Incorporation of lysine into Y base of phenylalanine tRNA in Vero cells

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ABSTRACT

Vero cells, a line derived from African green monkey kidney, contains a hypermodified base, called Y, adjacent to the 3' end of the anticodon of tRNA^Phe. Two types of evidence are presented suggesting that lysine is involved in biosynthesis of Y base in these cells. First, when Vero cells are starved for lysine, a new, early-eluting species of tRNA^Phe which lacks the fully modified Y base can be detected by reversed phase chromatography (RPC-5). After addition of lysine to the medium, this new species disappears. Second, when these cells are grown in low-lysine medium and then exposed to [3H]lysine, radioactivity from the lysine comigrates with tRNA^Phe. The Y base can be selectively excised from tRNA^Phe by incubation at pH 2.9, and extracted into ethyl acetate. Thin-layer chromatography of acid-excised material from these cells reveals that lysine-derived radioactivity comigrates with genuine Y base from calf liver tRNA^Phe and the acid-excised tRNA no longer contains radioactivity. These results are consistent with the model that lysine is a structural precursor of Y base in tRNA^Phe of Vero cells.

INTRODUCTION

In almost all tRNAs sequenced thus far, there is a modified nucleoside adjacent to the 3' end of the anticodon (1). Phenylalanine tRNAs isolated from a variety of eukaryotic cells contain at this position a highly modified, hydrophobic, fluorescent base, called Y. This base has one of the most complex structures characterized in any nucleic acid, consisting of a tricyclic imidazo derivative of guanine, to which a four-carbon side-chain is attached in higher organisms (2,3). It is synthesized in yeast by modification of guanosine (3). While the possible involvement of the 3-amino-3-carboxypropyl group from methionine has been reported in yeast (4), no data has been presented on the biosynthesis of Y base in mammalian cells.

As an approach to the study of Y base biosynthesis in mammalian cells, we determined whether any of the essential amino acids were direct precursors of Y base. This was done by growing Vero cells, a line derived from monkey kidney, in medium in which one of the fourteen essential amino acids was replaced by...
its radioactive counterpart, and measuring the extent of labeling of the Y base. Previously, this procedure was used to demonstrate the involvement of methionine in this synthesis (5). Vero cells were used because they retain viability under prolonged incubation in amino acid deficient media. In this study, we have explored the possible involvement of lysine in Y base biosynthesis.

EXPERIMENTAL PROCEDURES

Vero cells, a line derived from African green monkey kidney (6), were grown in Dulbecco's Modified Eagles medium (DME), Grand Island Biological Co., Grand Island, N.Y.), containing 10% dialyzed calf serum (v/v) (Flow Labs, Bethesda, Md.), and low lysine (0.04 mM, compared to 0.56 mM in normal DME) unless otherwise specified. The cells were plated at 4 X 10⁶ cells per 150 mm plastic tissue culture dish (Lux Scientific), and incubated at 37°C for the duration of the experiment. No mycoplasma contamination was detected throughout the course of these experiments by the method of Todaro (7), or by autoradiography of [³H]thymidine labeled cells. In some experiments, the medium was removed from the cells after eight days incubation (without change), and replaced with identical medium containing 0.56 mM lysine, or L-[4,5-³H(N)]lysine/plate (New England Nuclear, Boston, Mass.), and the incubation continued for an additional 48 hours.

At the end of the incubation period, cells were harvested and tRNA prepared as previously described (5). Briefly, medium was removed, the cells were washed twice with 0.9% NaCl in 0.01 M phosphate buffer (pH 7.2), scraped off the plates, and pelleted by centrifugation at 1500 rpm for ten minutes at 4°C. The pellet was resuspended in 0.01 M Tris-HCl (pH 7.5), with 0.01 M NaCl and 1.5 mM MgCl₂, and allowed to swell at room temperature for ten minutes. Nonidet P40 (Shell Chemicals, Chicago, Ill.) was added to a final concentration of 0.5% (v/v), and the suspension was vortexed to lyse the cells. Intact nuclei were removed by centrifugation for ten minutes at 10,000 rpm. The supernatant was then extracted several times with phenol-chloroform (1:1, v/v), chloroform, and ether. RNA was precipitated by addition of 0.1 volume of 2 M sodium acetate (pH 4.5) and two volumes ethanol. After 12 hours at -20°C, the RNA was collected by centrifugation at 10,000 rpm for 30 minutes. Ribosomal RNA was removed, and tRNA deacylated as previously described (8).

