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Analysis of Translational Fidelity of Ribosomes with Protamine Messenger RNA as a Template†

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ABSTRACT: A novel method was developed to estimate the translational fidelity of mammalian ribosomes in vitro with protamine mRNA of rainbow trout as template. Protamines are mixtures of basic proteins consisting of only seven types of amino acids (Arg, Ile, Val, Ser, Pro, Ala, and Gly), arginine (codon, A*G*R and C*G*N) being abundant. Taking advantage of the absence of lysine (codon, A*A*G) in the proteins, we determined the misincorporation of this amino acid into protamines in a cell-free translation system consisting of mouse liver ribosomes, protamine mRNA, [^{3}H]lysine, [^{14}C]arginine, and seven unlabeled amino acids: Ile, Val, Ser, Pro, Ala, Gly, and Met. After the reaction, translation products were analyzed by either sucrose gradient centrifugation or polyacrylamide gel electrophoresis. In the former method, radioactive protamines are mostly found on monosomes, but not on polysomes, probably because of the basic nature of the proteins. The error frequency was calculated from the molar ratio of [^{3}H]lysine to [^{14}C]arginine incorporated into protamines with an appropriate correction. The frequency was found to be 0.0006–0.002. This method enabled us to determine the frequency of misrecognition of purine bases at the second position of arginine codons in mRNA.

In all organisms, it is important to maintain high fidelity of transfer of genetic information. Transcription and translation of genetic messages must be highly accurate for synthesis of functional proteins for cellular activities. Therefore, for an understanding of fundamental biological processes, it is essential to have information on the mechanism of accurate translation of mRNA, i.e., the fidelity of protein synthesis (Kurland, 1979; Yarus, 1979).

In the early 1960's, when decoding of the genetic code was being studied intensively, it was realized that the translational fidelity of ribosomes is not perfect in a cell-free translation system derived from *Escherichia coli* and *Thermophilus* (Gorini, 1974). The frequency of mistranslation in vitro was on the order of 10^{-2} with synthetic polynucleotides such as poly(U) as template. Moreover, in some proteins, such as ovalbumin, hemoglobins, and flagellin, the incorporation of amino acids that are not expected to be present was found to occur at a frequency of 10^{-3}–10^{-4}, a value that is believed to be comparable to that in vivo (Loftfield, 1963; Loftfield & Vanderjagt, 1972; Edelmann & Gallant, 1977).

The discrepancy between the error frequencies in vitro and in vivo might be because synthetic polynucleotides without the specific sequences present in natural mRNAs were used for in vitro studies. Alternatively, there may be specific error-reducing mechanisms in vivo (Lake, 1981). For many years, synthetic messenger RNA has been used in studies on the error frequency of translation in vitro. It seems preferable to use natural mRNAs for this purpose, but no suitable systems with natural mRNA are available for studies on translational fidelity in vitro. One reason for this is that most proteins contain all 20 types of amino acids, making it difficult to distinguish incorrect incorporation of amino acids, if any, from correct incorporation. However, if a natural mRNA that does not code for 1 or more of the 20 amino acids were used, it should be easy to measure incorporation of incorrect radioactive amino acids. mRNAs of this type that could be used for this purpose

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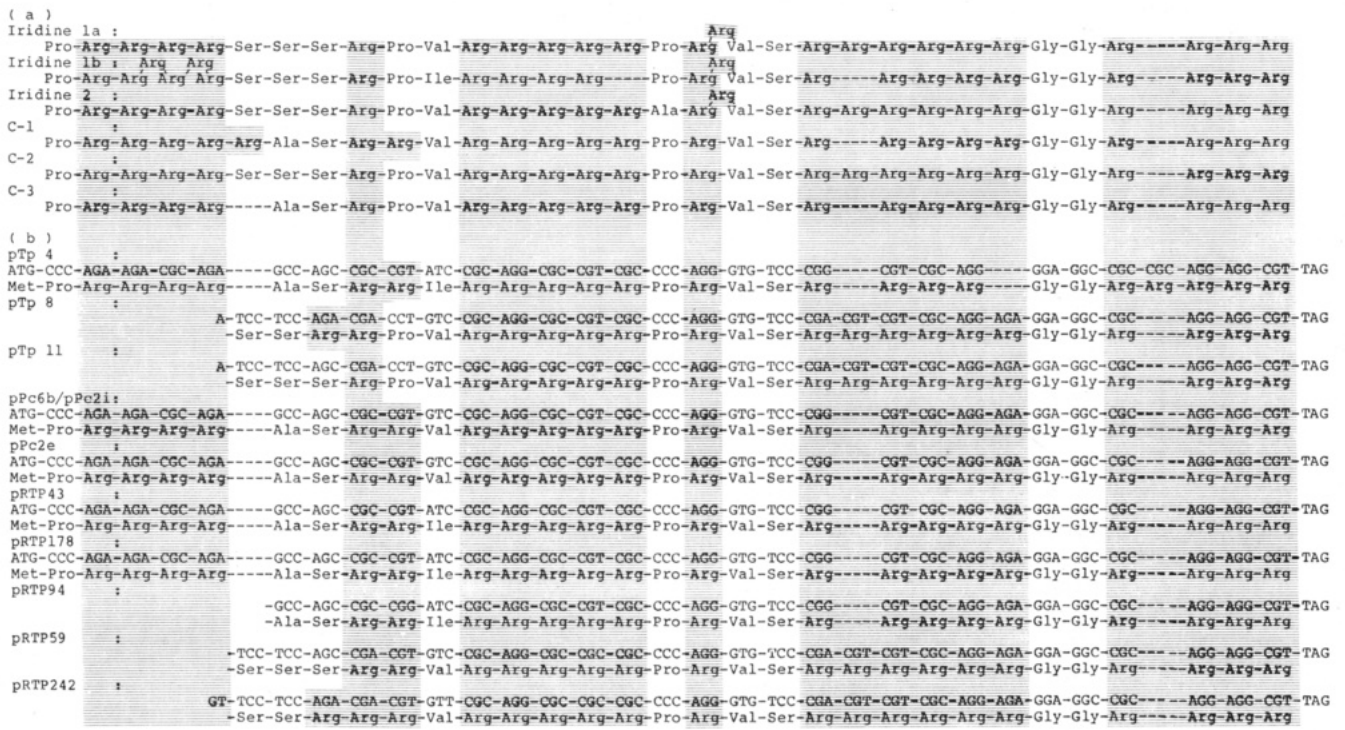

FIGURE 1: Amino acid sequences of 6 types of rainbow trout protamines (iridine) and nucleotide sequences of the amino acid coding portions of 10 cloned protamine cDNAs. Amino acid sequences (a) are cited from Ando & Watanabe (1969) and Gedamu et al. (1981). The nucleotide sequences (b) together with the predicted amino acid sequences of pTP 4, pTP 8, and pTP 11 are from Jenkins (1979), those of pPc6b, pPc2i, and pPc2e are from Sakai et al. (1981), and those of pRTP43, pRTP178, pRTP94, pRTP59, pRTP242, and pRTP131 are from Gedamu et al. (1981). Arginine is distinguished from other amino acids by shading. Dashes are used to indicate gaps, which were introduced to achieve optimal sequence alignment. In natural protamines, methionine at the NH_2-terminal is processed.

