

Defective Transfer RNA-Queuine Modification in C3H10T1/2 Murine Fibroblasts Transfected with Oncogenic *ras*¹

Claudia J. Morgan,² Frank L. Merrill, and R. W. Trewyn

Departments of Surgery and Biochemistry, Tulane University School of Medicine, New Orleans, Louisiana 70112 [C. J. M., F. L. M.], and Division of Biology, Kansas State University, Manhattan, Kansas 66506 [R. W. T.]

ABSTRACT

tRNA isoacceptors for aspartic acid, asparagine, histidine, and tyrosine are modified in the anticodon wobble position with the deazaguanine analogue queuine. Queuine modification is defective in many tumors and transformed cell lines, and the extent of hypomodification correlates with staging and outcome in numerous human tumors. The molecular role of queuine modification in normal cells and the mechanism of queuine hypomodification in tumors are unknown. We have characterized non-transformed C3H10T1/2 murine fibroblasts (C3H) and their *ras*-transfected counterparts (RasC4) with respect to the causes and effects of queuine hypomodification. RasC4 cells are hypomodified for queuine compared with C3H cells, despite increased tRNA-guanine ribosyltransferase activity. Excess exogenous queuine can cause repletion of tRNA queuine levels in RasC4 cells. Queuine modification of both C3H and RasC4 cells can be decreased by treatment with 7-methylguanine. This treatment does not affect growth in monolayer culture but enhances anchorage-independent growth of RasC4 cells greatly. These cell lines may be useful systems for the study of queuine function in normal cells and the causes and consequences of hypomodification for queuine in tumors.

INTRODUCTION

tRNA isoacceptors for aspartic acid, asparagine, tyrosine, and histidine are modified posttranscriptionally in the first (wobble) position of the anticodon by substitution of the deazaguanine analogue queuine for guanine. The modification occurs by base exchange and is catalyzed by the enzyme tRNA-GRT³ (EC 2.4.2.29). The physiological role of this modification is unknown, but evidence suggests that queuine modification is important for normal cellular regulation. In normal, nonneoplastic cells, tRNA isoacceptors for these four amino acids are predominantly queuine modified, but in many tumors, the corresponding tRNAs are deficient in queuine modification, having guanine in the anticodon first position (reviewed in Ref. 1). In human leukemias, lymphomas, and lung and ovarian tumors, the extent of hypomodification for queuine correlates with both histopathological staging and outcome (2-5).

The mechanism by which tRNA becomes hypomodified for queuine in neoplastic cells is not known. Bacterial cells are capable of synthesizing queuine, but cells of all higher eukaryotes must obtain queuine exogenously or by salvage (6). Several cell lines and tumors have been found to salvage queuine from the nucleotide; however, not all cell lines have this capability (5, 7). Uptake of exogenous queuine by cultured cells has both low and high affinity components and is influenced by agents that regulate protein kinase C (8-10). Some cell

lines, such as LM cell fibroblasts, do not seem to possess the high affinity uptake component (11).

It is unclear how queuine hypomodification contributes to neoplastic transformation. Conflicting observations have been reported from studies in murine models. Queuine-free mice, derived by maintaining animals on a queuine-free diet in sterile conditions for 1 year, did not have an increased risk of tumors (12). Because such animals were maintained in a sterile, protected environment, interpretation of these data with respect to tumor risk under normal environmental conditions is difficult. In contrast, perfusion of mice bearing Ehrlich's ascites tumors with queuine caused repletion of the tRNA-queuine content of the tumor cells and inhibition of tumor growth, suggesting that queuine depletion of tRNA may favor tumor growth (13). In tissue culture, a causative role for queuine in transformation has been observed (14). In primary Chinese hamster embryo fibroblasts exposed to the initiating agent 3-methylcholanthrene, culture for 6 weeks in the presence of m⁷G, an inhibitor of queuine modification by tRNA-GRT (15), permitted the emergence of cells with a transformed phenotype, characterized by focus formation in monolayer culture, and growth in anchorage-independent culture. After prolonged culture in m⁷G, stably transformed cell lines with tRNA hypomodified for queuine were obtained.

