Chapter 6

Stereoselectivity of Translation in Live Cells

Abstract

Translation fidelity in living cells is attained through several steps during protein synthesis. For example, the elongation factor 1A (EF1A) preferentially delivers L-amino acids versus D-amino acids (via aminoacyl-tRNA) to the ribosome. However, D-residues can be introduced into protein by puromycin analogs since they act in an elongation-factor independent mode. Here, various puromycin analogs with L- and D- stereochemistry are used to test the effect on cell viability. D-amino acid analogs are toxic to cells but it appears that ribosomal stereospecificity is variable and a function of amino acid side chain identity. For a flexible side chain (biocytin), there appears to be negligible selectivity for the L- versus D-analog. These data have broad implications for the *in vivo* synthesis of peptides or proteins bearing stereochemically altered residues.

6.1 Background

Proteins synthesized on the ribosome are comprised entirely of L-amino acids. Several proofreading steps ensure that only L-amino acids are incorporated into protein. For example, EF-Tu which escorts aminoacyl-tRNA (aa-tRNA) into the ribosome, preferentially binds L-aa-tRNA over D-aa-tRNA (1). In addition, the peptidyl transferase reaction was not previously recognized to accommodate D-residues since a puromycin analog with a D-configuration (D-phenylalanine-PANS) did not inhibit translation in an *E. coli* extract and in rabbit reticulocyte lysate (2). In addition, incorporation of D-residues was unsuccessful using the chemically misacylated-tRNA strategy with D-residues (3, 4). Based on these data, the toxicity of D-amino acids in live cells is expected to be insignificant. However, *in vitro* analysis of L- and D-puromycin analogs (**1** and **2**, respectively; Figure 6.1) suggested that D-residues are active in translation (5). But whether D-puromycin would also function in live cells remained unexamined.

There is utility in determining whether D-residues are incorporated into protein in live cells and what influences there potency. D-residues are fundamental residues in many cyclic peptides, some of which are antibiotics such as gramicidin A or bacitracin. However, these peptides are synthesized by nonribosomal peptide synthetases (NRPS), which can utilize both L- and D-amino acids (6). These enormous protein complexes are highly specialized for the sequence to be synthesized, yet require large individual protein complexes for each amino acid to be coupled. Therefore, reconstitution of biosynthetic pathways (7) for novel peptides or peptide libraries remains a challenge. However, the discovery that ribosomes do utilize D-residues *in vitro* may allow peptide libraries (8, 9) to be screened for novel antibiotics, immunosuppressants, or even D-peptide-based drugs that are amenable to oral administration.

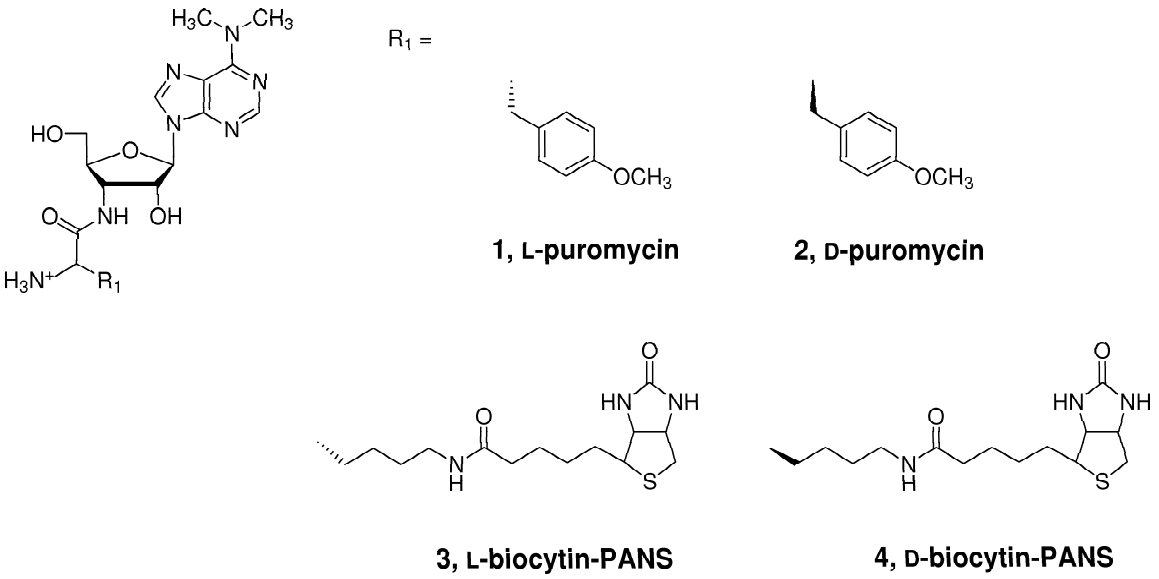

Figure 6.1: L- and D-amino acid analogs used in live cell assays.

