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Ribosomal Decoding Processes at Codons in the A or P Sites Depend Differently on 2′-OH Groups*

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The importance of 2′-OH groups of codons for binding of cognate tRNAs to ribosomal P and A sites was analyzed applying the following strategy. An mRNA of 41 nucleotides was synthesized with the structure C_{16}-GAA-UUC-GUC-C_{16} coding for glutamic acid (E), phenylalanine (F) and valine (V), respectively, in the middle (EFV-mRNA). A second template, the E(dF)V-mRNA, was identical except that it carried a deoxyribo-codon -dUdUdC- for phenylalanine. tRNA binding to the P site is totally insensitive to the presence or absence of the 2′-OH group of the P-site codon, and tRNA binding to the P site is also not affected if the A-site codon lacks the 2′-OH groups. However, binding is impaired if the deoxy-codon is present at the E site. In sharp contrast, the A-site binding of Ac-aminoacyl-tRNA was severely reduced in the presence of the deoxy-codon at the A site as well as at the P site. The results demonstrate that the correctness of base pairing is also "sensed" via a correct sugar structure of the codon, *e.g.* positioning of the sugar pucker (2′-OH), during the decoding process at the A site (elongation) but not during the decoding at the P site (initiation).

The transfer of the genetic message into the amino acid sequence of a distinct protein is based on the ribosomal decoding process, which occurs at the ribosomal P site during initiation and at the A site during elongation. Our knowledge concerning the mechanism of the decoding processes is sparse. Two models have been suggested. The prevailing model, which has been more or less generally accepted over the last 15 years, assumes that the discrimination energy responsible for the accuracy of the decoding process is derived from the stabilities of base pairing during codon-anticodon interaction. Studies on lifetimes of anticodon-anticodon interactions in solution revealed that the selection accuracy according to this model could hardly be better than 1:10 (one incorrect selection per 10 correct ones) and certainly not better than 1:100 (1). Since ribosomes select the amino acids with a precision around 1:1000 (2), it is clear that this experimental model does not properly reflect the ribosomal decoding process. These findings led to the suggestion that the discrimination energy inherent to base pairing between codon and anticodon has to be exploited more than once (3), and a possible mechanism for this was provided by proofreading theories (4, 5).

However, aqueous conditions may not apply for the decoding center, which may provide an environment where the energetics of the interaction are stronger and more discriminating. A detailed model was suggested about 10 years ago (6), according to which the ribosomal decoding center recognizes the codon-anticodon duplex in the same way as does the active center of an enzyme, *i.e.* it recognizes the correctness of the stereochemistry of the partial Watson-Crick structure formed by the codon-anticodon duplex. This model implies that the sugar-phosphate backbone of the codon-anticodon is recognized and is only bound if correctly positioned, thus contributing to the discrimination energy, which could be much larger than that predicted by the previous model.

A decisive experiment to discriminate between the two models would be a comparison of ribo-codons and 2′-deoxyribo-codons during tRNA binding, since base pairing in RNA-RNA duplexes is almost identical to that in DNA-RNA hybrids, which also adopt an overall A form typical for RNA duplexes. However, the sugar puckering in the DNA strand of hybrids is quite different from that of RNA duplexes (7). The first model predicts that DNA codons would be accepted just as well as RNA codons, whereas according to the second model the ribosomal decoding center should sharply discriminate between DNA codons and RNA codons.

It has been shown that single-stranded DNA could not be translated under normal conditions (8–12). Poly(dT), in contrast to poly(U) is not accepted as mRNA in enzymatic translation systems derived from the eubacteria *Escherichia coli* (11, 12) as well as from eukaryotic wheat germ and rabbit liver; only yeast ribosomes show some spurious activity (13). In the absence of ribosomes the $tRNA^{Phe}$ association constant for the deoxyribo-triplet p-dTdTdT is not less, but even slightly higher than that for the ribo-triplet p-UUU (14). Therefore, the absence of translational activity of poly(dT) cannot be explained by the weakness of the corresponding codon-anticodon interactions *per se*. Specifically the P site was tested by Ricker and Kaji (15), who reported that deoxy triplets p-dAdUdG or dAdTdG were as efficient as ribo-AUG in directing the nonenzymatic binding of fMet-tRNA to the P site.