To study the function of the tRNA-queuine modification in normal and neoplastic cells, we have used a murine fibroblast cell line, RasC4, produced by introduction of an oncogenic *ras* gene (pEJ6.6; Ref. 16) into the murine fibroblast cell line C3H10T1/2 (C3H Ref. 17). RasC4 cells exhibited a greatly reduced serum requirement for growth in culture, were epidermal growth factor independent for early G₁ traverse, and had a G₁ transit time reduced from 12 to 6 h (17). RasC4 cells have a raised, spindle shape in culture and do not form a density-arrested monolayer characteristic of C3H cells but continue to proliferate to form foci in monolayer culture. We have observed that RasC4 cells are queuine hypomodified relative to the untransformed C3H10T1/2 fibroblasts. In this report, we characterize C3H and RasC4 cells with respect to the potential causes and effects of queuine hypomodification of tRNA.

MATERIALS AND METHODS

Cells. RasC4 cells were provided kindly by Dr. Ed Leof (Mayo Clinic, Rochester, MN). C3H10T1/2 cells were obtained initially from Dr. Leof; low passage cells were supplied thereafter by the American Type Culture Collection (Rockville MD). Cells were cultured in DMEM containing 10% FBS (Hyclone, Logan UT). C3H cells were used between passages 9 and 15.

tRNA Purification. tRNA was prepared by a modification of our previous protocol (18). Briefly, cells were homogenized in TriReagent (Molecular Research Center, Inc., Cincinnati, OH), extracted with chloroform, and precipitated according to the manufacturer's instructions. Precipitates were solubilized in H₂O, made 2 M in LiCl, and refrigerated overnight. After centrifugation at 12,000 × g for 15 min at 4°C, tRNA in the supernatant was recovered by ethanol precipitation using a glycogen carrier. RNA yields were quantitated by absorbance at 260 nm.

RPC-5 Chromatography. tRNA was deacylated for 30 min at 37°C in 0.1 M Tris, (pH 8.0), 50 mM KCl, and 20 mM MgCl. For analysis of radiolabeled tRNA, deacylated tRNA (40 μg) was aminoacylated with ³H-labeled aspartic acid or ¹⁴C-labeled histidine and applied to a 1.0 × 20-cm column of *n*-octyl

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² To whom reprint requests should be addressed, at Department of Surgery, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, LA 70112-2632.

³ The abbreviations used are: GRT, guanine ribosyltransferase; m⁷G, 7-methylguanine; rQT₃, ³H-labeled reduced queuine; FBS, fetal bovine serum; TCA, trichloroacetic acid; RPC-5, reversed-phase column 5.

