Arginyl Transfer Ribonucleic Acid Protein Transferase and Endogenous Acceptor Proteins in Cultured Mammalian Cells*

BARRY GOZ AND PETER VOYTEK
From the Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

SUMMARY
The presence of arginyl-tRNA protein transferase has been shown in a variety of mammalian cells in culture. Analysis by sodium dodecyl sulfate acrylamide gel electrophoresis of the available endogenous acceptor proteins (proteins that can be arginylated at their amino terminus by this enzyme) reveals that there are at least two such proteins (designated Peaks I and II) with the same relative migration during electrophoresis in a variety of tissues. Analysis of baby hamster kidney cells (BHK), polyoma-transformed BHK, and herpes simplex-infected BHK showed Peak II, but in the general region of Peak I there were two peaks. Other peaks, which varied in migration and appearance, were also observed in the various cell preparations. The incorporation of labeled arginine was inhibited by ribonuclease, canavanine, hemoglobin, and hemin but not by puromycin nor cycloheximide. The specificity of the enzyme from different species for exogenous acceptor proteins of various species is reported.

EXPERIMENTAL PROCEDURE

Cells—BHK21/C131 (baby hamster kidney cells) and BHK21/C13/PYH3 (PYH3) a line cloned after polyoma virus transformation of BHK21/C13, were grown in Dulbecco’s Medium with 10% fetal calf serum. HeLa (human cervical carcinoma), Hep2 (human carcinoma of the larynx), African green monkey kidney cell lines (BSC-1, Vero, and CV 1), and MDBK cells (bovine kidney) were grown in Dulbecco’s Medium with 10% calf serum and L cells (mouse fibroblasts) in Minimum Essential Medium, Eagle (Hanks’ salts) with 10% fetal calf serum. L5178Y cells, a mouse leukemic cell line, were grown in suspension culture with Fischer’s medium containing 10% horse serum.

Preparation of Cell Extracts—The cultured cells were drained of medium and washed with phosphate-buffered saline (137 mM NaCl, 8.1 mM Na2HPO4, 2.6 mM KCl, and 1.4 mM KH2PO4). More phosphate-buffered saline was added and the cells scraped off with a rubber policeman. The cells were collected by low speed centrifugation and the sediment resuspended in 10 volumes of ice-cold suspension medium (250 mM sucrose, 100 mM mercaptoethanol, 150 mM Tris, pH 7.8, 30 mM KCl, 5 mM MgCl2, and 0.1 mM EDTA). The suspension was again centrifuged at low speed, the pellet collected and resuspended in a volume of suspension medium equal to the pellet volume. The suspension was frozen and thawed three times followed by three 10-s periods of sonication with a Brorwill Biosonic Ultrasoundicator. The disrupted cells were centrifuged for 2 hours at 50

* This investigation was supported by Public Health Service Grants CA-5262 and CA-10748 from the National Cancer Institute.

1 The abbreviations used are: BHK, baby hamster kidney cells; SDS, sodium dodecyl sulfate.
For preparation of extracts from mouse kidney, the method was modified. The kidneys were minced and then washed four to five times with phosphate-buffered saline to remove as much residual blood as possible. This procedure rendered the fluid decanted from the minced tissue colorless to the eye. An equal volume of suspension medium was added to the washed minced tissue which was homogenized by four or five strokes in an all glass homogenizer. The suspension of disrupted cells was centrifuged at 4°C for 30 min at 10,000 × g. The resulting supernatant fluid was centrifuged at 4°C for 2 hours at 39,000 rpm in a Spincor 50 Ti rotor. The supernatant fluid was collected.

Assay of Arginyl-tRNA Protein Transferase and Formation of Arginyl-tRNA—Arginyl-tRNA protein transferase activity in the 100,000 × g supernatant fluid was measured essentially as described by Dupras and de Lamiande (6). The contents of the incubation mixtures are indicated in the appropriate portions under "Results." Radioactivity was measured in a scintillation mixture with Triton X-100 as described by Patterson and Greene (11).

SDS-Acrylamide Gel Analysis of Endogenous Acceptor Protein—One-milliliter samples of 14C-arginine-labeled proteins formed by incubation of the 100,000 × g supernatant fluid of cell extracts as described under "Results" were mixed with an equal volume of 2% SDS solution containing sodium phosphate buffer (10 mM) at pH 7.0 and 2-mercaptoethanol (200 mM). The solutions were mixed, heated at 100°C for 1 min, and then dialyzed against two 500-ml changes of electrode buffer described below. Sucrose and bromphenol blue dye were added to concentrations of 10 and 0.01%, respectively.

