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DIFFERENCES IN AMINOACYLATION IN VIVO AND IN VITRO OF LYSINE ISOACCEPTING tRNAs FROM VIRUS-TRANSFORMED CELLS

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Summary. When murine sarcoma virus-transformed cells are labeled with [^{3}H]lysine in vivo for various periods, 5 of 6 isoaccepting lysine tRNAs separable by RPC-5 chromatography are aminoacylated in 1 hr to the same extent that they are aminoacylated in vitro. The sixth isoacceptor, $tRNA_6^{Lys}$, is not aminoacylated in vivo to a measurable extent in 1 hr, although it is present in the tRNA prepared from the cells. All six isoacceptors are aminoacylated with [^{3}H]lysine in vivo when the labeling period is 2 or 3 hr. These results further show that in vitro correlations of the amount of $tRNA_4^{Lys}$ with cell division accurately reflect the situation in vivo. Results of differential centrifugation indicate that $tRNA_6^{Lys}$ occurs in mitochondria.

Introduction. Juarez et al. (1) demonstrated seven isoaccepting lysine tRNAs by benzoylated DEAE-cellulose and reverse-phase chromatography (RPC-5). Six of the isoacceptors are separable routinely by RPC-5 chromatography, and one, $tRNA_6^{Lys}$, is found in polyoma and murine sarcoma virus-transformed cells but not in normal cells (2). $tRNA_6^{Lys}$ does not function as efficiently in protein synthesis in vitro as the other isoacceptors (3). Ortwerth and Liu (4) showed by aminoacylation in vitro that normal and neoplastic cells that are dividing have an isoacceptor, $tRNA_4^{Lys}$, that is absent in nondividing cells and that neoplastic cells have a large amount of that species. Juarez et al. (5) reported that rapidly dividing normal cells also can have a large amount of that isoacceptor. We are reporting now that $tRNA_6^{Lys}$ is not aminoacylated with [^{3}H]lysine added to culture medium as rapidly as the other isoacceptors and that this probably results, at least in part, from a mitochondrial location of $tRNA_6^{Lys}$. Also, $tRNA_4^{Lys}$ is aminoacylated in vivo to the same extent that is found when isolated tRNA is aminoacylated: in dividing cells in culture, a large proportion of lysyl-tRNA, in vivo, is lysyl-$tRNA_4^{Lys}$, whereas in slowly dividing or nondividing cells, lysyl-$tRNA_4^{Lys}$, in vivo, is only a small

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part of total lysyl-tRNA^{Lys}. Thus, data reported here substantiate that the changes in amounts of tRNA$_4^{Lys}$ revealed by aminoacylation *in vitro* accurately reflect what is happening *in vivo*.

Materials and Methods. [^{3}H]Lysine (75 Ci/mmol or 93 Ci/mmol) was obtained from Amersham. Murine sarcoma virus-transformed Balb/3T3 cells (KA31), obtained from Dr. George Todaro, were maintained in Dulbecco's modification of Eagle's minimum essential medium buffered with 20 mM Hepes[1] 10 mM N-tris(hydroxymethyl)methylglycine, and 24 mM $NaHCO_3$, pH 7.6 (6), supplemented with 10% calf serum (Grand Island Biological Company). For labeling with [^{3}H]lysine, cells were grown in 75 cm^2 plastic culture flasks in the same medium except that the lysine concentration was lowered to the level (400 µM) used in Eagle's minimum essential medium and the medium was adjusted to 10 µCi/ml in [^{3}H]lysine.

At the end of the labeling period, medium was removed, the cell monolayer was washed twice with phosphate-saline buffer (10 mM sodium phosphate, 150 mM NaCl, pH 7.2), and the monolayer was treated with 5 ml of a mixture, at 60° (7), of phenol and extraction buffer (0.14 M sodium acetate at pH 5.0, 6 mM 2-mercaptoethanol) (8) containing 0.5% sodium dodecylsulfate in a procedure modified from that of Chu-Der and Ortwerth (9). Each flask was rinsed with another 5 ml of the same mixture, and after 10 min of mixing, $CHCl_3$ was added before separating organic and aqueous phases by centrifugation (10). The residue of phenol and interphase was washed with fresh extraction buffer, and the combined aqueous phases were reextracted with phenol. The resulting aqueous phase was mixed with 1 ml of DEAE-Sephacel. After 10 min at 0°, the DEAE-Sephacel was poured into a column, and tRNA was recovered (11) and precipitated with 3 volumes of 95% ethanol at -20°. Recovered tRNA was stored in RPC-5 buffer (10 mM sodium acetate at pH 5.0, 10 mM magnesium acetate, and 6 mM 2-mercaptoethanol). A sample of the tRNA was used to determine the yield in A_{260} units and the amount of radioactivity, and another sample was saved for aminoacylation *in vitro*. The remaining [^{3}H]lysyl-tRNA from aminoacylation *in vivo* was used directly for RPC-5 chromatography.

