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7. Flaxseed acetone powder (20 g, 50 mg/ml) was extracted at 0°C with 50 mM phosphate buffer, pH 7.0 (buffer A) (*5*). The 10,000*g* supernatant was fractionated with ammonium sulfate (0 to 45% at 0°C). The precipitate was applied to a column of octyl-Sepharose CL-4B (Pharmacia, 50-ml bed volume) in 95 ml of buffer A with 0.5 M ammonium sulfate and 0.25% (w/v) Emulgen 911. After extensive washing with buffer A containing 0.5 M ammonium sulfate, we eluted the activity with buffer A containing 0.25% Emulgen 911. The active fractions were then applied to a Mono-Q HR 5/10 column (Pharmacia) in 20 mM triethanolamine (pH 7.3) and 0.1% Emulgen 911 (buffer B). The column was eluted at 1 ml/min with a gradient of buffer B containing 0.5 M NaCl (buffer C); activity eluted at 20% buffer C. Finally, the enzyme was purified on a Mono-P HR 5/20 chromatofocusing column (Pharmacia) (Fig. 1). Centriprep-30 concentrators (Amicon) were used for concentrating enzyme fractions and PD-10 columns (Pharmacia) were used for buffer exchange. Chromatography was carried out at room temperature; at all other times the enzyme was kept at 0°C.
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Specificity for Aminoacylation of an RNA Helix: An Unpaired, Exocyclic Amino Group in the Minor Groove

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An acceptor stem G3·U70 base pair is a major determinant of the identity of an alanine transfer RNA. Hairpin helices and RNA duplexes consisting of complementary single strands are aminoacylated with alanine if they contain G3·U70. Chemical synthesis of RNA duplexes enabled the introduction of base analogs that tested the role of specific functional groups in the major and minor grooves of the RNA helix. The results of these experiments indicate that an unpaired guanine 2-amino group at a specific position in the minor groove of an RNA helix marks a molecule for aminoacylation with alanine.

CONTACTS OF PROTEINS BOUND TO the B-form DNA helix are commonly made through base-specific hydrogen bond donor and acceptor groups that lie in the major groove, which is sufficiently wide to accommodate an α helix or other structural motifs. The groups in the major groove include guanine and adenine N-7, guanine O-6, adenine 6-amino, thymine O-4, and cytosine 4-amino. Amino acid side chains and the peptide backbone provide complementary hydrogen-bonding groups on proteins that enable all base pairs in DNA to be distinguished, in principle, on the basis of major-groove interactions (*1*). In contrast, RNA helices are A-form structures that have deep but narrow major grooves that limit accessibility of protein side chains on α helices or other motifs. Although there are fewer hydrogen-bonding possibilities in the minor groove to allow discrimination among the base pairs in duplex RNA, the minor groove of the A form is wide and shallow and is readily accessible to side chains that emanate from structural elements of proteins (*1, 2*).

These considerations have relevance to the molecular basis for the discrimination of tRNAs by aminoacyl tRNA synthetases. These discriminations provide the basis for the genetic code whereby amino acids are assigned to nucleotide triplets (anticodons) that are encoded by tRNAs. For Ala and His tRNAs, the anticodon and other parts of the tRNA are dispensable for aminoacylation (*3*). Small RNA hairpin helices that reconstruct the acceptor end of these tRNAs can be aminoacylated with complete specificity. In the case of Ala, aminoacylation requires a single G3·U70 base pair in both prokaryotic and eukaryotic Ala tRNAs (*4*). Alteration of this base pair to G·C, A·U, or U·G abolishes in vitro aminoacylation with Ala (*5*).

RNA duplexes composed of complementary single strands are also aminoacylated with Ala, provided that they contain G3·U70 (*6*). The complementary single strands can easily be synthesized by chemical methods that use fully protected diisopropylamino-β-cyanoethyl ribonucleoside phosphoramidites (*7*). These methods provide the opportunity to synthesize protected ribonucleoside phosphoramidites of two-base analogs that, when introduced in place of G3·U70, can be used to identify the atomic group (or groups) at the 3·70 position that are essential for aminoacylation.