Table I: Codon Usage in Cloned Protamine Genes[a]

UUU	Phe	0	UCU	Ser	0	UAU	Tyr	0	UGU	Cys	0
UUC	Phe	0	UCC	Ser	3	UAC	Tyr	0	UGC	Cys	0
UUA	Leu	0	UCA	Ser	0	UAA	END	0	UGA	END	0
UUG	Leu	0	UCG	Ser	0	UAG	END	10	UGG	Trp	0
CUU	Leu	0	CCU	Pro	4	CAU	His	0	CGU	Arg	35
CUC	Leu	0	CCC	Pro	15	CAC	His	0	CGC	Arg	66
CUA	Leu	0	CCA	Pro	0	CAA	Gln	0	CGA	Arg	8
CUG	Leu	0	CCG	Pro	0	CAG	Gln	0	CGG	Arg	7
AUU	Ile	0	ACU	Thr	0	AAU	Asn	0	AGU	Ser	0
AUC	Ile	3	ACC	Thr	0	AAC	Asn	0	AGC	Ser	8
AUA	Ile	0	ACA	Thr	0	AAA	Lys	0	AGA	Arg	26
AUG	Met	5	ACG	Thr	0	AAG	Lys	0	AGG	Arg	50
GUU	Val	0	GCU	Ala	0	GAU	Asp	0	GGU	Gly	0
GUC	Val	7	GCC	Ala	7	GAC	Asp	0	GGC	Gly	10
GUA	Val	0	GCA	Ala	0	GAA	Glu	0	GGA	Gly	10
GUG	Val	10	GCG	Ala	0	GAG	Glu	0	GGG	Gly	0

[a] Codon usage is tabulated for the five complete and five incomplete nucleotide sequences of the cDNA presented in Figure 1b.

are fibroin mRNA (Tsujimoto & Suzuki, 1979), lipotropic hormone mRNAs (Nakanishi et al., 1979), dog β-globin mRNA (Dayhoff, 1972), metallothionein mRNA (Glanville et al., 1980), and protamine mRNA (Gedamu et al., 1981; Jenkins, 1979; Sakai et al., 1981).

This paper reports studies on the translational fidelity in vitro with protamine mRNA of the rainbow trout. The reasons for choosing this mRNA were as follows: Protamines are a family of basic proteins each containing about 30 amino acid residues of only seven types, i.e., Arg, Ile, Val, Ser, Pro, Ala, and Gly. About two-thirds of these amino acid residues are arginine. Six types of protamines with different primary sequences have been found by amino acid sequencing (Figure 1a) (Ando & Watanabe, 1969), and five complete and five incomplete sequences have been deduced by nucleotide sequencing of protamine cDNAs from rainbow trout in Canada (Gedamu et al., 1981), England (Jenkins, 1979), and Japan (Sakai et al., 1981) (Figure 1b). The codon usage of the mRNAs is shown in Table I. Thus, it should be easy to determine misincorporation of amino acids not usually present in the proteins in a cell-free translation system dependent on these mRNAs.

On the basis of these considerations, our protocol for studying misincorporation of an incorrect amino acid by anomalous codon–anticodon interactions was as follows: (1) purification of protamine mRNA to homogeneity; (2) translation of the mRNA in a double-label assay with the ^{14}C-labeled correct amino acid, arginine, and the ^{3}H-labeled incorrect amino acid, such as lysine; (3) isolation of the monosomes translating the mRNA or (4) electrophoretic separation of the translation products; and (5) determination of the error frequency from the molar ratio of incorrect to correct amino acids incorporated.

We report here detailed conditions for determination of the fidelity of decoding in a cell-free translational system dependent on protamine mRNA. Application of the method to

study the translational fidelity of ribosomes derived from young and old mice has been reported elsewhere (Mori et al., 1983).

Experimental Procedures

Materials

Animals. The animals used in this work were mice (ddY strain, male), rats (Wistar STD, male), and rabbits. Reticulocytes were induced in the rabbits by treatment with β-acetylphenylhydrazine. The testes of rainbow trout (*Salmo gairdnerii*) were collected from freshly killed trout at Tokyo Metropolitan Fisheries Experimental Station, Okutama Branch, in December, frozen rapidly on dry ice, and stored at −80 °C.

Chemicals. The sources of materials used in this work were as follows: amino acids from Ajinomoto, Tokyo; radioactive amino acids from Amersham. Their specific activities were as follows: [^{14}C]arginine, 344 mCi/mmol; [^{3}H]lysine, 88 and 100 Ci/mmol; [^{3}H]histidine, 51 Ci/mmol; [^{3}H]cysteine, 0.92 Ci/mmol; [^{3}H]leucine, 118 Ci/mmol; [^{3}H]serine, 8.9 Ci/mmol; [^{3}H]methionine, 15 Ci/mmol. ATP and GTP were from Boehringer-Mannheim; RNase A, phosphocreatine, creatine phosphokinase, and puromycin were from Sigma; casamino acids were from Difco; ethidium bromide was from Aldrich; urea was from Schwarz/Mann. All other reagents were of analytical grade.

Methods

Preparation of Protamine mRNA. Protamine mRNA was extracted from rainbow trout testis essentially by the method and Gedamu & Dixon (1976). Frozen testis was thawed in 3 volumes of 0.02 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 7.4, containing 0.1 M NaCl, 0.003 M $MgCl_2$, and 0.1% Triton X-100 and homogenized in a mixer (Hitachi VA-833). The homogenate was centrifuged at 9000 rpm ($13000g_{max}$) for 10 min, and the supernatant was adjusted to 2% sodium dodecyl sulfate and 10 mM ethylenediaminetetraacetic acid (EDTA) and mixed with an equal volume of phenol/chloroform/isoamyl alcohol (50/49/1 v/v). The mixture was shaken vigorously and centrifuged. RNA in the aqueous phase was precipitated with ethanol and dissolved in 0.01 M Tris-HCl, pH 7.4, containing 0.5 M KCl. The solution was passed through a column (0.7 × 1 cm) of oligo(dT)–cellulose, and poly(A)-containing RNA bound on the column was eluted with 0.01 M Tris-HCl, pH 7.4. This RNA was rechromatographed after heat denaturation and purified further by centrifugation in a 5–20% sucrose gradient in NETS buffer (0.05 M NaCl, 0.01 M EDTA, 0.01 M Tris-HCl, pH 7.4, and 0.2% sodium dodecyl sulfate) at 38 000 rpm for 15 h at 22 °C in an RPS40T rotor of a Hitachi 65P-7 ultracentrifuge. Peak fractions of 6–7 S were collected, and the RNA was precipitated with ethanol. The recovery of poly(A)-containing 6–7S RNA (named protamine mRNA or P-mRNA) was 0.1–0.2 mg per 100 g of testis.

