THE CHEMICAL BASIS FOR THE ORIGIN OF THE GENETIC
CODE AND THE PROCESS OF PROTEIN SYNTHESIS

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INTRODUCTION

It is perhaps the defining characteristic of life that living systems respond to changes (stimuli) in the environment, not with direct, simple and predictable chemical reactions, but via indirect and complex nucleic acid-mediated responses requiring the synthesis first of both mRNA and protein. What had been required to reach the living state was that a catalog of previous experiences be made available (as nucleotide sequences) and a means for converting this experiential record into active principles, proteins. How did this genetic information arise and how did the process arise to convert the nucleic acid form into protein? Certainly, the secret as to how these matters came to be is the major unsolved mystery concerning the origin of life.

We are presently beginning our tenth year on a project, the major thrust of which is to understand just how the process of protein synthesis, including that very important aspect, genetic coding, came to be. Our work has, for many years, been concentrated on two aspects of the problem: 1) the chemistry of active aminoacyl species; and 2) affinities between amino acids and nucleotides, and specifically, how these affinities might affect the chemistry between the two.

The work prior to the present reporting period has successfully shown that essentially all of the component aminoacyl transfer reactions in protein synthesis are spontaneous and, under proper conditions, can occur in high yield. Active aminoacyl moieties (either N-blocked or free) can be transferred from the AMP anhydride to the aminoacyl imidazolide to the AMP ester (1) or to the 2'-OH groups of polynucleotides (2). These aminoacylated polynucleotides can yield peptides when incubated (3). However, no specificity between amino acid and nucleotides was found either in these transfer reactions or in the peptide formation. We did, however, find earlier indications of selective affinity between some amino acids and their anticodonic nucleotides by studying binding of thermal proteinoids
to homopolynucleotides (4). Based on these early studies, we searched the literature for further evidence supporting amino acid-anticodon relationships as the basis for the code origin. We found that the data of Garel et al. (5) showed a direct correlation between the hydrophobicities of amino acids and their anticodonic nucleotides (6). We extended these correlations to include the hydrophilicities of amino acids and their anticodonic dinucleoside monophosphates, and Jungck (7) found that the properties of bulkiness and polarity also correlated anticodonically.

We had, by this time, come to believe that relationships, and probably selective affinities, between amino acids and their anticodonic nucleotides formed the basis for the code origin. Table I is the genetic anticode arranged in a manner to give decreasing hydrophobicity from top to bottom and left to right. There is some order to the code. For example, the hydrophobic amino acids phe, leu, ile, val and met in the first vertical column all have the most hydrophobic nucleotide "A" as their central and most important letter. Conversely, the most hydrophilic nucleotide "U" serves as the central letter for the collection of hydrophilic amino acids in the far right column, with the most hydrophilic amino acid, lys, having the most hydrophilic trinucleotide UUU as one of its anticodons. However, belief in an anticodonic basis for the code origin based upon such circumstantial evidence as this is not sufficient. What seemed to be required, as Crick (8) suggested many years ago, was that we accumulate data on selective affinities between amino acids and on selective reactions.

We have, in fact, accumulated a number of indicators of selective affinities of the first column hydrophobic amino acids for adenine nucleotides, showing invariably that phe, the only one with AAA as an anticodon, has the greatest affinity for adenine derivatives (9). Furthermore, in studying the non-enzymatic activation reaction using ATP, Mg^{++} and the hydrophobic amino acids, phe was found
to react the most rapidly of any (10). We were then in a position to propose that affinity enhances chemistry. While this is certainly not a novel concept, it is new with respect to ideas about the origin of the genetic code. The principle, in fact, relates to the activation energy, but more particularly, to the activation entropy for reactions. For example, when two molecules collide, whether or not they react with one another is first a function of the lifetime of the complex between the two, and second, a function of the orientation of the two molecules in the complex. Optimal positioning results in a decrease in activation entropy. In the equation $\Delta G^* = \Delta E^* - T\Delta S^*$, which relates the free energy, total energy and the entropy of activation, one can see (after rearranging to the form $\Delta E^* = \Delta G^* + T\Delta S^*$), that decreases in $\Delta S^*$ (i.e., increased ordering = $-\Delta S^*$) mean that the activation energy, $\Delta E^*$, is reduced. Reductions in $\Delta E^*$ lead to accelerated reactions. In fact, catalysts operate by lowering $\Delta E^*$ in just this way. We can then properly view reactions involving certain combinations of amino acids and nucleotides as autocatalytic, the catalysts being based on affinity.