The G4·U69 base pair is in the wobble configuration in the three-dimensional structure of yeast $tRNA^{Phe}$ (*8*), and nuclear magnetic resonance experiments have established the wobble pairing for the G3·U70 base pair in an Ala minihelix (*9*). The N-7 and O-6 atoms of G3 and O-4 of U70 project into the major groove, whereas N-3, the 2-amino group of G3, and O-2 of U70 lie in the minor groove of the A-form RNA helix (Fig. 1). Inosine (I), which lacks an exocyclic 2-amino group, forms the same wobble-like pair with uracil as guanine does (Fig. 1). Except for the 2-amino group of G3, the I3·U70 base pair retains all major and minor groove base atoms in the same orientation as G3·U70.

The fully protected inosine phosphoramidite was synthesized using the same protecting group strategy used for the standard ribonucleosides A, C, G, and U (*7*). This method involves the use of the *tert*-butyldimethylsilyl protecting group for the 2′-hydroxyl and a 3′-β-cyanoethyl *N,N*-diisopropylamino phosphoramidite group for formation of the internucleotide linkage (*10*). Inosine was introduced by solid-phase chemistry separately into positions 3 and 4 of a duplex that is based on the sequence of the first nine base pairs of the acceptor TΨC stem of *Escherichia coli* $tRNA^{Ala/GGC}$ (Fig. 2). These molecules are designated I3·U70/

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G4·C69 and G3·U70/I4·C69, respectively. Although the "wild-type" 9-nucleotide (nt) duplex (G3·U70/G4·C69) can be quantitatively aminoacylated with Ala (*6*), there is no aminoacylation of the I3·U70/G4·C69 duplex, even after prolonged incubation with substrate concentrations of Ala tRNA synthetase (Fig. 3A). The sensitivity of these experiments is sufficient to estimate that the rate of aminoacylation of the I3-substituted duplex is reduced to no more than 1/600 of the original rate. In contrast, the G3·U70/I4·C69 duplex is aminoacylated (Fig. 3A) at a rate within a factor of three of that of the "wild-type" duplex (*11*). Thus, in the context of the wild-type duplex sequence (Fig. 2), the unpaired exocyclic 2-amino group of the G3·U70 base pair is essential for aminoacylation.

Previous results demonstrated that substrates with a G3·C70 base pair are not aminoacylated, even though the 2-amino group is present in the minor groove (*3, 5*). In this case, the 2-amino group is paired with O-2 of cytidine in the Watson-Crick configuration (Fig. 1). A G3·C70/G4·U69 duplex variant (Fig. 2) was synthesized and tested for aminoacylation. The lack of aminoacylation of this duplex (Fig. 3B) demonstrates the importance of the position of the free 2-amino group in the RNA helix for enzyme recognition. Furthermore, this result suggests that the enzyme does not simply recognize an irregularity in the helix structure induced by the G·U wobble pair, as has been proposed (*12*).

The chemical synthesis of a fully protected 2-aminopurine (2-AP) ribonucleoside phosphoramidite has been described (*13*). In order to confirm that Watson-Crick pairing of the 2-amino group prevents aminoacylation, 2-AP was incorporated at position 3 of a 9-nt oligoribonucleotide to give a 2-AP3·U70 base pair (Figs. 1 and 2). This base pair replaces the wobble pairing of G3 with the Watson-Crick pairing of 2-AP. The substitution of G3·U70 with 2-AP3·U70 abolishes aminoacylation with Ala (Fig. 3B).

