Behar, D., Czapski, G., Rabani, J., Dorfman, L. M., and Schwartz, L. M. (1970), *J. Phys. Chem. 74,* 3209.
Bradbeer, J. W., and Racker, E. (1961), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 20,* 88.
Chollet, R., and Ogren, W. L. (1975), *Bot. Rev. 41,* 137.
Egneus, H., Heber, U., Mattiesen, U., and Kirk, M. (1975), *Biochim. Biophys. Acta 408,* 252.
Eickenbusch, J. D., Scheibe, R., and Beck, E. (1975), *Z. Pflanzenphysiol. 75,* 375.
Elstner, E. F., and Kramer, R. (1973), *Biochim. Biophys. Acta 314,* 340.
Epel, B. L., and Neumann, J. (1973), *Biochim. Biophys. Acta 325,* 520.
Fridovich, I. (1975), *Annu. Rev. Biochem. 44,* 147.
Glidewell, S. M., and Raven, J. A. (1976), *J. Exp. Bot. 27,* 200.
Goldsworthy, A. (1970), *Bot. Rev. 36,* 321.
Greenstock, C. L., and Miller, R. W. (1975), *Biochim. Biophys. Acta 396,* 11.
Han, T. W., and Eley, J. H. (1973), *Plant Cell Physiol. 14,* 285.
Hewitt, J., and Morris, J. G. (1975), *FEBS Lett. 50,* 315.
Hirata, F., and Hayaishi, O. (1975), *J. Biol. Chem. 250,* 5960.
Jackson, W. A., and Volk, R. J. (1970), *Annu. Rev. Plant Physiol. 21,* 385.
Lorimer, G. H., Andrews, T. J., and Tolbert, N. E. (1973), *Biochemistry 12,* 18.
Lorimer, G. H., Osmond, C. B., and Akazawa, T. (1976), *Plant Physiol. (Suppl.) 57,* 32.
Massey, V., Strickland, S., Mayhew, S. G., Howell, L. G., Engel, P. C., Matthews, R. G., Schuman, M., and Sullivan, P. A. (1969), *Biochem. Biophys. Res. Commun. 36,* 891.
Misra, H. P., and Fridovich, I. (1971), *J. Biol. Chem. 246,* 6886.
Ort, D. R., and Izawa, S. (1974), *Plant Physiol. 53,* 370.
Radmer, R. J., and Kok, B. (1976), *Plant Physiol. 58,* 336.
Shain, Y., and Gibbs, M. (1971), *Plant Physiol. 48,* 325.
Tolbert, N. E. (1973), *Current Top. Cell. Regul. 7,* 21.
Zelitch, I. (1975), *Annu. Rev. Biochem. 44,* 123.

Histone Messenger RNA from HeLa Cells: Evidence for Modified 5′ Termini†

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ABSTRACT: The distribution of [^{3}H]methyl radioactivity in cytoplasmic histone mRNA, isolated during the DNA synthetic (S) phase of the HeLa S_3 cell cycle, has been investigated. Evidence is presented that approximately 30% of the radioactivity is in m^7GpppX^mpYp oligonucleotides, where X^m represents 2′-*O*-methylated adenosine and guanosine with a molar ratio of 4:1, respectively. The remainder of the radioactivity is present as $m^7GpppX^mpY^mpZp$ oligonucleotides, where X^m is again 2′-*O*-methylated adenosine and guanosine (4:1) and where y^m represents 2′-*O*-methylated adenosine, guanosine, cytidine, and uridine with ratios of 2:1:1:1, respectively. While 48.6% of the [^{3}H]methyl radioactivity was present as N^6-methyladenosine in poly(adenylic acid)-terminated mRNA from S-phase cells, no evidence for N^6-methyladenosine was found in histone mRNA. It thus appears that histone mRNA which lacks 3′-terminal poly(adenylic acid) sequences and functions on cytoplasmic polyribosomes during a limited portion of the cell cycle is capped but lacks internal-modified nucleosides.

Inverted dinucleotides have recently been found at the 5′ termini of a variety of poly(A)[1]-containing mRNA species. These modified termini have the general structure $m^7G5'ppp5'X^m$(cap 1) and $m^7G5'ppp5'X^mpY^m$(cap 2). While cap 1 structures, where X^m is limited to 2′-*O*-methylated adenosine or guanosine, are the predominant termini synthesized by viral cores in vitro (Abraham et al., 1975; Furuichi and Miura, 1975; Furuichi et al., 1975a; Urushibara et al., 1975; Wei and Moss, 1975), both cap 1 and cap 2 structures have been found in poly(A)-terminated mRNA isolated from virus-infected and -uninfected mammalian cells (Adams and Cory, 1975; Cory and Adams, 1975; Furuichi et al., 1975b; Lavi and Shatkin, 1975; McGuire et al., 1976; Rose, 1975; Shatkin, personal communication; Wei et al., 1975a). One example of the heterogeneity of modified sequences found at the 5′ termini of cellular mRNA has appeared recently. Cory and Adams (1975) present evidence that poly(A)-containing mRNA from mouse myeloma cells contains a minimum of 27 5′-terminal sequences, with any of the four 2′-*O*-methylated nucleosides present at positions X^m and Y^m. The 5′ termini of low-molecular-weight nuclear RNAs also contain methylated nucleosides (Ro-Choi et al., 1974, 1975). In addition to modified nucleosides at the 5′ termini, poly(A)-containing cellular mRNA (Adams and Cory, 1975; Cory and Adams, 1975; Furuichi et al., 1975b; Desrosiers et al., 1974; Perry et al., 1975) and mRNA isolated from cells infected by SV40 virus (Lavi and Shatkin, 1975) and adenovirus (Shatkin, personal communication) contain internal N^6-methyladenosine residues, which are probably located near the 5′ end.