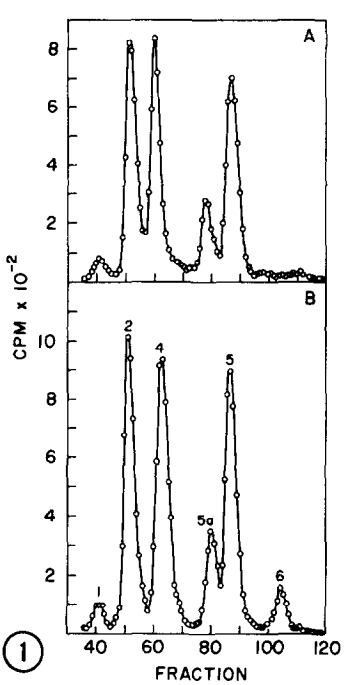
A subcellular fraction enriched in mitochondria was prepared by centrifuging a cell homogenate (prepared in 100 mM Tris, pH 7.8, 1 mM magnesium acetate, 10 mM potassium chloride, 6 mM 2-mercaptoethanol, and 250 mM sucrose in a Ten Broeck homogenizer) at 600 x g for 10 min, centrifuging the supernatant solution at 15,000 x g for 15 min, and washing the resulting pellet twice by resuspension and centrifugation at 15,000 x g for 15 min. tRNA was prepared essentially as described above.

[^{3}H]Lysyl-tRNA was prepared *in vitro* in a reaction mixture of 50 mM sodium cacodylate, pH 7.5; 20 mM magnesium acetate; 2 mM ATP; 10 mM 2-mercaptoethanol; 1 A_{260}/ml tRNA; 4 µM [^{3}H]lysine at 50 Ci/mmol; a mixture of 19 amino acids, not including lysine, each at 10 µM; and an excess amount of a preparation of aminoacyl-tRNA synthetases prepared essentially according to Jacobson *et al.* (2) After 20 min, [^{3}H]lysyl-tRNA was recovered on DEAE-Sephacel (11).

For chromatography of [^{3}H]lysyl-tRNA on a column (3 x 250 mm) of RPC-5 (12) sample was applied in 0.4 M NaCl in RPC-5 buffer, and the column was eluted with a 12-ml linear gradient of 0.50 M to 0.70 M NaCl in RPC-5 buffer at 45° (13). Fractions of 100 µl were collected and the amount of radioactivity was determined in a toluene-Triton X-100 scintillation cocktail.

Results. Cells were labeled with [^{3}H]lysine for 0.5, 1, 2, and 3 hr, and [^{3}H]lysyl-tRNA was isolated and chromatographed on RPC-5 for fractiona-

[1]Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.



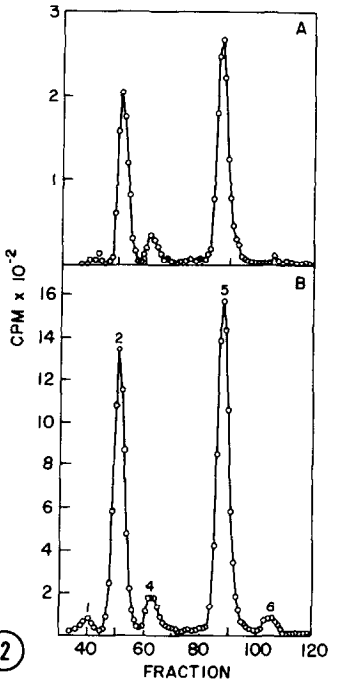


Fig. 1. RPC-5 profiles of $tRNA^{Lys}$ from midconfluency KA31 cells labeled for one hour *in vivo* or 20 min *in vitro*. Eight 75-cm^2 culture flasks were treated as described in Materials and Methods with [^{3}H]lysine (75 Ci/mmol) and 5.72 A_{260} of tRNA were obtained. Cell density was 7.1 x 10^4 cells/cm^2. For *in vitro* aminoacylation, 0.29 A_{260} of tRNA was saved. (A) *In vivo* aminoacylation; 5.32 A_{260} and 2 x 10^4 cpm applied. (B) *In vitro* aminoacylation; 0.043 A_{260} and 3 x 10^4 cpm applied.