6.2 Results and Discussion

The first experiment was to determine the cell sensitivity to to L- (**1**) and D-puromycin (**2**) (Figure 6.1). L-puromycin is known to be toxic to cells, but the concentration varies according to the cell line. The cell line used for these studies was a stable mammalian thymocyte 16610D9 cell line (D9) (10) which was used previously to test the labeling of protein by fluorescent puromycin conjugates (11). D9 cells were incubated with an L-puromycin concentration (10 μM) shown to be highly toxic to mammalian cells (optimal working concentration for L-puromycin varies from $2-20$ μM for mammalian cell lines). A D-puromycin concentration 100-fold greater (1000 μM) was used since *in vitro* analysis showed D-puromycin to have $\sim$150-fold reduced potency for inhibition of translation (5). Indeed, D-puromycin was also toxic to cells (24 h) and at a concentration that was only 100-fold greater than L-puromycin (Figure 6.2A). After a 48 h incubation with increasing amounts of D-puromycin, the concentration that killed 50% of the cells (EC_{50}) was 580 μM (Figure 6.2B).

These data establish for the first time that a D-amino acid analog is toxic to

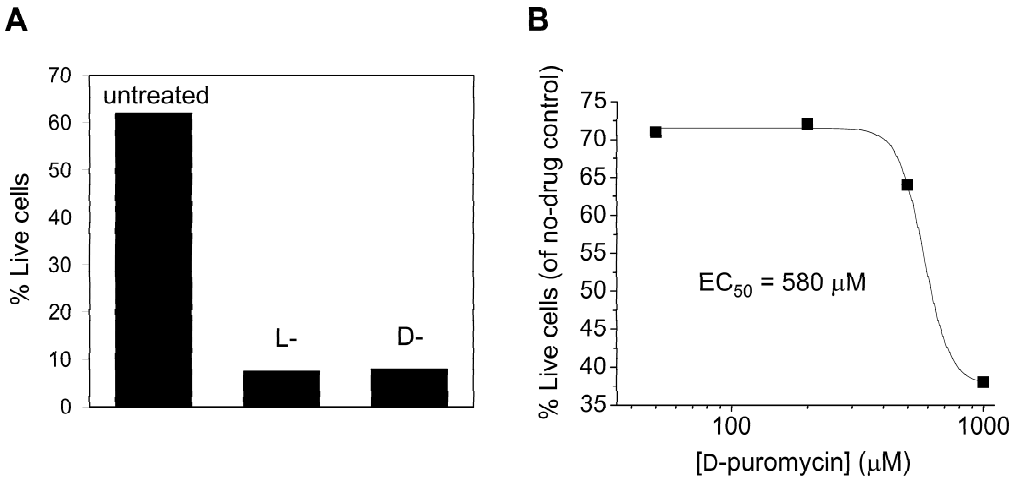

Figure 6.2: (A) Toxicity of L- (10μM) and D-puromycin (1000 μM) to live cells during a 24 h incubation. (B) D-puromycin concentration required to kill 50 percent of cells (EC_{50}) relative to an untreated control after a 48 h incubation.

mammalian cells. The concentration required for killing is rather high but reflects the fold discrimination (L- versus D-) obtained from *in vitro* translation experiments (5). L-puromycin toxicity is thought to result from participation in peptidyl transferase. This suggests that D-puromycin toxicity is also related to inhibition of translation through a similar mechanism.

The purity of the D-puromycin preparation is important in these experiments since any contaminating L-puromycin would cause the cells to die. Therefore, purity of the D-puromycin synthesis was assessed using HPLC chromatography. Isolated L- and D-amino acids are enantiomers and are expected to have identical chromatography profiles. But L- and D-puromycin derivatives are diastereomers and have unique physical properties which should be detectable from HPLC. Indeed, HPLC analysis of L- and D-puromycin shows that the D-puromycin sample is highly pure (Figure 6.3). This suggests that the effect observed results from D-puromycin toxicity, not from L-analog contamination.

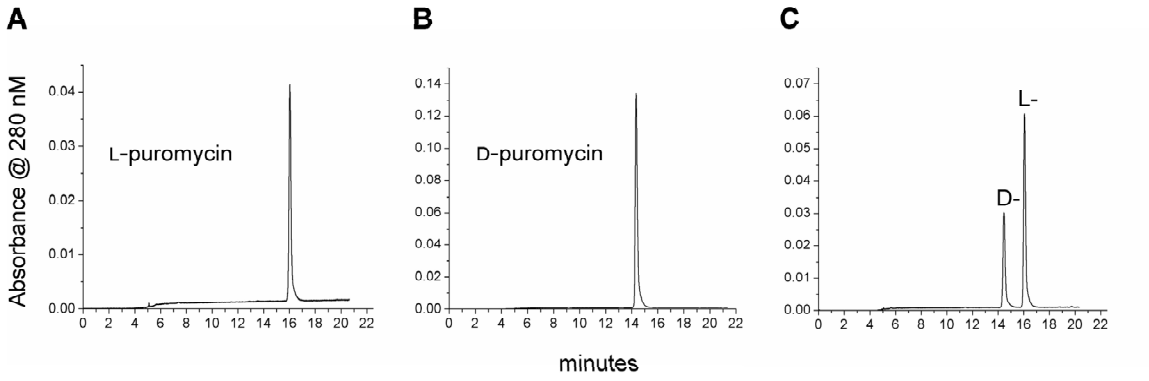

Figure 6.3: HPLC analysis of (A) L- and (B) D-puromycin and (C) L- and D-puromycin co-injection.

