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Kretz, Keith Allan

ANTICODON MODIFICATIONS OF TRANSFER-RNA AND CELL DIFFERENTIATION

The Ohio State University

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University Microfilms International
ANTICODON MODIFICATIONS OF TRANSFER RNA
AND CELL DIFFERENTIATION

A DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Keith A. Kretz, B.A.

* * * * *

The Ohio State University
1987

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For My Wife, Patricia
ACKNOWLEDGEMENTS

I am deeply indebted to Dr. Ronald W. Trewyn for his patience and guidance throughout the duration of this project. I am also sincerely grateful to Dr. Henri Grosjean, Dr. Bernard French, Eric Utz, and Dawn Patrick without whose help many of the technical aspects of this project could not have been completed. I would also like to thank Dr. Gerard Keith for his kind gift of yeast tRNA and Dr. Jon Katze for his gifts of queuine and RPC-5 materials.
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Research Publications


Abstracts


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INTRODUCTION

Following the demonstration that DNA is the genetic material of cells and the description of the DNA double helix by James Watson and Francis Crick, there was much debate as to the mechanisms by which the nucleic acids could code for cellular proteins. Watson and Crick quickly deduced the method of replication of DNA, but a mechanism for protein synthesis was not so readily apparent. An early clue came in 1957 when Hoagland et al. (1957) demonstrated a "pH 5 enzyme" activity which incorporated radioactive leucine into a low molecular weight RNA component of the enzyme fraction. They demonstrated that this RNA-bound leucine could then be transferred to microsomal protein when incubated with microsomes and GTP (Hoagland et al., 1957). In 1958, Crick proposed the idea of an "adaptor" molecule to bridge the gap between the genetic template and amino acids during protein synthesis. Today we know that the RNA component of Hoagland's system was Crick's adaptor molecule, namely transfer RNA.

Transfer RNAs are a group of small (ca. 25,000 Daltons) RNA molecules, 73-93 nucleotides in length, containing many modified bases. It is now known that
these molecules are not only used as adaptor molecules in protein synthesis, but they also have important functions in a wide variety of cellular control processes.

Research in the field of tRNA has moved quickly. In 1958, Zachau et al. (1958) determined that the amino acids are bound to the ribose of the 3' terminal adenosine of tRNA through an ester linkage. In 1959, Holley and Merrill (1959) reported the purification of a yeast tRNA^Ala species, and a few years later Holley et al. (1965) had determined the sequence. By 1961, it was generally believed that in the genetic code, a sequence of 3 nucleotides codes for an amino acid and these triplets (codons) were read in a non-overlapping, sequential manner to produce proteins (Crick et al., 1961). Marshall Nirenberg, in 1961, found the first clue to breaking the genetic code when he developed a cell-free protein synthesizing system and determined that poly-U coded for poly-phenylalanine (Nirenberg, 1963). It was quickly determined that poly-A led to the synthesis of poly-lysine and poly-C led to the synthesis of poly-proline. Soon the synthesis of random polyribonucleotides led to the deduction of the composition of the codons corresponding to each of the twenty amino acids, but the actual sequence of the individual codons was not yet determined. In 1964, Nirenberg and Leder (1964) discovered that trinucleotides promote the binding of specific tRNA molecules to
ribosomes in the absence of protein synthesis. Binding studies with all of the possible trinucleotides resulted in clearly determining the amino-acid coding of approximately 50 of the 64 codons. Not until H. Gobind Khorana developed a method for synthesizing polyribonucleotides with a defined repeating sequence was the entire code elucidated (Stryer, 1981).

Khorana initially used organic chemical methods to synthesize 2 complementary deoxyribonucleotides each with 9 residues \([d(TAC)_3 \text{ and } d(GTA)_3]\) which were used as templates for the synthesis of long DNA chains. These long DNA chains, existing in a double helix, were used as templates for transcription of the corresponding RNA strands using RNA polymerase. Because the DNA strands each contained only 3 of the 4 possible nucleotides, the strand to be transcribed could be selected by adding only 3 of the ribonucleotides. When GTP, UTP, and ATP were used, only the d(TAC) template could be used for RNA synthesis because to transcribe the d(GTA) strand, CTP is required. Alternatively, the d(GTA) strand could be transcribed exclusively by using CTP, UTP, and ATP. This resulted in the transcription of 2 long RNA strands with repeating sequences. These and other RNA polymers with repeating sequences were used to complete the table of tRNA codons and established conclusively the triplet nature of the code.
The genetic code was found to be highly degenerate in that there are 64 codons for only 20 amino acids. Except for methionine and tryptophan, each amino acid is coded for by 2 or more codons. In 1966, Francis Crick published his "wobble" hypothesis (1966) which proposes that the base pairing in the first two positions of the codon is strict Watson-Crick base-pairing (A:U, G:C), but the pairing at the third position of the codon (first position of anticodon) is not as stringent. Trends in the genetic code and examination of base-pairing possibilities led him to believe that several "new" base-pairs were possible when the bases were moved just slightly (wobbled) in orientation. He proposed additional base pairs according to the chart in Table 1. We now know that codon-anticodon pairing is not as simple as this, but the original hypothesis still serves as an excellent point from which to start.

The next major step in tRNA research was the development of new techniques to study the sequence and structure of the tRNAs. When Holley and co-workers first reported the sequence of yeast tRNA\(^{\text{Ala}}\), it was evident that there were many (9 in this instance) unusual nucleotides. Further evidence from other tRNAs confirmed the existence of many bases other than A, U, G, and C. With the accumulation of more sequences, several common features of tRNAs became evident (Figure 1): 1) They are
Table 1. Crick's wobble hypothesis.
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<td>C</td>
<td>G</td>
</tr>
<tr>
<td>A</td>
<td>U</td>
</tr>
<tr>
<td>U</td>
<td>A,G</td>
</tr>
<tr>
<td>G</td>
<td>U,C</td>
</tr>
<tr>
<td>I</td>
<td>U,C,A</td>
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Figure 1. Structure of transfer RNA.
Figure 1.
single-stranded RNA chains 73-93 nucleotides long. 2) The sequence is such that all tRNAs may be drawn in a cloverleaf pattern with approximately half of the nucleotides base paired. 3) They contain many unusual bases. 4) The 5' end is phosphorylated and the base is usually guanine. 5) The base sequence at the 3' end is CCA. 6) The adenosine in position 76 is the site of amino acid attachment.

Crystallographic studies to determine the three-dimensional structure of tRNAs took many years of intense work by several laboratories. Several years after the first crystals of tRNA were obtained, two groups, Quigley et al. (1975) and Ladner et al. (1975), reported the structure of yeast tRNA^Phe nearly simultaneously. Their studies elucidated several important features of the tRNA molecules: 1) The molecule is L-shaped. 2) There are two segments of double helix which are oriented nearly perpendicular to each other. 3) There are many unusual tertiary interactions between bases which help stabilize the molecules. 4) The CCA-amino acid attachment site and the anticodon loop are located at opposite ends of the molecule. These findings served to strengthen many of the hypotheses concerning the structure and function of the molecules. The most important of these was the confirmation of the cloverleaf structure and the demonstration that the amino acid attachment site and the
anticodon loop are on distinctly different sections of the molecule.

In addition to the work on the sequence and structure of tRNAs, many scientists turned to the task of defining the functions and mechanisms of tRNAs in protein synthesis. These studies continue yet today as many groups attempt to determine the interactions of the protein synthesizing machinery in ever greater detail. Much has been learned about the role of tRNA in this process. Transfer RNA molecules are aminoacylated by specific aminoacyl-tRNA synthetases using ATP-activated amino acids. These aminoacyl-tRNA synthetase molecules are very accurate in charging the proper amino acids on the proper tRNAs. Such accuracy is required for the high fidelity of protein synthesis because the codon in the mRNA is recognized by the anticodon of the tRNA and not by the amino acid. This high selectivity in the use of structurally similar molecules is probably aided by the presence of the large number of modified bases on the tRNAs. The aminoacylated tRNA molecules may then be used in the synthesis of proteins by the ribosomes.

There are two tRNA binding sites on the ribosome. The first is called the P (peptidyl) site where protein synthesis is initiated by a (formyl)methionine-tRNA molecule. The other site is the A (aminoacyl) site where incoming aminoacyl-tRNAs bind to elongate the protein
molecule. When both of these sites are filled with their appropriate aminoacyl-tRNAs, a peptide bond may be formed between the peptide [or (formyl)methionine] on the tRNA in the P site and the amino acid on the tRNA in the A site, with the resulting peptide linked to the tRNA in the A site. A translocation then occurs in which the uncharged tRNA in the P site is eliminated and the peptidyl-tRNA is moved from the A site to the P site. The eliminated tRNA is then available for aminoacylation again.

During these studies on the mechanisms of protein synthesis and related processes, tRNA molecules were found to be involved in several regulatory systems. In procaryotic cells, transcription and translation are tightly coupled and occur simultaneously. This is important in the demonstration by Oxender et al. (1979) of the transcriptional control of the tryptophan (trp) operon of E. coli by the amount of tryptophanyl-tRNA present in the cells. The trp operon codes for the synthesis of five enzymes responsible for the synthesis of tryptophan (Platt, 1978). The enzymes are transcribed and translated sequentially in equimolar amounts when the cells have a need for more tryptophan. Upstream from the structural genes are three important regulatory sites. The promoter and operator are the sites for the first level of regulation. When tryptophan is plentiful, it binds to a trp repressor protein and this complex binds to the
operator site of the operon. Binding of this trp-repressor complex at the operator prevents RNA polymerase from binding to the promoter site, thus restricting transcription and translation of the genes when tryptophan synthesis is unnecessary.

In addition to end-product repression, the level of tryptophanyl-tRNA also elicits control over synthesis of the trp genes. There is a short leader (L) sequence (162 nucleotides) prior to the first structural gene and some of this leader sequence is translated. In this fourteen amino acid leader region there are two tryptophan codons back-to-back which allow for additional attenuation. When tryptophan is plentiful, the leader sequence is translated in its entirety, but transcription is discontinued before reaching the structural genes. When tryptophan is scarce, the ribosome stalls on the mRNA at the site of the tandem tryptophan codons, causing a shift in the secondary structure of the mRNA, allowing the RNA polymerase molecule to continue to transcribe the structural genes of the trp operon. A similar situation is known to occur for the operons controlling histidine and phenylalanine synthesis. These two operons are even more biased to sensing the level of aminoacylated tRNA for each of the respective amino acids. The phenylalanine operon contains seven phenylalanine residues in the fifteen residue leader peptide (Stryer, 1981) and the histidine operon has a
string of seven histidine codons in a row in its leader (Stryer, 1981). These leaders appear to serve as very sensitive monitors of the level of aminoacylation of specific tRNAs for the regulation of amino acid synthesis.

In eucaryotic systems, transcription and translation are separate events, but it has been shown that tRNA may exert a direct influence on protein synthesis. Strehler et al. (1971) presented a hypothesis referred to as the "codon-restriction theory of aging and development", in which they discuss the possibility of the tRNA population of a cell controlling the synthesis of protein in those cells. Garel (1974) reported that changes in tRNA during silk production in *Bombyx mori* follow the demand for particular amino acid codons. Silk is made up of two proteins, fibroin and sericin. Fibroin is the predominant protein produced in the posterior part of the silk gland (70%) and 93% of the amino acids are glycine, alanine, serine, and tyrosine (Garel, 1974). Garel demonstrated that prior to induction of silk production, there was an induction of the tRNAs necessary for the efficient translation of the silk mRNA.

Litt and Kabat (1972) were able to use sheep reticulocytes to demonstrate that the tRNA in the reticulocytes is biased toward the codons used in the reticulocyte mRNA. The reticulocytes synthesize a preponderance of hemoglobin molecules. They demonstrated
that the tRNA population was shifted to maximize for the synthesis of these messages. Further analysis of these and other data in support of "functional adaptation of tRNA" was performed by Chavancy et al. (1979). They determined that in many instances in both animal cells and viruses, the distribution of tRNA isoaccepting species is matched to the codon usage in the corresponding mRNA.

Another interesting discovery was the finding that many viruses induce changes in the translational machinery of their host cells (for review see Littauer and Inouye, 1973). Changes have been found in tRNA, tRNA modifying enzymes, and aminoacyl-tRNA synthetases indicating the importance of tRNA for efficient protein synthesis. In addition to these changes in the host machinery, some viruses encode their own essential tRNAs. These additional tRNAs appear to be necessary to decode some of the virus codons which cannot be efficiently translated by the host cell tRNA. Viruses may also use tRNA as primers for their own DNA synthesis (Stryer, 1981).

Zilberstein et al. (1976) found a minor tRNA^{Leu} species to be required for translation of exogenous mRNA in mouse cells treated with interferon. The interferon-induced translation block was overcome by addition of the tRNA^{Leu} species to extracts used to translate Mengo virus RNA or hemoglobin mRNA (Zilberstein et al., 1976). In addition to this interferon-induced
block, Ortwerth et al. (1984) found a temperature sensitive mutant which is defective in the trnALys-5 to trnALys-4 modification. At the restrictive temperature, the cells were blocked in the G1 phase of the cell cycle and the modification enzyme necessary for the conversion of trnALys-5 to trnALys-4 was found to be altered (Ortwerth et al., 1984). In a possible related finding, Ghosh and Guhathakurta (1983) found that infection by phage ϕ149 of Vibrio cholerae resulted in synthesis of phage-specific tRNA. This may also be necessary for efficient translation of phage-encoded mRNAs.

Ali and Vedeckis (1987) also demonstrated that one of the three forms of the glucocorticoid receptor from mouse AtT-20 cells binds tRNA. The three basic tRNAs, Arg, Lys, and His, accounted for 78% of the tRNA bound by this receptor. They also proposed that this could act as a hormone-induced mechanism by which tRNAs could be preferentially sequestered to disrupt translation of specific proteins for regulatory purposes.

It is also interesting to note that recent evidence indicates that tRNA may not only be involved in the control of protein synthesis but also in the control of protein degradation. Ciechanover et al. (1985) found that tRNA is an essential component of the ubiquitin- and ATP-dependent proteolytic system. This group found that when RNA was degraded in their in vitro rabbit
reticulocyte system, proteolysis ceased to occur and only readdition of a tRNA fraction was able to restore the proteolytic activity. This proteolytic activity appears to be substrate and tRNA specific offering another potential regulatory function for tRNA.

One means of looking at differences in the tRNA populations of cells is by RPC-5 chromatography. RPC-5 chromatography was developed by Pearson et al. (1971), and it allows different tRNA species to be separated on a reversed phase column (Yang and Novelli, 1971). The family of tRNAs to be monitored is aminoacylated with the appropriate radiolabelled amino acid. The tRNA mixture is then run through the column and the eluate is collected in fractions or monitored by an on-line scintillation counter. The tRNA species (isoacceptors) corresponding to the radiolabelled amino acid used are separated and quantitated in this way.

Using the method of RPC-5 chromatography, several groups have shown that changes in the tRNA isoacceptor profiles may be related to different stages in cellular development and to the progression of carcinogenesis. Lin et al. (1980a, 1980b) monitored the levels of 64 tRNA isoaccepting species during the differentiation of Friend leukemia cells induced with dimethylsulfoxide. Major fluctuations of many of these isoacceptors occurred between 36 and 72 hours after induction of
differentiation. Changes in both predominant and minor tRNA species were apparent during the course of differentiation. Shindo-Okada et al. (1981) also reported changes in the tRNA isoacceptor profiles for several species of tRNA in murine erythroleukemia cells induced to differentiate with a variety of inducing agents. In addition, the isoaccepting species of tRNA from Drosophila melanogaster were evaluated by Hosbach and Kubli (1979) on days five and 35 during normal development. They also found significant changes in the isoacceptor patterns of several tRNA species. One feature which stands out in the changing isoacceptor patterns of the cells is that the alterations usually involve changes in modification of the tRNAs. In many cases, the presence or absence of a particular modified base is the difference between the pattern of isoacceptors observed.

Of the more than 60 different tRNA modifications identified to date, the most common are methylations, acetylations, and thiolations. In addition, there are very extensive modifications made in some cases. In Figure 1, some of the more unusual modified bases can be seen. An interesting fact concerning these modifications is that many of them occur in or near the anticodon where they have the potential to alter codon recognition. Thus, modifications in the anticodon may affect protein synthesis, either positively or negatively, by changing
the codon recognition potential of the tRNA population.

It would appear that these modifications in the anticodon loop are distinct from the modifications in the remainder of the tRNA. In eucaryotic cells, there is a subcellular division of modification enzymes. While the enzymes specific for modification of the anticodon loop are found in the cytoplasm, all of the other tRNA modifying enzymes appear to be located in the nucleus (Grosjean et al., in press).

Since all of the tRNA modifications occur post-transcriptionally, the projected 200 or more enzymes responsible for their synthesis are of much interest. While most modifications arise by addition of the modifying unit to the normal base, two modified nucleosides have been found which are synthesized by a different mechanism. Queuosine and inosine arise by a mechanism termed base exchange. The base exchange occurs when the enzyme removes a base from the primary transcript and replaces it with a new base without breaking the phosphodiester backbone.

The existence of queuine (the base of the nucleoside queuosine) in the wobble position of tRNA has been known for some time, but its complete structure and mechanism of biosynthesis were determined much more recently. Early in this research effort, many people thought queuine was simply a modified guanine that was merely "built" on an
existing guanine in the tRNA. The unidentified, modified nucleoside "Q" was originally found in the first position of the anticodon of E. coli tRNA^{TYR} by Goodman et al., RajBhandary et al., and Doctor et al. (1968; 1969; 1969). Harada and Nishimura (1972) later found Q in the same position in E. coli tRNA coding for Asn, Asp, and His. In 1975, Kasai et al. (1975) reported the structure of the modified nucleoside Q as 7-(4,5-cis-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanosine (Figure 2). With this data, Kuchino et al. (1976) proposed a replacement of guanine by queuine based on their findings that carbon-8 of guanine is not retained in queuine.

At the same time the structure of Q was being elucidated, the basis for the enzymatic synthesis of Q in tRNA was being worked out. In 1962, Marks et al. (1962) had determined that free guanine was incorporated into the 4S (tRNA) fraction of RNA by rabbit reticulocytes. Hankins and Farkas (1970) expanded on this work and determined that it was indeed guanine which was incorporated and that it was incorporated at a specific site in the tRNA. In 1973, Farkas et al. (1973) showed this incorporation to occur after transcription and determined that it was probably an enzymatic reaction because it was heat labile and concentration dependent. Farkas and Singh (1973) also demonstrated that the incorporating activity of the rabbit reticulocytes could
Figure 2. Transfer RNA-guanine ribosyltransferase reaction. The enzyme exchanges queuine for guanine in the first position of the anticodon of mammalian tRNAs for aspartic acid, asparagines, histidine, and tyrosine. The abbreviations are: Q, queuine; and G, guanine.
tRNA$_G$ → tRNA-guanine ribosyltransferase → tRNA$_Q$

**Figure 2.**
be recovered in a cell-free system which could label yeast tRNA as well as homologous tRNA. Farkas and Chernoff (1976) then demonstrated that only the hypomodified (G-containing) species of tRNA^{His} and tRNA^{Asn}, and not their Q-containing counterparts, were substrates for the "guanyling" enzyme.