The treated 14C-arginine proteins (0.05 ml) were analyzed on SDS-acrylamide gel with a Buchler analytical polyacrylamide vertical disc gel electrophoresis apparatus. Neutral 7.5% gels were prepared by mixing one portion of Solution A (30 g of acrylamide and 1.0 g of bisacrylamide in 100 ml of water) and one portion of Solution B (3.24 g of NaHPO4, 2.37 g of Na2HPO4·H2O, 0.4 g of SDS, and 0.4 ml of N,N′,N′,N′-tetramethyl ethylenediamine diluted to 100 ml) with two portions of Solution C (0.4 g of ammonium persulfate diluted to 100 ml). The mixture was poured into glass cylinders (0.5 × 7.5 cm) immediately covered with a layer of water and allowed to polymerize. The composition of the electrode buffer was 0.8 g of NaHPO4, 0.6 g of Na2HPO4·H2O, and 1.0 g of SDS in 1 liter of H2O. Electrophoresis was carried out at room temperature for approximately 2 hours at 200 volts. After completion of the electrophoresis the gels were frozen at −70°C, and cut into 1 mm sections with a Diversified Scientific Instruments, Inc., gel slicer. The slices were prepared for counting by the method of Dingman and Peacock (19), which gave a recovery of 80 to 90% of the counts per min put on the gels.

Amino-terminal Amino Acid Analysis—The preparation of NH2-terminal amino acids described here is similar to that carried out by Kaji (3). Ten milligrams of 14C-arginine-labeled protein in 2 ml were dialyzed against 500 ml of 0.6 M ammonium carbonate, pH 8.5, at room temperature for 4 to 6 hours. Two milliliters of 5% solution of 1-fluoro 2,4-dinitrobenzene in ethanol were added to 1 ml of the dialyzed sample, and the mixture was shaken for 18 hours at room temperature. The pH was then adjusted to 2 with concentrated HCl and the unreacted dinitrofluorobenzene was removed by three 10-ml washings with ether. An equal volume of 10% trichloroacetic acid was added to the aqueous phase, and the mixture was spun at 3000 × g for 5 min. The yellow precipitate was washed with 10 ml of ethanol followed by an equivalent quantity of ether, and then dried at room temperature over paraffin shavings. The dried powder was suspended in 3 ml of 6 N HCl, sealed in a glass tube under nitrogen, and digested at 105°C for 12 hours. The digestion mixture was centrifuged at 3000 × g for 10 min and the supernatant liquid was dried in vacuo in a small Petri dish. The powder was then dissolved in 1 ml of acetone which contained 1 mg of unlabeled dinitrophenyl-ω-arginine (Schwarz BioResearch Inc.) as a marker. It was necessary to add a drop of concentrated HCl to dissolve the arginyl derivative. Descending chromatography of 0.1-ml samples of this solution containing 600 cpm was carried out on Whatman No. 1 paper using the butanol-1-acetic acid-water (4:1:5) solvent system for 15 hours. The yellow spot was cut from the paper, placed in 10 ml of toluene 1,4-bis(2-(5-phenyloxazolyl)]benzene (POPOP) and the radioactive content determined.

14C-Arginyl-tRNA Preparation—14C]Arginyl-tRNA was prepared with stripped Escherichia coli B tRNA (General Biochemicals) and the supernatant fluid from an 80% ammonium sulfate-saturated solution of an E. coli B extract prepared as described by Voytek et al. (13).

RESULTS

Occurrence of Enzyme—The supernatant fluids from 2-hour, 100,000 × g centrifugations of sonically disrupted cells of various types were tested for arginyl-tRNA protein transferase activity. Apparent transferase activity was found in the 100,000 × g supernatant fluids of L, BHK, PyB3, HeLa, FEP-2, L5178Y, MDBK, Vero, BSC-1, and CV-1 cells. A typical experiment showing incorporation of 14C-arginine into a hot acid-insoluble product is shown in Fig. 1A. For BHK and L cells the rate of incorporation diminishes after about 2.5 hours. Similar time courses were obtained for the other cell lines tested. One explanation for the decrease in rate of incorporation could be that almost all of the available endogenous acceptor proteins have had arginine added. That such is the case, has been substantiated by the experiments depicted in Fig. 1, B and C. First, as shown in Fig. 1B, after 120 min of incubation of an L cell preparation, when the rate of incorporation of 14C-arginine is markedly decreased, if additional 14C-arginine, ATP, and ATP-generating components (equivalent to the amount present initially) are added to the mixture, no increase in 14C-arginine incorporation ensues. This observation indicates that the decline in incorporation is not due to exhaustion of the supply of arginine or ATP. Second, Fig. 1C shows that after a similar 120-min incubation, the addition of bovine serum albumin, an exogenous acceptor protein (9), results in a prompt and marked stimulation of the incorporation of 14C-arginine into hot acid-insoluble product, thus indicating that the other components of the assay mixture are active. The most feasible interpretation of these experiments is that the incorporation of labeled arginine essentially stops due to exhaustion of available endogenous acceptor protein. Moreover, the plateau level observed should represent a reasonable estimate of the amount of available acceptor protein in any particular preparation, provided arginine and ATP are present in excess.