Another strategy to ensure the purity of the D-analog sample is to carry out selective enrichment experiments of cells containing puromycin *N*-acetyl-transferase (PAC) (12). This enzyme *N*-acetylates the reactive amine on L-puromycin and blocks its ability to participate in peptide bond formation (13, 14). In a mixed population of cells, those that lack a vector expressing PAC can be selectively killed by long incubations ($\geq$48 hours) with puromycin, leaving only vector-containing cells alive. The marker for PAC activity in these experiments was GFP from the construct (MIG_{PAC}) used previously to confirm the chemical activity of puromycin-dye conjugates *in vivo* (Figure 6.4) (11). Cells infected with MIG_{PAC} should be enriched by only L-puromycin since PAC is a typical, highly stereospecific enzyme. If the D-puromycin synthesis contains any contaminating L-puromycin, then selective enrichment of GFP(+) cells should also be detected. Incubation of D9 cells infected with MIG_{PAC} with either L- or D-puromycin showed that L-puromycin (5 μM) enriched for 98% of MIG_{PAC} cells (Figure 6.5A). However, D-puromycin did not yield MIG_{PAC} cell enrichment, even up to a 1 mM D-puromycin concentration (Figure 6.5B).

A 0.05% L-puromycin contamination in the D-sample is equivalent to 0.5 μM of the

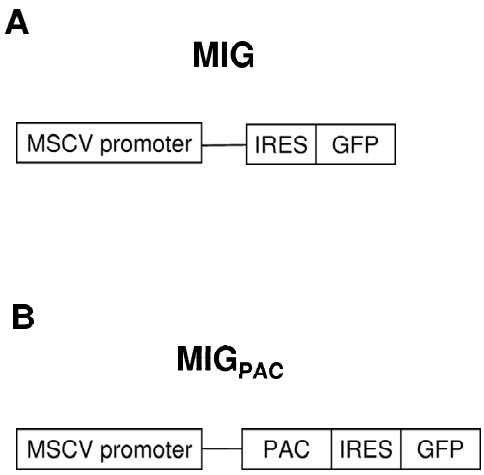

Figure 6.4: Constructs used to infect 16610D9 thymocyte cells. (A) MIG contains GFP and (B)MIG$_{\mathrm{PAC}}$ contains GFP and PAC rendering cells insensitive to puromycin action.

the L-species. Even this low level of L- contamination would yield detectable GFP(+) population enrichment, which is observed in the L-puromycin enrichment experiment (Figure 6.5A). This is strong evidence to support a highly pure D-puromycin preparation (<0.05%). Therefore, the toxic effect observed in the presence of D-puromycin results solely from the activity of the D-analog.

The stereoselectivity of ribosomes in mammalian cells (inferred from the toxicity of the analogs) showed ~100-fold discrimination between L- and D-puromycin (Figure 6.2). Previously, it was shown that *in vitro* ribosomal stereoselectivity falls over a broad range and is dictated by the size and geometry of the pendant side chain (5). Therefore, it was of interest to test the level of discrimination of other L- and D-amino acid analog pairs. The next pair tested was L-biocytin-PANS (**3**) and D-biocytin-PANS (**4**) (Figure 6.1).

The viability of D9 cells was determined after incubation with both L- and D-analogs. The % live cells in the presence of L- and D-puromycin at identical con-