The guanine incorporating activity was assayed in E. coli cell extracts and was purified to homogeneity (Okada and Nishimura, 1979). A similar enzymatic activity was purified from rabbit erythrocytes (Howes and Farkas, 1978). Subsequent studies have determined that the E. coli enzyme differs from that isolated from eucaryotic sources in substrate usage. Okada et al. (1979) concluded that the E. coli enzyme incorporated a precursor of Q [7-(aminomethyl)-7-deazaguanine] (not queuine itself), with Q synthesis being completed at the polynucleotide level. Katze and Farkas (1979) demonstrated that the rabbit reticulocyte enzyme incorporated queuine intact into tRNA in vitro, and subsequently Katze et al. (1984) demonstrated the incorporation of queuine into tRNA in vivo in mouse fibroblasts. These studies were followed by detailed studies of the parameters involved in this enzymatic reaction. Investigators have published results detailing the specificity of tRNA-guanine ribosyltransferase (EC 2.4.2.29) with respect to possible substrates and inhibitors as well as the kinetic
parameters for these substances (Shindo-Okada et al., 1980; Farkas et al., 1984; Farkas, 1983; Nishimura, 1983). Studies are currently underway to determine the effects of some of these inhibitors/substrates on cells.

The significance and function of the queuosine modification in tRNA is not yet fully understood, but evidence is accumulating which implicates this modification in cellular control processes. Many studies have demonstrated reproducible changes in isoaccepting patterns for the Q-containing tRNAs (Asn, Asp, His, Tyr) during differentiation of certain cell types (Lin and Agris, 1980; Lin et al., 1980; Shindo-Okada et al., 1981; Hosbach and Kubli, 1979), as well as documenting the existence of significantly greater quantities of Q-deficient tRNA in tumor tissues compared to the tRNA from matched normal tissues, which contains almost exclusively Q-modified tRNA (Okada et al., 1978; Katze and Beck, 1980).

These findings are very important because queuine is a dietary requirement in mammals (Farkas, 1983; Reyniers et al., 1981). Mammalian cells cannot synthesize queuine, but must obtain it from the diet or the gut flora. Mammalian cells in culture obtain queuine from the sera used to supplement the culture medium (Katze, 1978; Katze and Farkas, 1979). An E. coli mutant lacking tRNA-guanine ribosyltransferase, and therefore having no
queuosine in their tRNA, grows as well as normal cells until the cells are stressed (Noguchi et al., 1982). When these mutant cells were kept under unfavorable conditions for growth, a reduction in viability was observed. This was the only significant biological affect observed but it does suggest that queuosine in the tRNA does have a functional role in these cells.

Three studies lend support for the direct involvement of queuine in the mechanism of differentiation/carcinogenesis. First, changes in queuine containing tRNAs were reported during specific stages in the development of Dictyostelium discoideum (Schachner et al., 1984). Schachner et al. demonstrated that starvation for queuine actually blocked differentiation of the slime mold at one point in the life cycle, and that this blockade could be overcome by adding exogenous queuine. Second, Katze and Beck (1980) determined that they could reverse the queuine hypomodification of tRNA in Ehrlich ascites tumor cells by continuous infusion of queuine. Mice were inoculated with $10^6$ tumor cells and monitored for 7-9 days. Transfer RNA from tumors in these mice was evaluated. Transfer RNA from mice which received the continuous infusion of queuine had a significantly higher content of Q-modified tRNA than did the tRNA from the tumors of mice which did not receive queuine. Most importantly, the queuine infusion caused an inhibition of
tumor growth. The third line of support comes from studies of Elliott et al. (1984) which implicated the queuine modification of tRNA directly in the generation of an altered cell phenotype in normal human fibroblasts treated with a phorbol ester tumor promoter. Initial studies showed that tRNA from the phorbol-treated human fibroblasts became hypomodified for queuine with a concomitant increase in cell density and the expression of a transformed phenotype. This study also demonstrated that the addition of queuine to the culture medium was able to block the increase in cell density as well as maintain the tRNA in the Q-modified state. Subsequent studies showed that the phorbol ester was specifically inhibiting the transport of queuine into the cell, thereby depriving the tRNA-guanine ribosyltransferase of the needed substrate (Elliott et al., 1985).

A wide variety of agents (including phorbol ester tumor promoters) are known to induce the differentiation of leukemia cells in vitro (Bodner et al., 1981; Collins et al., 1980; Gusella and Housman, 1976; Huberman and Callaham, 1979; Lotem and Sachs, 1979), but the molecular mechanisms responsible for such chemically-induced maturation are, for the most part, obscure. With murine erythroleukemia cells (Gusella and Housman, 1976) and human promyelocytic leukemia (HL-60) cells (Gallagher et al., 1984; Schwartz et al., 1984), the purine
antimetabolite 6-thioguanine is a highly effective inducing agent only if the cells lack the purine salvage enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Since the HGPRT deficiency should impair the formation of 6-thioguanine nucleotides, Gusella and Housman (1976) proposed that the generation of these cytotoxic nucleotides, and their subsequent incorporation into DNA, is probably not involved in the differentiation sequence. Additional work by Ishiguro et al. (1984) showed that it was the free base of 6-thioguanine which was responsible for the induction of differentiation in the HL-60 cells and that cells which were not deficient in HGPRT metabolized this guanine analog to the cytotoxic nucleotide level. Although these studies demonstrated that unmetabolized 6-thioguanine was responsible for the induction of differentiation of HGPRT-deficient HL-60 cells, the molecular basis for this induction was not established. These studies along with earlier published reports that many purine analogs, including 6-thioguanine, may serve as substrates/inhibitors of the tRNA-guanine ribosyltransferase enzyme (Farkas et al., 1984; Shindo-Okada et al., 1980) offered the possibility that changes in the queuine modification of tRNA might be involved in the differentiation of these cells induced by 6-thioguanine. That possibility was investigated as part of this thesis project.
Just as queuine in the first position of the anticodon can alter codon recognition of the affected tRNAs, inosine can increase the recognition potential of the tRNAs in which it is found (Crick, 1966). The mechanism of synthesis of inosine in the wobble position of the anticodon of tRNA was unknown until recently. Kammen and Spengler (1970) demonstrated that the synthesis of inosine was a post-transcriptional modification, and they proposed that the mechanism was a site-specific, macromolecular deamination of adenosine. While Kammen and Spengler (1970) attempted to demonstrate the existence of this enzymatic activity, they were unable to do so. Recently, Elliott and Trewyn (1984) reported that inosine was synthesized instead by a base exchange reaction similar to the guanine for queuine exchange. The putative tRNA-hypoxanthine ribosyltransferase appeared to exchange hypoxanthine (the base of inosine) for some other base (probably adenine) in the wobble position of tRNA (Figure 3). This activity was separated from tRNA-guanine ribosyltransferase by DEAE-cellulose column chromatography and was assayed in rat liver and human leukemia cells (Elliott and Trewyn, 1984).

Based on the fact that inosine biosynthesis appears to occur via a base exchange reaction similar to that for queuine biosynthesis, it would seem that cellular events
Figure 3. Proposed tRNA-hypoxanthine ribosyltransferase reaction. The enzyme is believed to exchange hypoxanthine for adenine in the first position of the anticodon of mammalian tRNAs for alanine, arginine, isoleucine, leucine, proline, serine, threonine, and valine. The abbreviations are: A, adenosine; and I, inosine.
Figure 3.

*Ala, Arg, Ile, Leu, Pro, Ser, Thr, Val
which alter the intracellular concentration of hypoxanthine (the substrate for inosine biosynthesis) might affect cell functions. Several lines of evidence would seem to support this belief. First, specific enzymatic defects in the purine catabolic pathway have been associated with inherited immune deficiency diseases (Hirschhorn et al., 1978; Thompson and Seegmiller, 1980). Specifically, a lack of adenosine deaminase (ADA) or purine nucleoside phosphorylase (PNP) leads to an impairment of cell-mediated immunity, and ADA deficiency impairs antibody-mediated immunity as well. Studies have generally focused on finding a cytotoxic precursor which accumulates as a result of these defects. These studies have suggested that intracellular deoxyribonucleotide pools have been disrupted causing abnormal cell proliferation. Although cellular proliferation is necessary for normal lymphopoeisis, non-proliferative functions are also impaired when ADA is inhibited in cells in vitro, and these changes are independent of changes in nucleotide pools (Grever et al., 1982). In addition, when monocytes differentiate to macrophages in vitro the ADA activity increases nearly 10 fold, but when the ADA is inhibited, these cells can not differentiate (Fischer et al., 1976). Considering that hypoxanthine induces the differentiation of HL-60 cells (Collins et al., 1980) and murine erythroleukemia cells (Gusella and Housman, 1976;
Lacour et al., 1980), it has been proposed that the significant metabolic change is not an increase in some cytotoxic precursor but rather a decrease in the intracellular concentration of hypoxanthine, the substrate for tRNA-hypoxanthine ribosyltransferase and inosine biosynthesis in tRNA (Trewyn, 1984).

Additional evidence comes from studies with the antiviral drug inosiplex (methisoprinol, isoprinosine); a 3:1 molar complex of N,N-dimethylamino-2-propanol p-acetamidobenzoate and inosine (Ginsberg and Glasky, 1977). Extensive studies indicate that the demonstrated activity is not due to a strict antiviral activity but rather seems to act as an immunomodulating agent (Ginsberg and Glasky, 1977; Hadden and Giner-Sorolla, 1981; Hadden et al., 1983). Hadden et al. (1983) also ascribe an immunomodulating activity to inosine but studies show that inosine is much more effective when it is part of a complex (Hadden and Giner-Sorolla, 1981). The rates of ADA and PNP are such that the level of inosine is kept low while the production of hypoxanthine is favored (Hadden et al., 1983). Therefore, these immunomodulators may serve as a source of hypoxanthine for tRNA-hypoxanthine ribosyltransferase.

In addition, cells induced to differentiate with DMSO also have the ability to respond to added hypoxanthine (Trewyn et al., 1985). Both DMSO and hypoxanthine, used
separately, have the ability to induce the differentiation of leukemia cells in culture (Collins et al., 1980; Gusella and Housman, 1976). It was recently demonstrated that if used in combination, DMSO and hypoxanthine work in a synergistic manner (Trewyn et al., 1985). When HL-60 cells were treated with suboptimal concentrations of DMSO and hypoxanthine separately, little inhibition of growth or induction of differentiation was observed. When the same concentrations of DMSO and hypoxanthine were used together, a large increase in growth inhibition was observed as well as a significant increase in induction of differentiation.

Based on these findings, two different approaches were taken to study the putative tRNA-hypoxanthine ribosyltransferase and its effect on HL-60 cells. The first approach focused on changes in cell biology resulting from treatment of HL-60 cells with hypoxanthine and/or DMSO. This included studies of the induction of differentiation and changes in protein synthesis. The second approach utilized recombinant RNA techniques to reconstruct the anticodons of specific tRNAs (Fournier et al., 1983; Haumont et al., 1984). Because no tRNAs containing an unmodified adenosine in the first (wobble) position of the anticodon were available (Sprinzl et al., 1985), the recombinant RNA techniques were employed to prepare appropriate tRNAs for enzymatic studies with the
proposed tRNA-hypoxanthine ribosyltransferase. During the reconstruction procedure, it was possible to introduce a $^{32}\text{P}$ label adjacent to the nucleotide in the first position of the anticodon. In this way, it was possible to follow modifications at that position specifically. Yeast tRNA$^{\text{Ala}}$ (normal anticodon IGC) was chosen for reconstruction, after which it could be used as substrate both in vitro or in vivo (after microinjection into Xenopus laevis oocytes) to investigate some of the parameters of this enzymatic activity. The results of these reconstruction studies and the cell biology studies are presented below.

The wealth of published information offers support for the belief that tRNAs play an important role in cellular control processes. The work described below was undertaken to determine the role in leukemia cell differentiation of the queuosine and inosine modifications (catalyzed by tRNA-guanine ribosyltransferase and tRNA-hypoxanthine ribosyltransferase) in the tRNA anticodon wobble position. Considering the number of other modifications in tRNA, it is possible that more of these may be found to be important in cellular control mechanisms.
MATERIALS AND METHODS

Cell Culture.

Human Leukemia Cells. Human promyelocytic leukemia (HL-60) cells (Collins et al., 1977), obtained from Dr. Robert Gallo at the National Cancer Institute, and hypoxanthine-guanine phosphoribosyltransferase (HGPRT)-deficient HL-60 cells, obtained from Dr. Linda Thompson at the Scripps Clinic, were grown in suspension culture in RPMI-1640 medium (GIBCO, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS) (Sterile Systems, Logan, UT). These HL-60 cells were incubated at 37°C in a 10% CO₂ atmosphere. Human leukemic T-lymphoblasts (CCRF-CEM), obtained from Dr. Michael R. Grever at The Ohio State University, were grown in suspension culture in SMEM (GIBCO) supplemented with 10% bovine serum (BS) (Sterile Systems). CCRF-CEM cells were incubated at 37°C in a 4% CO₂ atmosphere. Human leukemia cells grown in suspension culture were seeded at densities of 5 x 10⁴ to 2 x 10⁵ cells/ml in 25 cm² tissue culture flasks containing 10 ml of medium plus serum or in 8 oz. glass penicillin bottles containing 20 ml of medium plus serum. Growth curves were determined by counting cells daily with a Coulter Counter Model ZM. CCRF-CEM and HL-60 cells achieved maximum
saturation densities of 1 to $3 \times 10^6$ cells/ml. After approximately 6 to 8 days of exponential growth, these cells entered the stationary phase of cell growth at which time they were subcultured to the desired density with fresh medium and serum.

Human leukemic T-cells (Jurkat), obtained from Dr. Charles Orosz at The Ohio State University, were grown in suspension culture in RPMI-1640 medium supplemented with 10% FBS. Jurkat cells were incubated at 37°C in a 10% CO$_2$ atmosphere. These cells were seeded at densities of $5 \times 10^4$ to $10^5$ cells/ml and counted as above. The Jurkat cells were subcultured every 3 - 4 days by a 1:5 dilution with fresh medium and serum.

When large numbers of cells were needed for enzyme isolation or tRNA, they were grown in 2 l spinner flasks containing 1 to 1.5 l of medium plus serum. The cells in the spinner flasks were incubated at 37°C in a 10% CO$_2$ atmosphere with the spinner bars rotating just fast enough to keep the cells in suspension. The growth curves were again monitored by daily counting of the cells using a Coulter Counter. Cells grown in this way passed through the growth cycle slightly faster (entering stationary phase at 5 to 7 days) than those cells grown in the flasks or bottles but they responded to the various treatment protocols in the same way.
Serum utilized in the RPC-5 studies was charcoal treated to remove queuine (Katze, 1978). A solution (50 ml) of dextran coated charcoal [25 mg/ml dextran (Sigma), 250 mg Norit A decolorizing carbon/ml (Pfanstiehl Laboratories, Waukegan, IL)] in 0.9% NaCl was added to 500 ml of fetal bovine serum. The treated serum was heated to 55°C for 30 minutes and stirred occasionally. This mixture was centrifuged at 1,000 rpm for 5 minutes in a Beckman TJ-6 centrifuge with a TH-4 swinging bucket rotor (200 x g) to remove the charcoal. The serum was then filtered and sterilized.

**Murine Erythroleukemia Cells.** Murine erythroleukemia cells (BB-88), obtained from the American Type Culture Collection were grown in RPMI-1640 supplemented with 10% FBS and 10 μM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO). The cells were incubated at 37°C in a 10% CO₂ atmosphere. These cells were seeded at densities of 5 x 10^4 to 2 x 10^5 cells/ml in 25 cm² flasks containing 10 ml of medium plus serum or in 8 oz. pharmacy bottles containing 20 ml of medium plus serum. Growth curves were determined by counting cells daily with a Coulter Counter. BB-88 cells achieved maximum saturation densities of 2 to 4 x 10^6 cells/ml after 3 to 4 days of exponential growth. When the cells reached stationary phase, they were subcultured to the desired density with medium and serum.
Verification of HGPRT-deficiency in HL-60 Cells.

Wild type HL-60 cells and HGPRT-deficient HL-60 cells were grown for 48 hours in the presence of 40 μCi of \( [\text{\textsuperscript{3}H}] \)hypoxanthine (Amersham, Arlington Hts., IL). At the completion of the incubation, the cells were harvested and washed as described below. After lysing and precipitating the nucleic acids with 1 ml 5% trichloroacetic acid (TCA) (Sigma) on ice for 30 minutes, the radiolabelled nucleic acids were collected on Whatman GFA glass fiber filters (Whatman Ltd., Maidstone, England) and washed with 30 ml of ice-cold 5% TCA. The filters were submerged in 5 ml of scintillation cocktail (Formula-963, NEN Research Products, Boston, MA) and counted in a liquid scintillation counter.

Harvesting Cells.

Cells grown in suspension culture were harvested by low-speed centrifugation of the culture media. Cell culture media was centrifuged in 50 ml conical centrifuge tubes at 1,000 rpm for 10 minutes in a Beckman TJ-6 with a TH-4 swinging bucket rotor (200 x g). Cell pellets were combined after resuspending in cold phosphate buffered saline (PBS). The cells were then washed one additional time with cold PBS and collected by centrifugation. Cells to be used for enzyme isolation were homogenized immediately, while cells to be used for tRNA isolation
were either stored frozen at -20°C or homogenized.

**Determination of Cellular Differentiation.**

**Differential Cell Counting.** Approximately $5 \times 10^5$ cells were collected on glass slides by cytocentrifugation (Shandon Southern Cytospin) for 10 minutes at 9,000 rpm. The cells were stained with Wright-Giemsa (Sigma) and differential cell counting was performed on a minimum of 200 cells to determine the percentage of total cells exhibiting mature morphology (Murao et al., 1983).

**OKM1 Monoclonal Antibody.** The OKM1 monoclonal antibody was used to follow the expression of a differentiation-specific cell surface antigen by indirect immunofluorescence (Breard et al., 1980; Murao et al., 1983). Cells ($10^6$) were suspended in 200 μl PBS and incubated with 10 μl OKM1 antibody (Ortho Diagnostic Systems, Raritan, NJ) for 45 minutes at 4°C. Blanks were run with PBS added in place of the OKM1 antibody. The cells were then washed with PBS and resuspended in 100 μl fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Ortho). After incubating in the dark for 30 minutes at 4°C, the cells were washed again and resuspended in PBS. The expression of the OKM1 antigen was then quantitated using an Ortho Diagnostic Systems Cytofluorograf System 50H.
Nitroblue Tetrazolium Reduction. Approximately 2 x $10^5$ cells were harvested by centrifugation at 1000 rpm for 5 minutes in a Beckman TJ-6 with a TH-4 swinging bucket rotor (200 x g) and resuspended in 100 µl of RPMI-1640 medium containing 15% FBS and 2 µg 12-O-tetradecanoyl phorbol-13-acetate (TPA)/ml. After addition of 100 µl of 0.1% nitroblue tetrazolium (NBT)(Sigma), the cells were incubated at 37°C for 20 minutes. A minimum of 200 cells were counted on a hemacytometer with those cells containing black formazan deposits being designated as positive (Sigma Bulletin 840, 1978).

Isolation and Purification of tRNA.

