Summer 1997

The Cellular and Molecular Dynamics of the Queuosine Modification in Transfer RNA: Definition, Modulation, Deficiencies and Effect of the Queuosine Modification System

Rana C. Morris

Old Dominion University

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THE CELLULAR AND MOLECULAR DYNAMICS
OF THE QUEUOSINE MODIFICATION IN TRANSFER RNA:
DEFINITION, MODULATION, DEFICIENCIES
AND EFFECT OF THE QUEUOSINE MODIFICATION SYSTEM

by

Rana C. Morris
B.S. May 1990, the College of William and Mary in Virginia

A Dissertation Submitted to the Faculties of
Old Dominion University and Eastern Virginia Medical School
in Partial Fulfillment of the Requirement for the Degree of

DOCTOR OF PHILOSOPHY
BIOMEDICAL SCIENCES
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and
EASTERN VIRGINIA MEDICAL SCHOOL

August 1997

Approved by:

Mark S. Elliott (Chair)
Frank J. Castora
Christopher Osseff
James H. Yuan
ABSTRACT

THE CELLULAR AND MOLECULAR DYNAMICS OF THE QUEUOSINE MODIFICATION IN TRANSFER RNA: DEFINITION, MODULATION, DEFICIENCIES, AND EFFECT OF THE QUEUOSINE MODIFICATION SYSTEM

Rana C. Morris
Old Dominion University and Eastern Virginia Medical School, 1997
Director: Dr. Mark S. Elliott

The presence of the queuosine modification in the wobble position of tRNA, tRNA, tRNA, and tRNA is associated with a decrease in cellular growth rate, an increase in the ability to withstand environmental stress, and differentiation of pleuripotent cells into mature phenotypes. The loss of this normal modification is strongly correlated with neoplastic transformation and tumor progression of a wide variety of cancers.

The "normal" system for formation of the queuosine modification in tRNA was studied in human fibroblast cell cultures and in mouse, rat and human liver tissues. The queuosine modification system is shown to be made up of three distinct mechanisms: uptake of the queuine base across the plasma membrane; incorporation of this base into cytoplasmic tRNA; and salvage of the base from products of normal tRNA degradation. The queuine membrane transporter and incorporation enzyme are activated via phosphorylation by protein kinase C and inactivated by the action of a phosphatase. This regulation by phosphorylation integrates the queuosine modification system into a very sensitive eukaryotic cellular switching mechanism already known to produce phenotypic alterations with strong correlations to changes in queuosine levels.

A comparative study of two "abnormal" human adenocarcinoma cell-lines (colon and breast) was performed to assess their queuosine levels and determine the malfunctioning system step(s) for the cause of the observed deficiencies. The 100% queuosine-deficient colon tumor cell-line possessed a null mutation for the queuosine incorporation enzyme, while the 50-60% queuosine-deficient breast tumor cell-line exhibited a strong deficiency in the queuine salvage mechanism. These results demonstrate the potential for determination of even multiple sites of lesions in the modification system that would yield queuosine-deficient tRNA characteristic of tumors.

Computational modeling was utilized to determine the biological function for the queuosine modification. Steric, electrostatic, and structural differences were observed for queuosine, queuosine-analogues and guanosine, the nucleosides incorporated into tRNA anticodon stem/loop structures, and in triad complexes of tRNA with mRNA and tRNA. The results of this research identify indistinguishable energetic parameters for complexes of queuosine-modified anticodon loops when paired with an mRNA containing cytosine- or a uridine-ending codon. However, guanosine-containing anticodon loops demonstrate much stronger energetic stability with cytosine-ending codons. The difference in codon
bias is shown to be due to the restriction of anticodon loop flexibility by a queuosine-induced extended intraloop hydrogen bonding network and only minimally due to a shift in hydrogen bonding pattern produced by an intraresidue hydrogen bond.

A key difference in the physiology of normal and neoplastic cells is in the increased expression of oncodevelopmental genes with respect to those housekeeping genes needed for survival. Sequence analysis of several oncodevelopmental and housekeeping transcripts suggests the presence of a contrasting bias in the usage of queuosine-related codons which end in cytosine or either cytosine or uridine, respectively. In combination with the mechanism proposed for tRNA decoding preferences, this codon usage bias suggests a potentially influential role for the queuosine modification system in the translational control of oncodevelopmental gene expression.
‘Turns out not where but who you’re with that really matters.

‘Turns out not where but what you think that really matters.

Whatever tears at us, whatever holds us down,
if nothing can be done we’ll make the best of what’s around.

paraphrased from “The Best of What’s Around” by the Dave Matthews Band
from their Album Under the Table and Dreaming (1994, The RCA Records Label)
ACKNOWLEDGMENTS

As much as I've toiled over the preparation of this document, I have procrastinated and postponed writing this “Acknowledgments” section for last because there is no way to adequately compensate the large number of extremely supportive people who have come into my life during my tenure in graduate school. Through the years of agonizing over coursework, written and oral comprehensive exams, research proposals, research itself, an oral defense of the research, and the preparation of this enormous document, there have been many people who have contributed to my success, as well as intellectual and emotional survival. I extend my heartfelt gratitude to a large number of people, only some of whom are listed below.

Foremost in their tolerance and patience have been my family. To the person who most strongly supported me throughout my tenure in graduate school and in life, I thank Jim for absolutely everything. There is not much else I can say to express my appreciation for your encouragement. To the two little rugrats (Jamie and Sean) who showed me that there are truly important things in life, and that distraction, oftentimes, can provide perspective that can solve even the most insurmountable of problems, I offer my biggest hugs and kisses. My mother and father (Steve and Louise), sister (Jo) and both sets of grandparents (Naomi & Sim and Ruth & Manny) have well practiced their heritage of affectionate nagging for tutorials, seminars, reprints and official graduation dates. Even my father-in-law (Jim, Jr.) learned the art of the nag, though not through genetic inheritance. But their love and devotion saw me through the endless reschedulings and postponements.

To the only other person who has had to deal with me day in and day out, in coffee and M-n-M surplus and in drought, through boring, incomprehensible seminar and defense, and with new creative ideas that work and that fail, I recognize the tireless, wit-filled, optimistic support from Dr. Mark S. Elliott my research advisor and cohort. However, an additional bucket-o-gratitude must also be extended to his family (Becky, Brent, Dave, and Matt...not to mention the three dogs, rabbit and hamster) who tolerated his absences and frustrations due to lab problems and successes.

My many thanks to my Dissertation Committee (Dr. Frank Castora, Dr. Chris Osgood, and Dr. James Yuan) for their patience, suggestions and guidance, and above all for the time spent reviewing this manuscript. Many faculty members within the Department of Biochemistry and Chemistry aided me not only in my academic education, but also in my professional and personal development. Among these extremely supportive folks are: Dr. Charles Bell, Dr. Ken Brown, Dr. Bob Ake, Dr. Allen Clark, Dr. Pat Pleban, Dr. Laura Moen, Dr. Roy Williams, and Dr. Keith Carson from the Biology Department. The large number of students who have come and gone over the years have been encouraging in their willingness to help, provide useful suggestions and criticisms, or just lend a patient ear. I thank a few in particular: (soon-to-be) Dr.s Thomas Heard, Miguel Santos and Lester Pretlow, (already) Dr.s Jackie Smith and Sandi Ward, and Julie Patrick, Robin Branche, Chris Hart, and Carlos Villar-Gosalvez.
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CHAPTER ONE
MUCH ADO ABOUT Q

Introduction

In 1953 the world of science changed because of James D. Watson and Francis H. C. Crick's publication on the structure of deoxyribonucleic acid (1). This treatise was not the first to describe the three-dimensional structure of a macromolecule, however it was the first attempt to describe the possible mechanism for a macromolecule's biological function based on the proposed conformation. Since this time, molecular biologists, biochemists, physiological chemists, and structural biologists have studied the impact that the structure of a compound would have on its function. In fact, the quote "structure is function" has often been regarded as the credo for modern biomolecular research.

For nucleic acids, the three-dimensional structure of each molecule is largely based on its primary structure, the nucleotide sequence. In the late 1950s, a primitive mechanism for determining the sequence of nucleic acids was developed. This procedure was comprised of a difficult and complicated series of nuclease digestions and analysis of overlapping fragment lengths. It was not until 1965, after seven years of work, that the first nucleic acid, yeast alanyl-transfer ribonucleic acid (tRNA<sub>B</sub>), was sequenced (2). In addition to the traditional nucleotide bases of RNA molecules [adenosine (A), cytosine (C), guanosine (G), and uridine (U)], the same group discovered that there exist, in tRNA, approximately 80 modified forms of these nucleotides (3). These modified nucleotides comprise up to 25% of all bases in a given tRNA. Most of these involve simple methylations of the four basic RNA bases, with a small number of other types of minor nucleotide alterations such as the reduction of uridine's C5-C6 double bond to form dihydrouridine and the deamination of adenosine to form inosine. There are a few large modifications that have been identified solely in tRNA, and these have been shown to exist only in the anticodon region—one of the two major functional centers of the molecule (4).

The development of a gas chromatography/mass spectrometry-based method allowed for the identification of the nucleotide composition of purified tRNAs (5). However, in 1968 a peak arose in the profile of an <i>Escherichia coli</i> tRNA<sub>B</sub> hydrolysate that could not be matched with previously established standards (6-8). Because this was a new and unknown base, it was given the designation "Q". In the vocabulary of biochemistry, the Q base needed to be assigned a name that could more definitively identify its nucleotidyl form. Since the free form of G was called guanine, for example, the free form of Q was to be called queuine. Thus, the written name for the presence of Q in a polynucleotide form was to be queuosine, again based on the example of guanosine for G. This development of a naming scheme was important for the validation of the existence of the Q nucleotide, as Q was eventually found to exist in the tRNA of every organism yet studied, with the notable exception of yeast (9-11).

The journal model for this dissertation is *Nucleic Acids Research.*

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In 1972, the Q modified nucleotide was localized to position 34 of tRNAs specific for aspartate, asparagine, histidine, and tyrosine (12) (Figure 1-1). The structure of queuosine was later determined by gas chromatography/mass spectroscopy (5) and elucidated by X-ray crystallography (13) (Figure 1-2). Queuosine was identified as a strangely modified guanosine analogue. The N-7 position of the purine ring is a C-7 in queuosine, which is the site of attachment for an aminomethyl tether to a dihydroxycyclopentenediol ring. This structure is known to be further modified with the addition of mannose and galactose residues to the cyclopentenediol ring hydroxyl groups of tRNA and tRNA15*, respectively (14). By chemical naming convention, the basic queuosine molecule was defined as 7-(3,4-trans-4,5-cis-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanosine. Since the early 1970s, focus on Q research has shied away from structural studies to branch into two main directions: sub-cellular biochemical research and physiological clinical research.

Biochemical Research

Basic biochemical research has characterized and localized the queuosine modification in the tRNA of intracellular organelles (mitochondria and chloroplast) and organisms as diverse as monerans (such as Escherichia coli, Zymomonas mobilis, Bacillus stearothermophilus, and Salmonella typhimurium), protistans (Chlamydomonas reinhardtii, Chlorella pyrenoidosa, Acanthamoeba castellanii, and Dictyostelium discoideum), and plants (Lupinus leuteus, tobacco, and wheat), as well as many animals [from marine life (lingula, starfish, tompinnow, flounder, and hagfish), insects (Bombyx mori, and Drosophila melanogaster), amphibians (Xenopus laevis), to mammals (mice, rats, opossum, rabbits, cows, and humans)]. Thus far, only one genus from the kingdom Fungi has been tested. However, several species of yeast have been identified as the only organisms currently known that do not contain the queuosine modification in their tRNA (10).

In biological systems, the Q base is known to exist in three forms: free queuine base, free nucleotide or nucleoside, and nucleoside incorporated in tRNA. The free base form, present in all cells and cell-types, has only recently been suggested to play a physiological role within a cell—modulation of receptor tyrosine kinases such as epidermal growth factor receptor and platelet-derived growth factor receptor (15). The free nucleoside or nucleotide exists in the cell due to the normal turnover of tRNA and appears to have no direct physiological role, although queuosine-5'-phosphate has been reported to serve as a source of salvaged queuine base in mammalian cells (16,17). The incorporated form of Q has been demonstrated in asparty1-, asparaginyl-, histidyl-, and tyrosyl-tRNA isoaccepting families of many organisms (11). These modified tRNA populations are further separated into queuosine-containing tRNA and tRNA5*, and the glycosylated forms of these queuosine-containing tRNAs; mannosylated queuosine-containing tRNA and galactosylated queuosine-containing tRNA5* (14,18,19). The purpose of these incorporated modification forms is not yet known.

Originally, it was thought that queuosine modifications in prokaryotic and eukaryotic tRNA were evolutionarily conserved. Thus, the enzyme that catalyzed the formation of this modification was assigned the
Figure 1-1. Molecular Skeleton, Ribbon Diagram, and Electron Density Surface of Aminoacylated Yeast Aspartyl tRNA. The main structure of the tRNA is shown in dark blue with the second and third anticodon bases in orange (light color, near the bottom) and the wobble base in red (medium color, at the bottom). The site for aminoacylation, the ribose of the adenosine at position 76, is shown in green on top. The Molecular Skeleton (representation on the left) is shown in ball and stick rendering with the hydrogen bonding sites of the anticodon and the 2' and 3' hydroxyl groups, for covalent attachment to aspartate, as space filling models. The Ribbon Diagram (center) shows the backbone structure of the tRNA with the anticodon and aminoacylated residues as ball and stick rendering. The Electron Density Surface (on the right) is shown with color mapping for the anticodon bases and the aminoacylated residue.
Figure 1-2. **Structures of Guanine and Queuine Bases.** A two dimensional representation of the nucleotidyl bases are drawn with an R representing the attachment site for an H for the base form (often abbreviated as G and Q) or a ribose for the nucleoside form (known as Guanosine and Queuosine). The classical nucleic acid numbering scheme is shown. Queuine is a modified guanine with the IUPAC designation 7-(3',4'-trans-4',5'-cis-dihydroxy-1'-cyclopenten-3'-ylaminomethyl)-7-deazaguanine.
name tRNA-guanine transglycosylase (TGTase; E.C. 2.4.2.29) regardless of the taxonomic classification of
the host organism. This enzyme was named based on its originally measured ability to transfer a guanine
base into the wobble position of these tRNA families (20-22). More recently the Enzyme Commission has
renamed this protein tRNA-guanine ribosyltransferase (TGRase), although the original designation is still in
wide use. With the further purification and identification of components of the prokaryotic and eukaryotic
systems, this assumption of conserved evolution is now in doubt.

The localization, structure, and metabolism of Q have been well studied in prokaryotes, and are now
under scrutiny in eukaryotes with the development of new methods to study the presence or absence of the
queuosine modification in mammalian tRNA.

Prokaryotes synthesize this nucleotide through a series of post-transcriptional steps catalyzed by
enzymes encoded in the "queuine operon" (23). First, tRNA-guanine transglycosylase (TGTase—the gene
product of tgr) inserts preQ1 (7-aminomethyl-7-deazaguanine) into the cognate tRNAs (24) via a base-
exchange reaction without disrupting the phosphoribosyl backbone (22). Then, a second enzyme, s-
adenosylmethionine: tRNA ribosyltransferase-isomerase (gene product of queA), adds the ribosyl group from
an s-adenosylmethionine to the transcript at the end of the 7-aminomethyl side chain of the inserted preQ1 and
isomerizes it to form the epoxycyclopentanediol moiety of what is called oQ (25,26). Finally, the epoxy
group of the oQ is reduced to form queuosine in an as-yet-unknown vitamin B12-dependent reaction (27).
This completes the prokaryotic queuosine synthesis.

It has been suggested that most eukaryotes, including humans, salvage the fully-formed queuosine base
from their diet or through absorption of metabolic turnover products from symbiotic bacteria living in the
intestines of the host (16,17,28,29). Once inside the circulatory system of the organism, queuosine is efficiently
taken up by a specific cellular transport mechanism (30). Then, a cytosolic enzyme often referred to as either
TGTase or TGRase inserts the pre-formed queuosine base into the four queuosine-specific tRNA isoacceptors
(21,31). Finally, in the case of eukaryotic tRNA*5' and tRNA*3', glycosylated queuosine-modified tRNA
isotypes have been documented (14). In 1977, a rat liver mannosyltransferase was identified which places
mannose residues on the hydroxyl groups of the cyclopentenediol ring of tRNA*5' (18). It is presumed that
there is a similar galactosyltransferase which exists that catalyzes the observed tRNA*5' glycosylations (19).

Many methods have been developed to detect queuosine-containing tRNAs. Early techniques to
study the nucleotide composition of tRNAs included the use of infrared analysis and ramen spectroscopy.
However, the most successful method to identify specific nucleotides, including Q, in tRNA samples was
traditional gas chromatography/mass spectrometry (5). Three different methods of analysis have since
been developed that have become the accepted procedures to measure the levels of queuosine-modified
tRNAs. These include high performance liquid chromatography strategies (HPLC), an in vitro enzymatic
radiolabeling assay, and, most recently, in vivo cell culture radiolabeling assays.

In the early days of "Q" research, several chromatographic matrices were utilized for the
separation of queuosine-containing tRNAs including dihydroxyboryl-substituted cellulose, acetylated-
dihydroxyborophenyl-succino-aminooethyl cellulose and concavalin A-sepharose affinity chromatography
(3,32,33). Eventually, with the development of HPLC technology, reversed phase HPLC matrices were
demonstrated to quickly and efficiently separate queuosine-containing from queuosine-deficient tRNAs
based on hydrophobic criteria (34,35). Studies on the chromatographic mobility of the queuosine-modified
tRNA (3) and x-ray crystal structure of queuosine (13) have suggested that the physiological state of the
molecule includes a positively-charged quaternary amine in the aminomethyl linker between the base and
the cyclopentenediol ring. Due to the presence of charged amine group and the two hydroxyl groups on
the cyclopentenediol moiety, the queuosine-containing tRNAs can be separated from other non-queuosine-
containing isoforms of tRNAs using RPC-5 HPLC (34). Thus, the total level of queuosine-containing
tRNAs can be determined with this method. Recently, a W-Porex C4 HPLC column was shown to resolve
not only queuosine-containing from non-queuosine-containing tRNA isoforms, but also to separate the four
isoaccepting families as well (36,37). Using the W-Porex C4 HPLC method, it is now possible to measure
the queuosine-modification levels for all four families of queuosine-containing tRNAs at once, as is shown
in the Appendix of this document. These HPLC methods require a large amount (1 to 2 mg) of tRNA and
are, therefore, only suitable for very large cell culture preparations or tissue analysis of substantial organ
biopsies.

A somewhat faster and more sensitive method for measuring queuosine levels was developed
utilizing the cloned Escherichia coli queuosine-incorporation enzyme, TGTase, in an in vitro assay (38,39).
The modification of tRNA with queuosine is an irreversible one, thus enzymatic incorporation of a
radiolabeled queuosine or guanine will "tag" the tRNAs that did not previously contain this modified base. The
amount of queuosine-containing tRNA will, therefore, be inversely proportional to the signal obtained. This
method requires extensive purification of the tRNA fraction from samples, but has been widely used to
measure the levels of queuosine in biopsy tissues (40-43), organ tissue lysates (40,43,44) and cell culture
lysatess (17,30,45).

Cell culture methods were more recently developed to measure cellular activities pertaining to the
queuosine modification system in vivo. In order to measure the permeability of or active transport ("uptake")
mechanism in cells for the queuosine base, a culture of cells is exposed to radiolabeled queuosine for a period of
time and the amount of radioactivity taken up by the cells is assessed (30). A modification of this strategy was
made to measure the amount of radiolabeled queuosine incorporated into cytoplasmic tRNA (46). The treatment
of cells with radiolabeled queuosine is the same in this method as with the uptake assay, however the cytosolic
fraction is filtered to retain any radiolabeled queuosine that has been incorporated into large macromolecules
such as tRNA. A method to measure the salvage of queuosine base from tRNA turnover products is similar to
that of the incorporation assay but involves a pulse-chase type of experimental design (16). Cell cultures are
pre-treated with queuosine-free media for varying lengths of time to produce a queuosine-free population of
tRNAs and to induce any cellular salvage mechanisms for the maintenance of cytosolic queuosine levels. Then,
a 24 hour exposure of the cells to radiolabeled-queuosine is performed to fully saturate the tRNAs with the
radiolabeled-base. Two sets of cultures are assayed for the ability of the cells to retain the label in macromolecular form: one is monitored for the rate of tRNA turnover (total levels of radiolabeled-tRNA) by chasing out the radiolabeled signal with the addition of an excess of unlabeled queuine to the media, while the other is treated with queuine-free media to determine the ability of the cells to maintain the radiolabel after the initial incorporation. The procedures for analysis of the timed samples are identical to that of the incorporation assay. These cell culture methods are highly sensitive (requiring limited cell culture samples to run) and, since these are in vivo assays, represent a method able to measure three facets of queuine metabolism (uptake, incorporation, and salvage) under relatively realistic and controllable physiological conditions.

Despite the years of biochemical research on the Q base and the queuosine modification of tRNA, a definitive description of the mammalian queuosine modification system itself, and in relation to the well characterized prokaryotic system, has not yet been produced. Unfortunately, the established method for monitoring queuosine modification levels, RPC-5 HPLC, is obsolete due to lack of availability of the column matrix and to natural degradation of the remaining stock. Therefore, alternative methods such as W-Porex C-4 HPLC and the in vitro and in vivo assays must be utilized in order to study the queuosine modification system in biochemical and physiological contexts.

**Physiological Research**

Although the biochemical function of and purpose for the Q nucleoside has not yet been established, there are strong correlations between queuosine levels and phenotypic variations in prokaryotic and eukaryotic organisms. The abilities of cells to survive stressed conditions, and undergo differentiation or neoplastic transformation have been shown to be indicative of and potentially caused by the presence or absence of queuosine-modified tRNA.

Studies with a mutant *E. coli* strain containing a null mutation for the TGTase enzyme suggested that queuosine-modified tRNA protects the organism from stress invoked by suboptimal growth conditions (47) and is necessary for the proper expression of nitrate reductase and an increased amount of b-type cytochromes under anaerobic conditions (48). This modification appears to allow these eubacterial cells to recover quickly from environmental strains to resume normal growth patterns. Eukaryotic *D. discoideum*, though able to grow in the absence of queuine, is not able to differentiate after either a nutritional or metabolic stress without a source of this base (49). These queuine deficient cells exhibit lower levels of lactate dehydrogenase (an anoxic stress protein) and the appearance of a novel cytochrome b with an absorbance maximum at 559 nm (50-52). However, with the addition of queuine to the culture media, the cells rapidly grow in size and number and are able to differentiate into stalk cells and spores. Thus, queuosine-modified tRNA may be involved with management of nutritional and oxidative stress in both prokaryotic and eukaryotic cells.

Cellular and clinical research has generally focused on establishing and strengthening the correlation between Q-modification levels and development, differentiation, and carcinogenesis in more complex eukaryotic organisms. In cells of plants, insects, and mammals a motif has been described noting the existence.
of low levels of Q-modified tRNAs in embryonic and undifferentiated tissues with a shift towards complete Q-modification in mature, differentiated tissues (53,54). In A. castellanii, vegetative cells (trophozoites) contain significantly lower levels of queuosine-modified tRNA than mature cysts (55). In D. melanogaster, the levels of queuosine-modified tRNAs increase dramatically in the first week after “birth” from non-existent to complete modification, but the rate of modification varies with genotypic strain and diet (56,57). The same pattern is observed during development in the rat, with maximal queuosine-modification levels plateauing at approximately 5 months post-birth (58). Human placental tissue has been shown to be deficient in this modification despite the availability of large amounts of free queuine within neighboring amniotic fluid (59), but normal, mature human tissue is completely modified with respect to Q (60). Thus, the level of queuosine-modified tRNA may be indicative of the developmental stage of an organism.

In 1980, a study was initiated to determine the effect of a “queuine-free” diet on conventional and germ-free mice (61). It was discovered that the formation of queuosine-modified tRNA did require the absorption of the dietary nutrient queuine, in that after one year the queuine-starved mice became entirely deficient in the queuosine modification (29,61). These animals appeared to be physiologically normal, although unchallenged by foreign immunogens. Thus, it was suggested that queuosine is not necessary for development, it may only be an artifact. However, it was discovered that, even after 4 weeks of the queuine-free diet, the mice retained 100% of queuosine in tRNA^sup and tRNA^ tyr, as well as 88% of the queuosine in tRNA^his and 85% of tRNA^sup (61). It has since been observed that tRNA^sup maintains the modification the longest of all the isoacceptors (37). Therefore, it is likely that the mice maintained very high levels of the queuosine modification throughout their formative weeks, significantly decreasing in the levels of the modification only after the cells became mature and static. Thus, the queuine-free diet study does not appear to rule out an effect for queuosine-modified tRNA in developing mouse embryos—particularly with information arising from studies involving cellular differentiation events both in situ and in cultured cells.

The differentiation of two organisms, D. discoideum and the topminnow, exhibit a dramatic increase in the levels of the queuosine modification from the onset of development to sporulation in the slime mold, and formation of the melanophoric system in this fish (62,63). Without exposure to queuine, the pleuripotent cells fail to differentiate and the organisms become senescent or die.

Cell culture studies examining differentiation patterns of mammalian cell-lines have shown a correlation of a rise in queuosine modification levels with chemically-induced differentiation events. While dimethylsulfoxide-induced erythroid differentiation of murine erythroleukemia cells occurs with an early decrease in the level of queuosine-modified tRNAs, the actual phenotypic change event concludes with a concomitant rise in the modification level during the late phases of differentiation (64,65). This experiment was repeated by another research group, this time with the use of multiple differentiation induction agents (66). Regardless of the agent added, a sharp increase in the level of the queuosine modification always preceded the final differentiation of these cells.

Additionally, the study of the differentiation of erythroleukemia cells was performed using a human
cell-line with several differentiation agents known to exhibit varying differentiation-induction capabilities (67). The use of 5-azacytidine only weakly affected the cells, causing a minor slowing in cell growth and no change in queuosine levels. Exposure to hemin induced a reversible differentiation event that was quickly reversed with removal of the agent by dialysis, and only a transient increase in queuosine-modified tRNA was observed. Sodium butyrate exposure caused a more stable differentiation event that required forty eight hours of dialysis for reversal. The initial differentiation correlated well with an early increase in the levels of the queuosine modification with a concomitant decrease after the long-term removal of the agent. 1-β-D-arabinofuranosylcytosine was exposed to the cultures to induce an irreversible differentiation event in the cells. The level of queuosine-containing tRNA increased immediately and was maintained when growth became inhibited. These studies firmly establish the linkage between an increase in the level of queuosine-modified tRNA and the phenotypic alteration toward a more differentiated state.

Unlike the strong evidence of the reactionary increase in queuosine levels during chemically-induced differentiation, there is some dispute about the effect of exposure of cell cultures to high levels of queuine. In studies utilizing the HeLa cultured human cervical carcinoma cell-line, the effect of supplementary exogenous queuine appeared to vary widely with regard to oxidative state of the cells from enhancing growth rate to blocking growth altogether (68,69). In specific leukemic and carcinoma cell-lines and under certain conditions, queuine was shown to prevent differentiation and increase the likelihood of proliferation. However the addition of queuine to other cultured cell-lines has shown strong anti-proliferative potential (70-72). During the early stages of chemical transformation of Chinese hamster embryo cells a decrease in the level of queuosine-modified tRNA was observed (73). Both the rapid growth seen during the transformation and the decrease in modification levels were reversed with the exposure of these cells to excess queuine added to the growth media. In addition, the rapid growth rate of neoplastic cells placed under the skin of a nude mouse was effectively blocked by the subcutaneous addition of a large dose of queuine (28,29).

Studies involving phorbol ester-induced transformation of normal human fibroblasts identified a transient decrease in queuosine levels just before a 5- to 10-fold increase in saturation density (74). With prolonged exposure of phorbol esters to these cultures, an increase in queuosine modification level was shown to precede a decrease in saturation density. The quantity of queuosine-modified tRNAs were comparable to those in “normal” cultures that had not been exposed to phorbol esters. Simultaneous treatment of cultures with both phorbol esters and additional exogenous queuine prevented the increase in cell growth.

A major facet of the neoplastic transformation (retrograde-differentiation) of cells involves the acquisition of anchorage-independent status. Thus, the ability of a cell-line to overcome cell density influences in its growth is a major step in the promotion of cancer. Two-stage initiation-promotion experiments for the transformation of Chinese hamster embryo cell cultures were undertaken to evaluate the effectiveness of the queuine analogue 7-methylguanine to promote transformation (75). 7-methylguanine was able to increase the formation of type III foci and anchorage-independent growth, thus functioning as a tumor promoter. Since this chemical serves as an inhibitor of the queuine-incorporation enzyme TGRase (76), it was
suggested that the queuosine modification serves as a suppressor of the promotion stage of carcinogenesis. Additional studies with phorbol ester-induced transformation of the Chinese hamster embryo cells showed a decrease in the levels of the queuosine modification in the early stages of their transformation effects (73). The addition of excess exogenous queuine to the culture media resulted in both an increase in queuosine modification levels and the reattainment of the anchorage-dependent phenotype.

It has long been known that cellular transformation can be induced in cultured cells when exposed to methylated purines and other purine analogues (77,78). In tissue culture studies, transformation of Chinese hamster embryo cells (75,79) and human fibroblasts (30,74,80) have been induced by exposure to either a classical phorbol ester tumor promoter or the methylated purine 7-methylguanine. These agents both appeared to promote a reduction of Q-modification levels in the course of their transformation effects.

Many of the studies mentioned with regard to differentiation and transformation and their correlation with fluctuating queuosine levels involve the treatment of cultured cells with phorbol ester tumor promoters. Phorbol esters are known to directly bind to and hyper-activate protein kinase C (PKC), an important regulatory enzyme in several cellular signal transduction cascades (81,82). Short-term exposure studies exploiting the ability of phorbol esters to activate PKC have been used as the classical cell culture method for the analysis of PKC-directed effects (83). However, under conditions of chronic exposure the cell's entire supply of endogenous PKC is activated. This permits the rapid catalytic degradation of the enzyme which effectively ends the "burst" of metabolic activation induced by the phosphorylation of PKC's target proteins (84). Chronic exposure to phorbol esters actually decreases the activity of this enzyme. This deactivation mechanism has been evolutionarily developed as a feedback-control loop for PKC, which plays an important role in cellular signal transduction pathways. The idiosyncrasy of this differential effect on PKC by a single compound has since been exploited 1) in experimentation to more strongly identify a PKC-catalyzed regulatory mechanism in certain cellular processes and 2) in explanations of why phorbol ester modulation of PKC activity has been correlated with both differentiation and retrograde differentiation events.

As mentioned previously, phosphorylation control mechanisms for the regulation of gene expression patterns have been strongly tied to phenotypic change events in many mammalian organisms. PKC, in particular, is an important regulatory switch in differentiation and neoplastic transformation of many cell types (85). This enzyme is directly tied to a large number of key metabolic processes within the cell by direct phosphorylation and activation or inhibition of the target's biological activity. PKC's ability to influence phenotypic alterations have been used to explain the effect of phorbol esters on cells, since it was discovered that these compounds directly hyper-activate PKC. Thus, if control of the queuosine modification system can be tied to PKC regulation, as suggested in a few studies, it would link tRNA metabolism and it's function to a list of other molecular events known to occur in the signal transduction pathway's control of gene expression and tumor promotion.

Abnormally low queuosine modification levels in tRNA have been strongly associated with tumor promotion and carcinogenicity (neoplastic transformation, a.k.a. retrograde-differentiation) both in vivo and in
situ. Transfer RNA isolated from neoplastic tissues and transformed cell-lines is queuosine-hypomodified to various degrees generally corresponding to the severity of the disease (38, 60, 86). In situ studies on multiple human tumor biopsies have shown a strong correlation between the loss of Q-modification and the progression and aggression of neoplastic disease. The degree of hypomodification has been used to develop diagnostic tests for staging of human neoplastic disease in lymphomas and leukemias (40), and in lung (41), ovarian (42) and brain (43) solid tumors. Explicit levels of decreasing queuosine in these cancerous tissues have been shown to directly correlate to increased aggression of the associated tumor's growth and metastasis as determined by histopathological grading.

Over the last ten years, physiological research has documented many cases describing abnormally low levels of the queuosine modification in undifferentiated and neoplastic cells. While addition of queuine base to culture media appears to induce differentiation and decrease growth rate in transformed cells, it also appears to reverse the transformation induced by chronic exposure of cells to phorbol esters. In addition, it has been suggested that the queuosine modification system might be in some way induced by PKC-catalyzed phosphorylation. PKC has been recognized as being adversely effected in many forms of transformed cells (85). Thus, PKC-modulation of the queuosine modification system might be deregulated in these cells, causing a cellular deficiency in the production of properly modified tRNA populations.

Each of the three steps of the queuosine modification system (cellular uptake of the free base, incorporation of queuine into tRNA, and salvage of the nucleoside) and modulation of their activity by PKC are all potential sites for deficiencies that can occur in neoplastic cells. These are likely to vary based on the origin of the tumor and may even involve a multiple lesion scheme that is often observed in cancers. However, only one queuosine deficient cell-line has so far been studied with respect to the actual malfunctioning step in the queuosine modification scheme (87). Thus, the likelihood of breakdown for any one or more of the steps of the queuosine modification system has yet to be determined. Also, a direct, or even indirect, causal relationship between queuine and cancer has not yet been established because of the lack of knowledge concerning the modified base's natural biological function and purpose.

Statement and Significance of Thesis Research

There have not yet been any attempts to describe the mammalian queuosine modification system in depth and only a few to mechanistically explain the effect of this modified nucleotide on cellular physiology. Thus, a global hypothesis describing the biological function of and purpose for the Q nucleotide has not yet been accepted.

To establish the foundations for a theory describing the reason for Q's existence, several preliminary studies must be completed. 1) A description of the queuosine modification system, in its entirety, in prokaryotic and eukaryotic hosts must be established. 2) The interrelatedness or connectivity of the queuosine modification system with other physiological processes within the cell must be ascertained. 3) Actual sites for queuosine modification system breakdown in abnormal cells must be demonstrated. 4) A plausible function
of the queuosine modification system with regard to the stability of macromolecular structures (tRNA) and to
effect on interactions within macromolecular complexes must be postulated and supported.

All methods utilized in the research described in this treatise are described in Chapter 2 with
literature reviews of the protocols and information concerning the experimental design strategies.

While the prokaryotic queuosine modification system in *E. coli* has been well described (23), various
parts of the eukaryotic system have been studied in many different organisms and in cell cultures. However,
all of the components of a single eukaryotic organism's queuosine modification system have not yet been
described in a single picture. Chapter 3 of this treatise describes methods used to study the eukaryotic
queuosine modification system. These techniques include *in vivo* assays that are able to dissect the uptake,
incorporation, and salvage steps within the system in a cell culture model system and an *in vitro* assay to
measure the presence of the queuosine incorporation enzyme, TGRase, which is useful for protein isolation
and characterization protocols. Cell culture studies were performed on normal human fibroblasts to measure
the abilities of these cells to perform the uptake, incorporation, and salvage components that comprise the
queuosine modification system. To further examine the incorporation step of this system, mammalian
incorporation enzymes from mouse, rat, and human liver were isolated and characterized. Finally, the
eukaryotic and previously characterized prokaryotic queuosine modification systems were compared in order
to determine an evolutionary relationship. This information is important to lay the framework for the choice
of proper model systems to be used in human disease etiology studies in the future.