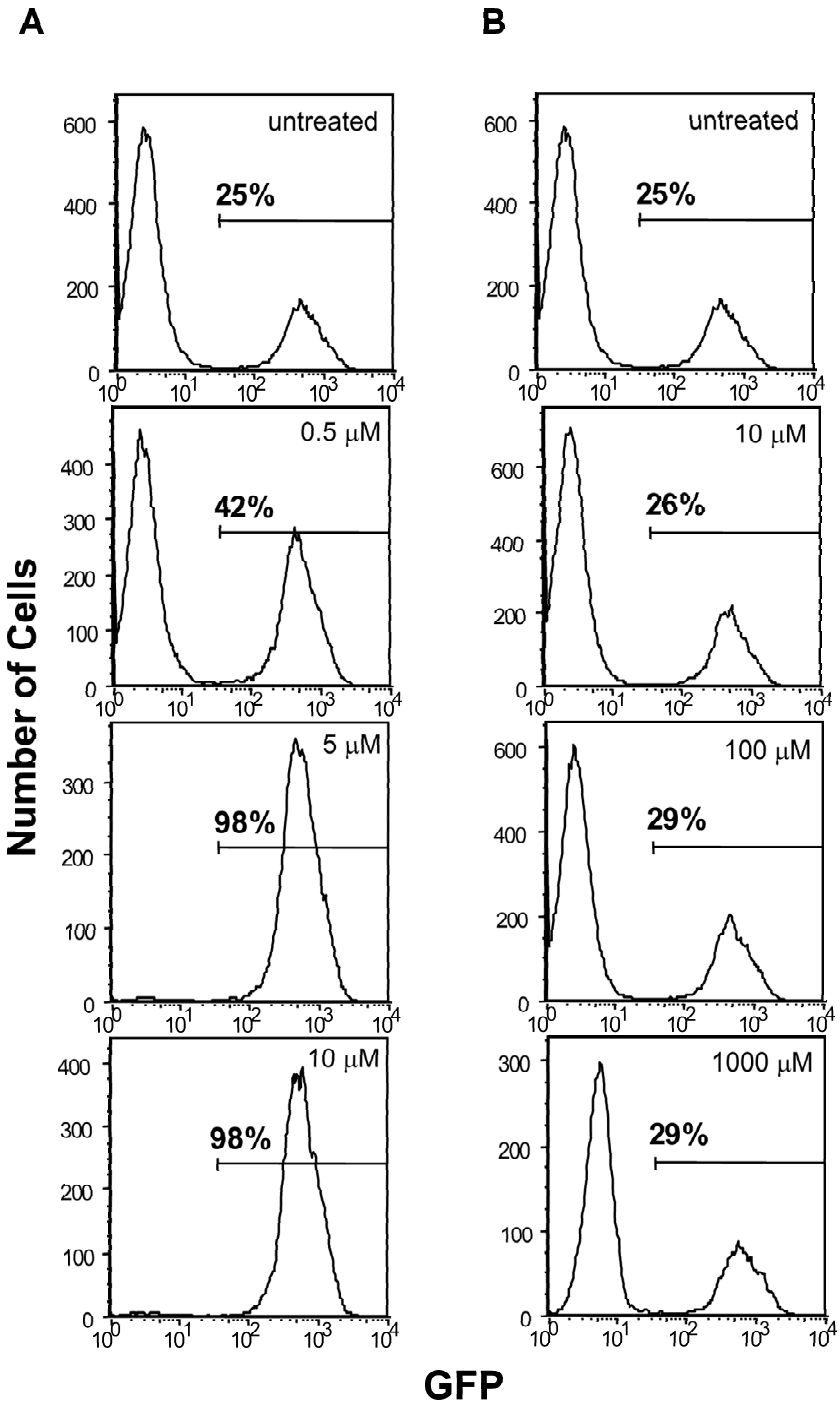

Figure 6.5: GFP(+) cell enrichment achieved with (A) L-puromycin but not with (B) D-puromycin.

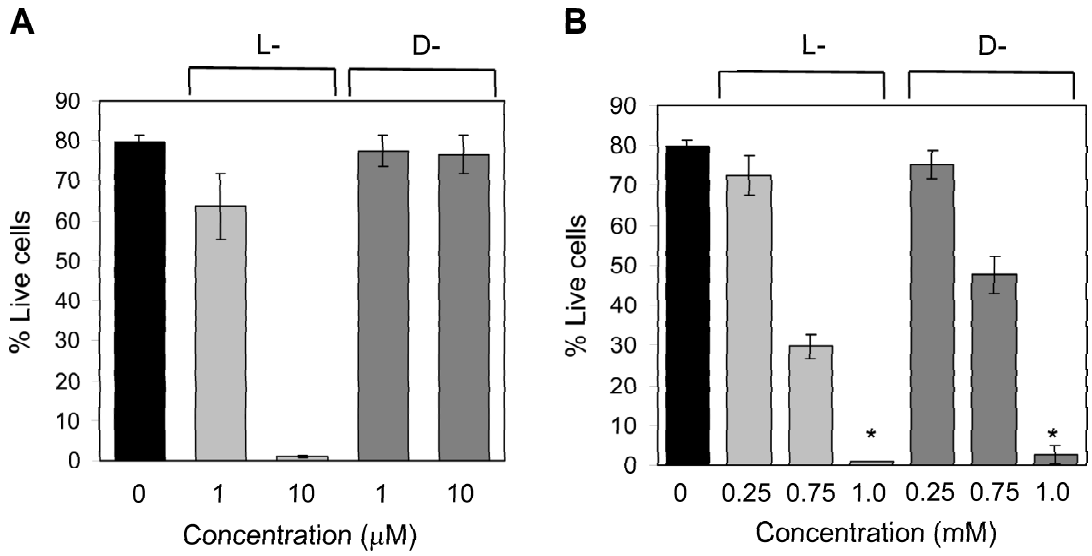

Figure 6.6: Viability of D9 thymocyte cells in the presence of L- and D-amino acid analogs. Cells are sensitive to the stereochemistry of (A) L- and D-puromycin but not (B) L- and D-biocytin-PANS. The mean ± standard error is calculated from three independent experiments except for *samples which were obtained from two independent experiments with minor changes (See Experimental Procedures).

centrations varies considerably (Figure 6.6A). However, for L- and D-biocytin-PANS at the same concentrations the % live cells is not dissimilar at each concentration tested (Figure 6.6B). In fact, the *in vitro* IC_{50} values for L- and D-biocytin-PANS are identical (Figure 6.7).

These data suggest that the side chain affects the level of discrimination the ribosome can make for the L-amino acid analog versus the D-analog (Figure 6.6). Assuming the loss in cell viability results from inhibition of translation, ribosomes in live cells also show a range of stereoselectivity. This reduced selectivity suggests that incorporation of D-residues may be possible with certain amino acids, given the ribosome shows little or no preference for the L- versus the D-variant.

