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Hematopoiesis and the Inosine Modification in Transfer RNA[1] (42129)

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Abstract. Human promyelocytic leukemia (HL-60) cells were used to begin to evaluate the role in hematopoiesis of inosine biosynthesis in the tRNA anticodon wobble position; a reaction involving the enzymatic insertion of preformed hypoxanthine. Dimethyl sulfoxide (DMSO) and hypoxanthine were found to induce the differentiation of HL-60 cells in a synergistic manner, and the induced differentiation was independent of changes in the purine catabolic enzymes adenosine deaminase and purine nucleoside phosphorylase. The short-term exposure of HL-60 cells to DMSO plus hypoxanthine resulted in enhanced leucine incorporation, and a model is presented showing how the inosine modification reaction in tRNA may be involved. A means by which hypoxanthine insertion into tRNA may modulate the synthesis of regulatory proteins (e.g., lymphokines and cell surface receptors) is also outlined.

It had long been assumed that the nucleoside inosine found in the first position of the anticodon of specific tRNAs was generated in the macromolecules by the selective enzymatic deamination of adenosine (1). However, our recent demonstration of an enzymatic insertion of preformed hypoxanthine into tRNA (2) indicates that inosine biosynthesis occurs by a base exchange mechanism similar to that catalyzed by tRNA-guanine ribosyltransferase (EC 2.4.2.29) (3, 4). The ramifications of this biosynthetic mechanism could be significant, since defects in the purine catabolic pathway responsible for generating hypoxanthine *in vivo* are associated with defects in cell-mediated immunity (5, 6). Therefore, the absence of adenosine deaminase (EC 3.5.4.4) or purine nucleoside phosphorylase (EC 2.4.2.1) may interfere with a tRNA modification reaction of importance for immune function by blocking formation of the required substrate hypoxanthine.

Hypoxanthine also induces the differentiation of murine erythroleukemia cells *in vitro,* while the further catabolites xanthine and uric acid do not (7). The hypoxanthine-induced differentiation does not involve salvage into the cellular nucleotide pool (7), and in addition, no changes in purine salvage were observed during differentiation of these cells induced by dimethyl sulfoxide (DMSO) (8). However, major changes in tRNA isoaccepting species were seen soon after inducing differentiation of cultured murine erythroleukemia cells with DMSO (9), and these included changes in some species ($tRNA^{Ala}$, $tRNA^{Leu}$, $tRNA^{Pro}$, $tRNA^{Ser}$, and $tRNA^{Thr}$) with the potential for being modified to contain inosine. Whether the macromolecular structural alterations actually involved the inosine modification was not established.

While a specific function in differentiation and/or cell-mediated immunity has not been demonstrated for inosine biosynthesis in tRNA, a role has been postulated (10). Inosine in the first position of the anticodon of a tRNA expands the codon recognition potential of that tRNA (11). Based on the wobble hypothesis for codon–anticodon pairing (11) and assuming inosine biosynthesis involves the exchange of hypoxanthine only for adenine (2), the codon reading capability should increase by threefold, i.e., inosine should be able to interact with uridine, cytidine, or adenosine in the third position of the mRNA codon while adenosine should only interact with uridine (11). Therefore, the inosine modification reaction was predicted to be

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required for mRNA codon translation involved in the synthesis of proteins and/or peptides of importance during hematopoiesis and in cell-mediated immunity (10).

A model depicting how inosine biosynthesis in tRNA might regulate protein synthesis is shown in Fig 1. In the example illustrated, a leucine tRNA is modified to contain inosine, and that tRNA should be able to read the three leucine codons depicted in bold print (CUC, CUA, and CUU). The unmodified tRNA (with adenosine in the wobble position) should only read the last leucine codon (CUU), so the ribosome would stall (thereby blocking translation of the mRNA) if the anticodon modification were not carried out and leucine tRNAs capable of reading both of the other codons (CUC and CUA) were not present. Regulating protein synthesis by this means would be possible only because the genetic code is degenerate, so other essential mRNAs could use codons not read by inosine-containing tRNAs. For example, different mRNAs in the same cell (or cell compartment) depicted in Fig. 1 could make use of the leucine codons CUG, UUA, and UUG, and as a result, their ability to function in protein synthesis would not be restricted.