In the new work reported later, we see another example of this principle.

Further study (11) of the activation reaction ($\text{ATP} + \text{aa} \rightarrow \text{aa-ADP} + \text{P}_i$) has shown that the rate of activation with a given nucleotide is a direct function of the $pK_a$ of the carboxyl group being activated. Since the carboxyl group of a peptide has a higher $pK_a$ than a free amino acid, the formation of the first peptides would appear to be autocatalytic with respect to further activation (of peptide) and formation of additional peptide bonds.

Still other studies (12) have shown that the N-blocked aminoacyl AMP anhydride (e.g. N-Acgly-AMP anhydride), while quite stable to hydrolysis, reacts very efficiently (i.e. essentially 100%) with nucleophilic agents, such as the amino group of amino acids.

The most pleasant and rewarding outcome of scientific research is that after sufficient data have been accumulated about a particular subject, one
reaches a point where general principles begin to emerge. The general principles that have emerged with respect to the present subject include:

1. the genetic code has an orderliness about it, and is not a random catalog;
2. the order seems to be reflected in correlations of properties between amino acids and their anticodonic nucleotides;
3. more importantly, this orderliness and these correlations are more fundamentally a function of selective affinities between amino acids and their anticodonic nucleotides; and
4. selective affinities between amino acids and nucleotides seem to result in selective reaction chemistry (i.e. affinity affects chemistry).

We believe it is the last two principles which are fundamental to the origin of the genetic code and the process of protein synthesis. The data presented in this report continue to support this idea.

EXPERIMENTAL

Our two major areas of investigation, affinity and chemical reactions, will be discussed separately.

Affinity Studies.

The earlier studies using thermal proteinoids had shown that such preparations which were rich in one of the homocodonic amino acids (phe, pro, gly and lys each has one homogenous codon) preferentially interact with their anticodonic homopolynucleotides. Referring to the genetic anticode in Table I, pro-, gly- and lys-rich proteinoids were shown to prefer to interact with their anticodonic nucleotides, poly G, poly C and poly U, respectively (4). Results with phe were ambiguous. These amino acids fall on a diagonal across the anticode. Although these results are far from trivial, they were obtained with thermal proteinoids, about which the precise nature is not clear. Regardless, they are polymers containing amino acid side chains, and these results show that if one changes the
composition of these side chains, the preference for association with polynucleotides changes in a statistically significant, anticodonically related way. In effect, with each of these four amino acids the question has been asked, which polynucleotide is preferred. In all cases, except phe, the answer was clear, the anticodonic polynucleotide was preferred, and even with phe the somewhat ambiguous results could be rationalized. At any rate, the results are supportive of the hypothesis that selective affinities between amino acids and their anticodonic nucleotides are the basis for the origin of the code and the process of protein synthesis. Other studies on the hydrophobic amino acids (phe, leu, ile, val, met) in the "A" column showed, using a variety of techniques, that phe (the only one with a nominal AAA anticodon) invariably had the greatest affinity for adenine derivatives.

**NMR studies.** We have now confirmed the results of these studies of the "A" amino acids using NMR, and have studied the interaction of phe with A, G, C and U.

We have found that NMR can be used in a great variety of approaches to study amino acid-nucleotide interactions. For example, monoamino acids can be studied with either mono- or polynucleotides. One can hold the amino acid at low concentration and increase the nucleotide concentration, following the effects on either line position or line width of the amino acid protons (or deuterons, if deuterated amino acids are used). Conversely, the nucleotide concentration can be held low and constant and the amino acid concentration varied, following the signals of the nucleotide. The amino acid itself may be used in such studies or, if solubility is a problem, the respective methyl ester or amide can be used.

