

## Effect of Point Mutations in the Decoding Site (C1400) Region of 16S Ribosomal RNA on the Ability of Ribosomes To Carry Out Individual Steps of Protein Synthesis

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**ABSTRACT:** In order to probe the relationship between structure and function of the ribosome, an *in vitro* system [Denman et al. (1988) *Biochemistry* (preceding paper in this issue)] was used to make a series of base changes around C1400, a residue known to be at the decoding site. Replacement of C1400 by U, A, or G, deletion of single bases at and to either side of C1400, and insertion of C or U next to C1400 were done. In a separate study, a mutant with seven extra nucleotides at the 3' end was constructed. The activity of these 11 mutants in A and P site binding and in initiation-dependent and initiation-independent peptide synthesis was analyzed. None of the base substitutions of C1400 were markedly inhibitory despite the almost complete conservation of this residue in ribosomal RNAs from a wide range of species. The insertions and deletions completely blocked initiation-dependent peptide synthesis but markedly stimulated the initiation-independent reaction. The effects on tRNA binding were variable. The extra stem and loop at the 3' end blocked initiation-dependent peptide synthesis but did not influence the other assays. The only modification to block all ribosomal function was the deletion of G1401. It appears that while the conserved and cross-linkable C1400 is not essential for function, the adjacent conserved G1401 is.

The *Escherichia coli* ribosome is a complex macromolecular structure consisting of two unequally sized subunits, 30S and 50S,<sup>1</sup> each of which contains a single large RNA, the 16S and 23S RNAs, respectively, complexed with a set of proteins. The 50S subunit contains in addition one molecule of 5S RNA. It has become increasingly evident that the role of the 16S and 23S RNAs is not simply that of a scaffold on which to spatially organize functionally active proteins but rather that the RNA itself is involved in the function of the ribosome. The first evidence in support of this concept was the finding by Shine and Dalgarno (1974) that the 3'-terminal nucleotides of 16S RNA were involved in selection of mRNAs by base pairing with RNA residues 5' to the initiation codon. Another example in 16S RNA was the detection of the close spatial proximity of C1400 (in *E. coli* 16S RNA) to the 5'-anticodon base of P site bound tRNA which was revealed by the ability to cross-link these two residues (Ofengand et al., 1988). This unique property appears to be a near-universal feature of the structure of ribosomes from all species (Nurse et al., 1987). Another example is the close proximity of U2584-U2585 of 23S RNA to the peptidyl transferase center (Barta et al., 1984). Still other examples are the numerous cases of ribosomes converted to antibiotic resistance by a single base change in either the large or the small ribosomal subunit [reviewed in Cundliffe (1987)] or by a specific base methylation (Helser et al., 1972; Cundliffe, 1987). In addition to these functionally identified regions of ribosomal RNA, two other features are worthy of note. These are the existence of long, evolutionarily conserved single-stranded sequences and the presence of methylated or otherwise modified residues at discrete sites (Noller, 1984). Interestingly, both of these features tend to be associated with functionally significant segments of ribosomal RNA. For example, the cross-linkable C1400 residue

is located in the center of a highly conserved 14-nucleotide sequence which also contains 2 of the 10 methylated residues of 16S RNA.

In order to probe the molecular details of the involvement of ribosomal RNA in ribosome function, we have developed a system for introducing single base alterations in 16S RNA in a way which allows the subsequent analysis of pure mutant ribosomes for their capacity to carry out all of the partial reactions normally involved in the process of protein synthesis (Krzyzosiak et al., 1987; Ofengand et al., 1988). Other workers have previously reported the construction of ribosomes mutant in their 16S RNA moiety [reviewed in Krzyzosiak et al. (1987)], but in those cases, assays of ribosome function in protein synthesis were limited by the presence of an unknown fraction of host cell ribosomes contaminating the mutant ribosome preparations. More recently, however, a system for directing a specific mRNA exclusively to the mutated ribosomes has been devised (Hui & de Boer, 1987), and preliminary results on mutant ribosome function *in vivo* have been reported (de Boer et al., 1988).

In this work, we report the behavior of 11 mutants, 10 of which involve the above-mentioned C1400 residue, or the single-stranded region around it in 4 *in vitro* assays which test different partial reactions of protein synthesis. Each mutant has been tested for P site binding of acylaminoacyl-tRNA, factor-dependent formation of fMet-Ser, EFTu-dependent A site binding of aminoacyl-tRNA, and oligopeptide synthesis. Comparison of results of the different assays for the various

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<sup>1</sup> Abbreviations: Phe-Phe, L-phenylalanyl-L-phenylalanine; TP30, mixture of all *E. coli* 30S proteins; GHG, human growth hormone; 30S, small ribosomal subunit of *E. coli*; 50S, large ribosomal subunit of *E. coli*; ribosome designations of NAT, C1400, U1400, A1400, G1400,  $\Delta$ 1397,  $\Delta$ 1398,  $\Delta$ 1400,  $\Delta$ 1401,  $\Delta$ 1402, U1400.1, C1400.1, and 3'(+7) are defined in Table I of Denman et al. (1988).

mutants has allowed conclusions to be drawn about the effects of the mutations studied on both the initiation and elongation phases of protein synthesis.

#### EXPERIMENTAL PROCEDURES

**Materials.** Purified *E. coli* tRNA<sup>Phe</sup>, tRNA<sup>Val</sup>, and tRNA<sup>Ser</sup> were from Subriden (Rolling Bay, WA). tRNA<sup>fMet</sup> was from Boehringer-Mannheim as were phosphoenolpyruvate, pyruvate kinase, GTP, poly(U), and poly(U<sub>2</sub>G). *E. coli* RNA polymerase, made according to Burgess and Jendrisak (1975), was a gift from T. Coleman (this institute). Poly(U<sub>3</sub>G) was from Pharmacia. RNasin was from Promega Biotec. Phe-Phe was from Cyclo Chemical Corp. CTP, UTP, ATP, and poly(ethylene glycol) 8000 were from Sigma. EFTu and EFG were obtained as described previously (Hsu et al., 1984). [<sup>3</sup>H]Phe, [<sup>3</sup>H]Val, and [<sup>14</sup>C]Val were from New England Nuclear. [<sup>14</sup>C]Met, [<sup>35</sup>S]Met, and [<sup>3</sup>H]Ser were from Amersham. Val-tRNA, AcVal-tRNA, Phe-tRNA, Ser-tRNA, and fMet-tRNA were prepared as described previously (Schwartz & Ofengand, 1978; Ofengand et al., 1979). 70S *E. coli* ribosomes and the ribosomal salt wash fraction containing initiation factors were prepared according to Hershey et al. (1979). Isolated 30S and 50S, natural reconstituted 30S, and synthetic wild-type and mutant 30S were obtained as described previously (Krzyzosiak et al., 1987; Denman et al., 1988).