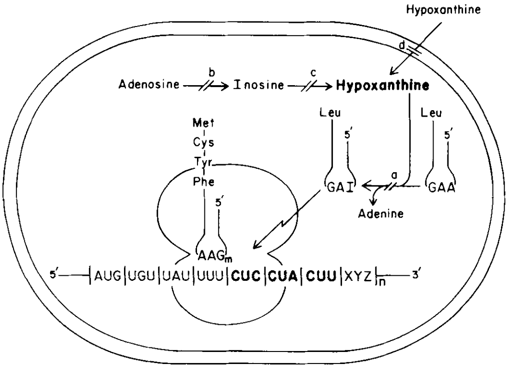

FIG. 1. Postulated model for how the hypoxanthine insertion reaction in specific tRNA anticodons may regulate protein synthesis. Transfer RNAs with the potential for having hypoxanthine inserted into the first position of the anticodon include those for alanine, arginine, isoleucine, leucine, proline, serine, threonine, valine, and perhaps glycine (2, 12). 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 (11), 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.

The model illustrated in Fig. 1 offers the basis for our studies of the potential role of inosine biosynthesis in tRNA in regulating (or modulating) hematopoiesis and cellular immunity. The intracellular availability of hypoxanthine is predicted to be a controlling factor as to whether inosine biosynthesis occurs and, therefore, whether specific mRNAs are translated.

Materials and Methods. *Cell culture.* Human promyelocytic leukemia (HL-60) cells, obtained from Dr. Robert Gallo at the National Cancer Institute, were grown in suspension culture in RPMI 1640 medium supplemented with 15% fetal bovine serum. Growth curves were established after HL-60 cells were plated at 6×10^4 cells/ml in 10 ml of medium per 25-cm^2 culture flask. Control cultures and cultures treated with 100 m*M* DMSO and/or 1 m*M* hypoxanthine were maintained in duplicate, and cells were enumerated with a Coulter counter. Based on the hypoxanthine content of the lot of serum utilized, the final concentration of hypoxanthine in the control and DMSO-containing medium was 12 μM. Cytospin preparations were used to assess morphological differentiation of the HL-60 cells treated as described above. The cells were stained with Wright-Giemsa, and differential cell counting was performed on a minimum of 200 cells to determine the percentage of total cells exhibiting mature morphology (13).

Enzyme assays. HL-60 cells in 1-liter spinner flasks were harvested by low-speed centrifugation, washed, and homogenized in reticulocyte standard buffer (RSB). The extract was then centrifuged at 30,000*g* for 20 min at 4°C, and the supernatant was used to assay adenosine deaminase and purine nucleoside phosphorylase. In both cases, a spectrophotometric, coupled enzyme system was used to monitor uric acid formation (14, 15). For adenosine deaminase, the standard reaction mixture contained 100 m*M* phosphate buffer (pH 7.2), 1.5 m*M* adenosine, 20 units of purine nucleoside phosphorylase, 20 units of xanthine oxidase, and cell extract in a total volume of 1 ml. For purine nucleoside phosphorylase, the same reaction mixture was used except 0.4 m*M* inosine replaced adenosine as the substrate and the commercial purine nucleoside phosphorylase was omitted. Control reactions contained everything except substrate or cell extract. The reaction mixtures were incubated at 37°C, and uric acid formation was monitored as an increase in absorbance at 293 nm using a Beckman DU-8 recording spectrophotometer. An extinction coefficient of 12.1/μmole/cm^3 for uric acid was used to calculate product formation, with units of enzyme activity in nanomole product/minute.

