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Failure to detect "cap" structures in mitochondrial DNA-coded poly(A)-containing RNA from HeLa cells

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ABSTRACT

The structure of the 5'-termini has been investigated in mitochondrial DNA-coded poly(A)-containing RNA from HeLa cells. For this purpose, mitochondrial RNA isolated from cells labeled for 3 hours with [<sup>32</sup>P]orthophosphate in the presence of 20 µg/ml camptothecin, and selected for poly(A) content by two passages through oligo(dT)-cellulose, was digested either with the nuclease P1 or with a mixture of RNases: the digestion products were then fractionated by two-dimensional electrophoresis. No "cap" structures were detected under conditions where the presence of such structures in one out of five to ten RNA molecules would have been recognized. It is, therefore, likely that "cap" structures are completely absent in HeLa cell mitochondrial poly(A)-containing RNA.

INTRODUCTION

Most eukaryotic cellular and viral mRNAs contain at the 5'-end an unusual nucleotide sequence, "cap", of the general structure m<sup>7</sup>G(5')ppp(5')XmpYp, where m<sup>7</sup>G represents a 7-methylguanosine residue linked by a 5',5'-triphosphate linkage to a nucleoside (X), which is most often 2'-O-methylated; in many mRNAs the third residue from the 5'-end (Y) is also 2'-O-methylated (see review by Shatkin<sup>1</sup>). The cap protects the mRNA at its 5'-terminus against attack by phosphatases and nucleases, and appears to promote mRNA function at the level of initiation of translation<sup>1,2</sup>.

No information is available as yet concerning the presence or absence of "cap" structures in mitochondrial mRNAs. These mRNAs represent a special class in the eukaryotic cell. The different environment in which they are utilized, and the prokaryotic characteristics of the mitochondria-specific translation apparatus, such as sensitivity to antibiotics, mechanism of initiation and nature of the initiation factors, suggest that the mitochondria-specific mRNAs may be subject to different regulation and may have different structural features from the remainder of cellular mRNA. Therefore, structural analysis of the 5'-terminal sequences of these RNAs is expected to provide valuable information as concerns both evolution

and regulation of the mitochondrial genome.

Previous work has demonstrated the occurrence in HeLa cell mitochondria of RNA species transcribed from mitochondrial DNA, which contain poly(A) stretches of about 55 nucleotides at their 3'-end<sup>3-6</sup>. Recently, this RNA has been resolved into a large number of discrete components by electrophoresis through agarose slab gels in the presence of the strong denaturing agent methylmercuric hydroxide<sup>7,8</sup>. It is likely, on the basis of circumstantial evidence, that these poly(A)-containing RNAs represent mitochondria-specific mRNAs or their precursors; however, direct proof for this is not yet available.

In the present work, the nature of the 5'-terminal structures in mitochondrial DNA-coded poly(A)-containing RNA from HeLa cells has been investigated. No evidence for "cap" structures has been found.

#### MATERIALS AND METHODS

(a) Cell Growth and Labeling Conditions: The method of growth of HeLa cells in suspension has been previously described<sup>9</sup>. For the labeling of mitochondrial RNA with [<sup>32</sup>P]orthophosphate, 5 to 6 x 10<sup>8</sup> exponentially growing cells, washed three times with warm modified Eagle's medium containing no phosphate, were resuspended in the same medium at the concentration of 1.5 x 10<sup>6</sup>/ml and incubated for 1 hour at 37°C. Cold phosphate (10<sup>-4</sup> M final concentration) and carrier-free [<sup>32</sup>P]orthophosphate (0.25 mCi/ml) were then added, and incubation was continued for 3 hours. Camptothecin (20 µg/ml), added 20 minutes before the [<sup>32</sup>P]orthophosphate, was used, unless otherwise specified, to inhibit high mol. wt. nuclear RNA synthesis<sup>10-12</sup>.

(b) Subcellular Fractionation and RNA Extraction: A crude mitochondrial fraction was prepared by differential centrifugation as previously described<sup>13</sup>, except that only one cycle of low and high speed centrifugation was used to minimize the possibility of RNA degradation; furthermore, EDTA (0.04 M) was added to the cytoplasmic extract before the high speed centrifugation, in order to reduce the contamination of mitochondria by cytoplasmic components<sup>13</sup>.