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[1] Abbreviations used are: poly(A), poly(adenylic acid); Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl.

While there is abundant evidence that the m^7G residue, which is common to all capped termini, is required for ribosome binding of mRNA and its efficient translation in vitro (Muthukrishnan et al., 1975; Both et al., 1975; Rao et al., 1975), recent results indicate that polio virus mRNA isolated from infected cells does not contain the m^7G residue (Hewlett et al., 1976; Nomoto et al., 1976). Although to date this is the only poly(A)-terminated mRNA isolated from mammalian cells without a cap, it appears that modification of the 5′ terminus of at least one mRNA species is not required for its efficient translation in vivo.

Thus far, no evidence has appeared concerning the 5′-terminal structures of RNA molecules with a known messenger function which lack poly(A) at their 3′ termini. Since both poly(A) and caps have been thought to extend the half-life of cytoplasmic mRNA by preventing degradation by termini-specific exonucleases, it would be of particular interest to examine the nature of the 5′ termini of an mRNA species which has a short half-life and lacks poly(A). We have chosen to investigate the 5′-terminal sequence of histone mRNA from HeLa S_3 cells, since it lacks 3′-terminal poly(A) sequences and functions on cytoplasmic polyribosomes with a relatively short half-life during a limited portion of the cell cycle (Borun et al., 1967; Stein, J. et al., 1975). By displaying the digestion products of [^{3}H]methyl-labeled histone mRNA on DEAE-cellulose paper following ionophoresis and subsequently characterizing these labeled oligonucleotides by determining their susceptibility to specific enzymes, it was possible to analyze the limited amount of radioactivity in the 5′ termini of histone mRNA. Evidence is presented that the pool of histone mRNA is capped but lacks any internal-modified nucleosides.

Experimental Procedures

Maintenance of Cells. HeLa S_3 cells were maintained at 37 °C in suspension culture in Eagle's medium (Eagle, 1959) supplemented with 7% calf serum and 2 mM glutamine.

Conditions for Radioactive Labeling and Isolation of mRNA from S-Phase Cells. HeLa S_3 cells were synchronized by treatment with two cycles of 2 mM thymidine (Stein and Borun, 1972). To obtain [^{3}H]methyl-labeled mRNA from S-phase cells, 1×10^9 total cells were grown in the presence of 30 μM adenosine for 1 h following release from the second exposure to 2 mM thymidine. The cells were then harvested and resuspended at a concentration of 2×10^6 cells/mL in medium lacking methionine and containing 30 μM adenosine, 20 μM guanosine, 20 mM formate, 5% dialyzed calf serum, and 20 μCi/mL [*methyl*-^{3}H]methionine (specific activity 3.7 Ci/mM, Schwarz/Mann, Orangeburg, N.Y.). After 5 min incubation unlabeled methionine was added to a final concentration of 20 μM.

Cells were harvested after 3.5 h of incubation at which time greater than 98% of the cells are undergoing DNA synthesis (Stein and Borun, 1972). The cells were washed with Earle's balanced salt solution and lysed in 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris (pH 7.4). After pelleting nuclei at 1500*g* for 5 min and mitochondria at 27 000*g* for 15 min, polyribosomes were pelleted from the postmitochondrial supernatant fraction by centrifugation at 100 000*g* for 90 min. Polyribosomes were suspended in 0.1 M NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate, 10 mM sodium acetate (pH 5.4), and the RNA was extracted twice at room temperature with 1 volume of phenol plus 1 volume of chloroform–isoamyl alcohol (24:1, v/v), followed by two extractions with 1 volume of chloroform–isoamyl alcohol. RNA was precipitated from the aqueous phase with 2 volumes of ethanol and was fractionated on 5–30% sodium dodecyl sulfate–sucrose gradients. RNA sedimenting in the 4S–18S region was pooled, concentrated by ethanol precipitation, and refractionated on a second 5–30% sodium dodecyl sulfate–sucrose gradient. RNA sedimenting in the 7S–11S region was pooled, concentrated with ethanol, and applied to a column of oligo(dT)-cellulose. Radioactive RNA eluting with application buffer (0.5 M KCl, 10 mM Tris (pH 7.5)) in the void volume was recycled through a second column of oligo(dT)-cellulose. Radioactive RNA which again eluted in the void volume was desalted on a column of Sephadex G-50 and concentrated by ethanol precipitation. We have previously shown (Thrall et al., 1974), in agreement with the work of others (Gallwitz and Breindl, 1972; Jacobs-Lorena et al., 1972), that such S-phase 7-11S RNA preparations direct the synthesis of histones in in vitro protein synthesizing systems. When the in vitro translated polypeptides are analyzed electrophoretically in a sodium dodecyl sulfate–polyacrylamide gel system where fractionation is principally by molecular weight (Laemmli, 1970) and in a acetic acid–urea–polyacrylamide gel system where fractionation is by charge as well as molecular weight (Panymin and Chalkley, 1969), detectable levels of proteins other than histones are not observed (Thrall et al., 1977). Additional criteria for purity of the histone mRNA preparation are derived from hybridization analysis with a ^{3}H-labeled DNA complementary to histone mRNAs prepared by the same procedure from S-phase cells not labeled with [*methyl*-^{3}H]methionine. The kinetics of the histone mRNA–histone cDNA hybridization reaction indicate a genetic complexity (3000 nucleotides) consistent with the presence of mRNAs for the five histone polypeptides. While polysomal RNA from S-phase HeLa cells hybridizes with histone cDNA, no significant level of hybrid formation between histone cDNA and G_1 polysomal RNA or polysomal RNA from S-phase cells treated with cytosine arabinoside is observed. These hybridization results indicate the absence of ribosomal RNA and tRNA sequences in the histone mRNA preparation and together with the translation data discussed above suggest the absence of other RNA species in the histone mRNA preparations. Other properties of the mRNA preparation have been reported (Stein, J. et al., 1975; Stein, G. et al., 1975; Park et al., 1976).

