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Relation of cell type and cell density in tissue culture to the isoaccepting spectra of the nucleoside Q containing tRNAs: $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$ and $tRNA^{Asp}$

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ABSTRACT

An examination, using reversed-phase chromatography and cyanogen bromide treatment, of $tRNA^{Tyr}$, $tRNA^{His}$, $tRNA^{Asn}$, and $tRNA^{Asp}$ from SV40-transformed mouse fibroblasts grown to different cell densities, untransformed cells grown to confluence, and mouse liver indicates that: (1) The tissue cultured mouse fibroblasts examined here are hypomodified with respect to nucleoside Q, while liver tRNA is almost completely modified with respect to Q. (2) Cell density and/or proliferative state do not present as major variables in controlling the expression of Q in the present system. (3) SV40 virus transformation is not a major variable controlling the expression of Q in the present system. The present results support previous use of cyanogen bromide effected shifts in chromatographic elution as an assay for nucleoside Q.

INTRODUCTION

Previous work from this laboratory has identified two growth variables which affect the expression of specific tRNA post-transcriptional modifications in tissue culture: a serum factor and cell density. Growth of SV40 virus transformed mouse fibroblasts (clone SVT2) in medium supplemented with fetal bovine serum rather than calf serum increases the nucleoside Q-containing isoaccepting species of $tRNA^{Asn}$, $tRNA^{His}$, $tRNA^{Asp}$ and $tRNA^{Tyr}$ (1). With increasing cell density of SVT2 cells, the degrees of the peroxy-Y modification in $tRNA^{Phe}$ and an undetermined modification(s) in $tRNA^{Lys}$ become more like that of liver (2). The effect of serum appears to be specific for the Q modification in that negligible serum induced differences are observed with other tRNAs examined (1). Likewise, the effect of cell density is significant only for $tRNA^{Lys}$ and $tRNA^{Phe}$ (1); however, small density-effected decreases in the apparent Q content of $tRNA^{Asp}$ and $tRNA^{Tyr}$ have been observed (1).

The present communication seeks to clarify the effect of cell density on the Q containing tRNAs. In addition, so that information derived from studies of tRNA isolated from tissue culture can be extrapolated to the *in vivo* state, the Q containing tRNAs from tissue culture (untransformed and SV40 virus transformed mouse fibroblasts) and mouse liver are compared.

It is important to note that eucaryotic tRNA exhibits not only nucleoside Q, but also Q*, derivatives of Q having mannose or galactose units linked to the cyclopentene diol moiety (3). Moreover, Q and Q* are distributed differently among rabbit liver tRNA species: apparently $tRNA^{Asp}$ contains only mannosyl Q, $tRNA^{Tyr}$ contains only galactosyl Q, and $tRNA^{Asn}$ and $tRNA^{His}$ contain only Q (4). In the present study, Q will be used generically to designate Q and/or Q*.

MATERIALS AND METHODS

Cells and culture methods. The BALB/3T3 (clone A31) mouse cell line (5) and an SV40-transformed subclone (SVT2) derived from it (6) were provided by G. J. Todaro, National Cancer Institute. During the period of this research these lines were found to be free of Mycoplasma contamination (Mycoplasma testing service of Microbiological Associates, Inc.). BALB/c mice were purchased from Flow Laboratories. General culture methods have been described (7). Preparative cell growth was performed in glass roller bottles (1400 cm^2 cell growing area) using 200 ml HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid)/TRICINE [N-tris-(hydroxymethyl)methylglycine]-buffered Dulbecco's medium (20mM HEPES, 10mM TRICINE, 24 mM $NaHCO_3$, pH 7.6) (ref.8), supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% calf serum. SVT2 cells were inoculated at $1 \cdot 10^7$ cells/bottle on day 1 and the medium changed on days 4, 6 and 8; cultures were harvested (18-21 h after medium change) on days 5, 7, and 9. A31 cells were inoculated at $2 \cdot 10^7$ cells/bottle, medium changed on days 4 and 7 (at which time they were confluent), and harvested on day 8.

