Changing the Identity of a tRNA by Introducing a G-U Wobble Pair Near the 3′ Acceptor End

WILLIAM H. MCCLAIN AND K. FOSS

Although the genetic code for protein was established in the 1960's, the basis for amino acid identity of transfer RNA (tRNA) has remained unknown. To investigate the identity of a tRNA, the nucleotides at three computer-identified positions in $tRNA^{Phe}$ (phenylalanine tRNA) were replaced with the corresponding nucleotides from $tRNA^{Ala}$ (alanine tRNA). The identity of the resulting tRNA, when examined as an amber suppressor in *Escherichia coli*, was that of $tRNA^{Ala}$.

A MAJOR UNSOLVED QUESTION IN the molecular biology of gene expression concerns the determination of the amino acid identity of transfer RNA (tRNA) molecules. These molecules function during protein synthesis by aligning the amino acids on the codons of the messenger RNA. To accomplish this, the tRNA must have two specificities, one for amino acid identity, and the other for codon recognition. The specificity of a tRNA for its cognate amino acid is mediated by an aminoacyl-tRNA synthetase enzyme. The fidelity of aminoacylation is crucial, for if the wrong amino acid is attached to the tRNA, it may be inserted in an improper place, leading to the synthesis of a faulty protein. Little is known about the nature and location of the nucleotides in tRNA that determine its aminoacylation identity (*1*). The identity is the net result of two types of interactions, the productive interaction of the tRNA with its cognate aminoacyl-tRNA synthetase and the nonproductive interaction of the tRNA with all other aminoacyl-tRNA synthetases.

The definitive structure of the anticodon could allow it to serve as an identity site in a tRNA for its cognate aminoacyl-tRNA synthetase. Biochemical studies have demonstrated that the anticodon is critical for the accurate aminoacylation of several tRNA's, in particular $tRNA^{Met}$ (*2*) and $tRNA^{Trp}$ (*3*), and that it is important for the rate of aminoacylation of $tRNA^{Tyr}$ (*4*). However, several nonsense suppressor tRNA's with changes in their anticodons, including $tRNA^{Tyr}$ (*5*), $tRNA^{Ser}$ (*6*, *7*), $tRNA^{Leu}$, $tRNA^{Cys}$, $tRNA^{Phe}$, and $tRNA^{Ala}$ (*7–10*), have been shown to retain identity with respect to the amino acid inserted into suppressed protein. Thus, the anticodon is important for the identity of only some tRNA's.

The tRNA identity problem has been addressed in several ways. Measurements of the kinetics of aminoacylation in vitro have demonstrated that certain nucleotide substitutions can adversely affect the reaction of a tRNA with its cognate aminoacyl-tRNA synthetase (*2–4*, *11*). Structural studies on cocrystals of a tRNA and aminoacyl-tRNA synthetase have begun to identify the contact sites between these macromolecules (*12*). The nucleotides responsible for identity have been studied genetically by means of suppressor tRNA's with altered identities (*7*, *13*, *14*). The main advantage of this approach is that it reflects the net outcome of 20 different aminoacyl-tRNA synthetase enzymes competing for suppressor tRNA.

We now describe several identity determinants in $tRNA^{Ala}$ of *Escherichia coli*. Clones of synthetic amber suppressor tRNA's were used to demonstrate that $tRNA^{Ala}$ identity changed with computer-selected nucleotides as they were introduced into other tRNA's. We were influenced by two previous observations. First, a mutation converting a G-C base pair to a G-U wobble pair at position 3–70 in *E. coli* $tRNA^{Lys}$ conferred ability on the tRNA to insert alanine or glycine into suppressed protein (*15*). Second, a suppressor based on the nucleotide sequence of $tRNA^{Ala}_{1A}$ (*17*) was inactive (*9* *10*); only $tRNA^{Ala}_{1B}$ contains a G-U wobble pair at position 3–70. After initiating our studies we learned from Paul Schimmel that conversion of a G-U wobble pair to a G-C base pair at position 3–70 in another *E. coli* $tRNA^{Ala}$ inactivated its suppressor activity. This information influenced us in our future research.