**Buffers.** RD is 20 mM Hepes, pH 7.5, 100 mM NH<sub>4</sub>Cl, 20 mM Mg(OAc)<sub>2</sub>, and 5 mM mercaptoethanol. TC is 50 mM Hepes, pH 7.5, 75 mM NH<sub>4</sub>Cl, and 75 mM KCl.

**A Site Binding.** This was performed in several steps. In step 1, a 1.7-fold molar excess of 50S was incubated with or without 150–200 nM 30S subunits in RD buffer at 40 °C for 30 min to ensure active 30S (Zamir et al., 1974). In step 2, the P site was filled with uncharged tRNA<sup>Phe</sup> by incubating 0.5–2.0 pmol of 30S equivalents of the step 1 mixture with 75 pmol of uncharged tRNA<sup>Phe</sup> in a 40-μL reaction mixture containing TC buffer, 14 mM Mg(OAc)<sub>2</sub>, and 25 μg/mL poly(U) for 10 min at 37 °C. In step 3, the ternary complex was prepared by incubating 1 mM GTP with or without 3 μM EFTu in TC buffer containing 5 mM Mg(OAc)<sub>2</sub> for 15 min at 37 °C. [<sup>3</sup>H]Phe-tRNA, 500 nM, was added and incubation continued for 5 min more. In step 4, 40 μL of the step 2 mixture was combined with 10 μL of the step 3 mixture and incubated for 20 min at 37 °C. The final concentrations were 12 mM Mg<sup>2+</sup>, 0.2 mM GTP, 20 μg/mL poly(U), 600 nM EFTu, 1500 nM tRNA<sup>Phe</sup>, 100 nM [<sup>3</sup>H]Phe-tRNA<sup>Phe</sup>, and 10–40 nM 30S in TC buffer. Binding was measured by cellulose nitrate membrane filtration as described previously (Ofengand et al., 1979). Values were calculated as moles per mole of added 30S after correction for the binding due to 50S alone.

**Phe-Phe Formation.** Formation of ribosome-bound Phe-Phe-tRNA during A site binding was measured as Phe-Phe released after alkaline hydrolysis. After A site binding as described above, 100-μL aliquots were adjusted to 88 mM KOH and incubated for 15 or 30 min at 56 °C. A third sample was incubated for 15 min at 56 °C in 130 mM KOH. Exposure of Phe-Phe to even the stronger alkaline condition caused <1% hydrolysis to Phe as shown by HPLC analysis (data not shown). The eluate was acidified with HOAc and applied directly to a C18 HPLC column (Merck Lichrosorb) as described by Gast et al. (1987). The positions of Phe and Phe-Phe were established with authentic standards. The fraction of ribosome-bound Phe present as Phe-Phe was taken as the dpm under the Phe-Phe peak divided by the sum of dpm under the Phe-Phe and Phe peaks.

**P Site Binding.** This was done as described by Krzyzosiak et al. (1987) except that Mg(OAc)<sub>2</sub> was present at 15 mM instead of 20 mM, 5 mM DTT was added, spermidine was not included, the 30S concentration was 67 nM, the 50S concentration was 100 nM, and the 10-min preincubation was omitted as it proved unnecessary. Values were calculated as moles bound per mole of added 30S after subtraction of the binding in the presence of 50S alone. Polynucleotide-dependent binding was about the same as 30S-dependent binding.

**Phe,Val Copeptide Synthesis.** This was performed in two steps. First, 150–200 nM 30S subunits were incubated with a 1.6-fold molar excess of 50S in RD buffer at 40 °C for 30 min. This mixture at the concentrations indicated in Figure 3 was assayed for Phe,Val copeptide synthesis as described by Ofengand et al. (1974) with the following modifications. EFTu was used at 70 nM, EFTs and unlabeled valine were omitted, EFG was used at 14 μg/mL, and there was a preincubation for 10 min at 37 °C before reaction was initiated by the addition of a mixture of [<sup>3</sup>H]Phe-tRNA and [<sup>14</sup>C]-Val-tRNA, both present at a final concentration of 300 nM. After 30 min at 37 °C, or as indicated in Figure 3A, reaction was stopped by hot TCA precipitation (Ofengand et al., 1974). Incorporation of label was quantitated by counting in a Beckman LS3801 scintillation spectrometer operating in the dual-isotope dpm mode. Values were corrected for the small amount of reaction in the presence of 50S alone.

**fMet-Ser Dipeptide Formation.** The incubation conditions were according to Nègre et al. (1986), except for the substitution of poly(ethylene glycol) 8000, [<sup>3</sup>H]Ser-tRNA<sup>Ser</sup>, twice as much EFTu, and a lower concentration of ribosomes in place of the originally described components. It was necessary to retitrate the Mg<sup>2+</sup> optimum for each stock solution mixture of salts and nucleoside triphosphates. While the optimum only varied from 8 to 9.5 mM Mg<sup>2+</sup>, it was relatively sharp, falling off to 75% of the maximum at 1.5–2 mM from the peak. Plasmid pBR322 was used as a source of DNA. After 90 min at 37 °C or as indicated in Figure 4A, 35-μL aliquots were treated with 6 μL of 1 M KOH, incubated 15 min at 37 °C, acidified with 0.5 mL of 0.5 M HCl, and eluted from a minicolumn of Dowex-50W-X8 (H<sup>+</sup>) as described by Weissbach et al. (1984). The amounts of 30S plus 50S or 70S ribosomes were as indicated. 50S was always present at a 1.5-fold molar excess over 30S. Values were corrected for the amount of reaction in the presence of 50S alone (see Figure 4).