Preparation, aminoacylation and reversed-phase chroma-

tography of tRNA. Isolation of tRNA and the preparation of a mixture of aminoacyl-tRNA synthetases have been described (7). A synthetase preparation from SVT2 cells grown to high density was used for aminoacylation, unless otherwise noted. In one instance a mouse liver synthetase preparation, prepared as described [7] but omitting the Sephadex G-100 step, was used. No differences in specificity between synthetase preparations from SVT2 cells and mouse liver were observed. Cyanogen bromide (BrCN) treatment of tRNA was performed as previously described [1]. tRNA was aminoacylated at 37°C as described [9], with either a ^{3}H- or a ^{14}C-labeled amino acid in the presence of 19 unlabeled amino acids (all amino acid concentrations were 10 μM, except that asparagine aminoacylation was performed in the presence of 50 μM unlabeled aspartic acid). Isoaccepting species were separated by reversed-phase chromatography using an RPC-5 column (0.9 cm by 20-21 cm) as described [1]. Cochromatographic comparisons insure that the peak numbers represent isoeluting species in each instance, and recoveries of labeled tRNA added were greater than 99% for $tRNA^{Asp}$ and $tRNA^{Tyr}$, 66-84% for $tRNA^{His}$, and 72-86% for $tRNA^{Asn}$.

RESULTS

$tRNA^{Asp}$. A comparison of $tRNA^{Asp}$ isolated from the untransformed A31 cell line grown to confluence (maximum density under these growth conditions) and mouse liver is shown in Figure 1A (four peaks are designated, for simplicity and for continuity with previously published studies of mammalian $tRNA^{Asp}$, though peaks 1, 2 and 4 are doublets). $tRNA^{Asp}$ from liver contains no more than trace quantities of peaks 2 and 4. By the criterion of BrCN-effected shifts in chromatographic elution for Q-containing tRNAs, $tRNA^{Asp}$ peaks 2 and 4 are Q-negative and peaks 1 and 3 have been shown to be Q-positive (1). Therefore, virtually all mouse liver $tRNA^{Asp}$ is Q-positive, a result in agreement with data of Okada *et al.* (4) and Roe *et al.* (10). A comparison (Fig. 1B) of $tRNA^{Asp}$ from mouse liver tRNA treated with BrCN prior to aminoacylation and untreated tRNA from SVT2 cells (1.6×10^{8} cells/bottle,