Transfer RNA was isolated and purified by a modification of the procedure of Buck et al. (1983). After collecting and washing the cells as above, the cell membranes were disrupted by vortexing the cells in 4 volumes of cold reticulocyte standard buffer (RSB) [10 mM Tris-HCl (pH 7.4), 10 mM NaCl, and 1.5 mM MgCl$_2$] containing 0.5% Nonidet P-40 (Sigma), a non-ionic detergent. The cell suspension was then centrifuged for 5 minutes at 5,000 rpm in a Beckman J2-21M centrifuge with a JA-20 rotor (3,000 x g) to pellet the nuclei. The resulting supernatant was centrifuged at 12,000 rpm (18,000 x g) for 30 minutes. This supernatant was extracted with an equal volume of phenol/water (5:1)
containing 0.1% quinolin (MCB, Norwood, OH) by frequent vortexing for 20 minutes. This mixture was centrifuged for 10 minutes at 12,000 rpm (18,000 x g) and the aqueous phase was collected. The phenol (Fisher Scientific, Fairlawn, NJ) was then reextracted with 1/2 volume of RSB as before. A 0.1 volume of 20% potassium acetate (pH 4.5) was then added to the combined aqueous phases and this mixture was made 2 M in LiCl by addition of 12 M LiCl (Boehringer Mannheim, Mannheim, W. Germany). This mixture was allowed to stand on ice for 4 hours to allow precipitation of high-molecular weight RNA before centrifugation for 20 minutes at 5,000 rpm (3,000 x g). The tRNA in the supernatant was precipitated by addition of glycogen (Boehringer) to 30 μg/ml and 3 volumes of 100% ethanol. After storage at -20°C overnight, the tRNA was collected by centrifugation at 12,000 rpm (18,000 x g) for 30 minutes and then dried under a vacuum. The tRNA was then dissolved in 1 ml of 0.1 M Tris-HCl (pH 7.5), 0.01 M MgCl₂ (Tris-Mg) buffer and applied to a small (1 x 2 cm) DEAE-cellulose column. The column was rinsed with 2 ml of Tris-Mg buffer followed by 7 ml Tris-Mg buffer containing 0.2 M NaCl. The tRNA was eluted with 5 ml Tris-Mg buffer containing 1 M NaCl and was precipitated with 0.1 volume of 4 M LiCl, 300 μg glycogen/ml, and 3 volumes of 100% ethanol.
**RPC-5 Chromatography.**

HGPRT-deficient cells were grown in queuine deficient FBS (charcoal treated as described above) to approximately $10^6$ cells/ml at which time they were split 1:2 to three sets; untreated control, 0.4 mM 6-thioguanine treated, and 0.4 mM 8-azaguanine treated. After 48 hours of growth in the presence of the various treatments, the cells were harvested and the tRNA was extracted (as described above). Purified tRNA was aminoacylated with $[^3]H$histidine (Amersham, Arlington Heights, IL; 49 Ci/mmol) using a mouse liver-derived aminoacyl-tRNA synthetase preparation. The histidyl-tRNA isoacceptors were separated on a column (0.9 x 20 cm) of RPC-5 support developed at 27°C with a 150 ml linear gradient of 0.475→0.80 M NaCl in 10 mM sodium acetate buffer (pH 4.5), 10 mM MgCl$_2$, 3 mM 2-mercaptoethanol, and 1 mM EDTA; the flow rate was 1.4 ml/minute, 1.5 ml fractions were collected, and the radioactivity was measured by scintillation counting in 10 ml of scintillation cocktail.

**Preparation of Enzyme Extracts.**

Cells, which had been harvested as described above, were resuspended in 5 ml of cold Buffer A [10 mM Tris-HCl (pH 7.4), 10 mM MgCl$_2$, 1 mM EDTA, 0.5 mM dithiothreitol (DTT), and 10% glycerol] and homogenized in an ice-cold, ground-glass tissue grinder (5 ml capacity, American
Scientific Products, McGaw Park, IL) until most of the cells were broken (as determined by trypan blue exclusion). The homogenate was centrifuged at 2,000 rpm for 10 minutes in a Beckman J2-21M centrifuge in a JA-20 rotor (500 x g). The resulting supernatant was then centrifuged at 12,000 rpm for 30 minutes (18,000 x g). This 12,000 rpm supernatant was used as the source of the tRNA-hypoxanthine ribosyltransferase and tRNA-guanine ribosyltransferase enzymes.

Escherichia coli tRNA-Guanine Incorporation Assay.

Transfer RNA, isolated by phenol extraction and DEAE-cellulose column chromatography as described above, was utilized as a substrate in the guanine incorporation assay of Okada et al. (1978). The assay involves the enzymatic exchange of radiolabelled guanine for the guanine in the first position of the anticodon of queuine unmodified tRNAs by the tRNA-guanine ribosyltransferase isolated from E. coli; an enzyme not able to utilize the queuine modified forms as a substrate. Therefore, radiolabelled guanine incorporation into tRNA is a measure of queuine hypomodification.

Transfer RNA-guanine ribosyltransferase was isolated from E. coli MRE600 cells (Grain Processing Corporation, Muscatine, IA) as described by Okada and Nishimura (1979). However, purification of the enzyme was carried only
through the DEAE-cellulose chromatography step, since the preparation was free of RNase activity when the RNase-deficient strain MRE600 was utilized (Elliott and Trewyn, 1982). The assay reaction mixture was a modification of that described by Okada and Nishimura (1979) containing: 70 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 0.2 μCi [14C]guanine-HCl (50-60 mCi/mmol)(ICN Radiochemicals, Irvine, CA), 60 μl E. coli enzyme, and 0.05-0.2 A₂₆₀ units of tRNA in a total volume of 0.2 ml. After 60 minute incubations at 37°C, the reactions were stopped by addition of 20 μl LiCl:glycogen (4 M:300 μg/ml) solution and 200 μl of ice-cold 10% TCA to each 200 μl aliquot. After allowing the tRNA to precipitate on ice for at least 30 minutes, acid precipitable, radiolabelled tRNA was collected on Whatman GFA glass fiber filters and rinsed with 30 ml of ice-cold 5% TCA. Filters were dried at 80°C for 30 minutes and after addition of 5 ml of scintillation cocktail, they were counted in a liquid scintillation counter.

Transfer RNA-Hypoxanthine Ribosyltransferase Assay.

This assay was a modification of the procedure of Elliott and Trewyn (1984). The standard reaction mixture contained 20 mM Tris-HCl (pH 7.4), 90 mM KCl, 3 mM MgCl₂, 0.3 mM EDTA, and 0.5 mM dithiothreitol (DTT) along with 0.5 μCi [³H]hypoxanthine (Amersham, 6.7 Ci/mmol), 0.4 A₂₆₀
units of unfractionated E. coli tRNA (Biogenics Research Corporation, Chardon, OH), and 100 µl of enzyme extract in a total volume of 200 µl. Incubation was at 37°C for 0-90 minutes. The reactions were stopped by addition of 20 µl LiCl:glycogen solution and 200 µl of ice-cold 10% TCA. After allowing the tRNA to precipitate on ice for at least 30 minutes, acid precipitable, radiolabelled tRNA was collected on Whatman GF/A glass fiber filters and rinsed with 30 ml of ice-cold 5% TCA. Filters were dried at 80°C for 30 minutes and after addition of 5 ml of scintillation cocktail, they were counted in a liquid scintillation counter.

When ³²P-labelled reconstructed tRNA was used as substrate in the modification reaction, unlabelled hypoxanthine (Sigma) (final concentration 10 µM) replaced the [³H]hypoxanthine in the reaction. The tRNA was precipitated with LiCl:glycogen and ethanol as described above and collected by centrifugation at 12,000 rpm (18,000 x g) for 15 minutes, after which the ethanol was decanted and the tRNA was dried under a vacuum. The tRNA was digested with 1 µg of nuclease P₁ from Penicillium citrinum (PL Biochemicals, Milwaukee, WI) in 10 µl of 50 mM ammonium acetate buffer (pH 5.3) for 2 hours at 37°C. The labelled 5' monophosphates were then identified by two-dimensional thin-layer chromatography as described below.
Two-dimensional Thin Layer Chromatography.

Two-dimensional thin layer chromatography (2D-TLC) was carried out according to the protocol of Nishimura (1972). After digestion of the tRNA to either the 5' or 3' monophosphates with nuclease P₁ or ribonuclease T₂ respectively, the reaction mixture was spotted onto the corner of a TLC square (6.7 x 6.7 cm) of 0.1 mm cellulose pre-coated on plastic and containing a fluorescent indicator (EM Science, Darmstadt, W. Germany). Each sample was spotted 2 μl at a time and dried after each application. After loading all of the individual sample, 2 μl of each of the unlabelled monophosphate standards (saturated solutions)(Sigma) were spotted on the square. The monophosphates were then run in the first dimension [66% isobutyric acid (Aldrich Chemical Co., Milwaukee, WI): 1% ammonia (EM Science): 33% water] until the solvent front was near the top of the TLC sheet (ca. 1 hour). The sheet was then removed and allowed to dry overnight. The second dimension [18% HCl (Mallinckrodt, Paris, KY): 68% isopropanol (Mallinckrodt): 14% water] was run until the solvent front was near the top of the sheet (ca. 2 hours). The sheet was allowed to dry and was then illuminated with a UV lamp. The dark spots corresponding to the unlabelled standards were marked and the squares were then autoradiographed (described below). After development of the autoradiograph, the radioactive spots were identified
by comparison with the standards.

**Autoradiography.**

Autoradiography was performed to locate radiolabelled nucleoside monophosphates separated by two-dimensional thin layer chromatography (as above) or radiolabelled proteins separated by polyacrylamide gel electrophoresis (see below). Either the thin layer chromatography sheets or the dried polyacrylamide gels were placed in a photographic cassette (Picker International, Highland Hts., OH) along with a sheet of Kodak X-Omat XAR-5 film (Kodak, Rochester, NY). After an appropriate period of exposure at room temperature (2 minutes to 1 day), the film was processed in a Kodak RP X-OMAT Processor. For samples containing small amounts of radiolabel (particularly the radiolabelled proteins), an intensifying screen (Spectra Blue Screen, Picker International) was included in the cassette on the side of the film opposite the sample. These samples were exposed at -70°C for an appropriate period of time (1-6 weeks), after which they were processed as described above.

**Radiolabelling of Cellular Protein.**

Cells to be radiolabelled for electrophoresis were harvested by centrifugation at 1,000 rpm for 5 minutes in a Beckman TJ-6 with a TH-4 swinging bucket rotor (200 x g)
and washed 2 times with calcium-magnesium free (CMF)-PBS. The cells were then resuspended at $10^6$ cells/ml in histidine-deficient RPMI-1640 medium containing 15% dialyzed FBS. The cell suspension was pre-incubated for 30 minutes at 37°C before adding the $[^{3}H]$histidine to a final concentration of 2 μCi/ml and incubating for 4 hours. The cells were again harvested by centrifugation (as above) and washed 2 times with CMF-PBS before lysing the cells in 1 ml of lysis buffer [9.5 M urea (Sigma) and 2% 2-mercaptoethanol]. Protein was precipitated with an equal volume of 10% TCA on ice for 30 minutes and collected by centrifugation for 5 minutes at 2,000 rpm in a Beckman Microfuge 12 (300 x g).

Cells to be tested for enhanced amino acid incorporation were harvested by centrifugation as above and washed 2 times with Hanks' balanced salts solution (HBS) (Gibco Laboratories, Grand Island, NY). The cells were resuspended at $5 \times 10^5$ cells/ml in HBS containing the appropriate chemical inducer (see below) and an aliquot was removed for protein determination by the method of Lowry et al. (1951). After a 10 minute pre-incubation, the $[^{14}C]$-labelled amino acid was added at 0.5 μCi/ml and 500 μl aliquots were removed at the designated times (0-60 minutes). These aliquots were precipitated with 2.5 ml 10% TCA on ice for 30 minutes. Radiolabelled protein was collected on Whatman GFA glass fiber filters and rinsed
with 30 ml of ice-cold 5% TCA. Filters were dried at 80°C for 30 minutes and after addition of 5 ml of scintillation cocktail, they were counted in a liquid scintillation counter. Inducers used included: DMSO (J.T. Baker, Phillipsburg, NJ), hypoxanthine, adenine (Sigma), cyclohexamide (Sigma), inosine (Sigma), adenosine (Sigma), inosiplex (Newport Pharmaceuticals International, Newport Beach, CA) polyinosinic-polycytidylic acid (Sigma), and polyadenylic-polyuridylic acid (Sigma). The labelling amino acids used include: \(^{14}C\)leucine (Amersham, 330 mCi/mmol), \(^{14}C\)phenylalanine (Amersham, 504 mCi/mmol), and \(^{14}C\)alanine (Amersham, 171 mCi/mmol).

**Discontinuous Polyacrylamide Gel Electrophoresis of Radiolabelled Proteins.**

This procedure was a modification of the procedure of Dreyfuss *et al.* (1984) for discontinuous polyacrylamide gel electrophoresis (PAGE) of radiolabelled proteins. The overall dimensions of the gel were 14 cm wide, 16 cm long, and 1.5 mm thick with a running gel of 12.5% acrylamide approximately 12.5 cm long and a stacking gel of 4% acrylamide approximately 2.5 cm long. Initially, the running gel was prepared as follows: 14.8 ml of acrylamide/bis-acrylamide (33.5%/0.3%) solution (International Biotechnologies Inc., New Haven, CT), 15.2 ml 1 M Tris-HCl (pH 9.1)(IBI), and 8.3 ml water were
degassed under vacuum for 5 minutes. After degassing, 0.4 ml 10% SDS (IBI), 0.5 ml 3% ammonium persulfate (Bio-Rad Laboratories, Richmond, CA) and 10 µl of N,N,N',N'-tetramethylethylene diamine (TEMED)(Bio-Rad) were added. This solution was poured to a height of 12.5 cm between the glass plates, overlaid with water saturated butanol, and allowed to polymerize for 2 hours. The butanol was poured off and the top of the gel was rinsed with water. The components of the stacking gel [1.3 ml acrylamide/bis (30%/0.44%), 2.5 ml 0.5 M Tris-HCl (pH 6.8), and 6.1 ml water] were degassed under vacuum for 5 minutes and 0.1 ml 10% SDS, 0.1 ml 3% ammonium persulfate, and 10 µl of TEMED were added. This solution was poured to the top of the glass plates and the comb was inserted approximately 1 cm. This was allowed to polymerize for 2 hours. Up to 25 µl of sample in 0.2 M Tris-HCl (pH 6.7), 4% SDS, 10% glycerol (Eton-Colby, Columbus, OH), and 5% 2-mercaptoethanol were then loaded into each sample well and run at 20 milliamps through the stacking gel and 30 milliamps through the running gel. At the completion of the run (ca. 3.5 hours), the gel was fixed and stained in a 0.3% Coomassie Blue (Bio-Rad), 50% methanol (Fisher), 30% acetic acid (EM Science) solution and destained with several changes of 10% methanol-10% acetic acid in water.

Gels to be autoradiographed (see above) were dried according to the following procedure. The polyacrylamide
gels were soaked in a 20% solution of 2,5-diphenyloxazole (PPO)(Fisher) in glacial acetic acid. The gel was transferred to an 18 x 34 cm piece of filter paper backing (Bio-Rad) and both the gel and filter paper were placed on the gel dryer (Bio-Rad Model 224). A piece of Saran Wrap (a clear plastic food wrap) was placed over the gel and this was covered with the rubber dryer cover. A vacuum was drawn on the apparatus for 2 hours with the heating unit of the dryer turned on. After the gel had dried, it was autoradiographed as described above.

Polyacrylamide Gel Electrophoresis of tRNA.

Preparative Polyacrylamide Gel Electrophoresis. A partially purified preparation of yeast tRNA obtained from Dr. Gerard Keith (Inst. Biol. Mol. Cell. CNRS, 67084 Strasbourg, France) was further purified by preparative polyacrylamide gel electrophoresis. This particular sample contained 3 yeast tRNA species: aspartic acid, glycine, and alanine. These three tRNA species were separated on partially denaturing polyacrylamide gels 14 cm wide, 16 cm long, and 1.5 mm in thickness.

The gel was prepared as follows: To 40 ml of gel solution [10% acrylamide, 0.5% bis-acrylamide, 4 M urea (Sigma), 100 mM Tris-borate (pH 8.3), and 2.5 mM EDTA (Sigma)] was added 400 ul of 10% ammonium persulfate and 40 ul of TEMED to start the polymerization process. This
solution was poured up to the top of the glass plates and allowed to polymerize for one hour with a 15 well sample comb inserted approximately 1 cm. The comb was removed and the sample wells were quickly rinsed with water. The gel was pre-run at 500 volts for one hour in 4.5 l pre-cooled tank buffer [100 mM Tris-borate (pH 8.3) and 2.5 mM EDTA]. The tank buffer was cooled to approximately 6°C by running cold tap water through a cooling coil in the lower buffer reservoir. The tRNA sample was dissolved in a sample buffer which was 4 M in urea, 15% sucrose, and 0.05% in both bromophenol blue (Bio-Rad) and xylene cyanol (IBI). Five microliters of sample containing 0.75 A260 units of tRNA were loaded into each sample well of the gel. The gel was then run at 500 volts for approximately 4 hours until the xylene cyanol marker reached the bottom of the gel.

The gel was removed from the glass plates and placed between 2 sheets of Saran Wrap. The gel was placed over a TLC sheet containing fluorescent indicator. The tRNA bands were then visualized by illuminating the gel with a UV lamp (Hassur and Whitlock, 1974). After the bands were identified, they were excised and cut into small pieces (<5 mm²). The tRNA was eluted and recovered by a modification of the method of Maxam and Gilbert (1977). The gel slices were suspended in just enough elution buffer (500 mM ammonium acetate, 10 mM MgCl₂, 0.1% SDS,
and 100 μM EDTA) to cover the slices. This suspension was rocked on a rocker platform (Bellco, Vineland, NJ) for 12-18 hours to elute the tRNA from the gel slices. The elution buffer was then pipetted off and the tRNA was precipitated with 3 volumes of ethanol.

**Analytical Polyacrylamide Gel Electrophoresis of tRNA Fragments.** Transfer RNA fragments, generated by enzymatic cleavage of pure tRNAs (described below), were purified by denaturing polyacrylamide gel electrophoresis. These analytical gels were 14 cm wide, 16 cm long, and 0.75 mm thick.

The gel was prepared as follows: To 20 ml of gel solution [0.75% bis-acrylamide, 8 M urea, 100 mM Tris-borate (pH 8.3), and 2.5 mM EDTA] was added 200 μl of 10% ammonium persulfate and 20 μl of TEMED to start the polymerization process. This solution was poured to the top of the glasses plates and allowed to polymerize for one hour with a 15 well sample comb inserted approximately 1 cm. The comb was removed and the sample wells were quickly rinsed with water. The gel was pre-run at 800 volts for one hour in 4.5 l of uncooled tank buffer (100 mM Tris-borate (pH 8.3) and 2.5 mM EDTA). The tRNA was dissolved in a sample buffer of 8 M urea, 15% sucrose, and 0.05% bromophenol blue and xylene cyanol. Five microliters of sample were loaded per well. The gel was
run at 800 volts until the xylene cyanol marker was approximately 2-3 cm from the bottom of the gel (ca. 3 hours). The gel was removed from the glass plates and stained for 5 minutes in a solution of 0.2% methylene blue, 3.9% sodium acetate, and 2% acetic acid. The gel was then destained with several changes of water. The tRNA was excised, eluted, and recovered as described above.

**HPLC Purification of tRNA.**

The partially purified preparation of yeast tRNA (described above) was sometimes further purified by HPLC instead of polyacrylamide gel electrophoresis. Using a Phenomenex W-Porex C-4 column (Rancho Palo Verdes, CA; 250 x 4.6 mm; 5 µm packing support) with a 0.2 M KH\(_2\)PO\(_4\)/K\(_2\)HPO\(_4\) (pH 7.0)/0.75% 2-propanol mobile phase (1 ml/min), large quantities (up to 30 A\(_{260}\) units) of tRNA were separated with a linear gradient of 2 M (NH\(_4\))\(_2\)SO\(_4\) (60%—>0%) over 40 minutes. Pooled fractions were dialyzed against two changes of two liters of water (12 hours each) and then two liters of 50 mM sodium acetate (pH 4.5) and 50% glycerol for 24 hours. The tRNA was precipitated with LiCl:glycogen and ethanol as described above.
Enzymatic Cleavage of tRNA.