Preparation of Liver mRNA. Mouse liver poly(A)-containing mRNA (L-mRNA), prepared by two cycles of oligo(dT)-cellulose column chromatography, was a gift from Dr. K. Anzai. The average size of the RNA was 10–18 S. With this mRNA preparation, over 200 different polypeptides were synthesized in a translation system from rabbit reticulocytes (Anzai et al., 1983).

Preparation of Globin mRNA. Globin mRNA (G-mRNA) was prepared by the method of Aviv & Leder (1972) from rabbit reticulocyte polysomes isolated essentially by the method of Crystal et al. (1974). Polysomal RNA was extracted with a phenol/chloroform/isoamyl alcohol mixture and passed through an oligo(dT)–cellulose column. Poly(A)-containing RNA bound on the column was eluted, precipitated with ethanol, and further purified by sucrose gradient centrifugation. Globin mRNA is 9S peak fractions were precipitated with ethanol and dissolved in water at a concentration of 0.1–0.3 mg/mL.

Preparation of Ribosomes. Ribosomes were prepared by the method of Falvey & Staehelin (1970) with some modification (Mori et al., 1979). Briefly, polysomes were prepared from livers of mice and treated with puromycin (Blobel & Sabatini, 1971). Ribosomes were precipitated through two layers of a discontinuous sucrose cushion containing 0.3 M KCl and 3 mM magnesium. Sedimentation analysis indicated that the ribosomes remained 80S monomers in buffer containing 5 mM magnesium but were separated into 60S and 40S subunits in buffer containing 3 mM magnesium and 0.3 M KCl. The ribosomes were stored at −80 °C at a concentration of 30–100 A_{260} units/mL in low-salt buffer containing 0.2 M sucrose.

Preparation of Soluble Translational Factors. Soluble translational factors were obtained from rabbit reticulocyte ribosomes by the method of Crystal et al. (1974). The ribosomal wash fraction was dialyzed and passed through a column of DEAE-cellulose. Proteins in fractions eluted with 0.3 M KCl were precipitated by adding ammonium sulfate to 68% saturation. The final precipitates were dissolved in 0.02 M Tris-HCl buffer, pH 7.4, containing 0.35 M KCl, 0.1 mM EDTA, and 1 mM dithiothreitol, and the solution was dialyzed against the same buffer and stored at −80 °C. The method was originally used to prepare initiation factors, but the final preparation contained sufficient activities for aminoacylation of tRNAs and for elongation of peptides with natural mRNA.

Preparation of Transfer RNA. Transfer RNAs were prepared from mouse and rat livers by the procedure of Nishimura (1971) with a few modifications. RNA was extracted with phenol from the cytoplasmic extract adjusted to 1 M NaCl. Contaminating DNA was removed by precipitation with isopropyl alcohol. RNA in the supernatant was precipitated with ethanol. The RNA precipitate was dissolved in 0.02 M Tris-HCl, pH 9.0, and deacylated tRNAs were purified by DEAE-cellulose column chromatography. tRNA was dissolved in water at a concentration of 22 A_{260} units/mL, and the solution was stored at −80 °C.

Preparation of Histones and Protamines. Histones and protamines were prepared as described by Jergil & Dixon (1970). Acid-soluble proteins were extracted from the washed and swollen nuclei of rainbow trout testis with 0.2 N HCl, and then with 0.1 N H_2SO_4. The proteins precipitated with perchloric acid were dissolved in 0.1 N acetic acid, and histones and protamines were fractionated on a Sephadex G-50 column by monitoring the absorbance at 224 nm. About 30% of the acid-extracted proteins was recovered as histones and 70% as protamines. Fractions containing both histones and protamines provided markers in the anlaysis of translational products by electrophoresis.

Translation Assay. The standard assay mixture (50 μL) contained 0.2–0.6 A_{260} unit of ribosomes, 0.2 μg of protamine mRNA, 3 μg of tRNA, 50 μg of protein equivalent factors, and the following reagents: 1 mM ATP, 0.4 mM GTP, 30 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid/KOH (Hepes/KOH), pH 7.4, 11 mM creatine phosphate, 12μg of creatine phosphokinase, and 0.03 mM amino acid mixture (Met, Ile, Val, Ser, Pro, Ala, and Gly). For determination of the ratio of misincorporation of lysine in place of arginine, 1 μCi of [^{3}H]lysine (10–12 pmol) and 0.1 μCi of [^{14}C]arginine (145 pmol) were added, and then the final

concentration of magnesium acetate was adjusted to 5 mM. The concentration of KCl was 90 mM after the addition of the factor preparation.

For translation of globin or liver mRNA, 19 amino acids (minus leucine) and 1 μCi of [^{3}H]leucine were added instead of the above amino acid mixture, and the magnesium ion concentration was adjusted to 3 mM.

Incubation was carried out for 120 min at 37 °C or as specified in the figure legends. The reaction was stopped by adding 5% trichloroacetic acid, pH 2, containing 0.25% sodium tungstate and 0.1% casamino acids (TCA solution) and heating the mixture for 15 min at 90 °C. The samples were filtered through GF/C (Whatman) and washed, and their ^{3}H and ^{14}C radioactivities were counted by using the external standard method in an Aloka LSC-900 or 700 liquid scintillation counter. For analysis of translational products, the reaction mixture was not treated with acid but was analyzed directly as described in the following sections.

Product Analysis by Sucrose Gradient Centrifugation. After incubation, the reaction mixtures were applied to 4.4 mL of linear gradients of 0.5–1.5 M sucrose in buffer A, and the gradients were centrifuged for 120 min at 50 000 rpm at 4 °C in a Hitachi RPS56T rotor, unless otherwise stated in the legends to the figures. Fractions (0.23 mL) were collected, monitoring A_{254}. Each fraction was mixed with 2 mL of TCA solution, and its hot acid-precipitable radioactivity was measured.