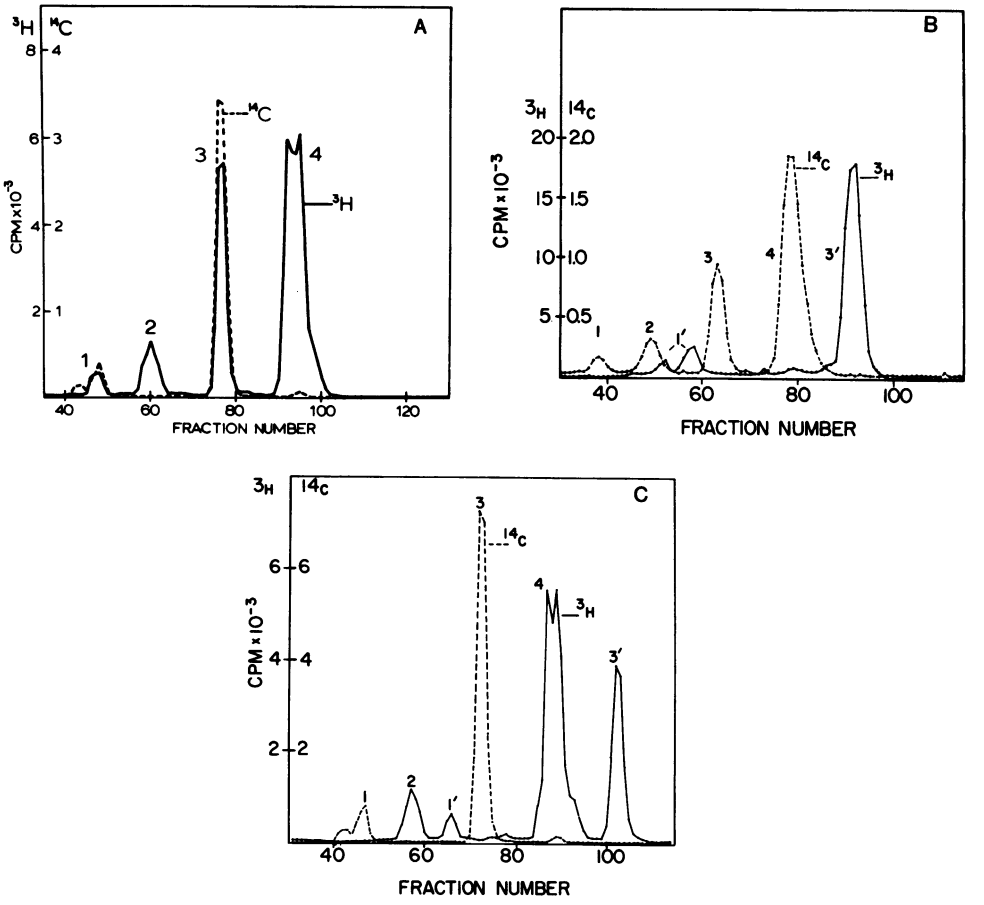

Figure 1. RPC-5 cochromatographic comparisons of Asp-tRNAs. (A) [^{3}H]Asp-tRNA from A31 cells and [^{14}C]Asp-tRNA from liver. (B) [^{3}H]Asp-tRNA, treated with BrCN prior to aminoacylation, from liver and [^{14}C]Asp-tRNA from SVT2 cells (1.6 x 10^8 cells/bottle, Table 1). (C) [^{3}H]Asp-tRNA, treated with BrCN prior to aminoacylation, from A31 cells and [^{14}C]Asp-tRNA from liver. Aminoacylation for (B) was performed with mouse liver synthetase; a 300 ml 0.48-0.75 M NaCl gradient at 27° was used for elution in each instance; additional experimental details are found in the text.

Table 1) supports this view. All of the $tRNA^{Asp}$ from mouse liver is shifted to later elution (designated peaks 1' and 3' to signify their origins) upon BrCN treatment. A comparison (Fig. 1C) of $tRNA^{Asp}$ from A31 cell tRNA treated with BrCN and untreated mouse liver tRNA shows again that peaks 1 and 3 are shifted to later elution (peaks 1' and 3') by BrCN, but that peaks 2 and 4 are unchanged by BrCN.

TABLE 1

Effect of cell density and cell type on the relative proportions of $tRNA^{Asp}$ resolved by RPC-5 chromatography.

tRNA Source	Cell Density[a]	Percent of Total $tRNA^{Asp}$ CPM in Peaks[b]				
		1	2	3	4	1.5 M NaCl
SVT2 Cells	1.6	1.9	10.5	23.8	61.6	2.2
	4.4	3.9	9.6	19.9	66.3	0.1
	6.6	4.5	9.9	21.0	64.5	0.0
A31 Cells	0.8	3.5	8.7	26.6	57.8	3.3
BALB/c Liver	---	13.7	0.2	84.8	1.2	0.0

[a] Cells per roller bottle x 10^8.

[b] Peak number designations correspond to those in Fig. 1. Percent values were determined by dividing the radioactivity in each peak by the total redioactivity recovered from all peaks.

When $tRNA^{Asp}$ profiles from SVT2 cells grown to different cell densities are compared (Table 1), an apparent density dependent increase is noted for peak 1. In that density dependent decreases for peaks 1 and 3 (with reciprocal increases in peaks 2 and 4) were found previously (1), the present data do not support the view that cell density is an important variable in the expression of nucleoside Q in $tRNA^{Asp}$ in tissue culture.