A computer comparison (*18*) of the published nucleotide sequences of *E. coli* tRNA's gave 54 combinations of nucleotides at two positions that are a distinguishing characteristic of $tRNA^{Ala}$ (Fig. 1). An earlier determination of ten combinations in $tRNA^{Ala}$ (*19*) was based on a larger data set that included the $tRNA^{Ala}_{1A}$ sequence (*17*) whose suppressor is inactive (*9*, *10*). The most frequent nucleotide positions in the 54 combinations were tested for a contribution to the identity of $tRNA^{Ala}$. These included positions 20 and 60, which lie in single-stranded regions, and position 70 which is base-paired with position 3 in a helical region of the molecule (Fig. 2).

The contribution of the nucleotides at positions 20, 60, and base pair 3–70 to the identity of $tRNA^{Ala}$ was examined by transplanting these nucleotides into a suppressor of *E. coli* $tRNA^{Phe}$. The $tRNA^{Phe}$ molecule was chosen for these studies because its cloverleaf secondary structure is similar to that of $tRNA^{Ala}$, while its primary sequence at positions 20, 60, and base pair 3–70 differs. In addition, the identity of $tRNA^{Phe}$ is partially determined by position 20 (*14*).

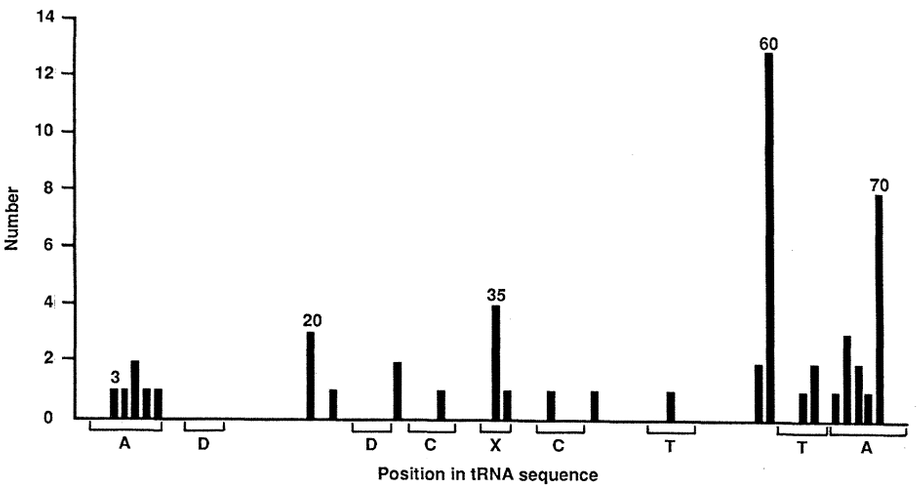

Fig. 1. Frequency of individual positions in the 54 two-position discriminators of $tRNA^{Ala}$ (*18*). Frequency on the vertical axis is a function of position in the tRNA on the horizontal axis. The prominent positions and position 3 are numbered. Cloverleaf stem segments are abbreviated as A, acceptor; D, dihydrouridine; C, anticodon (X marks the anticodon); and T, TΨ. The data set analyzed had 64 tRNA sequences from *E. coli* which consisted of 67 tRNA sequences less the sequences of $tRNA^{Ala}_{1A}$, $tRNA^{Glu}$, and $tRNA^{Arg}$ (*17*) that may have errors. The 54 two-position discriminators were 3, 70; 4, 60; 5, 20; 5, 35; 6, 60; 7, 60; 20a, 70; 20, 67; 20, 68; 26, 60; 26, 70; 30, 60; 35, 36; 35, 60; 35, 68; 40, 60; 44, 60; 51, 70; 59, 64; 59, 70; 60, 64; 60, 66; 60, 67; 60, 69; 60, 70; 63, 70; and 67, 70.

Department of Bacteriology, University of Wisconsin, Madison, WI 53706.