Product Analysis by Polyacrylamide Gel Electrophoresis. After 120 min of incubation, the reaction mixtures were treated with RNase A (5 μg/mL) for 5 min at 37 °C. The reaction was stopped by addition of an equal volume of 2 times concentrated solubilization buffer (0.2 N HCl, 8 M urea, 10% v/v β-mercaptoethanol, and 20% v/v glycerol containing methyl green). The samples were applied to 11% polyacrylamide [acrylamide/bis(acrylamide), 10/1 w/w] disk gels (0.5 × 10 cm) containing 7 M urea and 0.1 M sodium acetate buffer, pH 4.5. Electrophoresis was carried out at 1 mA/tube for 30 min and then at 4 mA/tube for 4 h. The reservoir contained 0.017 M sodium acetate buffer, pH 4.5, with 0.035 M β-alanine. The gels were stained with 0.1% Amido black 10B and destained with 7.5% acetic acid. At least five changes of the destaining solution were necessary for sufficient reduction of the background radioactivity due to free amino acids. Radioactivity in gels sliced at 5-mm intervals was measured by either of the following methods: (1) The slices were digested in 1 mL of hydrogen peroxide (36% w/v) for 10–16 h at 60 °C, and the radioactivity was measured in 10 mL of Triton/toluene scintillation cocktail (20% Triton X-100, 80% toluene, and 0.35% diphenyloxazole (method A). (2) The sliced gels were dried and oxidized with sample oxidizer (Packard Tri-Carb 3000), and ^{3}H and ^{14}C radioactivities were measured separately. (3) In separate experiments, hot TCA solution precipitable materials in translation products were obtained by centrifugation, and basic proteins were extracted from the precipitates with 0.1 N HCl. Then the extracts were subjected to electrophoresis, and the gels were treated as described in method A.

Quantitation of Error Frequency. Translational error frequency was determined as the molar ratio of lysine misincorporated in place of arginine. It was measured after separation of ribosomes on sucrose gradients or after separation of the products on polyacrylamide gels (see above). In the former case, the moles of [^{3}H]lysine and [^{14}C]arginine incorporated into the 80S monosome region, usually fractions 5–9, were calculated. The error frequency is expressed as the molar ratio of lysine to the sum of lysine and arginine incorporated for the AGR codons only, i.e., the molar ratio of lysine to the sum of the lysine residues and 76 out of 192 of the arginines incorporated (See Discussion for details). As endogenous incorporation of the amino acids were negligible in this case, the control without exogenous mRNA was not used. In the latter case, the moles of [^{3}H]lysine and [^{14}C]-arginine incorporated into slices containing protamines with or without mRNA were measured. The error frequency is expressed as the molar ratio of the net incorporation of lysine to the sum of the net incorporation of lysine and 76 out of 192 residues of arginine. With this method, subtraction of the endogenous incorporation was necessary, because the background radioactivity in the gels was not negligible.

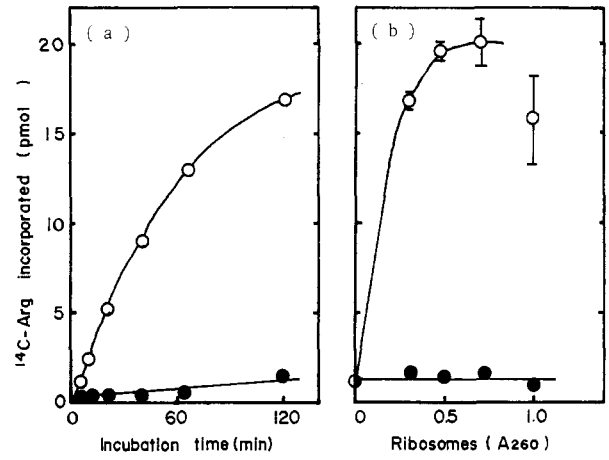

FIGURE 2: Incorporation of [^{14}C]arginine into hot trichloroacetic acid/tungstate-insoluble material in the presence (○) and absence (●) of protamine mRNA. (a) Time course of the reaction; reaction mixtures (125 μL) were incubated at 37 °C, and aliquots of 20 μL were withdrawn at the indicated times for radioactivity determination. (b) Effect of the concentration of ribosomes. Reaction mixtures (50 μL each) containing 0–1 A_{260} unit of ribosomes as indicated were incubated at 37 °C for 60 min with or without 0.2 μg of protamine mRNA and assayed for incorporation of arginine into hot acid-insoluble materials.

RESULTS

Cell-Free Translation Directed by Protamine mRNA. The components of the standard reaction mixture are described under Methods. They are essentially the same as those in usual reconstituted translation systems (Merrick, 1979; Staehelin et al., 1979) except that only the seven amino acids described above were included in addition to two radioactive amino acids. The optimal concentration of each component was determined experimentally.

Arginine was incorporated into hot acid-precipitable materials in the presence of protamine mRNA (Figure 2a,b). The [^{14}C]arginine found in the top regions of the gradients without exogenous message was probably due to arginyl-tRNA, which is partially resistant to hot acid treatment (Figure 4c,d). The translation was reduced to a certain extent when the concentration of ribosomes was more than 0.8 A_{260} unit per 50 μL of reaction mixture (Figure 2b). Otherwise, translation continued for at least 120 min at 37 °C (Figure 2a). In the absence of ribosomes, tRNA, factors, or mRNA, the incorporation of [^{3}H]arginine was less than 10% of that for the complete mixture (data not shown).

Translation of Protamine mRNA on Monosomes. The length of rabbit globin mRNA (G-mRNA) and rainbow trout protamine mRNA (P-mRNA) were shown to be about 550–600 and 250-300 nucleotides, respectively, by electrophoresis on an 11% polyacrylamide gel in the presence of 7

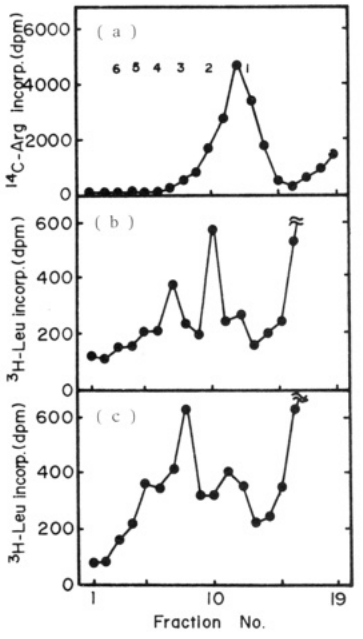

FIGURE 3: Sucrose gradient analysis of mono- and polysomes formed with protamine, globin, and mouse liver mRNA species in the cell-free translation system. About 0.2 μg of protamine (a), globin (b), and mouse liver (c) mRNAs, respectively, was assayed for translational activity in the standard assay (50 μL) with 0.1 μCi of [^{14}C]arginine (a) or 1 μCi of [^{3}H]leucine (b and c). After incubation at 37 °C for 90 min, 50 μL of the reaction mixtures was chilled and layered on 0.5–1.5 M sucrose gradients, and the gradients were centrifuged for 120 min at 50 000 rpm at 4 °C in an RPS56T rotor. Then gradient fractions (0.24 mL) were collected. The hot acid-precipitable radioactivity of each fraction was measured as described under Methods.