Using the methyl esters (which we synthesized) of the "A" amino acids, we obtained the data shown in Fig. 1 using poly A. From these results, we can calculate the binding constants in Table II using Scatchard analysis (i.e. plotting $\frac{\delta H_2}{[aa]}$ vs. $\delta H_2$). The slope of such lines is the binding constant in
reciprocal moles.

In still other studies using monoamino acids and mononucleotides, the results in Table II were obtained. In these studies the question has been: Taking one nucleotide, "A", which of the several amino acids having "A" as their central and most important anticodonic base, has the strongest preference for "A"? While there is some variation depending upon the method employed, within the data from any one method the answer again is clear and unambiguous. Phe, the only amino acid having AAA as a nominal anticodon, has the highest binding constant for "A". Although these binding constants are low, the differences are real, and our results are in essential agreement with those of Reuben and Polk (13) who studied the interaction of AMP with the methyl esters of amino acids.

In other studies, we have developed a method (14) based upon line width changes of deuterium signals from deuterated amino acids as the poly A or AMP concentration is increased. Fig. 2 shows the line broadening. Again, using these variations, the binding constant of phe for poly A is calculated to be $11 \text{ M}^{-1}$. This is somewhat higher than we found with the methyl ester binding to poly A in Table II.

Our next studies with NMR were designed to clarify the results with phe-rich thermal proteinoids. As pointed out earlier, those results were ambiguous — that is, they did not answer the question, "Which nucleotide does phe prefer?" We have now, by what is essentially the method of Reuben (15), used NMR with mononucleotides, equimolar Mg$^{++}$ and the methyl ester of phe (Me-O-phe) to answer this question. In these experiments, we followed the upfield movement of the nucleotide protons as the Me-O-phe concentration was increased. These upfield shifts in the nucleotide proton signals are shown in Fig. 3. Using these changes, we calculated the binding constants of phe for the four nucleotides from
the slope of the plots in Fig. 4. These are presented in Table III. Again, the results are clear and unambiguous, and show that phe does prefer its anticodon nucleotide, A. As revealed by the results reported herein, these NMR studies are obviously yielding the data necessary to elucidate the origin of the code, and need to be continued.

Our other hope was to use ultraviolet spectroscopy to determine binding constants. While a considerable amount of time has been expended on this effort using mainly methyl esters or amides of amino acids with polynucleotides, the changes in ultraviolet absorption due to the associations are so small as to be in the same range as the limit of errors in pipetting, i.e. 1%. Large numbers of experiments can iron out these differences, of course, but such a tack is somehow less than satisfying, and our need for confidence in the results is somewhat compromised.

Retardation chromatography. Earlier studies had shown that, using a 5% MgCl₂·6H₂O solution in ethanol as eluent, ATP would not move in paper chromatography. The hydrophobic amino acids, on the other hand, would move away from the origin with this solvent. If, however, we would apply these amino acids with ATP, the ATP would retard the amino acids at the origin, the amount retarded being a function of the ratio of ATP to the amino acid. We found in these studies that, of the "A" amino acids, phe was the easiest to retard with ATP. Those studies were made using radioactivity to quantitate the amount of amino acid retarded, although the procedure was complicated by the fact that ATP quenched during scintillation counting.

We have now extended these earlier studies, but using the ninhydrin assay for amino acids and simply cutting out the origin of the chromatogram and putting it directly into the assay. Using this procedure, we found that if we prepared the MgCl₂-ethanol mixture using MgCl₂·6H₂O which had been in a vacuum desiccator
for some time, phe would not move from the origin even in the absence of ATP, but if extra water was added, it would move, and at 4.5% H₂O (V/V) all the phe would leave the origin.