#### RESULTS

**A Site Binding.** The kinetics of EFTu-dependent binding to isolated 30S and 50S and to synthetic 30S and 50S are shown in Figure 1. Several features deserve comment. First, the use of appropriate amounts of uncharged tRNA<sup>Phe</sup> to fill the P site gave an excellent dependency of binding on EFTu. This is the operational definition of A site binding. Second, although the synthetic ribosomes lack all methylated bases about half have a functional A site which is occupied with essentially the same kinetics as isolated 30S. Third, the rate of binding was reasonably rapid, 75% of the maximum value being reached in 2.5 min. Essentially the same kinetics were obtained with 70S ribosomes (data not shown). The kinetics of Figure 1 do not reflect a rapid binding reaction followed by slow translocation and peptide bond formation. Direct analysis of the ribosome-bound peptide products by HPLC showed that less than 2% of the radioactive Phe present was in the form of Phe-Phe. If translocation and rebinding to the A site had occurred, dipeptide formation would have been expected.

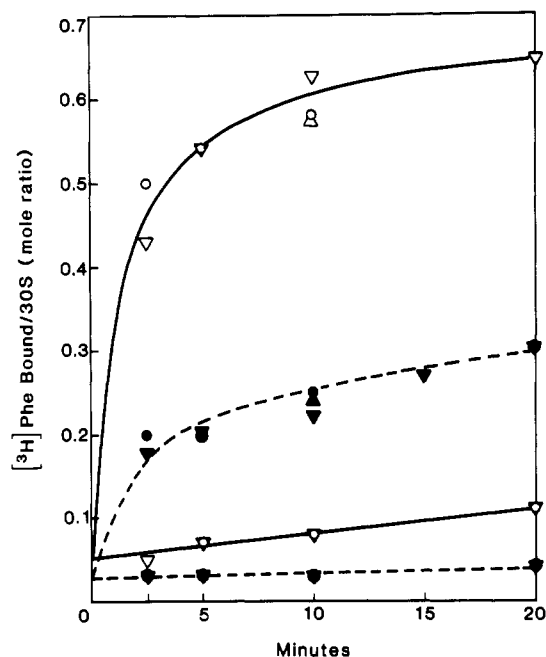


FIGURE 1: Kinetics of EFTu-dependent A site binding of reconstituted ribosomes. Binding assays were performed as described under Experimental Procedures except that the time of the final incubation was varied as indicated. Open symbols and solid line, isolated 30S; closed symbols and dashed line, synthetic (C1400) 30S. Upper curves, plus EFTu; lower curves, minus EFTu. The different symbols denote separate experiments. No blanks have been subtracted.

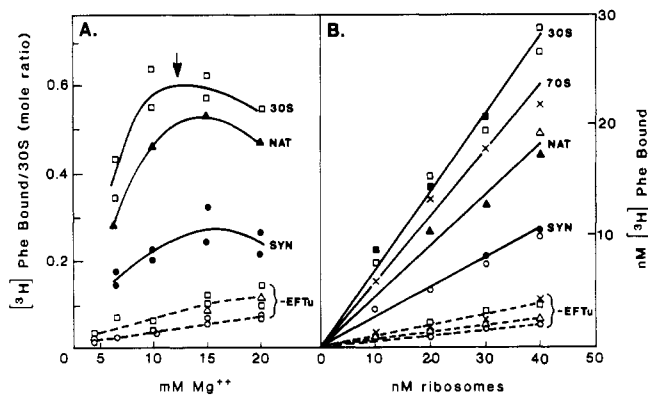


FIGURE 2:  $Mg^{2+}$  and ribosome concentration dependence of EFTu-dependent A site binding of reconstituted ribosomes. (Panel A) Dependence on  $Mg^{2+}$  concentration. (Panel B) Dependence on ribosome concentration. Binding assays were performed as described under Experimental Procedures with a final incubation of 20 min. 70S, times signs; isolated 30S, squares; NAT, triangles; SYN (C1400), circles. Closed or open symbols and upper solid curves, plus EFTu; open symbols and lower dashed curves, minus EFTu. The closed or open symbols denote two separate experiments. The arrow in panel A indicates the  $Mg^{2+}$  concentration used in panel B.

The  $Mg^{2+}$  concentration dependence of A site binding is shown in Figure 2A. The optimum was broad and did not differ significantly for isolated 30S or reconstituted 30S, whether made from natural or synthetic RNA. Moreover, under these conditions, the amount of tRNA bound was directly proportional to the amount of ribosomes added (Figure 2B). This was true not only for the 30S subunits shown but also for all of the mutant 30S (data not shown). In each case, binding was highly dependent upon added EFTu. Note that at the kinetic plateau the 30S plus 50S (denoted 30S on the figure) was as active as 70S. Values as high as 0.6–0.7 pmol bound/pmol of 30S were obtained. In this figure, 30S reconstituted from natural RNA was only 70% as active as 30S, but in more recent experiments, up to 90% activity has been obtained. The activity of the various mutant 30S in A site

Table I: Comparison of Functional Activities of Wild-Type and Mutant Synthetic 30S Ribosomes<sup>a</sup>

30S	P site binding (% of C1400) <sup>b</sup>	A site binding (% of C1400) <sup>c</sup>	(Phe,Val) copolypeptide (% of C1400) <sup>d</sup>	fMet-Ser (% of C1400) <sup>e</sup>
30S	250 ± 34	208 ± 28	161 ± 10	256 ± 19
NAT	210 ± 18	186 ± 24	142 ± 3	256 ± 2
C1400	100 ± 5	100 ± 8	100 ± 2	100 ± 15
3'(+7)	96 ± 10	93 ± 13	89 ± 2	18 ± 2
U1400	135 ± 15	127 ± 17	134 ± 5	113 ± 15
A1400	138 ± 13	80 ± 1	115 ± 15	141 ± 3
G1400	113 ± 20	64 ± 9	143 ± 11	55 ± 6
C1400.1	48 ± 1	52 ± 6	194 ± 19	6 ± 3
U1400.1	45 ± 1	68 ± 7	152 ± 11	1 ± 1
Δ1397	50 ± 7	94 ± 1	169 ± 10	2 ± 1
Δ1398	43 ± 5	58 ± 8	169 ± 6	2 ± 1
Δ1400	18 ± 3	60 ± 4	221 ± 2	2 ± 1
Δ1401	<5	<5	13 ± 2	<1
Δ1402	7 ± 2	31 ± 4	200 ± 7	<1