In the tRNA-dependent step of aminoacylation, nucleotide determinants influence both catalytic (k_{cat}) and binding (K_m) parameters. Although the effect of the missing exocyclic 2-amino group on k_{cat} cannot be determined, we attempted to assess the capacity of the I3·U70/G4·C69 duplex to bind to Ala tRNA synthetase. For this pur-

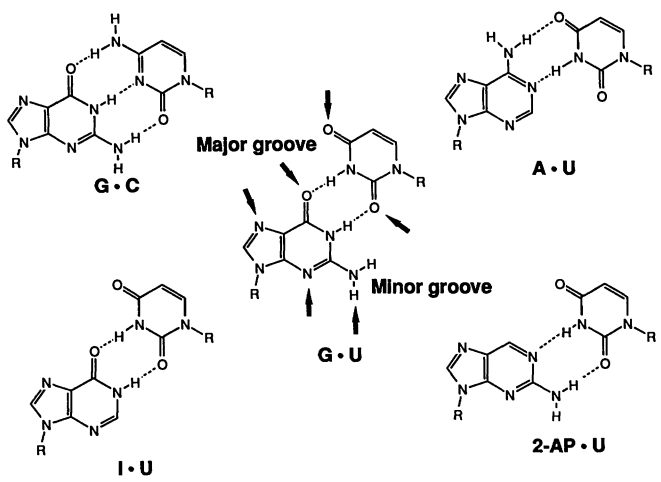

Fig. 1. Structures of base pairs incorporated at the 3·70 position of RNA molecules. The arrows indicate functional groups on both the major and minor groove sides of the G·U base pair that have the potential to form hydrogen bond contacts with protein side chains.

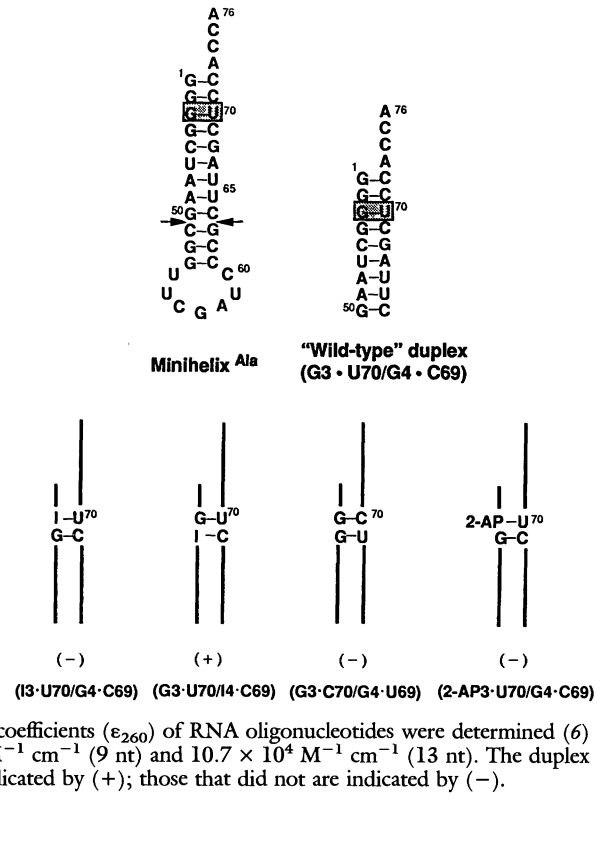

Fig. 2. Sequences of RNA substrates used in this study. The sequence of minihelix^{Ala} is based on the acceptor TΨC helix of *E. coli* tRNA^{Ala} [compare with (*3*)]. Numbering is based on that of full-length tRNA. The oligomers we used to construct the duplex substrates are derived from the stem region above the arrows. The sequence of the "wild-type" RNA 9-nt duplex (G3·U70/G4·C69) and the sequence variants containing modified bases at positions 3·70 and 4·69 are also shown. We prepared oligoribonucleotides by automated solid-phase synthesis either on a Gene Assembler Plus (Pharmacia) or a Cyclone (Milli-Gen/Biosearch) synthesizer using ribonucleoside phosphoramidite monomers as described (*7*). Syntheses of the fully protected inosine phosphoramidite and of the 2-aminopurine phosphoramidite are described in (*10*) and (*13*), respectively. Purification of the oligoribonucleotides was achieved on 16%-polyacrylamide gels, with elution and subsequent desalting procedures as described (*7*). Extinction coefficients (ε_{260}) of RNA oligonucleotides were determined (*6*) and are estimated to be 8.9×10^4 M^{-1} cm^{-1} (9 nt) and 10.7×10^4 M^{-1} cm^{-1} (13 nt). The duplex that resulted in aminoacylation is indicated by (+); those that did not are indicated by (–).

