*ISOACCEPTING TRANSFER RNA'S OF L-M CELLS IN CULTURE AND AFTER TUMOR INDUCTION IN C_3H MICE**

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Abstract.—Co-chromatography in a reversed-phase column was performed for 16 aminoacyl-tRNA's prepared from L-M cells grown in serum-free suspension culture and from tumors induced in irradiated C_3H mice by subcutaneous injection of L-M cells. The results showed that between the two sources there were (1) marked differences in aspartyl-, histidyl-, phenylalanyl-, and tyrosyl-tRNA's; (2) significant quantitative differences in isoaccepting species of alanyl-, isoleucyl-, seryl-, and threonyl-tRNA's; and (3) similar to minimally different patterns of arginyl-, methionyl-, prolyl-, tryptophanyl-, valyl-, glycyl-, leucyl-, and lysyl-tRNA's. The differences were evident when synthetases prepared either from L-M cells or from the tumors were used. When the L-M tumors were brought back into *in vitro* culture, their tRNA patterns were like those of L-M cells. Addition of serum to the culture medium caused the L-M cells to show very minute, but detectable, amounts of the isoaccepting tRNA's found in the tumors. The cellular mechanisms which may be related to the changes of tRNA patterns in the L-M cells are discussed.

The multiplicity of tRNA's accepting the same amino acid (isoaccepting tRNA's) has been well documented in lower and higher organisms (see Yang and Novelli[1] for review). Apgar and Holley[2] first reported that rat liver and yeast showed different patterns of isoaccepting tRNA's. Other studies have demonstrated that different chromatographic profiles of certain tRNA's were obtained during T2 phage infection in *E. coli*,[3, 4] and during shifts of culture conditions in microorganisms.[5, 6] Similarly, different profiles have been demonstrated in organisms at different phylogenetic levels,[7] in different mammalian tissues and organs,[8] in tissues of the same kind that produce different proteins,[9] in cells from different stages of development,[10] and in cytoplasmic and mitochondrial fractions of cells.[11, 12] In this communication, we will present the results of a study on tumor formation by L-M cells, a subline of the clone 929 of L cells[13] that has grown in a serum-free medium since 1958.[14] These cells still retain the capacity of tumorigenesis in the C_3H/Anf mice[15] from which the original explant of normal subcutaneous connective tissue was taken in 1940.[16]

Materials and Methods.—L-M cells and tumors: Detailed conditions and procedures for growing L-M cells and developing tumors have been described.[15] For tRNA preparation, L-M cells from a 4-day-old suspension culture, in medium 199 supplemented with 0.5% Bacto-peptone (199-P), were harvested by centrifugation and either used fresh or stored frozen at −70°C until used. To obtain L-M tumors, 1×10^7 viable cells in 1 ml volume were injected into the left thigh of 7-week-old C_3H/Anf male and female mice which had been irradiated with 425 R of X rays 4 hr previously. At 14 days the animals were decapitated and the tumors excised carefully from the surrounding tissue. The

tumors, about 2 to 3 gm per mouse, were always clean and free from any necrosis. They were identified morphologically as fibrosarcomas. The L-M cells, at the time of the present study, were in the 250th to the 270th passage in 199P medium.

tRNA and aminoacyl-tRNA synthetases: tRNA was prepared by phenol extraction, 1 *M* NaCl extraction, and DEAE-cellulose column chromatography in the manner described previously.[1] Direct phenol extraction was found satisfactory for L-M cells suspended in 10 volumes of 0.15 *M* NaCl, 0.01 *M* $MgCl_2$, 0.001 *M* EDTA, and 0.2 mg/ml washed bentonite. The tRNA prepared by this method gave the same chromatographic profile as the preparation from a postmitochondrial supernatant of the cells.

Synthetases were prepared from the postmicrosomal supernatant by following the published method,[1] except that 15% instead of 10% glycerol and 1 mM of reduced glutathione instead of 20 mM of β-mercaptoethanol were included in all solutions for chromatography. Small vials containing the synthetase preparation were stored in liquid nitrogen.