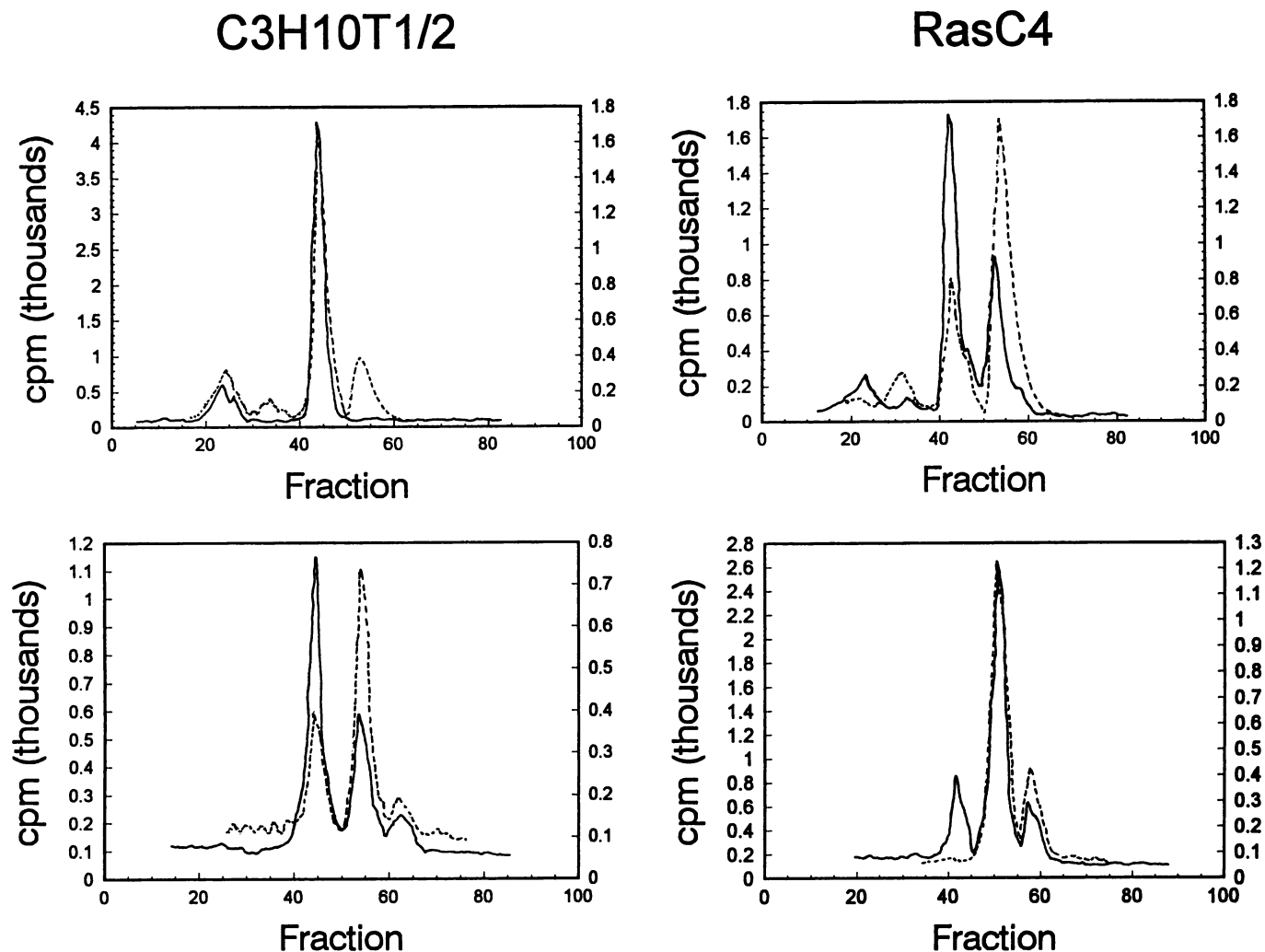


Fig. 1. tRNA-queueine modification in C3H and RasC4 cells cultured in the absence or presence of m^7G . tRNA was prepared from cells cultured in 10% serum alone (solid lines) or 24 h after the addition of $10 \mu M m^7G$ (broken lines). tRNA was aminoacylated with radiolabeled aspartic acid (upper panels) or histidine (lower panels) and resolved by RPC-5 chromatography on a 1×20 -cm column as described in "Materials and Methods." Fractions were analyzed by scintillation counting. In the upper panels, the two peaks observed in C3H cells cultured in serum alone represent queueine-modified tRNA^{Asp} isoacceptors. The two additional peaks observed in m^7G -treated C3H cells and RasC4 cells represent the corresponding unmodified (guanine-containing) forms. In the lower panels, the first peak represents queueine-modified tRNA^{His}, and the subsequent two peaks are unmodified forms (20).

quaternary ammonium polychlorotrifluoroethylene-coated resin (19) at $37^\circ C$ using a Rainin A-60-S pump (Woburn, MA). tRNA was eluted in 95 1.5-ml fractions with a linear gradient of NaCl (0.47–0.8 M) in 10 mM NaOAc₄ (pH 4.5). Fractions were analyzed by liquid scintillation counting. Using this separation protocol, tRNA isoacceptors containing guanine instead of queueine in the tRNA wobble position have been shown to elute at a higher salt concentration than their queueine containing counterparts (20).