Optimal Assay Conditions—The optimal conditions for measuring arginyl-tRNA protein transferase activity were determined for BHK and L cells when an ATP-generating system, arginine, and uncharged tRNA were used with the 100,000 × g supernatant fluid. The optimum for such a crude preparation reflects,
pH on showed a broad peak from about 7.6 to 8.6. Above pH 8.6, activity decreased gradually so that at pH 9.6 the activity was nearly 50% that of the maximum. Little can be made of the effect of pH on transferase activity since the change in activity may reflect an effect on arginyl-tRNA synthetase activity as well as increasing lability of arginyl-tRNA at the higher pH (6).

Specificity of Arginyl-tRNA Protein Transferase for Arginine—The specificity of the enzyme for transferring only arginine was tested to determine whether it was the same as reported for the transferase from excised tissue. First, as may be seen in Fig. 1D in a preparation of transferase from BHK cells in which there is marked incorporation of labeled arginine a similar incubation with 1\(^{14}C\)leucine shows no incorporation of radioactivity into the hot acid-insoluble fraction. Second, a mixture of several \(14C\)-labeled amino acids (New England Nuclear, \(14C\)-labeled, l-amino acid mixture) incubated with the BHK preparation also results in incorporation of radioactivity (Fig. 1E). Addition of excess unlabeled arginine alone to this incubation almost completely prevents measurable incorporation of radioactivity (Fig. 1E), thus showing that of all the labeled amino acids in the mixture only arginine is incorporated in significant amounts. The small residual incorporation may reflect a small incorporation of amino acid other than arginine as reported by Kaji (3). Addition of either unlabeled leucine or phenylalanine did not similarly diminish incorporation of radioactivity.

SDS-Acrylamide Gel Patterns of Endogenous Acceptor Protein—The SDS-acrylamide gel patterns of \(14C\)-arginine labeled acceptor protein incorporated via the usual incubation are shown for several of the cell lines (Fig. 2). Of note, is the great similarity of pattern in the extracts from the various species represented by the different cell lines. In all the cell extracts analyzed, a major peak is evident between Slices 15 to 20 (1T) of the electrophoretograms. The protein in this peak has a molecular weight of approximately 65,000 based upon the migration of bovine serum albumin under the same conditions.
the gels of the extracts from the various cells examined, one or two lesser peaks are also observable in gel Slices 9 to 13 and are assumedly of greater than 65,000 molecular weight. Two peaks in Slices 9 to 13 (Ia and Ib) were always seen with gels of incubations of extracts from BHK, herpes-infected, and polyoma-transformed (PyH3) BHK. In extracts from other cells only one peak (I) was always clearly discernible. The number and position of the peaks of radioactivity beyond the major peak are somewhat variable from preparation to preparation from even the same cells although a peak was almost always seen in the Slice 30 to 40 range. Treatment of the incubation mixture after incubation and prior to gel analysis with 50 μg per ml of pancreatic ribonuclease for 30 min did not noticeably change the gel patterns thus indicating that the observed peaks are not arginyl-tRNA. 2,4-Dinitrophenol analysis indicated that after correcting for 2,4-dinitrophenol quenching 80 to 90% of the [14C]arginine associated with the endogenous acceptor proteins is at the amino terminal.

The SDS-acrylamide gel pattern of incubations of extracts from polyoma-transformed (PyH3 cells) and herpes simplex-infected BHK cells were examined for differences (Fig. 3, top and bottom left). No striking difference was found in gel pattern in incubated extracts from these cells.

To show that the gel patterns observed are not an artifact of, or peculiar to, tissue culture-grown cells, freshly excised mouse kidney, processed as described under “Experimental Procedure,” was also analyzed by SDS-acrylamide gel. Incubation conditions were the same as for the L cells. Essentially the same pattern obtains with the mouse kidney preparation (Fig. 3, bottom right).