In the course of attempting to study queuine uptake in cultured human cells and to isolate the
mammalian queuosine incorporation enzyme it appeared that these systems may be modulated by phorbol
ester tumor promoters and protein phosphatase inhibitors, respectively. With the known linkage between
phorbol esters and PKC, and phosphatase inhibitors with an antagonist enzyme for PKC, studies were
undertaken to determine if this key secondary messenger system is involved in regulating the modification of
tRNA with queuine. Chapter 4 describes *in vivo* uptake, incorporation, and salvage assays performed on
human cell cultures in the presence of multiple kinase and phosphatase modulators. In addition, protein
isolation protocols were altered and *in vitro* assays were performed with the purified mammalian incorporation
enzymes to assess action of PKC directly on this enzyme. Thus, the interaction of the queuosine modification
system with PKC, a major cellular regulatory switch, would indicate connectivity of the queuosine
modification system with other important physiological processes.

It is well known that the queuosine modification is deficient in neoplastic cells, and that progression
of cancer is strongly correlated with staged decreases in modification levels. Chapter 5 describes *in vivo* cell
culture studies performed on human colon adenocarcinoma (HxGC3) and breast adenocarcinoma (MCF-7)
cell-lines to determine the source of their queuosine deficiencies which measure at 100% and 50-60%,
respectively. Uptake, incorporation, and salvage, as well as PKC-modulation assays were performed. These
results would indicate if a single step or multiple lesions of the queuosine modification system are the cause of
the deficiencies in these cell-lines. This information could have implications on the use of queuosine
modification levels for the designation of a tumor’s origin and may be important in any future diagnostic protocol or treatment schemes that might be proposed.

Despite the cellular and clinical evidence of the importance of the queuosine modification, there has not yet been a hypothesis suggested which links the presence of queuosine to a function that could cause the observed correlations with wide phenotypic effects, such as differentiation and neoplastic transformation. Chapter 6 describes the results of computational chemistry studies which attempt to explain potential effects of the queuosine modification on the rate and efficiency of protein translation by examining the effect of this modified base on the structure and dynamics of the tRNA. With the advent of molecular modeling techniques and comparison to previously published experimental results, structural studies of the queuosine base itself, of tRNA<sup>448</sup> with and without the queuosine modification, and in complexation studies within a A-site tRNA/mRNA/P-sitetRNA ribosomal model were performed. The results were used to assess queuosine’s effect on tRNA structure and function leading to an explanation of queuosine’s possible role in translational control of gene expression.

In the conclusion of this document, codon bias patterns for specific mRNA sequences were analyzed in order to correlate the structural effects of the queuosine-modified versus unmodified complex with the sequence of certain gene classes (housekeeping and oncodevelopment-related). Finally, a mechanism for the queuosine modification system with regard to the regulation of gene expression is proposed. This hypothesis attempts to mechanistically illustrate the role of the queuosine modification in normal cellular physiology and explain the effect of the lack of this base on development, differentiation, and neoplastic transformation.
CHAPTER TWO
EXPERIMENTAL DESIGN AND METHODS

Introduction

This chapter describes the use of several in vivo cell culture methods and an in vitro enzymatic assay to study the queuosine modification system. (A third technique based on high or medium pressure reverse phase liquid chromatography, is discussed in the Appendix of this document). The protocols have been optimized for the study of the system in mammalian tissues and cells. The in vivo studies utilized human foreskin fibroblast cultures to represent a "normal" mammalian tissue or human colon or breast adenocarcinoma cultures to represent neoplastic cells in assays for queuine uptake, incorporation, and salvage. In vitro assays were used to purify and further characterize the queuine incorporation enzymes from several mammalian sources (mouse, rat, and human liver tissues). This protein catalyzes the actual formation of the queuosine modification in what may be considered as the essential step in the synthesis of this modification in eukaryotes.

In addition, modified in vivo protocols were performed to identify the involvement of PKC in the regulation of queuine uptake, incorporation into tRNA, and salvage mechanisms. These involved exposure of the human fibroblast cell cultures to PKC activators (TPA and PDD) and inhibitors (H-7, staurosporine, sphingosine, and calphostin C) and phosphatase inhibitors (okadaic acid and calyculin A). Protein phosphatase activators are not commercially available, thus were not used in this study. Chronic exposure of cell cultures to TPA is known to induce proteolytic degradation and down-regulation of PKC activity. This phenomenon was exploited to verify the short-term exposure experiments by correlating the PKC and phosphatase inhibitor results and scrutinizing the PKC activator (TPA) results. PKC-modulation studies were performed in both cell cultures and in modified in vitro assays with semi-purified and purified queuine incorporation enzyme samples.

All human cell-lines and mammalian tissues were handled in accordance with Old Dominion University's policy regarding bloodborne pathogens.

In the final section of this chapter, computational chemistry methods for structure and property determination are described for guanosine, queuosine-analogue, and queuosine nucleotides. In addition, these nucleotides are studied for their effect on the structure and energetics of tRNA\(^{\text{A}^\text{P}}\) anticodon stem-loop molecules; as part of tRNAs in complexation with a translating mRNA; and located within an A-site tRNA/mRNA/P-site tRNA complex. The nucleotide analysis is based on a semi-empirical charge assessment and geometry optimization algorithm. Kollman all-atom force field parameters were employed for energy minimization and molecular dynamics simulation protocols utilized in tRNA anticodon stem/loop structure, tRNA/mRNA interactions, and tRNA/mRNA/tRNA complexations. In addition, a description of the methods used for gene transcript sequence analysis for codon bias patterns is outlined.
Cell Culture Methods

Eukaryotic cells have been shown to require the primary substrate for queuosine synthesis, the queuine base, to be imported into individual cells through a specific uptake mechanism. Uptake of queuine at the cellular level has been studied using cell culture techniques with mammalian cell-lines. These cells acquire the queuine base from serum that supplements the nutrient media (88). In 1985, queuine uptake studies were performed with diploid human fibroblasts using a tritiated queuine analogue to observe and measure queuine membrane transport (30). The cells were exposed to the radiolabeled nucleotide for a given period of time, then the culture was washed extensively to remove extracellular label. The cells were lysed and the internal components solubilized with ethanol. Finally, a liquid scintillation analysis of the radioactivity contained within this lysate indicated the amount of radiolabeled material transported into the cell.

An in vivo method for the investigation of radiolabeled queuine incorporation into tRNAs in cultured cells was developed by Muralidhar et al. in 1988 (46). The cells were exposed to radiolabeled queuine for a given period of time, then the culture was washed extensively to remove extracellular label. The cells were lysed and the internal components solubilized by incubation in a detergent-based lysis buffer. Then, an acid precipitation step was performed on the solubilized cytosolic solution in order to precipitate the large nucleotide component of the cytosol. A filter binding step was executed to remove the small molecular weight compounds, including unincorporated radiolabeled queuine base. Finally, the filter was analyzed by liquid scintillation. Using this method, the levels of intracellular queuine incorporation activity were able to be measured in a few cell lines.

Using a monkey kidney cultured cell-line, the maintenance of queuosine levels during queuine starvation has been assessed by a radiolabeled queuine pulse-chase methodology (17,31). This protocol involved the deprivation of queuine by treatment of cells with charcoal-stripped media, then saturation of cellular tRNAs with radiolabeled queuine by pre-incubation for 24 hours just prior to the initiation of the study. Then, cultures of cells were either maintained in charcoal-stripped media (to measure the ability of the cells to maintain the label—salvage the queuine base and reuse it in formation of the queuosine modification), or were exposed to media containing high levels of unlabeled queuine to flush out the label (which would indicate the turnover rate of the tRNAs specifically in the cells being studied). The final steps of the assay were similar to that utilized for the in vivo queuine incorporation assays.

Materials

The HFF cell-line was established from circumcision tissue by established methods (74). The HxGC3 human colon adenocarcinoma cell-line was obtained as a gift from Dr. Jon R. Katze (University of Tennessee, Memphis), and the MCF-7 human breast adenocarcinoma cell-line was obtained from the American Type Culture Collection (Bethesda, MD). Cell cultures were established and maintained in either 10% neonatal calf serum supplemented minimum essential media (HFF and HxGC3 cell-lines) or
10% neonatal calf serum supplemented Delbecco's modified eagle media (MCF-7 cell-line) (GIBCO, Grand Island NY). The DNA methylase inhibitor, 5-azacytidine (5-azaC), employed in the generation of the 5-azaC-treated HxGC₃ cultures was purchased from Sigma Chemical Company (St. Louis, MO). 5-azaC-treated HxGC₃ cultures were exposed to 5 μM 5-azaC for 24 hours, then incubated with minimal essential media containing 10% neonatal calf serum until confluence. Queuine-deficient cultures for the incorporation and salvage studies were generated by growing the cells in media supplemented with 10% charcoal-stripped calf serum (74), for at least three passages before the studies were initiated.

The radiolabeled reduced analog of queuine, tritiated dihydroqueuine (rQT₃), was a gift from Dr. Ronald W. Trewyn (Kansas State University). The addition of a radiolabeled analog of queuine, tritritiated dihydroqueuine (rQT₃), to cell cultures was employed to monitor cellular uptake of this base, its incorporation into tRNA, or salvage of the incorporated base during tRNA turnover. Two lots of lyophilized rQT₃ were dissolved in water to yield stocks concentration of 1 mM with specific activities of 0.19 μCi/μg and 3 μCi/pmol and stored at -20°C.

Studies involving protein kinase and protein phosphatase modulators were performed after treatment as specified. They were all received in powder form and dissolved in acetone at 1,000 times the working concentration to form stock solutions. cGMP- and cAMP-dependent kinase, and PKC activators were obtained from Sigma Chemical Company (St. Louis, MO). The cGMP and cAMP analogues (dibutyryl and 8-bromo) were applied to cell cultures at a concentration of 10 μM. 12-tetradecanoyl phorbol-13-acetate (TPA) and phorbol-12,13-didecanoate (PDD) were used at concentrations of 20 nM. The PKC inhibitors sphingosine (used at 20 μM), staurosporine (used at 100 nM), and calphostin C (used at 100 nM) were purchased from Biomol Research Laboratories of Plymouth Meeting, PA. The PKC inhibitor H-7 (1-[5-isoquinoline sulfonyl]-2-methyl piperazine dihydrochloride) (utilized at 10 μM) was obtained from Seikagaku America, Inc. (St. Petersburg, FL). The protein phosphatase inhibitors okadaic acid and calyculin A (utilized at varying nM concentrations) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA).

All culture work was performed in sterile polystyrene plasticware from Corning (Corning, NY).

**rQT₃ Uptake Assays**

Cells were subcultured into 35 mm dishes at a density of 4x10⁴ cells/mL in a final volume of 2 mL of media containing 10% serum. When required, 5-azaC treatment of HxGC₃ cultures was performed at this point. At confluence, the media was decanted from the cells and 1 mL of media supplemented with 10% calf serum and 100 nM rQT₃ (0.10 μCi) was added. In phosphorylation modulation studies, uptake of rQT₃ was compared between untreated control cultures and with cultures exposed to PKC and protein phosphatase modulating agents at concentrations listed above. The modulating agents were added to the culture at the same time as the rQT₃ for short-term exposure studies or for 7 days prior to the introduction of rQT₃ for long-term or chronic exposure studies. Incubations for rQT₃ uptake analysis were conducted at
37°C for up to 6 hours and terminated by rinsing the cell monolayer four times with 5 mL of ice-cold phosphate buffered saline. Then, the cells were lysed with 1.0 mL of 95% ethanol for five minutes. The lysate was aspirated and radioactivity determined by liquid scintillation. The amount of radioactivity in the cell lysate, normalized to the number of picomoles of rQT$_3$ per $10^5$ cells, was reflective of rQT$_3$ uptake into the cultured cells.

**rQT$_3$ Incorporation Assays**

Prior to the initiation of the incorporation assays, the generation of queuosine-deficient cultures was required to form queuosine-deficient tRNAs in order to effectively assess cellular TGRase activity. These cultures were produced by growing the cells in media supplemented with 10% charcoal-stripped calf serum for at least three passages before the start of the experiment. Cells were then subcultured into 35 mm dishes at a density of 4x10$^4$ cells/mL in a final volume of 2 mL of media containing 10% charcoal-stripped neonatal calf serum. When required, 5-azaC treatment of HxGC$_3$ cultures was performed at this point. At confluence, the media was decanted from the cells and 1 mL of media supplemented with 10% charcoal-stripped neonatal calf serum and 100 nM rQT$_3$ (0.10 pCi) was added. Incorporation of rQT$_3$ into the acid precipitable fraction (tRNA) of various control cultures and those treated with PKC and protein phosphatase modulating agents were compared in phosphorylation modulation studies. PKC and protein phosphatase modulators were added to the cultures either at the same time as the rQT$_3$ (short-term exposures) or 7 days prior to the rQT$_3$ addition (long-term or chronic exposures) at concentrations listed above. Incubations for all rQT$_3$ incorporation analyses were conducted at 37°C for timed intervals of up to 24 hours. Incubations were terminated by rinsing the cell monolayer four times with 5 mL of ice-cold phosphate buffered saline, followed by disruption of the cells with 0.5 mL of lysis buffer (10 mM Tris (pH 7.5), 0.01% SDS, 0.01% Triton X-100) for ten minutes at room temperature. The lysate was transferred to a small test tube, treated with 0.25 mL of ice-cold 30% trichloroacetic acid (TCA), and placed on ice for ten minutes. The resulting precipitate was collected by vacuum filtration through GFA 2.4 cm glass fiber filter disks. Each disk was thoroughly rinsed with 40 mL of ice-cold 5% TCA and a final rinse of 5 mL ice-cold 95% ethanol. The filters were analyzed for bound radioactivity by liquid scintillation. The amount of radioactivity on the filter disks, normalized to number of picomoles of rQT$_3$ per $10^5$ cells, was reflective of rQT$_3$ incorporation into the acid precipitable fraction (tRNA) from cultured cells.

**rQT$_3$ Salvage**

Fibroblasts and MCF-7 cells were subcultured into 25 mL culture flasks at a density of 1x10$^4$ cells/mL in a final volume of 5 mL of media containing 10% calf serum. When the cells reached confluence, the media was decanted and 5 mL of media supplemented with 10% charcoal-stripped calf serum for one day, one week, or three weeks to induce a possible latent queuine salvage mechanism. Before the beginning of the salvage study, 100 nM rQT$_3$ (0.10 μCi) was added to the cultures ant then
incubated for 24 hours to fully saturate the tRNAs with radiolabeled queuine. At the beginning of the study, the cell monolayer was washed 4 times with sterile phosphate-buffered saline and then incubated in either media containing 10% serum supplemented with 0.10 A_{260} units unlabeled queuine (89) or media supplemented with 10% charcoal-stripped serum and, if indicated, PKC and phosphatase modulators at conditions listed above. Incubations for rQT\textsubscript{3} salvage analysis were conducted at 37°C at 24 hour intervals for up to 4 days. Incubations were terminated by rinsing the cell monolayer four times with 5 mL of ice-cold phosphate buffered saline, followed by disruption of the cells with 0.5 mL of lysis buffer (10 mM Tris (pH 7.5), 0.01% SDS, 0.01% Triton X-100) for ten minutes at room temperature. The lysate was transferred to a small test tube and treated with 0.25 mL of ice-cold 30% trichloroacetic acid (TCA), and placed on ice for ten minutes. The resulting precipitate was collected by vacuum aspiration through GFA 2.4 cm glass fiber filter disks, and then thoroughly rinsed with 40 mL of ice-cold 5% TCA and a final rinse of 5 mL ice-cold 95% ethanol. The filters were analyzed for bound radioactivity by liquid scintillation. The level of radioactivity on the filter disks was reflective of the amount of remaining rQT\textsubscript{3}, in the acid precipitable fraction (tRNA) from cultured fibroblast cells over time.

**Methods for the Isolation and Characterization of the Mammalian Liver tRNA:Guanine Ribosyltransferase**

In an adaptation of an established *in vitro* method for the quantification of the queuosine modification in tRNAs, an enzymatic assay for the incorporation of queuine into tRNA is utilized to monitor the purification process of the queuine incorporation enzyme (tRNA-guanine ribosyltransferase; TGRase). This assay is a variation of the one developed by Okada, et al. in 1978 using the *E. coli* TGTase enzyme (38). This method has been used to measure the increasing levels of queuosine in tRNA during differentiation and development in different organisms (55,90,91), as well as to measure the decreasing levels of queuosine in tRNA during progressive stages of neoplastic transformation and metastatic progression (40-43).

In the *in vitro* enzymatic assay, a completely unmodified tRNA population with respect to queuosine is used as a substrate for the measurement of tritiated guanine or queuine incorporation (TGRase) activity of a sample. Since the yeast organisms are known to not synthesize queuosine nor utilize queuine (10), and since purified brewer's yeast tRNA is commercially available, this was the substrate of choice for the assay of TGRase activity. In the presence of the enzyme, incorporated radiolabeled queuine or guanine substrate in tRNA is separated from the free nucleotidyl bases by acid precipitation of the larger tRNA nucleic acid and filtration to remove the low molecular weight free queuine base. Since the modification of tRNA with queuosine is irreversible, the ability of the enzyme to incorporate tritiated guanine or queuine into tRNA is an inverse measure of the levels of queuosine-modified tRNA in a given sample.
Materials

Yeast tRNA and PKC purified from rat brain were purchased from Boehringer Mannheim (Atlanta, GA). ATP (biotech grade) was purchased from Fisher Scientific (Indianapolis, IN). Staurosporine and sphingosine were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). H-7 (1-[5-isoquinoline sulfonyl]-2-methyl piperazine dihydrochloride) was obtained from Seikagaku America, Inc. (St. Petersburg, FL). $^3$H-guanine (1 mCi, 168 Ci/mmol) and [$\gamma^{-32}$P]ATP (250 µCi, 3000 Ci/mmol) were purchased from Amersham Corporation (Arlington Heights, IL). The Silver Stain kit was purchased from BioRad, Inc. (Hercules, CA). All other chemicals or reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Buffers

Homogenization buffer (20 mM Tris-Cl [pH 7.5], 1 mM MgCl$_2$, 1.0 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 0.05% Triton X-100, and 10 µg/mL of the following protease inhibitors: aprotinin, calpain inhibitor I, chymostatin, leupeptin, pepstatin, phenylmethylsulfonylfluoride), Phosphate buffer (20 mM sodium phosphate [pH 7.2], 0.5 mM dithiothreitol, 1 mM MgCl$_2$, and 10% glycerol), Storage buffer (20 mM Tris-Cl [pH 7.5], 1 mM MgCl$_2$, 0.5 mM dithiothreitol, 1 mM EDTA, and 50% glycerol), Mono-Q buffer (10 mM Tris-Cl [pH 7.5], 1 mM MgCl$_2$, and 0.5 mM dithiothreitol), Reaction buffer (10 mM Tris-Cl [pH 7.5], 35 mM KCl, 1 mM MgCl$_2$, and 0.5 mM dithiothreitol), Denaturing Sample buffer (10 mM Tris-Cl [pH 7.4], 0.5% SDS, 20% glycerol, and 0.01% bromophenol blue), and Non-denaturing Sample buffer (10 mM Tris-Cl [pH 7.4], 20% glycerol, and 0.01% bromophenol blue).

Isolation of TGRase From Mammalian Liver

The purification of TGRase was performed by adaptation of previously published methods (44). Livers (42.5 g) were collected from five adult Sprague-Dawley rats that were sacrificed by cervical dislocation. 50.0 g liver tissue was obtained from adult BALB/c mice that had also been sacrificed by cervical dislocation. Approximately 43.0 g of human liver was obtained from autopsy of a 62 year old man who died of cardiac arrest. The fresh or flash-frozen livers were rinsed in ice-cold Homogenization buffer. The tissue was homogenized in 100 mL of cold buffer first by Waring blender and then by Dounce homogenizer, filtered through two layers of sterile gauze, and centrifuged at 10,000 x g for thirty minutes at 4°C. The resulting supernatant was centrifuged at 100,000 x g for one hour at 4°C. The supernatant was decanted through sterile gauze, brought to a final volume of 300 mL in Homogenization buffer, and loaded by gravity feed onto a 13 x 5 cm DE-52 ion exchange column in a 2 liter scinted glass funnel pre-equilibrated with Homogenization buffer. A two liter wash of Homogenization buffer lacking the protease inhibitors and triton X-100 was drawn through the column by low pressure vacuum using a water-aspirator. TGRase was eluted from the column with one liter of Homogenization buffer containing 0.2 M KCl. Protein in the 0.2 M KCl fraction was precipitated by adding ammonium sulfate to a final saturation of
55%. The solution was stirred on ice for 30 minutes, and then allowed to stand an additional 30 minutes. The protein precipitate was collected by centrifugation for 20 minutes at 10,000 x g.

The precipitate was redissolved in 25 mL of Phosphate buffer, and dialyzed against 4 liters of the buffer overnight at 4°C with at least two changes of buffer. Denatured precipitate in the dialysate was removed by centrifugation at 10,000 x g for 20 minutes, and the supernatant was collected and applied to a P-11 phosphocellulose column (2 x 10 cm) which had been pre-equilibrated at 4°C with Phosphate buffer. The phosphocellulose column was washed with 100 mL of Phosphate buffer at a flow rate of 1.0 mL/min. The sample was eluted with a 200 mL 0 to 1.0 M KCl gradient in Phosphate buffer. Fractions (2 mL) were collected and tested for the capability of 3H-guanine exchange with yeast tRNA. Those fractions that resulted in 3H-guanine incorporation were judged to contain TGRase activity. The pooled TGRase-active fractions (10 mL) from the 0.25 to 0.30 M KCl region of the gradient were either stored at sub-zero temperatures (-20°C or -70°C), or further purified using a 1 x 10 cm Pharmacia Mono-Q ion exchange column.

Samples to be stored were dialyzed against storage buffer for at least three hours and then stored in 1 mL aliquots at either -20°C or -70°C. Samples to be purified further by Mono-Q FPLC ion exchange chromatography were concentrated to 5 mL in an 50 mL Amicon pressure cell using a YM 10 membrane (10,000 MWCO) under 40 psi N2 gas. The sample was then diluted to 50 mL with Mono-Q buffer and reconcentrated to 5 mL.

Five to ten milligrams of protein (as determined by Bradford assay (92)) in 1.0 mL aliquots of the TGRase preparation were injected onto a pre-equilibrated 1 x 10 cm Mono-Q column. The column was eluted at a flow rate of 1.0 mL/min with a 100 mL 0 to 0.5 M KCl linear gradient in Mono-Q buffer. The fractions collected were assayed for TGRase activity. The protein preparation exhibiting TGRase activity eluted in the 0.20 to 0.25 M region of the gradient, and corresponded to the major peak of the A260 profile of the elution gradient. The Mono-Q fractions were concentrated to 3.0 mL in an Amicon pressure cell as before. The 3.0 mL samples were concentrated further using a Centricon 10 concentrator to volumes of 0.5 mL to 1.0 mL. These preparations were then used as samples in enzyme assays, electrophoresis and autoradiography, and Superose 6 size-exclusion chromatography.

*Reaction Conditions for TGRase Assays*

The assay for TGRase activity parallels one described previously(44), and is based on the enzyme's ability to reversibly exchange 3H-guanine for guanine in the first position of the anticodon of yeast tRNA. Yeast tRNA is unique in that it does not contain queuosine(10), therefore it is an excellent substrate for TGRase. TGRase assays were conducted in a total volume of 0.3 mL Reaction buffer with 20 μM 3H-guanine (1 μCi), 0.1 A260 unit of yeast tRNA (5 μg), and TGRase. Assays were incubated for one hour at 37°C, terminated by the addition of 100 μL of ice-cold 30% trichloroacetic acid (TCA), and chilled on ice for 10 minutes. The resulting precipitate was collected by suction filtration through glass fiber
filters, washed with 45 mL of ice-cold 5% TCA, and analyzed by liquid scintillation.

**Reaction Conditions for PKC Assays**

PKC reactions were performed in a total volume of 0.3 mL *Reaction buffer* containing 1 mM CaCl$_2$, 5.0 µg diolein, 5.0 µg phosphatidyl serine, and 100 µM [γ-$^{32}$P]ATP (1 µCi). Various concentrations of either histone fraction IV (positive control) or the TGRase preparation and PKC were added, incubated at 30°C for 30 minutes, and terminated by the addition of 100 µL of ice-cold 30% TCA. The precipitate was allowed to stand on ice for 10 minutes, collected by suction filtration through glass fiber filters, washed with 40 mL of cold 5% TCA and analyzed by liquid scintillation.

**Reaction Conditions for the Combined TGRase, PKC and Alkaline Phosphatase Assays**

TGRase, PKC, and Alkaline Phosphatase (AP) assays were combined using the aforementioned assay conditions. Reactions were performed in a total volume of 0.3 mL of *Reaction buffer* containing 1 mM CaCl$_2$, 5.0 µg diolein, 5.0 µg phosphatidyl serine, 20 µM $^{3}$H-guanine (1 µCi), 100 µM ATP, 0.1 A$_{260}$ yeast tRNA (5 µg), and various concentrations of TGRase, PKC, and/or AP as indicated. The reaction mixtures were incubated 30°C for 45 minutes. The reaction was terminated and prepared for liquid scintillation analysis as previously described.

**Electrophoretic Analysis**

Samples from each stage of the purification procedure were saved and applied to a denaturing or non-denaturing polyacrylamide electrophoresis experiment. The samples were boiled for five minutes and allowed to cool to room temperature. The samples were loaded onto parallel 16 x 16 cm SDS- and non-denaturing-PAGE gels prepared by the method of Laemmli (93). Each gel was run at 50 mA constant current for approximately 5 hours. The gels were then fixed and silver-stained using the BioRad Silver Stain kit to reveal banding patterns.

Combination reactions using the TGRase and PKC preparations were performed to assess the ability of PKC to incorporate $^{32}$P from [γ-$^{32}$P]ATP into TGRase. Reaction conditions were as described with the PKC assays with the differing reaction mixture samples containing 40 µg histone fraction IV (positive control), P-11 concentrated sample, or 10 µg of Mono-Q fraction samples. The radioactive reaction mixtures were incubated for 45 minutes at 30°C and terminated by the addition of Denaturing Sample buffer. The samples were boiled for five minutes and allowed to cool to room temperature. The samples were loaded onto a 16 x 16 cm SDS-PAGE gel. The gel was run at 50 mA constant current for approximately 5 hours. The gel was then wrapped in cellophane and exposed to X-ray film at -70°C for 48 hours. The X-ray film was developed by an automated film developer. Finally, the gel was simultaneously fixed and stained (40% methanol, 10% acetic acid, 0.05% Coomassie blue). Pictures were taken of the gel.
**Size-exclusion Chromatographic Analysis**

Weakly and strongly active Mono Q TGRase samples were analyzed by fast protein liquid chromatography (FPLC) on Pharmacia's Superose-6 high resolution size exclusion column with an LKB Bromma GT Chromatography System. Aliquots were initially separated by chromatography under native conditions in order to determine sizes of proteins and complexes in the sample. Approximately 800 µg of each sample was loaded onto a pre-equilibrated 1 x 30 cm Superose-6 column and eluted with 10 mM Tris-Cl [pH 7.5] at a flow rate of 0.5 mL/min. Molecular weights of major peaks were compared to standards on the basis of elution time as detected by UV monitor at 280 nm. Aliquots were then exposed to guanidinium-HCl to induce disassociation of protein complexes and separated to identify the sizes of protein monomers in the samples. Samples were incubated in a solution containing 4 M guanidinium-HCl for 30 minutes at room temperature. Then, the sample was applied to the Superose-6 column and eluted with 10 mM Tris-Cl [pH 7.5] at a flow rate of 0.5 mL/min.

**Computational Design and Methods**

In the past decade, computational chemistry methods for structure determination have been developed based on more and more accurate algorithms. In 1984 Harvey et al. began molecular dynamics simulations of a tRNA<sup>α</sup> using a very early Amber force field method (94-96). This was performed only on “heavy atoms” and required thousands of CPU hours on either a Cray or VAX supercomputer. In 1986, Nilsson and Karplus (97) modeled the dynamics of the anticodon stem/loop for tRNA<sup>α</sup> based on an early version of Karplus’ CHARMM software (98). This greatly decreased the CPU time required and produced results very similar to Harvey et al.’s computationally expensive experimentation. Both sets of results have maintained good credibility with the more recent examinations of the tRNA by experimental methods of temperature-jump experiments, the deciphering of X-ray crystallographic structures, and structural fluctuations based on multi-dimensional NMR interpretations.

In this research, Nilsson and Karplus’ methods have been adapted to a very different workstation system and software package. However, to incorporate non-standard modified nucleotides within the tRNA crystal structure model, full charge and geometric assessment of these nucleotides was first performed. Semi-empirical charge assessment and geometry optimization of nucleotides were determined by the most current algorithm that was included with the workstation’s software utilized in this study (AM1 and PM3 algorithm in MOPAC 6.0 software). Since these algorithms are notoriously inaccurate when assessing the geometry and charge distribution of amide linkages, an “mmok” command activating an amide correction factor was included in the calculations.

Energy minimization and molecular dynamics simulations of a tRNA anticodon stem-loop, based on Nilsson and Karplus’ older methods, are performed with the latest commercially available algorithms including the current version of the Amber force field—the Kollman all-atom force field (version 6.0). All atoms were treated in these studies and the solvent was implicitly included in the electrostatic calculations.
by use of a constant dielectric constant of 80. There are definite limitations using this new technology: among these the limited capacity to treat large complex macromolecular structures and the mathematical models that the experiments are based on. The computational system utilized in this study was not powerful enough to include solvation and counterion atoms in the simulations, nor to elongate the running time of the simulations due to limited processing speed and RAM, however the algorithms employed by this workstation are the most powerful to date when limiting the number of atoms explicitly treated. Despite these limitations, these calculations provide reasonable data that have been shown (when used appropriately) to provide accurate information about the system that is being monitored. When compared to a very recent publication reporting molecular dynamics simulation data of a similar molecule with counterions, solvation and much longer length of simulations (99), the results described in this treatise are virtually identical.

Instrumentation

The computational chemistry experiments performed in this study were performed on a Silicon Graphics Power Indigo\textsuperscript{2} workstation (MIPS R8000 processor) with 64 megabytes of RAM (Mountain View, CA). The modeling software was a commercially available package from Tripos and Associates, Inc. (St. Louis, MO) and included Sybyl base (6.2), Dynamics, Advanced Computation, MOLCAD, and Biopolymer modules, and MOPAC 6.0.

Nucleotide Analysis

In the initial analysis for determination of wobble base differences effecting tRNA anticodon decoding abilities, the structure and electrical potentials of four nucleotides were studied. Guanosine (G) is the unmodified “traditional” base found at position 34 in the primary transcript of the four queuosine-related tRNAs. Guanosine remains in these tRNAs unless enzymatically replaced by queuosine synthetic mechanisms. 7-deazaguanosine (7-deazaG) is a non-physiologically utilized guanosine analogue and was included in the study to examine structural effects caused solely by the lack of the N7 of guanosine which exists as a C7 in the queuosine modification. 7-aminomethyl-7-deazaguanosine (7-am-7-deazaG) is the first product in the prokaryotic queuosine synthetic pathway, yet only a minor physiological species. This residue was included in the study to account for discernible effects of the charged quaternary amine at the end of the 7-aminomethyl side chain which also exists in queuosine. Queuosine (Q) is the fully formed physiological modification found in prokaryotic and eukaryotic species. At physiological pH, the base contains a charged quaternary amine in the 7-aminomethyl side-chain which terminates in a cyclopentenediol ring.

The 5'-monophosphate nucleotidyl forms G, 7-deazaG, 7-am-7-deazaG, and Q were “sketched” and submitted to both AM1 and PM3 MOPAC (semi-empirical) charge and geometry optimization protocols using the “mmok” amide correction factor. A charged quaternary amine was utilized in the
design of the side chains for the Q and 7-am-7-deazaG molecules based on the recognized crystal structure of the Q nucleotide (13). An electron density surface was generated for each molecule and the electrical potential of each molecule was mapped onto the corresponding surface.

The three G analogues mentioned above (7-deazaG, 7-am-7-deazaG, and Q) were also incorporated into the Sybyl software's RNA monomer dictionary (Kollman All-atom Force Field parameters, including atom types and charges). These modified ribonucleosides were utilized for further experimentation using site-directed mutagenesis protocols for replacement of the G residue in position 34 of tRNA<sup>mp</sup> anticodon stem/loop structures.

**Anticodon Stem/Loop Analysis**

The ability to perform optimal dynamics simulations through explicit solvation or use of an entire tRNA molecule is prohibitive. Therefore, a method developed by Nilsson and Karplus for investigation of the tRNA<sup>mp</sup> anticodon stem and loop was adapted for use in this study (97). The Nilsson and Karplus method was originally based on a protocol published by Harvey, Prabhakaran, Mao and McCammon which studied motions of bases and lengths of hydrogen bonds in an intact yeast tRNA<sup>mp</sup> molecule (94).

The *Saccharomyces cerevisiae* transfer RNA<sup>mp</sup> crystal structure coordinates (2.8 Å resolution) were obtained from Brookhaven Protein Databank for this study (100,101). Two conformations of this molecule were identified in the crystal samples, A- and B-forms, which differ only in the DHU loop region between two dihydrouridine residues at positions 16 and 20. Wild-type *S. cerevisiae* tRNAs do not contain the queuosine modification due to the lack of any definable queuosine modification system in the host organism (10). Therefore, the yeast crystal structure for the tRNA<sup>mp</sup> contains a guanosine residue at position 34, and was the basis for a software-based mutagenesis protocol to produce anticodon stem/loop structures of tRNA<sup>mp</sup> containing 7-deazaG, 7-am-7-deazaG, or Q at the wobble position. With exception of the wobble base, the resulting structures were identical to that of the original molecule which contained a G in position 34.

The Nilsson and Karplus method utilized tRNA<sup>mp</sup> residues 26 through 44 for model computations which comprise the seven anticodon loop nucleotides and the six base-paired nucleosides of the stem. In this study, the modified and unmodified tRNA<sup>mp</sup> anticodon stem/loop residues 26 to 44 (identical in both A- and B-forms) were extracted in order to perform subsequent molecular dynamics simulations. Implicit aqueous solvation (constant dielectric function of 80) was added to all calculations in order to mimic an aqueous environment.

A steepest descent energy minimization protocol of 1,000 fs was performed on each molecule to relieve strain inherent in the crystallized structure. The resulting structures were compared with the original tRNA<sup>mp</sup> crystal structure which served as the reference template. The RMS differences in atomic position varied between 0.072 and 0.092 Å; therefore, it was assumed that molecular conformations were not significantly different before the dynamics run. The dynamics simulation was begun with gentle
warming of the system to prevent structural distortion and artifacts. Shake constraints were set for the stem base pairs between residues 26 to 30 and 40 to 44. The initial velocities and atomic vectors were assigned and reassigned for each preceding step by Boltzmann distribution. Temperature steps lasting for 100 fs beginning at 45 K and increasing to 273 K by 25 K intervals were performed and monitored at 5 fs intervals. A final step of 100 fs at 298 K was performed, then a 55 ps constant temperature plateau at 300 K was monitored at 1 ps intervals with rescaling of velocities at 25 fs intervals to enhance the temperature equilibration.

