

# RNA METABOLISM IN HE<sub>L</sub>A CELLS AT REDUCED TEMPERATURE

## II. Steps in the Processing of Transfer RNA

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### ABSTRACT

Incubation of HeLa cells at 24°C results in the modification of the processing of pre-tRNA to tRNA. Both methylation and size reduction were shown to take place *in vitro* when purified pre-tRNA was subjected to processing in a cytoplasmic extract of HeLa cells. The migration of pre-tRNA from the nucleus to the cytoplasm was not significantly altered at 24°C.

### INTRODUCTION

The presence of an apparent precursor to tRNA has been demonstrated in several mammalian cell systems (1-3). When HeLa cells are labeled for less than 30 min and the isolated RNA is separated by polyacrylamide gel electrophoresis, most of the labeled small molecular weight RNA is found between the 4S and 5S RNA peaks. If, after such a short labeling period, further RNA synthesis is prevented by actinomycin D, the RNA intermediate between 4S and 5S RNA is reduced in quantity with the concurrent appearance of label in the 4S RNA peak (2). The slower migration of the intermediate material under the conditions of electrophoresis is probably not due to secondary structure of the tRNA but due to a nucleotide sequence longer than that of tRNA (4). This intermediate material is deficient in methyl groups and has a lower content of pseudouridine than mature tRNA (5).

RNA precursor to tRNA is formed in the nucleus and transported into the cytoplasm where the formation of mature tRNA occurs via the cleavage of the polynucleotide and modification of individual nucleotides. Studies of this RNA *in vitro* have

strengthened the evidence that the heterogeneous RNA serves as a precursor to tRNA (4, 5).

In work previously reported (6), the accumulation of certain short-lived intermediates in the processing of 45S preribosomal RNA to mature 28S ribosomal RNA was demonstrated at reduced temperatures. Similar reduced temperature studies of the synthesis and processing of 4S and 5S RNA have provided a somewhat more detailed picture of the synthesis, modification, and transport of pre-tRNA.

### MATERIALS AND METHODS

#### *Cells*

The HeLa cells used were a Mycoplasma-free strain obtained from Microbiological Associates, Inc., Bethesda, Md. and were prepared as reported earlier (6).

#### *Isolation of RNA*

Cold phenol extraction was used for the isolation of RNA. The cells were washed twice with reticulocyte standard buffer (RSB) (0.01 M NaCl, 0.01 M Tris-HCl, 0.0015 M MgCl<sub>2</sub>; pH 7.4) and resuspended

in 5 ml RSB. 1 ml of a 7.5% sodium dodecyl sulfate (SDS) in 0.01 M ethylenediaminetetraacetic acid (EDTA) solution was added for cell lysis. The released DNA was sheared by several strokes in a Dounce homogenizer; 2 vol of phenol were added, and the mixture was shaken for 2 min at room temperature. Separation of the phenol and aqueous layers was accomplished by centrifugation at 1000 *g* for 3 min. The aqueous layer was removed and reextracted with an equal volume of fresh phenol as before. Residual phenol in the aqueous phase was removed by extraction with 1 ml diethyl ether. RNA was precipitated by the addition of 2 vol of cold ethanol to the aqueous layer. The RNA was allowed to precipitate at least 1 hr before centrifugation at 12,000 *g* for 15 min.

### *Electrophoresis*

Electrophoresis of the RNA and the radioactivity determinations were performed as reported earlier (6).

### *Radioisotopes*

L-(methyl-<sup>3</sup>H)-methionine (3.3 Ci/mm), uridine-5-<sup>3</sup>H (20 Ci/mm), <sup>3</sup>H-<sup>32</sup>PO<sub>4</sub> carrier-free, and S-adenosyl-methionine-methyl-<sup>3</sup>H (1.1 Ci/mm) were purchased from New England Nuclear, Boston, Mass.

The method of Mowshowitz (5) was used for the *in vitro* processing of pre-tRNA to tRNA. The reaction volume was 3.0 ml and consisted of the following: cytoplasmic extract in RSB, 1.8 ml; Tris, 0.5 M pH 9.0, 0.5 ml, ammonium acetate, 4.0 M, 0.2 ml, dithiothreitol, 2.5 mg/ml, 0.1 ml, pre-tRNA (varying amounts) 0.2 ml, and S-adenosyl-methionine-methyl-<sup>3</sup>H 50 μCi/ml. The incubations were performed at 37°C for the time specified in the results.