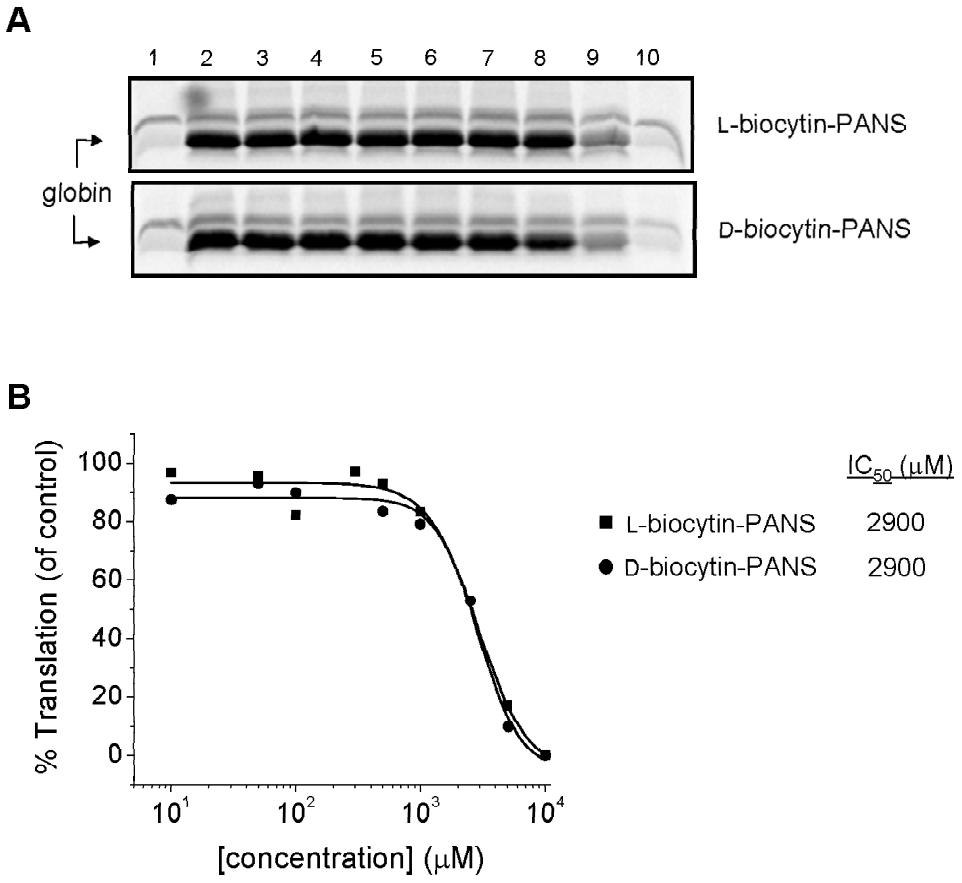

Figure 6.7: *In vitro* activity analysis (IC_{50}) for L- and D-biocytin-PANS. Globin mRNA was translated with increasing amounts of analog, resolved using tricine-SDS-PAGE, and quantitated by detection of [^{35}S]Met-globin as described (see Experimental Procedures). (A) Translation of globin with L-biocytin-PANS and D-biocytin-PANS: lane 1, no template; lane 2, globin alone; lanes $3-17$, concentrations from 0.01 to 10 mM. (B) % Translation of globin mRNA (of untreated control).

L- and D-alanine are also expected to be discriminated poorly by the ribosome in eukaryotic cells (yet this has yet to be tested) since there is a ~3-fold discrimination of L- and D-Ala-PANS by ribosomes *in vitro* (5). This low level of peptidyl transferase selectivity may allow D-alanine to be readily incorporated into protein. The following analysis demonstrates how measures can be taken to increase the recognition of D-amino acids via aa-tRNA-mediated protein synthesis.

Based on an analysis by Hopfield and co-workers partial selectivity at each stage of protein synthesis should exclude D-tyrosine from being incorporated into protein (1). For example, the EF-Tu-aa-tRNA complex of L-tyrosine-tRNA^{Tyr} is bound ~20 times more tightly than D-tyrosine-tRNA^{Tyr} (1). In addition, binding to ribosomes and subsequent peptide bond formation are favored for L-tyrosine-tRNA^{Tyr} by 3- and ~30-fold, respectively (1). Taken together the selectivity for L-tyrosine-tRNA^{Tyr} versus D-tyrosine-tRNA^{Tyr} is 10^4 (Figure 6.8; column I).

However, aminoacylation by a cognate synthetase is not required since a D-aa-tRNA could be prepared using the chemical misacylation strategy (15, 16). This decreases the discrimination of a D-aa-tRNA to 10^3-fold (Figure 6.8; column II). Further, the identification of an optimal tRNA body to use with D-amino acids to provide favorable thermodynamic compensation for EF-Tu or EF1A affinity would allow the tertiary complex to effectively compete with other naturally occurring aa-tRNA-EF-Tu complexes (17, 18). This improvement should further decrease the discrimination to $<10^2$-fold (Figure 6.8; column III).