The RNA was extracted directly from the crude mitochondrial fraction. For this purpose, the pelleted material was dissolved in 0.05 M Tris buffer (pH 7.4 at 25°C), 0.01 M EDTA, 1% sodium dodecyl sulfate (1 ml/1.5 x 10<sup>8</sup> cell equivalents), and incubated for 1 hour at room temperature in the presence of proteinase K<sup>14</sup> (100 µg/ml). After addition of NaCl (0.15 M final concentration), the RNA was extracted by shaking two times with an equal volume of a phenol-chloroform-isoamyl alcohol mixture (50:50:1 by volume) at room temperature. After ethanol precipitation of the aqueous phase and centrifugation, the pelleted nucleic acids were dissolved in 0.05 M Tris buffer (pH 6.7), 0.0025 M MgCl<sub>2</sub>, 0.025 M KCl (1

ml/1.5 x 10<sup>8</sup> cell equivalents), and incubated for 30 to 60 minutes at 2°C in the presence of 100 µg/ml pancreatic DNase I (RNase free, Worthington). Sodium dodecyl sulfate (1%) and proteinase K (100 µg/ml) were then added, and, after 20 minutes incubation at room temperature, the solution was brought to 0.15 M NaCl and the RNA reextracted, as described above. RNA was then purified from low mol. wt. [<sup>32</sup>P]-labeled molecules by chromatography (4°C) through a Sephadex G50 column equilibrated with 0.05 M sodium phosphate, pH 5.0. Free cytoplasmic polysomes were isolated from cells which had been labeled with [<sup>32</sup>P]orthophosphate either for 3 hours, as specified above, but in the absence of camptothecin, or for 48 hours (7 µCi/ml [<sup>32</sup>P]orthophosphate, 10<sup>-4</sup> M phosphate, initial cell concentration 10<sup>5</sup>/ml). Purification of polysomal mRNA was carried out as previously described<sup>15</sup>.

(c) RNA Fractionation and Analysis: RNA was fractionated by two consecutive passages through oligo(dT)-cellulose columns<sup>6</sup>. After the first chromatography, the bound material, collected by ethanol precipitation and centrifugation, was dissolved in 0.001 M Tris buffer (pH 8.0) containing 0.001 M EDTA, and subjected to a denaturation step, by heating for 5 minutes at 80°C, before the second passage through oligo(dT)-cellulose. The RNA bound twice to oligo(dT)-cellulose has been shown to contain poly(A) stretches corresponding to about 4S<sub>E</sub> (≈55 nucleotides)<sup>8</sup>, characteristic of mitochondrial RNA<sup>3,6</sup>. This RNA was utilized for 5'-end analysis as described below. A portion of each poly(A)-containing RNA preparation was routinely analyzed by electrophoresis through an agarose slab gel in the presence of methylmercuric hydroxide<sup>16</sup> in combination with autoradiography<sup>8</sup>.

(d) 5'-End Analysis of Poly(A)-Containing RNA:

1) Enzyme digestion

a. Nuclease P1

RNA (2-6 µg), in 25 µl of 50 mM ammonium acetate, 1 mM EDTA (pH 5.4), was digested with 0.5-1 µg of nuclease P1 (Yamasa Shoyu, Ltd., Japan). Incubation for 1 hour at 37°C gave complete digestion. Whenever alkaline phosphatase treatment had to follow the P1 digestion, the latter was carried out in 20 µl of 50 mM ammonium acetate, 0.5 mM EDTA (pH 5.4) for 1 hour at 37°C; the reaction mixture was then adjusted by addition of 1 µl 1 M ammonium hydroxide, 1 µl 50 mM ZnSO<sub>4</sub>, 2 µl 0.1 M MgCl<sub>2</sub> (final pH 8.0) and 5 µg alkaline phosphatase (Worthington, RNase-free), and incubated for another hour at 37°C.

b. Mixture of RNases

RNA (2-6 µg), in 25 µl of 50 mM ammonium acetate, 1 mM EDTA (pH 4.5), was digested by adding 2 µl of an RNase mixture [100 units/ml of

RNase T<sub>2</sub> (Sigma), 10 µg/ml RNase U<sub>2</sub> (CalBiochem), 2.5 µg/ml RNase A (Sigma), and 5000 units/ml of RNase T<sub>1</sub> (CalBiochem)], in 0.5 M ammonium acetate (pH 4.5). Digestion was carried out for 1 hour at 37°C.