Ribonuclease T1. Ribonuclease T1 from Aspergillus oryzae (PL Biochemicals) was used to cleave the tRNA anticodon specifically after guanine residues yielding a 5' hydroxyl group and a 3' phosphate at the cleavage site. In a modification of the method of Carbon et al. (1982), the reaction was carried out in a 20 mM cacodylic acid buffer (pH 6.5)(Sigma) with 10 mM MgCl2. Generally, 500 µg of tRNA were cleaved in 500 µl of reaction mixture containing 50 units T1 (100 units T1/mg tRNA). The reaction mixture was made without RNase-T1 present and was pre-incubated for 5 minutes at 4°C. After adding the enzyme and incubating for 45 minutes at 4°C, the reaction was stopped by adding 5 µl diethylpyrocarbonate (Sigma) with vigorous vortexing. The reaction mixture was then extracted with 500 µl of phenol saturated with 100 mM sodium acetate (pH 4.8). After vortexing for 1 minute, the mixture was centrifuged at 12,000 rpm (12,400 x g) in a microcentrifuge for 5 minutes. The aqueous phase was removed and the phenol was reextracted with 500 µl of water. The aqueous phases were combined and the tRNA fragments were ethanol precipitated. The cleavage fragments were then purified by analytical PAGE (described above).
Nuclease S₁. Nuclease S₁ from Aspergillus oryzae (PL Biochemicals) was used to cleave the tRNA anticodon yielding a 3' hydroxyl group and a 5' phosphate at the cleavage site. Nuclease S₁ has a slight preference for cleavage after uridine residues but may cleave in any single stranded region. The reaction was carried out in a 20 mM cacodylic acid buffer (pH 6.5) with 10 mM MgCl₂ according to the method of Fournier et al. (1983). Generally, 500 µg of tRNA were cleaved in 500 µl of reaction mixture containing 100-400 units S₁ [200-800 units S₁/mg tRNA]. The reaction mixture was made without nuclease-S₁ present and was pre-incubated for 5 minutes at 37°C. After adding the enzyme and incubating for 45 minutes at 37°C, the reaction was stopped by adding 5 µl diethylpyrocarbonate with vigorous vortexing. The reaction mixture was extracted with 500 µl phenol saturated with 100 mM sodium acetate (pH 4.8). After vortexing for 1 minute, the mixture was centrifuged at 12,000 rpm (12,400 x g) in a microcentrifuge for 5 minutes. The aqueous phase was removed and the phenol was reextracted with 500 µl of water as before. The aqueous phases were combined and the tRNA fragments were ethanol precipitated. The cleavage fragments were then purified by analytical PAGE (described above).
Mung bean nuclease. Mung bean nuclease (PL Biochemicals) was also used to cleave tRNA molecules after uridine residues in the anticodon using the procedure of Beauchemin et al. (1986). In a reaction mixture containing 30 mM sodium acetate (pH 4.5), 50 mM NaCl, and 1 mM ZnSO₄, 500 μg of tRNA (1 mg/ml) were cleaved with 10 units Mung bean nuclease (20 units/mg tRNA) at 37°C for 15 minutes. Phenol extraction and ethanol precipitation were performed as described above. The cleavage fragments were then purified by analytical PAGE (also described above).

End-labelling RNA.

5' End-labelling with [γ-32P]ATP.

T₄-polynucleotide kinase from infected E. coli cells (Amersham) was used to label the 5' end of tRNA fragments with 32P using [γ-32P]ATP following a method similar to that of Donis-Keller et al. (1977). This procedure [along with nuclease P₁ digestion (see below) and two-dimensional thin layer chromatography (see above)] was used to identify the 5' nucleotide of the tRNA fragments created by enzymatic cleavage (see above). The reaction was carried out at either pH 6.9 or pH 7.6 depending on the state of the 5' nucleotide. If the 5' nucleotide had a terminal phosphate group, the reaction was carried out at pH 6.9. At this pH, the enzyme transfers the 5' terminal phosphate of the RNA to ADP present in the
reaction mixture. Since the pH is 6.9, the kinase activity of the enzyme also acts to replace the removed phosphate using the $[^\gamma-32P]ATP$ present. Therefore, this reaction served to replace the 5' terminal phosphate of the RNA fragment with a $^{32}P$ label.

When the RNA to be labelled terminated in a hydroxyl group, the labelling reaction was carried out at pH 7.6 where the enzyme acts exclusively as a kinase. In this instance, the reaction was run in the same reaction mixture with the exception of the pH of the buffer. The method of labelling was the same for each of the above reactions except for the pH of the buffer used. Ethanol precipitated RNA samples (0.05-0.2 A$_{260}$ units) were collected by centrifugation at 12,000 rpm (12,400 x g) for 15 minutes in a microcentrifuge at 4°C. The ethanol was decanted and the RNA samples were dried under a vacuum. The RNA samples were then resuspended in 10 µl of a reaction mixture containing 50 mM imidazole (Sigma), 10 mM MgCl$_2$, 5 mM DTT, 0.1 mM EDTA, 30 µM ADP, 10 µCi $[^\gamma-32P]ATP$ (>3,000 Ci/mmol), and 1 unit of T$_4$-polynucleotide kinase. This mixture was incubated at 37°C for 2 hours. The reaction was stopped by adding 10 µl of 2x electrophoresis sample buffer. Labelled RNA was then purified by analytical PAGE, eluted, and digested with nuclease P$_1$ as described below. The labelled nucleotide was then identified by TLC as described above.
3' End-labelling With [5'-32P]pCp. T4-polynucleotide kinase and [gamma-32P]ATP were used to add a 32P label on the 5' side of cytidine 3' monophosphate (Cp) (Sigma). Using the method of England and Uhlenbeck (1978), 100 μCi of [gamma-32P]ATP (>3,000 Ci/m mole) were dried under a vacuum in an Eppendorf microcentrifuge tube. This ATP was suspended in 20 μl of a reaction mixture containing 50 mM Tris-HCl (pH 7.6), 10 mM MgCl2, 10 mM (DTT), 50 μg bovine serum albumin (BSA)/ml, 6.25 mM Cp, and 5 units of T4-polynucleotide kinase. The reaction mixture was incubated at 37°C for 1 hour, and the reaction was then stopped by heating to 65°C for 3 minutes. The [5'-32P]pCp was stored at -20°C until it was used.

T4-RNA ligase from infected E. coli cells (PL Biochemicals) was used to label the 3' end of RNA fragments with the [5'-32P]pCp as described by Bruce and Uhlenbeck (1978). This procedure [along with nuclease T2 digestion (see below) and two-dimensional thin layer chromatography (see above)] was used to identify the 3' nucleotide of the tRNA fragments generated by enzymatic cleavage. RNA, which had been dephosphorylated with T4-polynucleotide kinase at pH 6.0, was ethanol precipitated, collected by centrifugation, and dried under vacuum. To each dried tRNA sample, a 10 μl reaction mixture was added which contained 50 mM HEPES buffer (pH 7.6) (Research Organics, Cleveland, OH), 125 μM ATP, 20 mM
MgCl₂, 3.3 mM DTT, 10 μg BSA/ml, 50 μCi [5'-³²p]pCp, and 1.5 units of T₄-RNA ligase. The reaction mixture was incubated at 4°C overnight and the reaction was stopped by adding 10 μl of 2x electrophoresis sample buffer. The fragments were purified by analytical PAGE and eluted as described above. After hydrolysis with ribonuclease T₂ (described below), the 3' labelled nucleotides were then determined by TLC as described above.

**Hydrolysis of tRNA.**

**Nuclease P₁.** Nuclease P₁ from *Penicillium citrinum* (PL Biochemicals) was used to digest tRNA fragments to the 5' monophosphates following a modification of the method of Silberklang *et al.* (1977). Dried tRNA samples were suspended in 10 μl of a reaction mixture which was 50 mM in ammonium acetate (pH 5.3) and contained 1 μg of nuclease P₁. The reaction mixture was incubated at 37°C for 2 hours after which it was either frozen at -20°C for later use or was applied directly to a TLC sheet for resolution of the nucleoside monophosphates (described above).

**Ribonuclease T₂.** Ribonuclease T₂ from *Aspergillus oryzae* (PL Biochemicals) was used to digest tRNA fragments to the 3' monophosphates according to a modification of the method of Nishikura and De Robertis (1981). Dried
tRNA samples were suspended in 10 µl of a reaction mixture which was 50 mM in HEPES buffer (pH 4.5) and contained 0.1 unit of ribonuclease T2. The reaction mixture was incubated at 37°C for 2 hours after which it was either frozen at -20°C for later use or was applied directly to a TLC sheet for resolution of the nucleoside monophosphates (described above).

Periodate Oxidation.

Sodium metaperiodate and lysine were used according to the method of Keith and Gilham (1974) to cleave the base from the 3' end of tRNA fragments to generate a 3' phosphate. This was done to block that end of the fragment from reaction in the subsequent ligation reaction. The tRNA fragment to be modified was suspended in 300 µl water in a 1.5 ml Eppendorf tube and covered with foil. Sodium metaperiodate (30 µl, 100 mM)(Sigma) was added and incubated for 60 minutes in the dark at 4°C. Rhamnose (4 µl, 1 M)(Sigma) was then added and incubated for 20 minutes at room temperature to inactivate the excess periodate. Lysine (100 µl, 1 M, pH 8.9)(Sigma) was then added and incubated 45 minutes at 45°C to cleave the 3' base from the tRNA fragment. The fragment was then ethanol precipitated.
5' Phosphorylation.

T₄-polynucleotide kinase and ATP were used to phosphorylate the 5' hydroxyl group of tRNA fragments using the method of Donis-Keller et al. (1977). The tRNA fragment to be phosphorylated was suspended in 10 µl of a reaction mixture which contained 50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 10 mM DTT, 50 µg BSA/ml, and 10 mM ATP. Five units of T₄-polynucleotide kinase were added and the reaction mixture was incubated for 2 hours at 37°C. The reaction was stopped by heating the mixture to 80°C for 2 minutes, after which the tRNA was precipitated (as above).

Dephosphorylation.

Dephosphorylation of 3' RNA phosphate was accomplished using T₄-polynucleotide kinase at pH 6.0. Using the method of Carbon et al. (1982), the tRNA fragment was suspended in a small amount of buffer containing 100 mM imidazole (pH 6.0), 10 mM MgCl₂, 10 mM 2-mercaptoethanol, and 4 mM ATP. T₄-polynucleotide kinase was then added to a final concentration of approximately 1 unit/µg tRNA, and the mixture was incubated for 2 hours at 37°C. The reaction was stopped by heating at 80°C for 2 minutes. The reaction volume was then brought to 100 µl with water and was extracted with an equal volume of phenol saturated with 0.3 M sodium acetate (pH 4.5). The phenol was reextracted with 100 µl of water and the
aqueous phases were combined before ethanol precipitation. The tRNA was then washed several times with 70% ethanol to remove traces of phenol which could interfere with subsequent reactions.

**Renaturation and Reannealing of tRNA.**

Denatured tRNA may be returned to its native conformation by heating it to a high temperature and then allowing it to cool slowly (Fournier *et al.* 1983). Denatured tRNA was suspended in 50 mM Tris-HCl (pH 7.6) and 20 mM MgCl₂ to a concentration of approximately 1 A₂₆₀ unit/100 μl of solution. This solution was heated to 70°C for 5 minutes in a water bath after which the heating unit was turned off to allow the water to cool slowly to 45°C. The tRNA was then removed from the water bath and allowed to cool at room temperature for an additional 30 minutes. The tRNA was then ethanol precipitated.

It is also possible to reanneal 2 tRNA "half" molecules so they may combine to form an "anticodon deprived" tRNA. In this case, equal quantities of the two half molecules were suspended in the renaturation buffer. This solution was then heated and cooled as above.

**Radiolabelling Oligonucleotides.**

Dinucleotide monophosphates (Sigma) to be ligated into reconstructed tRNA were 5' end-labelled with
T₄-polynucleotide kinase and [gamma-³²P]ATP. This was done to label the nucleotide of interest in the tRNA anticodon so it might be monitored during modification studies in vitro or in vivo. After drying 100 µCi of [gamma-³²P]ATP (>3,000 Ci/mmol) under a vacuum, it was resuspended in 3 µl of reaction buffer [100 mM Tris-HCl (pH 7.6), 20 mM MgCl₂, 20 mM DTT, and 100 µg BSA/ml] and 2 µl of dinucleotide monophosphate (60 µM).

T₄-polynucleotide kinase was added to a final concentration of 150 units/ml of reaction volume and incubated for 2 hours at 37°C. The reaction was terminated by heating to 80°C for 2 minutes and the reaction mixture was used immediately in the subsequent ligation reaction (described below).

**Ligation.**

Following procedures similar to those of England and Uhlenbeck (1978), T₄-RNA ligase was used to covalently join 2 tRNA fragments. This ligase enzyme requires a 3' phosphate and a 5' hydroxyl group for ligation to occur. The reannealled tRNA was suspended in 2 µl of buffer [50 mM HEPES (pH 7.6), 120 µM ATP, 20 mM MgCl₂, 3.3 mM DTT, and 10 µg BSA/ml] and 6 µl of dinucleotide monophosphate labelling reaction mixture (described above). Two units of T₄-RNA ligase were added and incubated at 4°C for 18-24 hours. The reaction was stopped by addition of 10 µl of
2x sample buffer for electrophoresis (8 M urea, 30% sucrose, 0.1% bromophenol blue, and 0.1% xylene cyanol). The ligated tRNA was then purified on a denaturing analytical polyacrylamide gel.

**Microinjection of Oocytes.**

*Xenopus laevis* oocytes were microinjected following procedures similar to those of Carbon *et al.* (1983) and Haumont *et al.* (1984). After anaesthetizing the frog (hypothermia induced with 2 hours in a shallow ice water bath), the ovaries were surgically removed. Individual oocytes were extracted, cleaned, and stored in Barth's buffer (88 mM NaCl, 1.0 mM KCl, 2.4 mM NaHCO₃, 15 mM HEPES-NaOH (pH 7.6), 0.30 mM Ca(NO₃)₂·4H₂O, 0.41 mM CaCl₂·4H₂O, 0.82 mM MgSO₄·7H₂O, 10 µg sodium penicillin/ml, and 10 µg streptomycin sulphate/ml) at 19°C. Oocytes were injected in groups of 5 per data point with 50 nl of >200,000 cpm/ul of ³²P-labelled tRNA and were incubated for various periods of time (0.5-72 hours) at 19°C in Barth's buffer. After the appropriate period of incubation, the Barth's buffer was removed and the cells were crushed and resuspended in 200 µl of extraction buffer (200 mM sodium acetate (pH 4.5), 10 mM MgCl₂, 1 mM EDTA, and 1% SDS). Unfractionated *E. coli* tRNA (10 µl at 1 mg/ml) was added to the extraction mixture to aid in the recovery of the tRNA. The extraction buffer was extracted
with an equal volume of phenol saturated with 0.3 M sodium acetate (pH 4.8). After recovery of the aqueous phase, the phenol was reextracted with 200 μl water and the aqueous phases were combined, ethanol precipitated, and purified by electrophoresis on a denaturing, analytical polyacrylamide gel to ensure that only intact tRNA was hydrolyzed. The gel was then autoradiographed and the tRNA was eluted, hydrolyzed to the nucleoside monophosphate level, and the labelled nucleoside monophosphates were identified by TLC (described above).
RESULTS

**HGPRT Deficiency.** To verify that the mutant HL-60 cells were lacking in HGPRT, sets of both wild-type HL-60 cells and HGPRT-deficient HL-60 cells were incubated in the presence of $[^3]$H]hypoxanthine. After a 2 day incubation, it was clear that the wild-type HL-60 cells salvaged a large amount of the radiolabelled hypoxanthine whereas, the HGPRT-deficient cells salvaged very little (Figure 4).

**HL-60 Differentiation with Guanine Analog.** The structures of the guanine analogs used to treat the HL-60 cells are shown in Figure 5, and a dose-dependent cessation of proliferation was observed when wild-type HL-60 cells or HGPRT-deficient HL-60 cells were treated with increasing concentrations of either agent (Figures 6 and 7). In Figure 6(A), wild-type HL-60 cells were treated with 0.1 and 0.02 mM 6-thioguanine, while in Figure 6(B), the cells were treated with 0.1 and 0.02 mM 8-azaguanine. In Figure 7(A), HGPRT-deficient HL-60 cells were treated with 0.1 and 0.02 mM 6-thioguanine, while in Figure 7(B), the cells were treated with 0.1 and 0.02 mM 8-azaguanine.
Figure 4. Incorporation of $[^3H]$hypoxanthine by wild-type HL-60 cells and HGPRT-deficient HL-60 cells. Cells cultured for 48 hours in the presence of 40 μCi of $[^3H]$hypoxanthine were lysed and the nucleic acids were precipitated. Acid precipitable counts were collected and counted by liquid scintillation.
Figure 4.

[3H]Hypoxanthine Incorporation (kcpm)

Wild-type HL-60

HGPRT-deficient HL-60
Figure 5. Structures of 6-thioguanine and 8-azaguanine.
6-THIOGUANINE

8-AZAGUANINE

Figure 5.
Figure 6. Growth curves of wild-type HL-60 cells treated with guanine analogs. Cells in panel (A) were grown in the presence of various amounts of 6-thioguanine as follows: Control (○), 0.02 mM 6-thioguanine (△), and 0.1 mM 6-thioguanine (□). Cells in panel (B) were grown in the presence of various amounts of 8-azaquanine as follows: Control (○), 0.02 mM 8-azaquanine (△), and 0.1 mM 8-azaquanine (□).
Figure 7. Growth curves of HGPRT-deficient HL-60 cells treated with guanine analogs. Cells in panel (A) were grown in the presence of various amounts of 6-thioguanine as follows: Control (○), 0.02 mM 6-thioguanine (△), and 0.1 mM 6-thioguanine (□). Cells in panel (B) were grown in the presence of various amounts of 8-azaguanine as follows: Control (○), 0.02 mM 8-azaguanine (△), and 0.1 mM 8-azaguanine (□).
Figure 7.
After 8 days, the cells from Figures 6 and 7 were tested for morphological differentiation using differential cell counting. It was found that the wild-type HL-60 cells were too severely affected by the guanine analog treatment to be counted. A great deal of cell debris indicated extensive cell death had occurred and the intact cells exhibited unusual intracellular structure which could not be classified into any of the normal cell categories. In contrast, the HGPRT-deficient HL-60 cells were healthy-looking, but showed very little differentiated character when counted (Figure 8).

HGPRT-deficient HL-60 cells treated with even higher concentrations of 6-thioguanine or 8-azaguanine (Figure 9) again demonstrated the dose-dependent cessation of proliferation (Figure 9). In Figure 9(A), the cells were treated with 0.2 and 0.4 mM 6-thioguanine, while in Figure 9(B), the cells were treated with 0.1 and 0.5 mM 8-azaguanine.

The expression of a differentiation-specific cell surface antigen was examined with the OKM1 monoclonal antibody to determine whether the growth inhibition induced by the guanine analogs reflected a commitment to terminal cell differentiation. Control (untreated) cells expressed very little of the antigen [Figure 10(A)], while cells treated for 8 days with 0.4 mM 6-thioguanine [Figure 10(B)] or 0.4 mM 8-azaguanine [Figure 10(C)] expressed a
Figure 8. Differential counts of HGPRT-deficient HL-60 cells after incubation with guanine analogs. The cells were treated for 8 days with the indicated amount of guanine analog. The differential counts are from the experiment depicted in Figure 7, but similar results have been obtained in numerous independent experiments.
Figure 8.
Figure 9. Growth curves of HGPRT-deficient HL-60 cells treated with guanine analogs. Cells in panel (A) were grown in the presence of various amounts of 6-thioguanine as follows: Control (○), 0.2 mM 6-thioguanine (□), and 0.4 mM 6-thioguanine (▲). Cells in panel (B) were grown in the presence of various amounts of 8-azaguanine as follows: Control (○), 0.1 mM 8-azaguanine (□), and 0.5 mM 8-azaguanine (▲).
Figure 9.
Figure 10. Cytofluorographic analysis of a differentiation-specific cell surface antigen determined with the OKM1 monoclonal antibody. Indirect immunofluorescence staining was performed and evaluated with an Ortho Diagnostic Systems Cytofluorograf System 50H as described in the Materials and Methods. Cell number is plotted versus fluorescence intensity for untreated cells (A), cells treated with 0.4 mM 6-thioguanine (B), and cells treated with 0.4 mM 8-azaguanine (C). All cells were cultured for 8 days prior to analysis.
Figure 10.
significant amount. The percentage of CD1-positive HL-60 cells in each population depicted in Figure 10 were: Control 4.8%; 6-thioguanine, 78.2%; and 8-azaguanine 49.5%.