However, experiments with homopolymeric mRNA such as poly(dT) could not distinguish between effects of ribosome-mRNA interactions inside and outside of the decoding region. Precise estimation of codon effects at A, P, and E sites in the presence of homopolymeric mRNA is problematic as well. Here we show with a heteropolymeric mRNA containing one deoxyribo-codon that the tRNA binding to the A site depends on the presence of the 2′-OH groups of the corresponding codon, in contrast to tRNA binding to the P site. Furthermore, the A-site binding is impaired when the upstream P site displays a deoxyribo-codon. Likewise, the P-site binding is reduced when the upstream E site contains a deoxyribo-codon. The results argue

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TABLE I
Heteropolymeric templates and their deoxyribo-variants used in this study

	E F	V		
(5′)	C_{16}-GAA-**UUC**	-GUC	-C_{16} (3′)	**EFV-mRNA**
	Glu Phe	*Val*		
(5′)	C_{16}-GAA-**dUdUdC**	-GUC	-C_{16} (3′)	**E(dF)V-mRNA**
	M F	V		
(5′)	C_{16}-AUG-GUC	**-UUC**	-C_{16} (3′)	**MVF-mRNA**
	Met Val	*Phe*		
(5′)	C_{16}-AUG-GUC	**-dUdUdC**	-C16 (3′)	**MV(dF)-mRNA**

strongly in favor for the second model, which suggests that the decoding center at the A site recognizes the partial Watson-Crick structure formed by codon-anticodon interaction.

EXPERIMENTAL PROCEDURES

$tRNA_2^{Glu}$, $tRNA^{Phe}$, and $tRNA_2^{Val}$ (*E. coli*) were purchased from Subriden RNA, Rollingbay, WA. $tRNA^{Phe}$ (*E. coli*) was from Boehringer Mannheim. All radioactive amino acids and [γ-^{32}P]ATP were from Amersham Corp., HEPES was from Calbiochem, and polyamines were from Fluka. Reversed phase columns Nucleosil 300–5 C_4 and Nucleosil 300–7 C_8 were from Macherey-Nagel, Düren, FRG. All other chemicals were from Merck, Darmstadt, and Boehringer Mannheim, FRG.

Tightly coupled 70 S ribosomes were isolated from midlog-phase cells of *E. coli*, strain D10 (RNase 1$^-$, Met$^-$), as described elsewhere (16). 1 A_{260} unit of 70 S ribosomes was taken to be equivalent to 24 pmol, 1 A_{260} unit of tRNA to 1500 pmol.

Synthesis and Purification of mRNAs—Ribo-templates (C_{16}GAAUUCGUCC$_{16}$ and C_{16}AUGGUCUUCC$_{16}$) and ribo/deoxyribo-templates (C_{16}GAAdUdUdCGUCC$_{16}$ and C_{16}AUGGUC-dUdUdCC$_{16}$) were synthesized with dimethoxytrityl-cyanoethyl RNA/DNA phosphoramidites on an Applied Biosystems 392 DNA-RNA synthesizer. Deprotected mRNAs were purified by preparative electrophoresis in 17.5 or 20% PAAG with 7.5 M urea. Ribo- and ribo/deoxyribo-variants of the templates were prepared and analyzed in parallel. 1 A_{260} unit of the templates was taken to be equivalent to 3000 pmol.

Aminoacylation and Purification of tRNA—$tRNA^{Phe}$ and $tRNA_2^{Val}$ were aminoacylated, and Phe-tRNA and Val-tRNA were acetylated as previously described (16). Phe-tRNA, Val-tRNA, AcPhe-tRNA, and AcVal-tRNA were purified by reversed phase high performance liquid chromatography on Nucleosil 300–5 C_4 and Nucleosil 300–7 C_8 columns using a linear (0–30%) or step (60%) gradient of methanol in 400 mM NaCl, 10 mM Mg(acetate)$_2$, and 20 mM NH_4(acetate), pH 5.0. After the purification, charging levels of 1600–1700 pmol/A_{260} were obtained. AcPhe-tRNA and AcVal-tRNA were made free from the corresponding aa[1]-tRNAs by preparative deacylation of aa-tRNAs remaining in the Ac-aa-tRNA fractions. For this purpose, Ac-aa-tRNA fractions were treated with tRNA-free S-100 fraction used for aminoacylation. The treatment was done in the absence of ATP and the cognate amino acid but in the presence of AMP and pyrophosphate, both at a final concentration of 6 mM.