We then repeated the retardation experiments with the "A" amino acids with the results shown in Fig. 5. Although phe was most easily retained at the origin by ATP, val was also retained essentially to the same extent. Differential retardation is not a function of differential solubility, as we found the solubilities of these amino acids to be nearly the same, ise being the most soluble. This retardation process is obviously a complicated phenomenon and may not be continued. However, these observations suggested another idea—namely, to what extent is the interaction between two molecules influenced by the environment?

An initial look at this idea involved an investigation of the effect of various aliphatic alcohols on the ultraviolet spectrum of poly A. In such studies, an increase in the extinction coefficient can be interpreted as a destacking of the adenine residues. Methyl, ethyl, and propyl alcohols all caused an increase in the extinction coefficient (Fig. 6) as the alcohol concentration was increased, although methanol was the most efficient. In all cases, however, the extinction coefficient was observed to decline at higher alcohol concentrations, and eventually the poly A would become insoluble. These preliminary investigations will be continued, first to see if any of the amino acid-nucleotide interactions are enhanced, and second, to see if such alcoholic solutions, which are hypohydrous, would increase those rates of reaction which involve the elimination of water (e.g. activation and peptide bond formation).

Insoluble complexes. During the course of our NMR studies with poly A and the methyl esters of the amino acids, we found that if the phe concentration was carried too high, the complex became insoluble. Apparently the plus charged
amino group associates with the negatively charged backbone of the polynucleotide, and when the nucleotide charge is sufficiently masked, the complex precipitates. Moreover, we found a selectivity in this precipitation as shown in Fig. 7. With poly A the order of ease of precipitation was trp > phe > ile > leu > val > gly. Again in this sequence, we see phe displaying the greatest affinity for poly A of the "A" column amino acids (phe, leu, ile and val). One can make a rough estimate of the binding constants by assuming that at the midpoint of these curves, 50% of the poly A is complexed. When this is done, one obtains the values shown in Table II.

In summary, we may now consider all the affinity and correlation data collected thus far (i.e., including results presented in previous reports).

1. The thermal proteinoid data (4,16) showed that, of the homocodonic amino acids (phe, pro, gly, lys), pro, gly and lys preferentially precipitated with their anticodonic polynucleotides. Results with phe were ambiguous and suggested that the phe-rich proteinoid was perhaps "masking" its hydrophobic groups in much the same manner as a globular protein. Some other means of determining the phe-nucleotide preference was required. Solubility studies did show that, of the homocodonic amino acids, adenosine has the greatest affinity for phe (17).

2. NMR studies have shown that the binding constants of Me-O-phe for the nucleotides are AMP (6.6 M\(^{-1}\)) > GMP (3.5 M\(^{-1}\)) > CMP (2.1 M\(^{-1}\)) > UMP (1.1 M\(^{-1}\)). These studies would seem to clarify the ambiguities discussed in (1) above, and allow us to finalize the statement that the homocodonic amino acids preferentially interact with their anticodonic nucleotides.

3. A large body of data has been accumulated on the affinity of the "A" amino acids (phe, leu, ile, val, met) for adenine derivatives. These studies include binding constants determined by NMR (18) and UV (9), phase separation
experiments, retardation chromatography and adenosine solubility enhancement studies (9,17). Without exception, these studies have shown that phe, which has the only nominal AAA anticodon, also has the greatest affinity for adenine derivatives.

A broader summary statement about these affinity studies is that, in every case, the amino acid preferred to associate with its anticodonic nucleotide; in no case was the codonic nucleotide preferred.

In addition to these affinity studies, our own work shows direct correlations of the properties of hydrophobicity and hydrophilicity between amino acids and their anticodonic nucleotides (6), and Jungck (7) found that bulkiness and polarity were also correlated anticodonically.

All in all, these above studies are completely consistent with the hypothesis that the code origin was based on relationships and probably affinities between amino acids and their anticodonic nucleotides. While this may be true, one must then attempt to explain how the process of protein synthesis arose based on these affinities. That is the purpose of our chemistry studies.

Chemistry.