Macromolecular Complex Analysis

In order to analyze the effects of queuosine-modified versus unmodified tRNA in interactions with a neighboring tRNA (codon context) or cognate mRNA codon (codon bias), a model system of the tertiary structure for the tRNA/mRNA/tRNA complex needed to be chosen. Two possible conformations have been described for the placement of the tRNAs with respect to the mRNA on the ribosome: the Sundaralingham (S-) form (102) and the Rich (R-) form (103). Lim et al. investigated both the R- and S-form models based on the rigid crystal structure of two yeast tRNA molecules with a flexible 6-mer mRNA in order to study transcript phosphodiester backbone conformations between the two adjacent aminoacyl- (A-) and peptidyl- (P-) site codons (104). It was proposed that the R-form was the most likely arrangement on the ribosome due to relatively good agreement with cross-linkage data. MacDonald and Rein argued that the S-form was more correct due to a design also based on fixed crystal structures of yeast tRNA and tRNA with a flexible mRNA transcript (105). Prabahakaran and Harvey designed a model utilizing experimental cross-linkage data that was based on flexible anticodon loops with a fixed helical mRNA, instead of those parameters mentioned above (106). The results also describe a conformation that resembles the S-form, however the true ribosomally-bound configuration was suggested to be somewhere in between the previously described S-forms and this model due to the reliance of all of these models on one "fixed" component in each system.

In 1994 Easterwood, Major, Malhotra and Harvey built a model of the ternary complex nucleotide-by-nucleotide based on the cross-linkage data and crystal structure constraints for two tRNA molecules (107). This method did not require a fixed arrangement for either the mRNA or the tRNAs involved in the complex. The results of the protocol (the Easterwood structure) were compared with that of both Lim R- and S-forms for agreement with experimental fluorescent energy transfer data (108,109). Both the Lim S-form and the Easterwood structure (also an S-form) were closest in agreement with this information as compared with the Lim R-form. However due to the method of the model design, the Easterwood form contained less potential conflicts. Subsequently, a three dimensional structure of the Escherichia coli ribosome programmed with three tRNA molecules was described at a resolution of 18 Å (110,111). The positions of the A-, P-, and exit-site tRNAs were determined by high resolution cryo-electron microscopy and molecular modeling "fit" methods. This conformation was described as being
significantly different than either Lin R- or S- models. In comparisons with the cryoelectron microscopy structure and the aforementioned computational, the cryoelectron microscopy model and the Easterwood model constraints were in best agreement with the experimental data. Thus, the Easterwood model was chosen as the basis of the ternary complex conformation for analysis in this paper.

**Macromolecular Complex Analysis: Interaction of A- and P-site tRNAs (Codon Context)**

The Easterwood model for the ternary complex of A- and P-site tRNAs with 6-mer mRNA was used to assess the likelihood of interaction between the anticodon loops. Two of the largest modified nucleotides known are Q in position 34 of tRNA*-Q, tRNA*-Q, tRNA*-Q, and tRNA*-Q at position 37 in tRNA*-Q (existing in the crystal form of tRNA*-Q and utilized in the Easterwood model). Both of these modifications have been shown to be absent in clinical cases of rapidly growing neoplastic cells in which protein synthesis levels are increased (45). Therefore, steric interaction between these two large anticodon modification side-chains, if positioned next to each other on adjacent codons, has been suggested to have the potential of affecting the stability of the A- and P-site tRNAs in the translating ribosomal complex resulting in altered translational efficiency via codon context effects (112).

The Easterwood model’s poly-uridine RNA molecule was adapted to contain a GAU codon at either the 5’ end (the A-site) or the 3’ end (the P-site). The backbone and χ torsions were set to mimic that of the originally derived poly-uridine structure. Finally, a queuosine-modified tRNA*-Q anticodon stem/loop structure, as described above, was superimposed to replace either the A-site tRNA*-Q or the P-site tRNA*-Q of the Easterwood complex. A steepest descent energy minimization protocol of 1,000 fs was performed on these complexes to relieve torsional strain and produce reasonable structures for the study. Then, the two complexes were examined; A-site Q-modified tRNA*-Q anticodon stem/loop with P-site Y-containing tRNA*-Q, and vice versa. Distances were measured for the closest potentially interacting atoms for the Y and Q modifications in both complexes.

**Macromolecular Complex Analysis: Anticodon-Codon Association at the A-site (Codon Bias)**

The ternary Easterwood structure was utilized to develop a model for anticodon/codon interaction at the modeled A-site to assess the effects of specific wobble nucleosides on the association of tRNA with either of the two cognate codons. The original Easterwood 6-mer poly-uridine RNA molecule was mutated to contain a GAU or a GAC codon at the 5’ end of the mRNA (the A-site) and backbone and χ torsions were set to mimic that of the original poly-uridine structure. Finally, individual modified tRNA anticodon stem/loop structures derived from the site-directed mutagenesis protocol of the G-containing S. cerevisiae tRNA* crystal structure, as previously described, were superimposed into the A-site of the Easterwood complex. For this portion of the study, the P-site tRNA*-Q was deleted.

Four tRNA*-Q anticodon stem/loop structures were investigated; G-containing, 7-deazaG-modified, 7-am-7-deazaG-modified, and Q-modified molecules. Each anticodon stem/loop was complexed with

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either of two mRNA models (5'-GACUUU-3' (GAC-mRNA) or 5'-GAUUUU-3' (GAU-mRNA)). A steepest descent energy minimization protocol of 1,000 fs was performed on each of these eight complexes to relieve torsional strain and produce reasonable starting structures for the study. They were compared and exhibited significant similarity as modeled starting structures (RMS differences of 0.082-0.098). Then, each complex was subjected to a protocol of 100 fs per step ramping of 25 K increases from 48 K up to 323 K with a constant dielectric function of 80. “Actual” temperatures were monitored and levels for kinetic energy, potential energy, and total energy were calculated at 5 fs intervals. The results for each step were examined and when an equilibrium for the “programmed” temperature was approached, calculations were made for average “actual” temperature, kinetic energy, potential energy, and total energy. As expected, “actual” versus programmed temperatures averaged per step were shown to be linear with a correlation coefficient of 0.999 and a slope of 1.000 for all complexes.

**Codon Usage Analysis for Growth Control versus Housekeeping Transcripts**

Coding sequences for various mammalian growth control and housekeeping genes were acquired from published reports and manually analyzed for their content of codon triplets encoding asparagine, aspartate, histidine, and tyrosine amino acids. Then, the ratio of NAU versus NAC codons for each amino acid type was determined and an overall assessment of total NAU/NAC ratio was made for each sequence. Finally, a NAU/NAC ratio trend was determined and codon bias assessed for all transcript sequences examined.
CHAPTER THREE
DEFINITION OF THE QUEUOSINE MODIFICATION SYSTEM

Introduction

Prokaryotic synthesis of the queuosine modification has been well studied and is reasonably well understood, however until recently the design of the eukaryotic system was still relatively ambiguous. Due to the similarities in structure of the modification in organisms of these two Kingdoms, it was initially assumed that the systems were evolutionally conserved, thus similar. New information concerning the eukaryotic queuosine modification system in mammals and other classes of organisms has shaded this with doubt.

Based on the results of studies involving in vivo cell culture and in vitro protein assay methods, this document introduces a description of the mammalian queuosine modification system. This scheme is compared with the well characterized prokaryotic modification pathways of Escherichia coli, and Zymomonas mobilis. A hypothesis concerning the evolutionary relationship of prokaryotic and eukaryotic modification systems is presented and a suggestion of the importance of the queuosine modification in all organisms is offered. In the end an assessment is made for the appropriate choice of a model system to be used for future studies of human pathology.

Definition of the Queuosine Modification System in Prokaryotes

In prokaryotic systems, particularly the most studied organism E. coli, the queuosine modification is formed by a four-step intracellular pathway which involves enzymatic synthesis of the unique base in intact tRNA (Figure 3-1) (23). The first step involves the conversion of a guanosine triphosphate (GTP) molecule to a 7-aminomethyl- or 7-cyano-7-deazaguanine derivative of the guanine base. This step is performed by a GTP cyclohydrolase-like enzyme which involves the removal of both the N7 and C8 atoms of the nucleotide base and incorporation of some aminopropyl or cyanopropyl group, respectively, to reform the ring. The two currently known GTP cyclohydrolase enzymes (type I and II) have been ruled out as the catalyst for this reaction. GTP cyclohydrolase I is involved in pteridine biosynthesis for the conversion of GTP into dihydroneopterin triphosphate. However, there have been no intermediates found in this reaction which could account for possible 7-deazaguanine synthesis. GTP cyclohydrolase II is involved in riboflavin synthesis and catalyzes the formation of GTP into 2,5-diamino-6-oxy-4-(5'-phosphoribosylamino) pyrimidine. A ribA mutant of E. coli deficient for this enzyme has been isolated, but is still able to synthesize the queuosine modification. Thus, it has been proposed that there is an undiscovered GTP cyclohydrolase that catalyzes the formation of 7-aminomethyl-7-deazaguanine (preQ₁) and 7-cyano-7-deazaguanine (preQ₂).

The second and third steps for the synthesis of the queuosine modification are performed by two enzymes that have been shown to be encoded in a single operon (113). This queuine operon, as it has been
Figure 3-1. The Prokaryotic Queuosine Modification System in Escherichia coli and Zymomonas mobilis. The prokaryotic system is a synthetic one based on a four step process. I) Sep one is a reaction catalyzed by an unknown GTP cyclohydrolase, requiring guanosine triphosphate and an unknown aminopropyl or cyanopropyl donor to form preq, or preq₀, respectively. II) Reaction two is catalyzed by tRNA:guanine transglycosylase (from the gene tgt) which performs a base-exchange reaction with preq, or preq₀, for a transcriptionally-encoded guanine base (G) in position 34 of tRNA⁺, tRNA⁺⁺, tRNA⁺⁺⁺, or tRNA⁺⁺⁺⁺. The formation of incorporated preq₀ (known as preQ₀) terminates the synthetic reaction, but appears to be reversible. III) Incorporated preQ₀, however, is reacted upon by the enzyme s-adenosylmethionine:tRNA ribosyltransferase (from the gene QueA) with the aid of s-adenosylmethionine to convert preQ₁ into epoxyQ. IV) The formation of epoxyQ was only recently discovered and is reduced by an unknown mechanism with the aid of coenzyme B₁₂ to queuosine (Q). After the degradation of tRNAs by non-specific RNases, the fully formed nucleoside Q is secreted by the cell and is not reused in the prokarytic system.
called, includes five coding regions at nine minutes on the *E. coli* genome. The genes are *queA*, *tgt*, *yajC*, *secD*, and *secF*, in that order. The *secD* and *F* gene products are involved in protein translocation and export, while that of *yajC* is unknown. The gene products of *tgt* and *queA* are involved in the second and third steps of queuosine synthesis. The expression of this operon is regulated by the "factor of inversion stimulation" (FIS protein) which, when bound to an upstream activation sequence, increases the transcription of this operon (114). The FIS protein is also involved in the transcriptional activation of several tRNA genes as well as all rRNA operons. Thus, the synthesis of queuosine formation enzymes appears to be directly modulated with the production of the target substrate, the tRNA.

The product of the *tgt* gene, tRNA:guanine transglycosylase (TGTase), is a 43 kDa zinc-containing protein consisting of 369 residues with a pH of 4.6 to 4.8 (115). In vivo it functions as a monomer in a reaction requiring tRNA, guanine or guanine analogue, and magnesium ions, but does not need adenosine triphosphate-derived energy. The TGTase enzyme appears to utilize either guanine ($K_M = 5.3 \times 10^{-4} M$), preQ$_0$ ($K_M$ undetermined), or preQ$_1$ ($K_M = 1.4 \times 10^{-6} M$) as substrate for incorporation into tRNA (116). However, only the reactions with guanine and preQ$_0$ are reversible. Due to the $K_M$ value, the irreversibility of the reaction, and the relative abundance of the incorporated preQ$_1$, it was suggested that this compound serves as the natural substrate for this enzyme. It is noted, however, that for unknown reasons the recombinant form of TGTase appears to often act as a multimer and in this state can reverse the incorporation of preQ$_1$ as well as the other substrates (117). Site-directed mutagenesis studies on the tRNA$^G$ substrate have indicated that the identity determinants for the association of this molecule with the TGTase enzyme is the anticodon loop sequence $U_{33}G_{34}U_{35}$ (118). Since the $U_{33}$ is conserved in all tRNAs, it was thus determined that only the first and second anticodon bases were factors in tRNA recognition for this enzyme. Discovery of an *E. coli* TGTase mutant and subsequent site-directed mutagenesis studies on this enzyme have provided evidence that a major catalytic residue in the function of the enzyme is a serine residue at position 90. Sequence homology studies showed regions of the prokaryotic TGTase in common with the serine protease family, with the spatial orientation of residues immediately adjacent to serine 90 (asp89-ser90-gly91-gly92) similar to those found around the catalytic serine residues in trypsin and chymotrypsin (119). More recent evidence has shown the presence of an important aspartate residue at position 102 in prokaryotic TGTase (120). This amino acid is proposed to serve as a critical catalytic nucleophile in the prokaryotic queuosine synthesis reaction as suggested by the recent description of the crystal structure of the *Zymomonas mobilis* TGTase.

Once the preQ$_1$ is incorporated into tRNA, it can serve as a substrate for the gene product of *queA*, s-adenosylmethionine:tRNA ribosyltransferase isomerase (SAM:TGTase isomerase) (121). Although preQ$_0$ is often inserted into tRNA by TGTase, it is not a substrate for SAM:TGTase isomerase and has therefore been suggested to exist only in significant amounts as a side product within an *E. coli* mutant blocked in preQ$_1$ synthesis (122). The presence of the enzyme activity was discovered relatively early in queuosine research that demonstrated *E. coli* and *S. typhimurium* cells, when starved of methionine, do not
synthesize the queuosine modification. Only recently was it discovered that s-adenosylmethionine (SAM) plays an unprecedented and critical role as a ribose donor for the formation of queuosine (25,26). This compound, usually a methionyl or methyl group donor, serves a substrate for SAM: TGTase isomerase in the attachment of the cyclic ring to the end of the 7-aminomethyl side chain of the incorporated 7-deazaguanosine residue. With a weight of 39 kDa and a pH of 5.4, this enzyme has no sequence homology to currently known proteins. A reaction mechanism was suggested for this reaction which produces a 2,3-epoxy-4,5-dihydroxycyclopentyl-7-aminomethyl-7-deazaguanosine residue (epoxy-Q or oQ).

In the final reaction of the queuosine modification system identified in both E. coli and S. typhimurium, an unknown enzyme requiring coenzyme B\textsubscript{12} performs a complete reduction of the epoxy group to form the final prokaryotic queuosine (Q) modified nucleoside (123). This reaction appears to be environmentally regulated by the availability of the B\textsubscript{12} vitamin nutrient, as neither prokaryotic organism studied has the ability to synthesize this compound.

In the formation of the prokaryotic queuosine modification the only exogenous requirement is that of vitamin B\textsubscript{12}. The formation of the modified nucleotide appears to be regulated by the availability of the metabolic substrates (for example, GTP), FIS\textsuperscript{*} induced expression of the enzymes and queuosine-associated tRNAs, and the availability of vitamin B\textsubscript{12}. After synthesis of the fully-formed queuosine base, natural tRNA-turnover events occur to renew the important participants of the protein synthetic mechanism. The queuine base is not salvaged during degradation of the tRNA, as it is not a substrate for any of the four enzymes involved in queuosine synthesis, but is secreted into the environment by prokaryotes.

Definition of the Queuosine Modification System in Eukaryotes

Many of the mechanisms involved in the formation of the eukaryotic queuosine modification appear to be significantly different from that of the prokaryotic system. For example, unlike prokaryotes eukaryotes are unable to synthesize queuosine and must rely on uptake of the free queuine base from their diet or from secretions of symbiotic prokaryotes in gut flora. Eight week old mice that were fed specially prepared queuine-free diets were shown to be deficient in queuosine modification levels (29,61). After four weeks the queuosine levels in tRNA\textsuperscript{\textsuperscript{\textsubscript{W}}} and tRNA\textsuperscript{\textsuperscript{\textsubscript{H}}} decreased from a baseline of 85% to 15% modified tRNA, however tRNA\textsuperscript{\textsuperscript{\textsubscript{W}}} and tRNA\textsuperscript{\textsuperscript{\textsubscript{H}}} levels were maintained at 100%. It was not until after one year of exposure to the queuine-free diet that all queuosine modification levels in the tRNA decreased to zero percent. Thus, it was suggested that in order to create the queuosine modification in tRNA in a mammalian organism, queuine must be somehow taken into the system—presumably by ingestion.

The use of an \textit{in vivo} uptake assay method has been used to identify and determine the kinetics of a specific queuine transport system (74). The uptake of \textit{tritiated} queuine exhibited burst kinetics over two to four minutes of initial exposure, then a linear import until plateauing at three to four hours showing evidence of saturation. Lineweaver-Burke analysis indicated biphasic kinetics with a $K_M$ of $30 \pm 4 \text{ nM}$ and
a $V_{\text{max}}$ of 0.33 pmol queuine taken up per $10^6$ cells for physiological concentrations of 1 to 10 nM. At higher concentrations, 10 nM to 1,000 nM, the $K_m$ rose to 350 ± 150 nM and a $V_{\text{max}}$ of 22.5 ± 2.5 pmol queuine taken up per hour per $10^6$ cells. Treatment of the cells with guanine, 7-methyl guanine, adenine, hypoxanthine, or 7-deazaguanine did not affect the transport rate of queuine. However, addition of 10 µM dipyridamole (a generalized transport inhibitor) reduced the uptake by 50 to 70%. These results indicated that a specific queuine transport pathway exists for physiological levels of queuine, with additional transport by general nucleotide transporters at higher concentrations.

The second step of the queuosine modification system involves a queuine utilization mechanism for the formation of this modified base in eukaryotes rather than the synthetic mechanism demonstrated in prokaryotes. Incorporation of the base into tRNA has been studied in vivo and in vitro in many organisms, but has not been definitively explored in any one eukaryotic organism.

In another difference from the prokaryotic queuosine modification system, eukaryotic cells have the ability to salvage this base from tRNA turnover products to maintain queuosine modification levels under queuine deprivation. In the early 1980s, Güntüz and Katze developed an in vivo method to study what they suggested was evidence of a salvage capability for retrieval of queuine from tRNA turnover products (15). They discovered that these cells had the ability to salvage queuine from degraded tRNA molecules and reuse it to maintain queuosine levels in the absence of an exogenous supply of queuine. It was later discovered that only queuosine-5'-monophosphate was the substrate for the queuine salvage mechanism, as none of the other possible tRNA degradation products (queuosine, queuosine-3'-monophosphate, or mannosyl queuosine-5'-monophosphate) allowed for the maintenance of queuosine levels during queuine starvation (16).

In both prokaryotic and eukaryotic organisms, the key step for the formation of the queuosine modification is the only one so far studied in depth using in vitro methods. In a 1983 review of "Queuin, the Q-containing tRNAs and the Enzymes Responsible for their Formation", Farkas describes the three eukaryotic queuine incorporation enzymes (rabbit erythrocyte (20), rat liver (44), and wheat germ (124)) that had been purified to pseudo-homogeneity. He drew comparisons amongst these and to the well characterized E. coli enzyme (115). The plant TGRase, isolated from wheat germ in 1983, was shown to be a homodimer of 68 kDa subunits, and the prokaryotic monomer has been shown to be of 42.5 kDa.

Recently, published descriptions of isolations and characterizations of three additional mammalian TGRase enzymes; bovine liver TGRase (125), rat liver TGRase (126), and rabbit TGRase (127) have been reported. These purified mammalian proteins appear to exist as a heterodimer complex of $M_r$ 100 to 104. The larger subunit of the complex ranges from 60 to 66 kDa, with a smaller subunit of 34 to 45 kDa.

A 1980 protocol (44) for the partial purification of the rabbit liver TGRase by Shindo-Okada, et al. was the basis for purification strategy developed for the isolation of mouse, rat, and human liver samples described in this chapter. The isolation protocol used in this study involves several steps of gravity-based liquid chromatography, differential salt precipitation, and fast protein liquid chromatography.
(FPLC). After each of these steps an enzymatic assay was performed to assess the retention and purity of the TGRase enzyme activity in each sample collected. The enzymatic assay for the incorporation of queuine into tRNA was used to monitor the purification process for the isolation of the TGRase protein. Final characterization of the enzyme preparation involved non-denaturing and denaturing electrophoresis and size-exclusion FPLC.

In the studies presented here, a human fibroblast cell culture model is used to gain a better picture of the queuine uptake, incorporation, and salvage mechanisms in "normal" human tissues. The results of the purification and characterization of three mammalian liver queuine incorporation enzymes were used to strengthen the description of the incorporation step of the queuosine modification system. It is suggested that the basic design of the mammalian queuosine modification system is significantly different from that described for prokaryotes. However, the evolutionary relationship between the eukaryotic and prokaryotic systems has not yet been examined. The information gained in this research on the eukaryotic queuosine modification system of several mammalian organisms adds another dimension of knowledge to that of the well characterized prokaryotic queuosine modification system. A comparison of the two systems may help to ascertain the evolutionary development of the queuosine modification systems that have been detected in species as diverse as prokaryotes, eukaryotic algae, many fungi, plants, insects, fish, birds and mammals. In order to determine which model system to use for studies of human pathology, the assessment of a physiologically similar and experimentally viable system still needs to be determined. Furthermore, the details of how the queuosine modification system is tied into physiological metabolism within the mammalian cell is also unknown.

Results

Characterization of the Mammalian Queuosine Modification System via In Vivo Studies of Normal Human Fibroblast Cell Cultures

The results of this study with human foreskin fibroblast (HFF) cell cultures indicate that the maximal rQT$_3$ uptake rate occurs up to one hour, then gradually decreases until six hours at which time an equilibrium of uptake and export is established (Graph 3-1). Incorporation of 100 nM rQT$_3$ into cellular tRNA populations occurs in these cells in a linear fashion (approximately 0.25 pmol/10$^5$ cells/hour) until 12 hours, at which point the rate of incorporation decreases to an equilibrium at 24 hours (Graph 3-1). The initial rate of uptake for rQT$_3$ is ten-fold that of incorporation, and after 24 hours (at equilibrium) the amount of intracellular rQT$_3$ that is incorporated in tRNA remains at a constant level of approximately 30%.

Degradation of tRNA molecules occurs as a regular process of cellular component regeneration. The normal turnover of tRNAs in human cells occurs between 24 to 48 hours (128). The half-life of queuosine-modified tRNAs in the HFF cells studied was measured at 2 days by the pulse-chase salvage
Graph 3-1. Queuine Uptake and Incorporation into the tRNA of Human Fibroblast Cell Cultures. Culture flasks of human fibroblast cells (HFF) were grown to near confluence in normal media, fed with charcoal-stripped serum (lacking queuine) for 24 hours, then submitted to either an *in vivo* queuine uptake assay (●) or an *in vivo* queuine incorporation assay (■). A tritiated analogue of queuine (rQT₃) at a concentration of 100 nM was placed in the media at time zero. The uptake of this base into the cytosolic fraction of the cells was measured in multiple parallel cultures hourly starting at time zero until 6 hours, and the incorporation of this base into the acid-precipitable fraction of the cells (containing large proteins and nucleic acids, including tRNAs) was measured at 0, 3, 6, 9, 12, and 24 hours. The uptake of rQT₃ to 24 hours was extrapolated (shown as a dashed line), this line is based on other uptake studies in HFF cells that have been run for 24 hours. The counts per minute values obtained via liquid scintillation were normalized to the number of pmol rQT₃ taken up or incorporated in 10⁶ cells. Deviation bars indicated represent the standard deviation with \( n = 6 \).
method, regardless of the amount of time that the cells were exposed to charcoal-stripped media (Graph 3-2). Charcoal-stripped media treatment of the cells for one day, one week, or three weeks was used to induce queuine starvation. These cells were assessed for the ability to maintain rQT\textsubscript{3} levels in the incorporated state for up to 4 days. At one day of induction by queuine-starvation, rQT\textsubscript{3} is maintained in the tRNA population at 100\% for up to 4 days. One week of treatment appears to maximally induce the salvage mechanism with full maintenance of rQT\textsubscript{3} levels also for four days. After three weeks of queuine-starvation, the salvage of rQT\textsubscript{3} decreases to 60\% after four days.

Isolation and Characterization of Mammalian Queuine Incorporation Enzymes

In order to further characterize the queuine incorporation step, the queuine incorporation enzyme, TGRase, was isolated from mouse, rat, and human liver samples. The conditions of isolation for each step are summarized and outlined in Table 3-I. Samples of each TGRase preparative step were stored at -20°C or -70°C in two aliquots as either direct hard-freeze samples or with the addition of an equal volume of glycerol.

During early isolation procedures for the rat liver enzyme, it was observed that the activity of the enzyme preparation was generally labile regardless of storage conditions. Measurable TGRase activity in cold stored preparations decreased rapidly to a low background level within only one to four weeks (Graph 3-3). In one instance a TGRase preparation lost activity within only three days. A preliminary test for phosphatase activity in the TGRase preparation indicated phosphatase contamination (unpublished observations). This was measured by the sample’s ability to convert the colorless phosphatase substrate, p-nitrophenylphosphate, to the yellow colored p-nitrophenoxide anion under basic conditions. Addition of 10 \mu M phosphatase inhibitors sodium fluoride and sodium pyrophosphate to the homogenization buffer served to enhance the recovery of TGRase activity from rat liver isolates (Graph 3-4). From then on, these general phosphatase inhibitors were added to all homogenization buffers used for the mouse, rat, and human liver isolation procedures.

The optimized conditions (homogenization and initial centrifugation steps) indicate that this enzyme is likely to be located in the cytoplasm, and that it may have a slight association with cytosolic membranes. This supposition is based on the ability to increase yields of isolations performed with 0.01\% Triton X-100 added to the homogenization buffer.

Data in Table 3-II represents a typical isolation protocol and the resulting enzymatic characterization of the most evident TGRase-active fractions in the major steps of mouse, rat, and human liver TGRase isolation protocols. The rQT\textsubscript{3} incorporation percentage yield of TGRase-active protein, unit activity, specific activity, and purification factor of each isolation step was compared amongst mammalian species and exhibited similar values. The results are very similar to published results obtained for the isolation of bovine liver TGRase by Slany and Müller (125).
Graph 3-2. Queuine Salvage in Human Fibroblast Cell Cultures. Cultures of human fibroblast cells (HFF) were grown to near confluence in normal media, fed with charcoal-stripped serum (lacking queuine) for 1 day, 1 week, or 3 weeks with regular media changes, then submitted to an *in vivo* queuine salvage assay. A tritritiated analogue of queuine (rQT$_3$) at a concentration of 100 nM was placed in the media 24 hours before the start of the assay to saturate the tRNA with the radiolabeled base--"pulse". In two sets of parallel cultures at time zero, extracellular rQT$_3$ was washed away, then a media change with charcoal-stripped media was performed. In one set of cultures unlabeled queuine (0.10 A$_{260}$ units) was loaded (solid line) this represents the "chase" of the radiolabel out of the degraded tRNA population and is an indication of the tRNA turnover rate within those cells. No queuine was added to the other set of cultures (dashed line) which represents the ability of the cells to retain the radiolabel in the cell's tRNA despite the degradation processes involved in normal tRNA turnover (a measure of salvage ability). The counts per minute values obtained via liquid scintillation were normalized to the number of pmol rQT$_3$ contained within the acid-precipitable fraction of the $10^5$ cells that had been devoid of queuine for 1 day, 1 week or 3 weeks. Deviation bars indicated represent the standard deviation with $n = 4$. 
Table 3-1. Isolation Characteristics of Mammalian Liver tRNA:Guanine Ribosyltransferase in Key Steps of the Purification Protocol

<table>
<thead>
<tr>
<th>Fractionation Step</th>
<th>Separation Method</th>
<th>Characteristic Isolation Condition of TGRase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>cheesecloth filtration</td>
<td>soluble after homogenizing with a tissue grinder and filtering</td>
</tr>
<tr>
<td>Centrifuged Supematant</td>
<td>super- and ultra-centrifugation</td>
<td>soluble after 1 hour at 10,000 x g at 4°C and 1 hour at 100,000 x g at 4°C</td>
</tr>
<tr>
<td>DE-52 Cellulose LC</td>
<td>anion-exchange liquid chromatography: increasing linear or step gradient of 0 to 0.5 M KCl</td>
<td>eluted from the diethylaminomethyl-cellulose matrix at approximately 0.15 M KCl</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ Precipitant</td>
<td>salt precipitation at 55% saturation and super-centrifugation</td>
<td>insoluble at 55% salt saturation and 30 minutes at 10,000 x g at 4°C</td>
</tr>
<tr>
<td>P-11 LC</td>
<td>cation-exchange liquid chromatography: increasing linear gradient of 0 to 1 M KCl</td>
<td>eluted from the phosphocellulose matrix at approximately 0.25 M KCl</td>
</tr>
<tr>
<td>Mono Q FPLC</td>
<td>anion-exchange fast protein liquid chromatography: increasing linear gradient of 0 to 0.5 M KCl</td>
<td>eluted from the trimethyl aminomethyl-conjugated monobeads at approximately 0.15 M KCl</td>
</tr>
<tr>
<td>Superose 6 FPLC</td>
<td>size-exclusion fast protein liquid chromatography: isocratic elution</td>
<td>eluted from the cross-linked agarose beads at approximately 34.5 kDa</td>
</tr>
</tbody>
</table>
Graph 3-3. The Decay of Two Samples of Purified tRNA:Guanine Ribosyltransferase Activity. The graph indicates the decay of TGRase activity in two different rat liver Mono Q-FPLC preparations (● and ■) stored at -20°C and -70°C, respectively. Five micrograms of purified protein isolate was analyzed using the standard TGRase activity assay to measure the ability to incorporate $^3$H-guanine into 0.10 $A_{260}$ units of yeast tRNA in one hour at 37°C. An early sample (●) displayed residual TGRase activity of background levels after only four days, thus the activity trend after this time to 42 days is extrapolated (dashed line). Standard deviation values are indicated for each sample with $n = 4$. 
Graph 3-4. The Recovery of Crude tRNA:Guanine Ribosyltransferase Activity with the Addition of Protein Phosphatase Inhibitors to the Homogenization Buffer. The bar designated “No tRNA” indicates the nonspecific binding of $^3$H-guanine to protein within the sample (no tRNA substrate added). The “Control” bar shows the residual TGRase activity recovered from homogenization of a sample of rat liver tissue with no protein phosphatase inhibitors added. The remaining bars indicate the TGRase activity recovered from the homogenization of samples of the same rat liver tissue using homogenization buffer containing 10 μM sodium fluoride or 10 μM sodium pyrophosphate, respectively. Five micrograms of protein isolate from the phosphocellulose column step were used for each assay. All assays were performed using the standard TGRase activity assay method. Standard deviation values are indicated for each sample with $n = 4$. 

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<thead>
<tr>
<th>Table 3-2. Detailed Summary of Mammalian Liver tRNA:Guanine Ribosyltransferase Purification Steps</th>
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<tbody>
<tr>
<td>Rat Isolate</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Liver</td>
</tr>
<tr>
<td>Homogenate</td>
</tr>
<tr>
<td>Centrifuged Supernatant</td>
</tr>
<tr>
<td>DE-52 LC</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ Precipitant</td>
</tr>
<tr>
<td>P-11 LC</td>
</tr>
<tr>
<td>MonoQ FPLC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mouse Isolate</th>
<th>Total Volume (mL)</th>
<th>Unit Activity* (pmol/mL sample/hour at 37°C)</th>
<th>Concentration (mg/mL)</th>
<th>Total Mass (mg)</th>
<th>Percent Yield (%)</th>
<th>Specific Activity** (pmol 3H-G incorporated/mg protein/hour at 37°C)</th>
<th>Purification Factor***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>50,000</td>
<td>100</td>
<td>n/a</td>
<td>n/a</td>
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<tr>
<td>Homogenate</td>
<td>250</td>
<td>5.8</td>
<td>12.0</td>
<td>3,000</td>
<td>6.0</td>
<td>0.48</td>
<td>1</td>
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<tr>
<td>Centrifuged Supernatant</td>
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<td>58</td>
<td>9.25</td>
<td>1,850</td>
<td>3.7</td>
<td>6.3</td>
<td>13</td>
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<tr>
<td>DE-52 LC</td>
<td>500</td>
<td>224</td>
<td>2.12</td>
<td>1,060</td>
<td>2.1</td>
<td>106</td>
<td>221</td>
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<tr>
<td>(NH₄)₂SO₄ Precipitant</td>
<td>80</td>
<td>nd</td>
<td>nd</td>
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<tr>
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<td>25</td>
<td>341</td>
<td>1.09</td>
<td>27.2</td>
<td>0.054</td>
<td>313</td>
<td>652</td>
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<td>2255</td>
<td>5.30</td>
<td>3.74</td>
<td>0.0075</td>
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<table>
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<th>Human Isolate</th>
<th>Total Volume (mL)</th>
<th>Unit Activity* (pmol/mL sample/hour at 37°C)</th>
<th>Concentration (mg/mL)</th>
<th>Total Mass (mg)</th>
<th>Percent Yield (%)</th>
<th>Specific Activity** (pmol 3H-G incorporated/mg protein/hour at 37°C)</th>
<th>Purification Factor***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>43,000</td>
<td>100</td>
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<td>2,550</td>
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<td>0.34</td>
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<tr>
<td>Centrifuged Supernatant</td>
<td>100</td>
<td>63</td>
<td>15.8</td>
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<td>12</td>
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<tr>
<td>DE-52 LC</td>
<td>425</td>
<td>177</td>
<td>2.12</td>
<td>901</td>
<td>2.1</td>
<td>83</td>
<td>242</td>
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<tr>
<td>(NH₄)₂SO₄ Precipitant</td>
<td>65</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>P-11 LC</td>
<td>14</td>
<td>520</td>
<td>1.97</td>
<td>27.6</td>
<td>0.064</td>
<td>264</td>
<td>776</td>
</tr>
<tr>
<td>MonoQ FPLC</td>
<td>1</td>
<td>1360</td>
<td>3.10</td>
<td>3.1</td>
<td>0.0072</td>
<td>439</td>
<td>1250</td>
</tr>
</tbody>
</table>

*Unit Activity: pmol ³H-G incorporated/mL sample/hour at 37°C. **Specific Activity: pmol ³H-G incorporated/mg protein/hour at 37°C. ***Purification Factor: enzyme activity ratio of experimental sample as a factor of the homogenate specific activity.
Results of the phosphocellulose (P-11) cation-exchange liquid chromatography procedure demonstrate the first step indicating variance amongst samples. This occurs as an isolation-to-isolation variation between species and within tissue samples, and may be due to differences in levels of phosphorylation and possibly subunit stability in isolates. Early in the development of the rat liver enzyme preparations, TGRase activity assays of fractions from P-11 chromatography and succeeding steps indicated two distinct regions of TGRase activity, one major and one minor. Samples taken from mouse Mono Q anion-exchange liquid chromatography preparations and those found in human P-11 fractions also showed evidence of these two regions of TGRase activity. The major TGRase active fraction was used to determine specific activity and is the source of the data recorded in Table 3-II, with the second weaker TGRase activity fraction initially ignored. Much later studies with protein kinase C phosphorylation of these two peaks indicated that the second region of weaker TGRase activity is dramatically increased under phosphorylating conditions (see Chapter 4 for a more thorough explanation).