Leucine incorporation. HL-60 cells were harvested by low-speed centrifugation, washed twice with a Hanks' balanced salt solution, and resuspended at 5×10^5 cells/ml in Hanks' alone or Hanks' supplemented with 210 m*M* DMSO, 1 m*M* hypoxanthine, or 210 m*M* DMSO plus 1 m*M* hypoxanthine. After preincubating at 37°C for 10 min, [^{14}C]leucine (50 mCi/mmole) was added at 0.5 μCi/ml. At various time intervals up to 60 min, the amount of acid-insoluble radioactive leucine incorporated was determined by precipitation of 0.5-ml aliquots with 2.5 ml of 10% trichloroacetic acid (TCA) (16, 17). After 30 min on ice, the precipitates were collected on glass-fiber filters, washed with 40–50 ml of ice-cold 5% TCA, dried, and counted by liquid scintillation.

Materials. Fetal bovine serum (Lot No. 100430) was obtained from Sterile Systems, Logan, Utah, while the cell culture medium RPMI 1640 and the Hanks' balanced salt solution were from GIBCO, Grand Island, New York. Purine nucleoside phosphorylase and xanthine oxidase were purchased from Boehringer-Mannheim, Indianapolis, Indiana, while the [^{14}C]leucine was purchased from Amersham, Arlington Heights, Illinois.

Results. Growth curves for HL-60 cells treated with DMSO and hypoxanthine alone and in combination are shown in Fig. 2. Treatment with 100 m*M* DMSO alone diminished the growth of the HL-60 cells only slightly compared to the untreated controls, while 1 m*M* hypoxanthine was somewhat more effective. However, the combination of 100 m*M* DMSO plus 1 m*M* hypoxanthine resulted in an almost complete cessation of growth.

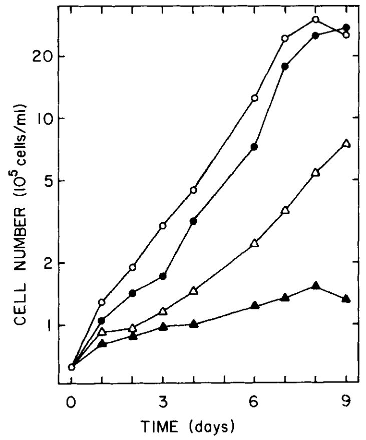

FIG. 2. HL-60 growth curves. Cells plated at an initial density of 6×10^4 cells/ml were monitored for 9 days as described under Materials and Methods. The curves depict untreated control cells (○) as well as cells treated with 100 m*M* DMSO (●), 1 m*M* hypoxanthine (△), and 100 m*M* DMSO plus 1 m*M* hypoxanthine (▲). On Day 9, differential cell counting was performed, and those results are presented in Table I.

Although 1 m*M* hypoxanthine inhibited the growth of HL-60 cells, it was not effective at inducing differentiation (Table I). Likewise, 100 m*M* 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 (Table I).

Adenosine deaminase and purine nucleoside phosphorylase were assayed in extracts of untreated HL-60 cells as well as in HL-60 cells induced to differentiate with DMSO plus hypoxanthine. The specific activity of purine nucleoside phosphorylase was almost threefold higher than adenosine deaminase in the HL-60 cells, but no differences were seen for the treated versus untreated cultures (Table II). Results similar to those for the 24-hr exposure to DMSO plus hypoxanthine were obtained at 72 hr (data not presented).

TABLE I. DIFFERENTIAL COUNTS OF HL-60 CELLS AFTER INCUBATION WITH DIMETHYLSULFOXIDE (DMSO) AND/OR HYPOXANTHINE (Hx)[a]

Inducer	Percentage of total cells			
	Promyelocytes	Myelocytes	Metamyelocytes	Neutrophils
None	89.4	7.8	2.8	0
DMSO	89.6	6.3	4.1	0
Hx	97.1	2.5	0.4	0
DMSO/Hx	46.2	32.4	19.5	1.9

[a] The cells were treated for 9 days with 100 m*M* DMSO, 1 m*M* Hx, or 100 m*M* DMSO plus 1 m*M* Hx. The differential counts are from the experiment depicted in Fig. 2, but similar results have been obtained in numerous independent experiments. See the Materials and Methods for additional details.