<sup>a</sup> Assays as defined below were performed 2–3 times on the reconstituted ribosome preparations shown in Figure 3 of Denman et al. (1988). These preparations were made at the same time by using a single batch of TP30. For synthetic (C1400) and Δ1402, two reconstitutions were performed from a single RNA preparation, and each one was assayed at least twice. All values are reported as percent of C1400 ± average deviation. When more than one reconstitution was available, the assay values for each reconstitution were first averaged, and then all reconstitutions were averaged. The average deviation is that of the reconstitution average. Where only one reconstitution was done, the average deviation of the assay values is given. <sup>b</sup> Isolated 30S was 0.4–0.7 pmol of tRNA bound/pmol of 30S. Duplicate samples were taken for each assay. <sup>c</sup> Isolated 30S was 0.5–0.7 pmol of tRNA bound/pmol of 30S. For each assay, two samples at a 2-fold different ribosome concentration were averaged. Final incubation was for 10 min (assay 1) or 20 min (assay 2). The values, which differed by <15%, were averaged. <sup>d</sup> Isolated 30S was 1.9–2.3 pmol of Phe incorporated (pmol of 30S)<sup>-1</sup> (30 min)<sup>-1</sup> with a Phe/Val ratio of incorporation of 1.3–1.4. The ratio for the insertion and deletion mutants was slightly higher; see Figure 8. For each assay, two samples at ribosome concentrations of 20 and 40 nM were averaged. <sup>e</sup> Isolated 30S was 3.2–4.2 pmol of fMet-Ser/pmol of 30S. For each assay, three samples over a ribosome concentration range of 9–26 nM were used to construct a linear curve, as in Figure 4B.

binding is listed in Table I and will be discussed below.

**P Site Binding.** This assay has been described previously by Krzyzosiak et al. (1987). The amount of binding was in all cases proportional to the amount of ribosomes added since AcVal-tRNA and 50S subunits were present in excess. To verify that binding was to the P site under these conditions, the bound tRNA was assayed for its ability to react with puromycin. This is the usual operational definition of P site binding. After binding was complete, 0.7 mM puromycin was added and incubation continued at 0 °C for up to 2 h. At this temperature, a negligible amount of translocation occurs (Geigenmüller et al., 1986) while peptidyl transferase is still active. More than 75% of the ribosome-bound Ac[<sup>3</sup>H]Val was released in a puromycin-dependent manner by 1 h of this treatment, when either 30S reconstituted from natural 16S RNA or the U1400 mutant synthetic 30S was used, confirming that the assay measures primarily P site binding. The activities of the various mutant 30S are listed in Table I.

**Phe,Val Copolypeptide Formation.** A simple in vitro system using only Phe-tRNA, Val-tRNA, and a synthetic mRNA, poly(U<sub>3</sub>G) which provided UUU and GUU codons, respectively, had been used in an earlier study to measure translocation plus peptide bond formation (Ofengand et al., 1974). The same system was used here as a means of bypassing the initiation process which, as shown below, was considerably more sensitive to mutational changes in this region of the 16S RNA. It was thus possible to study the effect of ribosomal RNA base changes on both the translocational and peptidyl transferase processes in mutants defective in initiation. As shown in Figure 3A, similar kinetic curves were obtained for both isolated 30S and synthetic 30S. Essentially the same kinetics were obtained for natural (NAT), 3'(+7), A1400,

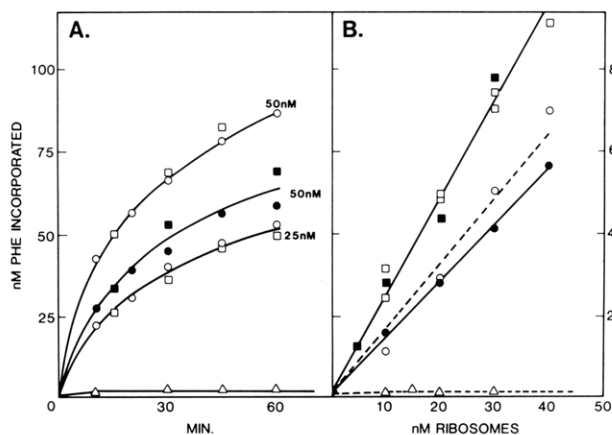


FIGURE 3: Phe,Val copolypeptide synthesis by synthetic and mutant ribosomes. (Panel A) Rate of reaction. The ribosome concentrations were as indicated. (○, □) Isolated 30S; (●, ■) synthetic (C1400) 30S. The two sets of symbols are two different experiments. (Δ) 50 nM isolated 30S with poly(U<sub>3</sub>G) omitted. (Panel B) Ribosome proportionality at 30 min of reaction: (□) isolated 30S; (■) Δ1400; (○) 3'(+7); (●) C1400; (Δ) no 30S added.

G1400, C1400.1, U1400.1, and Δ1400 reconstituted 30S (data not shown). The other mutants were not tested. No product was found in the absence of mRNA. The dependence of the amount of polypeptide produced in a 30-min reaction on the amount of 30S added is shown in Figure 3B along with several selected mutants. The figure demonstrates that this assay shows good ribosome proportionality and is completely dependent on the presence of 30S subunits, 50S subunits alone being inactive. Although only a few examples are displayed, all of the mutants yielded linear ribosome dependence curves. The relative activities shown here differ slightly from those listed in Table I which are the averages of several determinations.

In one experiment, synthesis of poly(Phe) dependent on poly(U) was compared with this assay by substituting poly(U) for poly(U<sub>3</sub>G). When poly(U) was used, the amount of synthesis increased from 1.5 to 5.5 pmol of Phe incorporated (pmol of 30S)<sup>-1</sup> (30 min)<sup>-1</sup> for the control 30S, and synthetic 30S was 64% of the control. The same preparation of synthetic 30S was 74% of the control in the standard assay. In the same experiment, the ability of Val-tRNA to recognize poly(U) was tested by including Val-tRNA in the assay. Thus, Val-tRNA recognition which requires first position miscoding was performed in competition with Phe-tRNA for the UUU codons. No incorporation of valine was detected for either the control or the synthetic ribosomes. The various mutant ribosomes were not tested.