Since there was evidence of the importance of phosphorylation levels for the success of isolation procedures, SDS-PAGE analysis and a protein kinase C catalyzed $^{32}$P-labeling study was performed with isolates from a TGRase-active P-11 fraction and two MonoQ regions exhibiting strong and weak rat liver TGRase activity to attempt to distinguish any differences between the highly active and weaker TGRase fractions. $^{32}$P-labeled and unlabelled P-11 and Mono-Q rat TGRase fractions in a protein kinase C-catalyzed reaction mixture were analyzed by denaturing and non-denaturing PAGE. The unlabeled denaturing gel was stained by Coomassie stain, destained and photographed (Figure 3-2). A parallel denaturing PAGE gel of the $^{32}$P-labeled samples was then exposed to film, and banding appeared in lanes containing the P-11 concentrate and in one of the consecutive Mono-Q fractions. A band at approximately 60 kDa was observed in the P-11 sample and in a very weakly TGRase-active Mono-Q fraction sample. A very faint band of potential $^{32}$P-incorporation was also observed in this region of the strongly TGRase active Mono Q fraction. These bands matched up well with the Coomassie-stained bands in the gel (Figure 3-2). The lane containing the P-11 fraction does not exhibit distinguishable banding patterns with respect to the autoradiograph, however in the lanes containing fractions of the Mono Q step two distinct bands fairly equal in intensity appear at approximately M, 60 and 34.5 in the fraction exhibiting weak TGRase activity, with a strong 34.5 kDa band and a faint 60 kDa band in the strongly TGRase-active Mono Q fraction. By comparison, a non-denaturing PAGE exhibited a very simple banding pattern in these lanes (unpublished observations). In the strongly TGRase-active Mono-Q fraction a single band was visible and was of small size (between 30 and 45 kDa). This correlated well with the denaturing PAGE results. However in the non-denaturing gel, the following Mono-Q fraction (which exhibited good $^{32}$P-incorporation, but limited TGRase-activity) exhibited a single very large molecular weight band. This suggested possible multiple subunit involvement evidenced as banding differences under non-denaturing vs. denaturing conditions, so a study using native and denaturing chromatography was undertaken.
Figure 3-2. SDS-PAGE Electrophoretic and Autoradiographic Analysis of Protein Samples During Isolation of the tRNA:Guanine Ribosyltransferase Enzyme. a) Two silver-stained 8% SDS-PAGE gels were run and the important lanes are shown together above. Each lane shows the separation of protein isolates from various steps of the human liver tRNA:guanine ribosyltransferase (TGRase) procedure. Fifty micrograms of protein were applied for the first four lanes and 40 μg for the P-11 eluent on one gel. On a second gel, 10 μg was applied for the MonoQ fractions. Molecular weight markers run for each gel were used for alignment of the lanes in this graphic, and their locations are shown on the left. On the right, double-headed arrows indicate the "interesting" bands at 60 and 34.5 kDa. b) A radiolabeling phosphorylation reaction assay was performed with 10 μU of protein kinase C (PKC) and γ32P-ATP, and either 40 μg of histone fraction IV (positive control for phosphorylation by PKC) or 40 μg of rat liver P-11 eluent or 10 μg of rat liver MonoQ fraction sample. The reactions were quenched with the addition of SDS-PAGE sample buffer and heating. They were then applied to an 8% SDS-PAGE gel and electrophoresis was performed. The gel was then exposed to X-ray film at -70°C for 48 hours and developed. Finally, bands of the gel were developed by simultaneous fixing and staining using 0.05% coomassie blue in 40% methanol, and 10% acetic acid. Molecular weight markers were also run and their migration positions are indicated on the left. Double-tailed arrows indicating "interesting" bands at 60 kDa and a group of four between 28 and 38 kDa (bracketed), corresponding to the positive control locations of histones, are shown on the right.
Comparisons were made between native Superose 6 FPLC chromatographs of the strongly TGRase-active and $^{32}$P-incorporating, weakly TGRase-active Mono-Q fractions (Figure 3-3). Compared to identifiable protein standards, the $^{32}$P-incorporating, weakly TGRase-active Mono-Q fraction maintains its major peak at approximately 100 to 110 kDa with minor peaks visible in the 55 to 65 kDa and 30 to 38 kDa ranges. Integration of the chromatograph showed 56% of the protein in the 104 kDa range with 32% and 12% in 60 and 34.5 kDa ranges, respectively. When this fraction was denatured by the presence of 4 M guanidinium-Cl a definitive shift in its peaks occurred. The 100 to 110 kDa peak virtually disappeared, while the main peak appeared to shift to the right and plateaued between 65 and 30 kDa. The TGRase-active Mono-Q fraction appeared to have the major peak at approximately 30 to 38 kDa, with a possible minor peak between 55 and 65 kDa. Integration of these peaks indicated that the strongly TGRase-active fraction contained approximately 80% of the protein in the smaller peak with less than 10% in the mid-sized peak. The retention time overlaps for the peaks in the chromatographs are suggested to be due to the presence of differing complexes of subunits. This is explained by the presence of TGRase as a large 104 kDa complex able to dissociate into subunits of 60 and 34.5 kDa, which corroborates the electrophoretic data.

Discussion

Eukaryotic organisms acquire the main substrate for the formation of the queuosine modification by either dietary ingestion of the queuine base or absorption of secreted queuine produced by intestinal gut flora. The queuosine modification system in mammalian cells is shown to consist of cellular uptake of the queuine base, incorporation of this nucleotide into tRNA, and salvage of the degraded transcript to recycle the base for reuse (Figure 3-4). The characteristic uptake activity of a queuine-specific transporter in human foreskin fibroblast cell cultures was determined using an in vivo assay method that approximates human physiological conditions. Linear uptake of queuine occurs at a rate of 2.5 pmol/10^5 cells/hour until saturation between four and six hours. The characteristic profile of the queuine incorporation mechanism was also assessed in the human fibroblast cells under the same conditions, with linear incorporation rates at ten-fold less than uptake. Not until 24 hours does the incorporation rate plateau, and it appears that approximately 30% of intracellular queuine is maintained in the incorporated queuosine-modified tRNA form. Finally, the ability of these cultures to salvage the incorporated queuine base and recycle was described. A wildtype baseline salvage activity is measurable in these cells, and can be induced to maximal activity by queuine starvation for up to one week. At three weeks and thereafter, the ability to salvage queuine decreases with an apparent acclimation of the cells.

The incorporation step of the queuosine modification system was studied in three mammalian organisms in order to more fully describe this important synthetic step. All three isolated mammalian (mouse, rat and human) liver TGRase enzymes appear to be composed of two subunits of approximately 60 and 34.5 kDa. These more recent results for the mouse and human TGRase enzymes confirm previous
Figure 3-3. Absorbance Profiles of Superose 6 FPLC Separation of Rat Liver tRNA:Guanine Ribosyltransferase-Active MonoQ FPLC Fractions. Two MonoQ-FPLC fractions were collected. Fraction one exhibited very high tRNA:guanine ribosyltransferase (TGRase) activity, and fraction two showed very weak activity but above the background level. An aliquot of each of these fractions were submitted to denaturing conditions (4 M guanidinium chloride at room temperature for 30 minutes). Injections of 800 µg protein onto Pharmacia's Superose 6 FPLC column were made for each sample and an on-line UV monitor measured the elution absorbance at 280 nm. The first minor peak at three minutes represents the injection peak. The first major peak indicated the void volume. Comparison's to molecular weight standards were made, and the three subsequent peaks were identified as being of approximately 104, 66 and 34.5 kDa, respectively. The denatured fraction two sample contained an extra peak at approximately 45 minutes that represents the elution of the guanidinium salt. The denatured fraction one profile is not shown due to limited differences from the nondenatured fraction’s profile.
The Proposed Eukaryotic Queuosine Modification System in Normal Human Fibroblast Cells. The eukaryotic system is based on usage of an available resource. Queuine (q) is a nucleotidyl base that is secreted by gut flora and is digested from virtually all dietary sources. It is absorbed in the intestines and distributed around the body in the vasculature. A specific queuine transporter is responsible for uptake of the base into the cell. In the cytosol tRNA:guanine ribosyltransferase incorporates it into tRNA\textsuperscript{G34}, tRNA\textsuperscript{G35}, tRNA\textsuperscript{G36}, or tRNA\textsuperscript{G37} via a base-exchange reaction with q for a transcriptionally-encoded guanine base (G) located in position 34. After the degradation of tRNAs by non-specific RNases during normal tRNA turnover, the q nucleotidyl base is salvaged by an unknown mechanism from queuosine-5'-monophosphate and reused for the incorporation step to form queuosine-modified tRNA.
findings regarding the rat liver enzyme (126) and are similar to that of the bovine liver TGRase (125).

The results of this experimentation and that of others suggest that mammalian TGRase enzymes are a heterodimer complex of a larger subunit (60 to 66 kDa) and a smaller subunit (31 to 35 kDa). There is speculation among laboratories both in the U.S. and in Germany about the stability of this dimer complex. In these studies, isolation of two regions of TGRase activity during the isolation protocols of both the P-11 cation-exchange liquid chromatographic and the Mono Q anion-exchange fast protein liquid chromatographic steps suggest that the large salt gradient required by these methods may induce disassociation of the TGRase subunits. The composition of the enzyme complex has also been suggested to be a problem for the isolation of TGRase by phosphocellulose cation-exchange as well as tRNA-affinity chromatography in the isolation of bovine TGRase (125).

Characterizations of mouse, rat, rabbit, bovine and human TGRase enzymes have shown these isolates to be heterodimers of very similar size (Table 3-III). However, a eukaryotic plant TGRase from wheat germ (124) and two bacterial TGTase enzymes from E. coli (39) and Z. mobilis (129) involved in the formation of the queuosine modification have been shown to contain subunit make-ups different from that of mammals or plants. The plant enzyme appears to be a homodimer of subunits similar in size to the larger subunit of the mammalian enzyme and utilizes queuine as its major substrate.

The prokaryotic systems are unique in that they are the only systems where the queuine-related enzymes have been purified, cloned and well-characterized. The E. coli version of TGRase has been suggested to be functional as either a monomer or as hexamer made up of two homotrimers (130). The monomers of both E. coli and Z. mobilis enzymes (Mr = 42.5) are of an identical size that does not appear to correspond with either of the eukaryotic subunits. More recent studies of these proteins indicate that they are both likely to function in vivo as 42.5 kDa monomers, rather than oligomers (129).

Partial nucleotide and amino acid sequences for the rabbit (Genbank accession number L37420) (127) and bovine (125) enzymes, respectively, have been compared with known nucleotide sequences of the prokaryotic enzymes from E. coli (Genbank accession numbers M63939 & M35286) (130) and Z. mobilis (Genbank accession number L33777) (129). There is no sequence homology between the eukaryotic and prokaryotic species.

Metal ion requirements for the function of prokaryotic, plant and mammalian enzymes also differ. The E. coli (131) and wheat germ (124) enzymes were suggested to require a divalent cation for activity. More recently, the E. coli enzyme has been shown to contain a structural Zn\(^{2+}\)-binding motif that appears to be required for tRNA association (132,133). This region of the prokaryotic enzyme is highly conserved in the Z. mobilis nucleotide sequence which has now been resolved to 1.85 Å by X-ray crystallographic methods (134). Therefore, the Zn\(^{2+}\) requirement for TGTase function is likely to be important in many prokaryotic forms of the enzyme. The bovine (125) and rat (44) liver enzymes have been shown not to require any metal ions to function.
<table>
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<tr>
<th>Source</th>
<th>$M_r$</th>
<th>#Subunits $(M_r^*)$</th>
<th>Nucleoside Substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
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<td>46</td>
<td>1 (46)</td>
<td>preQ$_1$</td>
<td>(22,130)</td>
</tr>
<tr>
<td>Zymomonas mobilis</td>
<td>45</td>
<td>1 (46)</td>
<td>preQ$_1$</td>
<td>(129)</td>
</tr>
<tr>
<td>Wheat Germ</td>
<td>140</td>
<td>2 (68 + 68)</td>
<td>queueine</td>
<td>(124)</td>
</tr>
<tr>
<td>Mouse Liver</td>
<td>104</td>
<td>2 (60 + 34.5)</td>
<td>queueine</td>
<td>(current work)</td>
</tr>
<tr>
<td>Rat Liver</td>
<td>80</td>
<td>?</td>
<td>queueine</td>
<td>(44)</td>
</tr>
<tr>
<td></td>
<td>104</td>
<td>2 (60 + 34.5)</td>
<td></td>
<td>(126) and</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(current work)</td>
</tr>
<tr>
<td>Rabbit Erythrocyte</td>
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<td>2 (60 + 43)</td>
<td>queueine</td>
<td>(20)</td>
</tr>
<tr>
<td>Bovine Liver</td>
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<td>2 (66 + 31)</td>
<td>queueine</td>
<td>(125)</td>
</tr>
<tr>
<td>Human Liver</td>
<td>104</td>
<td>2 (60 + 34.5)</td>
<td>queueine</td>
<td>(current work)</td>
</tr>
</tbody>
</table>

*Relative molecular weight ($M_r$) is listed in kilodalton units (kDa).
Differences in substrate specificity between the prokaryotic and eukaryotic enzymes are also in evidence. While both enzymes catalyze the exchange of guanine-for-guanine in the wobble position of tRNA, their substrates in the formation of the queuosine modification are very different. The *E. coli* enzyme utilizes 7-aminomethyl-7-deazaguanine as its primary substrate and does not associate with the fully formed queuine nucleoside at all (115). The incorporation of both prokaryotic substrates (guanine and 7-aminomethyl-7-deazaguanine) are reversible. Both plant (124) and animal (44,116) enzymes, however, preferentially bind queuine for incorporation into tRNA while exhibiting $10^2$- to $10^3$-fold less affinity for 7-aminomethyl-7-deazaguanine and 7-deazaguanine, respectively. Similar to the prokaryotic enzyme, the guanine-for-guanine reaction is reversible. However the eukaryotic enzymes incorporate queuine-for-guanine in an irreversible reaction. The removal of queuosine’s base from the tRNA in both prokaryotic and eukaryotic systems is only accomplished by turnover and degradation of the entire tRNA molecule.

The results of the comparisons between these prokaryotic and eukaryotic enzymes for sequence, complex structure, metal ion cofactor, and substrate specificity indicate significant differences in this family of enzymes. Although historically placed under one classification (E.C. 2.4.2.29) and name (tRNA-guanine transglycosylase), the prokaryotic and eukaryotic modification enzymes involved in the formation of queuosine appear to have arisen from distinct sources. The prokaryotic “queuine operon”, encoding at least two enzymes involved in the formation of the queuosine modification is not likely to be the evolutionary source of the eukaryotic queuosine modification enzyme. It is proposed that the prokaryotic enzyme should retain the historical name of tRNA-guanine transglycosylase, and that the eukaryotic enzyme should attain the new E.C. name of tRNA-guanine ribosyltransferase with a new E.C. number in sequence.

This description of the incorporation step of the queuosine modification system provides an important example for the differences between the prokaryotic and eukaryotic mechanisms. In addition, it is important to point out that the existence of the uptake and salvage steps described in the human fibroblast cells insinuate a dramatic design difference in these two systems. Prokaryotic cells cannot utilize the fully formed queuine base in the incorporation step and, instead of transporting a useable substrate for the synthesis of the modification into the cell, have developed a complicated series of reactions to form the 7-aminomethyl-7-deazaguanine (preQ1) substrate for the first step of the prokaryotic queuosine synthetic mechanism. In addition, the lack of a salvage mechanism in prokaryotes is not surprising due to the inability of the incorporation mechanism to utilize the fully formed queuine base. The differences in the prokaryotic and eukaryotic systems lead to the conclusion that these are not evolutionarily related, but may be products of convergent evolution. The development of two mechanisms to form the same strange tRNA modification also suggests that this product may have an important role in cellular function of all organisms, with the exception of yeast. Just what this role is, however, has not yet been determined.
With the discovery of the overwhelming differences between the prokaryotic and eukaryotic queuosine modification systems, the current focus of basic research regarding queuine and the queuosine modification may need to be reevaluated. Although the bulk of the enzymatic research has been focused on the prokaryotic queuosine incorporation enzyme for characterization, it may be necessary to concentrate on the examination of the mammalian queuosine modification system and the TGRase enzyme for application to human development and disease research. This research, involving the development of a human foreskin fibroblast cell culture model, provides a physiologically “normal” baseline for future comparisons for modulative effects within a cell, as well as a guideline for studying apparently “abnormal” neoplastic cells.
CHAPTER FOUR
MODULATION OF THE MAMMALIAN
QUEUOSINE MODIFICATION SYSTEM

Introduction

The queuosine modification of tRNA is universally conserved in mature, static cells of eukaryotic organisms from Dictyostelium discoideum to Homo sapiens. However, in rapidly dividing and undifferentiated cells the levels of this modification are dramatically decreased. Transfer RNA isolated from neoplastic tissues and transformed cell lines is also hypomodified with queuosine to various degrees (38,45,60,86). Neoplastic transformation of cells involves retrograde-differentiation events that are very similar but reverse processes of differentiation. Both differentiation and retrograde-differentiation events are induced and progress by the action of neighboring cell and environmental stimuli. These include such mechanisms as cytokine-induced signaling cascades and G-protein associated secondary messenger systems. The action of one class of experimental activators of an important intracellular signalling cascade has been linked to both phenotypic change events in mammalian cultured cells and an alteration in tRNA metabolism within those cells. There is some evidence that tumor promoting induction of cultured cells via exposure to phorbol esters also has a direct effect on the queuosine modification system.

In both murine and human cell culture experiments, chemically-induced differentiation, tumor promotion, and cellular growth potentiation have been inversely correlated with alterations in queuosine levels. Murine erythroleukemia cells are typically queuosine-deficient. These cells can be forced to differentiate and have been demonstrated to concurrently increase in levels of queuosine-modified tRNA (66,67). The differentiation of these cells and the increase in queuosine-modification level was effectively blocked by chronic treatment with the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA) (66). These observations suggest that the hypomodification of tRNA with regard to queuosine is related to growth enhancing or tumor promoting events. Chronic exposure of cultured normal human fibroblasts to phorbol-12,13-didecanoate (PDD), another classic phorbol ester, induces a large inhibition of the queuosine modification of tRNA (74). The decrease in queuosine content of tRNA precedes an increase in the population density of the fibroblast cultures. Concurrent addition of purified queuine effectively blocks the population density increase in fibroblast cultures exposed to PDD, and maintains the queuosine level in those cells at near normal levels. These results suggest that the queuosine modification system may be connected to controlling the growth rate of normal human fibroblasts and is regulated by the action of phorbol esters.

In the mid-1980s it was discovered that phorbol esters directly bind to and activate protein kinase C (PKC), a major player in the Ca²⁺ signaling cascades (81,83). Not long thereafter, it was demonstrated in in vivo cell culture studies of a few mammalian cell lines that cellular uptake of queuine is sensitive to
phorbol esters (30,80). Thus, it was suggested that queuine uptake may be modulated by PKC (135). Chronic exposure of cultured cells to phorbol esters has been shown to induce a proteolytic down regulation of PKC and its activity (84). Since a decrease in the level of queuosine-modified tRNA was also observed with chronic exposure of human fibroblasts to PDD (80), it was proposed that the down-regulation of PKC activity levels in cell culture may have decreased the rate of queuine uptake needed for the formation of queuosine-modified tRNA.

Over the many years of attempting to isolate and purify a mammalian queuine incorporation enzyme (tRNA:guanine ribosyltransferase; TGRase), it was observed that the activity of TGRase enzyme preparations were generally labile regardless of storage conditions. Measurable TGRase activity in cold stored (-20°C or -70°C) preparations would decrease rapidly to a low background level within only one to four weeks. In one instance a TGRase preparation lost activity within only four days (see Chapter 3, Graph 3-3). Since the suspected linkage between queuosine modification levels and the level of phosphorylation in cell culture studies appeared to be plausible, preliminary tests for phosphatase activity in the mammalian TGRase preparations were undertaken to see if this was the cause of the loss of TGRase activity. Tests indicated that there was significant protein phosphatase contamination in the samples. In addition, the addition of general protein phosphatase inhibitors sodium fluoride and sodium pyrophosphate to the homogenization buffer used in the initial isolation step dramatically increased the recovery of activity (see Chapter 3, Graph 3-4). Thus, it was suggested that the incorporation step of the queuosine modification system of mammals might also be influenced by the action of phosphorylation, possibly by PKC.

A multifaceted study, described here, was performed to investigate possible phosphorylation-based post-translational modification schemes using modified forms of both the in vivo and in vitro assay methods used in the experiments described in Chapter 3. These were undertaken in order to assess potential regulatory mechanisms concerning the queuosine modification system by phosphorylation. The description of the mammalian system, as described in the previous Chapter, is also revised to incorporate knowledge gained in these regulation control studies. Finally, the significance of this entire modification scheme is assessed with regards to the evolutionary relationship of prokaryotic and eukaryotic queuosine modification systems, to the ability of this system to be integrated within cellular metabolism, and to an explanation of possible deficiencies observed in many neoplastic cell-lines and tumor biopsies.

**Regulation of Cellular Metabolism by Phosphorylation and Protein Kinase C**

In 1992, the Nobel Prize for Physiology or Medicine was awarded to E. H. Fischer and E. G. Krebs for their work concerning “reversible protein phosphorylation as a biological regulatory mechanism” (136). They discovered a reciprocal kinase/phosphatase system that had the ability to regulate intracellular events such as mobilization of glucose from glycogen stores, prevention of organ transplant rejection, and development of chronic myeloic leukemia.
The list of protein kinases discovered since the early days of Fischer and Krebs' work has grown very large and has been grouped into families based on either a similar target for the enzyme (such as creatine kinase) or by the compound that activates the kinase's activity. Protein kinase C was discovered as a kinase that is activated by calcium ions (hence the "C"), therefore it is exquisitely sensitive to one of the major second messenger systems that exists in all cells (82). The activation of voltage-gated calcium pores as well as by the formation of inositol-triphosphate by phospholipase C and its subsequent induction of release of internal calcium stores both serve to activate the activity of protein kinase C. In addition, the list of targets for protein kinase C-catalyzed phosphorylation is very long and diverse, including cytoskeletal targets (such as vinculin, filamin, profilin, desmin, MARCKS, troponin T and I, and the C-protein associated with cardiac myofibrils), intracellular second messenger systems (protein kinase M-catalytic fragment, myosin light chain, neuromodulin and neurogranin), and transcription factors (CREB, CRE, C/EBPα, the vitamin D₃ receptor, and IκB in the IκB/NFκB complex) (137). Thus, protein kinase C has been implicated as playing a role in the regulation of the cell cycle, cellular growth rate, multidrug resistance, and phenotypic shifts during differentiation and neoplastic transformation (85).

Protein kinase C is a key regulatory enzyme in mammalian cells, however there is evidence of a protein kinase C-like activity in organisms from yeast to humans, and in all mammalian tissues sampled. In addition to the connection of aberrant PKC activity with cancer, abnormal levels of protein kinase C activity have been implicated in disorders ranging from learning disabilities (138) to diabetic retinopathy (Mandarino, 1992) to suicidal tendencies (139). Protein kinase C control over the queuosine modification system would provide a link between tRNA metabolism and a long list of other metabolic shifts known to occur during differentiation and neoplastic transformation.

In Vivo Studies of Phosphorylation Control of the Queuosine Modification System

Cell culture studies of human fibroblasts were used to measure the three facets of the queuosine modification system in normal cells (see Chapter 3). Modified in vivo uptake, incorporation, and salvage assays were repeated in these cells to assess the effect of different modulators of cellular phosphorylation on queuine and queuosine metabolism. The initial study involved exposure of the normal human fibroblast cultures to activators of three different protein kinases; cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), and PKC. The observations of cellular exposure to dibutyryl- and 8-bromo- analogues of cAMP and cGMP and two phorbol esters, TPA and PDD, on uptake and incorporation both suggested the involvement of PKC (140,141).

In this treatise, more comprehensive experiments were performed to verify the involvement of PKC in the regulation of queuine uptake and queuosine incorporation into tRNA. These involved exposure of the human fibroblast cell cultures to PKC activators (TPA and PDD), PKC inhibitors (H-7, staurosporine, sphingosine, and calphostin C), and phosphatase inhibitors (okadaic acid and calyculin A) (Figure 4-1). Phosphatase activators are not commercially available, thus were not used in this study.
Figure 4-1. Structures of Phosphorylation Modulating Agents. To ascertain if phosphorylation provides a mechanism for regulation of the queuosine modification system, an approach was developed using kinase and phosphatase modulators to measure their effect on queuine uptake, incorporation, and salvage rates. Several kinase and phosphatase modulators were used to identify the kinase and phosphatase families involved in the regulation scheme. Activators for protein kinase A (butyryl-cAMP and 8-bromo-cAMP), protein kinase G (butyryl-cGMP and 8-bromo-cGMP), and protein kinase C (TPA and PDD) were employed to identify the participating kinase family. Protein kinase C activators (TPA and PDD) and inhibitors (calphostin C, sphingosine, staurosporine, and H-7), and phosphatase inhibitors (okadaic acid and calyculin A) were studied to determine if phosphorylation, particularly by protein kinase C, is involved in the regulation of the queuosine uptake, incorporation, and/or salvage steps of the queuosine modification system.
Chronic exposure of cell cultures to TPA is known to induce proteolytic degradation and down-regulation of PKC activity (84). This phenomenon was exploited to verify the results of the short-term exposure experiments. While the effect of exposure to PKC and phosphatase inhibitors should not change with prolonged exposure to the agents, long-term exposure to the PKC activator TPA should instead inhibit the enzyme. In preliminary studies for the experimentation involving phosphatase inhibitors, concentration ranges were performed for exposure of agents to the cultures. This enabled the determination of IC$_{50}$ values which were compared to published standards of these compounds in order to suggest the family of phosphatases involved in the inhibition of the queuosine modification system at any of the uptake, incorporation, and/or salvage steps.

In Vitro Studies of Phosphorylation Control of the Incorporation Step of the Queuosine Modification System

Purification strategies for the isolation of mammalian TGRases were modified to assess the likelihood that direct phosphorylation of the enzyme helps to maintain their activity levels. Addition of 10 μM phosphatase inhibitors sodium fluoride and sodium pyrophosphate to a homogenization buffer used in the early stages of purification served to enhance the recovery of TGRase activity from rat liver isolates (see Chapter 3, Graph 3-4). Thereafter, when native in vitro TGRase incorporation assays showed minimal activity, PKC was added during a duplication of the assay. Through this procedural addition, it was possible to trace the purification of the enzyme through subsequent isolation steps. These efforts were able to eventually produce a homogeneous TGRase isolate.

To confirm direct phosphorylation and activation by PKC with regard to the TGRase enzyme, as was suggested by in vivo cell culture studies, additional experiments were performed with the addition of commercial PKC to the reaction assays. Control reactions were performed to assess the effect of PKC modulators directly on TGRase activity, and their effect on TGRase activity in the presence of PKC. Finally, reciprocal control of TGRase activity by a classical kinase/phosphatase regulatory mechanism was studied with the sequential addition of PKC and alkaline phosphatase to a single TGRase assay reaction.

Results

Regulation of Cellular Queuosine Uptake

Cultured human fibroblasts (passages 3-5) were exposed to the phosphorylation modulators to discern influences on rQT$_3$ uptake after a three hour incubation (Graph 4-1). The agents that are reported to stimulate the cAMP or cGMP dependent kinase systems had no effect on the rate of rQT$_3$ uptake. However, activators of PKC increased the rate of rQT$_3$ uptake by fifty percent when compared to untreated controls. By contrast, cellular exposure to inhibitors of PKC all decreased the rate of rQT$_3$ uptake. Additional fibroblast cultures were exposed to the protein phosphatase inhibitors in order to maintain
Graph 4-1. The Effect of Protein Kinase and Protein Phosphatase Modulators on Queuine Uptake in Human Fibroblasts. Each bar represents rQT<sub>3</sub> uptake in cultures exposed to various kinase and phosphatase modulators for three hours. The cultures were treated with modulators at the following concentrations: 10 μM dibutyryl-cGMP, 8-bromo-cGMP, dibutyryl-cAMP and 8-bromo-cAMP, 20 nM TPA and PDD, and 10 nM okadaic acid, 1 nM calyculin A, 100 nM calphostin C, 20 μM sphingosine, 100 nM staurosporine and 10 μM H-7. The uptake of the radiolabelled queuine in all cultures (4 x 10<sup>5</sup> cells) was measured after a three hour incubation with 100 nM rQT<sub>3</sub> and modulators at 37°C. These values were normalized to the number of pmol rQT<sub>3</sub> taken up by 10<sup>5</sup> cells, and converted to percentage uptake with the control culture value (5.5 ± 0.3 pmol/10<sup>5</sup> cells) serving as 100%. The value and standard deviation of the control culture is indicated as the line at the y-axis value 100% and in the shading surrounding this line. Deviation bars indicated represent the standard deviation with n = 6.
higher protein phosphorylation levels within the cell. These cultures showed a similar increase in queuine uptake when compared to those treated with PKC activators, further suggesting that phosphorylation is involved in the regulation of queuine transport.

Parallel time-course studies of rQT₃ uptake with short-term exposure to staurosporine, calphostin C, TPA, calyculin A, and okadaic acid were performed and compared to the rQT₃ uptake rate in control cells at hourly intervals over four hours (Graph 4-2). Treatment of the cells with the PKC inhibitors resulted in a decrease in the uptake rate for rQT₃ as compared to untreated control fibroblast cultures. The PKC activator (TPA) nearly doubled the rate of rQT₃ uptake, while the protein phosphatase inhibitors also stimulated the rQT₃ uptake rate. This further indicates that queuine uptake efficiency is linked to protein phosphorylation levels.

Fibroblast cultures were pre-treated for 7 days with TPA, staurosporine, and okadaic acid in order to measure effects of long-term exposure to these modulators on queuine uptake (Graph 4-3). This is particularly revealing since chronic exposure to TPA results in proteolytic down-regulation of PKC activity (84). As expected, exposures to the PKC inhibitor significantly decreased the uptake of queuine at 3 hours of exposure to rQT₃, while treatment with low levels of the phosphatase inhibitor induced a two-fold increase in rQT₃ uptake. Furthermore, chronic exposure to TPA resulted in an inhibition of rQT₃ uptake rate when compared with untreated control cultures. This is a reversal of the effect observed with short-term exposure to TPA, and supports the PKC modulation scheme for queuine uptake based on the idiosyncrasy of differential effects on PKC with short-term vs. long-term exposure to phorbol esters.

The relatively high concentrations of the protein phosphatase inhibitors used in the short-term exposure study was cause for concern given their reported toxicity levels (142-144). A range of concentrations of calyculin A and okadaic acid were studied to analyze their effects on the 3 hour time point for rQT₃ uptake relative to untreated control fibroblast cultures (Graph 4-4). These studies were performed for both short-term (simultaneous exposure to inhibitor and rQT₃) and for chronic exposure to the inhibitors (pre-treatment: 7 days prior to addition of rQT₃).

Concentrations of okadaic acid in excess of 100 nM proved to be cytotoxic for the 3 hour short-term exposure studies. However, increasing concentrations of okadaic acid from 5 to 100 nM demonstrated progressively increasing rQT₃ uptake. Similar trends were observed in studies of chronic exposure to this phosphatase inhibitor. Increasing concentrations of okadaic acid from 1 to 10 nM demonstrated progressively increasing rQT₃ uptake rates, while concentrations over 10 nM were cytotoxic to the fibroblast cultures. Comparable results were seen with studies using the protein phosphatase inhibitor calyculin A. However, the fibroblast cultures were much more sensitive to this compound. Exposure for 3 hours to 1 nM calyculin A effectively increased the rate of rQT₃ uptake. For chronic exposures (7 day pre-treatment), 0.2 nM calyculin A demonstrated equivalent maximal induction of rQT₃ uptake rate. Concentrations above these levels were cytotoxic in both short-term and chronic exposure studies, respectively.
Graph 4-2. The Effect of Short-Term Exposure to Protein Kinase C and Protein Phosphatase Modulators on Queuine Uptake in Human Fibroblasts. The graph on the left displays the uptake of rQT$_1$ during short-term exposure to a protein kinase C activator and protein phosphatase inhibitors. The bottom line (+) represents rQT$_1$ uptake in untreated control cultures, with the upper lines representing cultures treated with 10 nM okadaic acid (○), 0.2 nM calyculin A (●) and 20 nM TPA (□), respectively. The graph on the right illustrates the uptake of rQT$_1$ during short-term exposure to protein kinase C inhibitors. The top line (+) represents rQT$_1$ uptake in untreated control cultures, with the lower lines representing cultures exposed to 100 nM staurosporine (■) and 100 nM calphostin C (●), respectively. In these short-term exposure studies, modulators were added to the cultures at time 0. The uptake of the radiolabeled queuine in all cultures was measured after a 37°C incubation with 100 nM rQT$_1$ at 0, 1, 2, 3, and 4 hours. These values were then normalized to the number of pmol rQT$_1$ taken up by $10^5$ cells. Deviation bars indicated represent the standard deviation with $n = 4$. 
Graph 4-3. The Effect of Long-Term Exposure to Protein Kinase C and Protein Phosphatase Modulators on Queuine Uptake in Human Fibroblasts. This graph displays the uptake of rQT$_3$ after a week of exposure to a protein kinase C activator and inhibitor, and a protein phosphatase inhibitor. The solid line (+) represents rQT$_3$ uptake in untreated control cultures, with the other lines representing cultures treated with 20 nM TPA (□), 1 nM okadaic acid (◇), and 100 nM staurosporine (■), respectively. In these long-term exposure studies, cultures were pre-incubated with the modulators for 7 days before the initiation of the uptake assay with the addition of the rQT$_3$ at time 0. The uptake of the radiolabeled queuine in all cultures was measured after a 37°C incubation with 100 nM rQT$_3$ at 0, 1, 2, 3, and 4 hours. These values were then normalized to the number of pmol rQT$_3$ taken up by 10$^5$ cells. Deviation bars indicated represent the standard deviation with $n$ =4.
Graph 4-4. The Effect of Short-Term and Long-Term Exposure to Protein Phosphatase Inhibitors on Queuine Uptake in Human Fibroblasts. These bar graphs represent the effects of exposure of varying concentrations of okadaic acid (top graphs) and calyculin A (bottom graphs) on rQT$_3$ uptake in cultures. Short-term exposure studies (those graphs displayed on the left) were conducted with simultaneous initiation of the experiment and exposure of the cells to the modulator at time 0. Long-term exposure studies (graphs on the right) were conducted after pre-treatment of the cultures for 7 days before initiation of the experiment. The uptake of the radiolabeled queuine in all cultures ($4 \times 10^5$ cells) was measured after a three hour incubation with 100 nM rQT$_3$ at 37°C. These values were normalized to the number of pmol rQT$_3$ taken up by $10^5$ cells, and converted to percentage uptake with the control culture value ($5.5 \pm 0.3$ pmol/10$^5$ cells) serving as 100%. Deviation bars indicated represent the standard deviation with $n = 6$. 
It has been reported that calyculin A is a more effective inhibitor of protein phosphatase I than okadaic acid. In one study, IC₅₀ values of approximately 50 nM for okadaic acid and 0.3 nM for calyculin A were reported for muscle phosphatase I in studies of long-term exposure to the agents (142). Based on the reported sensitivity of protein phosphatases type I and II to okadaic acid and calyculin A with pretreatment exposure (142-144), the strong similarity of the data obtained in this study (Graph 4-4) to this reported characteristic suggests that protein phosphatases of the type I group are participating in the modulation of rQT₃ uptake in human fibroblasts.