Fig. 2. RPC-5 profiles of $tRNA^{Lys}$ from a very dense culture of KA31 cells labeled for one hour *in vivo* or 20 min *in vitro*. Four 75-cm^2 culture flasks were treated as described in Materials and Methods with [^{3}H]lysine (93 Ci/mmol) and 24.2 A_{260} of tRNA were obtained. Cell density was 7.8 x 10^5 cells/cm^2. For *in vitro* aminoacylation, 2.42 A_{260} of tRNA were saved. (A) *In vivo* aminoacylation; 6.05 A_{260} and 2 x 10^4 cpm applied. (B) *In vitro* aminoacylation; 0.04 A_{260} and 2 x 10^4 cpm applied.

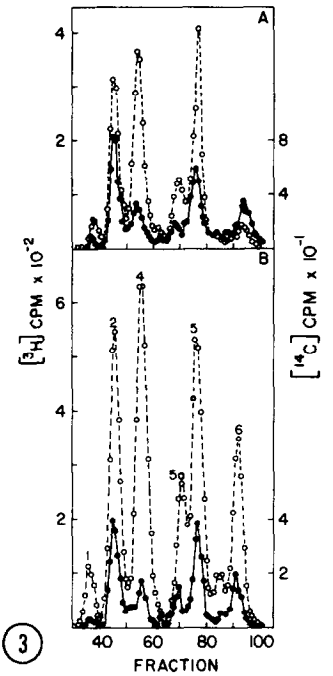
ting isoaccepting $tRNA^{Lys}$. tRNA from cells in midconfluence, labeled *in vivo* for 0.5 or 1 hr, had a typical distribution of isoacceptors of $tRNA^{Lys}$ except that $tRNA_6^{Lys}$ was not present or was just barely detectable (Fig. 1A). $tRNA_6^{Lys}$ was aminoacylated *in vitro* to an extent of about 5% of the total lysine isoacceptors (Fig. 1B), but it was labeled *in vivo* to less than 1% (Fig. 1A). $tRNA_1^{Lys}$ made up only 3% of the total, but it was clearly present under both conditions of labeling. $tRNA_4^{Lys}$ made up about 29% of the total, whether labeled *in vivo* or *in vitro*, and that amount is typical for rapidly growing cells (4,5).

The same results for $tRNA_6^{Lys}$ were obtained in an experiment involving labeling *in vivo* of cells in a heavy growth state in which there was considerable piling up of cells and in which cell growth slowed because of depletion of essential medium components or acidification of medium (Fig. 2A). Although $tRNA^{Lys}$ labeled *in vitro* had 3% of $tRNA_6^{Lys}$ (Fig. 2B), there was, at best, only a trace of $tRNA_6^{Lys}$ from the preparation labeled *in vivo*. In this experiment, labeling of $tRNA_4^{Lys}$ was the same *in vivo* and *in vitro*: 7% of the $tRNA^{Lys}$ isoacceptors, and that result is as expected for cells growing under less than optimal conditions (4,5).

When labeling *in vivo* was extended to 2 or 3 hr, $tRNA_6^{Lys}$ was clearly labeled. In 3 hr, $tRNA_6^{Lys}$ represented 4% of lysine isoacceptors labeled *in vivo* compared with 15% *in vitro* (Fig. 3). Similar results were obtained when cells were labeled for 5 or 17 hr. However, the RPC-5 profiles of [^{3}H]lysyl-tRNA formed during 5- and 17-hr labelings *in vivo* had uncharacteristic "backgrounds" through the regions of the major isoacceptors. This background was not present in these samples of tRNA aminoacylated *in vitro* and probably represents fragments of [^{3}H]lysyl-tRNA formed during normal degradation *in vivo*.

This apparent lag in aminoacylation *in vivo* of $tRNA_6^{Lys}$ with [^{3}H]lysine could be explained perhaps if this isoacceptor occurs as a constituent of an organelle. Thus, a crude preparation of mitochondria was used as a source of $tRNA^{Lys}$. The RPC-5 profile of lysyl-tRNA from mitochondria of KA31 cells has $tRNA_6^{Lys}$ as the major lysine isoacceptor (Fig. 4).