Radioactive poly(A)-containing RNA eluting from the oligo(dT)-cellulose column in 10 mM Tris (pH 7.5) was also recycled through a second column of oligo(dT)-cellulose after addition of KCl to a final concentration of 0.5 M. Poly(A)-containing mRNA isolated from the second column was desalted as above and concentrated by ethanol precipitation.

Analyses of Modified Nucleosides. [^{3}H]Methyl-labeled RNA was digested for 3 h at 37 °C in 15 μL of a solution containing 50 μg/mL pancreatic ribonuclease A (Worthington Biochemical Corp., Freehold, N.J.), 50 μg/mL ribonuclease T_1 (Calbiochem, La Jolla, Calif.), 2 units/mL ribonuclease T_2 (Calbiochem), and 50 mM ammonium acetate (pH 4.5). The hydrolysate was applied directly to a sheet of DEAE-cellulose paper 1-m long and ionophoresis was carried out in 50 mM sodium citrate buffer (pH 4.0) (Konrad et al., 1972) at 35 V/cm until the xylene cyanol dye migrated 30 cm from the origin. Radioactive products were located by counting 1-cm strips of dried DEAE paper in toluene-based scintillation fluid.

Digestion products were eluted from the DEAE paper with triethylamine carbonate (Barrell, 1971) and treated with *Penicillium* nuclease (Yamasa Shoya Co. Ltd., Tokyo, Japan), alkaline phosphatase (BAPF, Worthington Biochemical Corp.), and nucleotide pyrophosphatase (type III, Sigma

Chemical Co., St. Louis, Mo.), as described by Furuichi et al. (1975b). Hydrolysates were analyzed by electrophoresis on Whatman 3MM paper at 2600 V for 40 min in pyridine–acetate buffer (pH 3.5). Products were located as described above, eluted with water, and analyzed further by descending paper chromatography. Chromatograms were developed in either isobutyric acid–0.5 N NH_4OH (10:6, v/v) (system A); or 2-propanol–concentrated HCl–H_2O (68:17.6:14.4, v/v) (system B). After chromatography, radioactive products were located by counting 1-cm strips in 1-mL of water and 10 mL of Aquasol (New England Nuclear Corp., Boston, Mass.). Authentic marker compounds, which included 5′-adenosine monophosphate (pA), 5′-adenosine tetraphosphate (ppppA) (Sigma Chemical Co.), N^6-methyladenosine (N^6-mA), and 2′-*O*-methylated nucleosides (A^m, G^m, C^m, U^m) (P. L. Biochemicals, Inc., Milwaukee, Wis.), were located under ultraviolet light.

Results

Isolation of the 5′ Termini of S-Phase HeLa Cell mRNA and Characterization of the Internal-Modified Nucleoside from Poly(A)-Containing mRNA. HeLa cells exposed to [*methyl*-^{3}H]methionine during S-phase of the cell cycle contained radioactivity in both poly(A)-terminated mRNA and histone mRNA with approximately 1.2 and 0.6% of the total cytoplasmic ^{3}H radioactivity in each fraction, respectively. After digestion with a mixture of T_1, T_2, and pancreatic A ribonucleases, both mRNA pools yielded radioactive products that migrated during ionophoresis as two peaks on DEAE paper with mobilities slower than a marker with a net charge of −4 (Figure 1, panels a and b). Previous results have established that it is to this region of the DEAE paper that the 5′ terminus of reovirus (m^7GpppG^mpCp) migrates under the ionophoretic conditions used here (McGuire et al., 1976). In addition to the two broad peaks in the 5′-terminal region, digests of poly(A)-containing mRNA also contained radioactivity that migrated as a single sharp peak to the mononucleotide region of the paper (Figure 1, panel b). This radioactivity, which represented 48.6% of the total recovered, was eluted from fractions 52–55 and was characterized further by electrophoresis on 3MM paper. Most of the eluted radioactivity migrated with marker pA (Figure 1, panel c). After elution from the paper, this sample was digested with alkaline phosphatase and was reanalyzed by electrophoresis. Figure 1, panel d, illustrates that after removal of the phosphate, the pA-like material now migrated with the A^m and N^6mA markers. The identity of this modified nucleoside was subsequently confirmed as N^6-methyladenosine by descending paper chromatography (Figure 1, panel e). This same internal-modified nucleoside has been found in poly(A)-terminated mRNA isolated from both virus-infected and -uninfected interphase cells (Adams and Cory, 1975; Cory and Adams, 1975; Furuichi et al., 1975b; Lavi and Shatkin, 1975; Shatkin, personal communication). In contrast to poly(A)-terminated mRNA, the results illustrated in panel a of Figure 1 provide evidence that histone mRNA, which lacks poly(A), does not contain any internal N^6mA. Histone mRNAs also appear to differ from other nonpoly(A)-terminated RNA species such as ribosomal and transfer RNAs, which contain internal-methylated nucleotides. We were unable to detect any modified mononucleotides in digests of histone mRNA even after eluting the background radioactivity from the mononucleotide region of the DEAE paper with triethylamine carbonate and counting the eluates in 1 mL of water and 10 mL of Aquasol, that is, under conditions which increase the counting efficiency of ^{3}H radioactivity three- to sixfold.