**Regulation of Queuine Incorporation into tRNA**

Although the rate of uptake can be modulated, it was observed that maximal uptake of the queuine base to a saturating level occurs in six hours. After this point, uptake and efflux are balanced (30). It is suggested that the modulation of the uptake mechanism might not be solely responsible for the dramatic queuosine-modification deficiency seen in neoplastic transformation and progression of cancer cells to metastasis. Thus, the queuine incorporation mechanism (TGRase) was studied to determine if PKC acts in a dual role in modulating both components of the queuine modification system.

First, cultured human fibroblasts (passages 3-5) were exposed to dibutyryl and 8-bromo analogs of cAMP and cGMP, as well as TPA, PDD, calphostin C, sphingosine, staurosporine, H-7, okadaic acid or calyculin A for 8 hours in order to determine effects on the incorporation of rQT₃ into tRNA by TGRase (Graph 4-5). The queuine incorporation rates of cells exposed to these modulators were compared to an untreated control culture and three additional control (positive and negative) cultures. These additional control fibroblast cultures were utilized to establish both the maximum and minimum incorporation limits for comparison to the experimental samples. Queuosine-deficient tRNA cultures (cells treated with charcoal-stripped media for three passages) were used as substrate targets for demonstrating the maximal incorporation rate of rQT₃ into tRNA by TGRase (positive control). These cells demonstrated a nearly two-fold increase in rQT₃ incorporation rate when compared to normal serum-supplemented controls. The slower rate of incorporation in serum-supplemented control cells is likely due to partial queuosine-modification of the existing tRNA population in the cell. Two negative control cultures were grown in 10% serum-supplemented media. One culture was treated with additional 100 nM queuine and the other was treated with 10 μM 7-methylguanine. Addition of excess queuine completely and irreversibly saturates the cell's tRNA with queuosine and eliminates the cell's tRNA as a substrate for rQT₃ incorporation. 7-methylguanine is a competitive inhibitor of TGRase and blocks the ability of TGRase to incorporate rQT₃ into tRNA at concentrations used here (76). These two cultures represent the background levels of non-specific rQT₃ association to the acid precipitable fraction of solubilized cell cultures, which is expected in the absence of TGRase activity.

Cell cultures treated with agents that increase the activity of cAMP and cGMP dependent kinases appear to have no effect on the incorporation rate of rQT₃ (Graph 4-5). However, PKC activators and
phosphatase inhibitors increase the incorporation of rQT\textsubscript{3} toward levels observed in totally queuosine-deficient cells, while cells treated with inhibitors of PKC reduce the incorporation of rQT\textsubscript{3} to a base-line level just above that of the negative control cultures. These results demonstrate that PKC-catalyzed phosphorylation is important in regulation of TGRase activity, thus the formation of the queuosine modification of tRNA.

A 12 hour time-course for rQT\textsubscript{3} incorporation into tRNA of queuosine-deficient tRNA fibroblast cultures was performed in cells grown for three passages in 10% charcoal-stripped serum (queuine-free) supplemented media (Graph 4-6). In short-term exposure studies, rQT\textsubscript{3} and various modulating agents (staurosporine, sphingosine, calphostin C, TPA, okadaic acid and calyculin A) were simultaneously added to parallel cultures. Then, these were analyzed for rQT\textsubscript{3} incorporation into tRNA at four-hour intervals for 12 hours. Inhibitors of PKC decreased the rate of rQT\textsubscript{3} incorporation into tRNA to a base-line level as compared to untreated controls. The protein kinase activator and phosphatase inhibitors, when added to cell cultures at the same time as rQT\textsubscript{3}, both increased the rQT\textsubscript{3} incorporation rates when compared to untreated control cells.

In long-term exposure studies, queuosine-deficient cells pre-treated with various agents for 7 days were studied for rQT\textsubscript{3} incorporation rates into tRNA over a 12 hour time-course (Graph 4-7). Chronic exposure to TPA, which induces a proteolytic down-regulation of PKC (84), resulted in a decrease in rQT\textsubscript{3} incorporation into tRNA. However, treatment with staurosporine was more effective at inhibiting the rate of rQT\textsubscript{3} incorporation in cell cultures. It is both possible and likely that residual PKC activity may still be present in the cells chronically exposed to TPA. In addition, chronic pre-treatment of these cells for 7 days with okadaic acid showed rQT\textsubscript{3} incorporation levels lower than that of the control. Since this treatment maintains the phosphorylation level in the cell, the activity of TGRase should be enhanced during this preparative step and TGRase would effectively recycle queuine from tRNA turnover and incorporate it into new transcripts of tRNA. At the time of the rQT\textsubscript{3} addition, the cognate tRNA would be saturated with the queuosine modification and would no longer be used as a substrate for incoming rQT\textsubscript{3} due to the irreversible nature of the queuine incorporation reaction. Therefore, chronic exposure to okadaic acid should decrease the rate of rQT\textsubscript{3} incorporation into tRNA as compared to control cultures, and this is indeed the case.

**Regulation of Queuosine Salvage**

The final step of the queuosine modification system, salvage of the queuine base from queuosine-5'’-monophosphate (Q5P), was studied with regard to its ability to be modulated by PKC. The in vivo salvage assay method, described in Chapters 2 and 3, was modified to integrate phosphorylation modulator treatment into the standard assay much as was done for the in vivo uptake and incorporation assays (Graph 4-8). Cells were grown to near confluence, then a media change to charcoal-stripped (queuine-free) media for one day or two weeks was performed to induce any queuine salvage mechanism prior to the beginning.
Graph 4-5. The Effect of Protein Kinase and Protein Phosphatase Modulators on Queuine Incorporation into tRNA in Human Fibroblasts. Each bar represents rQT, incorporation into the tRNA of cultures exposed to various kinase and phosphatase modulators for eight hours at concentrations of 10 μM dibutyryl-cGMP, 8-bromo-cGMP, dibutyryl-cAMP and 8-bromo-cAMP, 20 nM TPA and PDD, and 10 nM okadaic acid, 0.2 nM calyculin A, 100 nM calphostin C, 20 μM sphingosine, 100 nM staurosporine and 10 μM H-7. The incorporation of the radiolabelled queuine into the tRNA of all cultures (4 x 10^5 cells) was measured after an eight hour incubation with 100 nM rQT, and modulators at 37°C. These values were normalized to the number of pmol rQT, incorporated into the acid-precipitable fraction of 10^5 cells. An untreated control represents rQT, incorporation into tRNA of untreated “normal” cultures (measured at 3.0 ± 0.2 pmol/10^5 cells), and was the basis for the normalization of experimental cultures’ values to percentage uptake with the untreated control culture value serving as 100%. The value and standard deviation of the control culture is indicated as the line at the y-axis value 100% and in the shading surrounding this line. In addition, negative control cultures for rQT, incorporation in cells treated with 10 μM 7-methylguanine, a competitive inhibitor for the queuine incorporation enzyme, and cultures of queuine-saturated cells (preincubated with 0.10 A_260 queuine for three passages) enabled the minimum amount of rQT, for a null incorporation baseline, which measure at 9 ± 3% and 5 ± 2%, respectively. These are shown in the line and shading at the y-axis value near zero. Finally, a sample of queuine-deficient cells that were preincubated with charcoal-stripped media for three passages established the maximum amount of rQT, able to be incorporated measured at 190 ± 10% (data not shown). Deviation bars indicated represent the standard deviation with n = 6.
Graph 4-6. The Effect of Short-Term Exposure to Protein Kinase C and Protein Phosphatase Modulators on Queuine Incorporation into tRNA of Human Fibroblasts. The graph on the left displays the incorporation of rQT₃ into acid-precipitable fractions of cultures during short-term exposure to a protein kinase C activator and protein phosphatase inhibitors. The bottom line (+) represents rQT₃ incorporation in untreated control cultures, with the upper lines representing cultures treated with 10 nM okadaic acid (△), 20 nM TPA (□) and 0.2 nM calyculin A (○), respectively. The graph on the right illustrates the incorporation of rQT₃ into acid-precipitable fractions of cultures during short-term exposure to protein kinase C inhibitors. The top line (+) represents rQT₃ incorporation in untreated control cultures, with the lower lines representing cultures exposed to 100 nM calphostin C (●), 100 nM staurosporine (■), and 20 μM sphingosine (♦), respectively. In these short-term exposure studies, modulators were added to the cultures at time 0. The incorporation of the radiolabeled queuine into tRNA in all cultures was measured after a 37°C incubation with 100 nM rQT₃ at 0, 3, 6, 9, and 12 hours. These values were then normalized to the number of pmol rQT₃ incorporated into the acid-precipitable fraction of 10⁵ cells. Deviation bars indicated represent the standard deviation with n =4.
Graph 4-7. The Effect of Long-Term Exposure to Protein Kinase C and Protein Phosphatase Modulators on Queuine Incorporation into tRNA of Human Fibroblasts. This graph displays the incorporation of rQT₃ into the acid-precipitable fraction of cultures after a week of exposure to a protein kinase C activator and inhibitor, and a protein phosphatase inhibitor. The solid line (+) represents rQT₃ incorporation in untreated control cultures, with the other lines representing cultures treated with 20 nM TPA (□), 1 nM okadaic acid (◇), and 100 nM staurosporine (■), respectively. In these long-term exposure studies, cultures were pre-incubated with the modulators for 7 days before the initiation of the uptake assay with the addition of the rQT₃ at time 0. The incorporation of the radiolabeled queuine into the acid-precipitable fraction of all cultures was measured after a 37°C incubation with 100 nM rQT₃ at 0, 3, 6, 9, and 12 hours. These values were then normalized to the number of pmol rQT₃ incorporated into the acid-precipitable fraction of 10⁵ cells. Deviation bars indicated represent the standard deviation with n =4.
Graph 4-8. The Effect of Short-Term Exposure to Phosphorylation Modulators on Queuine Salvage in Human Fibroblasts. Cultures of human fibroblast cells (HFF) were grown to near confluence in normal media, fed with charcoal-stripped serum (lacking queuine) for 1 day or 2 weeks with regular media changes, then submitted to an in vivo queuine salvage assay with the addition of phosphorylation modulators. A tritirradiated analogue of queuine (rQT$_3$) at a concentration of 100 nM was placed in the media 24 hours before the start of the assay to saturate the tRNA with the radiolabeled base. In two sets of parallel cultures at time zero, extracellular rQT$_3$ was washed away, then a media change with charcoal-stripped media was performed with the addition of 20 nM TPA (a protein kinase C stimulator), 1 nM okadaic acid (a protein phosphatase inhibitor), and 100 nM staurosporine (a protein kinase C inhibitor). In one set of cultures 0.10 A$_{260}$ units of unlabeled queuine was added (black bars) indicating the tRNA turnover rate within the cells. No queuine was added to the other set of cultures (light grey bars) which represent the ability of the cells to salvage and retain the radiolabel in the cell's tRNA. The counts per minute values obtained via liquid scintillation were normalized to the number of pmol rQT$_3$ contained within the acid-precipitable fraction of the $10^5$ cells that had been devoid of queuine for 1 day or 2 weeks. Deviation bars indicated represent the standard deviation with $n = 4$. 

Incorporated rQT$_3$ (pmol/10$^5$cells)
of the study. In short-term exposure studies, addition of TPA, staurosporine, H-7 or okadaic acid at the
start of the assay produced no dramatic effect on the maintenance of rQT<sub>1</sub> levels in the acid-precipitable
fraction of the human fibroblast cells over the four days of the study. The same was observed for both sets
of cultures that had been subjected to one day or two weeks of queuine starvation. Thus, it is suggested
that phosphorylation by PKC is not a regulating factor in the control of the salvage step of the queuosine
modification system.

Direct Action of Protein Kinase C on the Queuine Incorporation Enzyme In Vitro

In order to verify the results of the in vivo assay results with regard to the incorporation step of the
queuosine modification system, TGRase was isolated from rat liver as described in Chapter 3 and tested for
modulation of enzymatic activity and the ability to be directly phosphorylated by PKC. Early evidence of
the influence of PKC on TGRase activity was shown with an induction of the queuine incorporation
activity in cold-stored TGRase samples (see Chapter 3, Graph 3-3). In combined TGRase and PKC
enzyme assays on the P-11 phosphocellulose fraction of a rat liver preparation an increase was observed in
the activity of cold stored TGRase (Graph 4-9). Both partially active and totally inactive TGRase samples
were restored to their original P-11 phosphocellulose isolate activity by exposure to activated PKC. The
greater-than-100% return of activity seen in one isolate is suggested to reflect a loss of TGRase activity
during the isolation protocol from the original homogenate to the phosphocellulose fraction.

Purified TGRase preparations after the Mono Q FPLC step were subjected to a PKC
phosphorylation assays to determine the ability of the TGRase enzyme to serve as a substrate for PKC-
catalyzed phosphorylation. The PKC was able to incorporate <sup>32</sup>P from γ<sup>32</sup>P-ATP into the acid precipitable
component of the TGRase preparation derived from Mono-Q column, and at levels greater than that for
histones in the positive control sample (Graph 4-10). The phosphorylation of TGRase by PKC is
corroborated by the SDS-PAGE/autoradiograph discussed in Chapter 3 (Figure 3-3), where the larger of
the two subunits (approximately 60 kDa) appeared to be radiolabeled with <sup>32</sup>P by PKC.

In control experiments, the direct effect of PKC modulators on TGRase activity, PKC
phosphorylation of TGRase, and TGRase activity in the presence of PKC were tested. TGRase
preparations were assayed for <sup>3</sup>H-guanine incorporation into yeast tRNA in the presence of PKC activators
diacylglycerol, phosphatidyl serine, and CaCl<sub>2</sub>, as well as ATP) and PKC inhibitors (H-7, sphingosine, or
staurosporine). None of these agents directly effected the TGRase activity (Graph 4-11). The methylated
purine, 7-methylguanine, a known competitive inhibitor of TGRase demonstrated strong inhibitory effects
and served as the inhibited control for this assay. Direct phosphorylation of TGRase by PKC was
measured in the presence of PKC activators and PKC inhibitors. As expected, the phosphorylation of
TGRase by PKC was induced by the presence of PKC activators (diacylglycerol, phosphatidyl serine and
calcium) and dramatically inhibited by H-7, sphingosine and staurosporine (Graph 4-12). This inhibition
was observed even in the presence of the activators. The PKC preparations demonstrated no evidence of
Graph 4-9. *The Decay of Two Samples of Purified tRNA:Guanine Ribosyltransferase Activity and Reactivation by Protein Kinase C.* These graphs indicate the decay of residual TGRase activity in two different rat liver Mono Q-FPLC preparations and recovery of activity with the addition of protein kinase C. Five micrograms of purified protein isolate were analyzed using the standard TGRase activity assay to measure the ability to incorporate $^3$H-guanine into 0.10 $A_{260}$ units of yeast tRNA in one hour at 37°C (solid bars). Assay of these same samples by the combined TGRase and PKC activity assay was also performed under the same reaction conditions (hashed bars). Standard deviation values are indicated for each sample with $n = 4$. 
Graph 4-10. The Phosphorylation of Histones and tRNA:Guanine Ribosyltransferase by Protein Kinase C. The control bars of histones and tRNA:guanine ribosyltransferase (TGRase) only indicate the non-specific binding of [γ-32P]ATP and any contaminating kinase activity in 5.0 μg histone fraction IV and TGRase samples, respectively. The protein kinase C (PKC) bar indicates the background levels of [γ-32P]ATP binding to 10 μU PKC with no histone or TGRase substrate added. The remaining bars indicate the levels of 10 μU PKC-catalyzed phosphorylation of 5.0 μg histone and TGRase, respectively. All assays were performed by the combined TGRase and PKC activity assay method. Standard deviation values are indicated for each sample with \( n = 4 \).
Graph 4-11. The Effect of Protein Kinase C Modulators on the Activity of tRNA:Guanine Ribosyltransferase. The bar representing "no tRNA" indicates background levels of $^3$H-guanine adhering to protein within the tRNA:guanine ribosyltransferase (TGRase) sample (no tRNA substrate added). "TGRase only" indicates the TGRase activity level of the 5.0 μg protein sample with the addition of 0.10 A$_{260}$ units yeast tRNA substrate. Another negative control for the TGRase activity is shown in the "TGRase + 7mG" bar, with the TGRase activity in the presence of 10 μM 7-methylguanosine (7mG), a competitive inhibitor for TGRase. "TGRase + PKC act." indicates the TGRase activity in the presence of traditional protein kinase C (PKC) activators activators (1 mM calcium chloride, 5.0 μg diacylglycerol, and 5.0 μg phosphatidyl serine). Adenosine triphosphate (ATP), an important substrate for PKC, is also required for catalytic function of the phosphorylating enzyme PKC. So, the activity of TGRase in the presence of 10 μM ATP, and with PKC activators and ATP was assessed. Finally, the activity of TGRase in the presence of three different PKC inhibitors (50 μM H-7, 100 nM staurosporine and 25 μM sphingosine) was measured in the presence of calcium chloride, diacylglycerol, phosphatidyl serine and ATP. All assays were performed using the standard TGRase activity assay method. Standard deviation values are indicated for each sample with $n = 4$. 

![Graph showing the effect of protein kinase C modulators on TGRase activity.](image-url)
Graph 4-12. The Effect of Protein Kinase C Modulators on the Phosphorylation of tRNA:Guanine Ribosyltransferase by Protein Kinase C. The "PKC only" bar indicates the background level of $[\gamma-^{32}P]$ATP adherence to 10 µU protein kinase C (PKC) sample with no substrate added, but including the traditional PKC activators 1 mM calcium chloride, 5.0 µg diacylglycerol, and 5.0 µg phosphatidyl serine. The "TGRase only" bar indicates the presence of contaminating kinase activity in the tRNA:guanine ribosyltransferase (TGRase) sample, by assaying the TGRase sample without the presence of PKC. The "PKC + TGRase" indicates the incorporation of the radiolabeled phosphate into 5.0 µg TGRase by 10 µU PKC. The remaining bars indicate the ability of PKC to phosphorylate TGRase with the addition of three PKC inhibitors (50 µM H-7, 100 nM staurosporine and 25 µM sphingosine). All assays were performed using the standard TGRase activity assay method. Standard deviation values are indicated for each sample with $n \approx 4$. 
latent TGRase activity and the TGRase preparations exhibited no evidence of PKC activity. Finally, in assays that measured the effect of the presence of PKC on TGRase activity, \(^3\)H-guanine incorporation was increased when exposed to activated PKC as seen before (Graph 4-13). However, inhibitors of TGRase (7-methylguanine—negative control) and PKC (H-7, staurosporine, and sphingosine) all appeared to reduce the activity of PKC-activated TGRase to background. Thus, the effect of the PKC on \(^3\)H-guanine incorporation into tRNA is suggested to be due to direct phosphorylation causing activation of the TGRase enzyme.

Combined assays were run with TGRase, PKC and alkaline phosphatase (AP) (Graph 4-14). The results of TGRase activity assays with the presence of PKC and an antagonist phosphatase (AP) verify the phosphorylation-modulation that has been suggested so far. Again, the TGRase activity was greatly enhanced by the presence of PKC. This induction was reversed upon the addition of AP. In fact, baseline TGRase activity was decreased by the presence of the phosphatase enzyme. This suggests that during the isolation procedures there is some basal level of phosphorylation maintained in the isolated TGRase-active fractions.

\(^3\)P-labeled and unlabelled P-11 and Mono-Q TGRase fractions were analyzed by denaturing and non-denaturing PAGE as described in Chapter 3. To summarize, the mammalian liver TGRase enzyme is likely a dimer with a 60 kDa subunit that appears to be phosphorylated at a serine residue by PKC and a 34.5 kDa subunit that appears to contain the active site for the enzyme. Superose 6 FPLC analysis of weakly TGRase-active and strongly TGRase-active Mono Q fractions verified the composition suggested by SDS- and native-PAGE. TGRase assays were performed on the rat liver Mono Q fractions with and without the addition of PKC to assess the effect of the kinase on the dimer versus the active subunit monomer form of the TGRase enzyme (Figure 4-2). The fraction containing primarily the 34.5 kDa subunit exhibited almost three-fold greater TGRase activity than the dimer fraction without the addition of PKC, and did not appeared to be effected by it’s addition. The weak TGRase-active fraction, containing primarily the dimer form of the enzyme, showed a near tripling of it’s TGRase activity with the addition of PKC which increased it’s specific activity to that of the other fraction and both within the ballpark of that reported in the isolation protocols for the purified rat liver TGRase enzyme.

**Discussion**

The uptake of queuine into "normal" cultured human fibroblasts appears to be modulated by phosphorylation. Activators of cAMP and cGMP dependent kinases have no effect on \(rQT_3\) uptake, while increasing PKC activity with phorbol ester treatment elevates the rate of \(rQT_3\) uptake. Conversely, chronic exposure of TPA to fibroblast cultures, which is known to down-regulate PKC activity, decreases \(rQT_3\) uptake. A spectrum of protein kinase inhibitors, with various specificities to PKC, all inhibit \(rQT_3\) uptake into fibroblast cultures in concentration ranges reported to be most specific for PKC. These results make a strong case for the role of PKC in enhancing the uptake rate of queuine into fibroblast cultures.
Graph 4-13. The Effect of Protein Kinase C Modulators on tRNA:Guanine Ribosyltransferase Activity in the Presence of Protein Kinase C. The “no tRNA” bar indicates background levels of $^3$H-guanine that adheres to protein in a combined tRNA:guanine ribosyltransferase (5.0 µg TGRase) and protein kinase C (10 µU PKC) reaction mixture with no tRNA substrate added. The “TGRase only” bar indicates the TGRase activity level of the TGRase sample (5.0 µg) in the presence of 10 µU PKC, but without the addition of PKC activators (1 mM calcium chloride, 5.0 µg diacylglycerol, 5.0 µg phosphatidyl serine, and 10 µM ATP). “PKC only” represents a negative control for TGRase activity in the reaction mixture containing 10 µU PKC, PKC activators and TGRase assay reagents, but without the TGRase sample. The “TGRase + PKC” bar indicates the TGRase activity of the sample in the presence of PKC and PKC activators. Another negative control shows the “TGRase + PKC” reaction mixture’s activity in the presence of 10 µM 7-methylguanine, a competitive inhibitor for TGRase. The remaining bars represent the activity of TGRase in the presence of both PKC, PKC activators, and three PKC inhibitors H-7 (50 µM), staurosporine (100 nM) and sphingosine (25 µM), respectively. All assays were performed using the standard TGRase activity assay method. Standard deviation values are indicated for each sample with $n=4$. 
Graph 4-14. *The Modulation of Purified tRNA:Guanine Ribosyltransferase Activity by Phosphorylation.* The "no tRNA" bar indicates the non-specific binding of $^3$H-guanine to protein within the 5.0 µg tRNA:guanine ribosyltransferase (TGRase) sample (no tRNA was added). The bar identified as TGRase only indicates the residual TGRase activity in this sample. "TGRase + AP" indicates the TGRase activity with the addition of 0.40 mU alkaline phosphatase (AP), a general phosphatase. "TGRase + PKC" indicates the sample's TGRase activity with the addition of 10 µU protein kinase (PKC). The remaining bar indicates the TGRase activity present with the addition of competing PKC (10 µU) and AP (0.40 mU) enzymes.
Figure 4-2. Superose 6 FPLC Absorbance Profiles and Results of Assays Measuring MonoQ FPLC Fractions' tRNA:Guanine Ribosyltransferase Activity With and Without Exposure to Protein Kinase C. The two MonoQ-FPLC fractions, corresponding to the Superose 6 FPLC profiles shown in Figure 3-3, were examined for tRNA:guanine ribosyltransferase (TGRase) activity. Parallel assays were run using either the traditional *in vitro* TGRase assay method with or without the addition of 10 μU protein kinase C (PKC) and its activators (1 mM calcium chloride, 5.0 μg diacylglycerol and 5.0 μg phosphatidyl serine). Negative controls for TGRase activity in the PKC and the PKC activator samples indicated no contaminating activity (data not shown). *Specific activity for each protein sample was calculated based on the number of pmol of H-guanine incorporated into 0.10 A₂₆₀ units yeast tRNA per mg protein per hour at 37°C. The star on the FPLC profile over the peak, estimated at 66 kDa, indicates the corresponding size subunit identified by autoradiograph (Figure 3-2) as containing the phosphorylation site for PKC attachment of a phosphate.*
The inhibition of protein phosphatases by both okadaic acid and calyculin A also induces an increase in the uptake of rQT₃. These results indicate that maintenance of protein phosphorylation is important to maximize the rate of queuine uptake. Due to the concentrations of the two phosphatase inhibitors involved, it is suggested that protein phosphatases of type I are down-regulating queuine uptake activity by removing a phosphate group from the queuine-specific membrane transporter. This analysis is based on reported sensitivity of protein phosphatases type I and II to okadaic acid and calyculin A (142-144).

The incorporation of queuine into tRNA in cultured human fibroblasts also appears to be modulated by PKC and protein phosphatase activity. Activators of cAMP and cGMP dependent kinases have no effect on rQT₃ incorporation. However, activators of PKC and inhibitors of protein phosphatase both increase TGRase incorporation rates above a base-line incorporation level, while inhibitors of PKC decrease incorporation rates of rQT₃ to the base-line level. Chronic exposure to TPA, which is known to induce a proteolytic down-regulation of PKC, also decreases rQT₃ incorporation into fibroblast tRNA when compared to untreated controls. These results make a strong case for the role of PKC in enhancing the incorporation rate of queuine into tRNA in fibroblast cultures.

The TGRase enzyme, which is responsible for the production of the queuosine modification, has been under scrutiny since its discovery in 1973. However, most published reports document only partial purifications of the enzyme and limited storage half-lives (44, 115). Over the past thirteen years, TGRase isolated from rat, rabbit, and human tissues has always shown instability and loss of assayable activity with short term storage (three days to four weeks). The results of these *in vitro* studies suggest that PKC activity is essential in maintaining the activity of TGRase in addition to activation of the cellular uptake mechanism for it's substrate, queuine. Several stored TGRase preparations isolated from rat liver with low levels of activity all have been shown to be fully reactivated upon exposure to PKC. Inactivation of PKC in combined TGRase and PKC assays with H-7, staurosporine or sphingosine resulted in a large decrease in the activity level of TGRase as compared with the PKC activated control. Neither the PKC inhibitors nor the PKC activators exerted effects on TGRase directly. In addition, several ³²P incorporation assays were run demonstrating that PKC appears to be directly phosphorylating TGRase.

It is proposed that the TGRase enzyme exists as a heterodimer of approximately 104 kDa, with a PKC-substrate regulatory subunit of 60 kDa and a catalytic subunit of 34.5 kDa. The subunit composition of the fully active TGRase protein is still under debate. The 34.5 kDa subunit has been shown to contain significant TGRase activity, but it is not activated by PKC. A preparation containing primarily the dimer form exhibits very weak TGRase activity which is dramatically increased by the addition of functional PKC. It has been suggested that PKC phosphorylates the large subunit which induces dissociation of the dimer and releases the 34.5 kDa catalytic subunit which would then be free to perform it's apparent queuine-insertion activity into tRNA (126). However, Slany and Langgut have suggested that the dimer form is the active form an that phosphorylation just alters the conformation of the dimer to induce it's
activity (125).

Despite the strong circumstantial evidence that both queuine uptake and incorporation are regulated by PKC, the mechanism catalyzing the salvage of intracellular queuine does not appear to be effected by PKC.

Revised Description of the Mammalian Queuosine Modification System

It is proposed that PKC and protein phosphatase are important modulators of both queuine uptake and incorporation into tRNA in “normal” mammalian cells. These are likely to be independently regulated sites for PKC due to 1) the ability of the uptake mechanism to fully saturate the cell with queuine in six hours despite down-regulation by dephosphorylation, 2) the observation of the modulation of TGRase activity up to 24 hours in cultured cells, and 3) in vitro evidence that shows direct phosphorylation and regulation of rat liver TGRase activity by PKC.

When fully integrated into the description of the queuosine modification system in “normal” mammalian cells, the ability to be regulated by PKC adds another layer of functionality to the “big picture” of queuine metabolism (Figure 4-3). This regulatory enzyme is strongly connected to many other cellular and extracellular processes that serve to dramatically shape the metabolism and eventually the function of a cell. Any agent or treatment regimen that could lead to a down-regulation in PKC activity, such as chronic exposure to phorbol-ester tumor promoters, would have an inhibitory effect on both TGRase activity and queuine uptake into the cell leading to queuine-hypomodified tRNA.

In vivo cell culture experiments with human fibroblasts and in vitro assays with purified rat liver TGRase suggest that control of TGRase activity by phosphorylation may be a universal mammalian phenomenon. Due to organismal structure (multicellular and differentiated nature of eukaryotic organisms), different regulatory design schemes have been evolutionarily developed for eukaryotes and prokaryotes. In the well known E. coli (prokaryotic) model, the formation of queuosine is transcriptionally regulated by the FIS protein (114). There is no known PKC-like activity in prokaryotes and only vaguely similar PKC-like activities in yeast which are early evolutionarily transitional eukaryotes.

Additionally, as mentioned in Chapter 3, the TGRase and TGTase enzymes are composed of widely divergent physical compositions. It is reasonable to suggest that these two enzymes developed from different origins, with different substrates (preQ, for the prokaryotic system and Q for the eukaryotic system). Thus, the TGRase and TGTase enzymes are very likely derived from different genes and may have only a superficial connection through the production of what is a universally conserved highly modified nucleoside. Even the design of the two systems is based on different processes, the prokaryotic version is a synthetic mechanism that builds Q from intracellular compounds while the eukaryotic version is a utilization mechanism that imports and salvages an exogenous compound. The evolutionary development of two independent but convergent systems seems to indicate that the queuosine modification has great physiological importance for it’s existence, in that both the prokaryotic and eukaryotic kingdoms
Figure 4-3. The Revised Eukaryotic Queuosine Modification System in Normal Human Fibroblast Cells. The eukaryotic system is based on usage of an available resource as described in Figure 3-4. It involves an uptake step facilitated by a specific queuosine transmembrane transporter, tRNA:guanine ribosyltransferase which catalyzes the incorporation reaction of q into tRNA^mn, tRNA^sp, tRNA^bs, or tRNA^p, and a salvage step to recycle free queuosine base for the incorporation step from queuosine-5'-monophosphate. The rates of both the uptake and incorporation steps are shown to be stimulated by direct phosphorylation by protein kinase C (PKC), while preliminary studies of the salvage step do not appear to be effected by PKC or a PKC regulated modulation system.
have independently developed mechanisms for creating this nucleoside.

Cellular and Clinical Ramifications of Protein Kinase C Regulation of the Queuosine Modification System

Protein kinase-protein phosphatase reciprocal control systems are important components of the cell's signal transduction system, and are known to be critical components involved with differentiation, control of cell growth and neoplastic transformation (see Chapter 1). The results of the queuosine modification system modulation studies tie an important signal transduction network to mechanisms responsible for tRNA modification with queuine. Alterations in the signal transduction system can effect both the availability of queuine base and the activity of TGRase which can potentially lead to the queuosine-hypomodification of tRNA commonly observed in neoplastically transformed and undifferentiated cells. In addition to the effect that the shift in tRNA modification might on it's function in protein translation, queuosine deficiencies could be used as an indication of cellular metabolic changes that often lead toward neoplastic transformation and progression toward metastatic cancer.
CHAPTER FIVE

DEFICIENCIES OF THE QUEUOSINE MODIFICATION SYSTEM

Introduction

In normal adult human cells the tRNA population is known to be fully saturated with the queuosine modification (60). However, neoplastic cell-lines derived from human biopsies exhibit profound deficiencies in the levels of queuosine. The deficiencies vary in their degree of hypomodification, as seen in such cell-lines as T-84 colon carcinoma (17% deficient), MCF-7 breast adenocarcinoma (50 to 60% deficient), HL-60 promyelocytic leukemia (60 to 80% deficient), and HxGC3 colon adenocarcinoma (100% deficient) (45,87). The extent of this hypomodification may be exaggerated in successive passages by the conditions of tissue culturing, however this appears not to be an artifact of the method due to similar observed deficiencies in primary tissue biopsies.

There have been several published reports utilizing human biopsied tissue in order to determine the extent of the queuosine deficiency in tumors. In one study, breast adenocarcinomas were tested at 3% deficiency in intraductal and invasive cancer, progressing to 8% deficiency in metastatic invasive tumors, to 20% deficiency in a metastasis to the neck (87). Examination of ovarian tumors demonstrated an even greater decline in queuosine levels; 12% deficiencies in stage I cancers, progressing to 37% in stage III, and finally to 63% queuosine hypomodification in stage IV and metastatic ovarian cancer (42). Additionally, brain tumor biopsies were tested for their queuosine modification levels and demonstrated a similar trend of 29.2% deficiency in meningiomas progressing to 66.9% in metastatic astrocytomas (43). While diagnostic procedures have been developed based on queuosine deficiencies in leukemias and lymphomas (40), lung cancers (41), and ovarian tumors (42), it is still not known if the breakdown of a single component or multiple steps of the queuosine modification system are the cause of the deficiencies. In fact, only one published report regarding the examination of the incorporation step in a neoplastic human cell-line has yet come to light.

Studies characterizing the queuosine modification system have been performed in “normal” human cell culture experiments and with enzymes isolated from “normal” mammalian liver tissues. Chapter 3 of this treatise describes the three components of the queuosine modification system; transmembrane uptake of the queuine base, cytoplasmic incorporation of this base into tRNA to form the queuosine modification, and salvage of the base from the tRNA turnover product queuosine-5’-monophosphate (QSP). Chapter 4 provides evidence in “normal” cells that the rates of both the uptake and incorporation steps of the queuosine modification system are increased by protein kinase C (PKC)-catalyzed phosphorylation, while it appears that salvage is only minimally effected by PKC, if at all. Thus, the mammalian queuosine modification system has been relatively well described in “normal” fibroblast cells and other mammalian tissues, however the individual components of this system have yet to be
substantially studied in abnormal cells.

The studies presented in this chapter attempt to identify the malfunctioning sites of the queuosine modification system in two human adenocarcinoma cell-lines. As with many cancers, the human colon adenocarcinoma (HxGC3) and human breast adenocarcinoma (MCF-7) cell-lines are both significantly deficient in queuosine modification levels. The HxGC3 cell-line is completely unmodified with respect to this particular modification, while the MCF-7 cell-line is greatly diminished in its population of queuosine-modified tRNAs (50 to 60% deficient). Parallel in vivo uptake, incorporation, salvage, and modulation assays were performed on the colon and breast adenocarcinoma cell-lines and with human foreskin fibroblasts (HFF) as a "normal" reference. Comparisons between the results of the "normal" fibroblast cultures and those of the cancerous cell-lines were made in an attempt to determine the site or sites of the deficiency within the established components of the queuosine modification system.

These results can be used to explain the observed queuosine hypomodified levels found in these cultures. They can also be used to suggest sites for deficiencies in other known queuosine deficient tumors and cell-lines, as well as whether a multiple-site deficiency scheme is necessary to cause the substantial queuosine modification loss that is often seen in progressive neoplasia. Finally, results of the in vivo assay methods will likely give an indication if the cell culture methods utilized in this treatise are a viable and realistic model system for future basic and clinical studies.