$tRNA^{Tyr}$. A comparison of $tRNA^{Tyr}$ isolated from A31 cells and mouse liver is shown in Figure 2A. Only the early eluting forms are found in liver tRNA. Previously, from BrCN-effected shifts in chromatographic elution, SVT2 cell $tRNA^{Tyr}$ peaks 1 and 2 were determined to be Q-positive and the remaining peaks to be Q-negative (1). However, after BrCN treatment, the $tRNA^{Tyr}$ patterns (Fig. 2B) from A31 cell and liver tRNAs differ in an important respect: while peaks 1 and 2 are eliminated by BrCN from both tRNAs, a large amount of peak 3 remains in the BrCN-treated A31 cell tRNA and only a trace amounts of peak 3 remains in the BrCN-treated liver

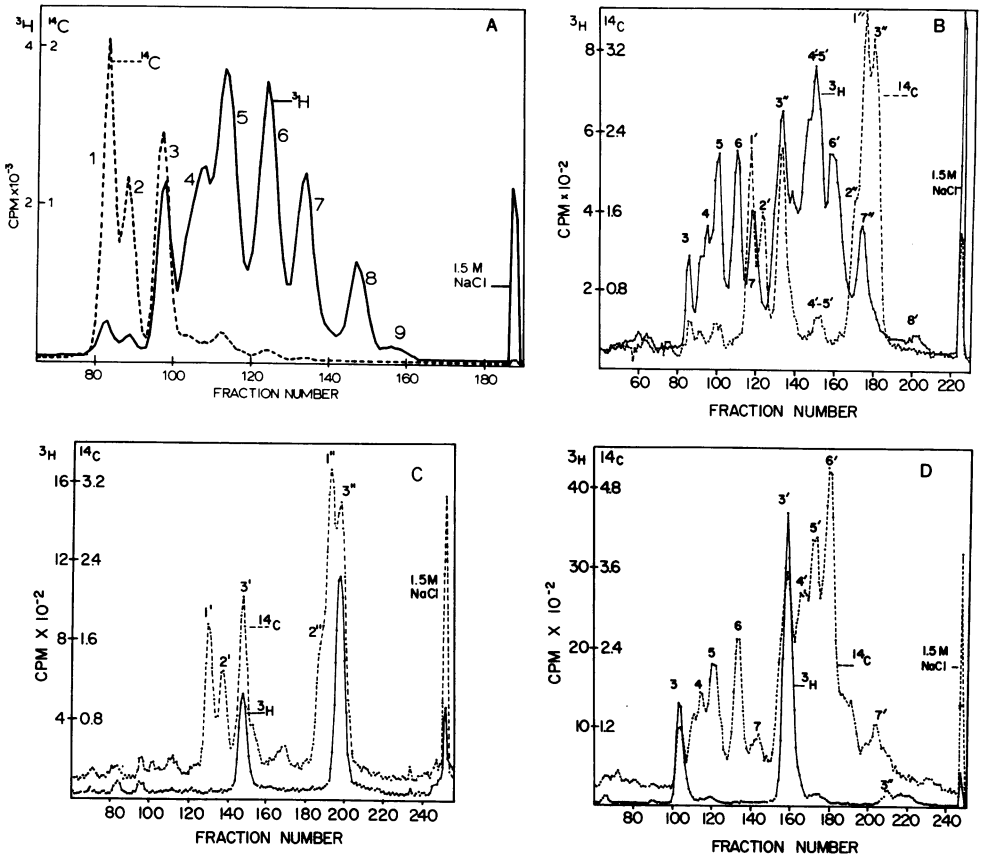

Figure 2. RPC-5 cochromatographic comparisons of Tyr-tRNAs. (A) [^{3}H]Tyr-tRNA from A31 cells and [^{14}C]Tyr-tRNA from liver; eluted with a 400 ml 0.52-0.68 M NaCl gradient at 37°. (B) [^{3}H]Tyr-tRNA, treated with BrCN prior to aminoacylation, from A31 cells and [^{14}C]Tyr-tRNA, treated with BrCN prior to aminoacylation, from liver; eluted with a 600 ml 0.52-0.68 M NaCl gradient at 37°. (C) [^{3}H]Tyr-tRNA$_3^{Tyr}$, resolved by RPC-5 chromatography and treated with BrCN prior to aminoacylation, from liver; [^{14}C]Tyr-tRNA, treated with BrCN prior to aminoacylation, from SVT2 cells (4.4 x 10^8 cells/bottle, Table 2); eluted with a 600 ml 0.52-0.76 M NaCl gradient at 37°. (D) [^{3}H]Tyr-tRNA$_3^{Tyr}$, resolved by RPC-5 chromatography and treated with BrCN prior to aminoacylation, from SVT2 cells; [^{14}C]Tyr-tRNA, treated with BrCN prior to aminoacylation, from SVT2 cells (4.4 x 10^8 cells/bottle, Table 2); eluted with a 600 ml 0.52-0.76 M NaCl gradient at 37°. Additional experimental details are found in the text.