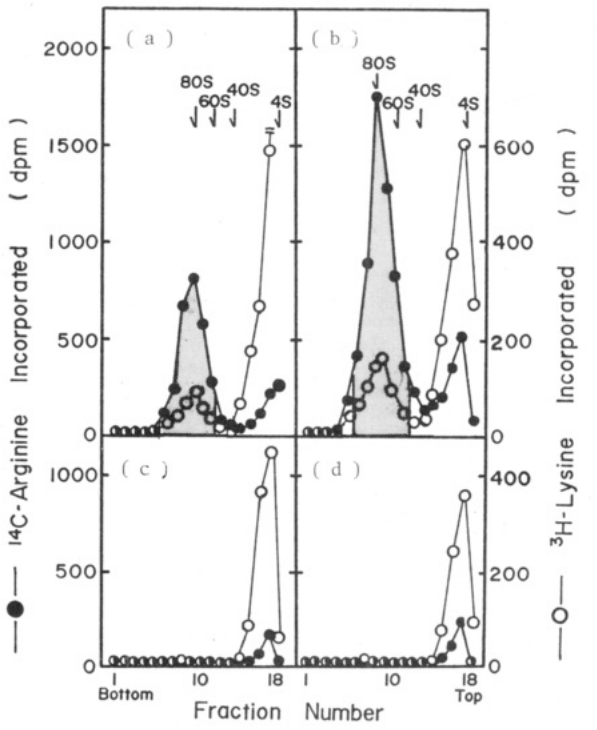

FIGURE 4: Incorporation of arginine and lysine into nascent polypeptides on ribosomes translating protamine mRNA. In vitro translation was carried out for 45 (a) and 90 min (b) with 0.2 μg of protamine mRNA in a total volume of 50 μL. Control incubations were carried out for the same time in the absence of mRNA (c and d). After incubation, the samples were chilled and applied to linear gradients of 0.5–1.5 M sucrose. Centrifugation was carried out for 90 min in this experiment. The absorbance at 254 nm was monitored continuously during the fractionation. Fractions of 0.24 mL were collected for measurement of radioactivity. The radioactivities of [^{14}C]arginine (●) and [^{3}H]lysine (○) incorporated into hot trichloroacetic acid/tungstate-precipitable materials are shown. The profile of adsorbance (A_{254}) is not shown, but the positions of 80S ribosomes and 60S and 40S subunits are indicated by arrows.

M urea (data not shown). Most of the poly(A)-containing RNAs extracted from the cytoplasm of mouse liver (L-mRNA) were longer than 800 nucleotides (data not shown). Judging from the length of each RNA and the distance between ribosomes in polysomes (about 100 nucleotides) (Lehninger, 1975), P-mRNA, G-mRNA, and L-mRNA are expected to be translated on monosomes, pentasomes, and heptasomes and longer polysomes, respectively. Although the polysome sizes observed (Figure 3) were smaller than those expected from the sizes of the mRNAs, they were roughly in proportion to the expected values.

It should be noted that in the case of P-mRNA only a small portion (less than 10%) of the polypeptides was released from the ribosomes during translation, while in the cases of G-mRNA and L-mRNA, 53% and 44%, respectively, of the polypeptides were released. Thus, most protamine molecules appeared to remain bound on ribosomes even after completion of translation in vitro (see Figure 5). This was probably because protamine is a highly basic protein and, therefore, tends to associate with rRNA.

Misincorporation of Lysine. The finding that newly synthesized protamines remained associated on monosomes suggests the interesting possibility of examining mistranslation of P-mRNA without purification of the products. We therefore examined whether amino acids not coded in P-mRNA, for example, lysine (see Table I), were incorporated into polypeptides on monosomes. In theory, the following amino acids could be incorporated into protamines by an error of one letter in the triplet codons of arginine (codons CGN, AGR), cysteine (codons *U*GY/*C*GY),[1] histidine (codons C*A*Y/C*G*Y), and lysine (codons A*A*R/A*G*R). We first examined the possibility of misincorporation of lysine.

After translation in the presence of [^{14}C]arginine and [^{3}H]lysine, assay mixtures were subjected to sucrose gradient centrifugation (Figure 4). In addition to [^{14}C]arginine, [^{3}H]lysine was incorporated into acid-insoluble materials in the monosome region only when the translation mixtures were incubated with P-mRNA (Figure 4a,b). Without the mRNA, no radioactivity was found in the monosome region, but significant radioactivity was detected at the top of the gradients (Figure 4c,d).

This was probably due to aminoacyl-tRNAs which are partially resistant to hot acid treatment, because similar radioactivity was also found without P-mRNA (see Figure 2). This finding suggests that with P-mRNA, 20–50% of the radioactivity at the top (4 S) of the gradients is due to aminoacyl-tRNAs not to protamines released from ribosomes. Therefore, the incorporation of amino acids into protamines cannot be determined by simply measuring the total radioactivity incorporated into acid-insoluble materials.

Then we determined the error frequency of translation by calculating the moles of arginine and lysine incorporated into monosome regions (Table II). In two sets of experiments, the error frequency was 0.0006–0.0017; i.e., misincorporation of one lysine molecule per 590–1700 arginine molecules. The values are apparently lower than those in the poly(U) system (Kurland, 1979; Yarus, 1979; Lengyl et al., 1961; Mori et al., 1979; Laughrea, 1981).

[1] This should be read as "cysteine would be incorporated by misrecognition of *U* for *C* at the first position of the arginyl codon CGY" and so forth.

Table II: Lys/Arg Error Frequency Determined by Sucrose Gradient Analysis

expt	reaction time (min)	[^{14}C]Arg on monosomes (dpm)	[^{3}H]Lys on monosomes (dpm)	error, Lys/(Arg + Lys)
1	45	2680	254	0.00109
	90	5540	292	0.00060
2	45	6640	949	0.00164
	90	7750	997	0.00134

[a] The ^{3}H or ^{14}C radioactivity in the monosome region (fractions 6–11) in Figure 4a,b was determined and the error frequency was calculated as described under Methods (experiment 1). In experiment 2, the reaction volume was 100 μL. Centrifugation was carried out for 90 min at 46 000 rpm, and the error frequency was calculated from the radioactivity in fractions 10–13.

The apparent error frequency varied with the conditions of assay, e.g., the concentration of Mg^{2+} ion, ionic strength, and tRNA concentration (M. Itoh, Y. Endo, N. Mori, and S. Goto, unpublished results). In the present experiment, translation was carried out at a magnesium ion concentration of 5 mM, which gave optimal efficiency and fidelity of protamine synthesis.