During the year, we completed a study of the stabilities of four activated forms of one amino acid, phe, in aqueous solution as a function of pH. The compounds we synthesized in our lab included N-acetylphe-AMP ester and anhydride and phe-AMP ester and anhydride. The results (Fig. 8) are plotted as the natural log of the pseudo first order rate constants. By far the most stable form is N-acetylphe-AMP ester, while the most unstable is phe-AMP anhydride. The N-blocked aminoacyl anhydride is the only form which becomes increasingly unstable at low pH as well as high. Interestingly, as the pH is increased, all forms have essentially the same reactivity, but it should be pointed out that in the case of the phe-AMP ester and phe-AMP anhydride, considerable peptide formation
occurs as higher pH's are reached. The results with those compounds then represent disappearance due to both hydrolysis and peptide bond formation. We are presently studying how much of each reaction is occurring.

Because of the instability of the phe-AMP anhydride and the relative stability of the N-acetyl compound, and especially because of the latter compound's efficiency at forming peptides, we have continued the study of peptide formation using N-acetylglu-AMP anhydride as donor and other compounds as acceptor. The high efficiency of the reaction of this compound with glu has already been mentioned. We next turned to aminoacyl AMP-esters as acceptors. These compounds are models of the peptide acceptors (aminoacyl-tRNA esters) in contemporary protein synthesis. Using glu-AMP ester and phe-AMP ester, we studied their reaction with N-acetylglu-AMP anhydride at pH 7 and 25°C. The results are shown in Fig. 9, and are compared with the earlier results using glu acceptor. We believe these results to be primarily a reflection of affinities, although other factors may be involved. For example, when the acceptor amino acid, glu, was not attached to a nucleotide, the reaction was slowest. When glu was esterified to AMP, the rate increased substantially. There are two possible reasons for this. One is that when an amino acid is esterified, its α-amino pKₐ drops considerably, making it a better nucleophile. The second is that when both donor and acceptor are attached to AMP, they then have the possibility of increased organization because of the tendency of adenine rings to stack with each other. In this case, though, the amino acid glu has very little affinity for the nucleotide, A.

Finally, if the acceptor is now phe-AMP ester, another significant increase takes place, and the reaction is essentially complete in less than a minute. We believe the principal reason for this latter increase in rate is that phe has a considerably greater affinity for A. Thus, upon collision, the phe moiety can serve to orient the donor N-Acgly-AMP and the phe-AMP ester acceptor by placing itself between
the two adenine rings. This appears to be yet another example in which affinity affects chemistry. Peptide bond formation was the fastest in the case where at least one of the amino acids bore an anticodonic relationship to the nucleotides present. This point must be explored further. For example, we need to determine the activation entropies for various combinations of amino acids and nucleotides. Does the amino acid-anticodonic nucleotide pair always react the fastest?

This particular idea holds great promise because when both the donor and acceptor are attached to nucleotides, a polynucleotide template might presumably serve as a further organizing principle, thus reducing the activation entropy even more. This is conceptually exciting because it means that the polynucleotide template is literally a catalyst for peptide bond formation. The entire picture, then, is that amino acid-nucleotide interactions are responsible for both enhanced activation of amino acids and autocatalytic peptide bond formation, and this catalysis can be further enhanced by association with a template. These studies are in progress.

An Hypothesis

The combined data above allow us to propose, with some confidence, the following hypothesis:

The origin of the genetic code and the process of protein synthesis were both based on selective interactions between amino acids and nucleotides which, in turn, resulted in selective reactions between them. This allowed selective activation of amino acids and yielded aminoacyl nucleotides which could then act as primitive tRNA's, base pairing with polynucleotides to yield organized complexes, and promoting the formation of selected peptides.
REFERENCES