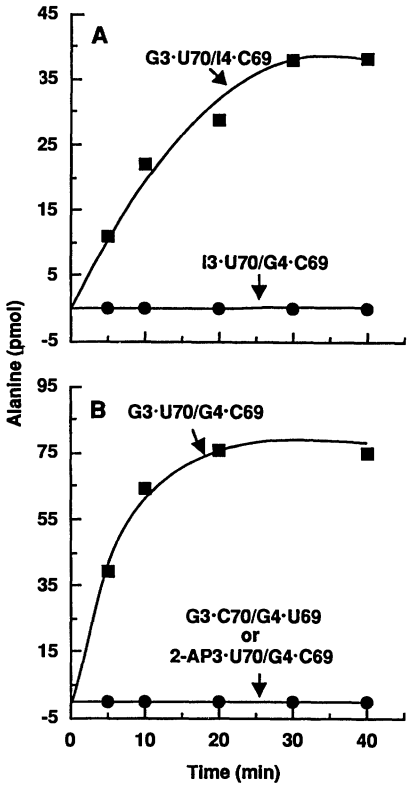

Fig. 3. Aminoacylation with alanine of 9-nt RNA duplexes by Ala tRNA synthetase. (**A**) Aminoacylation of a 13-nt RNA annealed to a 9-nt RNA containing the substitution G3→I3 (I3·U70/G4·C69) versus G4→I4 (G3·U70/I4·C69). (**B**) Aminoacylation of a wild-type duplex (G3·U70/G4·C69) versus a G3·C70/G4·U69 duplex or a 2-aminopurine–substituted duplex (2-AP3·U70/G4·C69). Assays were carried out at 25°C, pH 7.5, as described (*6*), with a final concentration of 5 μM annealed duplex and 0.5 μM purified *E. coli* Ala tRNA synthetase. Each time point represents the amount of alanine incorporated per 18-μl reaction aliquot.

pose, the duplex was added as a potential inhibitor of the aminoacylation of $tRNA^{Ala}$. At pH 7.5, no inhibition of aminoacylation was observed with a 50-fold excess of the I3·U70/G4·C69 RNA duplex. Therefore, although removal of the 2-amino group from position 3 of the duplex reduces the overall rate of aminoacylation (k_{cat}/K_m) to no more than 1/600 of the original rate, we estimate that the enzyme-RNA dissociation constant (K_m) is increased by at least 20-fold.

Transfer of the G3·U70 base pair into other tRNAs or into minihelices that are based on the sequences of other tRNAs results in aminoacylation with Ala (*3, 4, 14*). Although the N73 nucleotide (*14*) and the N2·N71 base pair (*15*) in particular modulate aminoacylation efficiency, no unique nucleotide or base pair at these positions is required for aminoacylation of the G3·U70-containing substrates. Thus, the exocyclic 2-amino group of G3·U70 is recognized by the enzyme in the context of different sequence variants in the acceptor stem. However, all of these variants presumably adopt the A-form helix where the 2-amino group is accessible from the minor groove. In view of the results reported here, the observation of the failure to aminoacylate a DNA helix (3′-rA) whose sequence is based on the acceptor stem of $tRNA^{Ala}$ (*6*) might be explained by its B-form geometry, where the 2-amino group in the minor groove is substantially less accessible than in the A-form structure (*1, 2*).

