Relative Efficiency of Anticodons in Reading the Valine Codons during Protein Synthesis in Vitro*

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Using a protein synthesizing in vitro system programmed with MS 2-RNA, the relative efficiency (in the presence of each other) of valine tRNAs with the anticodons U*AC (U* represents 5-oxyacetic acid uridine monophosphate), GAC, and IAC to read the valine codons was investigated. An anticodon which can read all three positions of the codon according to the rules of Watson-Crick base-pairing and the wobble hypothesis is an order of magnitude more efficient than an anticodon which misreads the codon by reading only the first two positions and presumably disregards the third nucleotide of the codon. There are two seeming exceptions to this behavior: the anticodon U*AC reads the codon GUU quite efficiently and IAC is as effective as U*AC in reading the codon GUG. The significance of these exceptions is evaluated with respect to the organization and evolution of the genetic code.

During protein synthesis in vivo, the first two positions of the three letter codons in the genetic code are read by the anticodons strictly according to the base-pairing rules of Watson and Crick. However, the discrepancy between the large number of codons in a degenerate code and the limited number of anticodons available to read them, introduces complications in the reading of the third codon nucleotide, which is recognized by the 5'-end nucleotide of the anticodon, the wobble nucleotide. To overcome this difficulty, Crick (1) introduced an additional set of base-pairing rules for the reading of the third codon position which allows for so-called wobble interactions: U in the wobble position can recognize G (as well as A) in the third position of the codon, I can recognize both U and A (as well as C), and G can recognize U (as well as C). This leaves a number of conceivable interactions that are not allowed even in the wobble position: U does not recognize U and C, does not recognize U, C, or A, I does not recognize C, and G does not recognize A. This set of rules represents what may now be called the classic concept of wobble pairing restrictions. However, such aberrations should rather, represent extensions of the wobble rules, which may not necessarily be regarded as misreadings. They could, for example, represent extensions of the wobble rules, which may in their original form have been too narrow. In the present work on this possibility was also sought.

This study of codon-anticodon recognition again made use of the in vitro protein-synthesizing system programmed with the viral message MS2-RNA. However, now, competition experiments between pairs of isoaacpeting valine tRNAs with different anticodons were performed to determine their relative efficiency in reading the valine codons of the MS2 coat protein cistron. The valine codons were selected for examination because they are all present in the coat protein cistron and several valine tRNAs were accessible. Moreover, the system largely synthesizes the coat protein, whose amino acid sequence is known (4). In this cistron, the valine codons are located favorably so that in the cryptic digest of the coat protein it is possible to readily determine the reading of the individual codons by the competing anticodons. Finally, the system is strictly dependent on added valyl-tRNA and is free of any valine tRNA ligase activity, which could lead to erroneous data by catalyzing transaminoacylations or transesterifications (2).

The results were as predicted by the wobble hypothesis, with two seeming exceptions: U*AC reads the codon GUG quite efficiently and IAC is as effective as U*AC in reading naphthalene-1-sulfonyle-3C, 2-thiocytidine monophosphate; U*, 5-oxyacetic acid uridine monophosphate; sU, 2-thiouridine monophosphate and derivatives thereof. A nucleotide triplet in parenthesis after a tRNA denotes the anticodon; tRNA\(^{\text{Val}}\)(IAC) is valine tRNA, with the anticodon IAC.

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1The abbreviations used are: tRNA\(^{\text{Val}}\), arginine tRNA; tRNA\(^{IAC}\), isoleucine tRNA; tRNA\(^{U*AC}\), valine tRNA; dansyl, 5-dimethylaminonaphthalene-1-sulfonyle-3C, 2-thiocytidine monophosphate; U*, 5-oxyacetic acid uridine monophosphate; sU, 2-thiouridine monophosphate and derivatives thereof. A nucleotide triplet in parenthesis after a tRNA denotes the anticodon; tRNA\(^{\text{Val}}\)(IAC) is valine tRNA, with the anticodon IAC.
the codon GUG. The U*U and I-G pairings at the wobble position which these interactions imply, may well represent legitimate translational interactions rather than misreadings. However, at least in the case of I seemingly reading G, certain restrictions must apply which are discussed below.