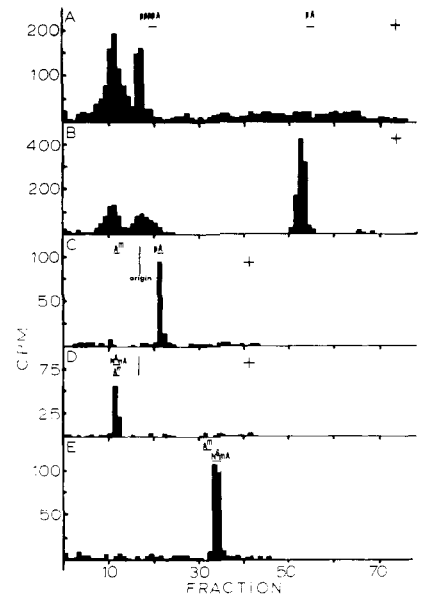

FIGURE 1: Analyses of hydrolytic products released from [^{3}H]radioactive histone mRNA and poly(A)-containing mRNA isolated from S-phase cells. RNA was digested with a mixture of T_1, T_2, and pancreatic A ribonucleases and analyzed by ionophoresis on DEAE-cellulose paper as described under Experimental Procedures. ^{3}H radioactive products were located by counting 1-cm strips in scintillation fluid. Nucleotides of interest were eluted from the paper with triethylamine carbonate, washed, digested with additional enzymes, and analyzed by paper electrophoresis or descending paper chromatography (see Experimental Procedures). (a and b) Distribution of radioactive products after ionophoresis of digests of histone mRNA and poly(A)-terminated mRNA on DEAE paper, respectively. (c and d) Electrophoresis on 3 MM paper of radioactivity eluted from fractions 52–55 of panel b before and after treatment with alkaline phosphatase, respectively. The vertical line at 17 cm represents the origin. (e) Analysis of radioactivity which migrated to fractions 12 and 13 (panel d) by descending paper chromatography in system A.

Characterization of the Modified Nucleosides in Cap 1 Termini of Histone mRNA. The [^{3}H]methyl-labeled oligonucleotides from three digests of histone mRNA, which migrated 17–19 cm from the origin after ionophoresis on DEAE paper (Figure 1, panel a), were eluted, pooled, and characterized further by digestion with additional enzymes. After sequential treatment with *Penicillium* nuclease and alkaline phosphatase, a broad peak containing the major portion of the radioactivity was found to migrate during electrophoresis to the pA region of the 3 MM paper (Figure 2, panel a). The two smaller peaks in fractions (12–13) and (16–17), which contained less than 8 and 5% of the recovered radioactivity, respectively, probably represent some Y^m nucleosides released from contaminating cap 2 structures ($m^7GpppX^mpY^m$) during *Penicillium* nuclease treatment (see Figure 3, panel a).

The nuclease resistant material which migrated toward the anode (fractions 20–24) was eluted, pooled, and digested with nucleotide pyrophosphatase and alkaline phosphatase. This treatment would be expected to cleave and remove the internal phosphates from the modified nucleosides of the cap 1 inverted dinucleotide and release m^7G and X^m. Three peaks were found after paper electrophoresis (Figure 2, panel b). The radioactive product which migrated most rapidly toward the cathode (peak 1) was eluted and subsequently identified as m^7G by descending paper chromatography (Figure 2, panel c). Recently, evidence has appeared that N^6,$O^{2'}$-dimethyladenosine residues have been found in the X^m position of capped termini of

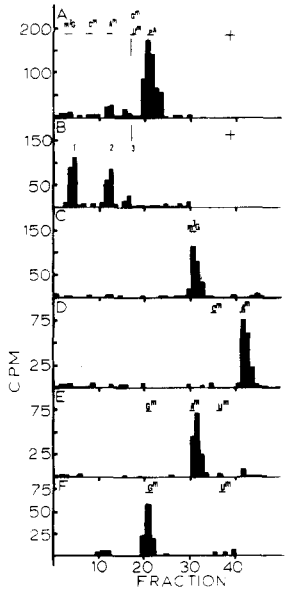

FIGURE 2: Analyses of cap 1 termini from histone mRNA. Radioactivity was eluted from fractions 17-19 after ionophoresis of three digests of histone mRNA (Figure 1, panel a). These ^{3}H-labeled oligonucleotides were pooled, washed, digested sequentially with ribonuclease, and analyzed further by paper electrophoresis and descending paper chromatography (see Experimental Procedures). (a) Distribution of radioactivity, after digestion of pooled eluates from the DEAE-cellulose fractions with *Penicillium* nuclease and alkaline phosphatase, on 3 MM paper following electrophoresis. (b) Material in the pA region of panel a (fractions 20-24) was eluted, treated with nucleotide pyrophosphatase followed by alkaline phosphatase, and analyzed by paper electrophoresis. (c and d) Analysis of radioactivity in peaks 1 and 2 of panel b by descending paper chromatography in system A, respectively. (e and f) Analysis of radioactivity in peaks 2 and 3 of panel b by descending paper chromatography in system B, respectively.