Radiolabeled leucine incorporation into TCA precipitable material was monitored with HL-60 cells treated short-term with DMSO plus hypoxanthine (Fig. 3). Compared to untreated control cells, these cells incorporated appreciably more leucine. The combination of DMSO and hypoxanthine also yielded significantly more leucine incorporation than did DMSO alone or DMSO plus adenine. In separate experiments, 1 m*M* hypoxanthine alone was found to have no effect (data not presented).

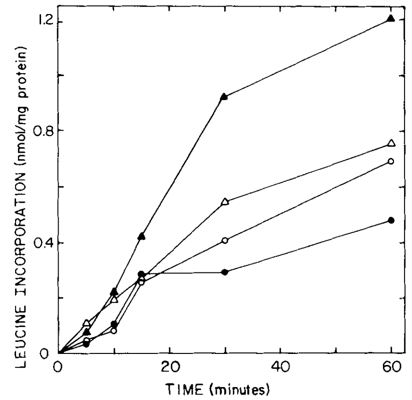

FIG. 3. Enhanced leucine incorporation by HL-60 cells treated with DMSO plus hypoxanthine. Cells (5 × 10^5/ml) preincubated for 10 min in a Hanks' balanced salt solution alone (○) or Hanks' plus 210 m*M* DMSO (●), 210 m*M* DMSO and 1 m*M* adenine (△), or 210 m*M* DMSO and 1 m*M* hypoxanthine (▲) were radiolabeled 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. See Materials and Methods for additional details.

Discussion. It has been known for sometime that, individually, DMSO and hypoxanthine will induce the differentiation of HL-60 cells (18). However, the concentrations required are higher than those utilized in much of this investigation. It was surmised that the mechanism of action for DMSO-induced differentiation might involve enhanced hypoxanthine transport into the cell or cell compartment where inosine modification in tRNA occurs (Fig. 1). Therefore, suboptimal concentrations of DMSO and hypoxanthine were evaluated in combination, and a synergistic induction of HL-60 differentiation was observed (Fig. 2 and Table I). While these results do not prove the mode of action of either agent, they are consistent with the postulated model.

Changes in purine catabolism are common during hematopoiesis, but the function of such changes remains obscure. Significant two- to threefold increases in purine nucleoside phosphorylase activity (without a change in adenosine deaminase) were reported during the phorbol ester-induced differentiation of chronic lymphocytic leukemia cells (19), while nearly a 10-fold increase in adenosine deaminase activity was reported when peripheral blood monocytes differentiated into macrophages *in vitro* (20). In the latter case, inhibiting the elevation of adenosine deaminase activity blocked the cellular differentiation, thereby indicating a fundamental role for the enzyme. No such differences in the purine catabolic enzymes were observed in the present study when HL-60 cells were induced to differentiate with DMSO plus hypoxanthine (Table II). However, if the

TABLE II. ADENOSINE DEAMINASE (ADA) AND PURINE NUCLEOSIDE PHOSPHORYLASE (PNP) ACTIVITY IN HL-60 CELLS AFTER INCUBATION WITH DIMETHYLSULFOXIDE (DMSO) AND HYPOXANTHINE (Hx)[a]

Inducer	Specific activity[b]	
	ADA	PNP
None	55.4 ± 3.5	139 ± 9
DMSO/Hx	51.1 ± 4.3	143 ± 26

[a] The cells were treated for 24 hr with 210 m*M* DMSO plus 1 m*M* Hx, after which ADA and PNP were assayed in crude cell homogenates as described under Materials and Methods.

[b] The results indicate the mean (± S.D.) of duplicate determinations from two independent experiments and are expressed as nmol/min/mg protein.

increases in purine catabolism are needed to generate the substrate for inosine biosynthesis in tRNA (Fig. 1), such changes should not be required when the substrate hypoxanthine is supplied exogenously.