**EXPERIMENTAL PROCEDURES**

**Microorganisms and Preparation of S30 Extract**—The growth of the ts-mutant Escherichia coli NF-29 and the preparation of the S30 extract from it has been described previously (2). Phage MS2 was grown with *E. coli* CR 63 u" as host and the MS2-RNA was prepared by phenol extraction (2, 5).

tRNA\textsuperscript{val} and tRNA\textsubscript{IAC} from *E. coli* and Yeast—Information about the preparation of these compounds and sources of other chemicals is given in the Ref. 2. The tRNA\textsuperscript{val} isoeospecies were esterified with L-[14C]valine (265 or 280 Ci/mol) or L-[3H]valine (15 or 30 Ci/mm) as described by Mitra and Mehler (6). The acceptor activity of the tRNA\textsuperscript{val} preparations was approximately 1 nmol of valine/\(A_{260}\) unit.

**Conditions for in Vitro Protein Synthesis and Analysis of Valine-containing Coat Protein Peptides**—Conditions for in vitro protein synthesis were as described by Nirenberg (7) with the following modifications. Magnesium acetate was not present in Mix I and was instead added to Mix II to give a final concentration of 11 mM in the incubation mixture. A mixture of 19 L-amino acids (without valine) was added to the reaction mixture to give a concentration of 0.08 mM for each amino acid. The S30 extract (30 to 70 mg of protein) was preincubated at 37°C for 5 to 10 min with Mix II, unlabeled amino acids, and water to a volume of 5 to 12 ml (after the final additions).

The preincubation reduced the valine tRNA ligase activity in the extract to a level where it could not be detected in the presence of valinyl-AMP. After the preincubation 80 to 200 \(A_{260}\) units of MS2-RNA, 100 to 200 \(A_{260}\) units of crude tRNA from *E. coli*, and valinyl-AMP to a final concentration of 20 \(\mu\)M were added to the incubation mixture which contained the competing valyl-tRNAs\textsuperscript{val} in the concentration of 6.4 \(\mu\)M each. One of them was esterified with \[^{14}C\]valine and the other with \[^{3}H\]valine. The incubation was at 37°C for 5 min and was terminated by precipitation with an equal volume of 1 M perchloric acid and the sample was heated for 20 min at 95°C in order to solubilize any aminocyl-tRNA. The insoluble protein was centrifuged out and washed several times as described by Nathans (8). The labeled coat protein was purified by chromatography on Sephadex G-75, digested with trypsin after addition of carrier coat protein, and the tryptic digest was fractionated by ascending paper chromatography and high voltage electrophoresis. The labeled peptides were visualized by autoradiography and the valine-containing peptides 3 and 4 were dapsylated and separated from undapsylated material by paper chromatography. The dapsylated peptides were hydrolyzed to produce dapsyl valine and free valine, which were separated by paper chromatography. The spots were cut out and eluted with 0.5 M HCl into the counting vials and the \(^{3}H/^{14}C\) ratio was determined in a Packard Tri-Carb liquid scintillation spectrometer, model 2425, after addition of 10 ml of Instagel. Experimental details of the procedures are given in Ref. 2.

**RESULTS**

The valine tRNAs used in the competition experiments were tRNA\textsuperscript{val} \((E. coli)\) with anticodon U*AC, tRNA\textsubscript{GAC} \((E. coli)\) with anticodon GAC and tRNA\textsubscript{IAC} \((yeast)\) with anticodon IAC. The general strategy was to esterify one tRNA in a competing pair with \[^{3}H\]valine and the other with \[^{14}C\]valine, and use sufficiently high concentrations of these competing tRNAs to ensure that 60 to 70% of the tRNA incubated was still in the aminocylated form at the end of the experiment. The two tRNAs, for instance \[^{3}H\]valyl-tRNA\textsuperscript{val} \((U*AC)\) and \[^{14}C\]valyl-tRNA\textsubscript{IAC} \((GAC)\), were then incubated together in the *in vitro* system in equimolar concentrations. As a further check, in an otherwise identical experiment the labeling was switched so that, in the example cited above, the \[^{3}H\]valine was now on tRNA\textsubscript{IAC} and the \[^{14}C\]valine on tRNA\textsubscript{val}.