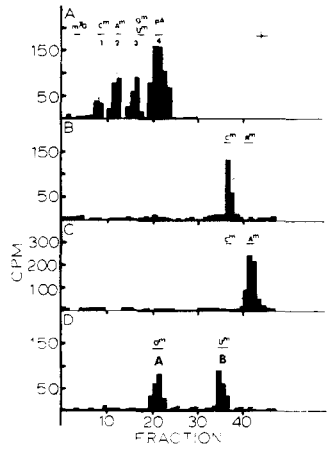

FIGURE 3: Analysis of cap 2 termini from histone mRNA. Radioactivity was eluted from fractions 9-14 after ionophoresis of three digests of histone mRNA, digested with ribonuclease, and analyzed as described in the legend of Figure 2. (a) Distribution of radioactivity after electrophoresis on 3 MM paper following digestion of the pooled eluates with *Penicillium* nuclease and alkaline phosphatase. (b) Analysis of the radioactivity which migrated with marker C^m (peak 1, panel a) by paper chromatography in system A. (c and d) Analysis of radioactivity in peaks 2 and 3 of panel a by paper chromatography in system B, respectively.

poly(A)-containing mRNA (Wei et al., 1975b). For this reason, the radioactive product in peak 2, which migrated with the A^m marker, was eluted and characterized further in two chromatographic systems. In both cases, this digestion product was found to migrate with an R_f identical to marker A^m (Figure 2, panels d and e). Finally, the radioactivity which remained near the origin with the U^m and G^m markers (peak 3) was found to migrate as a homogeneous peak with the G^m marker following paper chromatography (Figure 2, panel f), with no radioactivity above background detected in the U^m region.

TABLE I: Ratio of Radioactivity in Modified Nucleosides of Cap 1 Termini.[a]

Peak	Marker	Fraction no.	cpm	Ratio
1	m^7G	4-5	199	5.4
2	A^m	12-13	144	3.9
3	G^m	16-17	37	1.0

[a] Distribution of radioactivity in peaks 1, 2, and 3 of Figure 2, panel b. The sum of radioactivity in each of the three digestion products separated by electrophoresis on 3 MM paper was determined by counting 1-cm strips in toluene scintillation fluid. The ratio of radioactivity in each was determined by dividing the total cpm in each peak by the amount in peak 3.

In addition to the chromatographic analyses of the X^m residues, the amount of radioactivity in the digestion products illustrated in panel (b) of Figure 2 provides evidence for monomethylated nucleosides at this position. For example, if multimethylated residues were present in the penultimate position, the sum of radioactivity in peaks 2 and 3 would exceed that found in m^7G (peak 1). Table I provides evidence that the total amount of radioactivity in peaks 2 and 3 is approximately equal to that found in peak 1 and suggests a molar ratio of 4:1 for m^7GpppA^m and m^7GpppG^m in cap 1 termini of histone mRNA.

Characterization of the Modified Nucleosides in Cap 2 Termini of Histone mRNA. The [^{3}H]methyl-labeled oligonucleotides from histone mRNA which migrated between 9 and 14 cm from the origin during ionophoresis on DEAE paper (Figure 1, panel a) were eluted, pooled, and digested with *Penicillium* nuclease followed by treatment with alkaline phosphate. This regimen of enzyme digestion should release 3′-terminal nucleosides from cap 2 structures yielding cap 1 and Y^m residues. Four radioactive products were resolved by paper electrophoresis (Figure 3, panel a). Subsequent analyses of peaks 1-3 by descending paper chromatography demonstrated that, while material which was eluted from 1 and 2 migrated as homogeneous peaks with C^m and A^m markers, respectively (Figure 3, panels b and c), the material in 3 was resolved into two peaks (A and B) with equal molar amounts of radioactivity migrating with G^m and U^m markers (Figure 3, panel d). Since we again found no evidence for dimethylated mononucleosides being released from these structures, estimates of the relative amounts of radioactivity in peaks 1-3 from panel (a) of Figure 3 suggest a molar ratio of approximately 2:1:1:1 for A^m, G^m, C^m, and U^m as the Y^m nucleosides in the cap 2 termini of histone mRNA (Table II).

The radioactive product(s) in peak 4 were treated with nucleotide pyrophosphatase followed by alkaline phosphatase and were analyzed by paper electrophoresis (Figure 4, panel a). Three digestion products were found to migrate during paper electrophoresis to positions which were identical to those following similar treatment of cap 1 structures. Furthermore, the relative amount of radioactivity in each peak is similar to digests of cap 1, that is, approximately 5:4:1 (Table III). The products in peaks 1, 2, and 3 were identified again as m^7G, A^m, and G^m, respectively (Figure 4, panels b-e). These results suggest a sequence composition of $m^7GpppX^mpY^m$ at the

TABLE II: Ratio of Radioactivity in Modified Nucleosides of Cap 2 Termini at Position Y^m.[a]

Peak	Marker	Fraction no.	cpm	Ratio
1	C^m	8-9	73	1.0
2	A^m	12-13	162	2.2
3	G^m,U^m	16-17	145	2.0
A	G^m	20-23	205	1.0
B	U^m	34-37	196	1.0

[a] Distribution of radioactivity in peaks 1, 2, and 3 of Figure 3, panel a, and peaks A and B of panel d. The sum of radioactivity and ratio in each peak was determined as described in the legend of Table I. The sum of radioactivity in peaks A and B was determined by counting 1-cm strips in 1 mL of water and 10 mL of Aquasol following descending paper chromatography. The ratio was determined by dividing the total cpm in each peak by the amount in peak B.

TABLE III: Ratio of Radioactivity in Modified Nucleosides of Cap 2 Termini at Position X^m.[a]

Peak	Marker	Fraction no.	cpm	Ratio
1	m^7G	4-5	236	4.5
2	A^m	12-13	195	3.7
3	G^m	16-17	52	1.0

[a] Distribution of radioactivity in peaks 1, 2, and 3 of Figure 4, panel a. The sum of radioactivity and ratio in each peak was determined as described in the legend of Table I.