For small scale RPC-5 chromatography, deacylated tRNA (10 μg) was applied directly to a 1×7 -cm column of the resin and eluted in 95 0.25-ml fractions with a linear gradient of 0.4–0.8 M NaCl in 10 mM NaOAc₄ (pH 4.5). Fractions were analyzed by dot blotting as described (18). Briefly, fractions were denatured in 1 M deionized glyoxal and applied to a Magna nylon membrane (Micron Separations, Inc., Westborough, MA) using a 96-well dot blot manifold (Schleicher & Schuell, Keene, NH). An unfractionated control sample was dotted at position 96. Membranes were baked and washed to remove glyoxal then hybridized with ³²P end-labeled oligonucleotide probes for tRNA^{Asp}, tRNA^{His}, and 5S RNA (18). Autoradiographs were obtained using Kodak (Rochester, NY) BioMax film and scanned on a Pharmacia LKB Ultrascan XL laser densitometer (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) using GelScan software. All profiles were aligned according to the elution position of 5S RNA.

Assay of tRNA-GRT Activity. Cells were grown to subconfluent density in DMEM/10% FBS, harvested by trypsin digestion, washed once with PBS,

and solubilized in PBS containing 0.5% Nonidet P-40. Immediately, 100 μl containing the equivalent of 5×10^6 cells were added in duplicate to the assay mix (600 μl), containing (final concentrations) 50 mM Tris (pH 7.5), 20 mM MgCl₂, 18 μCi rQT₃, and 1 A₂₆₀ unit yeast tRNA (Sigma Chemical Co., St. Louis, MO). Immediately after mixing, 200 μl were removed and added to 1 ml cold 30% TCA. The remaining assay was incubated for 1 h at $37^\circ C$, and two additional aliquots of 200 μl were removed and added to 1 ml cold 30% TCA. After 30 min on ice, TCA precipitates were collected by filtration through glass fiber filters (GF/A; Whatman, Inc., Clifton, NJ), washed with 5% TCA and 95% ethanol, dried, and counted in scintillant.

Assay of Anchorage-independent Growth. Cells (5×10^4) were seeded in 60-mm plates in 1.5 ml MEM containing 0.33% agar, 10% FBS, and 10% tryptose phosphate broth with or without $10 \mu M m^7G$, on a layer of 7 ml 0.5% agar in MEM containing 10% FBS and 10% tryptose phosphate broth with or without $10 \mu M m^7G$. Colonies were photographed after 2 weeks.

RESULTS

Modification of tRNA by Queueine in C3H and RasC4 Cells. Queueine modification of tRNA in RasC4 cells cultured in 10% serum (the source of exogenous queueine) was compared with that of non-transformed C3H cells. tRNA prepared from RasC4 cells and from

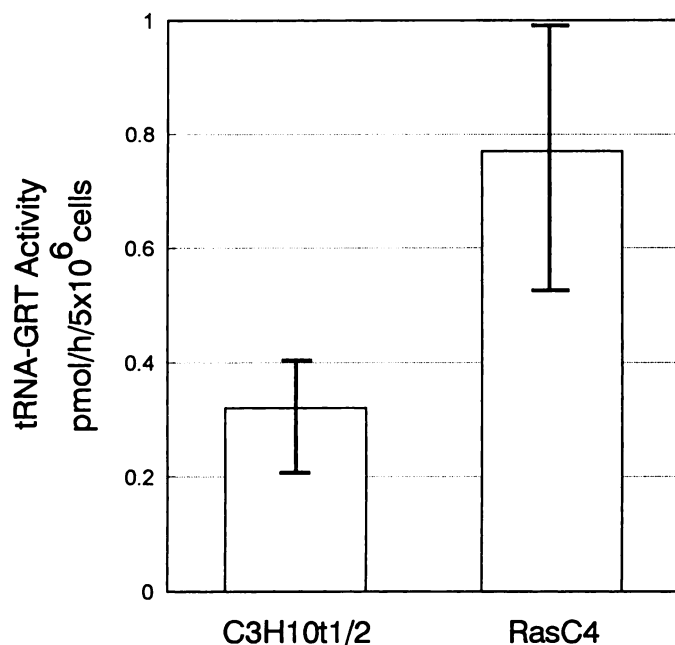


Fig. 2. Activity of tRNA-GRT. Total lysates of log phase C3H and RasC4 cells were assayed for tRNA-GRT activity as described in "Materials and Methods." Data, expressed as pmol rQT₃ incorporated into yeast tRNA/h/5 × 10⁶ cells, represent the average (±SE) of three independent experiments performed in duplicate.