Peptidyl transferase discrimination for an aminoacylated-tRNA with 4-*O*-methyl-tyrosine is expected to be 5-fold (1). This assumes that the discrimination between L- and D-tyrosine-tRNA and L- and D-4-*O*-methyl-tyrosine-tRNA in peptidyl transferase is equivalent, at 5-fold (1). Next, the stereoselectivity of peptidyl transferase between L- and D-alanine-tRNA can be calculated. The ~ 150-fold peptidyl transferase selectivity for L- and D-puromycin and the ~3-fold selectivity for L- and D-alanine-PANS (5) can be used to calculate the fold selectivity for peptidyl transferase

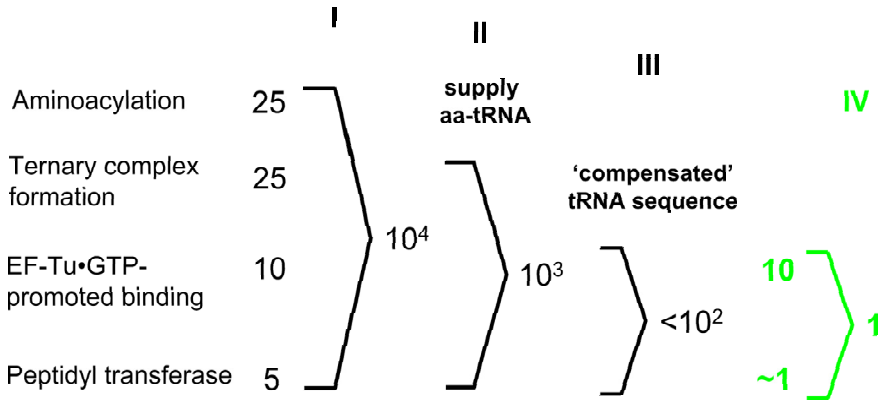

Figure 6.8: Analysis of ribosomal selectivity for D-alanine using an optimized tRNA body starting with the (I) maximal selectivity of L- versus D-tyrosine from (1).

activity in the ribosome for L- versus D-alanine-tRNA^{Ala} using the following equation:

$$\frac{[4\text{-}O\text{-methyl-tyrosine-tRNA-fold selectivity (1)}]}{[\text{puromycin-fold selectivity (5)}]} \times \frac{1}{[\text{alanine-PANS-fold selectivity (5)}]}$$

$$\frac{5}{150} \times \frac{1}{3} = 1$$

Replacing the 5-fold discrimination for peptidyl transferase between L- and D-tyrosine-tRNA (1) with that for L- and D-alanine-tRNA from this calculation changes the overall discrimination of D-residues from protein synthesis to ~1 (Figure 6.8; column IV). This calculation virtually eliminates the stereoselectivity of translation for incorporation of D-alanine-tRNA. This argument predicts that steps can be taken to reduce the stereoselectivity of protein synthesis at several levels: 1) supply excess of chemically misacylated-tRNA, 2) use a tRNA body that optimizes association with the elongation factor (EF-Tu or EF1A), and 3) use a D-residue that shows little peptidyl transferase selectivity, such as D-biocytin or D-alanine.

6.3 Conclusions

Until recently, D-amino acids were not considered to be important in the biology of higher organisms. The discovery that small peptides with D-amino acids are involved in development, hormone synthesis, and neurotransmission, as well as in age-related conditions such as Alzheimer's has challenged theories about the utility of D-amino acids. (reviewed in (19)). The data presented here highlight the possibility that D-amino acids do have biological relevance in live cells. This examination should facilitate the conception and design of experiments to elucidate the role that D-amino acids play both in normal and disease states *in vivo* as well as aid in construction of D-based peptide libraries for mRNA display selections (8).

6.4 Experimental Procedures

6.4.1 General information

Low-resolution mass spectra were recorded on a PE SCIEX API 365 triple quadruple electrospray mass spectrometer at the Beckman Institute Mass Spectrometry Laboratory, California Institute of Technology. Column chromatography was carried out on silica gel (40 – 63 μm, EM Science). Analytical HPLC was performed using a Vydac C18 column (5 mm, 4.5 x 250 mm) with buffer A (5 mM NH_4OAc, pH 5.5 with 10% acetonitrile) and buffer B (5 mM NH_4OAc, pH 5.5 with 90% acetonitrile); a linear gradient of 100% buffer B in 50 min was used with a flow rate of 1 mL/min. All reagents were of highest available commercial quality and were used without further purification. L-puromycin and puromycin aminonucleoside (3'-amino-3'-deoxy-*N*,*N*'-dimethyl-adenosine) (PANS) were purchased from Sigma Chemical Co. D-puromycin was prepared as described in (5). FMOC-L-biocytin was obtained from Novabiochem. FMOC-D-biocytin was prepared from coupling FMOC-D-lysine (Bachem) and D-biotin (>99.9%; Sigma Chemical Co.) as described below. Immobilized piperidine

beads (3-4 mmol/g) were purchased from Aldrich. Puromycin analog concentrations were determined with the following extinction coefficients ($M^{-1}cm^{-1}$) at 260 nm: Aromatic puromycin analogs such as L- and D-puromycin (**1** and **2**, respectively) [$\epsilon = 10,500$] in water; non-aromatic puromycin analogs such as L- and D-biocytin (**3** and **4**, respectively) [$\epsilon = 11,000$] in phosphate buffered saline (pH 7.3). Rabbit reticulocyte lysate was purchased from Novagen. Rabbit globin mRNA was obtained from Life Technologies Gibco BRL.