The results are partly summarized in Table I where the Rf values of the I, In, Ib, and II peaks are compared in electrophoretograms of the different cell preparations. Note the almost perfect agreement for the Rf values of Peaks I and II from the various cell extracts and the close agreement for the Peaks Ia and Ib in the gels of incubations of extracts from the BHK and related cells.

**Exogenous Acceptor Proteins**—As mentioned above, bovine serum albumin exhibits acceptor activity. Such activity is shown for BHK cells in Fig. 4. This is in agreement with Soffer’s work with rabbit liver (9) as is the apparent acceptor activity of bovine thyroglobulin (Fig. 4).

The fact that the activity has been shown in a variety of cells from different species allowed the investigation of species specificity of the enzyme activity. No such specificity was found. Preparations from cells of various species were tested with proteins (all albumins except for immunoglobulin G (IgG)) for acceptor activity. However, as may be seen (Fig. 5), there are differences amongst the various combinations of enzymes and acceptor protein. For example, mouse albumin acted as a much better acceptor protein for the transferase from human HeLa cells than mouse L cells, and bovine albumin-stimulated arginine incorporation by human HeLa cell extracts much better than

![Fig. 3. Comparison of SDS-acrylamide gel patterns of extracts of BHK cells, of polyoma virus transformed BHK cells (PyH3), herpes simplex (Rory II), infected BHK cells, and freshly excised mouse kidney cells. The 100,000 X g supernatant fluid of extracts of the indicated cells were incubated for 6 hours, 37° with the standard BHK mixture (Fig. 1A) but using 15 μM [14C]arginine with a specific activity of 250 Ci per mole. For the herpes simplex infection the cells were infected at a multiplicity of 10 plaque-forming units per cell and adsorbed for 1 hour. Infection was allowed to proceed for 12 hours before harvesting the cells for analysis. The ordinate is as in Fig. 2.](image)

![Fig. 4. Effect of exogenous proteins on the incorporation of [14C]arginine. BHK 100,000 X g supernatant fluid was incubated with the standard mixture (Fig. 1A) with no further additions (O—O), plus 80 μM bovine thyroglobulin (△—△), or plus 80 μM bovine serum albumin (●—●).](image)
The preparations from bovine kidney cells. All the exogenous acceptor proteins were tested at a final concentration of 5 ng per ml. Human albumin was a poor acceptor protein with extracts of all three cell lines tested. Using increased concentrations did not improve the acceptor protein activity of the human albumin. The poor activity may be a function of the structure of the human albumin (e.g., the tertiary conformation). Soffer and Capan (14) have reported evidence relative to arginine addition to immunoglobulins which also suggests that the conformation of the acceptor protein and the accessibility of the amino terminal are factors in acceptor protein activity. It may also be due to the amino terminal of most of the molecules already having an arginine residue, although Thompson (15) has reported that the amino-terminal amino acid of human albumin is aspartate and therefore has the requisite acidic amino-terminal amino acid (9). It is possible that the source, purity, or mode of preparation of the human albumin is a factor also.

Inhibition of Arginine Incorporation—Pancreatic ribonuclease (1 mg per ml final concentration) virtually prevents any incorporation of [14C]arginine into protein (Table II).

The properties of the enzyme in cultured mammalian cells reported here are similar to those described by other investigators.
The 100,000 × g supernatant fraction of an extract of BHK cells was incubated for 20 min with the standard BHK mixture (Fig. 1A) varying the arginine concentration, with or without bovine serum albumin and in the presence or absence of human hemoglobin. The amount of incorporation of [14C]arginine into hot acid-insoluble product in the absence of human hemoglobin is the control level (0% inhibition).

| Arginine | Albumin | Inhibition with hemoglobin |
|---------|---------|---------------------------|
| μM     | μM     | %                         |
| 15      |         | 78                        |
| 250     |         | 74                        |
| 320     |         | 85                        |

**Fig. 7.** Inhibition by human hemoglobin of incorporation of [14C]arginine into hot acid-insoluble product by a 100,000 × g supernatant fraction of BHK cells using [14C]arginine or [14C]arginyl-tRNA as substrate. The 100,000 × g supernatant fluid of a BHK extract was incubated as follows: with standard BHK mixture (Fig. 1A) alone (---) or with standard BHK mixture plus 80 μM human hemoglobin (●●●●●●●●); with 2 μM [14C]arginyl-tRNA (20 Ci per mole), 100 mM Tris buffer (pH 7.8), 100 mM mercaptoethanol, and 65 mM KCl alone (---) or with 80 μM human hemoglobin (○○○○○○). For arginyl-tRNA protein transferase from freshly excised tissues. The specificity for arginine, amino-terminal addition of arginine, acceptor protein activity of bovine serum albumin and thyroglobulin, sensitivity to ribonuclease, and lack of inhibition by puromycin have all been reported as properties of arginyl-tRNA protein transferase.