2) Separation and Analysis of Nuclease P1 and RNase Digestion Products

[<sup>32</sup>P]-labeled ribonucleotides and other products of digestion were separated by the two-dimensional electrophoretic technique of Sanger and Brownlee<sup>17</sup>, which had already been utilized for the isolation of 5'-oligonucleotides from the 5'-ends of eukaryotic mRNAs by Adams and Cory<sup>18</sup>. For the identification of the "cap" structures, authentic "cap" dinucleotides (P-L Biochemicals, Inc., 1 OD<sub>260 mµ</sub> unit of each) were added to the P1 digest of cytoplasmic poly(A)-containing RNA.

Each digest was applied directly onto a strip of Whatman No. 1 paper, and subjected to high voltage electrophoresis (1-2 kV, Gilson Electrophorator) in pH 3.5 buffer (0.5% pyridine (v/v), 5% acetic acid (v/v), 5 mM EDTA) until the xylene cyanol FF (blue) marker migrated to approximately 10 cm from the origin. The portion of the paper from 2.5 cm below to 33 cm above the origin (in the direction of migration) was used for transfer to the second dimension. The paper strip was trimmed to a 2 cm width, a second batch of dye marker mixture was applied to the center of each separated dye marker spot, and the strip was clamped onto a DEAE paper (Whatman DE 81) sheet using two Plexiglass (DuPont) strips. The DEAE paper sheet was wetted with water on both sides of the strip and left undisturbed for 30 minutes; during this time, the diffusing water transferred the nucleotides quantitatively from the paper strip onto the DEAE paper. The DEAE paper sheet was dried, and then subjected to electrophoresis at 1 kV in the same pH 3.5 buffer until the xylene cyanol FF marker migrated to about 10 cm from the origin. [<sup>32</sup>P]-labeled nucleotides were then located by autoradiography (about 1 week) using Kodak RP/R (14 x 17 inches) X-ray film. The "cap" dinucleotide markers were identified by their blue fluorescence under short-wave UV and their positions marked.

The DEAE paper areas containing radioactive spots were cut out and counted in a Toluene-Liquifluor mixture (New England Nuclear) using a Beckman LS 233 scintillation counter. The DEAE paper sections containing the spots to be eluted were then washed with toluene (2x), ether (2x) and dried. Elution was carried out in siliconized Pasteur pipettes plugged with a small piece of cotton to trap paper lint. One to 2 ml of 1 M triethylammonium bicarbonate was let to drain through each spot into a 15 ml centrifuge tube. Recovery of the counts was about 70%. The eluted nucleotides were dried under vacuum, redissolved in 20 µl of deionized water and subjected to further enzymatic treatment. For this purpose, the

following enzymes and conditions were used:

- a. E. coli alkaline phosphatase, 5  $\mu\text{g}$  in 50 mM Tris-HCl, 10 mM  $\text{MgCl}_2$ , pH 8, final volume 25-30  $\mu\text{l}$ ; 2 hour incubation at 25°C.
- b. Snake venom exonuclease (Worthington), 5  $\mu\text{g}$  in 50 mM Tris-HCl, 10 mM  $\text{MgCl}_2$ , pH 8.9, final volume 25-30  $\mu\text{l}$ ; 2 hour incubation at 25°C.
- c. Spleen exonuclease (Worthington), 0.5 units in 50 mM ammonium acetate, 10 mM NaF, 1 mM EDTA, pH 5.4, final volume 25-30  $\mu\text{l}$ ; 2 hour incubation at 25°C.
- d. Nuclease P1, 1  $\mu\text{g}$  in 50 mM ammonium acetate, pH 5.4, final volume 25-30  $\mu\text{l}$ ; 2 hour incubation at 25°C.
- e. Snake venom exonuclease (Worthington), 12  $\mu\text{g}$ , and nucleotide pyrophosphatase (from Crotalus adamanteus venom, Sigma), 0.1 unit, in 50 mM Tris-HCl, 10 mM  $\text{MgCl}_2$ , pH 7.5, final volume 33  $\mu\text{l}$ ; 2 hour incubation at 37°C.