Peptide 3 in the tryptic digest has an NH\(_2\)-terminal valine coded for by GUU and an internal valine coded for by GUC and the internal valine by GUG (the complete sequences of these peptides and the corresponding cistron sequences are given in Table IV in Ref. 2). By comparing the ratio of \(^{3}H\) to \(^{14}C\) in the incubation mixture to that in the peptide positions corresponding to the valine codons, it is possible to determine the relative efficiency of the competing anticodons in the reading of the different codons. The results are given in Tables I–IV and in the accompanying histograms. The \(^{3}H/^{14}C\) ratio in the incubation mixture was measured under exactly the same conditions as used for the determination of the same ratio in peptide valines. To facilitate the retrieval of information from the tables, the accompanying histograms present relative figures of merit for the competing anticodons in terms of their ability to read the valine codons. The merit figures were calculated as mean values from the relative efficiencies given in the table, using for each codon the lowest value as an arbitrary unit. Open columns denote codon-anticodon interactions involving Watson-Crick base pairs between the third codon position and the wobble nucleotide; hatched columns represent wobble interactions permitted by the wobble rules and closed columns interactions which are not allowed by the wobble rules. Comparisons are only meaningful between columns in the same panel.

The outcome of the competition between tRNAs\textsuperscript{val} with the anticodons U*AC, GAC, and IAC for the codons GUC and GUA (Tables II and III) is essentially as predicted by the wobble hypothesis. The anticodons GAC and IAC are an order of magnitude more efficient than U*AC in reading the codon GUC. In the same way, IAC and U*AC are more than ten times as effective as GAC in reading the codon GUU. On the other hand, the anticodon U*AC reads GUU, in violation of the wobble rules, with about half the efficiency of the anticodon GAC, which makes an interaction according to the wobble rules (Table I). Another violation of the wobble restrictions is shown in Table IV where IAC is as effective as GAC and the internal valine by GUG (the complete sequences of these peptides and the corresponding cistron sequences are given in Table IV in Ref. 2). By comparing the ratio of \(^{3}H\) to \(^{14}C\) in the incubation mixture to that in the peptide positions corresponding to the valine codons, it is possible to determine the relative efficiency of the competing anticodons in the reading of the different codons. The results are given in Tables I–IV and in the accompanying histograms. The \(^{3}H/^{14}C\) ratio in the incubation mixture was measured under exactly the same conditions as used for the determination of the same ratio in peptide valines. To facilitate the retrieval of information from the tables, the accompanying histograms present relative figures of merit for the competing anticodons in terms of their ability to read the valine codons. The merit figures were calculated as mean values from the relative efficiencies given in the table, using for each codon the lowest value as an arbitrary unit. Open columns denote codon-anticodon interactions involving Watson-Crick base pairs between the third codon position and the wobble nucleotide; hatched columns represent wobble interactions permitted by the wobble rules and closed columns interactions which are not allowed by the wobble rules. Comparisons are only meaningful between columns in the same panel.

### Table I

| Competition of anticodons with their codons | Codon GUU | \(^{3}H/^{14}C\) ratio | Relative reading efficiency of anticodons |
|------------------------------------------|----------|---------------------|---------------------------------------|
| [^{3}H]Valyl-(U*AC)                     | [^{14}C]Valyl-(GAC) | 7.3                  | GAC = 1.6                             |
| [^{3}H]Valyl-(GAC)                       | [^{14}C]Valyl-(U*AC) | 6.9                  | GAC = 2.0                             |
| [^{3}H]Valyl-(IAC)                      | [^{14}C]Valyl-(GAC) | 6.9                  | IAC = 2.5                             |
| [^{3}H]Valyl-(GAC)                       | [^{14}C]Valyl-(IAC) | 6.8                  | IAC = 2.5                             |
| [^{3}H]Valyl-(IAC)                      | [^{14}C]Valyl-(U*AC) | 7.0                  | IAC = 2.5                             |

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Reading of Valine Codons

**TABLE II**

| Codon GUC | ¹H/¹⁴C ratio | Peptide position corresponding to codon | Relative reading efficiency of anticodons |
|-----------|-------------|--------------------------------------|----------------------------------------|
| [³H]Valyl-(U*AC) | 7.3 | 9 | GAC |
| [³H]Valyl-(GAC) | 6.9 | 60.4 | U*AC |
| [³H]Valyl-(U*AC) | 7.7 | 0.5 | IAC |
| [³H]Valyl-(IAC) | 6.0 | 104.8 | U*AC |
| [³H]Valyl-(GAC) | 7.0 | 4.1 | IAC |