Aminoacylation and RPC-2 chromatography: The conditions for the aminoacylation of tRNA were the same as described previously,[1] except that 1 mM glutathione instead of β-mercaptoethanol was used. With the concentration of ATP fixed at 4 mM, the optimal mg/ATP ratios predetermined and used for the activation of the amino acids were: 0.8 for Leu; 2 for Gly, Met, Pro, Thr, and Tyr; 3 for Arg, His, Try, Ser, and Val; and 5 for Ala, Asp, Ile, Lys, and Phe. Nineteen other nonlabeled amino acids were always included with the labeled amino acid during the reaction. The accepting activity of tRNA was assayed by the paper disc method.[17] All the labeled amino acids were purchased from the New England Nuclear Corporation.

The labeled aminoacyl-tRNA was isolated from the reaction mixture by the method in which a miniature DEAE-cellulose column is used.[9]

The reversed-phase column (RPC-2§) was constructed by the procedure of Weiss and Kelmers.[1, 18] It was found that chromosorb W could be reclaimed from used RPC-2 packing by washing first with distilled H_2O and then with absolute ethanol until the washings were clear. In this study many chromatographic runs were performed in a column made from the regenerated chromosorb W, and satisfactory resolution of isoaccepting tRNA's was obtained. Chromatography and assay for the radioactivity in the fractions were done as reported elsewhere.[1, 9, 19] The recovery of the trichloroacetic acid precipitable radioactivity from the column was usually constant for identical aminoacyl-tRNA's carrying different labels, but varied from 75 to 95% in the different aminoacyl-tRNA's.

Results.—tRNA and aminoacyl-tRNA synthetases preparations were both active, either isolated from L-M cells or from the tumors. To ensure that tRNA preparations were free from artificial modifications, the isolation was completed in about 8 hours and preliminary tests performed.[1] With saturating amounts of synthetases, the charging of labeled amino acid usually reached a plateau in 10 to 20 minutes which persisted up to 70 minutes. Ribonuclease activity was not detected in the synthetase preparation. Comparison of the various amino acid-accepting activities of the L-M cell tRNA's with those of the L-M tumor tRNA's did not reveal any remarkable relative change for a particular amino acid tRNA.

Co-chromatography of aminoacyl-tRNA's from L-M cells and L-M tumors: tRNA's isolated from the washed L-M cells and from fresh solid tumor tissues were acylated by synthetase preparations from the same source with the same amino acids, but with different labels (^3H and ^{14}C) and then co-chromatographed on the RPC-2 column. Chromatographic comparison was made for 16 aminoacyl-tRNA's. In Figures 1*a–h*, chromatographic patterns are shown for arginyl-, prolyl-, tryptophanyl-, valyl-, glycyl-, leucyl-, lysyl-, and methionyl-tRNA's, and either similar or minor, but reproducible, differences in the quantity of iso-