Misincorporation Frequencies of Other Amino Acids. We next examined the incorporation of other amino acids coded by P-mRNA, i.e., serine and methionine, and not coded by P-mRNA, i.e., lysine, histidine, cysteine, and leucine (Table III; see also Table I). Almost the same amount of arginine was incorporated into the monosome region in all cases, i.e., 2500–3300 dpm per assay. As observed in experiments with arginine and lysine (Figures 4 and 3a), both coded and noncoded amino acids were incorporated into acid-insoluble materials in monosomes and the top region of sucrose gradients (not shown).

We then calculated and molar ratios of the two amino acids from the radioactivities found in monosome regions. As expected, the observed molar ratios, ^{3}H-amino acid/[^{14}C]arginine incorporated, were different for different sets of amino acids. Of the four noncoded amino acids, lysine and histidine were incorporated at low frequencies, while leucine and cysteine were incorporated more frequently. Moreover, as expected, serine and methionine, which are coded by P-mRNA, were incorporated more frequently, i.e., 1 mol of amino acid per 5.9 and 15.4 mol of arginine, respectively. These molar ratios are in good agreement with the values calculated from the codon frequencies of these amino acids in P-mRNA, i.e., 1 Ser/5.5 Arg and 1 Met/22 Arg (see Table I).

Evidence of Misincorporation of Lysine into Protamine Molecules. In the experiments described above, radioactive acid-insoluble materials associated with monosomes were regarded as protamines. To confirm that this was the case, we

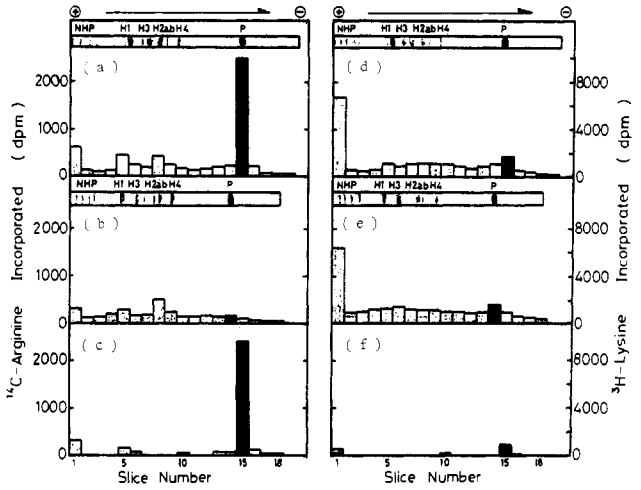

FIGURE 5: Analysis of the products of cell-free translation directed by protamine mRNA. The polypeptides synthesized in a reaction mixture (100 μL) containing [^{3}H]lysine and [^{14}C]arginine were treated with RNase A and separated by acid/urea–polyacrylamide gel electrophoresis. The gels were stained with Amido black 10B, destained, and cut into 5-mm-thick sections. The slices were digested with hydrogen peroxide, and the radioactivity was measured. [^{14}C]Arginine incorporation in the presence (a) or absence (b) of 0.4 μg of protamine mRNA; (c) net incorporation of [^{14}C]arginine after subtraction of the background radioactivity in (b) for the radioactivity in (a); incorporation of [^{3}H]lysine (d–f) under conditions similar to those for (a–c). Schematic patterns of staining in panels a, b, d, and e represent the positions of migration of histones and protamines used as carriers for extraction of translation products and as internal markers on electrophoresis.

analyzed the translation products by polyacrylamide gel electrophoresis (Figure 5). The mobilities of histones (H1, H2A, H2B, H3, and H4) and protamines relative to the tracking dye were about 0.3–0.6 and 0.9, respectively. The mobilities of basic proteins were inversely proportional to their molecular weights in this gel.

Over 80% of the radioactivity of arginine was found in the slices containing authentic protamines (Figure 5a), the rest being distributed all over the gels. This background radioactivity was also found without P-mRNA (Figure 5b). By subtraction of the background value, over 95% of the net incorporation of arginine was calculated to be in protamines (Figure 5c).

On the other hand, after subtraction of the background (Figure 5e), net incorporation of [^{3}H]lysine into protamine-containing slices was found to be small but definite (Figure 5f). The background radioactivity was also found in the gel when the assay mixture without incubation was examined (data not shown). Therefore, the radioactivity spread all over

Table III: Comparison of Molar Ratios of Amino Acids Incorporated into Monosomes Translating Protamine mRNA

amino acid	^{3}H-amino acid[a] concn (pmol/μCi)	^{3}H-amino acid[a] sp act. (dpm/fmol)	^{3}H-amino acid incorpd[b] (dpm)	[^{14}C]Arg incorpd[b] (dpm)	molar ratio obsd[c]	molar ratio expected[d]	error frequency[e]
Lys	10.0	177	585	3330	1/1260	0	0.00201
His	19.5	74.4	141	2970	1/1950	0	0.00130
Leu	8.5	191	1090	2520	1/552	0	0.00458
Cys	1090	1.6	416	2580	1/12.2	0	0.208
Met	1566	1.2	141	2970	1/15.4	1/22	
Ser	1612	0.7	585	3330	1/5.9	1/5.5	

[a] The specific activities of amino acids and their concentrations in the reaction mixture are shown. As the mixture contained 1.5 μCi of each amino acid, their concentrations are different. In the reactions with methionine and serine, the concentrations of each cold amino acid in the reaction mixture are considered in the calculations of the concentration and specific activity. [b] Total radioactivity incorporated into the monosome region. [c] Values are molar ratios of ^{3}H-labeled amino acid to ^{14}C-labeled arginine in the monosome region. Values are not identical with error frequencies, because they are not corrected for concentration by a factor of 76/192 (see text for details). [d] Molar ratios calculated from the frequencies of usage of each amino acid and arginine from the codon-usage table for protamines (see Table I). [e] Molar ratios corrected by a factor of 76/192 for the observed ratio of each amino acid to arginine.