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11. The melting temperature (T_m) of the wild-type 9-nt duplex (G3·U70/G4·C69) was determined under aminoacylation assay conditions to be 59°C at 2.5 μM and 64°C at 40 μM (*6*). In the present study, the T_m of the I3·U70/G4·C69 duplex was determined to be 52°C at 5 μM. No melting of the duplex was detected at the assay temperature of 25°C. Therefore, it is unlikely that the lack of aminoacylation of the I3·U70/G4·C69 duplex is due to duplex instability.
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Variable Effects of Phosphorylation of Pit-1 Dictated by the DNA Response Elements

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Pit-1, a tissue-specific POU domain transcription factor, is required for the activation of the prolactin, growth hormone, and Pit-1 promoters that confer regulation by epidermal growth factor, adenosine 3′,5′-monophosphate (cAMP), and phorbol esters. Pit-1 is phosphorylated in pituitary cells at two distinct sites in response to phorbol esters and cAMP. Phosphorylation of Pit-1 modifies its conformation on DNA recognition elements and results in increased binding at certain sites and decreased binding at other sites, dependent on DNA sequences adjacent to the core Pit-1 binding motif. One residue (Thr^{220}), located in the POU homeodomain within a sequence conserved throughout the POU-domain family, confers these responses.

PIT-1 APPEARS TO SERVE AS THE PITUITARY-specific activator of the growth hormone and prolactin genes during normal ontogeny (*1–6*) and is necessary for the development and proliferation of lactotroph, somatotroph, and thyrotroph cells in the anterior pituitary gland. Pit-1 autoregulates its own gene (*7, 8*). Its binding sites have been implicated in the activation of the growth hormone gene by cAMP (*9*) and the transcriptional regulation of the rat prolactin gene by cAMP, epidermal growth factor (EGF), and phorbol esters (TPA) (*10*). EGF, cAMP, and TPA, which are known to activate specific protein kinases, thus, might exert their effects on prolactin and growth hormone gene expression by way of phosphorylation of Pit-1.

Immunoprecipitation from a pituitary cell line (GC) labeled in vivo revealed that the amount of ^{32}P label in both the 33- and 31-kD Pit-1 variants (*11*) increased four- to eightfold in response to dibutyryl cAMP and three- to sixfold in response to TPA. The rate of synthesis of Pit-1, quantitated by [^{35}S]methionine labeling (*12*), was equivalent in control and treated cells (Fig. 1). After stimulation, >80% immunoprecipitated Pit-1 migrated as phosphoprotein by isoelectric focusing (*13*). Treatment with Co^{2+}, which inhibits the activation of protein kinase C and prolactin gene expression in GC cells (*14*), decreased basal Pit-1 phosphorylation twofold. Because of the prolonged exposure of Pit-1 to cellular phosphatases during immunoprecipitation, the extent of phosphorylation is likely to be underestimated. Two-dimensional (2D) chromatographic patterns of tryptic phosphopeptides generated from Pit-1 suggested that the sites of phosphorylation were similar in control, dibutyryl cAMP–, or TPA-

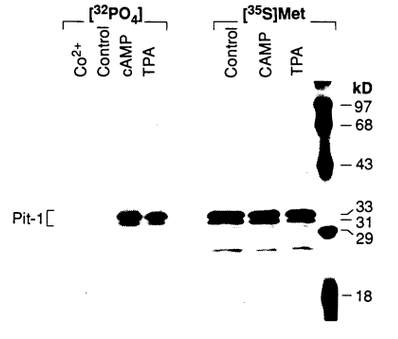

Fig. 1. Phosphorylation of Pit-1 in response to cAMP and phorbol esters. SDS-PAGE autoradiograms show [^{32}P]orthophosphate- and [^{35}S]methionine-labeled Pit-1 immunoprecipitated from GC cells that were unstimulated (control), TPA- or cAMP-induced, or treated with $CoCl_2$.

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*The contribution of M.S.K. and Y.F. to this manuscript were equivalent and both authors should be considered as primary authors.