6.4.2 Procedure for Preparation of FMOC-D-biocytin

FMOC-D-lysine (0.204 mmol) was dissolved in dried *N*,*N*-α-dimethylformamide (DMF) (0.700 mL) at room temperature. To this solution was added 1-H-benzotriazolium (HBTU) (0.204 mmol) and 6-chloro-1-hydroxy-1H-benzotriazole (HOBT) (0.204 mmol) and stirred for 10 min. FMOC-D-lysine (0.204 mmol) dissolved in diisopropylethylamine (DIEA) (2.55 mL) was added slowly with stirring. The solution was stirred at ambient temperature overnight and then dried *in vacuo*. The product was resuspended in MeOH/CH_3COOH/$CHCl_3$ (7:13:80) and purified by flash chromatography using MeOH/CH_3COOH/$CHCl_3$ (7:10:83). Homogenous product fractions were dried *in vacuo* to yield FMOC-D-biocytin as a yellow-brown oil (120 mg, 99.0%). LRMS, *m/z* $(M+H)^+ = 595.2$.

6.4.3 General Procedure for Preparation of Puromycin Analogs

N,*N*'-dicyclohexylcarbodiimide (DCC) (0.0539 mmol) was added to a cold (0 °C) solution of PANS (0.0520 mmol), FMOC-protected amino acid (0.0541 mmol), and *N*-hydroxysuccinimide (NHS) (0.0556 mmol) in dried *N*,*N*-α-dimethylformamide (DMF) (0.900 mL). The solution was stirred for 30 min in an ice-water bath and then for 42 h at ambient temperature. *N*,*N*'-dicyclohexylurea was filtered and washed (EtOAc,

4 mL), and the filtrate was concentrated *in vacuo*. The material was resuspended in triethylamine/MeOH/$CHCl_3$ (2:8:90) and purified by flash chromatography using triethylamine/MeOH/$CHCl_3$ (2:8:90). Homogenous product fractions were dried *in vacuo* to yield the FMOC-protected products. FMOC-deprotection was carried out using immobilized piperidine in DMF (5mL) with stirring overnight at ambient temperature for L- and D-biocytin-PANS (**3** and **4**, respectively). The beads were filtered and the solvent was removed *in vacuo*. The residue was purified using HPLC to afford the products L- and D-biocytin-PANS. LRMS, m/z $(M+H)^+ = 649.4$. L- and D-puromycin (**1** and **2**, respectively) were prepared as described previously (5).

6.4.4 *In vivo* Analysis of Puromycin Analogs

16610D9 thymocyte cells (D9 cells; 25,000 cells) were combined with puromycin analogs (dried initially and dissolved in RPMI media with 10% FBS) in total volume of 100 μL in 48-well microtiter plates. After incubation at 37°C in a humidified atmosphere with 5% CO2 for 24 h, cells were analyzed directly using a Beckman FACScabilur flow cytometer to collect forward and side scatter measurements, which shows a characteristic plot for viable cells. A slight modification of the procedure as indicated in Figure 6.6 was using a total volume of 300 μM in 24-well microtiter plates in a separate experiment.

6.4.5 Preparation of MIG_{PAC} Infected 16610D9 Cells

The PAC gene was cloned into MIG using BgII and EcoRI restriction sites to yield MIG_{PAC}. 293T-HEK fibroblasts (American Tissue Culture Collection) were cotransfected with pECL-Eco and MIG or MIG_{PAC} by calcium phosphate precipitation [see reference (11)]. After 12 hours, the precipitate was removed, cells were washed once with PBS, and 4 mL of fresh complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) was added. Viral supernatant was

removed 24 hours later and used for infection of D9 cells. One million D9 cells were spin-infected with 0.4 mL of viral supernatant suplemented with 5 μg/ml Polybrene (Sigma-Aldrich).

6.4.6 Enrichment of GFP(+) 16610D9 Cells using Puromycin and Puromycin Conjugates

D9 cells infected with either MIG of MIG_{PAC} were cultured in RPMI media with 10% FBS and grown at 37 °C in a humidified atmosphere with 5% CO_2. For each experiment, D9 cells (0.25 x 10^6/well) were added to 24-well microtiter plates along with puromycin and puromycin analog dissolved in the minimum amount of media. After a 48 h incubation, the cells were washed twice in 2 mL PBS + 4% FCS and resuspended in PBS + 4% FCS supplemented with 2% formaldehyde along with incubation at 37 °C for 10 min and analyzed using flow cytometery.

6.4.7 IC_{50} Determination

Translation reactions containing [^{35}S]Met were mixed in batch on ice and added in aliquots to microcentrifuge tubes containing an appropriate amount of puromycin, puromycin-conjugate, or oligonucleotide dried in vacuo. Typically, a 20 μl translation mixture consisted of 0.8 μL of 2.5 M KCl, 0.4 μL of 25 mM MgOAc, 1.6 μL of 12.5X translation mixture without methionine, (25 mM dithiothreitol (DTT), 250 mM HEPES (pH 7.6), 100 mM creatine phosphate, and 312.5 μM of 19 amino acids, except methionine) (Novagen), 3.6 μL of nuclease-free water, 0.6 μL (6.1 μCi) of [^{35}S]Met (1175 Ci/mmol), 8 μL of Red Nova nuclease-treated lysate (Novagen), and 5 μL of 0.05 μg/μL globin mRNA (Gibco). Inhibitor, lysate preparation (all components except template), and globin mRNA were mixed simultaneously and incubated at 30 °C for 60 min. Then 2 μL of each reaction was combined with 8 μL of tricine loading buffer (80 mM Tris-Cl (pH 6.8), 200 mM DTT, 24% (v/v) glycerol, 8% sodium dodecyl

sulfate (SDS), and 0.02 % (w/v) Coomassie blue G-250), heated to 90 °C for 5 min, and applied entirely to a 4% stacking portion of a 16% tricine-SDS-polyacrylamide gel containing 20% (v/v) (20) (30 mA for 1h, 30 min). Gels were fixed in 10% acetic acid (v/v) and 50% (v/v) methanol, dried, exposed overnight on a PhosphorImager screen, and analyzed using a Storm PhosphorImager (Molecular Dynamics).