**fMet-Ser Dipeptide Formation.** In this simplified plasmid-directed system, mRNA synthesis is coupled to protein synthesis which is limited to formation of the N-terminal dipeptide of the plasmid-borne gene product. The system has been shown to correctly reflect the initiation of both transcription and translation (Robakis et al., 1983; Cenatiempo, 1986). In its original form, a large excess of ribosomes was used so that the dipeptide formed was proportional to the amount of mRNA. We modified the assay by decreasing the amount of ribosomes until they became limiting. The mixtures of 50S and 30S were not heat-activated as in the above assay, since all the 30S particles were activated after reconstitution or isolation (Krzyszosiak et al., 1987) and control experiments explicitly showed that, at least for this assay, a second activation step was unnecessary. Figure 4A shows the time course of the reaction for isolated 30S, synthetic 30S, and two mutants. It is not known why the reaction comes to a stop after

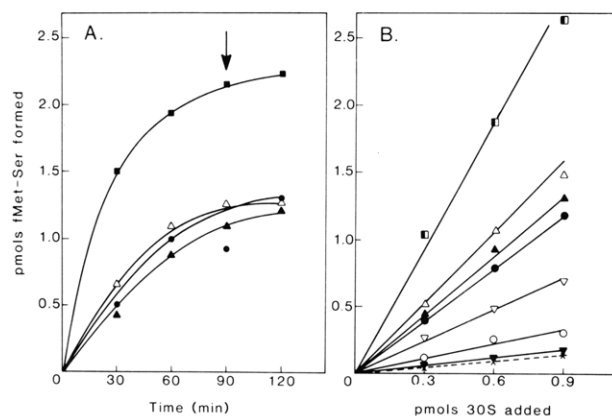


FIGURE 4: fMet-Ser dipeptide synthesis by synthetic and mutant ribosomes. (Panel A) Kinetics of reaction. 0.7 pmol of 30S was used. (Panel B) Ribosome dependence of a 90-min reaction. Assays were done as described under Experimental Procedures: (■) isolated 30S; (□) NAT; (▲) U1400; (●) C1400; (Δ) A1400; (▽) G1400; (○) 3'(+7); (▼) all deletions and insertions; (×) 50S with no 30S added.

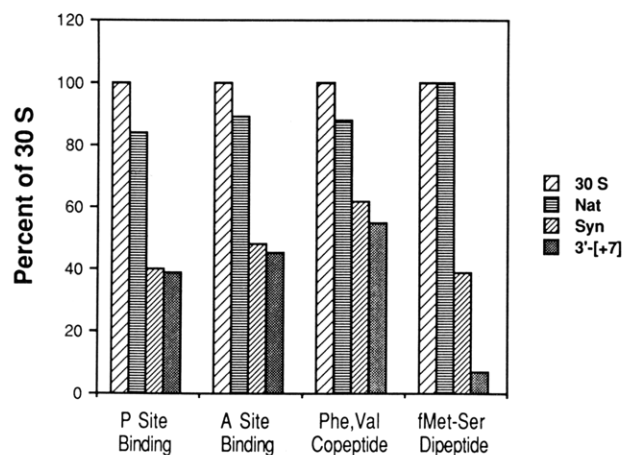


FIGURE 5: Functional activities of reconstituted 30S ribosomes. Values were obtained from Table I. SYN, C1400.

about 90 min. However, as this time gave the maximum yield of product, it was adopted for the standard assay. Figure 4B shows that the amount of product after 90-min reaction was strictly dependent on the amount of ribosomes added, that reconstituted natural 30S plus 50S was as active as isolated 30S plus 50S, and that essentially no reaction occurred with 50S alone. The activity of 70S ribosomes was the same as isolated 30S plus 50S (data not shown). The activity of all of the mutants is listed in Table I and discussed below.

**Functional Activities.** The relative activities of the 11 mutants in the 4 above-described assays are given in Table I and Figures 5, 7, and 8. The data of Table I were obtained by using freshly transcribed RNAs which were reconstituted over a 3-day period with a single preparation of TP30. The values are generally in good agreement with results reported previously (Krzyszosiak et al., 1987; Ofengand et al., 1988) as well as with a large body of data accumulated over a 1.5-year period using a series of RNA and TP30 preparations (data not shown). Some of the A1400 and G1400 results reported previously were lower due to the use at that time of a less extensive set of data.

**Synthetic and Natural 30S.** Figure 5 compares the activity in all of the above assays for isolated 30S, 30S reconstituted with isolated 16S RNA, the synthetic nonmutant RNA, and the mutant with additional 3'-nucleotides. In all of the assays, natural reconstituted 30S was about as active as isolated 30S, confirming the original findings of Held et al. (1973), albeit with a different reconstitution regimen. Synthetic 30S was

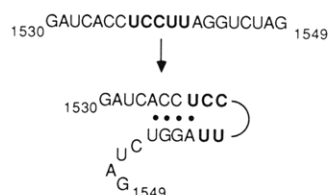


FIGURE 6: Possible folding at the 3' end of the 3'(+7) mutant. Residues 1537–1541 which can base pair with the  $\beta$ -lactamase Shine–Dalgarno sequence are shown in boldface. Dots denote potential intramolecular base pairs.

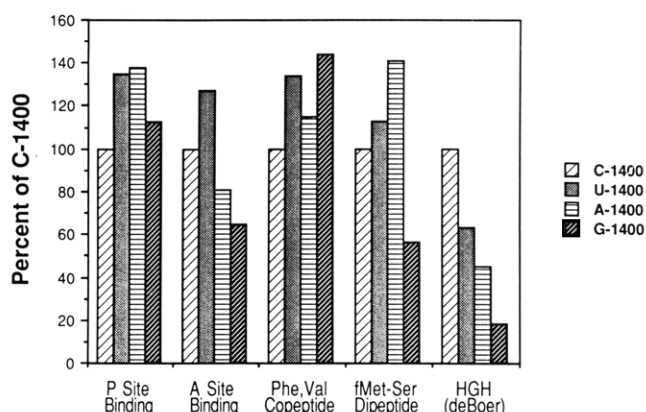


FIGURE 7: Functional activities of position 1400 mutants. Values were obtained from Table I. HGH (de Boer) values are those for the *in vivo* synthesis of immunoprecipitable human growth hormone polypeptide using the specialized ribosome system described by Hui and de Boer (1987), taken at approximately 1 h when the amount of protein produced was approximately proportional to time (de Boer et al., 1988).

about 40% as active as isolated 30S in every assay except the Phe,Val copeptide assay where a somewhat higher activity level was obtained. The value for P site binding is slightly lower than the 50% previously reported for synthetic 30S (Krzyszosiak et al., 1987), and the value for natural RNA is higher than that reported previously. These latter results appear to have been due to a hidden defect in the original natural RNA preparation, as all subsequent 30S reconstituted with natural RNA have been almost as active as the control 30S (Table I). Addition of seven nucleotides to the 3' end of 16S RNA had no effect on tRNA binding to either the A or the P sites nor did it affect initiation-independent peptide synthesis. It did, however, strongly inhibit (ca. 6-fold) the initiation-dependent reaction. A reasonable explanation for this rather specific inhibitory effect is shown in Figure 6. The seven extra nucleotides which were part of the contiguous *Mst*II and *Xba*I restriction sites created the possibility for base pairing with the anti-Shine–Dalgarno region by forming a four base pair stem and a four-base loop. Since the Shine–Dalgarno region of the  $\beta$ -lactamase mRNA (Sutcliffe, 1978) used in this assay would have base-paired with residues 1537–1541 present in the newly formed loop, it is easy to understand why protein synthesis initiation was severely inhibited.