Aminoacylation of tRNA was performed by incubating 10 A₂₆₀ (0.5 mg) of Vero tRNA with 3.5 mg of crude aminoacyl-tRNA synthetase, prepared from rat liver according to Grunberger et al. (8), in a 0.5 ml reaction mixture con-
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taining 10 mM KCl, 10 mM MgCl₂, 4 mM ATP (pH 7.5), 0.6 mM CTP (pH 7.5), 0.1 M Tris-HCl (pH 7.5), 0.2 mM each of nineteen cold amino acids (minus phenylalanine) and 5 μCi of [¹⁴C]phenylalanine (specific activity 424 Ci/mol or 100 μCi of [³H]phenylalanine (22 Ci/mMol) for 30 minutes at 37°C. The labeled Phe-tRNA was recovered by applying the entire reaction mixture to a DEAE-cellulose column (1 X 5 cm; DE-52, Whatman), and eluting as described (9). The charged tRNA was applied to an RPC-5 column (10), (0.6 X 30 cm) and eluted with a 100 ml linear gradient of 0.55 -0.7 M NaCl in 10 mM Na acetate (pH 4.5), 10 mM Mg acetate, 2 mM β-mercaptoethanol, at a pressure of 250 pounds per square inch. Fractions were collected at a flow rate of two ml per minute and aliquots were mixed with Hydroflour scintillation cocktail (National Diagnostics, Somerville, N.J.) and counted in a Searle liquid scintillation spectrometer. Results were plotted on a Hewlett-Packard computer-plotter programmed to subtract background and eliminate crossover contributions of [¹⁴C] into [³H] channel.

When the Vero cells had been incubated with [³H]lysine, Y base was excised from charged [¹⁴C]Phe-tRNA by incubation in 0.05 M citrate buffer (pH 2.9) for 18 hours at 37°C (9). The solution was then neutralized, the Y base recovered by extraction into ethyl acetate, and the tRNA precipitated from the aqueous phase with ethanol. This Phe-tRNA (-Y) was then analyzed by RPC-5. The excised Y base was concentrated by flash evaporation and loaded onto a silica gel thin-layer chromatography plate (silica gel 60, no fluorescent indicator, EM, Germany) along with standards prepared from yeast (Saccharomyces cerevisiae, Y₅₀) and calf liver (Y₇₅). The chromatogram was developed with ethyl acetate:1-propanol:water (4:1:2, v:v:v). Migration of the standards was determined by their fluorescence under ultraviolet light. The section containing the Vero Y base was divided into one cm fractions that were scraped off the plate and serially eluted with one ml portions of ethyl acetate and water. The eluates from each fraction were mixed with Hydrofluor and counted.

RESULTS AND DISCUSSION

We have previously shown that when Vero cells were grown in medium lacking methionine, the synthesis of Y base was blocked (5). An addition of methionine to these cells restored the synthesis of the Y base. We have used a similar approach to find out whether lysine is also involved in the biosynthesis of Y base. To do this, Vero cells were grown in medium that was limiting for lysine, and after eight days incubation the cells were given medium which contained [³H]lysine. An RPC-5 chromatography of the isolated tRNA
was then employed to determine whether radioactivity from $[^3H]$lysine cochromatographed with tRNA\textsuperscript{Phe} aminoaetylated with $[^1^4C]$phenylalanine. Figure 1A shows that when tRNA from Vero cells, incubated with $[^3H]$lysine, was fractionated on an RPC-5 column, radioactivity from lysine cochromatographed solely with tRNA\textsuperscript{Phe}. To eliminate the contribution of charged lysyl-tRNA to the profile, total tRNA was extensively deacylated prior to aminoaetylation.

The Y base can be selectively excised from tRNA\textsuperscript{Phe} without breaking the polynucleotide backbone of the molecule by incubating the tRNA at pH 2.9 (9). Phenylalanine-tRNA lacking the Y base elutes earlier from RPC-5 columns than normal Y-containing tRNA\textsuperscript{Phe} (8). Figure 1B shows an RPC-5 profile obtained after excision of the Y base from the tRNA shown in Figure 1A. The tRNA\textsuperscript{Phe} eluted earlier than in Figure 1A, indicating that the Y base had been excised. Lysine-derived radioactivity no longer cochromatographed with

![Figure 1. Elution profiles of Vero tRNAs from RPC-5 columns.](image)

(A) $[^1^4C]$-Phe-tRNA from Vero cells incubated with $[^3H]$lysine as described in Experimental Procedures.

