The Transfer RNA Methylases of Human Lymphocytes.
I. Induction by PHA in Normal Lymphocytes

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Assays were performed that measured both the rate and extent (or total capacity) of transfer RNA methylases in extracts of lymphocytes cultured in the presence and absence of phytohemagglutinin (PHA). The tRNA methylases of human peripheral blood lymphocytes undergoing blastogenesis in culture with PHA had a five- to sixfold increase in rate and a three- to sevenfold increase in extent of methylation of heterologous tRNA. These data suggest that PHA transformed lymphocytes not only contain elevated levels of tRNA methylases, but that the increase includes qualitatively different enzymes from those found in normal peripheral blood lymphocytes. Experiments in which lymphocytes were incubated for various times with PHA revealed that tRNA methylase induction occurred late in or after DNA synthesis and after morphologic transformation, but prior to mitosis. Rate and extent of tRNA methylation increased simultaneously. PHA induction of tRNA methylase activity was dependent on the synthesis of new RNA in lymphocytes cultured from 40 to 45 hours. The increase was not due to different levels of inhibitors or activators or preferential degradation of reaction components. The data suggest that quantitative and qualitative changes occur in the tRNA methylases of the normal human peripheral blood lymphocyte stimulated by PHA to undergo transition to an undifferentiated cell "in cycle." The possible significance of these findings to control of protein synthesis in PHA transformed lymphocytes is discussed.

A MAJOR ASPECT of cellular differentiation and proliferation involves selective synthesis of proteins. Regulation of protein synthesis may occur by the control of the types and quantity of messenger RNA (mRNA) transcribed. Another level of control is the translation of the mRNA codons. Among other possibilities, translational control can involve changes in species[1,2] or quantity[3-6] of the central component of protein synthesis, transfer RNA (tRNA). Control at this level has been invoked by several authors.[7-11]

Changes in the structure of tRNA may alter its acceptance of amino acids or its binding to the mRNA-ribosome complex, and thereby markedly alter protein synthesis. Changes in tRNA have already been found to occur during cellular differentiation,[12-14] after phage infection,[3,4] during variation of bacterial growth,[15] following hormone treatment,[16] between normal and neoplastic

cells,[17-20] between similar cells producing different immunoglobulins,[21] and between cells in culture and tumors derived from these cells.[22] Alterations in tRNA structure can occur by primary base-sequence change at the time of transcription of the "pre-tRNA" polynucleotide or by modification of the "pre-tRNA's" after their transcription, as well as by further modification of already mature tRNA. One major way "pre-tRNA"[23] and possibly mature tRNA[24,25] is modified is by methylation.

Stimulation of the human peripheral blood lymphocyte with PHA results in its transformation from a small "resting" cell to a large blastlike cell, which ultimately undergoes mitosis. This system is of great interest not only for its immunological aspects but also for the examination of the biochemical events involved in conversion of a resting cell to an actively growing cell. This transformation is preceded and accompanied by marked changes in the rates of RNA and protein synthesis.[26-28] In this communication we report our findings that transformation of normal human lymphocytes with PHA results in a marked induction in the enzymes that catalyze the methylation of tRNA, collectively termed tRNA methylases.

MATERIALS AND METHODS

Preparation of Lymphocyte Cultures

Peripheral blood was obtained from normal donors. Erythrocytes were sedimented with 6 per cent dextran at 37°C for 1 hour. Lymphocytes were separated from other leukocytes (less than 5 per cent polymorphonuclear leukocyte contamination) by the use of nylon columns as previously described.[29] The lymphocytes from 5–10 donors with O+ blood type were pooled for each experiment. In one experiment lymphocytes were obtained using an NCI-IBM continuous-flow Blood Cell Separator. The leukocyte fraction contained 85 per cent lymphocytes and 15 per cent polymorphonuclear and mononuclear cells.

Cells were suspended to 3 × 10⁶ cells per ml. in Eagle's minimum essential medium (Flow Laboratories) containing 30 per cent fetal calf serum (Grand Island Biological Co.), 3 mM glutamine, and 6500 units each of penicillin and streptomycin. RPMI Medium 1640 (Industrial Biological Laboratories) was used in later experiments since it was found to produce a greater percentage of transformed cells.