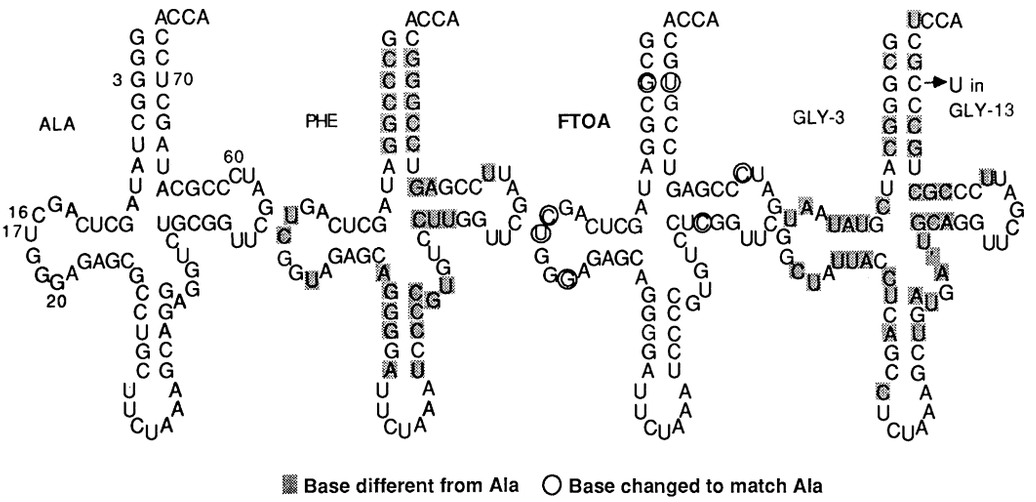

Fig. 2. Comparison between amber suppressors of *E. coli* $tRNA^{Ala}$, $tRNA^{Phe}$, the FTOA derivative of $tRNA^{Phe}$, and the GLY-3 and GLY-13 derivatives of $tRNA^{Gly}$. An arrow points to the nucleotide present in GLY-13. The shaded portions indicate the bases that are different from those in $tRNA^{Ala}$, and the circles indicate where the base was changed to match the $tRNA^{Ala}$. The tRNA's were not analyzed for nucleotide modifications present in the wild-type tRNA molecules. Genes encoding the suppressor tRNA's flanked by 5′ Eco RI and 3′ Pst I restriction enzyme sites were synthesized (*20*) at the UW Biotechnology Center, ligated into plasmid pGFIB, and cloned in *E. coli* strain XAC/A16 (*7*, *8*).

Although not predicted by the computer study, positions 16 and 17 were also changed as they combine with position 20 in determining $tRNA^{Phe}$ identity (*14*), and position 51 was changed to increase tRNA synthesis and suppression efficiency (*14*, *20*). The final nucleotide sequences of the resulting tRNA, termed FTOA, and of the two parent tRNA's are shown in Fig. 2. (F and A are the respective single-letter designations for phenylalanine and alanine.)

Genetic suppression tests suggest that the FTOA tRNA inserts some alanine into suppressed protein. Amber mutation alleles 211 and 234 in the *TrpA* gene of *E. coli* are diagnostic of the insertion of some alanine or glycine into suppressed protein. This amino acid requirement is more stringent at the 234 locus (*21*). The $tRNA^{Ala}$ suppressor relieved the auxotrophy of 211 but not of 234, the $tRNA^{Phe}$ suppressor was active for neither allele, and the FTOA suppressor relieved the auxotrophy of both 211 and 234 (Table 1). Also, the suppressors of $tRNA^{Ala}$, $tRNA^{Phe}$, and the FTOA tRNA gave positive responses with *TrpA* alleles 15 and 243, where many different amino acids are compatible with *TrpA* function, and negative responses with *TrpA* 49, where an acidic amino acid relieves auxotrophy (*21*). The higher suppression efficiency of the FTOA tRNA relative to $tRNA^{Ala}$ may explain why only it relieves the auxotrophy of *TrpA* 234. However, this suppression test does not distinguish between the insertion of alanine and glycine and, of greater importance, it cannot reveal whether alanine or glycine comprise only a minor fraction of several different amino acids inserted by a tRNA with a relaxed specificity for aminoacylation. Determination of the amino acid sequence of suppressed protein is necessary to establish the identity of the suppressor tRNA.