low passage (<15 passages) C3H cells was deacylated, aminoacylated with individual radiolabeled amino acids, and resolved by chromatography on RPC-5, eluting with a linear NaCl gradient. Under these conditions, queuine-modified isoacceptors elute at a lower salt concentration than their guanine-containing counterparts. tRNA isoacceptors for aspartic acid, which have the highest affinity for tRNA-GRT, are expected to be modified fully in C3H cells cultured in serum-containing medium. As shown (Fig. 1), two isoaccepting species were observed. In the RasC4 cells, these isoaccepting species were also observed; however, two additional peaks were present, corresponding to tRNA^{Asp} isoacceptors lacking the queuine substitution in the anticodon wobble position.

The affinity of tRNA-GRT for tRNA isoacceptors for histidine, asparagine, and tyrosine is relatively lower than that of tRNA^{Asp}, and these species are incompletely queuine modified in normal cultured cells. As shown (Fig. 1), both Q-modified and unmodified tRNA^{His} isoacceptors are observed in C3H and RasC4 cells, but the fraction of unmodified isoacceptors in RasC4 cells is higher than in the untransformed cells.

The purine analogue m⁷G is an inhibitor of tRNA-GRT. Treatment of both C3H and RasC4 cells with 10 μM m⁷G for 24 h caused a shift in the profile of tRNA isoacceptors toward the unmodified forms (Fig. 1). In C3H cells, the resulting profile of tRNA^{Asp} isoacceptors resembled that of RasC4 cells cultured in 10% serum (30% unmodified). In RasC4 cells, treatment for 24 h with m⁷G resulted in substantial hypomodification for queuine (70% unmodified). A similar analysis of tRNA^{His} isoacceptors showed that essentially all detectable tRNA^{His} isoacceptors in m⁷G-treated RasC4 cells contained guanine instead of queuine.

Activity of tRNA-GRT. The mechanism of queuine hypomodification of tumor tRNA is not clear. Possible causes include defective or absent tRNA-GRT, the presence of endogenous inhibitors of tRNA-GRT, impairment of queuine uptake, or rapid turnover of newly synthesized tRNA. To determine the cause of defective tRNA queuine modification in RasC4 cells, we evaluated the activity of tRNA-GRT in total lysates of C3H and RasC4 cells using a radiola-

beled analogue of queuine, rQT₃, and yeast tRNA, which does not contain the queuine modification as a substrate (Fig. 2). After 1 h, incorporation of rQT₃ into yeast tRNA by lysates of the RasC4 cells was slightly higher than in the nontransformed cells.

Effect of Exogenous Queuine on tRNA Modification in RasC4 Cells. We wanted to ask whether queuine modification of RasC4 cells could be restored to normal levels in the presence of elevated levels of exogenous queuine in culture. Because purified queuine is available in limited amounts, this experiment could not be done using standard techniques (see Fig. 1) because of the large quantities of cultured cells required. The miniaturized protocol for resolution of tRNAs results in some loss of resolution of individual isoaccepting species, but it permits analysis of tRNA-queuine modification from much smaller amounts of material. Using this technique, we asked whether tRNAs from RasC4 cells could be induced to become queuine modified by