tRNA. In order to resolve these conflicting data and to identify the source of the peaks designated 1', 2', 3' and 1", 2", and 3" in Figs. 2B, 2C, and 2D, liver tRNA^{Tyr} peaks

1, 2, and 3 were separated by RPC-5 chromatography, treated with BrCN, aminoacylated and cochromatographed with BrCN-treated SVT2 cell tRNA and BrCN-treated liver tRNA. Comparison of $tRNA^{Tyr}$ from BrCN-treated liver whole tRNA and BrCN-treated liver $tRNA_3^{Tyr}$ (Fig. 2C) shows an almost complete shift of $tRNA_3^{Tyr}$ to later elution (peaks 3' and 3"). The data for resolved peaks 1 and 2 are not shown, peaks 1-7 cochromatograph with untreated $tRNA^{Tyr}$, and the designation of the other peaks is inferred from relative elution order. When $tRNA_3^{Tyr}$ from SVT2 cells (4.4×10^8 cells/bottle, Table 2) is examined similarly [isolated by RPC-5 chromatography, treated with BrCN, aminoacylated and cochromatographed with BrCN-treated unfractionated SVT2 tRNA (Fig. 2D)], a large portion of peak 3 remains unchanged in elution, peak 3' is prominent, and only a trace amount of peak 3" is found.

Interpretation of data resulting from BrCN treatment of mammalian $tRNA^{Tyr}$ and $tRNA^{Asn}$, which contain the BrCN reactive nucleoside 3-(3-amino-3-carboxypropyl)uridine (acp^3U) (10-12) in addition to Q, is complicated because BrCN treatment effects later RPC-5 elution of tRNAs which contain either acp^3U or Q (1,13). However, the efficient reaction of BrCN with $tRNA^{Asp}$ and $tRNA_1^{His}$ (see below), which do not contain acp^3U, argues that incomplete reaction of BrCN with acp^3U (or a derivative of acp^3U) is responsible for the double peaks formed from $tRNA^{Tyr}$. This being the case, it is likely that $tRNA^{Tyr}$ peak 3" (Figs. 2B and 2C) is doubly modified by BrCN and derived from a $tRNA_3^{Tyr}$ which contains both Q and acp^3U, and that peak 3' (Figs. 2B, 2C, and 2D) is singly modified by BrCN, either at acp^3U alone (no Q content) or at Q alone (incomplete reaction with acp^3U or no acp^3U content). In any event, though $tRNA_3^{Tyr}$ from liver and tissue culture cells is indistinguishable by high resolution RPC-5 chromatography, the major component behaves as Q-positive in BALB/c liver tRNA [in agreement with other data suggesting that liver $tRNA^{Tyr}$ is almost completely modified with respect to Q (4,10)] and Q-negative in SVT2 and A31 cell tRNA.

When $tRNA^{Tyr}$ profiles from SVT2 cells grown to different cell densities are compared (Table 2), negligible density

TABLE 2

Effect of cell density and cell type on the relative proportions of $tRNA^{Tyr}$ resolved by RPC-5 chromatography.

tRNA Source	Cell[a] Density	Percent of Total $tRNA^{Tyr}$ CPM in Peaks[b]								
		1	2	3	4	5	6	7	8	1.5M NaCl
SVT2 Cells	1.6	2.3	1.4	19.4	17.3	28.5	16.0	10.3	3.9	0.1
	4.4	2.3	1.6	11.8	23.5	24.6	24.1	8.2	1.9	1.8
	6.6	2.3	2.1	8.8	21.0	21.5	29.7	11.1	1.6	1.7
A31 Cells	0.8	2.0	1.2	10.2	15.2	24.3	22.1	13.6	6.8	3.8
BALB/c Liver		38.3	21.0	29.5	4.8	4.7	1.5	0.0	0.0	0.1

[a]Cells per roller bottle x 10^8.