5′-termini of histone mRNAs, where X^m represents A^m and G^m (4:1) and where Y^m represents A^m, G^m, C^m, and U^m (2:1:1:1).

Discussion

Both cap 1 and cap 2 structures have been found in digests of [^{3}H]methyl-labeled histone mRNAs isolated from HeLa S_3 cells during the DNA synthetic (S) phase of the cell cycle. Approximately 30% of the [^{3}H]oligonucleotides migrate during ionophoresis as m^7GpppX^mpYp, where X^m represents A^m and G^m (4:1). The remainder of the radioactivity migrates in a more heterogeneous fashion with a slower mobility on DEAE paper and appears to have the sequence composition $m^7GpppX^mpY^mpZp$, where X^m is again A^m and G^m (4:1) and where Y^m represents A^m, G^m, C^m, and U^m (2:1:1:1). The common sequence composition of modified nucleosides through the X^m position in these two termini is consistent with recent kinetic data which argue that nuclear cap 1 structures are further modified at the Y position in the cytoplasm, during the biogenesis of mRNA (Perry and Kelley, 1976).

Recent results have demonstrated that the 5′ termini of poly(A)-containing mRNA isolated from interphase HeLa cells are relatively heterogeneous with regard to base composition, with all four 2′-*O*-methylated nucleosides present at position X^m, and G^m, C^m, and U^m present at the Y^m position. While the results from interphase cells differ from those reported here, they are not incompatible. For example, histone mRNA lacks poly(A) and therefore would not have been present in the pool of poly(A)-terminated mRNA analyzed previously. Secondly, histone mRNA, which directs the synthesis of five polypeptides on cytoplasmic polyribosomes during

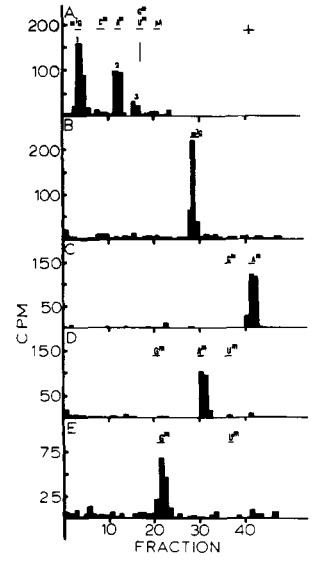

FIGURE 4: Analysis of ^{3}H-labeled oligonucleotide(s) in peak 4 of Figure 3, panel a. Radioactive digestion product(s) were eluted from fractions 20-24, pooled, digested with ribonuclease, and analyzed as described in the legend of Figure 2. (a) Distribution of radioactivity after digestion of pooled peak 4 eluates with nucleotide pyrophosphatase followed by alkaline phosphatase and analysis by paper electrophoresis. (b and c) Analysis of radioactivity in peaks 1 and 2 of panel a by descending paper chromatography in system A, respectively. (d and e) Analysis of radioactivity in peaks 2 and 3 of panel a by descending paper chromatography in system B, respectively.

S phase, would represent only a very small fraction of the total mRNA present in interphase cells and should, therefore, contain a less heterogeneous population of 5′ termini. Finally, by enriching for a small subset of messenger sequences, modified nucleosides may be found which were undetected during examination of a larger messenger pool. For example, results presented here provide evidence for the presence of A^m at the Y^m position of cap 2. While this modified nucleoside was not found at this position in poly(A)-terminated mRNA from interphase HeLa cells, a small amount of $m^7GpppA^mpA^mpAp$ has been found at the 5′ termini of poly(A)-containing mRNA isolated from mouse myeloma cells (Cory and Adams, 1975).

Several laboratories have reported that histone mRNA isolated from the polyribosomes of S-phase HeLa cells is heterogeneous, consisting of mRNAs for the five histone polypeptides (Thrall et al., 1974; Wilson et al., 1974; Borun et al., 1975). If one can make the assumption that each species, as isolated here, is present in equimolar amounts, then the ratio of 4:1 for the A^m and G^m residues in the X^m position would be consistent with the penultimate base being A^m in four histone mRNA species and G^m in one species. This assumes that no dimethylated nucleosides are present at this position. Recent evidence suggests that N^6,$O^{2'}$-dimethyladenosine is present in the X^m position of caps in poly(A)-containing mRNA isolated from HeLa cells, L cells, and adenovirus-infected cells (Wei et al., 1975b). We were unable to resolve dimethylated adenosine residues from A^m marker in digests of cap structures from histone mRNA using two chromatographic systems. Furthermore, data from Tables I and II are consistent with a monomethylated A residue in the X^m position, since the sum of radioactivity in products which migrated with A^m and G^m markers is equivalent to the amount of radioactivity in the m^7G peaks.

In addition to modified 5′ termini, approximately one-third

of the [^{3}H]methylmethionine label was found in N^6-methyladenylic acid as internal-modified mononucleotides which were not immediately adjacent to the capped termini of poly(A)-containing mRNA isolated from interphase HeLa cells (Furuichi et al., 1975b). Results presented here in panels b–e of Figure 1 demonstrate that approximately 48.6% of the total [^{3}H]methyl label in poly(A)-containing mRNA isolated from S-phase cells was also present as N^6-methyladenylic acid. In contrast to this situation with poly(A)-terminated mRNA, we were unable to detect any internal-modified mononucleotides in digests of histone mRNA, even under conditions which increase the counting efficiency of ^{3}H radioactivity by three- to sixfold. Recent results indicate that histone mRNA may not be unique in this regard. For example, globin mRNA, which contains poly(A), was also found to lack any internal N^6-methyladenylic acid residues (Perry and Scherrer, 1975). Furthermore, since 18S ribosomal RNA and transfer RNA contain an average of 1 methylated nucleotide per 48 and 12 residues, respectively (Brown and Attardi, 1965; Staehelin et al., 1968), and digests of histone mRNA lack methylated mononucleotides, the results in panel a of Figure 1 argue that ribosomal RNA and transfer RNA were absent from the pool of 7S–11S nonpoly(A)-containing transcripts analyzed here.