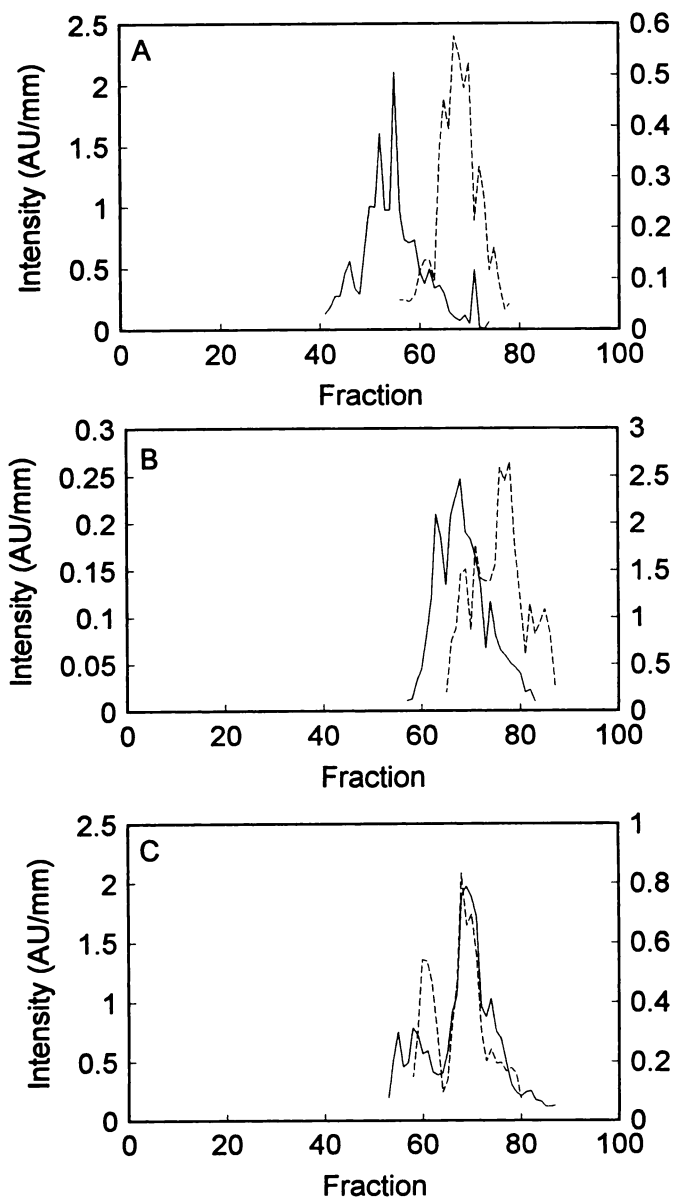


Fig. 3. Effect of excess exogenous queuine on tRNA modification in RasC4 cells. tRNA was prepared from cells cultured in 10% serum alone (broken lines) or after 24 h in serum plus 1 μM purified queuine (solid lines). Queuine was purified from bovine amniotic fluid as described (25). tRNA (10 μg) was resolved by RPC-5 chromatography on a 1 × 7-cm column, dot blotted, and probed for tRNA^{Asp} (A), tRNA^{His} (B), and 5S RNA (C) as described in "Materials and Methods." Profiles were generated from densitometric analysis of dot blots.