Queuine Hypomodification. To establish whether the guanine analogs were inhibiting queuine modification of tRNA, cells were grown in parallel sets, control and 0.4 mM 6-thioguanine treated, for tRNA isolation. The tRNA was then used as a substrate in the guanine incorporation assay. The points at which tRNA was isolated are indicated by the arrows in the growth curves depicted in Figure 11(A), i.e., day 0, day 2, and day 5. As shown in Figure 11(B), HGPRT-deficient HL-60 cells treated with 6-thioguanine were transiently hypomodified for queuine (the tRNA was a better substrate for radiolabelled guanine incorporation), whereas the control cell tRNA remained nearly totally queuine modified. The most significant queuine hypomodification occurred on day 2, prior to the time at which cell proliferation decreased significantly in the treated cells. The 8-fold difference in guanine incorporation on day 2 in the experiment depicted was the lowest difference between control and treated cells in three independent experiments (the others being 10- and 14-fold), and these differences were determined to be highly significant (p=0.001) by the Mann-Whitney U test. In one experiment, the guanine incorporation into tRNA was
Figure 11. Queuine hypomodification of tRNA induced by 6-thioguanine. Transfer RNA was isolated from cells at the times indicated by the arrows in the growth curve in panel (A). The cell populations are: Control (o) and 0.4 mM 6-thioguanine treated (Δ). Panel (B) shows the amount of queuine hypomodification (radiolabelled guanine incorporation) of the tRNA from control cells (cross hatch) and 6-thioguanine treated cells (open bars), at the times indicated. See Materials and Methods for details.
Figure 11.
Guanine Incorporation (pmol/hour/tRNA A₂₅₀ unit)

Cell Number (x₁₀⁻⁵)

Days

0 1 2 3 4 5
also evaluated on day 1, and a 2.5-fold increase was observed for the 6-thioguanine treated cells (data not presented). By day 5, when cell proliferation had ceased in the 6-thioguanine treated cell population, the tRNA was again nearly fully queuine modified [Figure 11(B)].

**Guanine Analog Insertion.** RFC-5 chromatography was performed on tRNA from HGPRT-deficient HL-60 cells grown in the presence of the guanine analogs to determine whether the analogs were substrates for insertion. Figure 12(A) is the histidine isoacceptor profile for tRNA from untreated, HGPRT-deficient HL-60 cells grown in charcoal treated serum (queuine deficient). When the cells were treated with 6-thioguanine, a very different histidine tRNA isoacceptor profile was obtained [Figure 12(B)], with new, late-eluting species appearing. Transfer RNA isolated from 8-azaguanine treated cells yielded another histidine tRNA isoacceptor profile [Figure 12(C)].

**Queuine Reversal.** To determine whether the changes in queuine modification of tRNA play some role in guanine analog-induced differentiation, the growth of cells treated with 6-thioguanine or 8-azaguanine with and without excess queuine was evaluated. The cells which were used for the queuine reversal tests were pretreated for 12 hours with 0.5 μM queuine added to the medium. The growth curves shown in Figure 13 demonstrate that queuine was able to reverse, at least partially, the
Figure 12. RPC-5 chromatography of the histidine isoacceptors from cells treated with guanine analogs. Transfer RNA from HGPRT-deficient HL-60 cells was aminoacylated with $[^3]$H]histidine and separated by RPC-5 chromatography. The profile presented in each of the panels is as follows: Panel (A), untreated control cells; Panel (B), 0.4 mM 6-thioguanine; Panel (C), 0.4 mM 8-azaguanine.
Figure 12.
Figure 13. Growth curves and differentiation markers of HGPRT-deficient HL-60 cells treated with guanine analogs plus or minus additional queuine. The cell populations in panel (A) were treated as follows: Untreated controls (○), 0.4 mM 6-thioguanine (□), and 0.4 mM 6-thioguanine plus 0.5 μM queuine (■). The cells in panel (B) were treated as follows: Untreated controls (○), 0.4 mM 8-azaguanine (△), and 0.4 mM 8-azaguanine plus 0.5 μM queuine (▲). The cells in panel (C) were treated as follows: Untreated controls (○), 300 mM DMSO (△), 300 mM DMSO plus 0.5 μM queuine (▲). Panel (D) presents the results of the differential cell counting and NBT reduction from the cells in Panel (A). The designations are: Untreated controls, solid bar; 0.4 mM 6-thioguanine, right hatch; 0.4 mM 6-thioguanine plus 0.5 μM queuine, cross hatch. Panel (E) corresponds to Panel (B) as follows: Untreated controls, solid bar; 0.4 mM 8-azaguanine, right hatch; 0.4 mM 8-azaguanine plus 0.5 μM queuine, cross hatch. Panel (F) corresponds to Panel (C) as follows: Untreated controls, solid bar; 300 mM DMSO, right hatch; 300 mM DMSO plus 0.5 μM queuine, cross hatch.
guanine-analog induced growth inhibition of HGPRT-deficient HL-60 cells. In Figure 13(A), queuine was added at a concentration 800-fold lower than 6-thioguanine (0.5 μM vs. 0.4 mM), and yet a significant reversal of the growth inhibition is obvious. In fact, queuine concentrations as much as 4,000-fold lower than 6-thioguanine had some effect (data not presented). In the case of 8-azaguanine [Figure 13(B)], queuine was also added at a concentration 800-fold lower (0.5 μM vs. 0.4 mM), and that resulted in a somewhat greater reversal of the guanine analog-induced response. Addition of 0.5 μM exogenous queuine had no effect on the growth of untreated control cells (data not presented).

Confirmation that the queuine mediated reversal of growth inhibition correlated to an effect on differentiation was obtained by examining the cells for morphological and functional differentiation after 5 days of treatment. In Figure 13(D), it can be seen that the cells treated with 0.5 μM queuine in addition to 0.4 mM 6-thioguanine were less differentiated (had a higher percentage of promyelocytes and fewer cells that could reduce NBT) than those cells not receiving excess queuine. Slightly different results were obtained with 8-azaguanine. The data in Figure 13(E) indicate little change in the morphological differentiation of these cells but a substantial reversal of the functional
differentiation (based on NBT reduction). Addition of excess exogenous queuine also had no effect on the morphological or functional differentiation of untreated control cells (data not presented).

For comparative purposes, the ability of excess queuine to reverse the DMSO-induced differentiation of HL-60 cells was also examined. As can be seen in Figure 13(C), 0.5 μM queuine had no significant effect on the growth inhibition of the HGPRT-deficient HL-60 cells induced with 300 mM DMSO, nor did it reverse the induction of differentiation as reported in Figure 13(F). Although these results would indicate that DMSO is not acting via tRNA-guanine ribosyltransferase, the possibility remains that it is acting via a similar mechanism. Experiments described below were undertaken to investigate the possibility that DMSO is acting via the tRNA-hypoxanthine ribosyltransferase modification (Figure 3).

The differences in cells observed by staining with Wright-Giemsa during differential cell counting are documented in Plates I - III. Untreated control cells [Plate I(A)] and cells treated with 0.5 μM queuine [Plate I(B)] show the common characteristics of promyelocytes including large rounded nuclei with nucleoli and cytoplasmic inclusions. Cells induced to differentiate for 5 days with 0.4 mM 6-thioguanine [Plate II(A)] show characteristics associated with more mature cells
Plate I. Untreated control cells (HGPRT-deficient HL-60) (A) or cells treated with 0.5 μM queuine (B) prepared by cytocentrifugation and stained with Wright-Geimsa.
Plate I.

(A)

(B)
Plate II. HGPRT-deficient HL-60 cells treated with 0.4 mM 6-thioguanine (A) or 0.4 mM 6-thioguanine plus 0.5 μM queuine (B) prepared by cytocentrifugation and stained with Wright-Giemsa.
Plate II.

(A)

(B)
Plate III. HGPRT-deficient HL-60 cells treated with 0.4 mM 8-azaguanine (A) or 0.4 mM 8-azaguanine plus 0.5 µM queuine (B) prepared by cytocentrifugation and stained with Wright-Geimsa.
Plate III.

(A)

(B)
including smaller nuclei with no nucleoli and some indenting of the nuclei. When cells were treated with 0.5 μm queuine in addition to the 0.4 mM 6-thioguanine, the cells show more immature cell characteristics than those induced to differentiate with 6-thioguanine [Plate II(B)], indicating a reversal of the inducing effects of the 6-thioguanine. Similar results were seen when 0.4 mM 8-azaguanine was used as the inducing agent [Plate III(A)] and the effects were reversed with 0.5 μM queuine [Plate III(B)].

Altered Protein Synthesis. Based on the fact that the queuine analogs are substrates for tRNA-guanine ribosyltransferase, it was felt that the presence of these modified bases in the tRNA anticodon might be affecting protein synthesis by changing the anticodon base-pairing. Therefore, HGPRT-deficient HL-60 cells (control and 6-thioguanine treated) were incubated in the presence of [3H]histidine. After lysing the cells and separating the proteins by SDS-polyacrylamide gel electrophoresis, it was found that a prominent protein of approximately 12,000 molecular weight was synthesized by cells treated with 6-thioguanine which is not present in the control sets (Plate IV).

HL-60 Differentiation with DMSO and Hypoxanthine. HL-60 cells, both wild-type and HGPRT-deficient, respond to induction of differentiation by DMSO or hypoxanthine in
Plate IV. Autoradiograph of proteins synthesized by HGPRT-deficient HL-60 cells treated with 6-thioguanine and resolved by SDS-polyacrylamide gel electrophoresis. After 48 hours of treatment with or without 6-thioguanine, the cells were resuspended in histidine depleted culture medium containing [3H]-histidine. After 4 hours at 37°C, the cells were harvested and lysed. The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. The lanes represent: untreated control cells, (A) and (B); 6-thioguanine treated cells, (C) and (D).
Plate IV.
a dose-dependent manner as shown in Figures 14 and 15. When these cells were examined by differential cell counting to determine the extent of morphological differentiation, it was found that only the higher concentrations of inducer were able to induce significant morphological changes (Figure 16). Interestingly, it can be seen that unlike the case with guanine analog induction, both the wild-type HL-60 cells and the HGPRT-deficient HL-60 cells respond to induction by DMSO or hypoxanthine in a similar manner.

Growth curves of HL-60 cells treated with DMSO and hypoxanthine alone and in combination are shown in Figure 17. Treatment with 100 mM DMSO alone diminished the growth of the HL-60 cells only slightly compared to the untreated controls, while 1 mM hypoxanthine was somewhat more effective. However, the combination of 100 mM DMSO plus 1 mM hypoxanthine resulted in an almost complete cessation of growth.

Although 1 mM hypoxanthine inhibited the growth of HL-60 cells, it was not effective at inducing the differentiation of these cells (Figure 18). Likewise, 100 mM DMSO alone caused no change in the proportion of cells exhibiting a more mature morphology when compared to the untreated controls. However, the combination of DMSO and hypoxanthine yielded a significant population of cells more mature than promyelocytes (Figure 18).
Figure 14. HL-60 growth curves. Cells plated at an initial density of $2 \times 10^5$ cells/ml were counted for 9 days. The curves in Panel (A) depict untreated control cells (●), as well as cells treated with 50 mM DMSO (○), 100 mM DMSO (△), 200 mM DMSO (●), and 300 mM DMSO (○). Panel (B) depicts cells treated with 0.1 mM hypoxanthine (○), 1.0 mM hypoxanthine (△), 2.5 mM hypoxanthine (●), and 5.0 mM hypoxanthine (○). Differential cell counting was performed on Day 9 and the results are presented in Figure 16.
Figure 14.
Figure 15. HGPRT-deficient HL-60 growth curves. Cells plated at an initial density of 2 x 10^5 cells/ml were counted for 9 days. The curves in Panel (A) depict untreated control cells (●), as well as cells treated with 50 mM DMSO (○), 100 mM DMSO (△), 200 mM DMSO (●), and 300 mM DMSO (○). Panel (B) depicts cells treated with 0.1 mM hypoxanthine (○), 1.0 mM hypoxanthine (△), 2.5 mM hypoxanthine (●), and 5.0 mM hypoxanthine (○). Differential cell counting was performed on Day 9 and the results are presented in Figure 16.
Figure 15.

Cell Number ($x \times 10^{-5}$)

Days

Days
Figure 16. Differential counts of HL-60 cells (wild-type and HGPRT-deficient) after incubation with DMSO or hypoxanthine. The cells were treated for 9 days with the indicated amount of inducer. The differential counts are from the experiments depicted in Figures 14 and 15, but similar results have been obtained in numerous independent experiments. Legend: Panel (A), wild-type HL-60 cells; untreated control, cross hatch; 50 mM DMSO, narrow right hatch; 100 mM DMSO, wide right hatch; 200 mM DMSO, narrow left hatch; 300 mM DMSO, wide left hatch. Panel (B), wild-type HL-60 cells; untreated control, cross hatch; 0.1 mM hypoxanthine, narrow right hatch; 1.0 mM hypoxanthine, wide right hatch; 2.5 mM hypoxanthine, narrow left hatch; 5.0 mM hypoxanthine, wide left hatch. Panel (C), HGPRT-deficient HL-60 cells; untreated control, cross hatch; 50 mM DMSO, narrow right hatch; 100 mM DMSO, wide right hatch; 200 mM DMSO, narrow left hatch; 300 mM DMSO, wide left hatch. Panel (D), HGPRT-deficient HL-60 cells; untreated control, cross hatch; 0.1 mM hypoxanthine, narrow right hatch; 1.0 mM hypoxanthine, wide right hatch; 2.5 mM hypoxanthine, narrow left hatch; 5.0 mM hypoxanthine, wide left hatch.

NC - not counted (contamination)
Figure 16.
Figure 17. HL-60 growth curves. Cells plated at an initial density of $6 \times 10^4$ cells/ml were counted for 9 days. The curves depict untreated control cells (o), as well as cells treated with 100 mM DMSO (□), 1 mM hypoxanthine (△), and 100 mM DMSO plus 1 mM hypoxanthine (★). Differential cell counting was performed on Day 9 and the results are presented in Figure 18.
Figure 17.
Figure 18. Differential counts of HL-60 cells after incubation with DMSO and/or hypoxanthine. The cells were treated for 9 days, stained with Wright-Giemsa, and counted. The differential counts are from the experiment depicted in Figure 17, but similar results have been obtained in numerous independent experiments.
Figure 18.
**Leucine Radiolabelling.** Because hypoxanthine incorporation into tRNA should expand the codon recognition potential of the species involved, the possibility existed that protein synthesis might be altered rapidly by treating cells with hypoxanthine. Radiolabelled leucine incorporation into TCA precipitable protein was monitored with HL-60 cells treated short-term with DMSO and/or hypoxanthine. Compared to untreated control cells, the cells treated with DMSO plus hypoxanthine incorporated appreciably more leucine than did DMSO alone or DMSO plus adenine (Figure 19). Similar results were obtained when radiolabelled leucine was replaced by radiolabelled alanine or phenylalanine (results not presented).

The possibility that processes other than protein synthesis were responsible for the large increase in radiolabelled leucine incorporation into TCA precipitable material was investigated. Duplicate assays were run in a typical leucine incorporation assay with one set of the reactions receiving 50 µg/ml cycloheximide, a specific inhibitor of protein synthesis. Figure 20 indicates that a change in protein synthesis was responsible for the observed effect. In the assays run in the presence of cycloheximide, the leucine incorporation was inhibited to comparable levels in both instances, whereas the duplicate assays run without cycloheximide showed the normal
Figure 19. Enhanced leucine incorporation by HL-60 cells treated with DMSO plus hypoxanthine. Cells (5 x 10^5/ml) preincubated for 10 minutes in a Hanks' balanced salt solution alone (○), Hanks' plus 210 mM DMSO (□), 210 mM DMSO and 1 mM adenine (*), or 210 mM DMSO and 1 mM hypoxanthine (△) were radiolabelled with [14C]leucine starting at time 0. At the time intervals indicated, samples were removed, and the amount of covalently incorporated leucine was determined by acid precipitation.
Figure 19.
Figure 20. Leucine incorporation by HL-60 cells treated with DMSO plus hypoxanthine in the presence of cycloheximide. Cells preincubated for 10 minutes in a Hanks' balanced salt solution alone (○), Hanks' plus 200 mM DMSO and 1 mM hypoxanthine (□), 50 μg/ml cycloheximide (△), or 200 mM DMSO, 1 mM hypoxanthine, and 50 μg/ml cycloheximide (★) were radiolabelled with [14C]leucine starting at time 0. At the time intervals indicated, samples were removed, and the amount of covalently incorporated leucine was determined by acid precipitation.
Figure 20.
stimulation of leucine incorporation by DMSO and hypoxanthine.

The dose dependence of the hypoxanthine effect was determined by treating HL-60 cells short-term with DMSO and various concentrations of hypoxanthine and again monitoring the incorporation of radiolabelled leucine into TCA precipitable protein. Compared to untreated control cells, the cells treated with DMSO and hypoxanthine demonstrated an increase in leucine incorporation into TCA precipitable protein (Figure 21). As was the case in the growth curves, the response was dose-dependent.

To determine if the position of the cells in the growth cycle had any bearing on the results of the DMSO/hypoxanthine induced increase, leucine incorporation in proliferating cells was compared to that in resting cells. HL-60 cells harvested in the exponential phase of the growth cycle were assayed for leucine incorporation in the presence of DMSO + hypoxanthine (Figure 22). The results show a 3-fold increase in leucine incorporation with DMSO/hypoxanthine treatment when compared to untreated or DMSO treated cells. When resting cells were tested, the results were somewhat different. As shown in Figure 22(B), these cells also demonstrated increased leucine incorporation when treated with DMSO plus hypoxanthine, but the difference was nearly 12-fold in this case as compared to a 3-fold increase in the
Figure 21. Enhanced leucine incorporation by HL-60 cells treated with DMSO plus various concentrations of hypoxanthine. Cells (5 x 10^5/ml) preincubated for 10 minutes in a Hanks' balanced salt solution alone (•), Hanks' plus 200 mM DMSO and 1.0 mM hypoxanthine (O), 200 mM DMSO and 0.5 mM hypoxanthine (A), 200 mM DMSO and 0.1 mM hypoxanthine (•), or 200 mM DMSO and 0.05 mM hypoxanthine (O) were radiolabelled with [14C]leucine starting at time 0. At the time intervals indicated, samples were removed, and the amount of covalently incorporated leucine was determined by acid precipitation.
Figure 21.
Figure 22. Comparison of enhanced leucine incorporation by HL-60 cells from different points in the growth cycle. Panel (A) represents cells removed from culture on Day 5 (late proliferating stage) preincubated for 10 minutes in a Hanks' balanced salt solution alone (o), Hanks' plus 210 mM DMSO (□), or 210 mM DMSO and 1 mM hypoxanthine (△) and radiolabelled with [14C]leucine starting at time 0. At the time intervals indicated, samples were removed, and the amount of covalently incorporated leucine was determined by acid precipitation. Panel (B) represents cells removed from culture on Day 6 (early resting stage) and treated as cells from Day 5.
Figure 22.
proliferating cells. Again, cells treated with DMSO alone showed no increase in leucine incorporation.