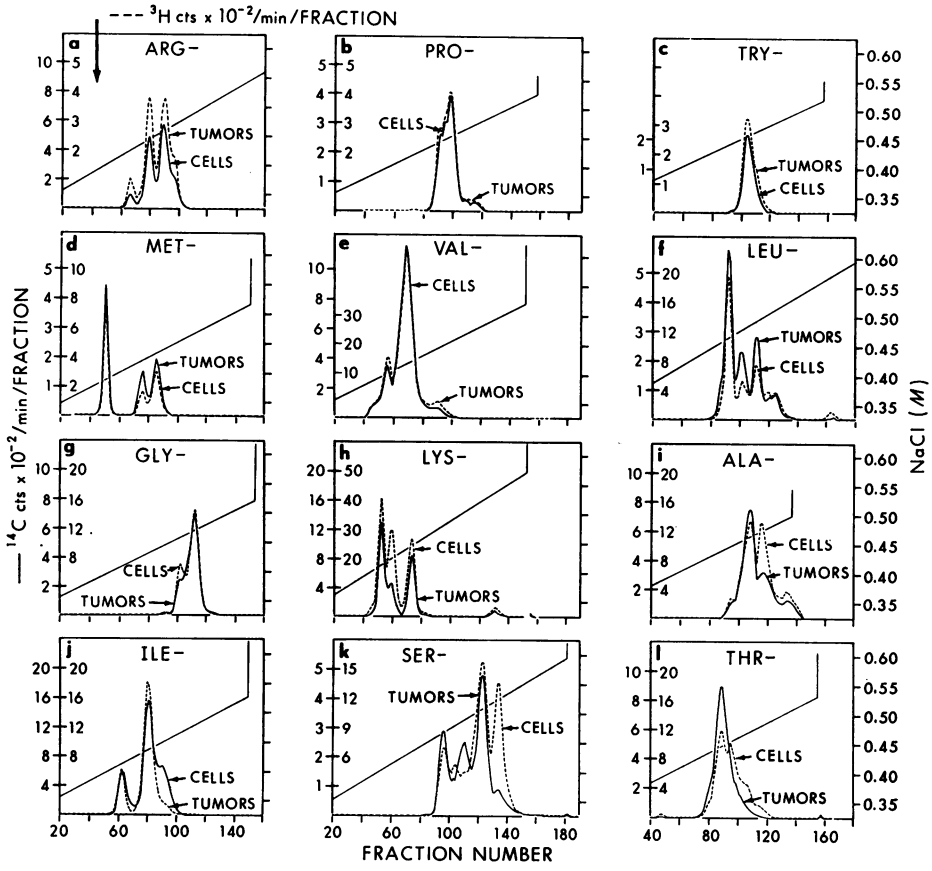

FIG. 1.—RPC-2 co-chromatography (*a-h*), of eight aminoacyl-tRNA's, showing similar to minimally different patterns in the 199P L-M cells and the L-M tumors, and (*i-l*) of four aminoacyl-tRNA's, showing quantitative differences among isoaccepting species in the L-M cells and tumors. The column, maintained at 20°C, was eluted at 1.5 ml/min with a linear gradient of 2-liter buffer containing 0.35–0.60 *M* NaCl, 0.01 *M* Na-acetate (pH 4.5), 0.01 *M* $MgCl_2$, 0.001 *M* EDTA, 0.003 *M* β-mercaptoethanol.

accepting tRNA peaks were observed. Significant quantitative differences were found in tRNA species accepting alanine, isoleucine, serine, and threonine (Figs. 1 *i–l*). There were also marked differences in the aspartyl-, histidyl-, phenylalanyl-, and tyrosyl-tRNA's of the L-M cells and tumors (Fig. 2). A tyrosyl-tRNA, migrating in the front of the four tyrosyl-tRNA's resolved on RPC-2, was very prominent in L-M tumors, but was completely absent in L-M cells. Of the two phenylalanyl-tRNA peaks detected, the first was about 60 per cent in L-M cells and below 5 per cent in L-M tumors; the second was about 40 per cent in L-M cells and more than 95 per cent in L-M tumors. L-M tumors have three histidyl-tRNA peaks, of which the most prominent one, in the front, was detected as the most minor in L-M cells. The L-M cells had two aspartyl-tRNA peaks of 17 and 83 per cent. The L-M tumors showed four aspartyl-tRNA peaks: two (2 and 10%) eluted from the column at the same salt concentrations