[b]Peak number designations correspond to those in Fig. 2. Percent values were determined as in Table 1. Peak 9 contained less than 1% of the CPM in all cases.

dependent differences are noted for peaks 1 and 2, the Q-containing species, though others, especially peak 3, show differences.

$tRNA^{Asn}$. A comparison of $tRNA^{Asn}$ isolated from A31 cells and mouse liver is shown in Figure 3A. $tRNA_1^{Asn}$ is a minor species in A31 cell tRNA, but the major species in liver tRNA. Virtually all of liver $tRNA_1^{Asn}$ is BrCN-sensitive, while only a portion of liver $tRNA_2^{Asn}$ is BrCN-insensitive (Fig. 3B). Again, the problem (see above) imposed by the presence of acp^3U in $tRNA^{Asn}$ must be kept in mind, but the results in Fig. 3B are consistent with previous data (1,10,14) suggesting that $tRNA_1^{Asn}$ is Q-positive and that at least a portion of $tRNA_2^{Asn}$ is Q-negative. Nonetheless, in agreement with others (4,10,14), most $tRNA^{Asn}$ from liver appears to be modified with respect to Q.

$tRNA^{His}$. A comparison of $tRNA^{His}$ isolated from A31 cells and mouse liver is shown in Figure 4A. The major species in liver, $tRNA_{1a}^{His}$ and $tRNA_1^{His}$, are absent and present in low amounts, respectively, in A31 cells. As predicted from

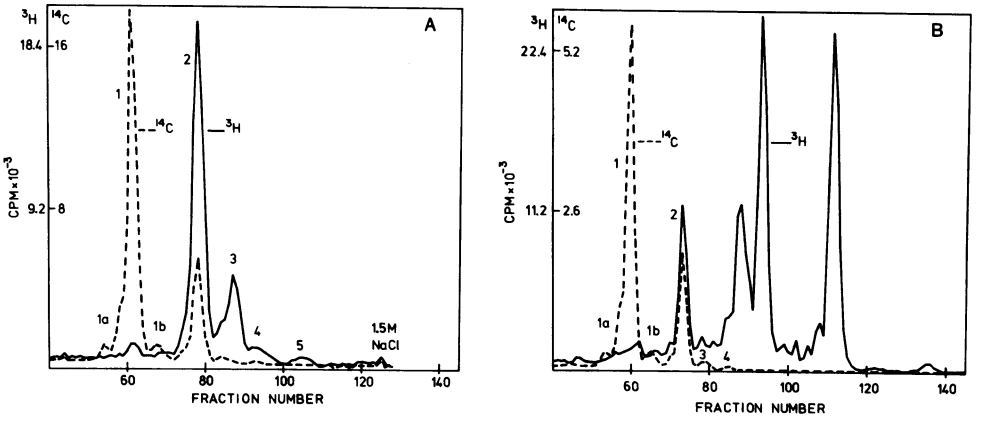

Figure 3. RPC-5 cochromatographic comparisons of Asn-tRNAs. (A) [^{3}H]Asn-tRNA from A31 cells and [^{14}C]Asn-tRNA from liver. (B) [^{3}H]Asn-tRNA, treated with BrCN prior to aminoacylation, from liver and [^{14}C]Asn-tRNA from liver. A 300 ml 0.48-0.75 M NaCl gradient at 37° was used for elution in each instance; additional experimental details are found in the text.

previous data on SVT2 cell tRNA (1), liver tRNA peaks 1a and 1 are BrCN-sensitive, while at least a portion of peak 2 (and perhaps, peak 3) is BrCN-insensitive (Fig. 4B). These results are consistent with other reports that the large majority of liver tRNA^{His} is modified with respect to Q (4,10,15), but that a minor, later eluting, Q-negative tRNA^{His} fraction exists as well (15).

DISCUSSION

Three conclusions can be drawn from the present data.