**Base Substitution Mutants.** The functional effect of base changes at C1400, the invariant C residue which is uniquely cross-linkable to tRNA, is shown in Figure 7. In this figure, the activity is given relative to the parent C1400 as 100%. Despite the almost complete conservation of C at position 1400 or its equivalent in small subunit RNA of all organisms examined (Ofengand et al., 1986; Dams et al., 1988), replacement by U, A, or G had only a moderate effect. The largest change was produced by G1400, but even in this case, the inhibition was only 2-fold and only for factor-dependent A site binding and for factor-dependent chain initiation. The extreme

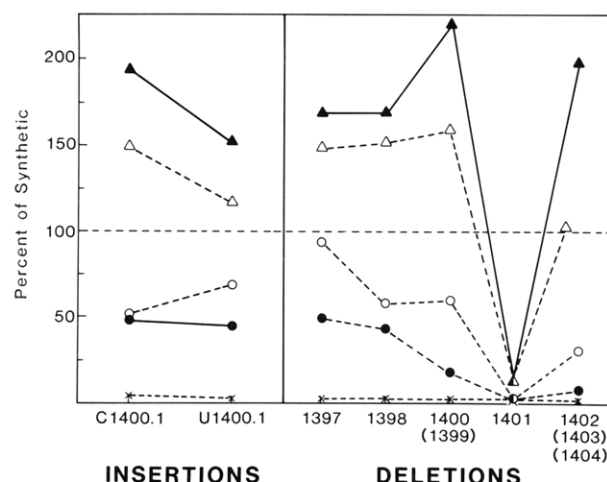


FIGURE 8: Functional activities of insertions and deletions between positions 1397 and 1404. Values are taken from Table I. (●) P site binding; (○) A site binding; (▲) Phe,Val copeptide synthesis, [<sup>3</sup>H]Phe incorporation; (Δ) Phe,Val copeptide synthesis, [<sup>14</sup>C]Val incorporation; (×) fMet-Ser dipeptide synthesis.

right set of values was calculated from the data of de Boer et al. (1988) for the *in vivo* expression of HGH-like polypeptide. These results show a lower activity for the same substitutions, but even G1400 possessed some activity. As the results were obtained *in vivo*, they reflect not only the ability to make a peptide large enough to be immunoreactive but also the ability of the mutant RNAs to be assembled into ribosomes. As we have shown that the G1400 mutant assembles poorly unless special conditions are used (Denman et al., 1988), it is perhaps not too surprising that our functional assays, which test preassembled ribosomes, should be higher than those observed by de Boer and co-workers.

**Insertion and Deletion Mutants.** The effect of a U or C insertion adjacent to C1400 and deletion of C1400 or single bases to either side of it is shown in Figure 8. The results were surprisingly varied. First, all of the changes blocked fMet-Ser synthesis. This result should be contrasted with those of Figures 5 and 7. Apparently, this area is critical for the initiation process. Second, G1401 was the only residue whose deletion blocked all functional activity. The contrast in the Phe,Val copeptide assay with the deletion of C1400 or C1402 was particularly striking. Third, there was a general large stimulation of initiation-independent peptide formation for all of the changes, with the exception of G1401. For example, deletion of C1400, which is the same as deleting C1399, stimulated Phe,Val copeptide synthesis 2.2-fold compared to synthetic nonmutant ribosomes and even surpassed isolated 30S by almost 1.4-fold. While there was a slight variation in the Phe/Val incorporation ratio among the various mutants, the effect was in general small. The meaning of the relatively greater variation with  $\Delta$ 1402 is not clear. Fourth, the various changes depressed both P and A site binding. P site binding was more affected by the deletions than A site binding, and both showed a gradation of effect, the inhibition seeming greater as the deletion moved in the 3' direction. Increasing the  $Mg^{2+}$  concentration from 15 mM (the standard assay) to 25 mM only slightly increased P site binding values for  $\Delta$ 1400 and  $\Delta$ 1402, but even that slight increase was not found for  $\Delta$ 1401. It is striking that even when A and P site binding was severely inhibited as in  $\Delta$ 1400 and especially in  $\Delta$ 1402, Phe,Val copeptide synthesis, which clearly requires a functioning A and P site, was markedly stimulated. There also appears to be a weak inverse correlation between P site binding and Phe,Val synthesis.



## DISCUSSION

The major partial reactions of protein synthesis that have been tested in this work are factor- and codon-dependent A site binding, codon-dependent P site binding, peptide bond synthesis which also requires translocation, and initiation factor dependent formation of the initial peptide bond. Translocation must occur in the Phe,Val copeptide assay since about seven Phe and Val peptide bonds were made per isolated 30S in 30 min if one assumes that only half of the ribosomes were functional. For synthetic 30S, the value was approximately four. Thus, this assay most probably measures the rate of translocation since that reaction is thought to be the rate-limiting step in peptide bond formation (Gast et al., 1987). Formation of the fMet-initiated dipeptidyl-tRNA also involves cycling. Table I indicates that three to four fMet-Ser were made per isolated 30S, if all of the 30S were active. This effect has been noted previously and ascribed to dissociation of the dipeptidyl-tRNA from the ribosome followed by reinitiation (Cenatiempo et al., 1982). Thus, a decrease in the overall amount of dipeptide synthesized could be due to a decreased ability to form the first dipeptide, to an increased stability of the ribosome-dipeptidyl-tRNA complex so that cycling does not occur as frequently, or to some combination of both effects.

The synthetic nonmutant ribosomes were active in all of the partial reactions just described as was shown previously for P site binding (Krzyzosiak et al., 1987) and for peptide synthesis (Ofengand et al., 1988). Thus, the 10 methylated bases of 16S RNA are not essential for any of these partial reactions, and the 5' end of 16S RNA can be modified as well. While the 3' end modification inhibited initiation of protein synthesis, this probably was due to the specific sequence used and not to a general requirement for a normal 3' end. This conclusion is currently being tested by substituting a non-base-pairing sequence at the 3' end in place of the 3'(+7) extra nucleotides.