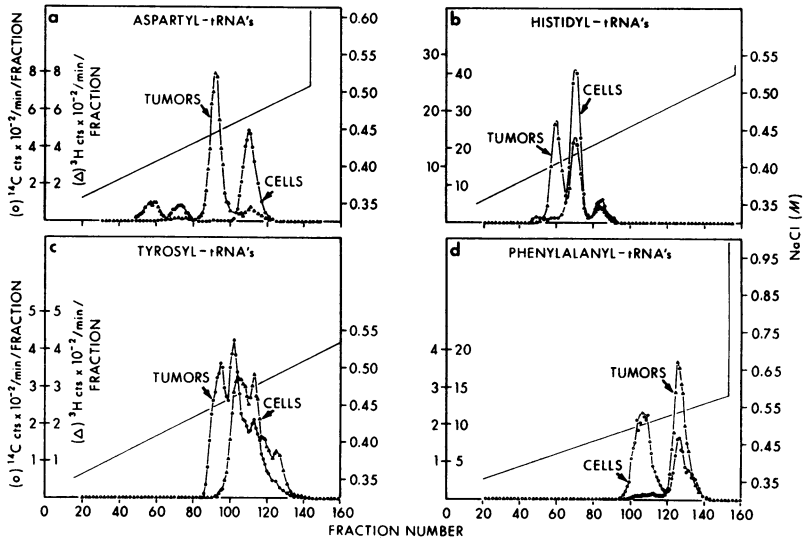

FIG. 2.—RPC-2 co-chromatography of four aminoacyl-tRNA's, showing marked differences in the L-M cells and tumors.

as those of L-M cells, and two (14 and 74%) eluted at different salt concentrations where nearly no aspartyl-tRNA's from L-M cells were found. It is noteworthy that the ratio of the two isoaccepting aspartyl-tRNA's in L-M cells, approximately 1:5, was the same as that found for the two pairs of aspartyl-tRNA's in the tumors (Table 1).

The authenticity of the differences between L-M cell and tumor tRNA's was verified in four other batches of L-M cells and tumors by several control experiments, which included reversing the labels of amino acid, exchanging the synthetase preparations (see below), pretreatment of tRNA at 85°C for six minutes before aminoacylation, and vital (*in vivo*) charging of tRNA with labeled amino acid in intact L-M cells.

L-M tumors grown in vitro: Tumor tissues were trypsinized and adapted to *in vitro* culture.[15] They were grown as a monolayer by one passage in medium 199 containing 10 per cent horse serum, and by several passages in 199P, and were

TABLE 1. *Percentages of four isoaccepting aspartyl-tRNA's resolved by RPC-2.*

Source of tRNA	Peak 1	2	3	4
L-M cells, 199P	0*	17.3	0.6	82.1
L-M cells, 199 + 5% mouse serum†	0.6	16.9	3.5	79.0
L-M cells, 199 + 5% horse serum†	0	18.2	0	81.8
L-M cells, 199 + 10% fetal calf serum‡	0.8	16.1	4.1	79.0
L-M tumors, female C_3H mice	14.3	2.1	74.1	9.5
L-M tumors, male C_3H mice	14.8	1.2	76.4	7.6
L-M tumors, *in vitro* 199P culture	0	17.8	0	82.2

* Sensitivity of the determination did not detect below 0.5%.
† After three passages.
‡ After ten passages.

then propagated in 199P suspension culture to obtain enough material for isolating tRNA. Chromatographic patterns of aspartyl-, phenylalanyl-, and tyrosyl-tRNA's from these *in vitro*-grown tumor cells were like those of the L-M cells in stead of those of solid L-M tumors (Figs. 3 *a* and *d*).

Effect of sera in culture medium on tRNA patterns of L-M cells: When 5 per cent C_3H/Anf mouse serum was substituted for 0.5 per cent Bacto-peptone in the culture medium for three passages, minute changes of aspartyl- and tyrosyl-tRNA patterns occurred in the L-M cells (Figs. 3 *b* and *e*), and tRNA species characteristic of L-M tumors appeared in very low quantity. Similar changes were found in the L-M cells after 10 passages in 10 per cent fetal calf serum. No such change, however, was observed in the L-M cells after three passages in 5 per cent horse serum.

Comparison with other C_3H mouse tissues: Figure 3*c* and *f* demonstrates that aspartyl- and tyrosyl-tRNA's similar to those of L-M tumors were obtained from the liver of the L-M tumor-bearing mouse. It was also found from other studies that phenylhydrazine-induced C_3H mouse reticulocytes contained seryl- and isoleucyl-tRNA's similar in RPC-2 chromatographic patterns to those of the tumors. However, the minor peaks of tumor tRNA's which were characteristic of the L-M cells were usually not detectable in the C_3H liver and reticulocytes.