Figure 1. Plot of downfield shift of H$_8$ proton signal of poly A as the function of increased concentration of amino acid methyl esters.
Fig. 2. Line broadening of deuterium signal from deuterated phenylalanine as the concentration of either 5'-AMP (left) or poly A (right) is increased. (BBRC, in press)
Fig. 3. Variation in chemical shift ($\Delta\delta$ Hz) of nucleotide proton signals as the concentration of phe-O-Me is increased. Nicolet 300 MHz spectrometer, pD 7.0, 25°C operated in fourier pulse mode.
Fig. 4. Data from Fig. 3 plotted as $\frac{\Delta \delta}{[\text{Me-O-Phe}]}$ vs. $\Delta \delta$ for the system mononucleotide plus Me-O-Phe at pH 7.0, 25°C. Slope equals the binding constant in M$^{-1}$. 
Fig. 5. Percent of amino acid retarded at the origin as a function of the molar ratio of ATP/aa in paper chromatography using 5% \( \text{MgCl}_2 \cdot 6\text{H}_2\text{O} \) in absolute ethanol plus 4.5% additional water as solvent. phe (●), val (○), ile (□), and leu (△).
Fig. 6. Variation in the $\varepsilon_{257}$, extinction coefficient of poly A with increasing concentration of methyl (○), ethyl (●), or propyl (□) alcohol.
Fig. 7. Generation of turbidity (apparent absorption at 400 nm) of $10^{-3}$ M poly A solutions in the presence of various concentrations of the methyl esters of several amino acids at pH 7.0 in 0.05 M phosphate buffer.
Fig. 8.
Plot of the natural log of the rate constants for disappearance of the active species at 25°C as a function of pH. Reaction conditions and assay procedures are described in text.
Fig. 9. Disappearance of ninhydrin color as peptide bond formation proceeds at pH 7 and 25°C using N-Acgly AMP anhydride as the donor in all cases and gly (Δ), gly-AMP ester (●) and phe-AMP-ester (○) as acceptors.
<table>
<thead>
<tr>
<th>3' end</th>
<th>A</th>
<th>G</th>
<th>C</th>
<th>U</th>
<th>5' end</th>
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<tbody>
<tr>
<td>A</td>
<td>phe</td>
<td>ser cys tyr</td>
<td></td>
<td>A</td>
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<td>phe</td>
<td>ser cys tyr</td>
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<td>ser try term</td>
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<td>thr arg lys</td>
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<td>U</td>
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The genetic anticode is presented 3' → 5' so that the anticodons can be more easily imagined as base pairs of their codon equivalents i.e., the codon-anticodon strands pair in an antiparallel fashion.

This tabulation is actually a simplification of the real anticodons which appear in the anticodonic loop of tRNAs. The difference resides principally in the nucleotide of the 5' end which is most often not the one predicted from Watson-Crick base paring with the codons, but usually a modified base, frequently inosine. However, since the first two letters in the anticode are the most important, this simplification does not alter the major thesis here.
Table II. Binding Constants of Several Amino Acids for 5'-AMP and Poly A

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Reuben and Polk&lt;sup&gt;a&lt;/sup&gt;</th>
<th>UAB&lt;sup&gt;b&lt;/sup&gt;</th>
<th>UAB&lt;sup&gt;c&lt;/sup&gt;</th>
<th>UAB&lt;sup&gt;d&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Phe</td>
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<td>6.8</td>
<td>6.9</td>
<td>5.0</td>
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<td>Leu</td>
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<td>3.4</td>
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<tr>
<td>Gly</td>
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<td>-</td>
<td>0.37</td>
<td>0.5</td>
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<sup>a</sup> Reuben and Polk (13)

<sup>b</sup> Low concentration of amino acid with increasing concentrations of 5'-AMP, as discussed in the text

<sup>c</sup> Constant poly A concentration and varying concentrations of the amino acid methyl esters

<sup>d</sup> Estimated from turbidity curves as described in the text for mixtures of poly A and the methyl esters of the amino acids
Table III. Binding Constants of Me-O-Phe for Mononucleotides

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Binding Constant, M(^{-1})</th>
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<td>5'-AMP</td>
<td>6.6</td>
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<td>3.5</td>
</tr>
<tr>
<td>5'-CMP</td>
<td>2.1</td>
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<tr>
<td>5'-UMP</td>
<td>1.1</td>
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</table>