Table IV: Misincorporation of Lysine Instead of Arginine into Protamine Synthesized in Vitro

	incorpn with P-mRNA[b]		endogenous incorpn[c]		net incorpn[d]		
expt[a]	[^{3}H]Lys (dpm)	[^{14}C]Arg (dpm)	[^{3}H]Lys (dpm)	[^{14}C]Arg (dpm)	[^{3}H]Lys (dpm)	[^{14}C]Arg (dpm)	error frequency[e]
1	1680	2480	1150	189	126	2290	0.000630
2	2790	2730	2380	302	412	2430	0.00195
3	1660	2420	1450	73	213	2350	0.00104

[a] In experiments 1 and 2, gel slices were treated by method 2 as described under Methods. In experiment 3, the sample was treated by method 1. [b] Radioactivity found in the slice containing protamine in assays with protamine mRNA. See slice 15 in (a) and (d) in Figure 5 for experiment 1. [c] Assays without exogenously added mRNA. Slice 14 in (b) and (e) in Figure 5 for experiment 1. [d] Radioactivity in *b* minus that in *c*. Slice 15 in (c) and (f) in Figure 5 for experiment 1. [e] Error frequency calculated from the net incorporation of amino acids. When the positions of protamine differed in gels with or without protamine mRNA, net incorporation was calculated from the radioactivities in the slices containing protamine. For example, slice 15 in (a) and (d) minus slice 14 in (b) and (e), respectively, in Figure 5 for experiment 1.

the gels (Figure 5b,e) is probably that of free radioactive amino acids, not polypeptides synthesized by comtaminating mRNA. It is, therefore, concluded that the acid-insoluble radioactivity found in the monosome region on sucrose gradients (Figure 4) is in fact due to lysine and other amino acids misincorporated into protamine molecules.

We calculated the lysine/arginine substitution frequency from the radioactivity in the slices containing protamine (Table IV). Three independent electrophoretic analyses were made, and calculations were made from results using different conditions for preparation of ribosomes and counting of radioactivity (see Experimental Procedures). The molar ratio of lysine/arginine was again found to be 0.00063–0.00195, values being of the same order of magnitude as the ratio observed on sucrose gradient analysis (Table II).

Discussion

Usefulness of Protamine mRNA for Study of Translational Fidelity. We have developed an in vitro assay method for estimating the translational fidelity of eukaryotic ribosomes using protamine mRNA of rainbow trout. The reasons for choosing this mRNA are described in the introduction. In addition, this mRNA has the practical advantages of being easy to purify in relatively large amounts, i.e., 0.1–0.2 mg/100 g of testis, and of being stable for at least 1 year at −80 °C, probably because of its short nucleotide length and its high GC content.

In determination of the fidelity of decoding protamine mRNA in a cell-free translation system, the purity of radioactive amino acids used in the experiments is important. In the present experiments, analysis of the [^{3}H]Lys preparation by paper chromatography revealed that it does not contain any detectable [^{3}H]Arg (data not shown). Thus, possible comtamination of [^{3}H]Arg in the [^{3}H]Lys preparation does not pose a problem in our experiments. In addition, it is important to exclude any possibilities of incorporation of [^{3}H]lysine due to contaminating mRNAs or their fragments with lysyl codons. Ribosomal preparations or protamine mRNA preparations could be comtaminated with other mRNAs or their fragments. However, the possibility of contamination could be excluded for the following reasons: (1) Ribosomes were obtained by puromycin treatment of polysomes and were dissociated completely into equimolar amounts of large and small subunits in a solution containing 3 mM Mg^{2+} and 0.3 M KCl, suggesting that no appreciable amounts of mRNA fragments or peptidyl-tRNA remained in the ribosomal preparations (Falvey & Staehelin, 1970). (2) Possible contaminating mRNAs or their fragments would not be efficiently translated, because there were only nine types of amino acids in the reaction mixture. (3) The protamine mRNA preparation seemed to be at least 97% pure as judged from the size distribution of translation products on the polyacrylamide gel, and it did not appear to contain mRNAs for histones (Figure 5), which would affect the results of this assay most, since histone mRNAs contain a large proportion of codons for lysine and histidine (Isenberg, 1979).

Thus, error due to contaminating messages in estimation of the translational fidelity of protamine mRNA seemed to be negligible. We therefore concluded that any amino acids incorporated in the present assay could be regarded as protamine mRNA dependent.

Evaluation of the Misreading Pattern in the Protamine System. In the protamine mRNAs or cDNA so far sequenced, there are 192 arginine codons, among which 76 could be misread as lysine codons by misrecognition of a single base at the second position of codons AGA and AGG. The other four arginine codons would hardly be misrecognized as lysine codons because two bases in a codon must be misrecognized for this type of error to occur. Therefore, we assumed that 76 out of 192 (=0.396) of the arginine molecules could be replaced by lysine. We took this value into consideration in estimation of the error frequency (see Methods).

There are, however, other possibilities to explain misincorporation of lysine into protamine. First, lysine may be incorporated into protamine by an error in the aminoacylation of tRNA. This type of error should contribute as a background value to the error frequency of decoding in this assay. The relative importance of this type of error must be determined experimentally in the future. However, when the translational fidelities of different ribosomal preparations are compared, it is possible to estimate the differences in error frequency due to misrecognition between the codon and anticodon, because in the test samples components other than ribosomes are the same. Second, lysine might be misincorporated by misrecognition at the first or third base in the codon. Bases at these positions are known to be more frequently misrecognized than those at the second position. This possibility, however, could be ruled out, because protamines do not contain any amino acids that could be replaced by lysine by misrecognition of the first or third base of the codons, i.e., glutamine, glutamic acid, or aspartic acid. A final possibility was that lysine was incorporated by misrecognition of the second base of codons for amino acids other than arginine. However, this possibility was also excluded by the fact that protamine synthesized in vivo does not contain AUA-, AUG-, or ACR-coded amino acids, i.e., isoleucine, methionine, or threonine, respectively. Of these codons, AUG must be present in protamine mRNA as an initiation codon of translation. It is therefore possible that this codon is misrecognized as a lysine codon, AAG. However, there is only one methionine codon in protamine mRNA, so the contribution of this type of error can be neglected in the present estimation. We therefore conclude that lysine was incorporated into protamine by misrecognition of guanine for adenine at the second position

of two of the six degenerated arginyl codons (AGR).

In addition to misrecognition of arginine codons for lysine codons, we also examined other types of misreading (Table III). The misincorporations of cysteine (codon, UGY) and leucine (codons, UUR and CUN) were about 20 and 2–3 times higher, respectively, than that of lysine. The high misincorporation of cysteine could be explained by the general tendency (Yarus, 1979; Jergil & Dixon, 1970) for errors to be more frequent at the first position of codons than at the second position. In this case, codon CGY for arginine might be recognized as UGY. Misreading of AGC (codon for Ser) or GGC (codon for Gly) as a codon for cysteine is also possible, but the contribution of this error must be small, because of the low frequencies of these codons in mRNA. Misincorporation of leucine can occur by misrecognition of the arginine codon (CGY) or proline codon (CCY) as CUY or of the isoleucine codon (AUC) or valine codon (GUC) as CUC.