Several other compounds were also evaluated to determine their effects on amino acid incorporation. It was determined that if nucleosides were used in place of the bases normally used (e.g. inosine and adenosine), similar results were obtained (Figure 23). However, when inosiplex (which contains inosine), polyinosinic-polycytidylic acid, and polyadenylic-polyuridylic acid were examined, no increase in leucine incorporation was observed (Figures 24 and 25).

**Inosine Modification of tRNA by BB-88 Cells.** Murine erythroleukemia cells (BB-88) were evaluated for the ability to incorporate radiolabelled hypoxanthine into tRNA in vitro. As depicted in Figure 26, a crude cell extract from these cells was able to incorporate radiolabelled hypoxanthine into unfractionated *E. coli* tRNA in a manner similar to that already reported for CCRF-CEM cells and HL-60 cells (Elliott and Trewyn, 1984).

Based on the mechanism of action of tRNA-guanine ribosyltransferase, in which a guanine for guanine exchange may occur (Okada et al., 1978), it was questioned whether a similar exchange of adenine for adenine might occur with tRNA-hypoxanthine ribosyltransferase. [³H]Adenine and unfractionated *E. coli* tRNA were used as substrates in a tRNA-hypoxanthine ribosyltransferase assay
Figure 23. Leucine incorporation by HL-60 cells treated with DMSO and various nucleosides. Cells preincubated for 10 minutes in a Hanks' balanced salt solution alone (o), 200 mM DMSO and 1 mM hypoxanthine (□), 200 mM DMSO and 1 mM inosine (*), or 200 mM DMSO and 1 mM adenosine (△) were radiolabelled with $[^{14}C]$leucine starting at time 0. At the time intervals indicated, samples were removed, and the amount of covalently incorporated leucine was determined by acid precipitation.
Figure 23.
Figure 24. Leucine incorporation by HL-60 cells treated with DMSO and inosiplex. Cells preincubated for 10 minutes in a Hanks' balanced salt solution alone (○), Hanks' plus 200 mM DMSO and 1 mM hypoxanthine (□), or 200 mM DMSO and 1 mM inosiplex (△) were radiolabelled with [14C]leucine starting at time 0. At the time intervals indicated, samples were removed, and the amount of covalently incorporated leucine was determined by acid precipitation.
Figure 25. Leucine incorporation by HL-60 cells treated with DMSO and polyinosinic-polycytidylic acid or polyadenylic-polyuridylic acid. Cells preincubated for 10 minutes in a Hanks' balanced salt solution alone (○), Hanks' plus 200 mM DMSO and 1 mM hypoxanthine (□), 200 mM DMSO and 10 μg/ml polyinosinic-polycytidylic acid (△), or 200 mM DMSO and 10 μg/ml polyadenylic-polyuridylic acid (*) were radiolabelled with [14C]leucine starting at time 0. At the time intervals indicated, samples were removed, and the amount of covalently incorporated leucine was determined by acid precipitation.
Figure 25.
Figure 26. Hypoxanthine insertion into transfer RNA.

$[^3H]$Hypoxanthine was incorporated into unfractionated E. coli tRNA by a murine erythroleukemia (BB-88) cell extract.
Figure 26.
using a BB-88 cell extract as an enzyme source. Figure 27 demonstrates the incorporation of adenine into the E. coli tRNA. It appears that the adenine was utilized by the tRNA-hypoxanthine ribosyltransferase assay and unlabelled hypoxanthine could inhibit the incorporation. It is evident that when the hypoxanthine was added at 40 minutes, it was capable of blocking further adenine incorporation by this extract (Figure 27).

Transfer RNA Reconstruction. Based on the possible regulatory implications of the inosine modification in tRNA, it was felt the modification enzyme should be characterized further. To do this, a pure tRNA isoacceptor with adenosine in position 34 was required. Since no suitable tRNAs were available commercially, tRNA anticodon reconstruction, using recombinant RNA technology, was employed to generate the tRNA-hypoxanthine ribosyltransferase substrate. It was decided that yeast tRNA_{Ala}, which naturally contains inosine in the first position (position 34) of the anticodon (Figure 28), would be used for anticodon reconstruction.

The partially purified preparation of yeast tRNA_{Ala} obtained for this study contained 3 tRNA species. These were initially purified by partially denaturing polyacrylamide gel electrophoresis on 10% acrylamide with 4 M urea (Plate V). The tRNA bands were identified by aminoacylation studies (Figure 29) on the partially
Figure 27. Adenine insertion into transfer RNA. 
[\textsuperscript{3}H]Adenine (1 \mu M) was incorporated into unfractionated E. coli tRNA by a murine erythroleukemia (BB-88) cell extract (O). Addition of 10 \mu M hypoxanthine at 40 minutes blocked further adenine incorporation (Q).
Figure 27.
Figure 28. Structure of yeast tRNA\textsuperscript{Ala}. Modified nucleosides: G1, 1-methylguanosine; D, dihydrouridine; G4, N2,N2-dimethylguanosine; I, inosine; I1, 1-methylinosine; F, pseudouridine; T, 5-methyluridine. (Sprinzl et al., 1985).
Figure 28.
Plate V. Preparative polyacrylamide gel electrophoresis. Three yeast tRNA species, partially purified by counter-current distribution, were resolved by gel electrophoresis on a 10% polyacrylamide gel containing 4 M urea. The bands of tRNA are: a) tRNA^Asp, b) tRNA^Gly, and c) tRNA^Ala.
Plate V.
Figure 29. Aminoacylation of tRNA from partially purified tRNA sample.
Figure 29.

[\[^3\text{H}\] Amino Acid Incorporation (cpm)]

- Alanine
- Glycine
- Aspartic Acid

12,000
9000
6000
3000
0
purified sample.

This electrophoretic method of purification proved too inefficient and not reproducible, so a new HPLC separation system was evaluated. Using a Phenomenex W-Porex C-4 column with an ammonium sulfate gradient, large quantities (up to 30 A260 units) of the partially purified tRNA preparation could be resolved (Figure 30). Purified tRNA from the column was dialyzed to remove most of the high salt in which it eluted from the column and was collected by ethanol precipitation for use as a substrate for reconstruction. Each of the peaks was identified by aminoaacylation studies (Figure 31). Although peak II seemed to accept glycine as well as alanine, gel electrophoresis (Plate VI) and enzymatic cleavage both indicate peak III to be pure tRNAAla.

The scheme for reconstruction is outlined in Figure 32. The objective was to remove the inosine from the first position of the anticodon and to replace it with adenosine labelled on its 5' side with a 32P molecule. The first step in the reconstruction was the enzymatic cleavage of the tRNA to generate the appropriate half molecules. Nuclease S1 was used initially to generate the 5' half molecule (A), with Mung bean nuclease being used subsequently with more success. The tRNA fragments were purified by analytical polyacrylamide gel electrophoresis in 15% acrylamide with 8 M urea. Typical cleavage
Figure 30. HPLC separation of yeast tRNA\textsubscript{Ala}. Partially purified yeast tRNA\textsubscript{Ala} (30 A\textsubscript{260}) was separated on a Phenomenex W-Porex C-4 column using a mobile phase of 200 mM potassium phosphate (pH 7.0) and 0.75% 2-propanol with a linear gradient of ammonium sulfate from 1.2 M \( \rightarrow \) 0.0 M over 80 minutes. In order of elution, the peaks are: tRNA\textsubscript{Gly}, tRNA\textsubscript{Ala}, and tRNA\textsubscript{Asp}.
Figure 31. Aminoacylation of tRNA fractions after HPLC purification. Amino acid incorporation designated by: Asp, right hatch; Gly, cross hatch; Ala, left hatch.
Figure 31.

$[^3]H$Amino Acid Incorporation (cpm)

Peak I

Peak II
Plate VI. Analytical gel electrophoresis of HPLC purified yeast tRNA^Ala fraction. Various amounts (0.375, 0.25, and 0.125 A260 units) of yeast tRNA^Ala were separated on a denaturing, 15% polyacrylamide gel containing 8 M urea.
Plate VI.
Figure 32. Schematic procedure for the construction of the AGC "anticodon-substituted" yeast tRNA$^{Ala}$. The tRNA was reconstructed via enzymatic cleavage and ligation as shown. Nuclease S1 or Mung bean nuclease was used to generate the 5' half-molecule (A) and T$_1$ nuclease was used to generate the 3' half-molecule (B). Fragments were purified by polyacrylamide gel electrophoresis. Fragment (B) was then phosphorylated with T$_4$-polynucleotide kinase (fragment C) and subsequently shortened using periodate oxidation to give fragment (D). Fragments (A) and (D) were then reannealed yielding molecule (E). The dinucleotide ApG (F) was labelled with $^{32}$P on the 5' end with T$_4$-polynucleotide kinase and $^{32}$P-ATP generating the radiolabelled dinucleotide (G). This dinucleotide was then joined to the anticodon-deficient tRNA (E) with T$_4$-RNA ligase generating an intact tRNA molecule containing a $^{32}$P labelled adenosine in the first (wobble) position of the anticodon. The reconstructed tRNA was purified by polyacrylamide gel electrophoresis. Modified bases: I, inosine; II, l-methylinosine; F, pseudouridine.
Figure 32.
patterns are shown in Plates VII (lane 1) and VIII. Mung bean nuclease produced fewer bands than $S_1$, with the major cleavage products representing a larger proportion of the reaction product. Nuclease $T_1$ was used to generate the 3' half molecule [Plates VII (lane 2) and IX] indicated as (B) in Figure 32. Plate VII (lane 2) represents cleavage with 50 units of nuclease $T_1$/mg tRNA. In this instance, there was a great deal of uncut tRNA still present in the reaction mix, but no extra fragments to indicate an excess of $T_1$ had been used. Therefore, the enzyme concentration was increased to 100 units/mg tRNA in Plate IX. It is apparent that a much larger percentage of the yeast tRNA$^{Ala}$ was cut at this enzyme concentration, without generating a prohibitive number of non-specific cleavage fragments.

After elution from the gel, a small portion of each of the fragments was 5' or 3' end-labelled to determine the terminal nucleotide at the cleavage site for each fragment. Using [5'32P]pCp and T4-RNA ligase to 3' end-label the Mung bean fragments, or [gamma-32P]ATP and T4-polynucleotide kinase to 5' end-label the $T_1$ fragments, the fragments were again purified by polyacrylamide gel electrophoresis. These labelled fragments were eluted and hydrolyzed completely to the monophosphate level using either nuclease $T_2$ for 3' monophosphates or nuclease P1 to yield 5' monophosphates. The labelled monophosphates were
Plate VII. Polyacrylamide gel electrophoresis of yeast tRNA Ala "half" molecules generated with nuclease S1 and T1 nuclease. The half molecules generated by enzymatic cleavage were separated by gel electrophoresis on a 15% polyacrylamide gel containing 8 M urea. Lane 1 contains the tRNA fragments generated by cleavage with nuclease S1 (1,000 units/mg tRNA). Lane 2 contains the fragments generated by cleavage of the tRNA with T1 nuclease (50 units/mg tRNA).
Plate VII.
Plate VIII. Polyacrylamide gel electrophoresis of yeast tRNA^Ala "half" molecules generated with Mung bean nuclease. The half molecules, generated by enzymatic cleavage with Mung bean nuclease (600 units/ml), were separated by electrophoresis on a 15% polyacrylamide gel containing 8 M urea.
Plate VIII.
Plate IX. Polyacrylamide gel electrophoresis of yeast tRNA Ala "half" molecules generated with T₁ nuclease. The half molecules, generated by enzymatic cleavage with T₁ nuclease (100 units/ml), were separated by electrophoresis on a 15% polyacrylamide gel containing 8 M urea.
Plate IX.
then identified by two-dimensional thin layer chromatography via cochromatography with nucleoside monophosphate standards.

As expected, the top band in the bottom set of Mung bean fragments (band A, Plate VIII) yielded uridine as the only labelled monophosphate indicating it was the desired fragment [Figure 32(A)] for reconstruction. The fragment just below (A) in this gel also yielded uridine as the 3' end nucleotide, but this fragment corresponds to cleavage after uridine in position 32 and not 33 as needed. The top band in the T1 digestion pattern (band B, Plate VII) yielded a cytidine as the only labelled monophosphate indicating it was the correct fragment [Figure 32(B)].

After identification, the 3' half molecule was prepared for the ligation reaction as shown in Figure 32. The 5' end of fragment B was phosphorylated with ATP and T4-polynucleotide kinase to yield a reactive end for the ligation reaction (C). The next step was to shorten the 3' end by one nucleotide using the periodate oxidation method (D). The reason for this nucleotide removal was to generate a 3' phosphate to block this end from being reactive in the final ligation reaction. The two half molecules were reannealed to yield an "anticodon-deficient" tRNA (E). The dinucleotide monophosphate (F) was prepared for ligation by adding a 5' 32P label using T4-polynucleotide kinase. The anticodon-deficient tRNA
(E) and labelled dinucleotide (G) were then ligated using
T₄-RNA ligase. This resulted in an intact yeast tRNA²⁴⁰ molecule being generated which contained a ³²P-labelled
adenosine in the first position of the anticodon. The
products of this ligation reaction were separated by
analytical polyacrylamide gel electrophoresis and
visualized by autoradiography (Plate X).

Modification of the Reconstructed tRNA. The
reconstructed tRNA was eluted from the gel and used in an
in vitro assay for tRNA-hypoxanthine ribosyltransferase
activity. The reconstructed tRNA was incubated with
hypoxanthine and a crude cytosolic enzyme preparation from
HL-60 promyelocytic leukemia cells. Transfer RNA was
ethanol precipitated and digested to the 5' monophosphates
with nuclease P₁. The monophosphates were separated by
two-dimensional thin layer chromatography and the labelled
nucleotides detected by autoradiography. As shown in
Plate XI, the control (unreacted) reconstructed tRNA
yielded only labelled adenosine monophosphate [XI(A)],
whereas the modified reconstructed tRNA yielded
predominately inosine monophosphate [XI(B)]. The other
product has not been identified.

After demonstrating that the reconstructed tRNA could
be modified using the in vitro tRNA-hypoxanthine
ribosyltransferase assay, the reconstruction efforts were
expanded to examine more parameters of the enzymatic
Plate X. Autoradiograph of reconstructed yeast tRNA\textsubscript{Ala}. The products of the ligation reaction in which the 5' \textsuperscript{32}P-labelled dinucleotide "pApG was covalently inserted into the reannealed "anticodon-deprived" tRNA were purified by denaturing, polyacrylamide gel electrophoresis. The tRNA and dinucleotide were joined using T\textsubscript{4}-RNA ligase. The arrow indicates the reconstructed tRNA\textsubscript{Ala} with 1 labelled dinucleotide inserted into the anticodon used in the subsequent in \textit{vitro} modification reaction.
Plate X.
Plate XI. Autoradiograph of 5' nucleoside monophosphates separated by two-dimensional thin layer chromatography. Reconstructed yeast tRNAAla was used as substrate for tRNA-hypoxanthine ribosyltransferase in an in vitro assay. The reconstructed tRNA was incubated at 37°C for 60 minutes with an enzyme preparation from HL-60 cells as described in "Materials and Methods". Modified tRNA was collected by ethanol precipitation and hydrolyzed with nuclease P₁ from Penicillium citrinum to the 5' monophosphates. The nucleoside monophosphates were separated by two-dimensional thin layer chromatography according to the procedure of Nishimura (1972) after which the labelled nucleotide was determined by autoradiography. Identification of the labelled 5' nucleoside monophosphate was made by comparison with known standards. A) Control (unreacted) reconstructed tRNAAla. B) Modified tRNAAla. C) Reference map of 5' nucleoside monophosphates.
Plate XI.
activity. In addition to the initial yeast tRNA\textsuperscript{Ala} reconstruction, \textit{E. coli} tRNA\textsuperscript{AlaII} was also reconstructed in a similar manner. \textit{E. coli} tRNA\textsuperscript{AlaII} has a primary structure very similar to the yeast tRNA\textsuperscript{Ala} (Figure 33). The reconstruction scheme for the \textit{E. coli} tRNA (Figure 34) was identical to that followed for the yeast tRNA\textsuperscript{Ala}.

As depicted in Plates XII and XIII, the \textit{E. coli} tRNA yielded very similar cleavage patterns when cut with Mung bean nuclease (Plate XII) or T\textsubscript{1} (Plate XIII). However, there was one significant difference which is not readily apparent in these pictures. When \textit{E. coli} tRNA\textsuperscript{AlaII} was cleaved with Mung bean nuclease, there was cleavage after the modified uridine residue in position 34 of the anticodon, whereas there was no cleavage after the inosine in that same position in the yeast molecule. This results in an extra major fragment (1) being generated, requiring the use of the second band (2) as fragment (A) in the reconstruction scheme.

Plate XIV is the autoradiograph of the gel used to separate the ligation reaction products in which the \textit{E. coli} tRNA\textsuperscript{AlaII} was reconstructed. The bands of reconstructed tRNA were excised and eluted from the gel and used in \textit{in vitro} modification studies.

The further tRNA-hypoxanthine ribosyltransferase studies using the reconstructed tRNAs were carried out with cell extracts from Jurkat cells. When the
Figure 33. Structure of *E. coli* tRNA^AlaII^. Modified nucleosides: D, dihydrouridine; V, uridine-5-oxyacetic acid; T, 5-methyluridine; F, pseudouridine. (Sprinzl et al., 1985)
Figure 33.
Figure 34. Schematic representation of the procedure for construction of AGC "anticodon-substituted" E. coli tRNAAla. The tRNA was reconstructed via enzymatic cleavage and ligation as shown. Mung bean nuclease was used to generate the 5' half-molecule (A) and T₁ nuclease was used to generate the 3' half-molecule (B). Fragments were purified by polyacrylamide gel electrophoresis. Fragment (B) was then phosphorylated with T₄-kinase (fragment C) and subsequently shortened using periodate oxidation to give fragment (D). Fragments (A) and (D) were then reannealed yielding molecule (E). The dinucleotide ApG (F) was labelled with ³²P on the 5' end with T₄-kinase and ³²P-ATP generating the radiolabelled dinucleotide (G). This dinucleotide was then joined to the anticodon-deficient tRNA (E) with T₄-RNA ligase generating an intact tRNA molecule containing adenosine in the first (wobble) position of the anticodon. The reconstructed tRNA was purified by polyacrylamide gel electrophoresis. Modified bases: V, uridine-5-oxyacetic acid.
Figure 34.
Plate XII. Polyacrylamide gel electrophoresis of E. coli tRNAAlaII "half" molecules generated with Mung bean nuclease. The half molecules, generated by enzymatic cleavage with Mung bean nuclease (600 units/ml), were separated by electrophoresis on a 15% polyacrylamide gel containing 8 M urea.
Plate XII.
Plate XIII. Polyacrylamide gel electrophoresis of E. coli tRNA$\text{Ala}^{\text{II}}$ "half" molecules generated with $T_1$ nuclease. The half molecules, generated by enzymatic cleavage with $T_1$ nuclease (100 units/ml), were separated by electrophoresis on a 15% polyacrylamide gel containing 8 M urea.
Plate XIV. Autoradiograph of reconstructed *E. coli* tRNA\textsubscript{Ala\textsuperscript{II}}. The products of the ligation reaction in which the 5' \textsuperscript{32}P-labelled dinucleotide "pApG was covalently inserted into the reannealled "anticodon-deprived" tRNA were purified by denaturing, polyacrylamide gel electrophoresis. The tRNA and dinucleotide were joined using \textit{T}_{4}\textendash RNA ligase. The arrow indicates the reconstructed tRNA\textsubscript{Ala} with 1 labelled dinucleotide inserted into the anticodon used in the subsequent \textit{in vitro} modification reaction.
Plate XIV.
reconstructed tRNAs were modified using Jurkat cell extracts, both the yeast tRNA^{Ala} [Plate XV(2)] and E. coli tRNA^{Ala} [Plate XV(5)] showed conversion of a significant amount of the adenosine to inosine (Plate XV).
Plate XV. Autoradiograph of 5' nucleoside monophosphates separated by two-dimensional thin layer chromatography. Reconstructed yeast tRNA\textsubscript{Ala} and \textit{E. coli} tRNA\textsubscript{Ala}\textsubscript{II} were used as substrate for tRNA-hypoxanthine ribosyltransferase in in vitro assays. The reconstructed tRNA was incubated at 37°C for 60 minutes with an enzyme prep from Jurkat cells as described in "Materials and Methods". Modified tRNA was collected by ethanol precipitation and hydrolyzed with nuclease P\textsubscript{1} from \textit{Penicillium citrinum} to the 5' monophosphates. The nucleoside monophosphates were separated by two-dimensional thin layer chromatography according to the procedure of Nishimura (1972) after which the labelled nucleotide was determined by autoradiography. Identification of the labelled 5' nucleoside monophosphate was made by comparison with known standards. Samples are as follows: 1) Control (unreacted) yeast tRNA\textsubscript{Ala} 2) Modified yeast tRNA\textsubscript{Ala} 4) Control \textit{E. coli} tRNA\textsubscript{Ala}. 5) Modified \textit{E. coli} tRNA\textsubscript{Ala}. 
Plate XV.
DISCUSSION

Transfer RNAs have been implicated in cellular regulatory processes for many years, but to date, most of the evidence has been circumstantial. Based on the number and complexity of modifications made to the macromolecular structure of tRNAs (especially in the anticodon loop), some regulatory role(s) might be indicated. The studies presented above support the belief that at least two of these modifications, those generating queuosine and inosine, are important in cell differentiation. Investigations with 6-thioguanine and 8-azaguanine suggest that changes in queuosine modification of tRNA are required for the differentiation of HGPRT-deficient HL-60 cells. Several possible mechanisms for this differentiation were presented. Experiments with tRNA-hypoxanthine ribosyltransferase indicate an enzymatic activity similar to tRNA-guanine ribosyltransferase, and more importantly, that this modification reaction may serve as the basis for the differentiation of HL-60 cells induced by hypoxanthine and DMSO.