Binding of tRNAs to Ribosomes—Binding of tRNAs and Ac-aa-tRNAs to ribosomes was performed as described elsewhere (16). Analysis was done in a buffer system containing 20 mM HEPES-KOH (pH 7.5), 6 mM $MgCl_2$, 150 mM NH_4Cl, 4 mM 2-mercaptoethanol, 0.05 mM spermine, and 2 mM spermidine. The standard assay (final volume of 25 μl) contained about 10 pmol of 70 S ribosomes, not less than 10-fold molar excess of a corresponding template, and different combinations of tRNAs. Binding of tRNAs to ribosomes was performed in two steps. During the first step, ribosomes and templates were incubated at 37 °C in the absence or presence of deacylated tRNA to prefill the ribosomal P site. During the second step, the preformed complex was incubated at 37 °C with added Ac-aminoacyl-tRNA. The presence of Ac-aminoacyl-tRNAs at the A or P sites was tested with the puromycin reaction, which was carried out at 0 °C for 15–16 h as described elsewhere (16). Before adding, the pH of the freshly prepared puromycin solution was adjusted to 7.5.

RESULTS

Table I presents the mRNAs used in this study. They all have the general structure C_{16}-three codons-C_{16} with a total length of 41 nucleotides, which is about the length of an mRNA protected by the ribosome against RNase attack (17, 18). Each of the three codons appears only once in the mRNA sequence. One pair of mRNA molecules codes for glutamic acid (E), phenylalanine (F), and valine (V), whereby the EFV-mRNA contains the Phe codon in the usual ribo-form UUC and the E(dF)V-mRNA in the 2′-deoxy form dUdUdC. The second pair of mRNAs, the MVF-mRNA and the MV(dF)-mRNA, also carry a ribo-codon and deoxyribo-codon for Phe, respectively, but in a different context.

In the first series of experiments we determine the binding of *N*-acetyl-Phe-$tRNA^{Phe}$ (AcPhe-tRNA) to the Phe-codon at the P site or the A site (Table II). In a control experiment we assess the binding to nonprogrammed 70 S ribosomes, which occurs exclusively at the P site as indicated by the puromycin reaction (see also Ref. 19 and references therein). The binding of AcPhe-tRNA to programmed P sites is increased by about 25% compared with the nonprogrammed P site (experiment 2, compare 2.9 and 3.0 with 2.2 pmol). The interesting point is that the reactions depending on the ribo-codon or on the deoxyribo-codon of Phe are indistinguishable, *i.e.* the P site is completely insensitive to the presence or absence of 2′-OH groups in its codon.

A strikingly different picture is found when AcPhe-tRNA is bound to the A site (Table II, experiment 3). In the presence of EFV-mRNA (ribo-codon for Phe) the binding value is the same as that found for P-site binding. In contrast, when a deoxyribo-codon is displayed at the A site, the total binding is reduced to one third, half of it being present at the A site. Considering the A-site fractions found in the presence of UUC or dUdUdC (2.55 and 0.5 pmol, respectively), the lack of the 2′-OH groups in the A-site codon reduces the binding to this site by a factor of 5.

Experiment 3 shows yet another detail. The low AcPhe-tRNA binding to the A site in the presence of a deoxyribo-codon is much lower than the binding to nonprogrammed P sites (1.0 *versus* 2.2 pmol). This means that the $tRNA_2^{Glu}$ (codon GAA) can efficiently bind to the ribo-codon in the P site, thus displaying the deoxyribo-codon at the A site. A control experiment with deacylated [^{32}P]tRNA confirmed that the P site can be equally well saturated whether or not a deoxyribo-codon is at the adjacent A site (data not shown).

In the next experimental series we bind an AcVal-tRNA to P or A sites displaying a Phe codon in the ribo- or deoxyribo-form upstream at E or P sites (Table III). The binding values to nonprogrammed (experiment 1) or programmed P sites (experiment 2) are relatively low. The low binding can be explained by the observations that nonprogrammed ribosomes have a low intrinsic affinity for $tRNA^{Val}$ (20). Other factors might also contribute to the low binding, *e.g.* a higher sensitivity of some nucleotides of the Val-$tRNA^{Val}$ against the acetylation procedure of the α-amino group of valine compared with Phe-$tRNA^{Phe}$. Since qualitatively the same results were obtained with $tRNA^{Phe}$ we show here the results with $tRNA^{Val}$ in order to demonstrate that the observed effects do not depend on the tRNA species used or on the codon present but rather on the decoding process at the site under observation.