Cell cultures were incubated at 37°C in glass-stoppered bottles without shaking. Two mg. of phytohemagglutinin M (Difco Laboratories) per 250 ml. of culture medium were added at the start of incubation. Control cultures were incubated for the same time as those receiving PHA. The proportion of transformed cells in cultures of various cell lots incubated with PHA for 40 hours varied from 10 to 40 per cent. Cells were harvested by placing the culture bottles in an ice bath and then centrifuging at 175 × g. The cells were then washed with normal saline, and residual red cells were removed by hypotonic lysis. The lymphocytes were then stored at −170°C until used.

Measurement of RNA and DNA Synthesis

RNA synthesis was estimated by determining the incorporation of ¹⁴C-uridine (62 mCi/mmole, Amersham/Searle Corp.) into RNA during 15 hours of incubation.[30] Under these conditions PHA produced a fivefold increase in uridine incorporation, which was completely suppressed by actinomycin D (10 μg./ml.).

DNA synthesis was determined by measuring the incorporation of ¹⁴C-thymidine (58 mCi/mmole, Amersham/Searle Corp.) into DNA during a 1-hour pulse as described previously.[30]

Preparation of Enzyme Extracts and Undermethylated tRNA

Preparation of enzyme extracts and undermethylated *Escherichia coli* $K_{12}W6$ tRNA has previously been described.[31] Equivalent numbers of cells from the cultures were used in preparing the extracts used for enzyme assays. The protein content was determined by the method of Lowry et al.[32] and was similar for all extracts.

Transfer RNA Methylase Assays

The tRNA methylases catalyze the methylation of tRNA by S-adenosyl-L-methionine according to the following scheme:

$$\text{S-adenosyl-L-methionine + undermethylated tRNA} \xrightarrow{\text{tRNA methylases}} \text{methylated tRNA}$$
$$\text{+ S-adenosyl-homocysteine.}$$
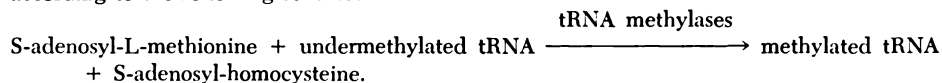
Transfer RNA methylase assays were performed as previously described.[31] The reaction mixture contained in a final volume of 0.3 ml.: 4.5 nmoles of ^{14}C-S-adenosyl-L-methionine (44.5 mCi/mmole, New England Nuclear Corp.); 25 μmoles of Tris-HCl, pH#9.0; 70 μmoles of ammonium acetate; 0.3 μmole of dithiothreitol; 0.3 μmole of tetrasodium EDTA; and variable amounts of enzyme extract. Rate assays were performed with excess *E. coli* tRNA (60 μg.) and limiting amounts of methylases. Extent assays (total capacity for methylation) were carried out with limiting amount of *E. coli* tRNA (1 μg.) and excess methylases. The activity of tRNA methylases was determined from the initial rate of reaction, where S-adenosyl-L-methionine incorporation into tRNA is a linear function of the protein content of the assay mixture. The tRNA-methylating rates of different extracts were compared by expressing rate as cpm per μg. of protein per 60-minute incubation (specific activity). The extents or capacities of tRNA methylases were determined from the plateau portion of the protein concentration curves, where addition of more protein did not result in further S-adenosyl-L-methionine incorporation into tRNA (maximal methylation of tRNA). Extents were expressed as cpm per μg. tRNA. Assays were performed in duplicate or triplicate with a 10–15 per cent variation in the experimental results.
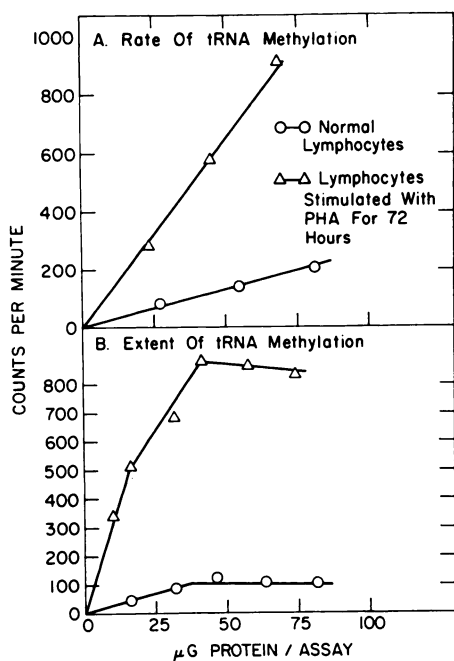