Recently, Furuichi et al. (1975b) have suggested that internal base modifications may be present at recognition sites of cleavage enzymes which process large poly(A)-containing heterogeneous nuclear RNA to smaller cytoplasmic mRNA by conserving those sequences adjacent to poly(A) segments. Since, to date, only purine residues have been found at the 5′ termini of nascent transcripts, the absence of internal base modifications and the presence of only A^m and G^m as the penultimate residues in the 5′ termini of histone mRNA are compatible with a model which would allow the conservation of nascent 5′ termini in nuclear histone transcripts which are the same size as cytoplasmic mRNA molecules. Experiments using DNA complementary to histone mRNA are presently under way to determine whether nuclear sequences can be detected which are larger than cytoplasmic histone mRNA.

Our observations regarding the presence of capped structures in histone messenger RNAs of S-phase HeLa S_3 cells are consistent with a recent report by Surrey and Nemer (1976) which indicates that sea urchin embryo histone messenger RNAs have capped 5′ termini and lack internal-methylated nucleotides.

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References

Abraham, G., Rhodes, D. P., and Banerjee, A. K. (1975), *Cell 5,* 51–58.

Adams, J. M., and Cory, S. (1975), *Nature (London) 255,* 28–33.

Barrell, B. G. (1971), *Proced. Nucleic Acid Res. 2,* 751–779.

Borun, T. W., Gabrielli, F., Ajiro, K., Zweidler, A., and Baglioni, C. (1975), *Cell 4,* 59–67.

Borun, T. W., Robbins, E., and Scharff, M. D. (1967), *Proc. Natl. Acad. Sci. U.S.A. 58,* 1977–1980.

Both, G. W., Banerjee, A. K., and Shatkin, A. J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1189–1193.

Brown, G. M., and Attardi, G. (1965), *Biochem. Biophys. Res. Commun. 20,* 298–302.

Cory, S., and Adams, J. M. (1975), *J. Mol. Biol. 99,* 519–547.

Desrosiers, R., Friderici, K., and Rottman, F. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3971–3975.

Eagle, H. (1959), *Science 130,* 432–437.

Furuichi, Y., and Miura, K. I. (1975), *Nature (London) 253,* 374–375.

Furuichi, Y., Morgan, M., Muthukrishnan, S., and Shatkin, A. J. (1975a), *Proc. Natl. Acad. Sci. U.S.A. 72,* 362–366.

Furuichi, Y., Morgan, M., Shatkin, A. J., Jelinek, W., Salditt-Georgieff, M., and Darnell, J. E. (1975b), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1904–1908.

Gallwitz, D., and Breindl, M. (1972), *Biochem. Biophys. Res. Commun. 47,* 1106–1111.

Hewlett, M. J., Rose, J. K., and Baltimore, D. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 327–330.

Jacobs-Lorena, M., Baglioni, C., and Borun, T. W. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2095–2099.

Konrad, M., Toivonen, J., and Nierlich, D. P. (1972), *Nature (London), New Biol. 238,* 231–233.

Laemmli, U. (1970), *Nature (London) 227,* 680–682.

Lavi, S., and Shatkin, A. J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2012–2016.

McGuire, P. M., Piatak, M., and Hodge, L. D. (1976), *J. Mol. Biol. 101,* 379–396.

Muthukrishnan, S., Both, G. W., Furuichi, Y., and Shatkin, A. J. (1975), *Nature (London) 255,* 33–37.

Nomoto, A., Lee, Y. F., and Wimmer, E. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 375–380.

Panyim, S., and Chalkley, R. (1969), *Biochemistry 8,* 3972–3979.

Park, W. D., Stein, J. L., and Stein, G. S. (1976), *Biochemistry 15,* 3296–3300.

Perry, R. P., and Kelley, D. E. (1976), *Cell 8,* 433–442.

Perry, R. P., Kelley, D. E., Friderici, K. H., and Rottman, F. (1975), *Cell 4,* 387–394.

Perry, R. P., and Scherrer, K. (1975), *FEBS Lett. 57,* 73–78.

Roa, M. S., Wu, B. C., Waxman, J., and Busch, H. (1975), *Biochem. Biophys. Res. Commun. 66,* 1186–1193.

Ro-Choi, T. S., Choi, Y. C., Henning, D., McCloskey, J., and Busch, H. (1975), *J. Biol. Chem. 250,* 3921–3928.

Ro-Choi, T. S., Reddy, R., Choi, Y. C., Raj, N. B., and Henning, D. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1548.

Rose, J. K. (1975), *J. Biol. Chem. 250,* 8098–8104.

Staehelin, M., Rogg, H., Baguley, B. C., Ginsberg, T., and Wehrli, W. (1968), *Nature (London) 219,* 1363–1364.

Stein, G. S., and Borun, T. W. (1972), *J. Cell Biol. 52,* 292–301.

Stein, G. S., Park, W. D., Thrall, C. L., Mans, R. J., and Stein, J. L. (1975), *Nature (London) 257,* 764–767.

Stein, J. L., Thrall, C. L., Park, W. D., Mans, R. J., and Stein, G. S. (1975), *Science 189,* 557–558.

Surrey, S., and Nemer, M. (1976), *Cell 9,* 589–595.