Description of the HxGC3 Human Colon Adenocarcinoma Cell-line

In 1978, the HxGC3 cell-line was derived from a biopsy of a 61 year old male suffering from colon polyps. The diagnosis of this cancer was described as a poorly-differentiated adenocarcinoma of the transverse colon. The cells were maintained as xenografts in male immune-deprived CBA/lac mice and eventually a cultured cell-line was established (145). In a 1986 article describing phorbol ester modulation of queuine uptake in diploid human fibroblasts, several transformed cell-lines, including the HxGC3 cells, were analyzed for queuine uptake kinetics and their responsiveness to phorbol ester-induced inhibition of uptake (80). In experiments similar to the in vivo uptake assays described in Chapters 3 of this work, the HxGC3 cells were shown to exhibit biphasic kinetics for their uptake mechanism in a similar pattern similar to that observed for the "normal" fibroblast cultures. The low $K_M$ for the HxGC3 cells was determined to be $40 \pm 2 \text{ nM}$ which is similar to $30 \pm 4 \text{ nM}$ for the fibroblasts, while the high $K_M$ was determined to be $440 \pm 60 \text{ nM}$ corresponding to $350 \pm 150 \text{ nM}$ for the fibroblast cultures.

In 1992, the same laboratory examined the HxGC3 cell-line for its ability to produce queuosine-modified tRNAs using an indirect RPC-5 medium pressure liquid chromatographic method for the quantification of queuosine-negative and queuosine-containing tRNAs (87). It was discovered that these cultures were dramatically deficient in queuosine-modified tRNA. However, if the cells were exposed in vivo to a treatment of the DNA demethylating agent 5-azacytidine (5-azaC), a small peak appeared at the same retention time as queuosine-modified tRNA. Since the uptake mechanism for queuine in the HxGC3
cells appeared to be normal, it was suggested that a defect in the incorporation or salvage components of the queuosine modification system might be present. Additionally, it was proposed that the 5-azaC treatment might be inducing the gene expression of an important protein in one of these two systems.

Activity of the HxGC<sub>3</sub> queuine incorporation enzyme was measured after partial purification by cell lysis, homogenization, centrifugation, separation on a DEAE-cellulose column, and simultaneous concentration and desalting of the active fractions with a microconcentrator (87). Finally, an <em>in vitro</em> assay was performed on the concentrate in order to measure its ability to incorporate tritiated-dihydroqueuine into bulk yeast tRNA. The HxGC<sub>3</sub> cell-line was not shown to contain the queuine incorporation activity, nor did the addition of HxGC<sub>3</sub> cell lysate to functional HL-60 TGRase preparations influence this activity. Thus, it was inferred that the HxGC<sub>3</sub> cell-line contained no functional queuine incorporation enzyme. Any <em>in vitro</em> assays that may have been performed on 5-azaC treated cell homogenates were not mentioned.

The salvage mechanism for Q5P was studied in a different method from that of the queuine incorporation enzyme. The HxGC<sub>3</sub> cells were lysed and centrifuged. The samples were added to an <em>in vitro</em> assay that measured the ability of the lysate to catalyze the formation of free tritiated dihydroqueuine base from tritiated QSP. There was concern that phosphatase contamination of the lysate would produce an inactive queuosine substrate from the Q5P in a competing reaction with the salvage enzyme. Therefore, sodium fluoride or α,β-methylene-ADP were added to the reaction mixtures to prevent dephosphorylation of the Q5P substrate. However, this also reduced the reaction to a semi-quantitative method due to the partial inhibition of the salvage mechanism by these compounds. After the completion of the reaction, the mixture was separated by CI<sub>18</sub> high pressure liquid chromatography to detect the presence of tritiated Q5P, queuosine or queuine. A mouse liver homogenate was prepared to serve as a salvage standard and the results of the HxGC<sub>3</sub> lysate reaction were determined as a percentage of the mouse liver salvage. The HxGC<sub>3</sub> cell-line was significantly deficient in both tRNA<sup>60</sup> and tRNA<sup>10</sup> levels and exhibited 18% efficiency in queuine salvage.

Since this cell-line was shown to be entirely deficient in queuosine and also to contain no observable queuine incorporation activity, it was proposed that the incorporation step of the queuosine modification system was malfunctioning in the HxGC<sub>3</sub> cells. The low efficiency of salvage, therefore, would not be a major player in the low queuosine modification levels because the maintenance of intracellular queuine concentrations would not be able to induce the queuosine synthesis to occur without the presence of a functional queuine incorporation enzyme. Since <em>in vivo</em> 5-azaC treatment of the cells produced measurable queuosine-modified tRNA levels, it was also suggested that the incorporation was the component likely effected by 5-azaC induced DNA demethylation and induction of gene expression.

These studies, with the exception of the HxGC<sub>3</sub> <em>in vivo</em> uptake assays, were not utilizing methods developed to measure the queuosine modification system components under near physiological conditions. The assay methods described in this treatise in chapters 2 and 3 may be a better model to examine these mechanisms. The <em>in vivo</em> techniques for assaying uptake, incorporation, salvage, and the PKC-modulated
steps are used to verify that the uptake mechanism is similar to that of "normal" fibroblasts. Additionally, these methods will be used to demonstrate that the incorporation mechanism is compromised in the HxGC₃ cells and is the mechanism that is directly effected by the 5-azaC treatment. *In vivo* salvage assays were not performed in these cells. This decision was made based on the futility of the *in vivo* method for measuring the salvage efficiency of cells which cannot create the modification, nor modify the tRNA at easily measurable levels even when induced by 5-azaC treatment.

*Description of the MCF-7 Human Breast Adenocarcinoma Cell-line*

The MCF-7 cell-line was derived from a pleural effusion excised from a 72 year old female that had previously undergone a radical mastectomy for malignant mammary adenocarcinoma (146). The cells were cultured into a stable cell-line and retained several differentiated characteristics of the mammary epithelium including the ability to express the cytoplasmic estrogen receptor and the capability of forming domes. The cell-line, at passage 138, was donated to the American Type Culture Collection by C.M. McGrath in April 1982. Since this time, the MCF-7 cell-line has become a well established standard for *in vivo* studies of estrogen receptor function and multidrug resistance development. Thus, while existing in many labs it has also been utilized for other studies as an example of a human transformed cell-line, and more specifically as an estrogen-dependent human breast adenocarcinoma model.

No known queuosine modification system studies have yet been performed using the MCF-7 cell-line. Since it is a well known and well studied example of neoplasia, it was selected for study as another transformed cell-line for contrast to or comparison with the HxGC₃ cell-line. The *in vivo* techniques for assaying uptake, incorporation, salvage, and PKC-modulated activity of the first two steps are utilized to describe and assess the three components of the queuosine modification system in these cells. The MCF-7 cell-line has been determined to be 50% to 60% undermodified with respect to queuosine (data not shown). The results of experimentation, described in this chapter, will be used to decipher the source of this deficiency as compared with results of "normal" human fibroblast cultured cells.

**Results**

**Uptake of Queuine**

The HxGC₃ and MCF-7 cell-lines exhibit rQT₃ uptake rates and levels that are comparable to that observed in normal HFF cells (Graph 5-1). Both neoplastic cultures tested demonstrate linear uptake of the radiolabeled queuine analogue through 60 minutes and plateau at approximately 120 minutes. In addition, the maximal quantities of rQT₃ taken up by the three cell-lines are all very close in range (7.4 to 7.8 pmol/10⁵ cells). 5-azaC-treated HxGC₃ cells were also assayed for the ability to take up the rQT₃ and were shown to exhibit the same uptake profile as the untreated HxGC₃ cell cultures. This culture set was studied to ensure that any incorporation differences observed in this cell-line were due solely to the incorporation...
step and not due to uptake insufficiencies.

Incorporation of Queuine into tRNA

It has been reported the HxGC₃ cell-line is deficient in the levels of queuosine-modified tRNA due to transcriptional deactivation of a component of the queuosine modification system (87). In these in vivo studies, despite the proper functioning of the queuine uptake step, direct time-course incorporation assays show only background levels of incorporated rQT₃ in HxGC₃ cells even after 24 hours of exposure to this queuine analogue (Graph 5-2). Treatment of these cells with the DNA methylase inhibitor and transcriptional activator 5-azaC does not appear to effect the uptake of rQT₃, however incorporation of rQT₃ into tRNA is distinguishable above the background as early as six hours after exposure to the rQT₃. The level of rQT₃-modified tRNA elevates to 4.5-fold background levels at 12 hours, and continues to rise through 24 hours. This pattern is in contrast to the HFF cell-line’s incorporation profile which is linear through nine hours and plateaus at twelve. The level of queuosine modification in the HxGC₃ cultures is 5-fold lower than the incorporation levels in the HFF cells at 24 hours, nonetheless it remains significantly above that of the background.

In contrast to the HxGC₃ cell-line, MCF-7 cultures exhibited no deficiency in queuine incorporation activity. Incorporation of rQT₃ in MCF-7 cells is linear to 6 hours and plateaus at 12 hours. This is very similar to the profile of HFF culture incorporation assay. Maximal incorporation levels of rQT₃ in these two cell-lines are also similar.

Modulation of Uptake and Incorporation by PKC-catalyzed Phosphorylation

In the rQT₃ uptake studies both neoplastic cell-lines exhibited sensitivity to modulators of PKC activity and inhibitors of protein phosphatase activity and to a similar extent as the “normal” HFF cells (Graph 5-3). However, exposure to phosphorylation modulators produced results for the rQT₃ incorporation step that were comparable in only MCF-7 and HFF cell-lines. Even after 5-azaC-induction of the queuine incorporation system in the HxGC₃ cell-line, the incorporation of rQT₃ does not appear to be effected by exposure to the PKC or phosphatase modulators (Graph 5-4). It is proposed that the absence of regulation is due to the 5-azaC-induced synthesis of only the smaller catalytic subunit and not the larger regulatory subunit that makes up the “normal” tRNA:guanine ribosyltransferase dimer.

Queuine Salvage

The queuine salvage mechanism of the HxGC₃ cell-line was not studied due to the requirement of the cell culture assay method to measure the salvage of queuine via maintenance of the level of incorporated nucleotidyl base. Previous reports, however, suggest that the queuosine-deficient HxGC₃ cells maintain a poor queuine salvage efficiency of 18% (17,31).
Graph 5-1. Time-Courses of Queuine Uptake in Human Foreskin Fibroblast (HFF), Colon Adenocarcinoma (HxGCj), 5-azaCytidine-Treated HxGCj, and Breast Adenocarcinoma (MCF-7) Cell-Lines. These cell-lines were grown to near confluence, then 100 nM rQT$_3$ (a tri-tritiated queuine analogue) was added at time 0, and uptake assays performed. The amount of radioactivity taken up into the cells was measured at 0, 0.5, 1, 1.5, 2, 2.5, 3, and 6 hours. The values were normalized to the number of picomoles of rQT$_3$ per $10^5$ cells. Deviation bars indicated represent the standard deviation with $n = 6$. 

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Graph 5-2. Time-Courses of Queuine Incorporation into tRNA of Human Foreskin Fibroblast (HFF), Colon Adenocarcinoma (HxGC3), 5-azaCytidine-Treated HxGC3, and Breast Adenocarcinoma (MCF-7) Cell-Lines. These cell-lines were grown to near confluence, then 100 nM rQT3 (a tri-trieiated queuine analogue) was added at time 0, and incorporation assays performed. The amount of radioactivity inserted into tRNA was measured at 0, 3, 6, 9, 12 and 24 hours. The values were normalized to the number of picomoles of rQT3 per 10^5 cells. Deviation bars indicated represent the standard deviation with n = 6.
Graph 5-3. *The Effect of Exposure to a Protein Kinase C Activator (TPA), Inhibitor (Staurosporine), and Phosphatase Inhibitor (Okadaic Acid) as Compared to Untreated Cultures on Queuine Uptake in Human Foreskin Fibroblast (HFF), Colon Adenocarcinoma (HxGC), 5-azaCytidine-Treated HxGC, and Breast Adenocarcinoma (MCF-7) Cell-Lines.* These cell-lines were grown according to methods. Then, rQT₃ (a tritiated queuine analogue) was added at time 0, and uptake assays performed. Uptake assays were measured at 3 hours. The amount of radioactivity taken up into cells was measured by liquid scintillation. The values were normalized to the number of picomoles of rQT₃ per 10⁵ cells. Deviation bars indicate the standard deviation with n = 6.

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Graph 5-4. The Effect of Exposure to a Protein Kinase C Activator (TPA), Inhibitor (Staurosporine), and Phosphatase Inhibitor (Okadaic Acid) as Compared to Untreated Cultures on Queuine Incorporation into tRNA of Human Foreskin Fibroblast (HFF), Colon Adenocarcinoma (HxGC), 5-azaCytidine-TreatedHxGC, and Breast Adenocarcinoma (MCF-7) Cell-Lines. These cell-lines were grown according to methods. Then, rQT, (a tri-tritiated queuine analogue) was added at time 0, and incorporation assays performed. The incorporation assays were stopped at 8 hours. The amount of radioactivity inserted into tRNA was measured by liquid scintillation. The values were normalized to the number of picomoles of rQT per 10⁶ cells. Deviation bars indicate the standard deviation with n = 6.
tRNA-turnover baselines measured for MCF-7 and HFF cells showed slightly faster degradation rates for the tRNA of the neoplastic cell-line, and the MCF-7 cell-line was observed to have a slightly faster growth rate over the four days of the experimentation. However, the values are still reasonably close (Graph 5-5). In these studies the HFF cells are able to retain nearly 100% of incorporated rQT₃ for up to four days with one day to over a week of queuine starvation, with only a small decline in rQT₃ retention after three weeks of starvation. In contrast to queuine salvage in the "normal" cells, the MCF-7 cell-line exhibits virtually no latent salvage capability. At only one day of queuine starvation, these cells show a similar turnover pattern of rQT₃-modified tRNA retention with and without a non-radioactive queuine chaser. After one week of queuine starvation approximately 50% salvage of rQT₃ is evident. Three week queuine starvation dropped rQT₃ maintenance levels to that of just above background.

Discussion

Due to the multistep process of the queuosine modification system, this research attempts to investigate four critical aspects to it's proper functioning. Studies were undertaken to address whether the malfunctions of the queuosine modification system in HxGC₃ and MCF-7 neoplastic cell-lines were due to lack of adequate uptake, incorporation ability, salvage capability or to aberrant PKC-catalyzed modulation of uptake or incorporation. Experiments involved experimental and control cultures of each cell-line in order to diffuse arguments regarding the modification differences of varying cell types (fibroblasts, and colon and breast adenocarcinomas). Comparisons of "normal" (HFF cells) and neoplastic (HxGC₃ and MCF-7 cell-lines) cells were performed verifying that the characteristic hypomodification observed in cancer cells (45) occurs in these cultures. Additionally, an investigation of each step of the queuosine modification system was executed to determine the site or sites for malfunction of this system in the two representative tumor cell-lines.

There appears to be no abnormality in the uptake mechanisms of either of the two neoplastic cell-lines which could create the queuosine deficiency observed in the cultures.

Results of the HxGC₃ cell-line incorporation studies directly support the hypothesis that there is no incorporation of queuine into tRNA in these cells, and confirms that this is due to the lack of a cellular TGRase activity. This result verifies the presence of 5-azaC-induced incorporation of queuine in the HxGC₃ cell-line that had been previously seen only indirectly by RPC-5 liquid chromatography and is much stronger evidence for the theory that the HxGC₃ cell-line is deficient in the queuosine modification due to a null mutation in the functional alleles for the TGRase protein. Malfunction of the incorporation step in the queuosine modification system is not the cause for the observed MCF-7 queuosine deficiency.

"Normal" HFF cells have been shown to contain a dual-modulation scheme for both the uptake and incorporation of queuine (Chapter 4, (126,147)). These two components of the queuosine modification system have been shown to be directly activated by PKC. In contrast, protein phosphatases are able to reverse this process by removing the phosphate group and thereby inhibiting both systems. Purified
Graph 5-5. Comparison of Queuine Salvage in “Normal” Human Fibroblasts and Mammary Adenocarcinoma Cell Cultures. Cultures of human fibroblast (HFF) or breast adenocarcinoma (MCF-7) cells were grown to near confluence in normal media, fed with charcoal-stripped serum (lacking queuine) for 1 day, 1 week, or 3 weeks with regular media changes, then submitted to an in vivo queuine salvage assay. A dose of 100 nM tritirated analogue of queuine (rQT₃) was placed in the media 24 hours before the start of the assay to saturate the tRNA with the radiolabeled base. For each cell-type, two sets of parallel cultures were established. At time zero extracellular rQT₃ was washed away, then a media change with charcoal-stripped media was performed. One set of cultures was exposed to 0.10 A₂₆₀ units of unlabeled queuine (solid line) to provide an indication of the tRNA turnover rate within those cells. No queuine was added to the other set of cultures (dashed line) which represents the ability of the cells to retain the radiolabel in the cell’s tRNA despite the degradation processes involved in normal tRNA turnover. The counts per minute values obtained via liquid scintillation were normalized to the number of pmol rQT₃ contained within the acid-precipitable fraction of the 10⁶ cells that had been devoid of queuine for 1 day, 1 week or 3 weeks. Deviation bars indicated represent the standard deviation with n =4.
mammalian queuine incorporation enzymes from mouse, rat, cow, and human liver tissues have been isolated and shown to exist in a dimeric form with a regulatory and a catalytic subunit (Chapter 3 (125,126)). In order to determine if similar regulation schemes are functional in the neoplastic cell-lines, in vivo uptake and incorporation assays were performed with cells exposed to a PKC activator (TPA), a PKC inhibitor (staurosporine), and a protein phosphatase inhibitor (okadaic acid) in concentrations most specific for modulation of these cellular PKC and phosphatase enzymes. The response of the queuine uptake mechanisms in both HxGC3 and MCF-7 cell-lines when exposed to these modulators was comparable to that of the “normal” HFF cultures. In studies of the incorporation step of 5-azaC-treated of HxGC3 cells, however, the induced queuine incorporation activity effected by exposure to PKC or phosphatase modulators. This may be due to the transcriptional activation of only the mRNA for the active subunit of the enzyme. The MCF-7 cell-line, however, exhibited a comparable response to the “normal” HFF cell-line upon exposure to phosphorylation modulators.

While it was reported that the HxGC3 cell-line maintains a level of only 18% salvage activity (17,31), it was not possible to measure the levels using the in vivo assay method employed in these studies. However, queuine salvage studies demonstrated a distinct deficiency in the salvage ability of the MCF-7 cell-line. With no latent salvage activity to only mildly inducible salvage activity available to the cells, a deficiency in the concentration of the queuine substrate for the incorporation step could arise. If either a decrease in the availability of dietary queuine or an increase in cell division rate were to occur, these cells would not be able to sustain high levels of queuosine-modified tRNA by salvage of the queuine base alone. The lack of an efficient salvage mechanism in the MCF-7 cell-line could help to explain the relatively moderate level of queuosine deficiency (50 to 60%) exhibited as compared with the HxGC3 cells (100%) which exhibit no queuine incorporation activity.

The results of this research suggest that the deficiencies of the queuosine-modified tRNA population found in cancer cells, such as the HxGC3 and MCF-7 cells used in this study, may arise from the malfunction of any of a number of steps. Due to the known importance of PKC in tumor biology and existence of defects of this enzyme in several neoplastic systems (85), it is also likely that ineffective phosphorylation-based modulation of the uptake and incorporation systems of other tumor cell-lines might also be a cause for observed queuosine hypomodification levels. However, two specific and distinct sites for queuosine modification system breakdown have been identified in this study. The HxGC3 cell-line is an example of a cell-type containing a transcriptional inability to produce the queuine incorporation enzyme that is required to create this modification (Figure 5-1), while the MCF-7 cell-line is an example of the disruptive effect of an inefficient queuine salvage mechanism (Figure 5-2).

In addition to the identification of the multifocal nature of the queuosine modification system and its malfunction, the identification of the etiology for queuosine deficiency has suggested three potential benefits for this knowledge. First, biopsied tumors that are identified as containing contrasting sites for queuosine modification system deficiency may be ruled out as a metastatic byproduct of a primary mass.
In addition, sites for potential chemotherapeutic treatments concerning queuine (if developed) can be more explicitly targeted to the specific neoplastic cells of a patient. Finally, a human cell-line with a null genetic mutation for a major component of the queuosine modification system has been found—the HxGC3 cell-line. This appears to mimic the situation seen in only the yeast organism where the queuosine modification does not exist (10). The discovery of a mammalian cell-line that does not contain a functional queuine incorporation enzyme provides a good subject for gene expression studies that allow for the experimental investigation of the effect of the queuosine modification on cells in general, and especially in determining the importance of the observed queuosine hypomodification in human neoplastic cells.
Figure 5-1. The Eukaryotic Queuosine Modification System in HxGC3, Human Colon Adenocarcinoma Cells. The eukaryotic system is based on usage of an available resource and is regulated by the direct action of protein kinase C (PKC) on both uptake and incorporation steps as described in Figure 4-3. In HxGC3 cells, which contain no queuosine in their tRNA, the incorporation step appears to be entirely deficient. Despite a very weak ability to salvage incorporated queoine from degraded tRNA, these cells lack the ability to synthesize the modified nucleoside in any capacity. Treatment with the DNA methylase inhibitor 5-azacytidine for 24 hours induces a weak but measurable induction of tRNA:guanine ribosyltransferase (TGRase) activity in these cells and provides evidence of the formation of queuosine-modified tRNAs. This induced activity, however, is not susceptible to stimulation by PKC. Thus, it is suggested that at least one allele of the catalytic subunit of TGRase is inactivated by the methylation of the promoter region of its gene in these cells.
Figure 5-2. The Eukaryotic Queuosine Modification System in MCF-7 Human Breast Adenocarcinoma Cells. The eukaryotic system is based on usage of an available resource and is regulated by the direct action of protein kinase C (PKC) on both uptake and incorporation steps as described in Figure 4-3. In MCF-7 cells, which contain a significant deficiency of 50-60\% queuosine in their tRNA, the salvage step appears to be deficient. Despite the ability to take up queuine from the surrounding environment and incorporate it into tRNA, these cells show a pronounced inability to salvage the modified nucleoside. Latent salvage activity does not occur in these cells with only one day of queuine starvation. In addition, strong induction of the salvage mechanism by queuine starvation for one week, which is seen in "normal" human fibroblasts, appears to be compromised in these cells (approximately 50\% effective). After three weeks of queuine starvation the ability to salvage queuine from tRNA is completely lacking. Thus, despite the ability to take up exogenous queuine and incorporate it into tRNA, reduced salvage efficiency predisposes these cells to significant queuosine deficiencies in a shortage of exogenous supply or under fast growing conditions.
CHAPTER SIX
EFFECTS OF THE QUEUOSINE MODIFICATION SYSTEM
ON THE STRUCTURE AND FUNCTION OF tRNA

Introduction

Queuosine is a modified nucleoside found in position 34 (the wobble position) of the anticodon hairpin loop of tRNA<sup>sup</sup>, tRNA<sup>an</sup>, tRNA<sup>his</sup>, and tRNA<sup>pr</sup> in every organism yet studied with the sole exception of yeast (10,11). Chapters 3 and 4 of this treatise fully describe the mechanism and regulation of the queuosine modification system in human systems. The control of this system by protein kinase C-catalyzed phosphorylation allows the formation of queuosine to be coordinated with other cellular processes that control the life cycle of a cell. The aberrant functioning of the queuosine modification system is directly correlated with neoplastic transformation and metastatic progression of abnormal cell lines. Chapter 5 describes a series of assays used to determine the exact point at which the system failure occurred in two cancer cell-lines. However, despite the understanding of the normal cellular dynamics of the queuosine modification, there is still relatively little subcellular knowledge regarding the function of this modified nucleoside within a tRNA.

Modifications of RNAs have been shown to have a strong influence on the alteration of steric and electrostatic properties of these important molecules. Aspects of RNA structural stabilization and of anticodon hydrogen bonding potentials are crucial components for tRNA function. Weiss proposed that a mispairing of the anticodon wobble base could dramatically effect the binding kinetics of the tRNA to its mRNA codon—which, he suggested, has the potential to regulate the rate of translation (148). Grosjean, Houssier & Cedregen have demonstrated the presence of different conformers of anticodon regions (i.e. flexibility) in temperature-jump relaxation experiments due to the presence of altered binding kinetics for queuosine versus guanosine-containing tRNAs (149). It was further proposed that the formation of the queuosine nucleoside is involved in "a mechanism of translational control (coordinated modulation) relying on the interdependence of metabolic pathways, modified nucleotide synthesis and codon usage...".

The crystal structure of the 5'-monophosphoryl-queuosine nucleotide indicates the likely presence of a hydrogen bond between the aminomethyl group of the modification's side-chain and the O6 of the purine ring structure (13). This intramolecular association may have a role in altering the hydrogen bond accepting ability of this position in tRNA. Additionally, the anticodon loop is the most flexible region of the tRNA molecule, with the most variable configuration adjacent to the site of the queuosine-modification at the phosphoryl linkage between residues 33 and 34 (150). Thus, it is likely that the effect of a modified wobble base (position 34) on the function of tRNA would be influenced by alteration of either electrostatic or steric properties of the anticodon based on the mobility of residues in the anticodon loop. In view of the extended multi-functional group structure of queuosine and its location in the wobble position of tRNA, it
is likely that this modified nucleoside plays a significant role in the decoding properties of tRNA through adjustment of the molecular dynamics of the anticodon or anticodon loop structures.

The structural stability of RNA loops have been shown to be influenced by changes in the nucleoside sequence of the molecule. Singh and Kollman have recently studied the relative mobility of RNA hairpin loop residues based on thermodynamic properties of a molecule before and after the introduction of a point mutation (151). Their calculations indicated that a simple C to U point mutation in the RNA loop was capable of dramatically increasing the flexibility of the loop by dissolution of an intramolecular (cross-loop) hydrogen bond.

The alteration of nucleosides by modification in tRNA also plays a significant role in the structural dynamics of RNA stem/loop structures. Due to steric hindrance of functional groups, 2′O ribose methylation and base thiolation of non-anticodon residues have been shown to cause the shift of ribose puckering toward the C3′-endo conformation which stabilizes base stacking interactions throughout neighboring residues (152). These modifications enhance the structural stabilization of the anticodon while influencing proper codon recognition and preventing misreading of non-cognate codons (152). Recently, an alternative ribose puckering conformation (C2′-endo) was shown to be induced by the presence of dihydrouracil (153). In addition to this modified base’s non-aromatic nature, the promotion of the different ribose configuration caused a disruption of base-stacking interactions within the RNA molecule. This causes increased regional flexibility which is important in the hinge region of the tRNA molecule.

An increase in the strength of base-stacking interactions (154) and the formation of extended hydrogen bonding networks was shown to be promoted by the presence of the anticodon loop pseudouridine nucleotide (155). Both of these factors caused an increase in the rigidity of the entire anticodon. Thus, modification of RNA primary transcripts has been shown to be involved with the overall structural dynamics of the molecule through the modulation of hairpin loop stability. Due to the extended multi-functional group structure of the queuosine modification, this wobble base may play a role in RNA loop stabilization based on potential extended intra-anticodon loop interactions.

It has been suggested, due to the size of a few large modified nucleosides in the anticodon loops of various tRNA molecules, that some modified bases have the potential to sterically interact with a neighboring tRNA anticodon loop in aminoacyl-/peptidyl-site tRNA interactions (112). The lack of the normal wyebutine modification at position 37 of tRNA\textsuperscript{\textit{bc}} molecules, a tricyclic purine analogue with a large branched side-chain, has been suggested to increase the speed of translation around UUU and UUC phenylalanine codons, while the fully modified tRNA exerts unspecified restrictive codon context effects (156). Experimental evidence supporting context effects exerted by the queuosine modification has been reported in studies demonstrating the ability of unmodified (guanosine-containing) form of tRNA\textsuperscript{\textit{G}} to read-through UAG stop codons (157,158). Queuosine-modified tRNA\textsuperscript{\textit{G}} do not appear to possess this amber suppression capability. Additional codon context effects have been proposed for both queuosine-modified tRNA\textsuperscript{\textit{Am}} and wyebutine-modified tRNA\textsuperscript{\textit{bc}} in frameshifting events essential to the translation of
gag-pol gene products of human immunodeficiency virus (HIV), human T-cell leukemia virus (type I) (HTLV-1), and bovine leukemia virus (BLV) (159). It was demonstrated that absence of the queuosine modification in tRNA\textsuperscript{am} enhanced the ribosomal frameshifting around an AAC codon at the shift signal in HTLV-1 and BLV. Wyebutine-deficiency in tRNA\textsuperscript{acr} enhanced frameshifting at a UUU codon frameshifting signal in HIV. These frameshifting events are essential to the production of gag-pol products for these viruses, and codon context effects exerted by these two modified nucleotides appear to have important modulating effects in the efficiency of the frameshifting mechanism. Since neighboring tRNA loop interactions are plausible in the framework of adjacent ribosomal aminoacyl- and peptidyl-sites, and since bulky tRNA modifications, such as wyebutine and queuosine, could be positioned in immediate proximity with sequential UUPy and GAPy codons, it is conceivable that steric interference at these codon pairs (restrictive codon context) could be significant. Thus, codon context-based steric restriction of tRNA association by anticodon loop modified nucleosides could effect the translation efficiency of mRNAs for proteins based on the existence of specific codon pairs and the levels of modified-tRNAs.

The function of queuosine in the wobble position of the anticodon has also been investigated with regard to its decoding properties. Results of experimentation, which included the injection of drosophila tRNA\textsuperscript{his} and turnip yellow mosaic coat protein mRNA into \textit{Xenopus} oocytes, demonstrated that guanosine-containing tRNA\textsuperscript{his} has a strong preference for codon sequences of CAC over CAU. However, queuosine-modified tRNA\textsuperscript{his} does not exhibit a preference for either histidine codon, indicating that queuosine eliminates codon bias between histidine codons in this system (160). Another study examined the effects of rabbit reticulocyte queuosine-modified tRNA\textsuperscript{his} on the decoding properties for histidine codons found in rabbit globin mRNA using a reticulocyte lysate translation system (161). The authors also demonstrated that the queuosine-modified tRNA\textsuperscript{his} has no preference for either U or C in the third position of the histidine codons. Grosjean, Houssier and Cedregen utilized temperature-jump relaxation experiments and verified that while guanosine-containing and queuosine-modified tRNAs are equally stable in complexes with N\textsuperscript{U}A\textsuperscript{U} codons (162), guanosine-containing tRNAs are significantly more stable in complexation with N\textsuperscript{A}C\textsuperscript{U} codons (149,163,164). Thus, it is suggested that the queuosine modification serves to alter the codon binding properties of tRNAs by reducing the C over U codon bias apparent with guanosine-containing tRNAs.

This study was initiated to explore the effects of queuosine on the decoding properties of the tRNA using computational chemistry approaches of molecular modeling, including molecular dynamics simulations. The experimentation was designed to study the intramolecular effects of the modification on the electrostatic and steric structure of the tRNA anticodon loop, as well as the effects of the modification on the complexation of tRNAs with a neighboring tRNA (codon context effects) or mRNA (codon bias effects).

Initially, nucleotide analysis was performed to assess the electrical potential of the hydrogen-bonding regions for guanosine and modified guanosine analogues, including queuosine. The method
involved semi-empirical charge assignment and geometry optimization algorithms, with visualization of an electrical potential map on the electron density surface for each molecule. In addition, anticodon stem/loop analysis was performed on tRNA^"p" anticodon stem/loop structures modified or unmodified at position 34 (the wobble position) in order to determine the effect of guanosine and guanosine analogues, particularly the queuosine modification, on the configuration and flexibility of the anticodon loop. This method involved the use of energy minimization techniques and extended molecular dynamics simulations.

Accordingly, macromolecular complex analysis was performed on a tertiary organization of tRNA^"p" anticodon stem/loop molecules, whole tRNA^"p" molecules, and a mRNA transcript in a configuration consistent with that associated at the aminoacyl- and peptidyl-sites of a translating ribosome (107). Evidence of potential interaction between aminoacyl- and peptidyl-site tRNAs at the queuosine and wybutine modifications of the anticodon loop were visually analyzed in order to determine the presence of a potential codon context phenomenon based on steric interaction. In addition, the A-site tRNA and mRNA association was analyzed to explore potential differences between queuosine-modified and guanosine-containing tRNA^"p" anticodon stem/loop structures with cognate GAC and GAU mRNA codons. Energy minimization and molecular dynamics simulations and energy calculation algorithms were used to determine relative tRNA anticodon/mRNA complex stabilities and evidence of preferred (biased) pairings.

Results and Discussion

Nucleotide analysis

Initially, structural analysis of guanosine (G) and the guanosine nucleoside analogues 7-deazaguanosine (7-deazaG), 7-aminomethyl-7-deazaguanosine (7-am-7-deazaG), and queuosine (Q) (Figure 6-1) was studied using semi-empirical methods. AM1 and PM3 charge assessment with geometry optimization was performed. Both MOPAC algorithms provided essentially the same values for electrostatic potential and for geometric arrangement of atoms. Since the PM3 method is more recent and appears to be more accurate when applied to nucleic acids with significant hydrogen bonding potential (165), the results of these calculations were used for further modeling.

Conformational search via simulated annealing was employed to determine reasonable conformations and differences in intermolecular associations and in the hydrogen bonding abilities of the three functional groups for nucleoside base-pairing of purine residues. After an extensive search, the quaternary amines of both the Q and 7-am-7-deazaG molecules appeared to be in reasonable positions for hydrogen bonding interactions with respective carbonyl oxygens at the C6 position (Figure 6-2). A hydrogen bond prediction algorithm built into the Sybyl software, which takes into account both distance and bond angle parameters, verified the likely presence of this interaction. This corroborates preliminary molecular dynamics simulations of the bases under gas phase, and under implicit and explicit solvation conditions, which indicated greater than 60% of the structures observed during the simulation were likely
Figure 6-1. Modified Nucleoside Bases Studied in this Treatise. Guanine is the traditional base found at position 34 (wobble position) of tRNA$^{AAA}$, tRNA$^{AUC}$, tRNA$^{AU}$, and tRNA$^{AU}$. Queuine, a guanine analogue, is a physiological modified nucleoside that replaces guanine in these sites in "normal" mature cells of every prokaryotic and eukaryotic organism with the exception of yeast (10). 7-deazaguanine and 7-aminomethyl-7-deazaguanine are intermediates in the formation of queuosine in prokaryotes. The three major structural differences between guanine and queuine involve position 7 of the base, the charged quarternary amine of the aminomethyl side-chain, and the cyclopentenediol ring of the side chain. In these studies, the intermediates were included to serve as a control for the effects of each of these differences. Arrows indicate the position of n-glycosidic linkages to ribose.

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Figure 6-2. Skeletal Structures of Guanosine and its Analogues. Models are shown of guanosine (upper right), 7-deazaguanosine (upper left), 7-aminomethyl-7-deazaguanosine (lower right), and queuosine (lower left) after MOPAC 6.0 semi-empirical energy minimization and geometry optimization. The orange arrows indicate the sites for internal hydrogen bonding from the quaternary amine of the side chain to the O6 of the base's carbonyl in 7-aminomethyl-7-deazaguanosine and queuosine.
to contain parameters consistent with the presence of this hydrogen bond.