The amino acid present in suppressed protein was established by sequencing suppressed dihydrofolate reductase protein translated from a plasmid-encoded messenger RNA with an amber mutation at codon ten (*7*). The protein was purified by methotrexate-affinity chromatography. Protein synthesized in the presence of the FTOA tRNA contained alanine and a minor amount of lysine at position 10; phenylalanine was absent (Table 1). This establishes that the combination of nucleotide changes present in the FTOA tRNA are sufficient to confer an alanine identity on this molecule.

The G-U wobble pair was examined by itself in $tRNA^{Phe}$ (variant PHEG3-U70) to determine its contribution to alanine identity. The result shows that G-U makes an important contribution as 24% alanine and 76% phenylalanine were present in the suppressed protein (Table 1). In contrast, variants of $tRNA^{Phe}$ containing transplants of $tRNA^{Ala}$ nucleotides at positions 16, 17, and 20 (variant PHEC16U17G20) or the $tRNA^{Ala}$ nucleotide at position 60 (variant PHEC60) did not insert alanine into suppressed protein (Table 1). The PHEC-16U17G20 variant did, however, show a marked reduction in suppressor gene efficiency and a dramatic change in amino acid identity.

Variants of *E. coli* $tRNA^{Ala}$ which change position 3–70 from G-U to either A-U or G-C (variants ALAA3 and ALAC70, respectively) demonstrate the importance of the G-U wobble pair to the function of $tRNA^{Ala}$. Both of these variants showed a marked reduction in suppressor gene efficiency (Table 1), even though tRNA synthesis was essentially normal (*22*), indicating the loss of a productive interaction with the alanine aminoacyl-tRNA synthetase. In contrast, variants of $tRNA^{Ala}$ containing transplants of $tRNA^{Phe}$ nucleotides at positions 16, 17, and 20 (variant ALAU16C17U20) or the $tRNA^{Phe}$ nucleotide at position 60 (variant ALAU60) had normal suppressor gene efficiency. Both variants inserted alanine and minor amounts of lysine into suppressed protein (Table 1) indicating the retention of the productive interaction with the alanine aminoacyl-tRNA synthetase even though minor negative elements for the nonproductive interaction with the lysine aminoacyl-tRNA synthetase may have been lost.

The fact that there is less alanine in suppressed protein from the PHEG3-U70 suppressor tRNA than from the FTOA suppressor tRNA could mean that the $tRNA^{Ala}$ nucleotides at positions 16, 17, 20, and 60 in the FTOA construct contribute to $tRNA^{Ala}$ identity in one or both of two ways: by serving as positive elements in combination with G3–U70 for the productive interaction of the FTOA tRNA with the alanine aminoacyl-tRNA synthetase, or by serving as negative elements for the nonproductive interaction of the FTOA tRNA with the phenylalanine aminoacyl-tRNA synthetase. It would be instructive to have variants of $tRNA^{Phe}$ that combine G3–U70 with C16, U17, G20, or C60 individually, but this has not been achieved. Nevertheless, the results obtained indicate that the G-U wobble pair can serve as an important determinant of the alanine identity of a tRNA. In contrast, the nucleotides at positions 16, 17, and 20, and at 60 have only minor roles in determining identity in $tRNA^{Ala}$, but assume larger importance in the FTOA tRNA where they may exert a negative influence toward the $tRNA^{Phe}$ component of the identity. These experiments provide no information on the contribution to $tRNA^{Ala}$ identity for positions in $tRNA^{Ala}$ and $tRNA^{Phe}$ where the nucleotides are identical (Fig. 2).