Thrall, C. L., Lichtler, A., Stein, J. L., and Stein, G. S. (1977), *Methods Cell Biol. 18* (in press).

Thrall, C. L., Park, W. D., Rashba, H. W., Stein, J. L., Mans, R. J., and Stein, G. S. (1974), *Biochem. Biophys. Res. Commun. 61,* 1443–1449.

Wei, C. M., Gershowitz, A., and Moss, B. (1975a), *Cell 4,* 379–386.

Wei, C. M., Gershowitz, A., and Moss, B. (1975b), *Nature (London) 257,* 251-253.
Wei, C. M., and Moss, B. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 318-322.
Wilson, M. C., Melli, M., and Birnstiel, M. L. (1974), *Biochem. Biophys. Res. Commun. 61,* 404-409.
Urushibara, T., Furuichi, Y., Nichimura, T., and Miura, K. I. (1975), *FEBS Lett. 49,* 385-389.

Modification-Deficient Transfer Ribonucleic Acids from Relaxed Control *Escherichia coli:* Structures of the Major Undermodified Phenylalanine and Leucine Transfer RNAs Produced during Leucine Starvation†

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ABSTRACT: The structures of the major, chromatographically unique phenylalanine and leucine tRNAs produced during leucine starvation of a relaxed control (rel⁻) mutant of *E. coli* have been determined. The results demonstrate that the unique species are modification-deficient forms of the major, normally occurring isoacceptor species. The unique $tRNA^{Phe}$ differs from the fully modified species at nucleotide positions 16, 37, 39, 47, and 55 from the 5′ terminus. The unique species contains uridine (U) in place of dihydrouridine-16 (D16), isopentenyladenosine in place of 2-thiomethyl-N^6-(δ^2-isopentenyl)adenosine-37, a mixture of U and pseudouridine (ψ) in position 39, a mixture of U and 3-(3-amino-3-carboxypropyl)uridine at position 47, and a mixture of U and ψ at position 55. The chromatographically normal isoacceptor from amino acid starved cells is deficient in D16 and ψ55, indicating that that species is a mixture of mature and undermodified tRNAs. The unique $tRNA^{Leu}$ isoacceptor consists of two subspecies which are undermodified forms of the major, normally occurring isoacceptor, $tRNA^{Leu}_1$. Both unique subspecies lack the D and ψ residues which occur at positions 16 and 39 from the 5′ terminus; one subspecies also lacks D17. Compared with the $tRNA^{Leu}_1$ from wild-type strains of *E. coli* B and K12, both $tRNA^{Leu}_1$ from nonstarved cells and the unique, rel⁻ $tRNA^{Leu}$ are deficient in the modified guanosine which normally occurs adjacent to the anticodon and the pseudouridine in the GTψC sequence of the ψ loop. Both the unique $tRNA^{Phe}$ and the unique $tRNA^{Leu}$ lack dihydrouridine residues which occur in the 5′ half of the D loop and pseudouridines which occur in the 3′ half of the anticodon loop and adjoining stem. Taken together, these findings suggest that the same enzymes are responsible for the formation of these particular modified bases in both tRNAs. The results further suggest that several, perhaps most, of the tRNAs from cells cultured under conditions in which RNA and protein synthesis are uncoupled will be similarly deficient in dihydrouridine and pseudouridine and other minor nucleosides which occur less frequently. Because both modification-deficient rel⁻ tRNAs have dihydrouridine at position 20 and pseudouridine in the ψ loop (and at position 41 in the unique $tRNA^{Leu}$), the results support the view that there are multiple D- and ψ-forming enzymes in *E. coli,* some of which may turn over rapidly or are selectively inactivated when protein synthesis is blocked. The results are discussed with a view toward understanding the structural basis for the altered biological activity of the unique $tRNA^{Phe}$ species and the order of events in the posttranscriptional modification of newly synthesized tRNA.

Various perturbations of cellular metabolism result in the appearance of chromatographically unique species of tRNA. In *Escherichia coli,* these conditions include: deprivation of essential amino acids (Fleissner, 1967; Capra and Peterkofsky, 1968; Stern et al., 1970; Waters et al., 1973; Fournier and Peterkofsky, 1975; Kitchingman and Fournier, 1974, 1975; Juarez et al., 1975; Katze and Mosteller, 1976; review by Littauer and Inouye, 1973), or iron (Wettstein and Stent, 1968; Rosenberg and Gefter, 1969; Juarez et al., 1975), culturing in the presence of antibiotics (Waters, 1969; Mann and Huang, 1973; Kitchingman and Fournier, 1975) or amino acid analogues (Chase et al., 1974), deviation from standard culturing temperatures (Chase et al., 1974), and reduced rates of aeration (Wettstein and Stent, 1968). The biochemical basis for the formation of these species is known in some instances. Starvation of relaxed control (rel⁻) mutants of *E. coli* for methionine, cysteine, or threonine results in the production of tRNA deficient in methylated nucleosides (Mandel and Borek, 1963), sulfur-containing nucleosides (Harris et al., 1969), or *N*-(purin-6-ylcarbamoyl)threonine (Powers and Peterkofsky, 1972), respectively; tRNA from methionine starved rel⁻ cells is also deficient in 3-(3-amino-3-carboxypropyl)uridine (Nishimura et al., 1974). Production of the undermodified species in these cases is a direct result of the removal of a substrate required for base modification. Removal of iron from the culture medium results in the formation of tRNA deficient in the methylthio moiety of 2-methylthio-N^6-(δ^2-isopentenyl)adenosine (ms^2i^6A) as iron is a necessary cofactor for one of the enzymes involved in the conversion of i^6A to ms^2i^6A (Rosenberg and Gefter, 1969).

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