### *In Vitro Conversion of Pre-tRNA to tRNA*

The method of Mowshowitz (5) was used for the *in vitro* processing of pre-tRNA to tRNA. The reaction volume was 3.0 ml and consisted of the following: cytoplasmic extract in RSB, 1.8 ml; Tris, 0.5 M pH 9.0, 0.5 ml, ammonium acetate, 4.0 M, 0.2 ml; dithiothreitol, 2.5 mg/ml, 0.1 ml; pre-tRNA (varying amounts) 0.2 ml, and S-adenosyl-methionine-methyl-<sup>3</sup>H 50 μCi/ml. The incubations were performed at 37°C for the time specified in the results.

### *Cell Fractionation*

Nuclear and cytoplasmic fractions were prepared by the method of Penman (7). In brief, the cells after suitable labeling were harvested by centrifugation in the International PR2 (International Equipment Co., Needham Heights, Mass.) for 10 min at 700–1000 *g*. They were then washed and resuspended in cold RSB for 10 min before being ruptured

with a Dounce homogenizer. The nuclei were centrifuged for 2 min at 2000 *g* and resuspended in 2 ml RSB to which 0.2 ml of a 2:1 mixture of 10% Tweens 40 and 10% desoxycorticosterone was added. The nuclei were further processed with additional detergent treatment if clean nuclei were desired. The supernatant from the centrifugation to sediment nuclei was recentrifuged for further separation of nuclei before use as cytoplasmic extract. After the second centrifugation no nuclei as such were detected in Giemsa-stained smears of the supernatant fraction.

## RESULTS

### *Synthesis and Methylation of 4S and 5S RNA at Reduced Temperatures*

The synthesis and methylation of 4S RNA and the synthesis of 5S RNA at reduced temperatures were measured by labeling cells at various temperatures with <sup>32</sup>PO<sub>4</sub> and L-(methyl-<sup>3</sup>H)-methionine. Unlabeled adenosine (10<sup>-6</sup> M) and guanosine (10<sup>-6</sup> M) were added to reduce the incorporation of the methyl label into purines. Labeling was performed at 37°, 33°, 26°, 20°, and 15°C for 4 hr, after which electrophoresis of the RNA was performed in 10% acrylamide gels. The synthesis or accumulation of 4S and 5S RNA was reduced as the temperature was lowered (Fig. 1). As previously reported for 45S rRNA (6), methylation of 4S RNA appeared to be less sensitive to temperature reduction than the synthesis of 4S RNA since the ratio of <sup>32</sup>PO<sub>4</sub>:<sup>3</sup>H does not increase and in fact decreased as the temperature was reduced (Table I). Furthermore, at 15°C when little synthesis of 4S RNA was detectable as measured by <sup>32</sup>PO<sub>4</sub> incorporation, <sup>3</sup>H counts appeared in the 4S region of the gel, suggesting that methylation was still occurring.

As the temperature was reduced there was an increase in the proportion of labeled RNA migrating between the 4S and 5S RNA bands (Fig. 1). Material migrating in a similar manner after a short pulse of radioactive label at 37°C has been shown to have the characteristics of a precursor to 4S RNA (2, 3).

### *Precursor to tRNA Formed at 27°C*

During the conversion of pre-tRNA to tRNA there is a reduction in the nucleotide content of the RNA, increased methylation, and further modification of pre-existing nucleotides (3–5, 8). The possibility that the RNA formed at reduced temperatures migrating between 4S and 5S RNA was a precursor to tRNA was explored. Cells were