1. The tissue cultured mouse fibroblasts examined here are hypomodified with respect to Q, while liver tRNA is almost completely modified with respect to Q. A factor in bovine serum appears to increase the Q content of SVT2 cell tRNA (1), but other variables must exist as well, because all tissue culture cells are not hypomodified with respect to Q (16).

2. Cell density and/or proliferative state are not major variables in controlling the expression of Q in A31 and SVT2 cells. This conclusion differs from a previous report that the Q content of SVT2 tRNA^{Asp} and tRNA^{Tyr} decreases with increasing cell density (1). In that the previous study employed bicarbonate-buffered cell culture medium, which

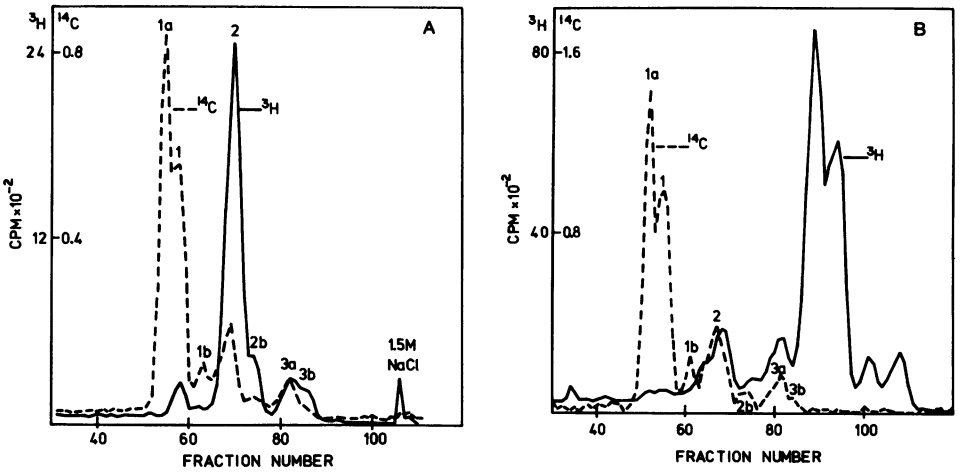

Figure 4. RPC-5 cochromatographic comparisons of His-tRNAs. (A) [^{3}H]His-tRNA from A31 cells and [^{14}C]His-tRNA from liver. (B) [^{3}H]His-tRNA, treated with BrCN prior to aminoacylation, from liver and [^{14}C]His-tRNA from liver. A 300 ml 0.50-0.75 M NaCl gradient at 27° was used for elution in each instance; additional experimental details are found in the text.

allows cultures to become acidic (pH 6.8-7.0) at high cell densities, while the present study has controlled culture pH more effectively by the use of HEPES plus TRICINE buffering (8), the previously reported effect of cell density on Q-containing tRNAs appears to have been an artifact resulting from decreased culture pH with increased cell density. 3. SV40 virus transformation is not a major variable controlling the expression of Q in the present system. This finding is surprising because tRNA isoaccepting profile changes consistent with decreased levels of Q-content are a common feature associated with SV40 or polyoma virus infection and transformation, both in tissue culture and *in vivo* (1,17,18). The A31 and SVT2 cells may not show this phenomenon relative to each other or it may have been masked by the present growth conditions.

Differences in tRNA isoaccepting spectra consistent with differences in the extent of Q-base modification have been observed in a number of *in vivo* and *in vitro* mammalian cell systems (1,17-19). However, no other biochemical or morphological phenotype has yet been linked with these differences.

In order to correlate differences in the extent of Q-base formation with a physiological function, a sensitive assay for Q in unfractionated tRNA is required and the indirect BrCN-effected shift in chromatographic elution is such an assay. The present data support previous use of the BrCN assay for Q (1,10). BrCN appears to be specific for the Q-base in $tRNA^{Asp}$ and $tRNA^{His}$ and to be useful for Q determination in $tRNA^{Tyr}$ and $tRNA^{Asn}$, bearing in mind that BrCN reacts with acp^3U as well. In addition, from the present results, both Q and Q* react with BrCN, in agreement with Roe *et al.* (10).

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