It has been known for some time that guanine analogs can induce the differentiation of HL-60 cells (Collins et al., 1980), but these same guanine analogs are also highly
cytotoxic (Ishiguro et al., 1984). Various studies showed that cells which were selected for 6-thioguanine resistance were deficient in HGPRT (Gusella and Housman, 1976; Gallagher et al., 1984). However, the HGPRT-deficient leukemia cells could still be induced to differentiate with guanine analogs, as well as a number of other agents (Gusella and Housman, 1976; Gallagher et al., 1984; Schwartz et al., 1984; Ishiguro et al., 1984). Ishiguro et al. (1984) recently verified that the cytotoxic action of 6-thioguanine in wild-type HL-60 cells was due to generation of 6-thioguanine nucleotides via HGPRT, while the ability to induce differentiation was due to the free base, 6-thioguanine. Figure 4 demonstrates that the mutant cells utilized in this investigation were in fact HGPRT-deficient.

Figures 6(A) and 7(A) demonstrate the difference between wild-type HL-60 cells and HGPRT-deficient HL-60 cells in their response to treatment with low doses of 6-thioguanine. With these concentrations of the guanine analog, the wild-type HL-60 cells were severely affected, i.e., 6-thioguanine was highly cytotoxic. While these concentrations of 6-thioguanine resulted in some growth inhibition (cytostasis) with the HGPRT-deficient cells, there was virtually no induction of differentiation (Figure 8). In addition, treatment with another purine analog, 8-azaguanine, gave similar results (Figures 6(B),
Subsequent studies with the HGPRT-deficient HL-60 cells employed higher concentrations of 6-thioguanine and 8-azaguanine. Figure 9 demonstrates a dose-dependent inhibition of growth in response to the guanine analog treatment. However, Schwartz et al. (1984) found that while 6-thioguanine treatment of wild-type HL-60 cells resulted in growth inhibition, it did not induce significant differentiation of these cells. Therefore, the expression of a differentiation-specific, cell surface antigen was examined using the OKM1 monoclonal antibody. Various studies have demonstrated that the cell surface antigen recognized by the OKM1 antibody is highly expressed in HL-60 cells which have undergone differentiation, but it is only slightly expressed by undifferentiated HL-60 cells (Breard et al., 1980; Murao et al., 1983). Figure 10 shows that cells treated 8 days with either 6-thioguanine or 8-azaguanine were much more reactive with the OKM1 antibody than were the control cells. These results serve to confirm the suggestion from Figure 9 that the HGPRT-deficient HL-60 cells treated with 6-thioguanine or 8-azaguanine undergo terminal differentiation.

Since 6-thioguanine and 8-azaguanine are both inhibitors/substrates for tRNA-guanine ribosyltransferase (Farkas et al., 1984; Shindo-Okada et al., 1980; Farkas,
The potential involvement of queuine modification of tRNA (Figure 2) in the induction of cell differentiation by these compounds was investigated. Figure 11 demonstrates how the queuine content of the tRNA changed in the HGPRT-deficient HL-60 cells which were treated with 6-thioguanine. Transfer RNA, extracted at the times indicated on the growth curve (Figure 11(A)), was assayed for queuine content. The tRNA from the cells treated with 6-thioguanine and extracted on day 2 clearly exhibited an increased ability to incorporate [14C]guanine [Figure 11(B)], thereby demonstrating a decreased level of queuine modification. This queuine hypomodification was transient, with the queuine content returning to the control level by day 5. While these results alone do not prove that the tRNA undermodification for queuine is involved in the differentiation process, they represent the first demonstration of a significant molecular perturbation induced in HGPRT-deficient HL-60 cells by the guanine analog.

The findings, depicted in Figure 11, are consistent with those of Lin et al. (1980) who reported changes in tRNA isoacceptors for the queuine family of tRNAs (asparagine, aspartic acid, histidine, and tyrosine) from murine erythroleukemia cells during differentiation induced by DMSO. Transient queuine hypomodification was
also reported in that case, with both the undifferentiated and terminally differentiated cells being queuine modified. However, in a similar study with murine erythroleukemia cells, Shindo-Okada et al. (1981) reported a shift from queuine undermodified to queuine modified tRNA during the differentiation process, and indicated that queuine limitation was probably responsible for tRNA hypomodification in the undifferentiated cells. Considering that queuine must be obtained from the sera utilized to supplement the cell culture media (Katze, 1978; Katze and Farkas, 1979), the conflicting results for the undifferentiated cells could merely represent variations in queuine content for different lots of serum (Katze et al., 1982), alterations in queuine transport for different cell lines (Elliott et al., 1985), or some other normal biological variation in queuine metabolism (Katze, 1983). Therefore, if queuine hypomodification of tRNA is required for some early event in the terminal differentiation of murine erythroleukemia cells, that condition still would have been met in both of the published erythroleukemia studies (Lin et al., 1980; Shindo-Okada et al., 1981).

In the present studies with guanine analogs, another explanation for the mode of action (in addition to queuine hypomodification) is also possible. Both 6-thioguanine and 8-azaguanine have been reported to be substrates (not
just inhibitors) for tRNA-guanine ribosyltransferase (Shindo-Okada et al., 1980; Farkas et al., 1984), so the analogs may be incorporated into the anticodon wobble position in place of queuine. This possibility would still be consistent with the transient queuine hypomodification in Figure 11, since only 7-deazaguanine analogs (e.g., queuine) are incorporated irreversibly (Farkas et al., 1984). Therefore, the tRNA on day 2 (Figure 11) could have had 6-thioguanine in the anticodon, but by day 5, the analog could have been replaced by queuine. RPC-5 chromatography was performed on tRNA from cells treated with the guanine analogs for 2 days to determine whether they were in fact, incorporated. The histidine isoacceptor profiles in (Figure 12) demonstrate that both 6-thioguanine and 8-azaguanine can be inserted into the tRNA as previously reported (Shindo-Okada et al., 1980; Farkas et al., 1984). As can be seen, the profiles of tRNA^His from the treated cells was shifted differentially in each case, indicative of analog containing species.

Since 6-thioguanine induced transient changes in queuine modification of tRNA in the HGPRT-deficient HL-60 cells (Figure 11), it was possible that excess exogenous queuine might block the induction of differentiation by 6-thioguanine and 8-azaguanine. As already stated, 6-thioguanine and 8-azaguanine are incorporated reversibly
into tRNA by tRNA-guanine ribosyltransferase, while queuine is incorporated irreversibly (Farkas et al., 1984; Shindo-Okada et al., 1980). Therefore, additional substrate (queuine) should overcome the transient queuine hypomodification (analog insertion) caused by the guanine analogs. The growth curves depicted in Figures 13(A) and 13(B), in which the cells treated with 6-thioguanine or 8-azaguanine also received excess queuine, show a significant reversal of growth inhibition even with queuine present at a concentration 800-fold lower than the inducing agents. Figures 13(D) and 13(E) are the results of evaluations to determine the extent of differentiation of the cells depicted in Figures 13(A) and 13(B). Figure 13(D) summarizes the data for the 6-thioguanine treated cells and it shows extensive reversal of both morphological and functional differentiation with the addition of exogenous queuine. The data for 8-azaguanine [Figure 13(E)] are somewhat different. The exogenous queuine again reversed the ability of the cells to reduce NBT (to differentiate functionally), but since 8-azaguanine induced no significant morphological maturation, there was little for queuine to influence with regard to that parameter.

The molecular basis for the differences in differentiation induced by 6-thioguanine and 8-azaguanine has not been determined, but it may be due in part to the
fact that 6-thioguanine is actively transported into the cells whereas 8-azaguanine appears to enter in a non-mediated manner (Plagemann et al., 1981). More importantly, 6-thioguanine in the anticodon of tRNAs is likely to have a greater effect than 8-azaguanine on codon recognition during protein synthesis, since the 6 position is involved in hydrogen bonding. In any event, the overall results support the contention that added queuine can inhibit the commitment to terminal differentiation induced by the guanine analogs, and that changes in queuine modification of tRNA are involved in an early commitment step in HGPRT-deficient HL-60 cells.

Considering that queuine reverses the growth inhibition induced by the guanine analogs (Figure 13), it is possible changes in queuine modification of tRNA are involved in the 6-thioguanine-induced resistance of HL-60 cells to differentiation reported by Gallagher et al. (1984). The cytodifferentiative resistance they reported arose subsequent to, and independently of, the cytotoxic resistance due to the loss of HGPRT, and it was associated with the acquisition of double minute chromosomes (Gallagher et al., 1984). If these double minute chromosomes caused altered queuine metabolism [for example by increasing the expression of the high affinity queuine transporter (Elliott et al., 1985)], the ability of 6-thioguanine to induce differentiation might be reduced,
since the guanine analog would be less available to tRNA-guanine ribosyltransferase if the preferred substrate were present in excess.

The presence of queuine in the anticodon of tRNA isoacceptors for asparagine, aspartic acid, histidine, and tyrosine has been known for a number of years (Nishimura, 1983; Farkas, 1983), but as yet, few important biological roles for this modification have been demonstrated (Katze et al., 1983). However, changes in queuine modification of tRNA have been observed during differentiation in a wide variety of cell systems. In addition to the changes reported here and those already described during the differentiation of murine erythroleukemia cells (Lin et al., 1980; Shindo-Okada et al., 1981), changes in queuine-containing tRNAs have been reported during specific stages in the development of Drosophila melanogaster (Hosbach and Kubli, 1979) and Dictyostelium discoideum (Schachner et al., 1984). In the latter case, Schachner et al. (1984) demonstrated that starvation for queuine actually blocked differentiation of the slime mold at one point in the life cycle, and that this blockade could be overcome by adding exogenous queuine. In addition, it was reported previously that phorbol ester tumor promoters inhibit queuine transport into human cells (Elliott et al., 1985) and thereby induce queuine hypomodification of tRNA (Elliott et al., 1984), and
phorbol esters also induce the differentiation of HL-60 cells (Huberman and Callaham, 1979; Lotem and Sachs, 1979), although down a different lineage than guanine analogs (monocyte/macrophage versus granulocyte). These various studies, in conjunction with those presented here, indicate the importance of queuine metabolism in the process of differentiation.

Perhaps the easiest to visualize molecular mechanism by which queuine might mediate some fundamental process associated with differentiation involves its normal location in the first position of the anticodon of tRNA^Asn, tRNA^Asp, tRNA^His, and tRNA^TyT, since in that position, the queuine modification might affect codon recognition during the translation of mRNA. While early reports showed no evidence of this, recent work has demonstrated some important differences in the decoding properties of tRNAs modified to contain queuine or unmodified containing guanine. Beinz and Kubli (1981) showed that when Drosophila tRNA was coinjected with tobacco mosaic virus RNA into Xenopus oocytes, tRNA^TyT which was unmodified for queuine (containing guanine) misread a leaky UAG stop codon in the viral RNA, while the modified tRNA^TyT (containing queuine) did not. Beier et al. (1984a; 1984b) further demonstrated that tobacco protoplasts synthesize two high molecular weight proteins from tobacco mosaic virus RNA. They determined that in
the cell normally infected by the virus, the larger of the two proteins was generated by efficient readthrough of the UAG stop codon by tobacco cell tRNA^TYR with guanine in the anticodon wobble position. In addition, Meier et al. (1985) reported that queuine modified histidine tRNAs exhibit a codon preference in vivo different from that of the unmodified (guanine containing) forms of the same tRNA. Their studies demonstrated that a Drosophila tRNA^His with a GUG anticodon preferred the histidine codon CAC to the codon CAU in vivo, while the same tRNA^His modified to contain a QUG anticodon showed little codon preference. Such differences could have a major impact on the rate and/or extent of translation of specific messenger RNAs in vivo. Recent work by Schachner and Kersten (1984) supports this contention. When protein synthesis in Dictyostelium discoideum was monitored by two-dimensional gel electrophoresis, changes were noticed between cells grown in the presence or absence of queuine. Specifically, they monitored the synthesis of several proteins during development, and major differences were observed with and without queuine. These findings suggest that the level of queuine modification of tRNA might play a role in regulating protein synthesis, and by this means one could explain the requirement for changes in queuine modification of tRNA for the commitment to terminal differentiation by HGPRT-deficient HL-60 cells.
The results of protein labelling experiments (Plate IV) lend support to the possibility that changes in the tRNA anticodon modification can change protein synthesis. When control and 6-thioguanine treated cells were labelled with $[^3H]$histidine and subjected to SDS-polyacrylamide gel electrophoresis, a prominent protein of approximately 12000 molecular weight was synthesized by cells treated with 6-thioguanine which is not present in the control cells. It is likely that the synthesis of this protein was the result of altered codon recognition by 6-thioguanine containing tRNAs, and it may have been due to readthrough of a stop codon as demonstrated previously with unmodified, guanine containing tRNAs (Bienz and Kubli, 1981; Beier et al., 1984a). Shindo-Okada et al. (1985) recently showed that replacing queuine with 6-thioqueuine in the anticodon converts tyrosine tRNAs into highly active amber (UAG) suppressors, so a similar situation may exist for 6-thioguanine. As shown in Figure 35, 6-thioqueuine and 6-thioguanine have identical hydrogen bonding regions, and as a result they may function in an identical manner. In the case where 8-azaguanine is used as the inducing agent, its presence in the first position of the anticodon should mimic the effect of an unmodified guanine in that position. 8-Azaguanine has the same hydrogen bonding region as guanine, but without the bulky side group of queuine.
Figure 35. Structures of queuine, 6-thioqueuine, 6-thioguanine, and 8-azaguanine.
Figure 35.
(Figure 35).

In fact, differences in codon-anticodon interactions, depending on which analog (6-thioguanine or 8-azaguanine) is present in the tRNA wobble position, could explain the differences in analog-induced differentiation (Figure 13). The data presented in this portion of the study demonstrate the involvement of altered queuine modification of tRNA in the differentiation of HGPRT-deficient HL-60 cells induced by the guanine analogs 6-thioguanine and 8-azaguanine. These results suggest that the presence of one or more tRNAs, modified to contain 6-thioguanine or 8-azaguanine, is necessary for an early step in the commitment to terminal differentiation. Considering the potential uses of 6-thioguanine and 8-azaguanine as chemotherapeutic agents, it may be possible to design similar antimetabolites targeted specifically to tRNA-guanine ribosyltransferase which are not substrates for HGPRT. If such compounds affected queuine modification of tRNA without being converted to cytotoxic nucleotides by HGPRT, they might induce the terminal differentiation of leukemia cells without being toxic to normal proliferating cells in vivo. The possibility of using 6-thioqueuine as a chemotherapeutic agent has already been suggested (Shindo-Okada et al., 1985, Shindo-Okada et al., 1984).

Identifying a role for queuosine modification of tRNA
in cellular differentiation offered the distinct possibility that changes in the inosine modification may be important in cellular differentiation processes as well. Because both of these modification reactions utilize a base exchange mechanism (Okada et al., 1979; Elliott and Trewyn, 1984), it was felt that a mechanism similar to that found in the guanine-analog induction might also occur in which the enzyme tRNA-hypoxanthine ribosyltransferase was involved. Findings supporting this idea include the demonstration of hypoxanthine induced differentiation of HGPRT-deficient erythroleukemia cells (Gusella and Housman, 1976) and HGPRT-deficient HL-60 cells (Ishiguro et al., 1984). Because hypoxanthine is not a substrate for tRNA-guanine ribosyltransferase (Shindo-Okada et al., 1980; Farkas et al., 1984), hypoxanthine can not be functioning in the same manner as the guanine analogs. The observations of Trewyn et al. (1985) show a synergistic action when hypoxanthine is used in conjunction with DMSO to induce the differentiation of HL-60 cells. Considering that excess exogenous queuine was not able to reverse the induction of differentiation by DMSO (Figures 13(C) and 13(F)), it is also likely that the mechanism of action of DMSO does not involve tRNA-guanine ribosyltransferase. However, these findings do not rule out a mechanism utilizing tRNA-hypoxanthine ribosyltransferase. Therefore, analyses were undertaken
to look for similarities in the mode of action of hypoxanthine and DMSO.

It was determined that both the wild-type HL-60 cells and the HGPRT-deficient HL-60 cells responded to DMSO or hypoxanthine induction in a dose-dependent manner (Figures 14 and 15), with the highest concentration of inducer resulting in some morphological differentiation in each case (Figure 16). While DMSO and hypoxanthine, used separately, resulted in partial growth inhibition and differentiation, it was found that these agents were able to act in a synergistic manner to induce the differentiation of these cells. The growth of HL-60 cells treated with suboptimal concentrations of DMSO or hypoxanthine alone was decreased only slightly (Figure 17). When the these cells were treated with the same concentrations of DMSO and hypoxanthine in combination, a much greater reduction in growth was obtained (Figure 17).

In addition to testing for growth inhibition, the treated cells were also evaluated for evidence of differentiation. The results in Figure 18 demonstrate that the DMSO or hypoxanthine treatment alone did not effectively induce the differentiation of the HL-60 cells. However, in the case where DMSO and hypoxanthine were used in combination, there was a significant increase in the proportion of cells exhibiting a morphology more mature than promyelocytes (i.e., metamyelocytes, myelocytes, and
banded neutrophils).