Qualitative comparison of the synthetase preparations: Despite the fact that isoaccepting tRNA patterns for several amino acids were different in the L-M cells and the tumors, there was no alteration in the aminoacylation properties of the synthetases from these two sources. This was suggested by two kinds of experimental results: (1) RPC-2 chromatographic fractions of deacylated tRNA from the L-M cells or the tumors, when assayed separately by the L-M cells and the tumor synthetase preparations, showed exactly superimposed profiles of accept-

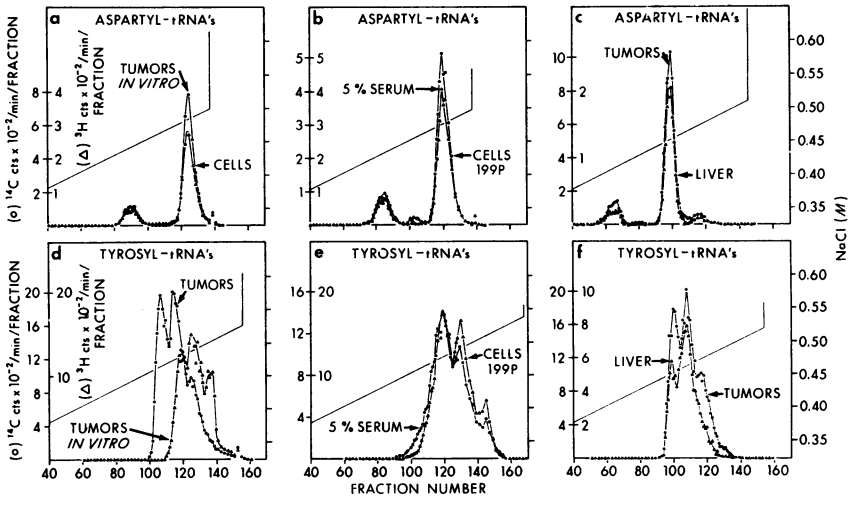

FIG. 3.—RPC-2 co-chromatography of aspartyl- and tyrosyl-tRNA's prepared from the L-M cells or L-M tumors with those from L-M tumor cells cultured *in vitro* in 199P (a and d), from L-M cells cultured in medium 199 including 5% mouse serum (b and e), and from livers of the L-M tumor-bearing C_3H/Anf mice (c and f).

ing activities for Ala, Arg, Asp, Gly, Ile, Leu, Lys, Met, Phe, Ser, Tyr, and Val; (2) exchanging the synthetase preparation for the precolumn aminoacylation did not alter the differences of aspartyl- or tyrosyl-tRNA's described previously for the L-M cells and the tumors.

Discussion.—The transfer of L-M cells from a serum-free medium *in vitro* to the body of the mouse *in vivo*, or vice versa, undoubtedly creates a very drastic change in their nutritional environment. The appearance of the different isoaccepting tRNA species in the L-M tumors and the disappearance of them upon *in vitro* culturing of the tumor cells, therefore, may indicate an adaptive response to these nutritional changes. This notion is supported by the demonstration that the isoaccepting tRNA species characteristic of L-M tumors become evident in L-M cells after serum is added to the culture medium. Too, these same species are present in the liver and reticulocytes, which presumably have the same nutritional environment as the tumors. These findings are similar to those observed in microorganisms following a shift of culture conditions.[5, 6] However, a more complicated situation may be involved in the conversion of tRNA patterns from those of L-M cells to those of L-M tumors, since there are a greater number of possible cellular mechanisms.