The failure of C1400 substitutions to affect function more strongly was surprising. This residue is almost entirely conserved among small subunit rRNAs from widely diverse species and is even more highly conserved than its surrounding residues of the conserved 1394-1408 sequence (Ofengand et al., 1986; Dams et al., 1988). Moreover, it is uniquely positioned in the 30S ribosome since it is the only residue which can be cross-linked to the tRNA anticodon under P site binding conditions (Ofengand et al., 1988). Nevertheless, no strong functional effects of base substitution could be detected in vitro. Perhaps C1400 is more important for correct assembly than for function. For example, the G1400 substitution interfered even with in vitro assembly (Denman et al., 1988), and possibly the other substitutions also interfere in vivo in more subtle ways. Alternatively, our assays may not be sufficiently sensitive to detect a kinetic effect which may, nevertheless, confer a key advantage to a growing cell in a competitive environment. It is also possible that some other part of the translation process, such as termination or fidelity, is dependent on C1400.

Deletion of C1400, on the other hand, resulted in a more complex pattern of effects. fMet-Ser synthesis was completely blocked whereas substitution of C1400 by G, the base showing the largest effect, only reduced this activity in half. Deletion of C1400 also strongly depressed P site binding yet at the same time markedly stimulated Phe,Val copeptide synthesis (discussed below). The substitution mutants did not greatly affect the same assays. These results suggested that the length of the single-stranded region from 1394-1408 might be the critical factor and prompted us to construct the other deletion and insertion mutants.

Deletion of each residue from 1397 to 1404 was examined. It was not necessary to construct deletions at C1399 or at C1403/C1404 since deleting C1400 was the same as deleting C1399 and deleting C1402 was the same as deleting C1403-C1404. The results (Table I and Figure 8) lead to three main conclusions. First, since all of the insertion and deletion mutants inactivate peptide chain initiation, the initiation complex appears to be very finely tuned in this region. Possibly, the length of this single-stranded region is important for the initiation process. The fact that no detectable amount of fMet-Ser was made means that not even one cycle of fMet-Ser synthesis was possible. Second, all but one of the changes markedly stimulated initiation-independent peptide synthesis to levels even above that of isolated 30S, and there appeared to be an approximate inverse correlation with P site binding. If, under our assay conditions, the relative affinity of tRNA for the P site controls the rate of translocation, then since translocation is the rate-limiting reaction in peptide formation (Gast et al., 1987), such a correlation might be expected. Since the P site assay is simply filter binding, a low-affinity association would not be readily detected. An extreme example of this effect is  $\Delta$ 1402. Such a mutant should make longer peptides, on the average, than the nonmutated synthetic ribosome, but this has not yet been confirmed by experiment. Third, deletion of G1401 blocked all ribosomal activity although the mutant particle still sedimented at 30S, contained all the 30S proteins (Denman et al., 1988), and could associate with 50S (see below). It is striking that a single base deletion could have such a marked effect, especially since the deletions on either side did not. This differential effect argues against the length of this region being important for the protein synthesis assays tested other than initiation. However, it is still possible that deletion of G1401 produces its functional effects by induction of a specific conformational rearrangement of the tertiary structure of the RNA. This could be examined by a structural analysis of the RNA in the mutant ribosomes and by making base substitutions at G1401. Both approaches are currently being pursued.

The functional results obtained are not the result of a failure of 30S and 50S to associate, a reaction which must precede all of the assays used in this work. Association was explicitly tested (Denman et al., 1988), and all mutants, except  $\Delta$ 1401, were shown to associate well under conditions similar to those used for functional assay. Although  $\Delta$ 1401 formed 70S with an efficiency clearly different from the rest, its association ability still far exceeded its functional activity. Therefore, the failure of  $\Delta$ 1401 to function in all of the tests applied cannot be due to its failure to associate with 50S but instead must be due to a more direct effect of the removal of that base on 30S structure or function.

Recently, Mélançon et al. (1987) reported the independent development of a system for the reconstitution of 30S ribosomes from rRNA transcribed in vitro which is very similar to that described by us (Krzyzosiak et al., 1987). Thus far, no point mutations have been described although a ribosome with residues 1510/1542 deleted was prepared which still possessed considerable polyphenylalanine synthesizing activity. The protein synthesizing activity of the full-length and truncated synthetic ribosomes prepared by these authors cannot be directly compared to our assay values because unlike our assays, which were strictly linear with the amount of 30S added, not only was linearity not demonstrated but also a 5-fold excess of synthetic 30S over 50S was used.

Other workers have also mutated this region of 16S RNA. Rottmann et al. (1988) reported that the double mutants

A1399/U1401 and A1399/C1401 were strongly inhibited in protein synthesis, a result which is consistent with the results reported here. de Boer et al. (1988) studied this region by measuring the *in vivo* production from HGH mRNA and mutant ribosomes of peptides immunoreactive with HGH antibody. In contrast to the native C1400 and the U1400 mutant, the A1400 and G1400 mutants made protein only for about 1 h and then stopped. The values given in Figure 7 were calculated for the beginning of the plateau. Obviously, the G and A substitutions would yield even lower relative values if a later time were chosen. We did not detect such a strong effect of A1400 and G1400 substitution in our assays. The large difference in the results of de Boer and colleagues and ourselves for G1400 could be partially explained as a difficulty in *in vivo* assembly similar to what we have described *in vitro* (Denman et al., 1988), but the differing effects seen with A1400 must reflect other differences between the *in vitro* and *in vivo* assays used. de Boer et al. also deleted the segment 1400–1404 and found almost no activity remaining in agreement with our finding that all of these bases are needed for initiation. They also substituted the segment UUGUUU1404 for CCGCCC1404. Although some activity might have been expected since U1400 was functional, both *in vivo* and *in vitro*, the mutant was virtually inactive. Lastly, C1402 was changed to A, and this mutant was also inactive. We found that deletion of C1402 blocked initiation but have not yet examined base substitutions at this position. However, Jemiolo et al. (1985) reported earlier that a change of C1402 to U had only a slight effect on the growth rate of cells harboring the mutant ribosomes.

In related studies, Beauclerk and Cundliffe (1987) found that methylation of G1405 to m<sup>7</sup>G or A1408 to m<sup>1</sup>A led to kanamycin plus gentamycin or kanamycin plus apramycin resistance, respectively, while Moazed and Noller (1987) found that neomycin, paromomycin, kanamycin, or gentamycin bound to 70S or 30S ribosomes strongly protected A1408 from chemical probes. The location of these residues near the decoding site in the vicinity of C1400 is consistent with the fact that these antibiotics are known to induce miscoding.