GLY-13 is a weak amber suppressor de-

rived from *E. coli* $tRNA_2^{Gly}$ (*23*) with a U instead of a C at position 70; consequently, GLY-13, like $tRNA^{Ala}$, has a G-U wobble pair at position 3–70 (Fig. 2). This derivative was obtained as a nonprogrammed variant (*24*) during oligonucleotide synthesis and cloning of an *E. coli* $tRNA^{Gly}$ suppressor. Sequencing showed 5% glycine and 95% glutamine in the suppressed protein produced by GLY-13; alanine was not detected in the sequence, and is thus present at less than 5%. The protein sequence obtained from the parental $tRNA^{Gly}$ suppressor (GLY-3) also gave a mixture of glycine and glutamine (Table 1). The partial loss of glycine identity by the parental suppressor $tRNA^{Gly}$ was anticipated from earlier work (*9*, *10*, *23*). The important observation from this experiment is that the G-U wobble pair does not confer alanine identity on this $tRNA^{Gly}$ molecule.

Several factors may contribute to the different functions observed for the G-U wobble pair in $tRNA^{Phe}$ and $tRNA^{Gly}$. First, an inspection of the sequences of $tRNA^{Ala}$, $tRNA^{Phe}$, and $tRNA^{Gly}$ (Fig. 2) shows that $tRNA^{Gly}$ differs extensively from $tRNA^{Ala}$ and $tRNA^{Phe}$ in the dihydrouridine region and in the variable loop, with differences in both primary and secondary structures. The dihydrouridine region consists of the base-paired stem and single-stranded loop containing nucleotides 16, 17, and 20, while the variable loop consists of the single-stranded nucleotides between the anticodon stem and the T stem (see Fig. 1). In the secondary structure of the dihydrouridine region, $tRNA^{Gly}$ is distinguished by the number of base pairs on the stem and the number of nucleotide residues flanking the constant GG residues in the loop. In the variable loop, $tRNA^{Gly}$ contains four rather than the five residues found in $tRNA^{Ala}$ and $tRNA^{Phe}$. The secondary structure present in $tRNA^{Ala}$ and $tRNA^{Phe}$ is found in many other tRNA species, and is correlated with a slightly different three-dimensional structure from that for molecules like $tRNA^{Gly}$ (*25*). The second factor to consider is the initial heterogenous identity of the parental $tRNA^{Gly}$ molecule. This heterogenity may restrict identity transformation to a third amino acid type, as simultaneous blocking of both components of the parental identity would be required. In addition, it may be difficult to block glutamine identity, as the glutamine aminoacyl-tRNA synthetase is active on tRNA's with many different primary sequences (*2*, *3*, *13*).

In summary, our experiments show that the G-U wobble pair near the 3′ acceptor end of *E. coli* $tRNA^{Ala}$ is a determinant of the amino acid identity of this molecule. We do not know how the G-U wobble pair contributes to this identity, although it could be specifically recognized (*25*). The nucleotides at position 60 and one or more of those at positions 16, 17, and 20 in $tRNA^{Ala}$ have a minor role in this identity. Additional nucleotides must also contribute since none of the derivative suppressor tRNA's assumed a complete identity. Continued analysis of $tRNA^{Ala}$ and of the well-characterized alanine aminoacyl-tRNA synthetase (*26*) may provide a model for tRNA identity.

Finally, the $tRNA^{Ala}$ sequences in other organisms (*27*) show several similarities to the sequence of *E. coli* $tRNA^{Ala}$. The most striking of these is the presence of the G-U wobble pair at position 3–70. In yeast, the G-U is again a distinguishing feature of $tRNA^{Ala}$ among the known tRNA sequences in this organism (*28*). In the $tRNA^{Ala}$ sequences of several other organisms—including *Bacillus subtilis*, *Mycoplasma mycoides*, and *Spiroplasma meliferum*—the G-U wobble pair appears in combination with a C at position 60. Such regularities suggest that a number of organisms use similar nucleotides and positions to establish the identity of $tRNA^{Ala}$.