We have considered the following three possible cellular mechanisms, which may relate to our finding of isoaccepting tRNA changes: (1) selection of L-M cell clones *in vivo*, (2) viral activation during growth of cells in the serum environment, or (3) development of "differentiated" progeny as a result of *in vivo* homeostatic influence. These imply, respectively, that the different isoaccepting tRNA's observed in the tumors are gene products of the selected clones, are induced by the action of a foreign genome, or are related to the expression of different gene functions. Although derived from the single-cell clone 929, L-M cells were shown to be heterokaryotic by chromosomea nalysis; after *in vivo* passages, they changed to a population of cells with higher chromosome number and increased level of alkaline phosphatase activity.[20] These *in vivo* properties, however, persisted and did not disappear upon returning to culture *in vitro*. This phenomenon contrasts with our findings for the isoaccepting tRNA peaks. Certain clone 929 L cells grown in serum-containing medium have been shown to contain type C particles, which could react with antiserum against murine leukemia virus.[21] Further, the addition of fetal calf serum was shown to induce a gross activation of "C" particle secretion by the L-M cells.[22] Electron microscopic examinations of the materials used in our studies, however, did not reveal the presence of "C" particles in L-M cells grown in serum-containing medium, and only two such particles in one section of several L-M tumors examined.[23] Our histological sections demonstrate that the cellular composition of the L-M tumors is mainly of fusiform type, usually with the formation of interlacing bundles, intercellular reticulum, and fibroglial fibers—the characteristic functional features of fibroblast reported for the L-cell sarcoma.[24] These features differ greatly from those of the L-M cells, which assume a spherical shape and have a seemingly undifferentiated morphology in the suspension culture. In two reviews of experimental work involving single tumor cell transplantation with teratocarcinoma and epidermoid carcinoma, Pierce[25] has pointed out that some

tumor cells are continually differentiating *in vivo* into cells incapable of tumor formation. Our preliminary results showed that, for a comparable transplantation of the tumors, at least ten times more dispersed viable cells from the primary L-M tumors than L-M cells from 199P culture were required, indicating that a large portion of the cells in the L-M tumors might have lost their proliferating activity. All of this circumstantial evidence, though insufficient to rule out the aforementioned first and second cellular mechanisms, tend to support the third, namely, that L-M cells may "differentiate" *in vivo* into progeny characterized by different isoaccepting tRNA profiles. A direct correlation of tRNA patterns and the cellular functions, however, remain to be established.

Using reversed-phase column chromatography, two laboratories[26] have shown that tRNA's with differing codon recognition properties can be successfully resolved although differing chromatographic tRNA peaks may recognize the same condon. Gefter and Russel[27] recently reported that modifications on a single base of an *E. coli* suppressor tyrosine tRNA molecule can affect its capacity to bind to ribosomes and that these modified forms of tyrosine tRNA can be separated by reversed-phase column chromatography. Hence, the differences in chromatographic profiles of isoaccepting tRNA's from L-M cells and L-M tumors may represent base exchanges, resulting in a different codon recognition, or base modifications, or both. For this distinction, three particular chromatographic features seem helpful. First, L-M tumors have 4 aspartyl-, 4 to 5 tyrosyl-, and 3 histidyl-tRNA peaks—each is more than 2, which is the number of codons recognized by these aminoacyl tRNA's in *E. coli* as well as in livers of the frog and the guinea pig.[28] Furthermore, the aspartyl-tRNA's can be paired into duplex sets. Second, the tRNA's of the L-M cells tend to elute in the middle of the chromatogram, a feature consistent with what has been found for undermethylated *E. coli* tRNA's.[4, 29] Third, our observation shows that the late-eluting peaks of Ser- and Ile-tRNA's, which are marked in the L-M cells, become relatively obliterated when reducing agent is omitted from the gradient buffer. These observations suggest that the L-M cells and the tumors may differ in tRNA base modifications involving a methyl group,[30] or an oxidizable side chain,[6, 31] or both. Further investigation along this line is in progress.

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‡ Head, Biohazard Section, National Cancer Institute, National Institutes of Health.

§ The reversed phase chromatographic system which utilizes 5% triacprylylmethylammonium chloride in tetrachlorotetrafluoropropane for the coating of chromosorb W is designated as RPC-2, to differentiate it from RPC-1 which utilizes 4% dimethyldilaurylammonium chloride in isoamylacetate, RPC-3 which utilizes trioctylpropylammonium bromide and RPC-4 which utilizes dimethyldilaurylammonium chloride without isoamylacetate.

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