Fig. 1.—Rate and extent of tRNA methylation in lymphocytes. Extracts of normal peripheral blood lymphocytes incubated in the presence and absence of PHA for 72 hours were assayed for tRNA methylase activity. See text for details of the assay. An excess of undermethylated *E. coli* $K_{12}W6$ tRNA (60 μg.) and limiting enzyme was used for rate assays (top) and a limiting amount of tRNA (1 μg.) with excess enzyme for extent assays (bottom). Activity was expressed as cpm per 60-minute assay.
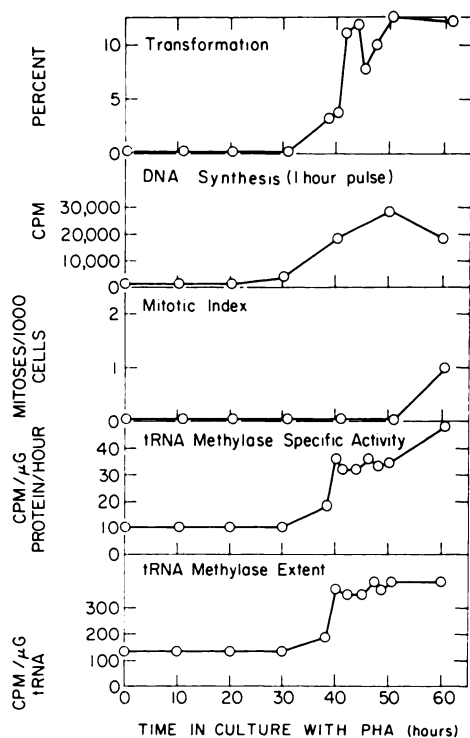
Fig. 2.—Time of tRNA methylase induction by PHA in normal lymphocytes and relation to DNA synthesis, transformation, and mitosis. Normal peripheral blood lymphocytes were incubated with PHA for various times. Per cent transformation and mitotic index were determined from smears of cultures. Rate of DNA synthesis was determined by measuring the incorporation of ^{14}C-thymidine into DNA during a 1-hour pulse. See text for details of tRNA methylase assays.

RESULTS

Rate and Extent of tRNA Methylation

The rate and extent of tRNA methylation were both higher in extracts from PHA transformed lymphocytes than in extracts from control lymphocytes. Data from a typical experiment are shown in Fig. 1. The tRNA methylase activities were compared with lymphocytes cultured in the presence and absence of PHA for 72 hours. Transformed lymphocyte extract tRNA methylase rates were approximately 600 per cent and tRNA methylase extents 700 per cent greater than those of control lymphocyte extracts. Since the amount of protein per lymphocyte increases after PHA addition, tRNA methylase specific activity based on the amount of cellular protein underestimates the per-cell increase in tRNA methylase activity.

The specific activities (rates) quantitate tRNA methylase activity in relation to the amount of other cellular proteins. The incorporation of S-adenosyl-L-methionine into tRNA is a linear function of the amount of protein added under the conditions of the rate assay. Extent or capacity, on the other hand, is measured on the plateau portion of the curve and as such the values are not affected by the addition of more enzyme. Thus, the relative methylating capacity of these enzymes governs the height of the plateau. Assuming equal amounts of cofactors (or other possible variables, such as inhibitors or ribonucleases) in the enzyme preparations, the extents are a valid comparison of the ability of enzyme extracts to recognize and methylate different sites on

tRNA. These data showing increases in both specific activity and extent of tRNA methylation in the transformed lymphocytes suggest not only that PHA-transformed lymphocytes contain elevated levels of tRNA-methylating enzymes, but that species of tRNA methylases are present that are not found in normal, unstimulated peripheral blood lymphocytes.