Table 1. Functional properties of suppressor tRNA's. Dihydrofolate reductase protein was purified by methotrexate affinity chromatography (*7*), desalted by reversed-phase high-performance liquid chromatography (HPLC) and sequenced through residue 15 (Applied Biosystems model 470A sequenator; at the USC Microchemical Core Laboratory). The percentage of amino acid at position 10 was calculated from yields in protein and standard samples. The calculation included a correction for carry-over from residue 9 to residue 10 based on carry-over observed in earlier cycles. Alanine is present at residue 9, and the lower limit for detecting alanine at residue 10 is about 5%. The values for glutamine in PHEC16U17G20, GLY-3 and GLY-13 include 2, 13, and 9%, respectively, from glutamic acid. Cysteine was not analyzed. The amino acid percentages reported are consistent with the HPLC profiles of the respective proteins (*22*). HPLC partially resolves protein species having either lysine, alanine, phenylalanine, or glutamine at residue 10; proteins containing glutamine or glycine are not resolved (*14*). Suppression efficiencies were determined with the use of amber mutations A16 and A30 in the *lacI-Z* fusion system of *E. coli* (*8*). Values are the average of triplicate measurements and are reported as the percentage of the wild-type *lacI-Z*40 fusion which averaged 153 units. For suppression of auxotrophy at the *TrpA* locus of *E. coli*, strains carrying a suppressor gene on plasmid pGFIB, a *TrpA* amber mutation on an F′ episome and a chromosome with mutations in *TrpA* and *MetB* were spot-tested for growth at 37°C on minimal media agar plates containing methionine and ampicillin, either with or without tryptophan (*21*). The response without tryptophan was scored after 48 hours as: +, growth; −, no growth; +/− and −/+, partial growth.

Suppressor gene	Amino acid (%) in suppressed protein	Suppression efficiency (%)		Suppression of auxotrophy in *TrpA*				
		A16	A30	15	49	211	234	243
ALA	Ala, 100	6	15	+/−	−	+	−	+/−
ALAU16C17U20	Ala, 96; Lys, 4	4	11	+	−	+	−/+	+
ALAU60	Ala, 94; Lys, 6	3	11	+	−	+	−/+	+
ALAA3		<1	2	+/−	−	+/−	−	+/−
ALAC70		<1	<1	+/−	−	−	−	+/−
PHE	Phe, 100	10	22	+	−	−	−	+
FTOA	Ala, 96; Lys, 4	14	22	+	−	+	+	+
PHEG3-U70	Ala, 24; Phe, 76	10	31	+	−	+	+	+
PHEC16U17G20	Phe, 67; Lys, 11; Gln, 7; Tyr, 15	<1	1	+	−	−	−	+
PHEC60	Phe, 94; Tyr, 6	5	14	+	−	−/+	−	+
GLY-3	Gly, 16; Gln, 84	24	92	+	−	+	+	+
GLY-13	Gly, 5; Gln, 95	3	10	+	−	+	+	+

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Middle Mississippian Blastoid Extinction Event

WILLIAM I. AUSICH, DAVID L. MEYER, JOHNNY A. WATERS

The Middle Mississippian blastoid (Phylum Echinodermata) extinction event (about 340 million years ago) was a rapid, habitat-specific extinction. Blastoids became rare or absent in shallow-water environments after the extinction, and this change was probably synchronous worldwide. Onshore-offshore habitat shifts have been recognized as an important historical trend among marine benthos. Unlike trends exhibited by other groups, blastoids appear to have repopulated shallow-water habitats after a period of diminished diversity and abundance.

MAJOR EVOLUTIONARY PATTERNS have received considerable study in recent years, including rapid mass extinctions and longer duration onshore-offshore habitat shifts. Important questions concerning mass extinctions include, among others, timing of the extinctions, proximate causes, periodicity of events, and the environmental response of affected organisms. Many community types and specific clades apparently originated in nearshore habitats and migrated through time to deep-water habitats (*1*).