Notably, the binding to programmed P sites is reduced by more than 50% when the upstream E-site codon is in the deoxy-form, *i.e.* P-site binding to ribo-codons is impaired when the codon at the E site lacks the 2′-OH groups but not when the deoxyribo-codon is at the A site (Table II).

An unexpected result is found in experiment 3 (Table III). A 5-fold increase of binding to the A site over that of the P site was observed in the presence of the EFV-mRNA (1.75 and 0.36 pmol, respectively). A similarly strong increase was already reported earlier, when the codon was positioned in the middle

[1] The abbreviations used are: aa, aminoacyl; IF, initiation factor; EF, elongation factor.

TABLE II

The 2'-deoxy-codon at the P or the A site; binding of AcPhe-tRNA

The UUC codon is given in bold letters since this codon is present either as ribo-codon or as 2'-deoxy-codon. The site location of AcPhe-tRNA (A or P) was assessed by the puromycin reaction as described elsewhere (16, 20); the A-site value obtained in the presence of E(dF)V-mRNA (experiment 3) is given in parentheses because the total AcPhe-tRNA binding is very low. One assay (25 μl) contained 11.5 pmol of 70 S ribosomes and 13 pmol of Ac[^{3}H]Phe-tRNA. The specific activity of Ac[^{3}H]Phe-tRNA was 951 dpm/pmol, the counting efficiency was 50.3%. In experiment 3, the P sites were blocked with deacylated $tRNA_2^{Glu}$ (100 pmol/assay) before AcPhe-tRNA was added. The data from the binding assays and the puromycin reaction were highly reproducible, two single values deviated from the average value by less than ±5%. Background value (no ribosomes) of the binding assays was 0.03 pmol and was subtracted. For more details, see "Experimental Procedures."

Exp. no.	binding state	mRNA	binding of AcPhe-tRNA [pmol]	binding site according to the puromycin reaction
	P		(Ψ)	
1	Ψ	no	**2.2**	P 100%
	E P A			
2	GAAUUCGUC Ψ	EFV	**2.9**	P 100%
		E(dF)V	**3.0**	P 100%
	E P A			
3	GAAUUCGUC Ψ Ψ	EFV	**3.0**	A 85%
	pre	E(dF)V	**1.0**	(A 52%)

TABLE III

The 2'-deoxy-codon at the E or the P site; binding of AcVal-tRNA

In experiment 3, the P site was occupied with deacylated $tRNA^{Phe}$ (50 pmol) before 18 pmol of Ac[^{14}C]Val-tRNA were added. The specific activity of Ac[^{14}C]Val-tRNA was 585 dpm/pmol. The standard deviation is given only when it is larger than the normally observed, ±5%. For further details, see legend to Table II. Background value (no ribosomes) was 0.08 pmol and has been subtracted.

Exp. no.	binding state	mRNA	binding of AcVal-tRNA [pmol]	binding site according to the puromycin reaction
	P		(Ψ)	
1	Ψ	no	**0.08**	P 100%
	E P A			
2	GAAUUCGUC Ψ	EFV	**0.36**	P 100%
		E(dF)V	**0.14** ±0.04	P 100%
	E P A			
3	GAAUUCGUC Ψ Ψ	EFV	**1.75**	A 100%
		E(dF)V	**0.2** ±0.02	(A 100%)