**TABLE III**

| Codon GUA | ¹H/¹⁴C ratio | Peptide position corresponding to codon | Relative reading efficiency of anticodons |
|-----------|-------------|--------------------------------------|----------------------------------------|
| [³H]Valyl-(U*AC) | 7.3 | 78.4 | U*AC |
| [³H]Valyl-(GAC) | 6.9 | 0.6 | GAC |
| [³H]Valyl-(U*AC) | 7.7 | 6.7 | IAC |
| [³H]Valyl-(IAC) | 6.9 | 7.8 | U*AC |
| [³H]Valyl-(IAC) | 6.8 | 106.8 | IAC |
| [³H]Valyl-(GAC) | 7.0 | 4.5 | IAC |

U*AC in reading the codon GUG in spite of the fact that the I-G interaction is strictly forbidden by the wobble rules. Finally, in the competition for the codons GUU and GUC (Tables I and II), the anticodon IAC is more effective than the anticodon GAC both in wobble interactions and in interactions according to the base-pairing rules of Watson and Crick. The possible significance of this finding will be discussed below.

To summarize the results of these experiments, a tRNA with an anticodon, which reads the codon according to the rules of the classic translation scheme is an order of magnitude more efficient than a tRNA which can only read the first two positions of the codon. However, there are two conspicuous exceptions to this generalization. The anticodon IAC seems to read the codon GUG as efficiently as the anticodon U*AC does. Furthermore, when the anticodons U*AC and GAC compete for the codon GUU, the difference in reading efficiency is only a factor of 2 in favor of GAC. These results are clearly not as predicted by the wobble hypothesis. On the other hand, they are consistent with reports that the anticodon U*AC recognizes the triplet GUU in ribosomal binding experiments (9), IAC recognizes GUG (10), and the anticodon ICG binds to the triplet CGG (11).

**DISCUSSION**

The possibility of misreading a three letter codon by reading only the first two nucleotides and disregarding the third position of the codon may be considered an intrinsic property of the present genetic code. At the same time, it is almost a forgone conclusion that an anticodon, which misreads a codon by "two out of three," should be less effective in protein synthesis than an anticodon able to read all three positions of the codon. In other words, the misreading anticodon should not be used with the same preference as a competing antico-
don able to read all three codon positions. Any aberration of the classic codon reading scheme, which gives a codon reading efficiency of the same order of magnitude as codon readings within the framework of the scheme, should probably not be considered a misreading. One could rather think of such aberrations as translationally meaningful, legitimate interactions. Hence, the finding of such behavior may warrant modification of the classic codon reading scheme so as to include some interactions not originally provided for in the wobble hypothesis. Viewed in this context, our present findings can be explained by two simultaneously operating mechanisms. "Two out of three" misreading would account for the anticodon U*AC reading the codon GUC, as well as the anticodon GAC reading both GUA and GUG. In all these cases, the misreading anticodon is an order of magnitude less efficient than the anticodon, which reads the codons according to the classic codon reading scheme. This is, nevertheless, a very high misreading frequency compared to the actual translational error frequency in the in vitro system, which must be orders of magnitude lower, judged by the ability of the system to produce most of its protein as what appears to be authentic coat protein. Superimposed on the effects of "two out of three" misreading are the results of interactions between the wobble nucleotide and the third codon position, which are outside the framework of the classic view of codon reading. Such interactions may, nevertheless, represent a legitimate and translationally effective reading. This is exemplified in the present work by the anticodon U*AC reading the codon GUC, and the anticodon IAC reading GUG with approximately the same efficiency as an anticodon which reads according to the classic rules.