References

[1] T. Yamane, D.L. Miller, and J.J. Hopfield. Discrimination between D-tyrosyl and L-tyrosyl transfer ribonucleic-acids in peptide-chain elongation. *Biochemistry*, 20:1897–1904, 1981.

[2] D. Nathans and A. Neidle. Structural requirements for puromycin inhibition of protein synthesis. *Nature*, 197:1076–1077, 1963.

[3] J.D. Bain, D.A. Wacker, E.E. Kuo, and A.R Chamberlin. Site-specific incorporation of nonnatural residues into peptides - Effect of residue structure on suppression and translation efficiencies. *Tetrahedron*, 47:2389–2400, 1991.

[4] J. Ellman, D. Mendel, S. Anthony-Cahill, C.J. Noren, and P.G. Schultz. Site-specific incorporation of novel backbone structures into proteins. *Science*, 202:301–336, 1992.

[5] S.R. Starck, X. Qi, B.N. Olsen, and R.W. Roberts. The puromycin route to assess stereo- and regiochemical constraints on peptide bond formation in eukaryotic ribosomes. *J. Am. Chem. Soc.*, 125:8090–8091, 2003.

[6] J.W. Trauger, R.M. Kohli, H.D. Mootz, M.A. Marahiel, and C.T Walsh. Peptide cyclization catalysed by the thioesterase domain of tyrocidine synthetase. *Naure*, 407:215–218, 2000.

[7] T.A. Keating, C.G. Marshall, and C.T. Walsh. Reconstitution and characteriza-

tion of the Vibrio cholerae vibriobactin synthetase from VibB, VibE, VibF, and VibH. *Biochemistry*, 39:15522–15530, 2000.

[8] T.T. Takahashi, R.J. Austin, and R.W. Roberts. mRNA display: ligand discovery, interaction analysis and beyond. *Trends Biochem. Sci.*, 28:159–165, 2003.

[9] A. Frankel, S.W. Millward, and R.W. Roberts. Encodamers: Unnatural peptide oligomers encoded in RNA. *Chem. Biol.*, 10:1043–1050, 2003.

[10] L. Van Parijs, Y. Refaeli, J.D. Lord, B.H. Nelson, A.K. Abbas, and D. Baltimore. Uncoupling IL-2 signals that regulate T cell proliferation, survival, and Fas mediated activation-induced cell death. *Immunity*, 11:281–288, 1999.

[11] S.R. Starck, H.M. Green, J. Alberola-Ila, and R.W. Roberts. A general approach to detect protein expression *In Vivo* using fluorescent puromycin conjugates. *Chem. Biol.*, page In press, 2004.

[12] S. de la Luna and J. Ortin. Pac gene as efficient dominant marker and reporter gene in mammalian cells. *Methods Enzymol.*, 216:376–385, 1992.

[13] J.N. Porter, R.I. Hewitt, C.W. Hesseltine, G. Krupka, J.A. Lowery, W.S. Wallace, N. Bohonos, and J.H. Williams. Achromycin: A new antibiotic having trypanocidal properties. *Antibiotics and Chemo.*, 11:409–410, 1952.

[14] J.A. Pérez-González, J. Vara, and A. Jiménez. Acetylation of puromycin by *Streptomyces alboniger* the producing organism. *Biochem. Biophys. Res. Commun.*, 113:772–777, 1983.

[15] S.M. Hecht. Probing the synthetic capabilities of a center of biochemical catalysis. *Acc. Chem. Res.*, 25:545–552, 1992.

[16] C.J. Noren, S.J. Anthony-Cahill, M.C. Griffith, and P.G. Schultz. A general method for site-specific incorporation of unnatural amino-acids into proteins. *Science*, 244:182–188, 1989.

[17] F.J. LaRiviere, A.D. Wolfson, and O.C. Uhlenbeck. Uniform binding of aminoacyl-tRNAs to elongation factor Tu by thermodynamic compensation. *Science*, 294:165–168, 2001.

[18] O. C. Asahara, H.; Uhlenbeck. The tRNA specificity of *Thermus thermophilus* EF-Tu. *Proc. Natl. Acad. Sci. U.S.A.*, 99:3499–3504, 2002.

[19] H. Yang, G. Zheng, X. Peng, B. Qiang, and J. Yuan. D-Amino acids and D-Tyr-tRNA^{Tyr} deacylase: Stereospecificity of the translation machine revisited. *FEBS*, 552:95–98, 2003.

[20] H. Schagger and G.V. von Jagow. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.*, 166:368–379, 1987.