Time of tRNA Methylase Induction

Morphologic transformation, rates of DNA synthesis, mitotic index, and tRNA methylase activities were measured in lymphocytes incubated with PHA for various times (Fig. 2). This particular experiment was performed with lymphocytes obtained by the use of an NCI-IBM continuous flow blood cell separator. Similar results were obtained with lymphocytes obtained from whole blood and purified by nylon columns.

Figure 2 illustrates that the induction of transfer RNA methylases occurred after full development of transformation and after the onset of DNA synthesis but prior to the onset of mitosis. Both transformation and tRNA methylases reached their maximal levels after 38–42 hours of culture with PHA. The increases in transfer RNA methylase rates and extents were concomitant. The per cent increase in activity was less than in the previous experiment and varied from 300 per cent to 800 per cent in various cell lots.

It has been demonstrated that lymphocytes cultured with PHA do not behave as a synchronized cell population even after release from a block in S phase.[33] Nevertheless, since resting lymphocyte tRNA methylase activities are low, it is reasonable to assume that the first cells that proceed through S and G_2 are responsible for the increases in activity found at this time—i.e., the induction of tRNA methylases by PHA occurs in S or G_2 phase of the cell cycle in human peripheral blood lymphocytes.

Actinomycin D Block of tRNA Methylase Induction

With concentrations that produced complete inhibition of RNA synthesis (10 μg./ml.), actinomycin D added at 40 or 42 hours of culture blocked the induction of tRNA methylases by PHA (Table 1). When actinomycin D was added at 45 hours of incubation with PHA, the induction was not affected. Actinomycin D added to PHA cultures at 40 hours did not affect the percentage

Table 1.—Actinomycin D Block of PHA Induction of Lymphocyte tRNA Methylases

Additions [a]	Per Cent of tRNA Methylase Specific Activity in Cultures Receiving Only PHA
None	35
PHA at 0 time	100
PHA at 0 time Actinomycin D at 40 hours	46
PHA at 0 time Actinomycin D at 42 hours	43
PHA at 0 time Actinomycin D at 45 hours	104

[a] PHA was added at the start of incubation, actinomycin D (10 μg./ml.) at the indicated times. All cultures were terminated at 48 hours.

Table 2.—Incorporation of ^{14}C-S-adenosyl-L-methionine (cpm) into 60 μg.
Undermethylated tRNA by Equal Amounts of Control and Transformed
Lymphocyte Extracts

(A) Control Lymphocyte (cpm)	(B) Transformed Lymphocyte (cpm)	(A) + (B) Predicted (cpm)	(A) + (B) Observed (cpm)
344	1157	1501	1516

of cells transformed. These data indicate that the increase in tRNA methylase activity in lymphocytes is dependent upon new RNA synthesis in the period from 40 to 45 hours of culture with PHA, and that morphological transformation occurs in the absence of tRNA methylase induction.

Mixing Experiments

The possibility that the apparent PHA induction of tRNA methylases in lymphocytes was due to the loss of an inhibitor or the presence of an activator was considered in the following experiments. Equal amounts of control and transformed lymphocyte extract were mixed and the assay was performed in the usual manner. As can be seen from the results of a typical experiment (Table 2), activities were additive. Thus, apparently neither extract contained factors that protentiated or inhibited the tRNA methylase activity of the other.

Delayed Addition of tRNA and S-adenosyl-L-methionine

The preincubation of either control or transformed lymphocyte extract in the standard assay mixture 1 hour before the addition of either tRNA or ^{14}C-S-adenosyl-L-methionine resulted in a similar reduction in tRNA methylase activity in both (Table 3). Thus, there was no significant difference between control or transformed lymphocytes in degradation or inactivation of substrates (tRNA or S-adenosyl-L-methionine) or of tRNA methylases under these assay conditions.