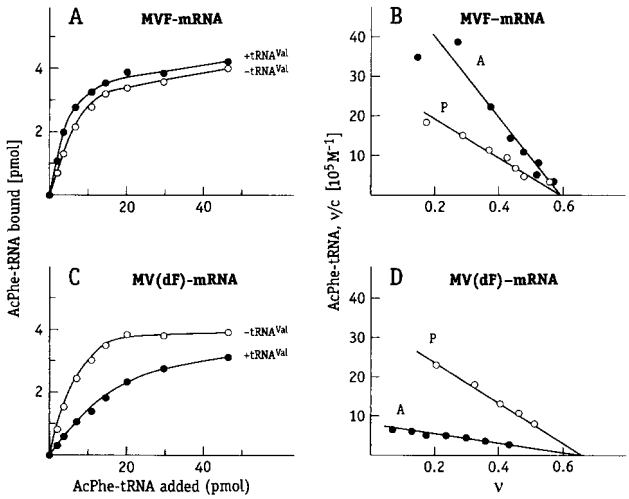

FIG. 1. **Binding of AcPhe-tRNA to the A and P sites of 70 S ribosomes programmed with either MVF or MV(dF) templates.** *A* and *C*, saturation curves; *C* and *D*, the corresponding Scatchard plots (21): ν is the number of bound AcPhe-tRNA molecules per ribosome, c is the concentration of free AcPhe-tRNA. One assay (25 μl) contained 7.4 pmol of 70 S ribosome and either 157 pmol of MVF (*A*, *B*) or 154 pmol of MV(dF) (*C*, *D*). After a preincubation for 30 min at 37 °C in the absence or the presence of 50 pmol of $tRNA_2^{Val}$ the indicated amounts of Ac[^{14}C]Phe-tRNA (1054 dpm/pmol) were added, and the systems were incubated for 60 min at 37 °C.

of a heteropolymeric mRNA (20) as is here the case. Surprisingly, if a deoxyribo-codon is located at the P site, the A-site binding directed by a ribo-codon drops by about 9-fold (1.75 and 0.2 pmol, respectively). The decoding center at the A site thus recognizes a deoxy-codon at the adjacent upstream P-site codon as does the P-site center with the upstream E-site codon, but the A site reacts in a much more sensitive manner, as indicated by the more strongly restricted binding.

The binding experiments with AcPhe-tRNA to A and P sites depending on a UUC or dUdUdC codon were repeated with the MVF- and MV(dF)-mRNA, respectively. The same results were found as those demonstrated in Table II obtained in the presence of EFV- or E(dF)V-mRNA, respectively (data not shown). It follows that the observed effects are solely due to the presence or absence of the 2'-OH groups at the corresponding codon and are not modulated by different context nucleotides. As an example conducted with the MVF/MV(dF) pair of mRNAs, saturation curves of AcPhe-tRNA are shown in Fig. 1, *A* and *C*. In the absence of deacylated $tRNA^{Val}$, AcPhe-tRNA binds to the P sites, whereas in the presence of $tRNA^{Val}$ it binds to the A sites. The binding values were processed according to Scatchard (21) (Fig. 1, *B* and *D*), which gives the corresponding association constants. These data are summarized in Table IV. The binding to the P sites occurs with the same affinity regardless of whether a ribo-codon or a deoxyribo-codon is present at the P site in agreement with the qualitative data shown in Table II, experiment 2, with the EFV/E(dF)V pair of mRNAs. The saturation curves also reveal an A-site affinity (ribo-codon), which is twice as high as the corresponding P-site affinity (11×10^6 and 5.1×10^6 M^{-1}, respectively). However, the affinity at the A site drops 10 times (from 11×10^6 to 1.1×10^6 M^{-1}) if a deoxyribo-codon is displayed at the A site, in qualitative agreement with Table II, experiment 3.

TABLE IV
Apparent association constants (K_{ass}) for AcPhe-tRNA binding to 70 S ribosomes programmed with MVF or MV(dF)

Site	mRNA	
	MVF	MV(dF)
	K_{ass} (10^6 M^{-1})	
P	5.1	5.3
A	11	1.1

DISCUSSION

A tRNA binds to the ribosomal P site totally independently of the presence of 2'-OH groups in the cognate codon at this site (Table II, experiment 2). The binding is still significant even in the absence of any codon (Table II, experiment 1) but is severely reduced in the presence of a near- or noncognate codon at the P site indicating codon-anticodon interactions at the P site (20, 22, 23). The binding to the P site is also not affected if the adjacent downstream codon at the A site is of the 2'-deoxyribo-form (Table II, experiment 3; see "Results"), but is unexpectedly impaired if the adjacent upstream codon at the E site lacks the 2'-OH groups (Table III, experiment 2). The decoding center at the P site, which is involved in the selection of the initiator tRNA, evidently does not recognize the 2'-OH groups at the P-site codon, but appears to be sensitive to the lack of these groups at the E-site codon.

However, tRNA binding to the A site requires the presence of the 2'-OH groups of the A-site codons (Table II, experiment 3), since the lack of the 2'-OH groups causes a drop in the tRNA-binding affinity by an order of magnitude (Table IV). A-site binding is also strongly reduced if a deoxyribo-codon is present at the adjacent P site (Table III, experiment 3). The decoding center at the A site, where the decoding of the genetic message during the elongation cycle takes place, is therefore recognizing 2'-OH groups of codons at both A and P sites. tRNA binding to both A and P sites is thus impaired if respective upstream codons are lacking the 2'-OH groups. This observation reveals a new kind of codon-context effect during translation and supports the idea (24) that the decoding center of the elongating ribosome interacts with the sugar-phosphate backbone of two adjacent codons either in the A and P sites (pretranslocational complex) or in the P and E sites (post-translocational complex).