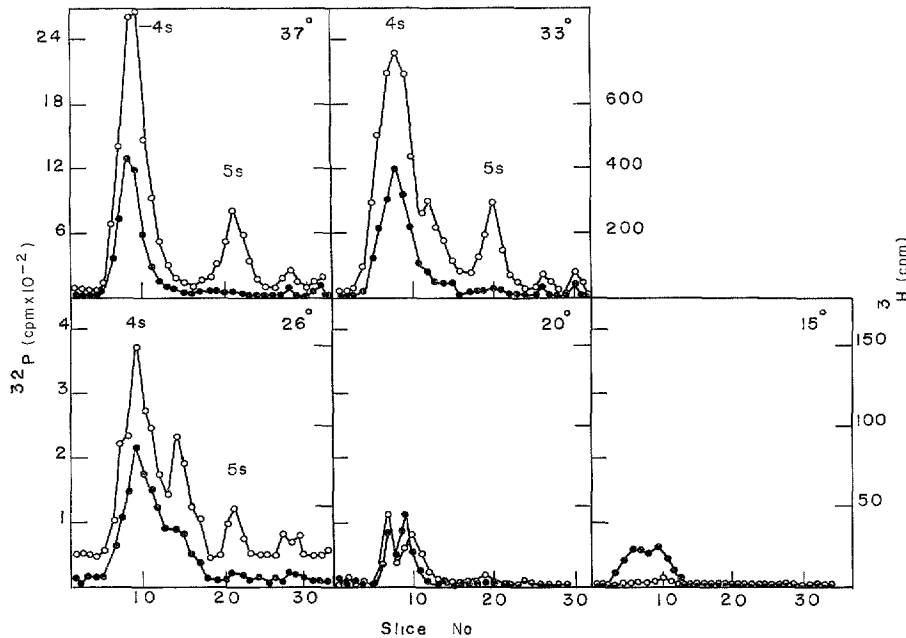


FIGURE 1 Synthesis and methylation of 4S RNA at reduced temperatures.  $5 \times 10^6$  cells were resuspended in 35 ml methionine and phosphate-free medium. L-(methyl- $^3\text{H}$ )-methionine ( $50 \mu\text{Ci/ml}$ ) and  $^{32}\text{PO}_4$  ( $200 \mu\text{Ci/ml}$ ) were added and equal numbers of cells were incubated at  $37^\circ$ ,  $33^\circ$ ,  $26^\circ$ ,  $20^\circ$ , and  $15^\circ$  for 4 hr. The RNA was extracted and electrophoresis was performed on 10% acrylamide gels for 10 hr. Radioactivity:  $\circ$ ,  $^{32}\text{PO}_4$ ;  $\bullet$ ,  $^3\text{H}$ .

labeled with  $^{32}\text{PO}_4$  for 3 hr at  $27^\circ\text{C}$  to allow accumulation of labeled precursor-like material. The culture was then divided into three samples (a) The first was incubated an additional 40 min at  $27^\circ\text{C}$  (Fig. 2 a) and the RNA was isolated by phenol extraction. The gels from this treatment serve to demonstrate the shoulder of material migrating between the 4S and 5S peaks on continued incubation at  $27^\circ\text{C}$  (b) In the second (Fig. 2 b), further RNA synthesis was prevented by actinomycin D ( $10 \mu\text{g/ml}$ ) and incubation continued for 40 min at  $37^\circ\text{C}$  to observe the possible precursor-product relationship of the RNA formed at  $27^\circ\text{C}$ . The methyl-acceptor capacity of the RNA formed at  $27^\circ\text{C}$  was assessed at the same time by incorporation of L-(methyl- $^3\text{H}$ )-methionine in the incubation medium.

The results of the above experiments (Figs 2 a and b) are interpreted to mean that RNA migrating between the 4S and 5S regions of the gel accumulates at  $27^\circ\text{C}$ . On further incubation at  $37^\circ\text{C}$ , some of the formed RNA can be methylated, and the methylated product after 40 min of incubation now migrates principally in the 4S region.

Direct methylation of presumptive precursor to tRNA was attempted by preparing a cytoplasmic extract from the third part of the culture. S-adenosyl-methionine-methyl- $^3\text{H}$  was added and the mixture was incubated 40 min at  $37^\circ\text{C}$  before phenol extraction of the RNA. Electrophoresis of

TABLE I  
Accumulation of 4S and 5S RNA at Reduced Temperatures

Temp	$^{32}\text{P}/^3\text{H}$	5S% of $37^\circ\text{C}$ control	4S% of $37^\circ\text{C}$ control
$37^\circ\text{C}$	8.28	100	100
$33^\circ\text{C}$	7.80	95	102
$26^\circ\text{C}$	4.72	10	17
$20^\circ\text{C}$	2.58	1	3

$^{32}\text{PO}_4$  and the methionine- $^3\text{H}$  cpm in 4S and 5S RNA of Fig. 1 were summed for each temperature, and the ratio of  $^{32}\text{PO}_4/^3\text{H}$  was determined for 4S RNA. The  $^{32}\text{PO}_4$  cpm in 4S and 5S RNA were further expressed for each temperature as the percentage of the  $37^\circ\text{C}$  control.