Addition of PHA to Enzyme Assays

A direct effect of PHA on tRNA methylase activity in lymphocytes was ruled out in experiments in which PHA was added to enzyme reaction mixtures. These results, as well as those of the previous three experiments above, demonstrate that the increase in tRNA methylases in lymphocytes treated with PHA is a true induction rather than an activation of enzyme activity.

Table 3.—The Effects of Preincubation on tRNA Methylase Specific Activity
of Lymphocyte Extracts

Addition After 1 Hour Incubation *	Specific Activity cpm/μg. Protein/Hour	
	Control Lymphocyte	Transformed Lymphocyte
(A) None (control)	28.1	81.7
(B) tRNA	10.6	32.8
(C) ^{14}C-S-adenosyl-L-methionine	12.6	37.6

* Incubations carried out with (A) all components (control), (B) without tRNA or (C) without S-adenosyl-L-methionine. After 60 minutes, control samples were terminated and 60 μg. tRNA were added to (B), 4.5 nmoles S-adenosyl-L-methionine were added to (C), and incubations were continued for an additional 60 minutes.

DISCUSSION

A series of biochemical changes occur prior to blastogenesis in lymphocytes cultured in the presence of PHA. After an initial interaction of PHA with lymphocyte membranes,[34] the following changes have been found: alterations of membrane phospholipid metabolism;[35] acetylation of histones;[36] increases in tRNA[37] and ribosomal RNA (rRNA) synthesis,[38] including decreased 18S rRNA "wastage;"[39] increases in nuclear template activity for RNA transcription[40] and increases in protein synthesis;[26] increases in lysosomal acid hydrolases[41] and shift of these enzymes to the cytoplasm;[42] induction of uridine kinase[43] and DNA polymerase;[44] alterations of glucose metabolism;[45,46] increases in LDH,[47] G6PD, acid phosphatase, and α-glucosidase activities;[48] and increases in DNA synthesis.[49] Whereas most of these changes take place early in lymphocyte transformation, as discussed below, the induction of tRNA methylases occurs late in transformation.

The tRNA methylases have generated a great deal of interest for several reasons. (1) Some tumors,[31,50-54] fetal tissues,[55] undifferentiated cells,[31] and oncogenic virus[56-58] or spontaneously[58,59] transformed cells have been shown to contain higher levels of tRNA methylases than adult normal tissues, differentiated cells, or nontransformed cells, and in a few cases to contain more methylated bases in their tRNA.[60-62] (2) Differences in the elution of amino-acyl-tRNA's on reverse phase partition columns between tumors and normal tissues[17-20] and between normal tissues at different stages of differentiation have been demonstrated.[12-14] (3) The degree and type of methylation appear to play a role in determining the function of the tRNA in acceptance of amino acids[24] and response to codons.[25]

The in vivo scheme of tRNA methylation is illustrated in Fig. 3. "Pre-tRNA" is transcribed from DNA in the nucleus. Kay and Cooper demonstrated unmethylated "pre-tRNA" in the cytoplasm of cultured lymphocytes within 30
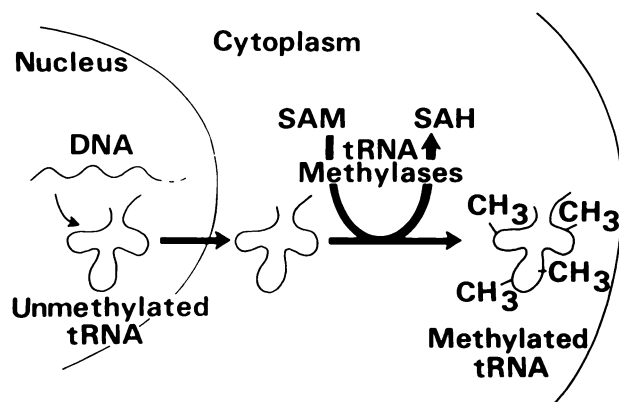

Fig. 3.—In vivo scheme of tRNA methylation. Unmethylated tRNA is transcribed from DNA in the cell nucleus. Although tRNA is represented as a "cloverleaf," its true conformation in the unmethylated state is not known. For illustrative purposes, the site of tRNA methylation is shown as the cytoplasm although this is controversial. The tRNA methylases catalyze the transfer of the methyl group from S-adenosyl-L-methionine (SAM) to tRNA forming methylated tRNA and S-adenosyl-L-homocysteine (SAH).