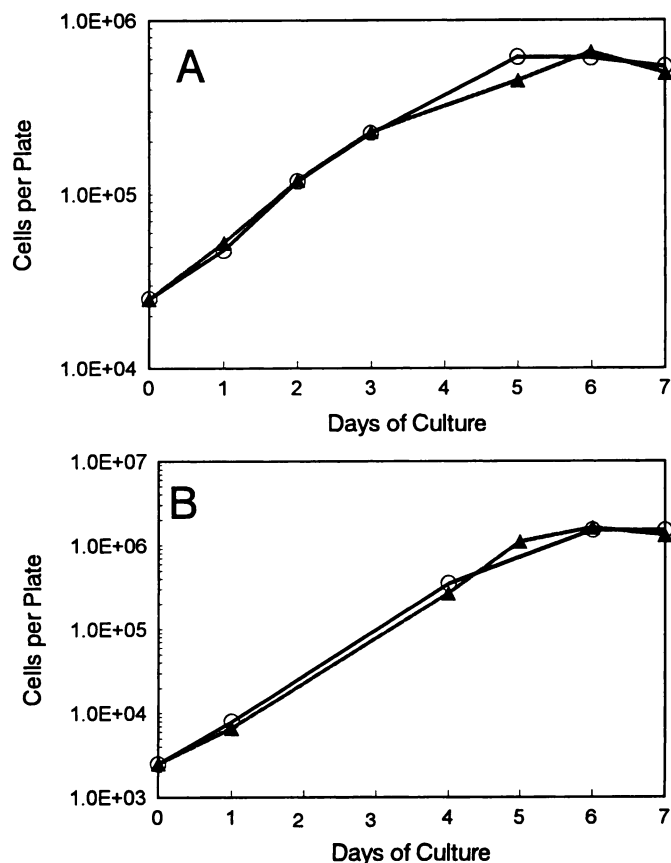


Fig. 4. Effect of m^7G on monolayer growth of C3H and RasC4 cells. C3H cells (A) and RasC4 cells (B) were plated in 35-mm tissue culture plates in DMEM containing 10% serum in the absence (circles) or presence (triangles) of $10 \mu M m^7G$. At each time point, cells were removed by trypsin treatment and counted. Points represent the average values obtained from triplicate plates at each point.

culture in added exogenous queuine. As shown in Fig. 3, RasC4 cells were able to incorporate exogenously added queuine into tRNA isoacceptors for both aspartic acid and histidine, causing a shift in the profile of tRNAs toward more modified forms.

Effect of Queuine Hypomodification on Phenotype in Monolayer and Anchorage-independent Culture. In monolayer culture, RasC4 cells proliferate with a shorter doubling time than their nontransfected counterparts. To determine whether the extent of tRNA modification for queuine influenced the rate of proliferation in monolayer culture, C3H and RasC4 cells were cultured in 10% serum in the presence or absence of m^7G (Fig. 4). No effect on doubling times was observed. In addition, depletion of queuine in RasC4 cells by the addition of exogenous queuine did not alter the rate of cell accumulation (data not shown).

Anchorage-independent proliferation is recognized as an *in vitro* hallmark of cell transformation. To determine the ability of C3H and RasC4 cells to grow in anchorage-independent culture, cells were plated in agar in the absence or presence of m^7G . As expected, C3H cells did not proliferate in agar culture under any conditions. In the absence of m^7G , RasC4 cells displayed a limited ability to form colonies. However, m^7G enhanced the ability of RasC4 cells to form colonies greatly; a slight increase in colony number and a pronounced increase in colony size were observed in 2 weeks of culture in the presence of $10 \mu M m^7G$ (Fig. 5).

DISCUSSION

Despite ample evidence associating defects in queuine modification with neoplasia, little is understood about the function of queuine

modification of tRNA in normal cells, and the mechanism and consequences of defective queuine modification in transformed cells. Investigation of the biological role of tRNA-queuine modification has been hampered by the technical difficulty of monitoring tRNA modification and the lack of a suitable *in vitro* model for controlled studies of the effects of queuine modification on cellular processes. The availability of a miniaturized protocol for analysis of queuine modification (Fig. 3; Ref. 18) has facilitated the study of tRNA modification in cultured cells and tissues. In this report, we characterize the C3H10T1/2 murine fibroblast cell line and its EJ-*ras*-transfected derivative, RasC4, as a potential *in vitro* system in which to study the biological role of normal and defective queuine modification.

We observed that RasC4 cells were relatively queuine hypomodified with respect to their nontransformed counterparts. The mechanism by which expression of an oncogenic *ras* gene leads to queuine hypomodification is unclear. Elucidation of the mechanism may be informative regarding the oncogenic mechanism of mutated *ras* as well as hypomodification for queuine. We found (Fig. 2) that the activity of the tRNA-GRT was not deficient in RasC4 cells. This observation is consistent with that of Muralidhar *et al.* (21) that the activity of tRNA-GRT in m^7G -transformed Chinese hamster embryo cells was slightly higher than in their nontransformed counterparts. Gunduz *et al.* (5), however, have identified a cell line derived from human colon cancer that lacks measurable tRNA-GRT activity completely. Decreased queuine modification of tRNA in RasC4 cells could be caused by failure of these cells to take up exogenous queuine or salvage endogenous queuine efficiently or by enhanced rates of