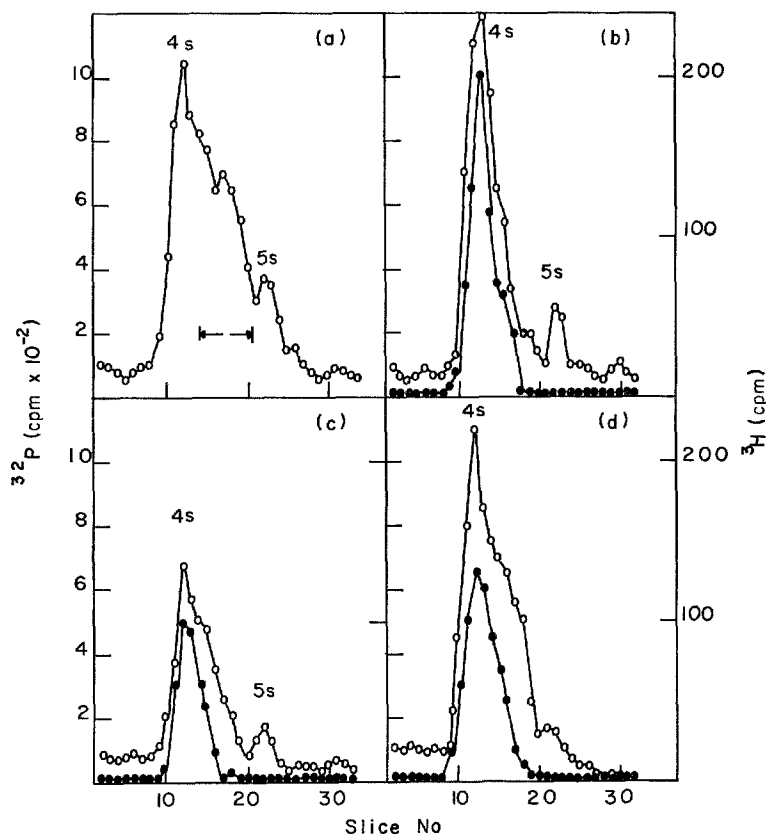


FIGURE 2 Production of pre-tRNA at reduced temperatures. (a)  $1 \times 10^7$  cells were resuspended in 10 ml of phosphate-free medium containing  $^{32}\text{PO}_4$  (100  $\mu\text{Ci/ml}$ ). The cells were incubated for 3 hr, 40 min at  $27^\circ\text{C}$  and the RNA was phenol extracted. Electrophoresis of the RNA was performed on 10% acrylamide gels for 11 hr. Radioactivity:  $\circ$ ,  $^{32}\text{PO}_4$ . (b)  $1 \times 10^7$  cells were incubated at  $27^\circ\text{C}$  for 3 hr in 10 ml phosphate and methionine-free medium containing  $^{32}\text{PO}_4$  (100  $\mu\text{Ci/ml}$ ). Actinomycin D (10  $\mu\text{g/ml}$ ) and L-(methyl- $^3\text{H}$ )-methionine (150  $\mu\text{Ci/ml}$ ) were added and incubation continued for 40 min at  $37^\circ\text{C}$ . Electrophoresis of the extracted RNA was performed on 10% acrylamide gels for 11 hr. Radioactivity:  $\circ$ ,  $^{32}\text{PO}_4$ ;  $\bullet$ ,  $^3\text{H}$ . (c)  $1 \times 10^7$  cells were labeled for 3 hr at  $27^\circ\text{C}$  in phosphate-free medium containing  $^{32}\text{PO}_4$  (100  $\mu\text{Ci/ml}$ ). A 2.2 ml cytoplasmic extract was formed from these cells added to the *in vitro* process mixture as described in Materials and Methods and incubated for 40 min at  $37^\circ\text{C}$ . The RNA was extracted and electrophoresis was conducted on 10% gels for 11 hr. Radioactivity:  $\circ$ ,  $^{32}\text{PO}_4$ ;  $\bullet$ ,  $^3\text{H}$ . (d) Preparative electrophoresis of the RNA from Fig. 2 a was performed on three 7.5% acrylamide gels for 11 hr. The gels were sliced into 1 mm sections and the RNA in the pre-tRNA region of the gels was extracted by shaking each slice 24 hr at  $4^\circ\text{C}$  in 1 ml buffer by the addition of 2 vol of cold ethanol. The purified RNA was added to 1.8 ml of a cytoplasmic extract prepared from  $2 \times 10^6$  cells which had been incubated for 30 min in the presence of actinomycin D (10  $\mu\text{g/ml}$ ). *In vitro* processing and methylation of the pre-tRNA was performed as in part c. The RNA was extracted and analyzed after electrophoresis on 10% acrylamide gels for 11 hr.

the RNA (Fig. 2 c) demonstrated the methylation of RNA migrating principally in the 4S region. The shoulder (Fig. 2 c compared to Fig. 2 a) between the 4S and 5S RNA indicates only partial loss of the intermediate material.