A study of the three sites for base pairing complexation (O6, N1, and N2) was performed to assess the effect of the hydrogen bond between the O6 and the quaternary amine of the aminomethyl side chain. The MOLCAD-generated electron density surface plots of G and 7-deazaG exhibited identical electrical potential maps, with strong hydrogen donor sites at the N1 and N2 positions and a moderate hydrogen accepting site at the O6 (Figure 6-3). However, as a result of the intramolecular hydrogen bonding, the O6 was observed to possess diminished electronegative character comparably in the Q and 7-am-7-deazaG molecules. This suggests a compromise of their Watson-Crick type hydrogen bond accepting status. Additionally, the relative strength of the electronegativity at the traditional hydrogen donating sites of the N1 and N2 in both these nucleotides appears to be reduced.

The results of the structural and charge studies performed for these molecules indicate the importance of the interaction between the quaternary amine and the O6 in the two molecules with the aminomethyl side chains. It appears that this intramolecular hydrogen bond alters the hydrogen donating/accepting profile of the base. Whereas the G and 7-deazaG appear to have a traditional (Watson-Crick type) ADD hydrogen bonding pattern, Q and 7-am-7-deazaG are suggested to have a DD pattern which may even be weaker in strength than the imino form of an adenosine. Of course, in biochemical and chemical systems, interactions such as hydrogen bonding are freely reversible. Therefore, both guanosine-like and adenosine-like interactions are likely to be possible with Q and 7-am-7-deazaG.

The quaternary amine of the aminomethyl side chain, existing in both Q and 7-am-7-deazaG, provides an electrostatic difference manifesting itself in hydrogen bonding potential differences between these two nucleosides and G or 7-deazaG. However, the aminomethyl side chain is not likely to have any steric effect on the codon-anticodon interaction, since the configuration of the nucleotide appears to position this extension away from the site for base-pair hydrogen bonding—confirming results seen in the original crystal structure of the Q nucleotide (13). Thus, the a possible codon bias effect is suggested based on differential electrical potential of tRNAs for wobble bases with and without the aminomethyl sidechain.

Anticodon Stem/Loop Analysis

Transfer RNA anticodon stem/loop structures with modifications of the wobble residue were analyzed using molecular dynamics simulation to determine differential intramolecular associations caused by the 7-deazaG, 7-am-7-deazaG, and Q bases. Unfortunately, due to computational limitations the addition of counterions and solvation to the system was not possible. In addition, a reasonably long length of simulation (up to ms) was also not possible. However, these preliminary studies were performed based on a qualitative method developed by Nilsson and Karplus on very different software and hardware computer systems for the anticodon stem-loop of tRNA^phe (97). The data generated was validated by the duplication of the original study and a favorable comparison between the original and duplicated results for tRNA^phe and the present study of tRNA_w.
Figure 6-3. Electrical Potential Surface Studies of Guanosine and its Analogues. Electron density surfaces were generated for each nucleotide in observed in (Figure 6-2). An electrical potential map for each was generated and is displayed on the surfaces with a red color representing the strongest positive charge proton donor capacity and purple indicating the strongest negative charge (proton acceptor capacity).
Using molecular dynamics simulations, molecular rigidity was assessed to determine queuosine’s effects on the overall flexibility of the anticodon loop: a region critical for the tRNA’s function as an adaptor for the mRNA codon. At 10 ps into the temperature plateau the RMS fluctuations of kinetic versus total energy were calculated for the three modified and the G-containing stem/loop structures and were determined to be less than 2%. This indicated a stable simulation for data collection and analysis. Transfer RNA\textsuperscript{amp} anticodon stem/loop molecules mutated to contain 7-am-7-deazaG and 7-deazaG produced minimization and dynamics results that were essentially identical to that observed for the G-containing tRNA. However, residue mobility and molecular flexibility differences were observed in the loop region of these molecules as compared with the Q-modified RNA loop.

The stem of the tRNA molecule from residue 26 through 31 and 39 through 44 maintains its base-pairing and A-helix structure throughout the simulation. The radius of gyration remains fairly constant, fluctuating from 9.85 to 10.20 Å for G-containing, 7-deazaG-modified, and 7-am-7-deazaG-modified tRNA\textsuperscript{amp} molecules and 9.70 to 10.10 Å for Q-modified tRNA\textsuperscript{amp}\textsuperscript{inc} during the 15 ps to 55 ps time frame over the course of the simulation plateau, as compared to 10.6 to 10.9 Å for tRNA\textsuperscript{inc} in the original study (97). The helix does appear to condense slightly over the length of the run and corresponds well to Nilsson and Karplus’ tRNA\textsuperscript{inc} anticodon stem/loop data. The RMS deviation from starting positions over the simulation was 0.54 to 0.70 Å on average for all G-containing, 7-deazaG-modified, and 7-am-7-deazaG tRNA\textsuperscript{amp} stem atoms, while 0.64 to 0.75 Å for all Q-modified tRNA\textsuperscript{amp} stem atoms. This parameter could not be compared with Nilsson and Karplus’ data because of the differences in available reference structures (initial dynamics structures (97) versus X-ray crystal coordinates) due to the limitations of the number of programmable steps in the software utilized. Overall, the stem structures of the molecules were well maintained with the only significant structural deviation at the ends (residues 26 and 44, and to a limited degree 27 and 43). This “end-effect” artifact was brought about by the artificial termination of the structure and was described by Nilsson and Karplus in the original work with the tRNA\textsuperscript{inc} anticodon stem/loop (97). This artifact is a necessary but unfortunate side-effect of the structural truncation required for minimizing the computation time required for these complex calculations.

The resulting RMS differences in atomic positions of the G-containing, 7-deazaG-modified, and 7-am-7-deazaG-modified tRNA\textsuperscript{amp} anticodon stem/loop structures closely mimic that of truncated tRNA\textsuperscript{inc} model (97) in comparisons of data at 5, 10 and 40 ps timepoints (data not shown). However, in the calculations of the Q-modified tRNA\textsuperscript{amp}, the resulting RMS difference values are dramatically different in the movement of specific loop residues (residues 32 through 37). The variance in these nucleoside positions are far lower in intensity than that observed in either the G-containing, 7-deazaG-modified, or 7-am-7-deazaG-modified tRNA\textsuperscript{amp} or the tRNA\textsuperscript{inc} anticodon stem/loop structures (97). This suggests possible structural and electrostatic influences on intramolecular mobility in the anticodon loop caused by the queuosine residue.

During the molecular dynamics simulation, it was observed that the greatest fluctuation of residue
positions of all tRNA\textsuperscript{up} anticodon stem/loop molecules exists at residues 34 and 35 (Graph 6-1). The great flexibility at the wobble position and that of the middle anticodon residue (position 35) are important for the association of the anticodon/codon triplet interaction. The movement of each of the anticodon bases is known to be influenced through direct covalent linkages as well as base-stacking interactions. In addition, the slowly decreasing mobility of residues through the anticodon bases (34, 35, & 36), as compared with those of residues 33 and 32 at the 5’ side of the anticodon, corroborates the long-held assumption concerning the role of base-stacking interactions in the global motility of the 3’ side of the anticodon loop. However, it was observed that distinct differences exist in the mobility of several Q-modified loop residues as compared with those in the G-containing, 7-deazaG, or 7-am-7-deazaG-modified loops.

The magnitude of RMS fluctuation of the residue at the wobble position (34) is greatest in the case of G-containing, 7-deazaG-modified, and 7-am-7-deazaG-modified loops, but in the Q-modified loop this falls to a level below that of residue 35 (Graph 6-1). The extent of fluctuation for residue 35 appears to be unchanged between the two anticodon stem/loop molecules, however the decrease in RMS fluctuations over the anticodon bases is terminated much more quickly in the Q-modified loop, indicating a great decline in mobility after residue 36 and before the queuosine wobble residue. Additionally, the sharp decrease of RMS fluctuations on the 5’ side of the wobble base at residues 33 and 32 is more dramatic in Q-modified tRNA with virtual immobility of base 32. These observations of differences in residue fluctuations and mobility can be explained by intramolecular hydrogen bond associations observed during the molecular dynamics simulation.

Visual inspection of the anticodon loop residues over the molecular dynamics simulation suggests that several potential intramolecular hydrogen bonding interactions influence the traditional base-stacking interactions of the anticodon nucleotides. In both G-containing and Q-modified anticodon loops, a hydrogen bond was observed between the N3 position of the uridine residue at position 33 (U33) and the backbone phosphate of the cytidine residue at 36 (C36) during the molecular dynamics simulation (Figure 6-4). Confirmation of this cross-loop hydrogen bond in the anticodon loop of guanosine-containing tRNA\textsuperscript{up} was published after the completion of this research in a solvated system with counterions, and for a simulation running roughly 10-fold longer than this simulation by Auffinger and Westhof (99).

Interestingly, the number of timepoints exhibiting the cross-loop U33 to C36 hydrogen bond in the Q-containing loop was 68%, while its presence in G-containing-, 7-deazaG-modified, and 7-am-7-deazaG-modified loops was less than 36%. The increased frequency of intramolecular hydrogen bonding interaction between these residues in the Q-modified anticodon loop may explain the decreased mobility (decline in slope) of the 3’ side of the loop (residues 37 and 38), as well as the previously discussed 5’ side of the loop (residues 33 and 32).

On closer examination of the Q-modified tRNA\textsuperscript{up} anticodon loop, it was observed that the hydroxyl groups of the cyclopentenediol ring were maintained in close proximity to the 3’O and 2’OH of the backbone ribose of U33, as well as the phosphate of the Q residue at 34 (Q34). Further analysis
Graph 6-1. *Fluctuations of Anticodon Loop Residue Positions During the Molecular Dynamics Simulations.* The root mean square (RMS) fluctuation of the position of each nucleotide in the anticodon stem loop structure was determined using a molecular dynamics simulation. A tRNA\(^{\text{rep}}\) anticodon loop containing guanosine in position 34 is represented by the graph on the left. A queuosine modified loop (position 34) is represented in the graph on the right. The flexibility of the entire anticodon region is greater in the guanosine-containing model than in the queuosine-modified anticodon model, with positions 32, 34, and 37 showing the greatest difference in flexibility.
Figure 6-4. Queuosine Induced Stabilization of Anticodon Flexibility Through Hydrogen Bonding Networks. Representation of ribbon backbones with ball and stick nucleoside bases for anticodon residues 34, 35, and 36 as well as the backbones of residues 33 and 36. a) The structure on the left depicts the queuosine modified tRNAAsp anticodon loop (green). Queuosine (green base and orange side-chain) engages in several stabilizing hydrogen bonded interactions between its cyclopentendio) hydroxyl groups and the phosphoribosyl backbone of U33 (indicated by solid yellow arrows). These interactions promote the stability of the additional cross loop U33 to C36 hydrogen bond interaction (indicated by yellow arrow outline). b) The structure on the right depicts the guanosine-containing tRNAAsp anticodon loop (blue). In this anticodon loop, there is no additional hydrogen bonding network observed due to the lack of the wobble base’s cyclopentenediol ring on the side-chain. As a result the U33 to C36 cross loop hydrogen bond is less frequently observed during the molecular dynamics simulations, thus appears to be much weaker in strength than the corresponding bond in the queuosine-modified complex.
revealed that allowable hydrogen bonding parameters exist between the hydroxyl groups of the queuosine cyclopentenediol ring to the 3' O during approximately 55% of the 55 ps simulation run and to the 2' OH for approximately 35% of the simulation run. Though it was initially proposed that these hydroxyl groups could interact with the phosphate group (13), in these studies the 3' O/2' OH associations were observed even more frequently than interactions with the phosphate group. In several instances, two or three of these hydrogen bonding associations (networks) formed simultaneously which would increase the stability of each interaction. In one example, the one hydroxyl group of the cyclopentenediol ring was close enough to interact with both the 3' O and the O of the phosphate simultaneously, with the other hydroxyl group interacting with the 2' OH. The presence of this hydrogen bonding network would explain the decreases in RMS fluctuation for the residues from 32 to 36, as these intramolecular bonds would greatly restrict the movement of these nucleotides.

The full structure of the Q modification appears to have great intramolecular influence on the flexibility of the anticodon loop. The intraresidue hydrogen bond between the quaternary amine and the O6 of queuosine, in addition to the potential electrostatic influences on base-pairing ability, appears to position the cyclopentenediol ring near the backbone of the anticodon loop between Q34 and U33. The presence of the dihydroxyl groups of the cyclopentenediol ring appears to be the major cause of this restriction of anticodon loop flexibility. Neither the 7-am-7-deazaG nor 7-deazaG residues contain the cyclopentenediol moiety nor comparable hydroxyl groups, and the modified anticodon loop forms of these bases show RMS fluctuation profiles virtually identical to that of G-containing tRNA. The two hydroxyl groups of Q's cyclopentenediol ring are then able to interact with the 3' O and 2' OH of the U33 residue, and the O of the backbone phosphate of residue Q34. This effectively locks the flexibility of the 5' side of the anticodon loop which is suggested to position the U33 in a more favorable conformation for its association with the backbone phosphate of C36. Thus, a proposed hydrogen bonding network is suggested to be stabilized by the queuosine modification; initially forming on the 5' side of the anticodon between Q34 and U33, and across the loop with the interaction between U33 and C36 on the 3' side of the loop. This network would likely produce the decreases in overall anticodon loop flexibility seen in these molecular dynamics simulations.

Since the completion of this work a report has been published of a significantly longer molecular dynamics simulation of the flexibility of the anticodon stem-loop of tRNA^*9 containing guanosine in position 34 of the anticodon (99). In addition, the system was solvated and contained sodium counterions for the charges present in the phosphodiester backbone. The calculations were performed using the CHARMM force field (98) on a different computer system with different simulation software. However, the anticodon stem-loop structures, flexibility, and predicted presence of the cross-loop hydrogen bond were virtually identical to those shown here. The necessary design weaknesses of the computational methods used in this experimentation (namely the length of simulation and lack of solvent and counterions) are acknowledged, but the results obtained are validated by this published report.
Macromolecular Complex Analysis: Interaction of A- and P-site tRNAs (Codon Context)

In an initial study of macromolecular associations, a 5'-GACUUU-3' transcript was modeled with either an A-site Q-modified tRNA\textsuperscript{ap} anticodon stem/loop region of tRNA\textsuperscript{ap} and P-site tRNA\textsuperscript{phe} or A-site tRNA\textsuperscript{phe} and A-site Q-modified tRNA\textsuperscript{ap} anticodon stem/loop region of tRNA\textsuperscript{ap}. This was performed to assess any possible steric or electrostatic interaction between the anticodon loops and stems of side-by-side tRNAs on a translating mRNA (codon context). These tRNAs were chosen because they contain two of the largest RNA nucleosides currently known—queuosine (Q) in tRNA\textsuperscript{ap} and wyebutine (Y) in tRNA\textsuperscript{phe} (Figure 6-5).

Observation of the two complexes indicates that there is no interaction between the anticodon stems of either of the two orientations of tRNAs. As for possible anticodon loop interaction, with Q-modified tRNA\textsuperscript{ap} in the A-site and Y-tRNA\textsuperscript{phe} in the P-site, the terminal methyl of the side-chain acetyl of wyebutine comes closest to the hydrogen of the N2 amine of queuosine at approximately 6.9 Å (Figure 6-6). This is too great a distance to suggest that steric hindrance might be exerted between these two tRNAs. With a relatively uncharged methyl group being a participant in any possible interaction, 6.9 Å is also too great a distance to support any electrostatic interaction. A recent cryoelectron microscopic model of the ribosome (110,111) suggests that the sites for the placement of the A- and P-site tRNAs are “buried” within a fairly tight cavern, this is likely to exclude a great deal of water for hydration of even charged atoms.

Finally, with the tRNAs in reversed orientation, Y-modified tRNA\textsuperscript{phe} in the A-site and Q-modified tRNA\textsuperscript{ap} in the P-site, the modified nucleotides are on opposite sides of the complex (Figure 6-6). This conformational model suggests that there is no potential for anticodon loop interaction in this configuration. These findings effectively eliminate the possibility of sterically or electrostatically-based restrictive codon context in A- and P-site:Q-Y interacting anticodon loop pairs.

A caveat must be included here, however. That we did not see anticodon stem or loop interaction in this computational modeling study does not preclude the fact that the interactions may occur in the “real” world. Our study is based on the best three dimensional model known at this time and our findings appear to be correct based on the predicted “kink” in the mRNA between the A- and P-site codons. This was predicted by Fuller and Hodgson in 1969 (166) and has been recently confirmed by the cryoelectron microscopy studies of Agrawal et al. (111). The mechanism for predicted codon context effects may then be answered by the possibility of interactions based on the proximity of aminoacyl acceptor ends, DHU loops, or even specific variable loops (167) of A- and P-site tRNAs. Additionally, recent cross-linking studies with ribosomal components have suggested a strong potential for rRNA interactions to influence the stability of tRNA/mRNA complexes by direct interaction with the wobble base of A-site tRNA (168). Thus, the previous codon context observations may be explained by tRNA/rRNA interactions which were not under investigation in this study.
Figure 6-5. Structures of Hypermodified Nucleotidyl Bases: Queuosine and Wybutine. These two modified nucleosides are currently the largest ones that have been identified in any nucleic acid. The wybutine base (often designated Y) is found in all organisms next to the anticodon of tRNA^pec in position 37. The queuine base (Q) is found in the wobble position (34) of tRNA^nes, tRNA^eup, tRNA^nem, and tRNA^ev in all organisms except for yeast.
Figure 6-5. Interaction Between Anticodon Loops Complexed with mRNA on Ribosomes. a) Shows the ternary complex of queuosine-modified (Q34) tRNA\textsuperscript{\textasciitilde} in the A-site adjacent to a wyebutine-modified (Y37) tRNA\textsuperscript{\textasciitilde} (blue) in the P-site of a ribosome complexed to a pair of cognate codons in a hexameric mRNA (red). b) Shows the reverse orientation of wyebutine-modified (Y37) tRNA\textsuperscript{\textasciitilde} (blue) in the A-site adjacent to a queuosine-modified (Q34) tRNA\textsuperscript{\textasciitilde} (green) in the P-site of a ribosomal complex. The highly modified Q and Y bases are drawn in yellow. The models are consistent with the structural parameters established by Easterwood and Harvey for tRNA-mRNA-ribosomal interactions. Results indicate that a bend occurring in the mRNA between the A- and P-sites of the ribosome prevents the queuosine and wyebutine modifications from sterically interfering with each other.
In an additional study of macromolecular associations, the G-containing and three modified anti-'
codon stem/loops of tRNA were modeled onto the A-site of either a 5'-GACUUU-3' (GAC-mRNA) or 5'-GAUUUU-3' (GAU-mRNA) transcript to form eight possible tRNA/mRNA complexes. The strength of association of each of these complexes was assessed by temperature-ramping molecular dynamics simulations to determine the effects of the unmodified and modified wobble bases on anticodon-codon interactions. Visual and computational examinations of G-containing, 7-deazaG-modified, 7-am-7-deazaG-modified, and Q-modified tRNA complexes were performed.

Our results suggest that the G-tRNA/GAC-mRNA, 7-deazaG-tRNA/GAC-mRNA, and 7-am-7-deazaG-tRNA/GAC-mRNA complexes were tightly associated through all three anticodon-codon bases from 48 K through 323 K. Through a ramping protocol for increasing temperatures, the GAU-mRNA complexes and both Q-tRNA/GAC-mRNA and Q-tRNA/GAU-mRNA complexes were observed to "retract" the 5' end of their anticodon loops away from the codon beginning at the wobble (34) position. This indicated that G-, 7-deazaG-, and 7-am-7-deazaG-containing anticodon stem/loops associated in a more stable complex with the GAC- over that of GAU-programmed mRNAs. In contrast, the Q-containing anticodon stem/loop did not appear to have a preference for association with either GAC- or GAU-programmed mRNAs.

Total, potential, and kinetic energies for each system were determined during the simulations (Graph 6-2). The total energy of each complex was observed to increase with the temperature ramp. However, there are distinct differences in the energetics patterns of complexes involving the G-containing, 7-deazaG-modified, and 7-am-7-deazaG-modified tRNAs as compared with the Q-modified tRNA complexes. With the rise in temperature during the simulation, those complexes involving a G/C or modified-G/C interaction (traditional Watson-Crick base-pairing) at the wobble position exhibited a more gradual increase in kinetic and potential energies than that observed for queuosine-containing complexes. The rate of increase in total energy for the G-tRNA/GAC-mRNA, 7-deazaG-tRNA/GAC-mRNA, and 7-am-7-deazaG-tRNA/GAC-mRNA complexes was approximately half that of the G-tRNA/GAU-mRNA, 7-deazaG-tRNA/GAU-mRNA, 7-am-7-deazaG-tRNA/GAU-mRNA, and both Q-tRNA complexes with GAC or GAU-mRNAs. The slow rise of energy levels for the second grouping of complexes indicates that the initial geometry assumed for all complexes is less favorable for the non-traditional Watson-Crick pairing structures. Each tRNA associates with either of it's cognate codons by exploiting the flexibility of the anticodon region. In comparing the energy patterns for the four nucleosides studied, we suggest that the reason for the difference in kinetic, potential and total energy pattern of the queuosine-containing complex is not due to the replacement of the guanosine base's N7 atom with a carbon atom, since the G-tRNA and 7-deazaG-tRNA complexes exhibit identical energetics patterns. The effects observed are also unlikely to be due to the charged aminomethyl side chain of the queuosine modification, since the 7-am-7-deazaG-
Graph 6-2. Energetics Analysis of Eight Complexes During Temperature Ramping Studies. The grey lines show the efficiency of the temperature steps during the molecular dynamics simulation. The red line (●) indicates the rise in kinetic energy, the blue line (○) represents the rise in potential energy and the purple line (+) indicates the rise in total energy during the simulations' increases in temperature (❖). These were calculated by a programmed algorithm within the Sybyl software (Advanced computation module). Each temperature step was examined for equilibration time (generally within 10 fs) and the remaining data points for each of 5 fs intervals during the 100 fs step were averaged. Note the similarities in all lines for the complexes involving a guanosine-, 7-deazaguanosine-, or 7-aminomethyl-7-deazaguanosine-containing tRNA and the traditional Watson-Crick partner for the mRNA's codon, cytosine. Also note the similarities in those complexes with the wobble pairing of the mRNA's codon containing uridine with both quenosine-modified tRNA complexes involving cytosine or uridine in the third position.
tRNA energetics patterns also duplicate that observed for G-tRNA and 7-deazaG-tRNA. Instead, the differences detected for complex stability of the G-, 7-deazaG-, and 7-am-7-deazaG-tRNAs versus the Q-tRNA systems are suggested to be caused by the presence of the cyclopentenediol ring moiety that exists only in the queuosine modification.

These results demonstrate that tRNA*sp/mRNA complexes containing the queuosine modification and all those with GAU-programmed codons exist in a higher energy state than those with the more traditional Watson-Crick type interaction. Additionally, we have shown that the modification of tRNA with Q greatly increases structural rigidity of the anticodon loop which is suggested to cause the observed destabilization of the anticodon/codon interaction. These computational modeling studies provide similar results to published experimental data (149,163,164) which indicate that the queuosine modification eliminates the G-containing tRNA's strong codon bias for codons ending in cytidine over uridine. Thus, alterations in RNA structure, represented by these sequence variations, appear to have a significant impact on the ability of the tRNA to efficiently associate with (decode) the sequence of the mRNA transcripts.

The results of this study provides evidence of differences in the hydrogen bonding potential of the Q-modified versus G-containing anticodon triplet in addition to differential rigidity of anticodon loops of Q-modified and G-containing forms of tRNA*sp. Either or both effects of the queuosine modification are suggested to have the potential to restrict the "wobbling ability" of the queuosine base and the anticodon when compared to the G-containing form.

Codon context effects by A- and P-site anticodon loop interactions do not appear to be a factor in systems containing queuosine-modified tRNA anticodon stem and loop structures due to the modeled "kink" in the mRNA between these two sites used in this study. The axes of A- and P-site tRNA molecules sharply angle away from each other which prevents even highly modified nucleosides on adjacent tRNAs, such as that seen for queuosine and wyebutine, from interacting.

Potential coding biases, however, do appear to exist between G-containing and Q-modified tRNA*sp anticodon stem/loop structures for the cognate and pseudo-cognate codons. G-containing tRNA*sp demonstrates a much more stable and energetically favored association with the traditional Watson-Crick type codon GAC, than with the wobble codon GAU. However, Q-modified tRNA*sp molecules exhibit no difference in complex stability with either of mRNA codons GAC or GAU. In addition, both Q-modified tRNA*sp complexes exhibit virtually identical structural and energetic associations as the G-containing tRNA*sp/GAU complex. The bias difference between guanosine- and queuosine-containing systems appears to be primarily due to the inflexibility of the Q-modified anticodon loop and its inability to accommodate the proper positioning required of tight Watson-Crick type associations. The presence of altered electrostatic potential surface (hydrogen bonding) characteristics of the wobble base appears to be subordinate to the strong differences observed in loop flexibility for realistic effects on overall function of Q-modified anticodon loops but may play a synergistic role in the overall function of queuosine-modified tRNA.
In 1982, Yarus proposed an “extended anticodon hypothesis” whereby many of the nucleosides in the anticodon loop are likely to have influence over the structure and the function of the anticodon, particularly in regard to anticodon/codon interaction at the A-site of the ribosome (169). In the following years, his proposition has been verified and extended to the functions of modified nucleosides in stabilizing or destabilizing the anticodon loop dynamics of tRNA (149). Thus, the efficiency and accuracy of translation is not solely limited to the participation of the three anticodon nucleosides. Moreover, modifications of nucleosides in and around the anticodon appear to be important factors effecting the coding potential and efficiency of tRNAs (170).

The results of this research suggest that the queuosine modification is involved with the regulation of gene expression rates in cells via a codon bias mechanism in tRNA-mRNA associations on the ribosome. The proposed mechanism is based on the decreased flexibility of the anticodon produced by the queuosine-induced extended intramolecular hydrogen bonding network of the anticodon loop. This appears to restrict the modified anticodon loop’s ability to accommodate proper positioning for tight Watson-Crick type associations with cognate codons, thereby limiting wobbling capabilities for queuosine-modified tRNA. In eukaryotic tRNA<sup>Met</sup> and tRNA<sup>Tyr</sup>, the cyclopentenediol ring of the queuosine modification is often modified with one or several mannose or galactose moieties, respectively (14,18,19). Individual oligosaccharides or chains of these glycosidic additions may have the potential to establish supplementary hydrogen bond interactions through the loop and up along the groove of the A-type RNA helix of the anticodon stem. If this proves to be true, it would undoubtedly further enhance the structural stabilization of the anticodon loop providing a significantly decreased bias in the coding potential of tRNA<sup>Met</sup> and tRNA<sup>Tyr</sup> molecules.

The cellular level of Q-modified tRNA appears to have strong implications with regard to translational control of gene expression for “biased” genes involved in cellular differentiation, stress response and neoplastic transformation. These events are tightly regulated by the production levels of specific growth control, differentiation inducing and stress response proteins. It is doubtful that the queuosine modification alone is responsible for the dramatic effects needed for phenotypic alterations of cells. However, it is likely that the costly and complex enzymatic formation of this modification has a measurable effect on global cellular physiology, particularly in collaboration with well known mechanisms for transcriptional regulation of specific growth control gene products.
CHAPTER SEVEN
THE EFFECT OF Q ON TERRAN LIFE-FORMS

The research discussed in this document has produced a description of the mammalian queuosine modification system which has been largely based on that found in human cells. This system is dramatically different from the well understood prokaryotic version. For many years it has been assumed that the production of the queuosine modification in tRNA of all organisms was by the same or a similar mechanism.

Since 1973 the transglycosylase enzyme that incorporates the modified base into tRNA has been designated as tRNA:guanine transglycosylase in both *Escherichia coli* and rabbit reticulocytes where it was first found to reversibly exchange a guanine-for-guanine in position 34 of asparagine-, aspartate-, histidine- and tyrosine-encoding tRNAs (60). The first indications that the eukaryotic and prokaryotic queuosine modification systems are different were published in 1976 with evidence of glycosylation of the queuosine residues of tRNA\(^{\text{as}}\) and tRNA\(^{\text{av}}\) in rabbit reticulocyte lysates (14). In 1978 the substrate of the base-for-base exchange leading to the synthesis of queuosine in *E. coli* was determined to be preQ, and not Q (24), while in 1980 it became evident that mammals required the fully-formed Q base for incorporation (11,61).

A comprehensive description of the prokaryotic queuosine modification system has been published (23) and is explained in depth in the introduction of Chapter 3 of this document. It is a synthetic mechanism which uses readily available substrates within the cell to build the modification on intact tRNA. The mammalian system, discussed in Chapters 3 and 4 of this treatise, is based on a utilization scheme which incorporates a specific transport mechanism for the queuine base, an incorporation enzyme that places it directly into position 34 of the involved tRNAs, and a salvage mechanism which can recycle the queuine base from tRNA turnover products for reuse by the incorporation enzyme (Figure 7-1). Even the generalized structures of the incorporation enzymes, which may catalyze a similar reaction with the two different substrates, are dramatically different.

Control of both the prokaryotic and eukaryotic queuosine modification systems is also dissimilar. The modulation of the prokaryotic system is based on transcriptional regulation of the operon which contains two of the four enzymes required for queuosine synthesis. The FIS (factor of inversion stimulation) serves to activate the gene expression for both the “queuine operon” and operons containing sequences for the four substrate tRNAs (114). The mammalian system is shown to be regulated by a post-translational phosphorylation-based mechanism in Chapter 4 of this document. Two of the three components are activated by protein kinase C-catalyzed phosphorylation while deactivated by the action of a protein phosphatase. The kinase-phosphatase reciprocal scheme is a common paradigm observed for the control of important metabolic events in virtually all eukaryotic systems, with protein kinase C a key regulatory switch for cellular growth and differentiation mechanisms.
Figure 7-1. The Cellular Design for the Eukaryotic Queuosine Modification System. This depiction of the eukaryotic system is based on the three step mechanism with modulation described in chapters three and four of this document. It involves an uptake step facilitated by a specific queuosine transmembrane transporter (shown in blue) which is activated by the phosphorylation of protein kinase C (PKC) (shown in green). Once inside the cell, tRNA:guanine ribosyltransferase (TGRase) catalyzes the incorporation reaction of the queuosine base (Q) into tRNA^{am}, tRNA^{am}, tRNA^{am}, or tRNA^{am} with concomitant release of guanine (G). The TGRase enzyme is a heterodimer made up of a larger regulatory subunit (shown in black) which is the target for phosphorylation by PKC, and a smaller catalytic subunit (shown in red). This enzyme is shown as being loosely associated with the membrane since the addition of detergents to the homogenization buffer during protein purification increases the yield of the isolate dramatically. The stoichiometry of phosphorylation and the functional state of the enzyme (dimerized or dissociated, associated with the membrane or soluble in the cytosol) is still in doubt. So a range of possibilities are shown here. Finally, the last step of the queuosine modification system is a salvage step (catalyzed by an unknown mechanism represented in orange) which recycles free queuosine base for the incorporation step from queuosine-5'-monophosphate.
Malfunctions of various steps of the queuosine modification system have been found to correlate with drastic phenotypic changes in cellular appearance and behavior. These include the inability to survive environmental stresses and to undergo developmental differentiation. In addition, queuosine deficiencies have been strongly correlated with increased growth rate, retrograde differentiation, and neoplastic transformation and metastatic progression of mammalian cells. In Chapter 5 of this document, two different human adenocarcinoma cell-lines (colon and breast), both strongly deficient in queuosine levels, were determined to contain lesions of the incorporation and salvage steps, respectively. The \textit{in vivo} methods developed in this research could be used to screen many other cell-lines and biopsies and determine sites of queuosine insufficiency in their queuosine modification systems. In addition, along with the \textit{in vitro} assay methods, these techniques could be used to fully characterize a biopsy's pathological phenotype for neoplastic grading as has been previously suggested (40-43).

Despite this new understanding of the queuosine modification system in eukaryotes and prokaryotes, a realistic physiochemical mechanism for queuosine's effect on cellular metabolism has still not been developed in either set of organisms. In order to discover the purpose and biological function of the queuosine modification of tRNA and explain queuosine's connection to the control of cellular maintenance, differentiation and growth, it is important to determine the most logical place for the greatest influence of this molecule in the cell. There has been some discussion of the queuine base as an activator for tyrosine kinases (15,171), however this has not been pursued as a significant function of the nucleotidyl base and would not account for the correlations of queuosine level fluctuations with such a wide variety of phenotypic alterations that have been observed.

Transfer RNA molecules are involved in many minor cellular functions, including serving as cofactors in retroviral priming, RNA polymerase priming, ATP-dependent ubiquitin-directed proteolysis, sequestration of the glucocorticoid receptor, chloroplast synthesis of δ-aminolevulinic acid and chlorophyll, synthesis of heme and vitamin B_{12}, and prokaryotic cell wall biosynthesis (172). However, \textit{in situ} the tRNA molecule's major role is in protein synthesis. Thus, the most logical site for the global effect of queuosine-modified tRNAs in areas as diverse as development, differentiation, stress-management, and neoplastic transformation is in the control of the translation stage of gene expression.

There are a few defined reactions of the tRNA which might be influenced by the presence or absence of modified bases. Transfer RNAs are involved with both initiation and elongation phases of protein translation, however those molecules involved with the queuosine modification (asparaginyl-, aspartyl-, histidyl-, and tyrosyl-tRNAs) are only utilized during elongation. The tRNA is complexed with several macromolecules during the elongation phase of translation: the cognate aminoacyl synthetase; the transport-specific elongation factor; and the ribosomal complex which includes the mRNA, aminoacyl (A-) site, peptidyl (P-) site, and exit (E-) site tRNAs, rRNAs and rRNPs.

In translation, the first major reaction for tRNA is it's association with a cognate tRNA synthetase. Several researchers have shown that a key identity determinant for proper association of the tRNA with it's
cognate aminoacyl synthetase is in the anticodon (173). However, many tRNAs are identified based on specialized base presence in the DHU or TVC loop and especially (even in the case of the aspartyl-tRNA) in the aminoacyl acceptor arm (174). Kinetics studies of guanosine-containing versus queuosine-modified tRNAs show no significant difference in aminoacylation rates by cognate aminoacyl synthetases (175).

Aminoacylated tRNAs are then required to associate with the ribosome, the site for the translation process. Movement through the ribosome is facilitated by translational elongation factors (EFs). Prokaryotic EF-Tu and eukaryotic EF-1α appear to have the same function in cells. They transport all aminoacyl-tRNAs to the ribosome for association with the ribosomal complex. The EFs do not discriminate when binding to any tRNA family member or isoacceptor. Recently, EF-tRNA complexes have been crystallized and are shown to bind solely to the aminoacyl acceptor arm of the tRNA (176). Therefore, it can be inferred that the presence or absence of a modified base in the anticodon should not have an effect on the association or transport of tRNAs by EFs.

The association of the tRNA with the ribosome is currently a focus of a great amount of research. *E. coli* ribosomal complexes have been the predominant subject for cross-linkage, nuclease protection, and tRNA binding studies (168), with their global structure recently clarified by high resolution cryoelectron microscopy (111). tRNA association with the translation platform appears to be due to the interactions of the EF and aminoacyl acceptor stem of the tRNA with the 23S rRNA, and the tRNA-anticodon with the mRNA (177). Cross-linkage studies have strongly suggested the presence of a ternary interaction between the tRNA-anticodon, the mRNA-codon and the C1400 residue of the 16S rRNA at the wobble position, which may be a stabilizing influence on the complex (168). No evidence of direct rRNP-tRNA interactions have yet been reported.