minutes after the addition of PHA.[37] Unmethylated "pre-tRNA" has also been found in other mammalian cells.[63,64] The tRNA methylases catalyze the transfer of the methyl group from S-adenosyl-L-methionine to tRNA-producing methylated tRNA and S-adenosyl-L-homocysteine.[65] Although illustrated as a single reaction, there are tRNA methylase species with several different base specificities.[66] When "pre-tRNA" becomes mature tRNA, it will accept amino acids and bind to appropriate messenger RNA–ribosome complexes to take part in protein synthesis.

PHA induces tRNA methylases in lymphocytes either late in DNA synthesis or in G_2 (as illustrated schematically in Fig. 4), but in either case after full morphologic transformation (Fig. 2). As noted above, new tRNA synthesis begins within 30 minutes of PHA addition.[37] Thus, apparently the time of tRNA synthesis is remote from that of tRNA methylase induction. In this regard, Tidwell et al.[67] recently reported in another system that tRNA synthesis and methylation were not synchronous.

Why are tRNA synthesis and tRNA methylase induction so widely separated in time? It may be that "pre-tRNA" remains unmethylated until new tRNA methylases appear some 37 hours later. However, the increase in mature tRNA observed by Kay and Cooper within 90 minutes of PHA addition[37] makes this unlikely. Another possibility is that preexisting tRNA methylases (constitutive) methylate the early appearing new "pre-tRNA." It is reasonable to assume that some specific proteins are required for the cell to enter mitosis. Perhaps the mRNA's for these proteins cannot be translated until some new and particularly modified (methylated) tRNA's are available. Thus, the new tRNA methylases arising late in transformation may have as their function either methylation of new tRNA species or further modification of already mature tRNA so that this mRNA may be translated and the cell can proceed in its transition from a "resting" to a proliferative cell. However, appreciation of the significance of tRNA methylase induction in PHA-transformed lymphocytes will have to await a clearer understanding of the role of these enzymes.

We have previously reported that blastic cells in culture derived from normal individuals and patients with various malignancies have tRNA methylase
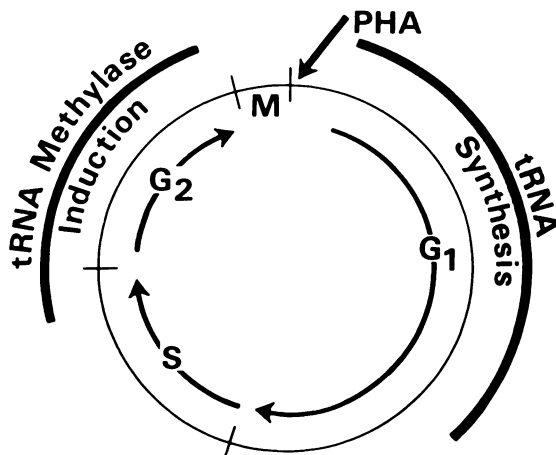

Fig. 4.—Schematic relationship of tRNA methylase induction to the cell cycle. Transfer RNA synthesis begins within 30 minutes after PHA addition.[37] Transfer RNA methylase induction occurs during or close to S phase and transformation.

activities similar to those observed in PHA transformed lymphocytes.[31] It appears on the basis of these data that undifferentiated normal, as well as neoplastic, cells may have high tRNA methylase activities. However, before it can be said that the tRNA's of normal undifferentiated cells are hyper-methylated with respect to those of normal differentiated cells, the methylated base composition of the tRNA's will have to be determined.

Little is known about the level of individual enzymes throughout the cell cycle in mammalian cells. The cycle-specific inducibility of tyrosine-amino-transferase in synchronized HT3 cells[68] is a notable exception. The apparent S or G_2 induction of lymphocyte tRNA methylases by PHA is another example of the cell-cycle specificity of a mammalian enzyme.

ACKNOWLEDGMENT

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