A refinement of the direct methylation achieved above was attempted with a fraction of the RNA designated as the control (Fig. 2 a). Preparative electrophoresis was performed on three 7.5% acrylamide gels. The gels were sliced into 1 mm sections,

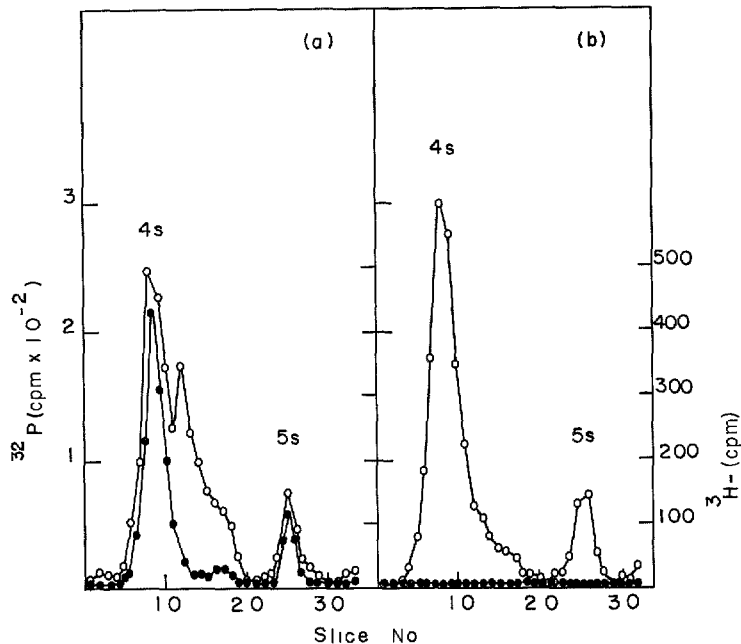


FIGURE 3 Control gels for the in vitro conversion of pre-tRNA into tRNA. (a) RNA from Fig 2 d was mixed with marker RNA from cells labeled at 37°C for 24 hr in the presence of 15  $\mu\text{Ci}/\text{ml}$  uridine- $^3\text{H}$ . Radioactivity:  $\circ$ ,  $^{32}\text{PO}_4$ ;  $\bullet$ ,  $^3\text{H}$ . (b) A culture of HeLa cells ( $10^8$  cells) was resuspended in 25 ml phosphate-free medium containing  $^{32}\text{PO}_4$  (100  $\mu\text{Ci}/\text{ml}$ ) and incubated at 37°C for 6 hr. Actinomycin D (10  $\mu\text{g}/\text{ml}$ ) was added and the incubation continued for an additional 2 hr. The RNA was isolated and added to an in vitro methylase assay as in Fig 2. After 40 min at 37°C the RNA was extracted and electrophoresis performed on 10% acrylamide gels for 11 hr. Radioactivity:  $\circ$ ,  $^{32}\text{PO}_4$ ;  $\bullet$ ,  $^3\text{H}$ .

placed into 1 ml of electrophoresis buffer, and the radioactivity was determined. The slices in the region of the proposed pre-tRNA (see middle of curve, section between vertical bars, Fig 2 a) were extracted overnight in electrophoresis buffer with shaking at 4°C. The RNA was precipitated from the buffer with 2 vol of ethanol. The precipitated RNA was resuspended in RSB and added to a cytoplasmic extract prepared from unlabeled HeLa cells which had been incubated 30 min at 37°C in the presence of actinomycin D (10  $\mu\text{g}/\text{ml}$ ) to allow conversion of residual pre-tRNA to tRNA. *S*-adenosyl-L-methionine-methyl- $^3\text{H}$  was added and the mixture was incubated at 37°C for 40 min. As seen in Fig 2 d, the labeled RNA now migrates more rapidly than upon initial isolation and there is methyl label associated with the 4S region of the gel.