The most significant findings are those relating to the endogenous acceptor proteins. In Soffer's recent report (10) on isolation of an acceptor protein from rabbit liver, he finds a protein which appears to be albumin based on its electrophoretic mobility, molecular weight, and amino acid composition. As stated in the introduction, it is not surprising to find an albumin acceptor protein in liver at the site of albumin synthesis. The major available endogenous acceptor protein (Peak II) that we find migrates in SDS-acrylamide gels similarly to albumin. It is not known if this protein is albumin. In these experiments the cells are incubated in medium with bovine serum; however, cells kept in serum-free medium for as long as 48 hours still have the particular acceptor protein at issue. Moreover, it would be surprising to find albumin in, for example, HeLa or L5178Y leukemic cells. Further speculation on this point should await more information.

If it is assumed that the average molecular weight of monomers of cellular proteins in BHK cells is 31,700 as found by Kiehn and Holland (18) by SDS acrylamide gel analysis of extracts of HeLa cells and if the amount of arginine added to endogenous acceptor protein as shown in Fig. 1A gives a valid estimate of available acceptor protein, then it can be calculated that the available endogenous acceptor protein represents 0.5% of total cellular protein on a molar basis. This represents a significant percentage of total cellular protein. The arginine-labeled protein found in Peak II which accounts for approximately 25% of the available endogenous acceptor protein is therefore also present in significant proportion (0.13% total cell protein). The fact that available endogenous acceptor protein appears to be present in this amount argues that the form of these proteins without arginine at the amino terminal is the predominant species as it is not likely that the total acceptor protein with or without arginine represents 1% or more of total cellular protein. Unfortunately, there is at present no means for measuring endogenous acceptor protein with arginine already added at the amino terminus. In this regard, it should be mentioned that terminal analysis of soluble proteins in HeLa cells (19) shows no arginine and 8 and 9% aspartate-asparagine, glutamate-glutamine, respectively, at the amino terminus.

At present, no cellular role has been attributed to the enzyme arginyl-tRNA protein transferase. Hopefully, the search for the role of this enzyme will be facilitated by the use now of tissue culture grown cells in which conditions may be more readily and precisely monitored and controlled than in the intact animal.

Acknowledgments—We wish to acknowledge the excellent technical assistance of Mrs. Evelyn B. Lentz. We are grateful to Doctor H. Vacken Apolobian, Wilona Summers, and Sherman Weissman for starter cultures of cells.

REFERENCES

1. Kaji, H., Novelli, G. D., and Kaji, A. (1963) Biochem. Biophys. Acta 76, 474
2. Gill, D. M. (1967) Biochim. Biophys. Acta 145, 792
3. Kaji, H. (1968) Biochemistry 7, 3844
4. Soffer, R. L., and Hornishi, H. (1969) J. Mol. Biol. 43, 163
5. Soffer, R. L. (1970) J. Biol. Chem. 245, 731-737
6. Dupuis, M., and de Lamerland, G. (1970) Cancer Res. 30, 1506
7. Soffer, R. L., and Mendelson, N. (1966) Biochem. Biophys. Res. Commun. 25, 292
8. Soffer, R. L. (1968) Biochim. Biophys. Acta 165, 228-240
9. Soffer, R. L. (1971) J. Biol. Chem. 245, 1481-1484
10. Soffer, R. L. (1971) J. Biol. Chem. 246, 1602-1606
11. Patterson, M. S., and Greens, R. C. (1965) Anal. Chem. 37, 854
12. Dingman, C. W., and Peacock, A. C. (1968) Biochemistry 7, 699
13. Votter, P., Chang, P. K., and Prusoff, W. H. (1971) J. Biol. Chem. 245, 1482-1485
14. Soffer, R. L., and Capala, J. D. (1971) Nature New Biol. 233, 44
15. Thompson, E. O. P. (1954) J. Biol. Chem. 200, 565
16. Metz, S. K., and Neihouse, A. H. (1967) J. Biol. Chem. 242, 5490-5494
17. Allende, C. C., and Allende, J. E. (1964) J. Biol. Chem. 239, 1102
18. Kiehn, E. D., and Holland, J. J. (1970) Nature 225, 544
19. Brown, J. L. (1970) Biochem. Biophys. Acta 221, 480