Fontana et al. (1980) had demonstrated an increase in protein synthesis (based on radiolabelled leucine incorporation) during HL-60 differentiation in vitro and Feuerstein and Cooper (1984) had reported a significant increase in leucine radiolabelling of specific membrane-associated proteins during HL-60 differentiation into macrophage-like cells induced by phorbol esters. Since some leucine tRNA isoacceptors can be modified to contain inosine and the resulting increase in codon recognition potential could lead to enhanced leucine radiolabelling of proteins, analyses with DMSO and hypoxanthine were undertaken. When HL-60 cells were treated short-term with DMSO and/or hypoxanthine, significant differences in the leucine incorporation into TCA precipitable material were found (Figure 19). Treatment with DMSO alone or DMSO plus adenine resulted in little change in leucine incorporation when compared to untreated controls, but the combination of DMSO plus hypoxanthine yielded significantly more leucine incorporation than any of these treatments.

More detailed characteristics of this enhanced leucine incorporation were then explored. To verify that the enhanced leucine radiolabelling was in fact a result of incorporation into protein, cycloheximide was used to inhibit protein synthesis. As expected, there was no
enhanced leucine incorporation without protein synthesis (Figure 20).

It was found that the addition of hypoxanthine at various concentrations, with a fixed concentration of DMSO, resulted in a dose-dependent increase in the amount of leucine incorporated (Figure 21). While the DMSO/hypoxanthine enhancement of leucine incorporation was reproducible, the extent of this enhancement from one experiment to the next was not. Therefore, cells at different stages in the growth cycle were examined for leucine incorporation. Figure 22 demonstrates a significant difference in the enhancement of leucine incorporation for proliferating cells compared to resting cells, with the resting cells showing the more dramatic increase in leucine incorporation. It is noteworthy that proliferating cells are less susceptible to induction of differentiation by DMSO and hypoxanthine than are cells in early stationary phase, so the latter cells may be "primed" to synthesize differentiation-specific proteins.

Other types of inducers were surveyed in an attempt to find additional compounds which could influence protein synthesis. When adenosine and inosine, the nucleosides of adenine and hypoxanthine, were used as inducers, they gave results comparable to those obtained with the bases (Figure 23). This is not surprising because purine nucleoside phosphorylase, which would convert adenosine to
adenine and inosine to hypoxanthine, is active in these cells (Trewyn et al., 1985). However, the immunomodulator inosiplex, which contains inosine, did not enhance leucine radiolabelling (Figure 24). While it was proposed that inosiplex may serve as a source of hypoxanthine for cells (Trewyn, 1984), a longer time frame for dissociation of the complex and metabolism of the inosine moiety to hypoxanthine may be required. Polyinosinic-polycytidylic acid and polyadenylic-polyuridylic acid were also evaluated, since with catabolism they might act as sources of inosine and adenosine respectively. Again, no enhancement was observed (Figure 25), but a longer time frame might be required.

Concurrent with the cellular studies of hypoxanthine effects, the tRNA-hypoxanthine ribosyltransferase activity was being characterized further in vitro. Murine erythroleukemia cells (BB-88) were assayed for the ability to incorporate radiolabelled hypoxanthine into unfractionated E. coli tRNA, and results similar to those already published for CCRF-CEM and HL-60 cells (Elliott and Trewyn, 1984) were obtained (Figure 26).

It has been shown that tRNA-guanine ribosyltransferase will catalyze a guanine for guanine exchange in the first position of the anticodon of the appropriate unmodified (guanine containing) tRNAs (Okada et al., 1979). Therefore, the possibility that
tRNA-hypoxanthine ribosyltransferase might carry out an adenine for adenine exchange was investigated. The results in Figure 27 demonstrate that when [³H]adenine and unfractionated E. coli tRNA were used as substrates in the tRNA-ribosyltransferase assay using a BB-88 cell extract, adenine was incorporated into the tRNA. Further studies with BB-88 cell extracts demonstrated that the adenine incorporation into tRNA could be inhibited by adding unlabelled hypoxanthine (Figure 27). Therefore, both of the tRNA anticodon modifications employing base exchange mechanisms (those involved in generating queuosine and inosine) can carry out a futile exchange (guanine for guanine and adenine for adenine) in the absence of the normal substrates, queuine and hypoxanthine. In the case of the leucine radiolabelling experiments with DMSO plus adenine (Figure 19), the adenine for adenine exchange should thus have prevented the inosine modification as predicted.

Although inosine is found in tRNA exclusively in the first position of the anticodon (position 34), it had not been demonstrated that this was the site of the inosine modification generated in vitro. Because an unmodified tRNA substrate was not available commercially, it was decided that recombinant RNA techniques would be utilized to reconstruct tRNAs to contain specifically labelled, ³²P-containing anticodons. It had already been
demonstrated that tRNAs reconstructed to contain adenosine in the first position of the anticodon, could be used as substrates for the inosine modification reaction in vivo after microinjection into *Xenopus laevis* oocytes (Fournier *et al.*, 1983; Haumont *et al.*, 1984).

The strategy for tRNA anticodon reconstruction is as follows: The tRNA molecule of interest is enzymatically cleaved in the anticodon loop to generate specific tRNA half molecules. Two appropriate tRNA half molecules are then reannealed to yield an anticodon-deficient tRNA in which the nucleotide to be monitored has been removed. A $^{32}$P label is then added adjacent to the nucleotide of interest, and the appropriate fragment is ligated into the anticodon loop of the reannealed tRNA half molecules. After modification in vitro or in vivo, the nucleotide of interest can then be determined by hydrolyzing the tRNA to the nucleoside monophosphate level and identifying any labelled species by two-dimensional thin layer chromatography (Grosjean *et al.*, in press).

Yeast tRNA$^{\text{Ala}}$ was chosen for reconstruction for 3 reasons: 1) Yeast tRNA$^{\text{Ala}}$ is a natural, inosine-containing tRNA [normal anticodon IGC (Figure 28)] (Sprinzl *et al.*, 1985). 2) A partially purified preparation of yeast tRNA$^{\text{Ala}}$ was available. 3) Yeast tRNA$^{\text{Ala}}$ contains 1-methylinosine adjacent to the 3' end of the anticodon (position 28) (Sprinzl *et al.*, 1985), and the
biosynthetic mechanism for this modification has not been determined.

Before the reconstruction could be undertaken, purification of the yeast tRNA\textsubscript{Ala} was required. The partially purified preparation contained 3 tRNA species, tRNA\textsubscript{Ala}, tRNA\textsubscript{Gly}, and tRNA\textsubscript{Asp}. Initially, fractions of this preparation were purified by partially denaturing polyacrylamide gel electrophoresis on 10% polyacrylamide gels containing 4 M urea (Plate V). This separation procedure was difficult to reproduce and inefficient for tRNA recovery. For these reasons, a new HPLC separation of the tRNAs using a Phenomenex W-Porex C-4 column with an ammonium sulfate gradient was tested (Figure 30). This method proved much better in both resolution and efficiency as compared to the electrophoretic separation.

The pure yeast tRNA\textsubscript{Ala} was reconstructed as diagrammed in Figure 32. The ultimate goal of this procedure was to replace the IGC anticodon with an AGC anticodon in which a \textsuperscript{32}P label had been introduced on the 5' side of the adenosine residue in the first position of the anticodon. After the generation of the tRNA half molecules via enzymatic cleavage, and their subsequent manipulation, they were reannealed to give a tRNA molecule devoid of the first and second positions of the anticodon. After labelling the dinucleotide AG, it was ligated into the tRNA to yield an intact yeast tRNA\textsubscript{Ala}
with an AGC anticodon. A commercially available *E. coli* alanine tRNA was reconstructed by similar means (Figure 34).

The labelled, reconstructed yeast tRNA was used as a substrate in an *in vitro* tRNA-hypoxanthine ribosyltransferase reaction with an extract from HL-60 cells. Plate XI confirms that this reconstructed tRNA can act as a substrate for the adenosine to inosine modification. The unmodified tRNA substrate yielded only AMP after hydrolysis to the 5' monophosphates, whereas the tRNA which had been used in the modification assay yielded predominately IMP after hydrolysis.

Further studies with reconstructed yeast tRNA<sub>Ala</sub> and *E. coli* tRNA<sub>Ala</sub>II were performed using a Jurkat cell extract. These studies demonstrated that both of these tRNAs could be modified to contain inosine *in vitro* (Plate XV). The fact that both tRNAs could be modified by the Jurkat cell extract suggests that the 1-methylinosine modification in position 37 is not required for hypoxanthine insertion in position 34 *in vitro*, since the *E. coli* tRNA lacks this modification. In some instances *in vivo* (at least in *Xenopus* oocytes), position 37 must be modified prior to 34 (Grosjean *et al.*, in press). The reason for this discrepancy remains to be established.

As discussed previously, evidence is building to support the belief that hypoxanthine can influence
cellular regulatory processes. Many purines and purine analogs, including hypoxanthine, can induce the differentiation of HL-60 cells (Collins et al., 1980) and murine erythroleukemia cells (Gusella and Housman, 1976). It now appears likely that both of these cell types insert preformed hypoxanthine into tRNA to generate inosine in the anticodon wobble position (Figure 26; Plate XI; Elliott and Trewyn, 1984). Enzymatic defects in the purine catabolic pathway, specifically adenosine deaminase and purine nucleoside phosphorylase, lead to an impairment in cellular immunity (Hirshhorn et al., 1978; Thompson and Seegmiller, 1980). These deficiencies should lead to a decrease in the intracellular concentration of hypoxanthine. Inhibition of adenosine deaminase inhibits the differentiation of monocytes to macrophages in vitro (Fischer et al., 1976). In addition, studies with the antiviral drug inosiplex, a 3:1 molar complex of N-N-dimethylamino-2-propanol and inosine, appears to act as an immunomodulating agent (Ginsberg and Glaskey, 1977; Hadden and Giner-Sorolla, 1981; Hadden et al., 1983). It is possible that this drug is broken down in the body and the inosine can act as a source of hypoxanthine via purine nucleoside phosphorylase. Hadden and Giner-Sorolla (1981) have also shown that inosine is an immunomodulator, but they also determined that it is more effective when administered as part of a complex. All of these
Figure 36. Postulated model for how the hypoxanthine insertion reaction in specific tRNA anticodons may regulate protein synthesis. Transfer RNAs with the potential for having hypoxanthine inserted into the first position of the anticodon include those for alanine, arginine, isoleucine, leucine, proline, serine, threonine, valine, and perhaps glycine. Any of these tRNA species with adenine in the primary transcript wobble position could be involved in regulating translation as shown. However, in the example illustrated, a leucine tRNA is modified by tRNA-hypoxanthine ribosyltransferase (a) which, according to the wobble hypothesis, should allow the resulting inosine-containing tRNAs to read the three leucine codons depicted in bold print (CUC, CUA, and CUU). The unmodified tRNAs should only read the last leucine codon (CUU), so the ribosome would stall if the anticodon modification were not carried out. The substrate for inosine biosynthesis in tRNA, hypoxanthine, could be generated endogenously from adenosine and inosine by the enzymes adenosine deaminase (b) and purine nucleoside phosphorylase (c), or it could be supplied exogenously and transported (d) into the cell or cell compartment
observations would be consistent with inosine-containing tRNAs being necessary for normal cellular function, whereby the enzymatic defects or inhibition could be decreasing the number of inosine-containing tRNAs and thereby disrupting proper cellular function.

How can inosine (or the absence of inosine) in the anticodon of tRNA affect cell functions? Trewyn et al. (1985) have proposed a theory referred to as "wobble attenuation of translation" to explain how this might happen (Figure 36). This theory proposes that specific inosine-containing tRNAs are required for the synthesis of some important cell proteins. Based on the wobble hypothesis for codon binding (Crick, 1966), the three leucine codons depicted in bold print could be read by an inosine-containing tRNA. However, if for any reason the level of hypoxanthine available for the inosine modification drops, there would be a decrease in the number of inosine-containing tRNAs and a subsequent decrease in the translation of any proteins requiring these tRNAs. This mechanism of attenuation is somewhat similar to that found in bacterial operons (Kolter and Yanofsky, 1982) and described previously. In the bacterial system, where transcription and translation are coupled, the level of aminoacylated tRNA present determines whether structural genes are transcribed or not. In the wobble attenuation model, the control
operates directly at the level of translation.

This theory is supported by two lines of evidence. First, a survey of sequences found a preponderance of codons requiring inosine-containing tRNAs in the short signal sequences for several important regulatory proteins. Many eucaryotic cells use short leader peptides to tag a protein for secretion or transmembrane positioning (Blobel et al., 1979). These leader sequences at the amino terminus are removed during the subsequent processing steps. Table 2 lists the leucine codons present in the signal peptides of human interleukin-2 and a mouse T-cell receptor polypeptide. Of the 20 amino acids in the interleukin-2 signal peptide, 5 are leucine codons. Based on Crick's wobble hypothesis, the possible leucine anticodons capable of reading each of the leucine codons are designated by an X. The anticodons depicted in parentheses have not been reported in eucaryotic tRNAs (Sprinzl et al., 1985). Therefore of the 5 leucine codons of the interleukin-2 signal polypeptide, 4 could be read by an inosine-containing tRNA and 3 out of 5 would require one, while only 5 of 18 leucine codons in interleukin-2 itself could be read by inosine-containing tRNAs. In the mouse T-cell receptor polypeptide the numbers are even more polarized. In this instance 6 out of 6 leucine codons in the signal could be read by an inosine-containing tRNA (5 of 6 require one), whereas only
Table 2. Leucine codons in signal sequences for T-cell proteins (Taniguchi et al., 1983; Hedrick et al., 1984).

а The AUG initiator codon is designated as position number S1. The signal sequences for interleukin-2 and the receptor polypeptide code for 20 and 19 amino acids, respectively.

β Based on the wobble hypothesis of Crick (1966). All possible leucine tRNA anticodon base sequences are included. The anticodons in parentheses have not been reported in eucaryotic tRNAs (Sprinzl et al., 1985). The anticodons (and codons) are written using the standard 5'→3' orientation.
Table 2.

POTENTIAL ANTICODON RECOGNITION

<table>
<thead>
<tr>
<th>Position</th>
<th>Codon</th>
<th>CAA</th>
<th>UAA</th>
<th>CAG</th>
<th>(AAG)</th>
<th>UAG</th>
<th>(GAG)</th>
<th>IAG</th>
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<tr>
<td><strong>Interleukin-2:</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>S6</td>
<td>CUC</td>
<td></td>
<td></td>
<td></td>
<td>(X)</td>
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<td>CUG</td>
<td></td>
<td></td>
<td>X</td>
<td>(X)</td>
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<tr>
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<td></td>
<td></td>
<td>(X)</td>
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<td>X</td>
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<tr>
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<td></td>
<td></td>
<td>(X)</td>
<td></td>
<td>(X)</td>
<td>X</td>
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<td>CUU</td>
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<td></td>
<td></td>
<td>(X)</td>
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<td>(X)</td>
<td>X</td>
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<tr>
<td><strong>Receptor Polypeptide:</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td>(X)</td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>(X)</td>
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</tr>
<tr>
<td>S7</td>
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<tr>
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<td>(X)</td>
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</tr>
<tr>
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<td>(X)</td>
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<td>X</td>
<td></td>
</tr>
</tbody>
</table>
6 of 21 in the remainder of the mRNA could be. In these cases, the level of inosine-containing tRNA could regulate the synthesis of these proteins. While this may not seem like a large obstacle for a cell to overcome, Hsu et al., (1985) found that the ilvGEDA operon of Serratia marcescens is attenuated by a single leucine codon. While this is a bacterial system, it demonstrates the levels of attenuation obtainable with little used codons.

The second line of evidence comes from the evaluation of the nucleoside composition of tRNA from human leukemia cells (Trewyn, 1984). Transfer RNA from these immature cells was purified and hydrolyzed to the nucleoside level prior to separation by HPLC. From this data, it was estimated that the tRNA from this sample contained only 0.2 inosine-containing tRNAs out of 20. This inosine content is surprising because inosine could be found in tRNAs for 8 of the 20 amino acids (bold print in Table 3 minus glycine). It is possible that the lack of inosine-containing tRNAs is responsible for the block in differentiation in these cells.

Since the level of hypoxanthine in the cell can be regulated at several points (Figure 36), the wobble attenuation theory is a plausible mechanism for controlling the synthesis of regulatory proteins. In this case, hypoxanthine could act as a regulator of cell function by controlling the synthesis of important cell
Table 3. Genetic code. Amino acid coding by triplet codons is depicted. Amino acids able to utilize inosine-containing tRNAs are designated by bold print. Inosine-containing tRNAs have been found for all of these designated amino acids with the exception of glycine.
Table 3.

<table>
<thead>
<tr>
<th>First position</th>
<th>Second position</th>
<th>Third position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U</td>
<td>C</td>
</tr>
<tr>
<td>U</td>
<td>Phe Ser Tyr Cys</td>
<td>Phe Ser Tyr Cys</td>
</tr>
<tr>
<td>C</td>
<td>Leu Pro His Arg</td>
<td>Leu Pro His Arg</td>
</tr>
<tr>
<td>A</td>
<td>Ile Thr Asn Ser</td>
<td>Ile Thr Asn Ser</td>
</tr>
<tr>
<td>G</td>
<td>Val Ala Asp Gly</td>
<td>Val Ala Asp Gly</td>
</tr>
</tbody>
</table>

- Phe: Phenylalanine
- Ser: Serine
- Tyr: Tyrosine
- Cys: Cysteine
- Stop: Stop Codon
- Trp: Tryptophan
- Pro: Proline
- His: Histidine
- Arg: Arginine
- Asp: Aspartic Acid
- Asn: Asparagine
- Glu: Glutamic Acid
- Gly: Glycine
- Leu: Leucine
- Ser: Serine
- Thr: Threonine
- Met: Methionine
- Val: Valine
- Ala: Alanine
- Lys: Lysine
- A: Arginine
proteins. For example, if interleukin-2 is required for the amplification of an immune response and the intracellular level of hypoxanthine is low, the response may be decreased or absent all together. On the other hand, the release of hypoxanthine could act as a trigger to increase production of an important cell protein to start a regulatory process. The radiolabelling experiments presented above appear to support this theory (Figure 19). Hypoxanthine treatment caused enhanced leucine incorporation into cellular protein, whereas adenine did not. It is proposed that the added hypoxanthine would allow the modification of specific tRNA species to contain inosine, expanding the codon recognition potential of the tRNA. When adenine is added, it may actually inhibit the inosine modification reaction to some extent by competing with hypoxanthine. In addition, when hypoxanthine was used in conjunction with DMSO, a synergistic effect was observed (Figure 19). In this instance, it is possible that changes in membrane permeability may be caused by the DMSO allowing more hypoxanthine to enter the cells (d, Figure 36).

While it is now known that Crick's wobble hypothesis is not always correct in predicting in vivo coding patterns, it is known that modified nucleotides in the first position of the anticodon can alter codon recognition (Bienz and Kubli, 1981; Beier et al., 1984a;
Meier et al., 1985; Kuchino et al., 1985). Anticodon-anticodon interactions with tRNAs having complimentary sequences have been performed to try to elucidate the effects on bonding caused by some of these anticodon modifications (Grosjean et al., 1985; Romby et al., 1985). Using the method of T-jump relaxation, it has been determined that many of modified bases found in the first position of the anticodon of tRNA alter the codon recognition potential of that tRNA to some extent. While many of these alterations seem to be slight, they offer a means whereby subtle changes in protein synthesis could be obtained; others offer the potential for major changes.

Based on the studies presented here using the HL-60 cell system to study the effect of the queuosine and inosine modifications of tRNA and the literature cited, it appears that the "wobble attenuation of translation" theory may offer a useful model for studying post-transcriptional regulation of cell processes. While the role of gene activation and repression can not be overlooked, it would appear that modifications in the "wobble" position of the anticodon may be responsible for some of the more subtle changes observed in protein synthesis. Based on the number of modifications found in this position of the tRNA (Figure 1), it would appear that there are many possibilities left to explore regarding this theory.
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