Decoding at the P site (probably related to the initiation process) does not take into account the 2'-OH groups of the corresponding codon but does so at the A site. The recognition pattern at the P site therefore seems to be simpler than that at the A site. This is not unexpected, since the discrimination constraints weighed on codon-anticodon interaction at the P site are relaxed compared with those at the A site. During initiation the discrimination energy is not solely provided by codon-anticodon interaction, but the initiation factors IF-2 and IF-3 (25) contribute as well as some unique features of the initiator-tRNA such as the three G-C pairs closing the anticodon loop (26). It has indeed been shown that the codon-anticodon-dependent accuracy for Ac-aminoacyl-tRNA binding is significantly lower at the P site than at the A site (about 6-fold lower under the conditions applied) (27).

During elongation the discrimination problem is significantly more serious at the A site, since (*a*) discrimination is restricted to codon-anticodon interaction and (*b*) in *E. coli*, for example, one out of 41 different tRNAs has to be selected (here tRNAs are considered as different when they differ in their anticodon) (28). The problem is aggravated by the fact that it is not aminoacyl-tRNA molecules which are selected but rather ternary complexes, where EF-Tu accounts for 2/3 of the total mass (about 70 kDa) and increases the affinity to the A site by more than 30-fold (19). However, EF-Tu adds nothing to the discrimination energy, since it is identical in all ternary complexes.

Considering on one hand the problem of selection and, on the other hand, the accuracy of translation being usually better than 1:1000 (one wrong amino acid incorporated per 1000

amino acids), it is clear that the discrimination energy provided by the lifetimes of base pairing during codon-anticodon interaction is not sufficient. The latter allows an accuracy of only 1:10 up to 1:100 (1). As outlined in the Introduction, this dilemma can be solved in two ways. (*a*) The discrimination energy derived from the stabilities of base pairing has to be used repeatedly, an idea which has provoked the proofreading concepts for translation (4, 5, 29, 30). (*b*) The discrimination energy is larger than previously assumed, since the nonaqueous conditions possibly established by the decoding center may strengthen the energetics of interactions, and other parameters than the lifetimes of codon-anticodon interactions are exploited for the recognition process. One possible concept has been proposed by Potapov (6), *i.e.* the stereochemistry of a correct partial structure of a Watson-Crick duplex formed by codon-anticodon interactions is recognized by the decoding center at the A sites. Correctly positioned sugars and phosphate groups are contact sites of the decoding center and thus contribute to the discrimination energy.

A comparison of the effects of ribo-codons *versus* 2′-deoxyribo-codons represents a decisive experiment, since an RNA-DNA hybrid forms an overall A-form structure as does an RNA-RNA duplex, but the sugar pucker of the DNA strand is significantly distorted in the hybrid (7). Concept (*a*) should be insensitive to the lack of 2′-OH groups in the A-site codons, whereas concept (*b*) should be sensitive. Our results give a clear-cut answer. The sugar structure is recognized at the A-site decoding center during tRNA binding. This observation provides strong evidence that the partial Watson-Crick structure is bound and recognized, whereas incorrect duplex structures are not stably bound. It readily explains why the accuracy is almost independent of the base pair character, *i.e.* whether or not a codon is rich in A/Us or G/Cs. It is clear that the lifetime concept is not an appropriate model for decoding. Since the recognition of Watson-Crick structures allows many ribosome-tRNA contacts and thus large discrimination energies, one has to envisage the possibility that the accuracy of translation is achieved with no need of proofreading mechanisms. The well documented fact that misincorporation of an amino acid is accompanied by an increased GTPase turnover has been taken as the crucial argument in favor of the existence of proofreading mechanisms in tRNA selection by ribosomes. However, alternative scenarios have been described which explain the excess of GTP cleavages during the selection of near-cognate aminoacyl-tRNA without assuming proofreading mechanisms (31); furthermore, the excess of GTP cleavages is much lower than previously assumed (32, 33). Transcriptases probably also recognize Watson-Crick structures during RNA synthesis (34), and they provide an example where high accuracy is achieved (better than 1:50,000) (35) without proofreading.

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