A duplicate sample of the above RNA (Fig 2 d) was electrophoresed with marker RNA from HeLa cells labeled for 24 hr at 37°C with uridine- $^3\text{H}$  (Fig. 3 a). The electrophoresis pattern shows a

homogeneous peak of RNA- $^3\text{H}$  in the 4S region which represents both the long-labeled HeLa RNA and the in vitro methyl-labeled RNA from the fraction represented in Fig. 2 d. The  $^{32}\text{P}$  pattern of Fig. 3 a demonstrates that a portion of the pre-tRNA fraction remained unmethylated and uncleaved during the methylation procedure.

To rule out spurious methylation as a source of the reaction products, 4S RNA formed during long-term (6 hr) incubation at 37°C (Fig 3 b) followed by 2 hr in actinomycin D (10  $\mu\text{g}/\text{ml}$ ) was subjected to methylation in a cytoplasmic extract. No incorporation of methyl- $^3\text{H}$  groups from *S*-adenosyl-L-methionine-methyl- $^3\text{H}$  could be demonstrated with such material (Fig 3 b).

#### *Distribution of Pre-tRNA between the Nucleus and Cytoplasm at 24°C*

It has been suggested that the conversion of pre-tRNA to tRNA occurs within the cytoplasm (4, 5). The increased accumulation of pre-tRNA at re-

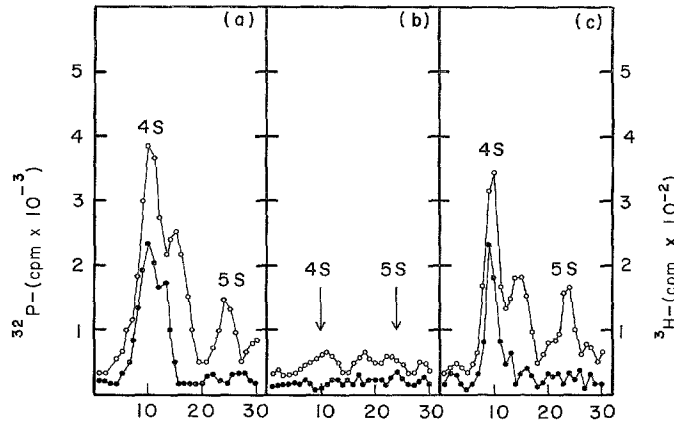


FIGURE 4 Distribution of pre-tRNA between nucleus and cytoplasm.  $1 \times 10^7$  cells were labeled for 3 hr at  $24^\circ\text{C}$  with  $^{32}\text{PO}_4$  ( $100 \mu\text{Ci/ml}$ ) and L-(methyl- $^3\text{H}$ )-methionine ( $50 \mu\text{Ci/ml}$ ). Total cellular RNA was extracted from one-half the cells, and the second was separated into nuclear and cytoplasmic fractions (7). The RNA was isolated by phenol extraction and separated by electrophoresis on 10% acrylamide gels for 10 hr. (a) Cytoplasmic; (b) nuclear; (c) whole cell extracts. Radioactivity.  $\circ$ ,  $^{32}\text{PO}_4$ ;  $\bullet$ ,  $^3\text{H}$ . Approximately equivalent total counts were applied to each gel.