(B) $[^1^4C]$-Phe-tRNA from the same cells as 1A after acid excision of the Y base.

$[^3H]$lysine (○); $[^1^4C]$phenylalanine (●)
tRNA\textsuperscript{Phe}, demonstrating that the counts were excised along with the Y base.

To prove that lysine-derived label resided in the Y base, the excised base was extracted into ethyl acetate, concentrated by flash evaporation, and chromatographed on silical gel, along with genuine Y base standards prepared from yeast and calf liver. The results of this experiment are shown in Figure 2. Radioactivity derived from lysine was seen to comigrate with the form of Y base isolated from calf liver, as would be expected for a mammalian cell line. Under the solvent conditions, free lysine remains at the origin (data not shown). We conclude from these results that the lysine molecule was incorporated into the Y base.

The quantity of labeled Y base obtained from these experiments was too small for spectrophotometric or gravimetric analysis. However, we were able to estimate the yield of Y base by subjecting a known amount of tRNA\textsuperscript{Phe} to extensive acid excision. From this estimate, the amount of radioactivity comigrating with genuine Y base on thin-layer chromatography, we arrived at an approximate specific activity. Comparing this specific activity with the experimentally-determined specific activity of the extracellular medium (via amino acid analysis) revealed that the newly synthesized Y base contains 60% of the specific activity of the extracellular $[^3\text{H}]$lysine. This number represents a minimum estimate of the specific activity of the newly synthesized Y base, since the proportion of total Y base that was pre-

![Figure 2. Thin-layer chromatography of Y base from Vero tRNA. Excised Y base (from Figure 1B) was recovered and chromatographed as described in Experimental Procedures, along with standards from yeast (YS.C.) and liver (YL).](2213)
sent before the addition of the radioisotope was not known, and also, the intracellular pool of lysine might have a lower specific activity because of lysine arising from protein degradation. Therefore, these data are consistent with the model that lysine is a direct precursor of the Y base. The possibility that radioactivity from lysine appeared in the Y base by way of tritium exchange could be rejected because the experiments reported in Figure 1 demonstrate that it is the Y base, and only the Y base, that is labeled. Such a limited tritium exchange is considered very unlikely. Since lysine labeled at positions 4 and 5 was used and no known metabolic reaction cleaves lysine at those positions, it seems very probable that the entire lysine molecule is being incorporated.

If lysine is being used as a Y base precursor by Vero cells, it should be possible to block the formation of Y base by depriving the cells of lysine. In analogous experiments, depriving Vero cells of methionine induced the formation of a new tRNA^Phe species which lacked the Y base (5). The cells were plated in medium containing a low concentration of lysine (0.04 mM), and incubated for ten days at 37°C. At the end of this period, the medium was replaced with media containing either a normal level of lysine (0.56 mM) or no lysine, but identical in all other respects. Twenty-four hours later, tRNA was harvested from both groups of cells, aminoacylated with [3H]phenylalanine, and fractionated on separate RPC-5 columns, as shown in Figure 3. Single-label conditions utilizing high-specific activity [3H]-phenylalanine were employed due to the severely restricted cell growth and concomitant low yields of tRNA imposed by the low lysine media. This composite figure reveals that the tRNA from cells given media without lysine contained an additional isoaccepting species of tRNA^Phe. The earlier elution of this additional species suggests that it lacks the Y base, as had been shown previously for tRNA^Phe from several tumors (11,12), and for methionine-starved Vero cells (5). The relatively low level of the early-eluting peak could be due to the fact that even the medium lacking lysine was found to contain 9.4 μM lysine (by amino acid analysis) after 24 hours of incubation.

Taken together, these results show that lysine is involved in the biosynthesis of Y base in tRNA^Phe of mammalian cells. The structure of lysine is compatible with a proposed biosynthetic pathway. Since it is established that in liver and kidney α-aminodipic acid semialdehyde is a metabolite of lysine (13), and that the 2-amino group of guanosine readily reacts with aldehydes (14), we propose that the first step of Y base biosynthesis is a
Figure 3. Effect of lysine starvation on elution profile of Phe-tRNA from RPC-5 columns.

Cells grown and treated as described in Experimental Procedures. This Figure is a composite of two separate column profiles.

condensation between those two groups. Closing of the third ring and methylation of the appropriate positions could complete the biosynthesis of the Y base. This hypothesis is currently under investigation. The technique of using Vero cells in culture deprived of amino acids or other constituents may also prove to be of value in future studies on the biosynthesis of other hypermodified nucleosides in tRNA of mammalian cells.

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