Discussion. These experiments were initiated because $tRNA_6^{Lys}$ does not function as well as the other isoacceptors in protein synthesis *in vitro*, at least in the wheat germ system with tobacco mosaic virus RNA as messenger (3). Thus, we intended to show whether the isoacceptor is fully aminoacylated *in vivo*. The results showed that it is aminoacylated but that the appearance of [^{3}H]lysine in $tRNA_6^{Lys}$ takes longer than for the other isoacceptors and that endogenous unlabeled lysyl-$tRNA_6^{Lys}$ is not fully exchanged for



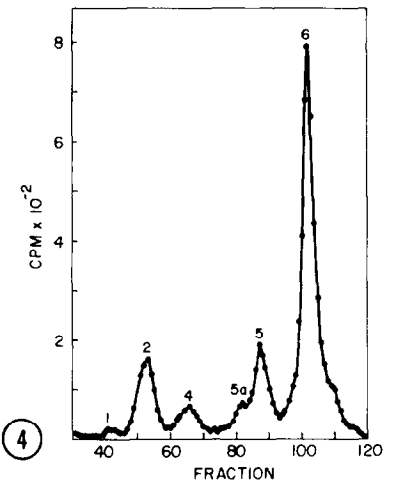


Fig. 3. RPC-5 profiles of $tRNA^{Lys}$ from KA31 cells labeled for three hours *in vivo* or 20 min *in vitro*. Six 75-cm^2 culture-flasks were treated as described in Materials and Methods with [^{3}H]lysine (93 Ci/mmol) and 4.20 A_{260} tRNA were obtained. Cell density was 9.0 x 10^4 cells/cm^2. For *in vitro* aminoacylation, 0.25 A_{260} of tRNA was saved. (A) *In vivo* aminoacylation; 4.02 A_{260} and 1 x 10^4 cpm applied. (B) *In vitro* aminoacylation; 0.025 A_{260} and 2.0 x 10^4 cpm applied. o---o, *in vivo* or *in vitro* aminoacylated [^{3}H]lysyl-tRNA; o——o, marker [^{14}C]lysyl-tRNA from a total preparation of KA31 tRNA.

Fig. 4. RPC-5 profile of $tRNA^{Lys}$ from a mitochondrial preparation from KA31 cells. Mitochondria were prepared and tRNA was obtained and aminoacylated *in vitro* as described in Materials and Methods.

[^{3}H]lysyl-$tRNA_6^{Lys}$ in labeling periods up to 3 hr or that only partial aminoacylation occurs. The sequestering or compartmentation of $tRNA_6^{Lys}$ in the cell so that its available lysine pool is different from the pool available to the other isoacceptors could explain, at least partially, these results. This explanation is supported by the demonstration of an enrichment of $tRNA_6^{Lys}$ in a mitochondrial preparation.

Costantino and Attardi (14) failed to obtain significant incorporation of [^{3}H]lysine and seven other amino acids into mitochondrially synthesized protein of HeLa cells. When the specific activity of [^{3}H]lysine was increased and its concentration was increased over ten-fold, label was incorporated

into protein synthesized in mitochondria (15). Thus, the sizes of amino acid pools of cytosol and mitochondria may influence the time of appearance of label in mitochondrial aminoacyl-tRNA and mitochondrially synthesized protein. Because we used culture medium normally containing lysine, [^{3}H]lysine available to the cells had a very low specific activity. If $tRNA_6^{Lys}$ is located in mitochondria, dilution of [^{3}H]lysine by cytosolic and mitochondrial pools may retard the rate of appearance of [^{3}H]lysine in lysyl-tRNA. Also, the rate of mitochondrial protein synthesis relative to cytosolic protein synthesis for KA31 cells is not known. A slower rate of mitochondrial protein synthesis would retard the appearance of label in mitochondrial lysyl-tRNA. The turnover of lysyl-$tRNA_6^{Lys}$, regardless of its cellular location, may be considerably slower than for the other isoacceptors because of a defectiveness in protein synthesis *in vivo* as well as *in vitro* or because of inefficient aminoacylation *in vivo*.

The results reported here add support to the importance of $tRNA_4^{Lys}$ in cell division (4,5) by demonstrating for the first time that results obtained by aminoacylating this isoacceptor *in vitro* accurately reflect the situation *in vivo*.

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