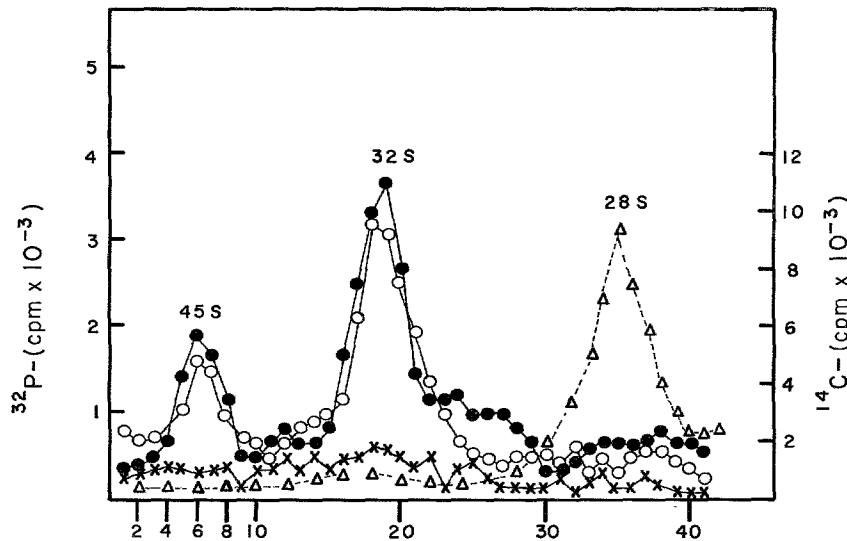


FIGURE 5 Samples of the extracts prepared for pre-tRNA and tRNA detection in Fig 4 were run on 2.6% acrylamide gels to separate 45S and 32S RNA. The whole cell, nuclear, and cytoplasmic distribution of 45S and 32S RNA is shown. Equivalent total counts were applied to each gel.  $^{32}\text{P}$  counts only are recorded. Marker RNA and the cytoplasmic fraction were run on the same gel.  $\bullet$ , nuclear fraction;  $\circ$ , whole cell;  $\times$ , cytoplasm;  $\triangle$ , 28S adenosine- $^{14}\text{C}$  marker 28S RNA from 48 hr pulse of HeLa cells.

duced temperatures was determined by first labeling cells with  $^{32}\text{PO}_4$  and L-(methyl- $^3\text{H}$ )-methionine for 3 hr at  $24^\circ\text{C}$ . The culture was then divided into two samples. Total cellular RNA was extracted from one part, while the second half of the culture was separated into nuclear and cytoplasmic frac-

tions. The RNA was isolated and separated by electrophoresis on 10% acrylamide gels. At  $24^\circ\text{C}$  (Fig 4) all the  $^3\text{H}$  label and most of the  $^{32}\text{PO}_4$ -labeled pre-tRNA was found in the cytoplasm, suggesting that the transport of pre-tRNA from the nucleus is not impaired at reduced tempera-

tures. The absence of significant contamination of 45S and 32S RNA in the cytoplasmic fractions (Fig. 5) of the cells was taken as evidence for integrity of the nuclei during the separation. Admittedly, the retention of the pre-tRNA as a much smaller species could vary from that of the larger rRNA. At 24°C there is no labeled 28S or 18S RNA detectable in the cytoplasm either.

#### DISCUSSION

The possible mechanisms accounting for reduced synthesis and processing of 45S RNA at temperatures below 37°C were discussed in the first paper of this series (5). Similar mechanisms may be involved in the synthesis and processing of 4S and 5S rRNA and their precursors. The absence of evidence establishing intimate association between pre-tRNA or 4S RNA and protein, such as is observed in the preribosomal ribonucleo-protein (RNP) particle, minimizes the role of RNA-protein association as a normal protector or stabilizer of the mature or immature 4S or 5S RNA molecule. It appears more likely that the temperature sensitivity observed is closely associated with transcription.

A comparison of the synthesis of 4S and 5S RNA as a function of temperature does not permit any distinction between polymerases or other potential common elements of their synthesis and control. On the other hand, ribosomal RNA synthesis responds quite differently to reduced temperature. From other evidence (9), it is virtually certain that the site of synthesis of 4S and 5S RNA is different from that of ribosomal RNA and that transcription is performed by polymerases distinguishable from the rRNA polymerase.

Methylation of tRNA is less sensitive to reduced temperature than its synthesis as demonstrated by the decreased <sup>32</sup>P:methyl-<sup>3</sup>H ratio. The relatively greater degree of methylation obtained at 15°C after a shift from 37°C is probably attributable to unmethylated pre-tRNA made at 37°C, then methylated at 15°C.

As the temperature is lowered, there is a progressive increase in the proportion of pre-tRNA to tRNA. Identification of the RNA migrating between 4S and 5S RNA as pre-tRNA is based upon two criteria. The first is the ability to chase this intermediate material formed at reduced temperatures into RNA migrating with tRNA after

incubation at 37°C. The second criterion is based on the observation that pre-tRNA is undermethylated and its size reduction is accompanied by increased methylation. Methylation of the heterogeneous material proceeded *in vivo* during the actinomycin D chase of the precursor material. Similarly, methylation was demonstrated *in vitro* with *S*-adenosyl-methionine-methyl-<sup>3</sup>H, using either a cytoplasmic extract prepared from HeLa cells incubated at reduced temperature or by using purified pre-tRNA made at reduced temperature with methylase supplied in a cytoplasmic extract prepared from HeLa cells devoid of pre-tRNA.

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