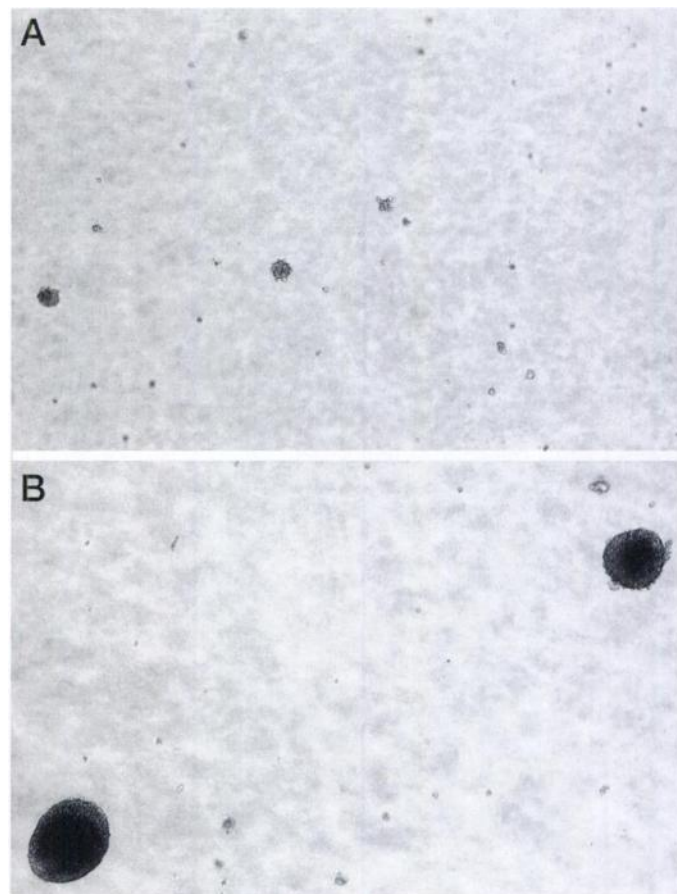


Fig. 5. Effect of m^7G on anchorage-independent growth of RasC4 cells. Cells (5×10^4) were seeded in 60-mm plates in 0.33% agar on a layer of 0.5% agar as described in "Materials and Methods." A, cells plated in control medium; B, cells plated in $10 \mu M m^7G$. Colonies were photographed after 2 weeks; magnification, $\times 40$.

tRNA synthesis or turnover in the transfected cells. These possibilities are the subjects of ongoing studies.

The extent of queuine modification of tRNAs in both C3H and RasC4 cells can be altered by culture in m⁷G or in medium containing elevated queuine (Fig. 1). Thus, queuine levels in tRNA from C3H cells can be made to approximate those in RasC4 cells, and vice versa, and queuine levels in RasC4 cells can be depleted extensively. By manipulating queuine levels in tRNA in the cell lines, we may be able to define the importance of queuine modification of tRNA in the *ras*-transfected phenotype. We observed that depletion for queuine did not affect the rate of monolayer growth of C3H and RasC4 cells (Fig. 4). In addition, repletion for queuine by culture in excess exogenous queuine did not affect the rate of proliferation of RasC4 cells (data not shown). However, further depletion of RasC4 cells for queuine enhanced growth in anchorage-independent culture greatly (Fig. 5), suggesting that queuine depletion may contribute to this characteristic of transformed cells. This observation is supported by that of Muralidhar *et al.* (21) that the anchorage-independent growth of m⁷G-transformed Chinese hamster cells was inhibited in the presence of excess exogenous queuine.

Although the molecular role of tRNA-queuine modification in normal cells is unclear, a likely possibility is that queuine modification is important in the restriction of translational wobble (22–24). The amino acid isoacceptors that are subject to queuine modification, tRNA^{Asp}, tRNA^{Asn}, tRNA^{Tyr}, and tRNA^{His}, translate codons that are not entirely degenerate: NAC and NAU encode the appropriate amino acid, and NAG and NAA encode a different one. Thus, a mechanism may exist for restriction of translational wobble by these tRNAs. If so, defective tRNA queuine modification might lead to an increase in the frequency of point mutations introduced during translation, which could be a contributing factor among the many processes that lead to neoplasia. Using the C3H and RasC4 cell lines described here, we should be able to test the effects of tRNA queuine modification on translational fidelity and efficiency by introducing appropriately engineered genetic constructs. These studies, currently in progress, may contribute to our understanding of the biological role of queuine modification of tRNA in maintenance of the normal cell phenotype.

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