position A35, as

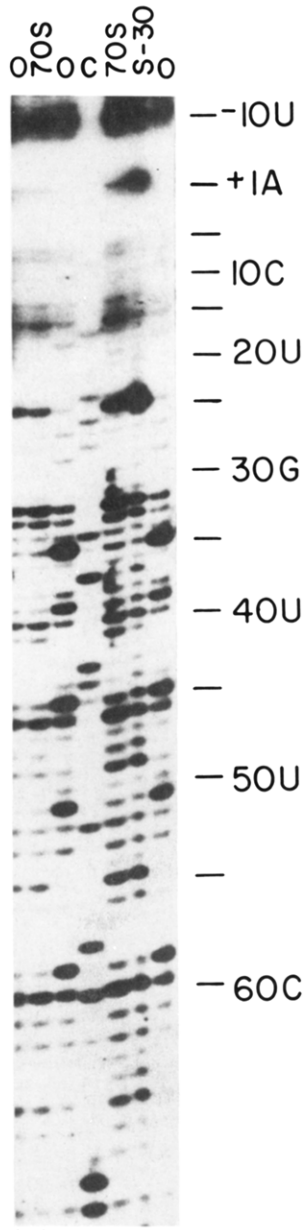

FIGURE 3: Footprinting with DMS. The DMS reaction probes for A and C residues. Samples were incubated with and without 70S ribosomes and treated with DMS (lanes 0 and 70S). An additional sample was incubated with cell-free extract and treated with DMS (lane S-30). The U's at 38 and 50 (bands at 39 and 51) appear strongly reactive. Such reactive U's can be seen occasionally on the gels of others although U itself is not reactive with DMS (Inoue & Cech, 1985). Since we do not know the basis of these reactions, we have not included them in Figure 4B.

well as enhancements at G47 and A48. The DEP data are summarized in Figure 4B. It should be noted that several residues (e.g., 48 and 53) that were protected in the Cu–OP reaction are enhanced in their reactivity with DEP (and DMS; see below). This seems explicable because DEP and DMS react with the bases, whereas Cu–OP, based on its known oxidation of deoxyribose residues of DNA, probably reacts with the ribose backbone.

Dimethyl sulfate is an effective reagent, providing a number of particularly strong cleavage sites (Figure 3). A general area of protection is seen in the region of nucleotides 25–58; here one sees a dramatic protection at many residues including the C's at 25, 27, 34, 37, 44, and 58. In addition to the protected sites, there are also sites whose reactivity with DMS was not altered by ribosomes or was weakly enhanced. The enhancements are seen at residues 45–48; these sites are distinct from bands, as seen in Figure 1, caused by the cleavage of the transcript by nucleases present in the ribosome preparations (e.g., at position −1). A summary of these results is shown in Figure 4C.

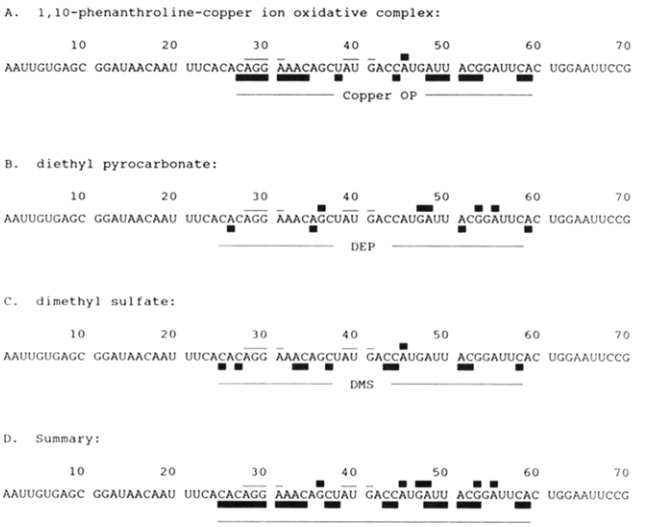

FIGURE 4: Summary of RNA footprinting data. The sites of protections and enhancements are shown for each of the chemical probes (A–C). A composite panel (D) shows the overall pattern generated by all three probes. Filled boxes below the sequences represent protected sites, those above represent sites of enhanced reactivity. The S–D sequence (28–31) and the *lacZ* start codon (39–41) are overlined.