A theory, which predicts the probability of "two out of three" misreading of different codons based on simple assumptions about the relative strength of the base pairs in codon-anticodon interactions involving the first two codon nucleotides, has already been presented (3). We must now consider possible explanations for U* in the wobble position reading U in the third codon position and for I seemingly reading G. In the wobble hypothesis, Crick excludes the U-U pair because the glycosyl bond separation distance is too short. Topal and Fresco (12) have come to a similar conclusion in their recent reinvestigation of the wobble interactions based on structural considerations and model building. They find that a large backbone distortion is required in order to establish a U-U pair, which would bring the phosphates closer together and give an energetically unfavorable base pair. These objections are undoubtedly well founded but it would seem that the structural disadvantages of this base pair can be compensated for by the introduction of an oxacyclic acid group in position 5 of the uracil ring. Given the assumption that U* is an extensive wobbler, i.e. can make more than one wobble interaction, one would predict that it should only be found in anticodons which read families of codons since it would otherwise represent a threat to translational fidelity. On examining the tRNA sequences available today, we find that this is indeed the case; U* is only used in the decoding of codon families (Fig. 1).

A wobble interaction between I and G is difficult to rationalize on the basis of our present understanding of the structural elements involved in codon-anticodon recognition. It is forbidden by the wobble rules, and Topal and Fresco (12) have found that it is impossible to form more than one hydrogen bond between I and G unless one resorts to the use of minor tautomers which would make the pair energetically unfavorable. Perhaps, in such instances where this pair occurs, the structural context of the anticodon is unusually favorable for stacking which could stabilize the codon-anticodon interaction. This is possibly so in the case of E. coli tRNAArg (ICG) recognizing the arginine codon CGG in ribosomal binding experiments (11). In tRNAArg modification of a sC at the 5'-end of the anticodon loop has been found to make this recognition impossible, presumably because of loss of stacking interactions. Rabbit liver tRNAVal (IAC), which recognizes the codon GUG in binding assays (10) has 2'-O-methylcytidine at position 5, which suggests that 2'-O-methylcytidine might give better stacking interactions (13). It is difficult to see why 4 should have the same effect.

Operationally, we cannot distinguish between the mechanism discussed above and a "two out of three" reading stabilized by certain features of the tRNA structure. There is also the additional problem that, while I can be permitted to read G in the third position of any codon belonging to a codon family, in at least one anticodon where I has actually been found, it cannot read G; I in the wobble position of tRNAArg from Torulopsis utilis cannot be allowed to read G in the codon AUG (Fig. 1). Clearly, much more experimental data will be needed before we can make a decision on the structural requirement for the apparent I-G reading.

Finally, it is important to bear in mind that the results which we have discussed were obtained exclusively from experiments in vitro and, in the case of the seeming I-G interaction, with a yeast tRNAVal in a protein synthesizing system from E. coli. One must consider the possibility that the heterologous tRNAVal, with anticodon IAC, could possess structural features outside the anticodon which would make this unusual interaction possible. It is also conceivable that the greater efficiency of the anticodon IAC, compared to GAC, in the reading of the codons GUA (wobbling) and GUG (Watson-Crick base-pairing) could have a similar explanation. On the other hand, as we have already pointed out, tRNAArg with anticodon ICG, recognizes the arginine codon CGG in a homologous ribosomal binding system from E. coli.

In any case, it is an open question as to what extent conclusions derived from our results would apply in vivo. It may, nevertheless, be appropriate to consider what biological advantages could accrue from the exceptions to the classic codon reading scheme which we have observed. "Two out of

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**Fig. 1.** The genetic code. Codon families are boxed in by heavy lines. The nucleotides U, U*, sU, and I are shown together with their corresponding codons to indicate the anticodons where they have been identified in the wobble position. The data have been compiled from wild type E. coli, and yeast as well as mammals. Consequently, the fact that in this figure U* and I appear together in several codon families, as in the valine codon family, does not mean that the same organism has valine anticodons with both U* and I in their wobble positions.
three" reading should probably be considered mainly as a misreading. It may, nevertheless, have been of considerable importance as a restriction operative in the evolutionary selection of the present organization of the code (3).

In the case of U* reading U it could be argued that U* and G, the combination of wobble nucleotides found in, for instance, E. coli valine tRNAs, would be more effective in codon reading than the combination U and G. The reason would be that U* can wobble with U in the third position so that this codon nucleotide is now read by each of the wobble nucleotides U* and G, which might give a more efficient reading.

An extension of the wobble rules to permit reading of codons ending in G by anticodons having I in the wobble position would mean that a single tRNA could read all codons in a codon family. Conceivably, this might represent a selective advantage in some instances. In such cases, the tRNAs would have a broader reading spectrum than envisaged in the classic codon reading scheme.

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