After the association of the tRNA with the A-site of the ribosome, the formation of the peptide bond (transpeptidation) and translocation of the tRNA to the P-site and then to the E-site takes place at a relatively steady state rate (177). Transpeptidation appears to be catalyzed by the 23S rRNA in the aminoacyl acceptor stem region of the A- and P-site tRNAs, with the translocation of the mRNA and associated tRNAs caused by the shifting of the tRNA molecules causing the subsequent dragging of the entire ribosomal complex along the mRNA transcript (178). Therefore, it is suggested that the presence of the modified wobble base does not have an effect on the rate of transpeptidation or translocation.

Finally, the potential effect of the wobble modification on translational rate is narrowed to either or both the interaction of the incoming tRNA with the P-site tRNA and the association of a tRNA with the mRNA. These are described by well known theories of “codon context” and “codon bias”, respectively. Interaction between neighboring tRNAs in the A- and P-sites of the ribosome have been suggested to be due to the close proximity of the successive codons on the mRNA and the extended nature of a couple of modified bases located near the margins of the anticodon loops: such as wybutine located at position 37 of tRNA^p^he and queuosine in position 34 of tRNA^ms^, tRNA^op^, tRNA^ps^, and tRNA^op^ (4,112,179). However, in the case of tRNA^p^he and tRNA^op^ with the currently accepted models of A-site tRNA/mRNA/P-site tRNA

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conformations, it is shown in Chapter 6 of this treatise that these two bases do not interact. A very recent report has been published indicating that the neither presence nor the absence of the queuosine-modification in tRNA\(^{\text{51}}\) has a dramatic effect on translational frameshifting at the coronavirus frameshift signal (180). This supports the computational model which disputes the biological function of queuosine in codon context effects for such events as ribosomal frameshifting.

Harvey’s group demonstrated that the anticodon loop of tRNAs are functionally and structurally separated from the rest of the molecule by the extended anticodon stem (94). Differences in the strength and stability of the anticodon-codon interaction, caused by an alteration in the flexibility of the anticodon loop, could influence the speed of tRNA placement into the A-site of the ribosome (169). Thus, the flexibility of the anticodon loop may have an effect on the rate of translation in general. Since the wobble base is often modified in tRNA, a modified nucleoside could effect the rate of translation due to it’s ability to associate with the codon’s corresponding base.

Modified bases have been shown to greatly effect the flexibility of the tRNA molecule by influencing ribose puckering, base stacking, and intraloop hydrogen bonding networks (151-155,181). These factors have been shown to greatly increase the ability of the tRNA to efficiently decode their corresponding tRNAs (149,170,182). Results discussed in Chapter 6 of this document show that the presence of the queuosine modification in tRNA\(^{\text{51}}\) does alter both the structural flexibility of the anticodon loop and the complex stability of the anticodon with the mRNA. In the case of tRNA\(^{\text{51}}\), the addition of the queuosine modification dramatically decreases the flexibility of the anticodon loop by establishment of an extended cross-loop hydrogen bonding network, thereby decreasing the stability of a tRNA complex with either the cognate or pseudo-cognate mRNA. In a ribosomally-based context, the modification of the tRNA\(^{\text{51}}\) with queuosine decreases the strong bias of guanosine-containing tRNAs for the traditional Watson-Crick pairing (cognate) over the wobble pairing (pseudo-cognate) for association with the codon on the mRNA.

In a final study performed in this research, a preliminary analysis of codon usage for several oncodevelopmental control and housekeeping transcript sequences was made. This was attempted in order to assess possible effects of the presence or absence of the queuosine modification on protein synthesis of these important proteins. Over time, the identity of the tRNA wobble base (queuosine or guanosine) could have a global effect on the metabolism and, potentially, phenotypic alteration of a cell.

Queuosine-modified tRNAs (asparaginyl-, aspartyl-, histidyl-, and tyrosyl-) translate four specific codons ending in either the traditional Watson-Crick base cytosine (C) or wobble base uridine (U) (Table 7-I). An analysis of the codon usage for these codons was performed for a sampling of several housekeeping and oncodevelopmental genes. The results are shown in Table 7-II. The percent usage of queuosine-involved from all transcript codons varies from 10 to 17 percent with no consistent trend based on housekeeping versus oncodevelopmental status. Although this does not seem to be a large percentage of residues, these four amino acids often play a major role in structural stabilization and function of
Figure 7-2. The Genetic Code

Start and stop codons are indicated. Queuosine-related codons, darkly shaded, are encoded by the letters NAU or NAC (N indicates any nucleotide base). Tyrosine-encoding codons begin with uridine (U), histidine-encoding codons begin with cytosine (C), asparagine-encoding codons begin with adenosine (A), and aspartate-encoding codons begin with guanosine (G).
proteins based on either their proton or electron transfer capabilities. An important observation of these codon patterns, however, is that the ratio of U (wobble base) to C (Watson-Crick base) in the wobble position of these codons differs between the housekeeping versus oncodevelopmental transcripts. In the overall nucleic acid sequence comparisons for the involved amino acid codons, the U to C wobble ratios demonstrate a large gap between the housekeeping transcripts (greater than 0.71) and the oncodevelopmental transcripts (less than 0.32) for those sequences examined. Thus, it is suggested that there may be a bias between these two classes of gene sequences which is able to be exploited by the guanosine-containing versus queuosine-modified tRNA translational machinery.

In developing a queuosine-associated codon bias hypothesis, normal and cancer cells would have different protein synthetic levels for specific proteins based on the codon usage of each mRNA sequence and the levels of queuosine-modified tRNA levels within the cells. Cells exhibiting normal queuosine-modified tRNA levels would produce housekeeping proteins such as ornithine decarboxylase, phospholipase A₂, β-galactosidase, α-actin, and α- and β-globin in similar quantities to oncodevelopmental protein assuming a comparable number of transcript copies. This is suggested to occur due to the lack of preference of queuosine-modified tRNAs for either NAC or NAU codon. In neoplastic cells, which contain elevated levels of guanosine-containing tRNAs, oncodevelopmental proteins such as c-fos, N-myc, c-Jun, and c-myc would be translated much more efficiently and rapidly than “usual” due to the more stable association of guanosine-containing tRNA to NAC over NAU codons.

Current dogma accepts that transcriptional regulation of oncodevelopmental genes in normal cells greatly reduces the quantity of these transcripts when compared to housekeeping mRNAs. Dual control of the expression of these growth control genes through both transcriptional and translational regulation could effectively modulate the levels of these important gene products. Thus, fully queuosine-modified normal cells would produce the housekeeping proteins in much larger quantities relative to the highly influential oncodevelopmental proteins which must be maintained at very low intracellular levels.

This document proposes a codon bias-directed hypothesis explaining the effect of the queuosine modification on cellular physiology. The organization of the queuosine modification system in prokaryotes and eukaryotes is now known. In addition, regulatory mechanisms for wiring the modification systems in with cellular metabolism have been identified. Thus, the cellular dynamics of the queuosine modification’s formation in tRNA, as well as the molecular dynamics of queuosine’s effect in protein translation are regulated in a cellular physiological context. This theory appears to propose a logical mechanism for how the loss of normal queuosine levels could cause the shift of the cellular life-cycle from stasis to retrograde-differentiation, and neoplastic transformation to tumor progression.
Table 7-1. *Wobble Base Bias of Codons Decoded by* tRNA<sup>asn</sup>, tRNA<sup>asp</sup>, tRNA<sup>his</sup> and tRNA<sup>tyr</sup> *in Selected Human Gene Transcripts*

<table>
<thead>
<tr>
<th>Human Gene Transcript</th>
<th>Total Number of Codons</th>
<th>Number of Q Codons (%)</th>
<th>Wobble U/C Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ornithine Decarboxylase</td>
<td>461</td>
<td>73 (16%)</td>
<td>2.04</td>
</tr>
<tr>
<td>Phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
<td>749</td>
<td>126 (17%)</td>
<td>1.93</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>677</td>
<td>114 (17%)</td>
<td>1.00</td>
</tr>
<tr>
<td>N-ras</td>
<td>189</td>
<td>32 (17%)</td>
<td>0.88</td>
</tr>
<tr>
<td>α-Actin</td>
<td>337</td>
<td>58 (17%)</td>
<td>0.81</td>
</tr>
<tr>
<td>β-Globin</td>
<td>107</td>
<td>24 (22%)</td>
<td>0.71</td>
</tr>
<tr>
<td>Fos</td>
<td>364</td>
<td>37 (10%)</td>
<td>0.32</td>
</tr>
<tr>
<td>N-myc</td>
<td>456</td>
<td>53 (12%)</td>
<td>0.29</td>
</tr>
<tr>
<td>Leukemia Inhibitory Factor</td>
<td>202</td>
<td>35 (17%)</td>
<td>0.25</td>
</tr>
<tr>
<td>JunA</td>
<td>331</td>
<td>33 (10%)</td>
<td>0.14</td>
</tr>
<tr>
<td>C-myc</td>
<td>439</td>
<td>63 (14%)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

All analyses were performed manually. The total number of codons, Q codons (those encoding for asparagine, aspartate, hisidine, and tyrosine), and those Q codons that ended in U or in C were determined based on visual inspection of the coding sequences for the gene transcripts. The percentage of total codons that involved Q and wobble U/C ratio was calculated based on these observations.
REFERENCES


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36 Witte, C. D. (1990), Department of Chemistry and Biochemistry. Old Dominion University, Norfolk, Virginia USA, pp. 67.

37 Santos, J. M. (1993), Department of Chemistry and Biochemistry. Old Dominion University, Norfolk, Virginia USA, pp. 59.


135 Elliott, M. S. and Crane, D. L. (1990) *Biochemical and Biophysical Research Communications*, 171, 393-400.


140 Crane, D. L. (1988), Department of Chemistry and Biochemistry. Old Dominion University, Norfolk, Virginia USA, pp. 124.

141 Brooks, B. J. (1989), Department of Chemistry and Biochemistry. Old Dominion University, Norfolk, Virginia USA, pp. 122.


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APPENDIX

SEPARATION OF QUEUOSINE-MODIFIED tRNA ISOACCEPTOR FAMILIES
BY RPC-5 AND W-POREX C4 LIQUID CHROMATOGRAPHY

Introduction

An early method developed to directly measure levels of the queuosine modification in tRNA is based on reversed phase C-5 (RPC-5) medium pressure liquid chromatography. The protocol employs the use of an RPC-5 column matrix composed of 10 micron polychlorofluoroethylene particles coated with trialkylmethylammonium chloride-containing alkyl groups ranging in length from C_4 to C_10. The RPC-5 methodology has been applied to the separation of tRNA species from many sources (183, 184), and is the standard technique applied to the chromatographic analysis of queuosine-modified tRNAs (21, 91, 185). Despite its successful application, the RPC-5 methodology presents some serious limitations and disadvantages. The RPC-5 matrix does not separate the different isoacceptor families with high efficiency. For example, it is possible to resolve the queuosine-modified from unmodified forms of aminoacylated tRNAs. However, the separation of tRNA Exp, tRNA Arg, tRNA His, and tRNA Tyr species is not possible. Furthermore, the RPC-5 matrix does not tolerate repeated pressurized applications with reproducible resolution. In addition, the use of organic solvents for optimizing separations on the RPC-5 column can lead to degradation of the matrix, where alkyl groups are gradually stripped away from the inert support. The rate of this deterioration can be decreased by the addition of a trace amount of trialkylammonium chloride (Adogen 264) to the running buffer (Farkas, pers. commun.). Ultimately, the matrix must be removed from the column to be regenerated. This takes time and compounds problems with reproducibility between reconditioned batches. Finally, and maybe most importantly, the RPC-5 matrix is no longer commercially available.

An alternative high pressure liquid chromatography (HPLC) column matrix has shown promise as a potential replacement for RPC-5 in analysis of queuosine modification levels in tRNA. The W-Porex C4 HPLC column has been successfully applied to the separation of bulk tRNAs isolated from bovine liver and wheat germ, as well as snRNA from L929 cells (35). The column matrix consists of an inert support of 5 micron porous silica particles with a uniform pore size of 30 nm. A C_4 carbon chain bound to the support provides the hydrophobic reversed phase for separation. The W-Porex C4 matrix has been demonstrated to significantly improve separation of bulk tRNAs over that of RPC-5 (35). This column matrix also has a high capacity for tRNA binding (1 to 20 mg), high durability, and excellent resolution over high pressure applications (60 to 90 BAR at a flow rate of 1 mL/min). In studies shown here, the W-Porex C4 column was tested for the ability to resolve the four families of the queuosine-containing tRNAs, and for the ability to separate individual queuosine-modified from unmodified tRNA isoacceptors.
Materials and Methods

Transfer RNA from promyelocytic leukemia (HL-60), chronic lymphocytic leukemia (CLL), acute myelocytic leukemia (AML), and normal human foreskin fibroblast (HFF) cells were isolated using established methods (21,91).

In vivo labelling of queuosine-containing tRNA from HL-60 cells was performed by exposure of the cell cultures to a radiolabeled queuine analog (rQT₃; specific activity 0.19 Ci/mmol, 0.1 µCi/mL). The cells were grown in a 150 cc culture flask to a density of 2 x 10⁶ cells/mL, then exposed to 100 nM rQT₃ for 24 hours. Finally, the tRNA was isolated as mentioned above.

Enhancement of queuosine-deficient HL-60 tRNA was performed by treatment of cultures with 7-methylguanine, an inhibitor of TGRase activity. Enhancement of queuosine-modified HL-60 tRNA populations was performed by the addition of excess queuine to the media. These tRNA populations were used to assess shifts in the levels of queuosine-modified versus unmodified tRNA isoacceptors when compared to untreated HL-60 tRNA. All cells were grown in 500 mL cultures to a density of 2 x 10⁶ cells/mL and exposed to 10 µM 7-methylguanine or 0.10 A₃₆₀ units of queuine for 24 hours. Again, the tRNA was isolated as above.

In vitro aminoacylation of tRNA with ³H-aspartic acid, ³H-histidine, and ³H-tyrosine or unlabeled amino acids was performed using isolates from 0.10 A₃₆₀ queuine-treated and 10 µM 7-methylguanine-treated HL-60 cells. Mouse liver aminoacyl-tRNA synthetase, isolated by established methods, was utilized to catalyze the aminoacylation reaction. Enzyme isolation and aminoacylation reaction conditions were performed as previously described (21,91).

Unlabelled tRNA, rQT₃-labeled tRNA, and ³H-aminoacyl tRNA were analyzed by W-Porex C4 HPLC. Generation of tRNA profiles was accomplished using a 250 x 4.6 mm i.d. W-Porex C4 column purchased from Phenomenex (Rancho Palos Verdes, CA). A programmable LKB Ultrachrom GTi Bioseparation system was used to develop running conditions for the column. All buffers were filtered through 0.22 µm filter and degassed prior to column equilibration and sample analysis. The optimal conditions for separation and analysis of queuosine-containing tRNAs were developed at a flow rate of 1.0 mL/min with a back pressure of 70 to 90 BAR based on elution conditions described by Dudock (35). A linear decreasing salt gradient was applied for separation of tRNAs consisting of 1.0 M to 0 M ammonium sulfate in 0.10 M potassium phosphate buffer (pH 7.0) modified with 0.75% isopropanol. The column was equilibrated in 1.0 M ammonium sulfate containing buffer at 1.0 mL/min for 10 minutes prior to analysis of tRNA samples. A linear descending salt gradient from 1.0 M ammonium sulfate to 0.20 M ammonium sulfate was applied for 80 minutes, then the salt concentration was reduced to 0 M over 20 minutes. The column was rinsed an additional 10 minutes with no salt buffer, brought back to 1.0 M salt over a one minute interval and re-equilibrated in 1.0 M ammonium sulfate containing buffer for ten minutes prior to the next analysis. On the final run of the day, the column was left in no salt buffer and the flow rate was
reduced to 0.1 mL/min and allowed to run continuously overnight. The rate of gradient change could be altered to enhance resolution of specific peaks either early or late in the chromatogram, but the simple linear gradient demonstrated adequate efficiency at separating the queuosine-containing tRNA isoacceptors.

The HPLC profiles of tRNA from rQT<sub>3</sub>-treated HL-60 cells were used to identify the retention times of the four rQT<sub>3</sub>-modified tRNAs. Isolated 0.10 A<sub>260</sub> queuine-treated HL-60 tRNA samples were radiolabeled in an aminoacylation reaction and the retention times determined. The tRNA isolated from 7-methylguanine treated HL-60 cells was subjected to a radiolabeling aminoacylation reaction and analyzed. Comparisons between differentially <sup>3</sup>H-aminoacylated tRNA profiles of 0.10 A<sub>260</sub> queuine-treated and 7-methylguanine-treated HL-60 tRNAs in order to determine the isoacceptor identity of each "peak" and the relative amounts of queuosine-modified and unmodified tRNAs were determined based on the established retention times for HL-60 tRNA.

Results and Discussion

Transfer RNA, isolated from several human cultured cell-lines (HL-60, AML, CLL, and HFF), was subjected to HPLC using a W-Porex C4 column in order to determine the best running conditions for effective resolution of different populations of tRNA isoacceptors (Figure A-1). The ability of this column matrix to perform good separation of bulk tRNA populations is a particular feature of this resin, and has been shown to be more proficient than the RPC-5 matrix (35). The optimal running conditions for the separation of the HL-60 tRNA isoacceptors was established at 1.0 mL/min which provided a back-pressure of 70 to 90 Bar for the HPLC system (36). The elution is controlled using a linear decreasing ammonium sulfate gradient from 1.0 M to 0.2 M in 0.10 M potassium phosphate buffer (pH 7.0) over 80 minutes, and finally to 0.0 M over an additional 20 minutes (37).

Parallel tRNA samples were isolated from HL-60 and CLL cultures that were either exposed to non-radiolabeled queuine or to a radiolabeled queuine analogue, rQT<sub>3</sub>, for 24 hours. The unlabeled control tRNA was aminoacylated in three reaction mixtures with one of the three available tritiated amino acids (<sup>3</sup>H-aspartate, <sup>3</sup>H-histidine, or <sup>3</sup>H-tyrosine). The tRNA containing the rQT<sub>3</sub> label was aminoacylated with non-radioactive amino acids. Each reaction mixture was applied to the W-Porex C4 HPLC system. The profile overlays for tritiated aminoacylated (Figure A-2) or tritiated queuosine-containing tRNAs (Figure A-3) are shown with identifying labels. Four distinct regions of radioactivity for queuosine-containing tRNAs were detected at retention times of 6, 21, 55, and 70 minutes. This is suggested to be due to the separation of the four different possible queuosine-modified tRNA isoacceptors. RPC-5 medium pressure liquid chromatography does not have the ability to resolve tRNA isoacceptors, and can only differentiate glycosylated from non-glycosylated forms of queuosine-modified tRNAs (87).

To verify that the four peaks detected with the <i>in vivo</i> rQT<sub>3</sub> labeling experiment were due to four distinct tRNA isoacceptors, another study was begun to detect both specific tRNA isoacceptors and
Figure A-1. W-Porex C4 HPLC Profiles of Transfer RNA Isolated from Four Human Cell Cultures. Purified tRNA samples (0.5 mg) from chronic lymphocytic leukemia (CLL), acute myelocytic leukemia (AML), promyelocytic leukemia (HL-60), and normal human fibroblast (HFF) cells were separated by an 80 minute decreasing salt gradient of 1.0 M to 0.2 M (NH₄)₂SO₄ in 0.10 M potassium phosphate buffer (pH 7.0) modified with 0.75% isopropanol. The running buffer was then held at 0.2 M (NH₄)₂SO₄ for 20 minutes. The samples were eluted on a 250 x 4.6 mm Phenomenex W-Porex C4 column at 1 mL/min and monitored by an on-line UV detector which measured the absorbance of the flow at 254 nm.
Figure A-2. Aminoacylated Transfer RNAs from Chronic Lymphocytic and Promyelocytic Leukemia Cells. Purified transfer RNAs from CLL and HL-60 cells exposed in vivo to 0.10 A_{260} units of queuine were each aminoacylated, in three separate reactions, with 3H-aspartate, 3H-tyrosine, or 3H-histidine. In each reaction 50 μg of tRNA was charged with 3H-amino acid (0.5 μCi) in the presence of semi-purified mouse liver aminoacyl-tRNA synthetase in a total volume of 150 μL. Each sample was separated by W-Porex C4 HPLC by a decreasing salt gradient of 1.0 M to 0.2 M (NH₄)₂SO₄ over 80 minutes, then held at 0.2 M (NH₄)₂SO₄ for 20 minutes at a flow rate of 1 mL/min. Elution fractions were collected at 0.5 minute intervals and analyzed by scintillation. This figure represents an overlay of the three chromatograms (one for each 3H-amino acid) for tRNA isolated derived from CLL and HL-60 cells, respectively. Backgrounds were subtracted to normalize the baselines relative to each other.
Figure A-3. Queuosine-Containing Transfer RNAs from Chronic Lymphocytic and Promyelocytic Leukemia Cells. Transfer RNA from CLL and HL-60 cells exposed to 100 nM rQT in culture was isolated and a sample (0.5 mg) was added to a reaction mixture for aminoacylation with non-radiolabeled amino acids. The reaction mixture was separated by a decreasing salt gradient of 1.0 M to 0.2 M (NH₄)₂SO₄ over 80 minutes, then held at 0.2 M (NH₄)₂SO₄ for 20 minutes at a flow rate of 1 mL/min. Eluent fractions were collected at 0.5 minute intervals and analyzed by liquid scintillation. The figure shows four major radioactive peaks returned from each cell-type corresponding to the four queuosine-containing tRNA species. The peaks were assigned identities by comparison to the elution patterns of ³H-aminoacylated tRNAs seen in Figure A-2. *The tRNA⁸⁶ peaks were assigned by process of elimination, since no ³H-asparagine was available at the time of these studies.
visualize each queuosine-modified and deficient forms. The HL-60 cell-line is known to be naturally 60 to 80% queuosine-deficient, so the cells were exposed in culture to two effectors of the queuosine modification system in order to exaggerate the queuosine-modified and queuosine-deficient populations of tRNA. Parallel cultures of HL-60 cells were treated for 24 hours with either an additional 0.10 A<sub>260</sub> queuine to induce saturation of the queuosine-modified tRNA isoacceptors, or 10 μM 7-methylguanine which is a competitive inhibitor (used at 10 times the K<sub>i</sub>) of the queuine incorporation enzyme. The tRNA fractions of these cultures were isolated and in vitro aminoacylation reactions were performed with each of three specific radiolabeled amino acid substrates (<sup>3</sup>H-histidine, <sup>3</sup>H-tyrosine, and <sup>3</sup>H-aspartate). Radiolabeled asparagine, the remaining queuosine-related isoacceptor, was not commercially available at the time of this study. Overlays of each amino acid’s chromatographic profiles for both queuine-treated and 7-methylguanine-treated tRNA samples are shown in Figure A-4.

The <sup>3</sup>H-histidyl tRNA profiles of queuine-saturated and queuine-deficient both exhibit three peaks of identical retention times. The 6 minute peak is likely to be the flow-through of excess radiolabeled amino acid remaining from the aminoacylation reaction. The peaks at 57 and 67 minutes correspond to tRNA<sup>his</sup> isoacceptors. Integration of the two peaks indicates that the 0.10 A<sub>260</sub> queuine-treated sample contains a 62 to 38% ratio of area from the first to the second peak, while the 7-methylguanine-treated sample has a ratio of 48 to 52% area. Therefore, the 57 minute peak corresponds to the queuosine-modified tRNA<sup>his</sup> isoacceptor, with the unmodified tRNA<sup>his</sup> eluting at 67 minutes. The incomplete saturation of the modification as seen in these percentages of shift was not unexpected given unpublished observations in this laboratory. However, the limited extent of the shift is somewhat disconcerting, since these conditions have been published as being effective concentrations of effectors of the queuosine modification system and utilized in many cell culture studies (116).

The <sup>3</sup>H-tyrosyl tRNA profiles of queuine-saturated and queuine-deficient samples both exhibit three peaks of identical retention times. The 7 minute peak is the flow through of excess radiolabeled amino acid remaining from the aminoacylation reaction. The peaks at 18 and 25 minutes correspond to tRNA<sup>tyr</sup> isoacceptors. Integration of the two peaks indicates that the 0.10 A<sub>260</sub> queuine-treated sample contains a 82 to 18% ratio of area from the first to the second peak, while the 7-methylguanine-treated sample has a ratio of 47 to 53% area. It is concluded that the 18 minute peak corresponds to the queuosine-modified tRNA<sup>tyr</sup> isoacceptor, with the unmodified tRNA<sup>tyr</sup> eluting at 25 minutes. As seen with the histidyl tRNA profiles, incomplete saturation and inhibition of the queuosine modification system is in evidence with this aminoacyl tRNA also, although not quite to the same extent as with tRNA<sup>his</sup>.

The <sup>3</sup>H-aspartyl tRNA profiles of queuine-saturated and queuine-deficient samples both exhibit two peaks of identical retention times. The 5 minute peak may be due to either the flow through of excess radiolabeled amino acid remaining from the aminoacylation reaction, or it may also be a significant peak with a second peak eluting at a retention time of 8 minutes. Assuming (for sake of argument) that the first peak is due entirely to one form of tRNA<sup>asp</sup> isoacceptor, integration of the two peaks indicates that the 0.10
A_{360} queuine-treated sample contains a 99 to 1% ratio of area from the first to the second peak, while the 7-methylguanine-treated sample has a very similar ratio of 98 to 2% area. This suggests that the 5 minute peak corresponds to the queuosine-modified tRNA^{\*} isoacceptor, with the unmodified tRNA^{\*} eluting at 8 minutes. As seen with both the histidyl and tyrosyl tRNA profiles, incomplete inhibition of the queuosine modification system is in evidence with this isoacceptor. Therefore, years of unpublished observations in this laboratory and elsewhere (29) regarding a non-uniform and preferential loss of the queuosine modification by certain isoacceptors are corroborated by the lack of complete inhibition of the queuosine incorporation enzyme expected at these published concentrations for 7-methylguanine (76). In addition, the inhibition of queuosine modification for aminoacetyl tRNA isoacceptors is not a global effect but occurs in a specific order with the tRNA^{\*} and tRNA^{\*} isoacceptors exhibiting the greatest resistance to queuosine decreases.

A final comparison of all three profiles and table of values (Table A-I) suggests that one combined aminoacylation reaction (\(^3\)H-histidyl, \(^3\)H-tyrosyl, and \(^3\)H-aspartyl labeled tRNA) mixture run on the W-Porex C4 HPLC system can separate both the tRNA isoacceptors and the queuosine-modified from unmodified forms. An experiment reacting the rQT\(_3\) labeled tRNA population with individually labeled amino acids and a mixed reaction of all four amino acids is currently underway. However, the results of this preliminary study also suggests that, were radiolabeled asparagine available, a combined reaction assay of all four aminoacetyl tRNAs would resolve all four possible isoacceptors and their queuosine-modified and unmodified forms. This contention is based on the resolution of four distinct regions of rQT\(_3\) incorporated tRNAs from \(in vivo\) studies. The results of the W-Porex C4 HPLC method is in direct contrast with chromatographs from established RPC-5 protocols which separate queuosine-modified from unmodified forms but do show acceptable resolution between isoacceptors. The RPC-5 methodology can, however, identify glycosylated from unglycosylated queuosine-containing tRNAs, while the W-Porex C4 HPLC system does not appear to be able to do this.

The W-Porex C4 HPLC method is, therefore, a very effective method for the separation and quantification of queuosine-modified tRNAs. Although it does not resolve glycosylated from unglycosylated modifications, it does have the advantage over RPC-5 medium pressure liquid chromatography of the ability to simultaneously detect the four tRNA isoacceptors and each queuosine-modified from unmodified form. This is important due to the current limitations of the RPC-5 methodology which is no longer commercially available and has several other drawbacks including rapid deterioration of the matrix by both solvent exposure and pressurized running conditions. Both liquid chromatographic procedures require rather large amounts of sample to analyze. Although the chromatographs shown in this chapter were run on the W-Porex C4 column with sample sizes of as little as 23 \(\mu\)g tRNA, most protocols would require 1 to 10 mg tRNA for efficient resolution. Therefore, it is important to note that this suggested sample size can limit the type of applications that could utilize this technology.
Figure A-4. Aminoacylated Transfer RNAs from Queuosine-Modified and Queuosine-Deficient Promyelocytic Leukemia Cells. The shift of $^3$H-aminoacylated tRNA peaks was analyzed for tRNA isolated from HL-60 cells exposed to 0.10 A$_{260}$ units of unlabeled queuine to help produce a fully queuosine-modified compliment of tRNAs (solid lines) and those who were pre-exposed for 24 hours to 10 μM 7-methylguanine, a competitive inhibitor for the queuine incorporation enzyme (dashed lines). HL-60 tRNA samples (50 μg) were placed in aminoacylation reactions with partially-purified mouse liver aminocyl-tRNA synthetase in three separate reactions with either $^3$H-aspartate, $^3$H-tyrosine, or $^3$H-histidine (0.5 μCi each). The samples were separated by W-Porex C-4 HPLC by a decreasing salt gradient of 1.0 M to 0.2 M (NH$_4$)$_2$SO$_4$ over 80 minutes, then held at 0.2 M (NH$_4$)$_2$SO$_4$ for 20 minutes at a flow rate of 1.0 mL/min. Elution fractions were collected at 0.5 minute intervals and analyzed by liquid scintillation. Backgrounds were subtracted to normalize the baselines relative to each other.
Table A-1. Retention Times and Percent Compositions of Major Peaks in HL-60 tRNA Samples.

<table>
<thead>
<tr>
<th></th>
<th>Queuine-treated HL-60 tRNA Isolates</th>
<th>7-methylGuanine-treated HL-60 tRNA Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_t$ = 5 minutes</td>
<td>$R_t$ = 8 minutes</td>
</tr>
<tr>
<td>$^3$H-aspartyl peaks</td>
<td>99%</td>
<td>1%</td>
</tr>
<tr>
<td>$^3$H-tyrosyl peaks</td>
<td>$R_t$ = 18 minutes</td>
<td>$R_t$ = 25 minutes</td>
</tr>
<tr>
<td></td>
<td>82%</td>
<td>18%</td>
</tr>
<tr>
<td>$^3$H-histidyl peaks</td>
<td>$R_t$ = 57 minutes</td>
<td>$R_t$ = 67 minutes</td>
</tr>
<tr>
<td></td>
<td>62%</td>
<td>38%</td>
</tr>
</tbody>
</table>

The retention times ($R_t$) for each of the three aminoacylated HL-60 tRNA isoacceptor pairs were based on the results depicted in Figures A-3 and A-4. The first peak suggested to contain queuosine-modified tRNA and the second the unmodified guanosine-containing form. The tRNA populations were purified samples from parallel HL-60 cultures, that had been exposed for 24 hours to either 0.10 $A_{400}$ units of exogenous queuine (to attempt to fully saturate the tRNA) or 10 $\mu$M 7-methylguanine (a strong competitive inhibitor for the queuine incorporation enzyme responsible for the formation of queuosine). Percent composition was ascertained from integration of the chromatographic profile shown in Figure A-4. Despite the assumption that the queuine-saturated and 7-methylguanine-treated cells should contain homogenous populations of queuosine-modified or queuosine-deficient tRNAs, this was not observed. A shift in the peak areas, however, was determined and exhibited the expected trend.
CURRICULUM VITAE

EDUCATION
1997
Old Dominion University/Eastern Virginia Medical School, Norfolk, Va
Ph.D Biomedical Sciences, Biological Chemistry Track
GPA 3.94, Selected for Phi Kappa Phi Honor Society
1990
College Of William And Mary In Virginia, Williamsburg, Va
B.S. Major in Biology, Minor in Music History
GPA 3.25, Selected for Alpha Lambda Delta and Phi Eta Sigma Honor Societies

TEACHING EXPERIENCE
1996-1997
Department Of Chemistry And Biochemistry, Old Dominion University, Norfolk, Va
Adjunct Assistant Faculty in the Chemical and Biochemical Sciences
Teaching Assistant
Instructor and Demonstrator for National Science Foundation “Young Investigator’s Program”
Summer 1994
Department Of Sciences, Tidewater Community College, Portsmouth, Va
Adjunct Faculty in the Biochemical Sciences

LABORATORY EXPERIENCE
1991-1997
Department Of Chemistry And Biochemistry, Old Dominion University, Norfolk, Va
Ph.D Student/Special Doctoral Research Assistant
1989-1990
Department Of Biological Sciences, College Of William And Mary In Virginia, Williamsburg, Va
Research Assistant
Summer 1989
Walter Reed Army Institute Of Medical Research, Washington, D.C.
Contract Research Assistant
1988-1989
Department Of Biological Sciences, College Of William And Mary In Virginia, Williamsburg, Va
Research Assistant

GRANTS, FUNDED PROPOSALS AND AWARDS
Department Of Chemistry And Biochemistry, Old Dominion University, Norfolk, Va

- Summer Graduate Student Research Grant Program, 1992 & 1993
- “Isolation and characterization of mammalian tRNA-guanine ribosyltransferases.” 1995
- “Determination of computer literacy knowledge required of successful graduates of physical and
  life sciences programs and Development of a course in scientific computing.” 1996

Submitted 1994
National Science Foundation-ILI Proposal, Washington, D.C.
“Molecular Modeling Workstations,” Co-authored (not Co-PI) with Dr. M.S. Elliott
Funded 1996-1997
Horsley Cancer Foundation Proposal, Charlottesville, Va
“Isolation and characterization of a cDNA for mammalian tRNA-guanine ribosyltransferase,”
Co-authored (not Co-PI) with Dr. M.S. Elliott

PUBLICATIONS
Refereed Articles
  “Activation of transfer RNA-guanine ribosyltransferase by protein kinase C”, Nucleic Acids
- Morris, R.C., Brooks, B.J., Hart, K.L. and M.S. Elliott. “Modulation of queuosine uptake and
  incorporation into tRNA by protein kinase C and protein phosphatase”, Biochimica et Biophysica
- Morris, R.C., Brown, K.G. and M.S. Elliott. “The effect of queuosine on tRNA structure and
  function”, under Review at Journal of Molecular Biology.
- Morris, R.C., Galicia, M.C., Clase, K.L. and M.S. Elliott. “Specific abnormalities in the queuosine
  modification systems of two neoplastic human cell-lines”, under Review at “Biochimica et
  Biophysica Acta .
- Santos, J.M., Witte, C.D., Morris, R.C. and M.S. Elliott. “Separation of queuosine-containing
  transfer RNA isoacceptors by reversed phase W-Porex C4 high pressure liquid chromatography”.
- submitted to Analytical Biochemistry, currently under revision.
- Morris, R.C., Gresham, J.K. and M.S. Elliott. “Isolation and Characterization of Two Mammalian
  transfer RNA-guanine ribosyltransferases”, manuscript in preparation for submission to Biochimie.

Textbooks
- Bell, Clark, Rodig and Taber. Organic Chemistry Laboratory: standard and microscale
  Graphics & Illustrations, Layout & Design, and Editing by Rana C. Morris.

PRESENTATIONS
- “The IQ of tRNA: The Effect of the Wobble Base on Function,” seminar, Department of Chemistry
  and Biochemistry, Old Dominion University, Norfolk, VA. November, 1993.
- “Modulation of the Queuosine Modification System by Protein Kinase C and Protein Phosphatase
  1a,” seminar. Department of Chemistry and Biochemistry, Old Dominion University. Norfolk, VA.
  April, 1995.