To determine if additional factors might affect the association of ribosomes with the translational initiation site, a second experiment was carried out with an S-30 cell-free extract replacing the ribosomes, and using DMS as a probe. The results were the same a those obtained with purified ribosomes (Figure 3, right).

Footprinting with RNases. We have also carried out experiments with ribonucleases A, PhyM, *B. cereus*, and T_1 as probes (not shown). The results were consistent with those obtained with the chemical probes, but because so few sites were cut at the low concentrations needed to prevent overdigestion of the template, we obtained much less information from these experiments.

Discussion

We have used RNA footprinting to examine a ribosome binding site. Use of RNases as probes provided only limited information. At the low concentrations of enzyme required for footprinting, only very few sites on the substrate RNA were reactive. The chemical probes, like the enzymes, also showed site specificity, as has been seen in other studies (Inoue & Cech, 1985; Stern et al., 1986; Ehresmann et al., 1987). Nonetheless, the chemical probes, particularly DMS and Cu–OP, were more evenly reactive and thus were more useful, in addition to allowing sites within the complex to be studied. However, none of the probes were reactive with double-stranded regions of the RNA substrate. In spite of the fact that Cu–OP is reactive with double-stranded but not single-stranded DNA (it was chosen for this reason), it did not react with bases in the double-stranded regions of RNA (Murakawa et al., 1989). Finally, it must be said that none of the reagents gave as clear a footprint of the ribosome on RNA as one often finds for a site-binding protein on DNA. While this may be due to different factors, it is at least in part due to the presence of uncomplexed mRNA in the reaction mixtures. Filter binding assays showed that only half of the mRNA was bound

at the concentration of ribosomes used. Unfortunately, use of higher concentrations led to excessive degradation of the mRNA by ribonucleases associated with the ribosomes (Materials and Methods). Since we have not examined sites other than that on the *lacZ* mRNA, as we intend, it remains to be seen if some complexes give a stronger footprint. Studies of other ribosome binding sites will also determine if the features determined for *lacZ* are unique or common.

Figure 4D shows a summary of the footprinting data. The region protected by the 70S ribosome spans approximately 35 bases from position 25 to 59 on the *lac* messenger, that is, 14 bases preceding the initiation codon A and 20 following it. This result supports the earlier work of Maizels (1974), who used RNase A and RNase T_1 to isolate the *lac* ribosome binding site, as well as that of several other workers with other mRNAs (Steitz, 1980).

The three chemical reagents revealed internal features of the ribosome binding site. In most general terms, the footprint shows that sequences on the messenger that are protected by ribosome binding are interspersed by sequences that are not. We interpret this to mean that certain sequences are specifically involved in establishing the complex and others are not. This interpretation gains support from the statistical analysis by Gold and his associates of the sequences present at translation initiation sites (Gold et al., 1981; Schneider et al., 1986). They have shown that in addition to the S-D sequence and initiation codon, nucleotides in three other regions are not random. These are the bases flanking the initiation codon as well as residues 8–11 beyond it (49–52 in *lac*). Strikingly, this pattern coincides with sequences protected by the ribosome (Figure 4D).

The pattern of protection obtained indicates that the mRNA interacts with elements of the initiation complex in four areas: the region of the S-D sequence (the "left" side of the complex); flanking the fMet codon; at nucleotides 48–52; and at nucleotides 58–59 (the "right" margin). In addition, residues whose reactivity with the reagents was enhanced in the presence of ribosomes were observed. It appears that the messenger is held on the surface of the ribosome in such a way as to expose these regions, i.e., near residues 36, 45–48, and 53–55.