Enhanced protein synthesis (based on radiolabeled leucine incorporation) was reported during the differentiation of HL-60 cells *in vitro* (17). In addition, the leucine radiolabeling of specific membrane-associated proteins was increased significantly during HL-60 differentiation into macrophage-like cells induced by phorbol esters (21). These data are consistent with the model presented in Fig. 1, and further support was obtained in this investigation (Fig. 3). The short-term (10 min) exposure of HL-60 cells to DMSO plus hypoxanthine prior to adding radiolabeled leucine resulted in a significant increase in leucine incorporation into TCA-precipitable material. Neither DMSO alone nor DMSO plus adenine (a purine which should not effect an increase in tRNA inosine biosynthesis) caused similar increases. Considering our previous demonstration that immature leukocytes have very little inosine in their tRNA (10), the results in Fig. 3 might best be explained by hypoxanthine insertion into leucine tRNAs which should enhance radiolabeled leucine incorporation into polypeptides as depicted in Fig. 1.

As a means for regulating gene expression, the model in Fig. 1 is somewhat analogous to the attenuation mode described for various bacterial operons (22). With that system, the level of aminoacylation of a particular tRNA determines whether ribosomes stall or translate leader sequences preceding structural genes in mRNA. The model presented here relies instead on a post-transcriptional structural modification in the tRNA anticodon. This modification is predicted to allow stalled ribosomes to proceed, thereby releasing a block in protein synthesis. Since the hypoxanthine insertion reaction expands the wobble capability of the tRNA, this type of control might be described as wobble attenuation of translation. Again, such a form of regulation would be possible only because the genetic code is degenerate, so other essential mRNAs could use different codons which are read by tRNAs not subject to similar controls.

An interesting possibility for how wobble attenuation of translation might be involved specifically in the control of hematopoiesis or immune function deals with the translocation of regulatory proteins through or into the cell membrane. In many such cases, the initial translation product has a signal peptide sequence at the amino terminus which allows the translocation of the protein (23). The signal peptide is then removed during or after the translocation process. Examination of the leucine codons in signal sequences for two T-cell proteins [interleukin-2 (T-cell growth factor) and a cell surface receptor polypeptide] demonstrates a preponderance of codons (CUU, CUC, CUA) with the potential to be read by an inosine-containing leucine tRNA (Table III). Four of five leucine codons in the interleukin-2 signal sequence could be translated by this tRNA, while only 5 of 18 leucine codons in interleukin-2 itself could be (24), i.e., other leucine tRNAs would be required to read 13 of the latter codons (CUG, UUA, UUG). The situation for the receptor polypeptide is even more polarized where 6 of 6 leucine codons in the signal sequence could be read by the inosine-containing tRNA while only 6 of 21 in the remainder of the mRNA could be (25). Therefore, the synthesis of such proteins might be regulated, at least in part, by wobble attenuation of translation in the signal sequences as depicted in Fig. 1. The data reported here for radiolabeled leucine incor-

TABLE III. LEUCINE CODONS IN SIGNAL SEQUENCES FOR T-CELL PROTEINS[a]

		Potential anticodon recognition[c]						
Position[b]	Codon	CAA	UAA	CAG	(AAG)	UAG	(GAG)	IAG
Interleukin-2								
S6	CUC						(×)	×
S7	CUG			×		×		
S12	CUA					×		×
S14	CUU				(×)		(×)	×
S16	CUU				(×)		(×)	×
Receptor polypeptide								
S5	CUU				(×)		(×)	×
S6	CUC						(×)	×
S7	CUC						(×)	×
S11	CUA					×		×
S13	CUU				(×)		(×)	×
S18	CUC						(×)	×

[a] Based on nucleotide sequences published by Taniguchi *et al.* (24) for human interleukin-2 and Hedrick *et al.* (25) for a mouse T-cell receptor polypeptide.

[b] 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.

[c] Based on the wobble hypothesis of Crick (11). All possible leucine tRNA anticodon base sequences are included. The anticodons in parentheses have not been reported in eucaryotic tRNAs (12). The anticodons (and codons) are written using the standard 5′ → 3′ orientation.

poration (Fig. 3) and that previously published (17, 21) appear to be compatible with such a model. The implications of this mechanism with regard to hematopoiesis and immune function could be far reaching, since the tRNA modification reaction may be subject to modulation by extrinsic agents.

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