Discovery of an abundant and diverse blastoid fauna (Phylum Echinodermata) in relatively deep-water cratonic sediments of the Mississippian (Lower Carboniferous, about 360 to 320 million years ago) Fort Payne Formation of south-central Kentucky (*2*) and recent analysis of the stratigraphic distribution of European blastoids (*3*) allow us to interpret the ecologic fabric of a significant blastoid extinction event. Blastoids underwent a major shift in habitat preference, from shallow water to deep water, at the middle to late Osagean boundary (Chadian to Arundian, or conodont zone divisions Vla to Vlb) blastoid extinction event. This extinction and habitat restriction appear to have occurred synchronously (within limits of biostratigraphic resolution) in both North America and Western Europe (*4*). The shift to deeper water habitats is especially striking because blastoids were primarily shallow-water benthos throughout their existence from the Middle Ordovician to the Late Permian (Caradocian to Kazanian) (*5*).

In North America, Kinderhookian blastoids were never dominant faunal elements; however, they were successful in both shallow-water and deep-water cratonic sea carbonate settings. Examples include the Hampton Formation of central Iowa (two genera) and the McCraney Formation of northeastern Missouri (two genera). Blastoids were also common locally in deep-water settings associated with Kinderhookian Waulsortian mounds in the western United States. Five blastoid genera are recognized from these deep-water facies in the Lodgepole Limestone of central Montana (*7*).

During the early to middle Osagean, blastoids were still present in deep-water settings, but they became important faunal elements in shallow-water carbonate settings (Fig. 1). Deep-water occurrences include facies associated with Waulsortian mounds in the Lake Valley Formation (early to middle Osagean) of New Mexico (six genera) and sediment-starved cratonic basin conditions of the early Osagean New Providence Shale in western Tennessee (one genus). Shallow-water lower to middle Osagean carbonate platforms with blastoids include the Redwall Limestone of northern Arizona (five genera) (*8*) and the Burlington Limestone of Iowa, Illinois, and Missouri. Blastoids attained their maximum diversity in the Burlington seas, where crinoids and blastoids dominated the entire benthic community. Approximately 17 blastoid genera (20 percent of all described blastoids) are known from the Burlington Limestone (*5, 9*). In addition to a high diversity, blastoids were relatively abundant in the Burlington.

A significant extinction and change in habitat preference among blastoids occurred after deposition of the Burlington. Although a regression and a transgression event have been proposed at this interval (*10*), carbonate sedimentation continued on the Burlington platform to yield the late Osagean Keokuk Limestone. In marked contrast to the abundance and diversity of Burlington blastoids, only three genera of blastoids are known from the Keokuk Limestone. Crinoids remained abundant and diverse during Keokuk deposition. Deeper water cratonic habitats in the late Osagean continued to support moderately diverse and abundant blastoid faunas (Fig. 1), such as from the prodeltaic sediments of the New Providence Shale in northern Kentucky and southern Indiana (five genera) (*11*), and the Fort Payne Formation of south-central Kentucky composed of Waulsortian buildups, crinoidal buildups, and several allochthonous facies (seven genera) (*2*). Blastoids in the Fort Payne are relatively common.

In Western Europe Late Tournaisian (Courceyan) blastoids are known from shallow carbonate settings, where they are relatively common and moderately diverse (Fig. 1). The best documented lower to middle Courceyan fauna is from Tournai, Belgium (five genera) (*12*). Lower to middle Courceyan faunas are also known in shallow-water carbonate facies in Ireland and Britain (*3*), such as the Hook Head Formation (County Wexford, Ireland) (four genera).

Upper Courceyan and Chadian (lower Visean) blastoids were commonly associated with the widespread, deep-water Waulsortian facies, particularly in the Craven Basin

W. I. Ausich, Department of Geology and Mineralogy, Ohio State University, Columbus, OH 43210.
D. L. Meyer, Department of Geology, University of Cincinnati, Cincinnati, OH 45221.
J. A. Waters, Department of Geology, West Georgia College, Carrollton, GA 30117.