Several of the residues studied here were protected by ribosome-bound tRNA in footprinting experiments (Meier & Wagner, 1984; Moazed & Noller, 1986). Both groups reported the strong protection of the N7 of G1401 by P site bound tRNA, consistent with the strong inhibition effects we observed on all functional assays upon deletion of this residue. Indeed, the strongest protection observed by Moazed and Noller in this region was of G1401. In addition, these authors found protection of the N3 of C1399 and C1400 by bound tRNA, which is again consistent with our findings that these residues are involved in the protein synthesis process. Thus, the tRNA footprinting studies are in excellent agreement, insofar as they are applicable, with our studies.

Comparison of our results with those of others illustrates some clear differences between our *in vitro* system and systems using intact cells. Most important among these is our ability to measure independent partial reactions of protein synthesis on a purified population of ribosomes of a defined composition. This advantage is illustrated by our ability to test the G1400 mutant, once conditions were found for its assembly. *In vivo* systems, on the other hand, have the ability to detect complex interactions among cellular components which may be omitted in defined systems. For this reason, we are currently involved in transferring the mutants described here into a system suitable for *in vivo* analysis. Additional mutagenesis studies are also under way, at these and other sites, in order to obtain

new insight into the way ribosomal RNA participates directly in the protein synthetic process.

Registry No. Mg, 7439-95-4; guanine, 73-40-5.

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## Sequence-Specific, Strand-Selective, and Directional Binding of Neocarzinostatin Chromophore to Oligodeoxyribonucleotides<sup>†</sup>

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**ABSTRACT:** The sequence-specific interaction of neocarzinostatin chromophore (NCS-C) has been evaluated with a series of synthetic oligodeoxyribonucleotides of defined sequences containing the most preferred nucleotide cleavage site, T or A, or both. NCS-C preferentially cleaves T or A residues in the sequence GN<sub>1</sub>N<sub>2</sub>, where N<sub>2</sub> is T or A. Greater cleavage occurs on the strand enriched with G residues, provided that they are adjacent to other G residues, but not at N<sub>1</sub>. These results are compatible with a model for drug binding in which the naphthoate moiety of NCS-C preferentially intercalates at GN<sub>1</sub>. This is accompanied by electrostatic binding interaction provided by the positively charged amino sugar moiety so as to place the reactive bicyclo[7.3.0]dodecadienediyl epoxide moiety in an appropriate orientation in the minor groove enabling, upon thiol activation, attack at C-5' of T or A. At certain sequences, such as GCT-AGC, a similar binding mode is also able to generate abasic lesions at the C residue on the opposite strand, forming a bistranded lesion. Although the reactions with glutathione generally show the same strand selectivity and sequence specificity as those with dithiothreitol, the former is usually more efficient than the latter.

The labile nonprotein chromophore of neocarzinostatin (NCS-C)<sup>1</sup> (Napier et al., 1979, 1981; Albers-Schönberg et al., 1980; Hensens et al., 1983; Edo et al., 1985a,b, 1986; Myers et al., 1988), an antitumor antibiotic produced by *Streptomyces carzinostaticus*, is solely responsible for its DNA-damaging activity. NCS-C binds to DNA by an intercalative mechanism and has a relatively strong affinity for DNA ( $K_d = 10^{-6}$  M) (Povirk & Goldberg, 1980; Povirk et al., 1981; Dasgupta et al., 1985) with two types of binding sites (Povirk et al., 1981; Dasgupta & Goldberg, 1985). Its binding stoichiometry ( $r_b$ ) for the high-affinity binding sites is 0.12 (approximately one NCS-C per four base pairs), and that for the low-affinity binding sites is 0.23 (approximately one NCS-C per two base pairs). Thiol-activated NCS-C abstracts a hydrogen atom from the C-5' of deoxyribose at T and A residues (Kappen & Goldberg, 1985), forming either O<sub>2</sub>-dependent single-stranded nicks with nucleoside 5'-aldehyde at the 5'-end of the break (Kappen et al., 1982; Kappen & Goldberg, 1983) or O<sub>2</sub>-independent stable adducts on the deoxyribose of these same residues (Povirk & Goldberg, 1982a,b, 1984, 1985a).

NCS-C consists of three main structural subunits: a substituted naphthoic acid, an amino sugar (*N*-methyl- $\alpha$ -D-fucosamine), and a bicyclo[7.3.0]dodecadienediyl epoxide (Figure 1). We previously proposed that the naphthoic acid moiety intercalates DNA in the minor groove and that the positively charged amino sugar moiety interacts with the negatively charged sugar phosphate backbone of DNA (Napier

& Goldberg, 1983). These two anchors assist in the juxtaposition of the bicyclo[7.3.0]dodecadienediyl epoxide moiety with the deoxyribose of chiefly T residues in the minor groove of DNA (Goldberg, 1986). The epoxide, or its equivalent, has been found to be relatively important in its biological activity, probably in a cooperative manner with the highly strained unsaturated bicyclo[7.3.0]dodecadienediyl moiety (Lee & Goldberg, 1988), whereas the cyclic carbonate (1,3-dioxolan-2-one) moiety appears to play a role in the passage of NCS-C through cellular and nuclear membranes (Napier et al., 1981; Lee & Goldberg, 1988). However, neither the nucleotide sequences for the preferential intercalative binding by NCS-C nor its relationship with major cleavage sites, which mainly occur at T residues, has been identified so far. In the presence of  $\beta$ -mercaptoethanol, the preferred dinucleotides for the NCS-C-induced cleavage of DNA restriction fragments occur in the relative probability order of AT > CT > TT > GT, where T residues are the target sites (Takeshita et al., 1981).

A recent hypothetical model, based on theoretical computations on the binding of NCS-C to a limited number of self-complementary tetramers (CGCG, GCGC, TATA, and ATAT), proposes that purine-(3',5')-pyrimidine sites are more favorable for intercalation than pyrimidine-(3',5')-purine in the minor groove (Chen et al., 1987). Unfortunately, however, it fails to provide a clear picture of how the binding mode

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<sup>1</sup> Abbreviations: NCS, neocarzinostatin (holoprotein); NCS-C, neocarzinostatin chromophore; DTT, dithiothreitol; GSH, glutathione; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.