The pattern in the region of the initiation codon is not so easily explained. Here we obtained protection of the residues surrounding the AUG, but not of the AUG itself. The failure to detect protection of the AUG codon may be due either to the weakness of the codon–anticodon interaction or to the actual state of the initiation complex formed in vitro. The fMet-tRNA in such complexes is in the P site, and the codon–anticodon interaction may not be maintained. It must be added that while complex formation in our system was stimulated by fMet-tRNA addition, we have not shown that the tRNA was present in treated complexes.

The three areas of protection discussed above—in the S-D region, around the AUG codon, and in the region 48–52—and one additional small region defining the downstream border of the ribosome binding site (58–59) are each separated by residues that show enhanced reactivity. It is possible that such enhancements occur in a trivial fashion by the unfolding of regional secondary structures in the mRNA, which otherwise suppress the access of a probe. On the other hand, the enhancement seen at nucleotides around 45–47, the third codon of the *lac* mRNA, was strong and observed with each of the three reagents. Most likely, this represents a point at which the messenger is looped or bent on the surface of the ribosome. It has been suggested, on the basis of the topology of the binding of tRNAs in the P and A sites, that the mRNA might bend between the sites [discussed in Offengand (1980)]. The enhanced site does not correspond to that position (no enhancement was seen between the first two codons, in the region 41–42) but rather is downstream to it. Bending has also been proposed on the basis of microscopy of ribosome–poly(U) complexes (Evstafieva et al., 1983), and Kang and Kantor (1985) interpreted the results of experiments on the T_2 ribonuclease sensitivity of poly(U) in ribosome complexes in terms of such bends. Indeed, it is worth noting that this site might be the same as that observed to divide the ribosome complex with poly(U) into domains of 20 and 16 nucleotides (Kang & Cantor, 1985). One might also speculate that such an exposed site provides for the interaction of an elongation factor or incoming tRNA in advance of the A site.

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Purification of Recombinant Human Tissue Factor

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ABSTRACT: Tissue factor (TF) is a 263 amino acid membrane-bound procoagulant protein that serves as a cofactor for the serine protease factor VII (fVII). Recombinant human TF (rTF) produced in both human kidney 293 cells and *Escherichia coli* has been immunoaffinity purified by using a TF-specific monoclonal antibody. Recombinant TF produced in 293 cells is glycosylated and migrates on reducing SDS–PAGE with an apparent molecular weight (M_r) of 45K. Some interchain disulfide-bonded rTF dimers are observed under nonreducing conditions. The *E. coli* produced rTF has a molecular weight of 33K and 35K, with the 33K band missing nine amino acids at the carboxy terminus. Although the *E. coli* produced rTF does not contain any carbohydrate, it is fully functional in both a chromogenic assay and a one-stage prothrombin time assay. A variant has been constructed wherein the cytoplasmic cysteine (residue 245) has been mutagenized to a serine residue. The amount of disulfide-linked aggregates is dramatically reduced following immunoaffinity purification of this four-cysteine variant (C2455), which is active in the chromogenic and prothrombin time assays.

Tissue factor (TF)[1] is a membrane-bound glycoprotein cofactor that functions in the initiation of blood coagulation. Following vascular injury, TF forms a complex with both the zymogen and activated forms of the serine protease factor VII (fVII) and modulates the catalytic activity of this enzyme toward its substrates. The importance of this interaction has been further demonstrated by the observation that this TF–fVII(a) complex, which was previously thought to activate only fX, also activates fIX (Osterud & Rapaport, 1977). Because the TF–fVII(a) complex has been shown to activate factors in both the extrinsic and the intrinsic pathways of blood coagulation, this complex is now believed to be the physiological inducer of blood clotting (Nemerson & Bach, 1982). TF apoprotein has been purified from bovine brain (Bach et al., 1981; Carson et al., 1985) as well as from human brain and human placenta (Broze et al., 1985; Guha et al., 1986; Morrissey et al., 1988; Rao & Rapaport, 1987). The apo-

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[1] Abbreviations: TF, tissue factor; fVII, factor VII; TFA, trifluoroacetic acid; DTT, dithiothreitol; TMAC, tetramethylammonium chloride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HPLC, high-performance liquid chromatography; MTX, methotrexate; dhfr, dihydrofolate reductase; r, recombinant.

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