Optimized Conditions for Translation. For examination of the translational fidelity of mouse liver ribosomes, we used an assay mixture consisting of mouse liver ribosomes, soluble factors of rabbit reticulocytes, mRNA, tRNA of mouse or rat, and other components necessary for translation. The optimal or sufficient concentrations of each component were determined experimentally. The optimal concentration of magnesium ion was about 3 mM for translation of globin or liver mRNA but 5 mM for translation of protamine mRNA (data not shown). Natural mRNAs are, in general, translated most efficiently at lower concentration (2–5 mM) of magnesium ion than those required by synthetic polynucleotides (5–20 mM) (Merrick, 1979; Staehelin et al., 1979; Laughrea, 1981). With protamine mRNA, however, 5 mM was optimal not only for the translational efficiency but also for the accuracy. In this point protamine mRNA differs from poly(U) template, with which higher accuracy was achieved at lower magnesium concentration than the optimum concentration for activity (Mori et al., 1979). The efficiency and fidelity of the present experimental system may be improved by adding polyamines or some additional divalent cations as has been done in a prokaryotic system (Jelenc & Kurland, 1979).

Application of the Method for Studies of the Mechanisms of Achieving High-Fidelity Translation. In the present system, the translational error is the sum of the errors in charging and decoding. Hopfield (1974) and Ninio (1975) independently pointed out that proofreading mechanisms would act at both these steps under certain circumstances. Yamane & Hopfield (1977) have shown that energy from ATP hydrolysis is used to reduce mischarging. This type of proofreading is not limited to aminoacylation. Recently, reports from Thompson's laboratory (Thompson & Stone, 1977; Thompson et al., 1981) have shown that GTP hydrolysis associated with poly(U)-dependent binding of aminoacyl-tRNA to *E. coli* ribosomes could allow a second check to eliminate a noncognate amino acid before it is incorporated into nascent polypeptides. Thus, bacterial ribosomes seem to be able to check some *near cognate*[2] aminoacyl-tRNA species at least by the first base of a codon. What about eukaryotic ribosomes and their recognition of the first or second position of codons? The recognition of pyrimidine bases at the second position could also be examined in their poly(U)-dependent system by using seryl-tRNA$_1$, which is cognate for UCY codons. The protamine mRNA directed translation system should be useful in further study of base recognition of the first and second positions of codons other than UUU, using isoaccepting species of lysyl, histidyl, and other tRNAs.

[2] This word is used as in Thompson's papers.

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Dicyclohexylcarbodiimide Inhibits the Monoamine Carrier of Bovine Chromaffin Granule Membrane†

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ABSTRACT: The monoamine carrier of bovine chromaffin granule membrane catalyzes a H^+/neutral amine antiport. Dicyclohexylcarbodiimide (DCCD) inhibits this carrier in a time- and concentration-dependent manner as shown by the following evidence: (i) it inhibits the carrier-mediated pH gradient driven monoamine uptake without collapsing the pH gradient; (ii) it affects the binding of the specific inhibitors [2-^{3}H]dihydrotetrabenazine and [^{3}H]reserpine. The DCCD inhibition of the carrier occurs in the same concentration range as that of the ATP-dependent H^+ translocase. Saturation isotherms of [2-^{3}H]dihydrotetrabenazine binding indicate that DCCD decreases the number of binding sites without any change of the equilibrium dissociation constant. Kinetic studies of DCCD inactivation indicate that the modification of only one amino acid residue is responsible for the inhibition. Preincubation of the membranes with tetrabenazine protects the carrier against inactivation by DCCD: in this case, [2-^{3}H]dihydrotetrabenazine binding and pH gradient driven monoamine uptake are restored after washing out of DCCD and tetrabenazine. We suggest the existence in the monoamine carrier of a carboxylic acid involved in H^+ translocation, similar to those demonstrated not only in F_0–F_1 ATPases but also in cytochrome *c* oxidase, mitochondrial cytochrome b–c_1 complex, and nucleotide transhydrogenase. Protonation–deprotonation of this group would affect the binding of [2-^{3}H]dihydrotetrabenazine by the carrier.

In the adrenal medulla, the catecholamines adrenaline and noradrenaline are mainly located in specific organelles, the chromaffin granules. The large concentration gradient existing between these organelles and the cytosol is the result of an ATP-dependent active transport system located in the granule membrane (Kirshner, 1962; Carlsson et al., 1963). This system involves (i) an inwardly directed ATP-dependent H^+ translocase (H^+ pump), which generates an electrochemical proton gradient $\Delta\mu_{H^+}$ (inside acidic and positive) (Casey et al., 1977; Phillips & Allison, 1978; Johnson & Scarpa, 1979; Scherman & Henry, 1980a), and (ii) a specific monoamine carrier driven by the $\Delta\mu_{H^+}$, which catalyzes an electrodissipative H^+/neutral monoamine antiport (Johnson & Scarpa, 1979; Apps et al., 1980a; Scherman & Henry, 1980b; Kanner et al., 1980; Knoth et al., 1980). The monoamine carrier is inhibited by the drugs tetrabenazine (TBZ)[1] and reserpine (Pletscher, 1976; Scherman & Henry, 1980c).

In order to elucidate the mechanism of amine translocation, we have investigated the effect of chemical modifications of the catecholamine carrier. The activity of the carrier of chemically modified resealed chromaffin granule ghosts was estimated under conditions where the H^+ pump was bypassed: a pH gradient (ΔpH, inside acidic) obtained by a pH jump in the absence of ATP was imposed on ghosts, and the carrier-mediated (tetrabenazine sensitive) amine accumulation inside of the vesicles was followed (Schuldiner et al., 1978; Phillips, 1978). By this technique, we have already described an inhibition of the monoamine carrier by the histidine-specific reagent diethyl pyrocarbonate (Isambert & Henry, 1981). In the present paper, we describe an effect of dicyclohexylcarbodiimide (DCCD), a reagent rather selective for carboxylic acid. This reagent is known to inhibit H^+ translocases of various origins, including that of chromaffin granule membranes (Bashford et al., 1976; Giraudat et al., 1980; Apps et al., 1980b, 1983; Cidon & Nelson, 1983). We now provide evidence that it inhibits also the monoamine carrier in the same concentration range.

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[1] Abbreviations: DCCD, *N*,*N*′-dicyclohexylcarbodiimide; TBZ, tetrabenazine (2-oxo-3-isobutyl-9,10-dimethoxy-1,3,4,6,7,11b-hexahydro-2*H*-benzo[*a*]quinolizine); [^{3}H]TBZOH, 2-[^{3}H]hydroxy-3-isobutyl-9,10-dimethoxy-1,3,4,6,7,11b-hexahydro-2*H*-benzo[*a*]quinolizine; 5-HT, serotonin (5-hydroxytryptamine); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; ATPase, adenosinetriphosphatase.

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