The individual enzyme digests were examined for their product composition by applying one-half on Whatman No. 1 paper and one-half on Whatman DE 81 DEAE paper. The one-dimensional electrophoresis was performed in the same manner (pH 3.5 buffer) as described above for the two-dimensional separation. [ $^{32}\text{P}$ ]mononucleotides and dye markers were run in parallel to aid identification.

## RESULTS

In previous work, the mitochondrial poly(A)-containing RNA from HeLa cells has been characterized in the size of the poly(A) stretches<sup>3,5,6</sup>, and in the homology to mitochondrial DNA of the non-poly(A) portion<sup>6,19</sup>. By polyacrylamide gel electrophoresis in the presence of formaldehyde, several discrete species of mitochondrial DNA-coded poly(A)-containing RNA could be recognized<sup>19</sup>. More recently, by using agarose slab gel electrophoresis in the presence of methylmercuric hydroxide as a denaturing agent, in combination with autoradiography, a larger number of discrete components than previously observed have been identified in the poly(A)-containing RNA labeled for 3 hours with [ $^{32}\text{P}$ ]orthophosphate in the presence of 20  $\mu\text{g}/\text{ml}$  camptothecin<sup>7,8</sup>. Fig. 1A shows a standard electrophoretic pattern obtained with such [ $^{32}\text{P}$ ]-labeled mitochondrial RNA. It has been established that the discrete species resolved in this RNA are products of the normal mitochondrial RNA metabolism in HeLa cells<sup>8</sup>; furthermore, all these species, as well as most of the heterogeneous material, have been shown to be transcription products of mitochondrial DNA on the basis of the ethidium bromide sensitivity of their synthesis (Fig. 1B) and of their base sequence complementarity to separated mitochondrial DNA strands<sup>7,8</sup>. In Fig. 1A, at least 30 to 40% of the  $^{32}\text{P}$ -label is associated with discrete species, as estimated from analysis of a densitometric tracing.

A      B

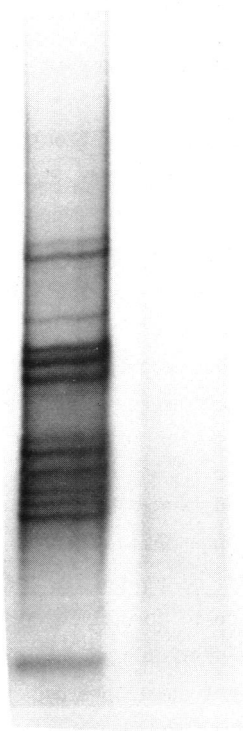


Figure 1. Autoradiogram, after 4 hour electrophoresis through a 1.2% agarose-5 mM  $\text{CH}_3\text{HgOH}$  slab gel, of the oligo(dT)-bound RNA from the mitochondrial fraction of cells labeled for 3 hours with [ $^{32}\text{P}$ ]orthophosphate in the presence of 20  $\mu\text{g}/\text{ml}$  camptothecin and in the absence (A) or presence (B) of 1  $\mu\text{g}/\text{ml}$  ethidium bromide.

1) Characterization of Nuclease P1 Digests

[ $^{32}\text{P}$ ]-labeled poly(A)-containing RNA isolated from free cytoplasmic polysomes was digested with nuclease P1, which cleaves phosphodiester linkages in nucleic acid to yield 5'-mononucleotides<sup>20-22</sup>, and the resulting products were separated as described above. As shown in Fig. 2, the P1 digest fingerprint exhibits the four 5'-mononucleotides and four spots corresponding in position to the "cap" dinucleotides  $\text{m}^7\text{G}(5')\text{ppp}(5')\text{X}$  resolved in this electrophoretic system (cap 1:

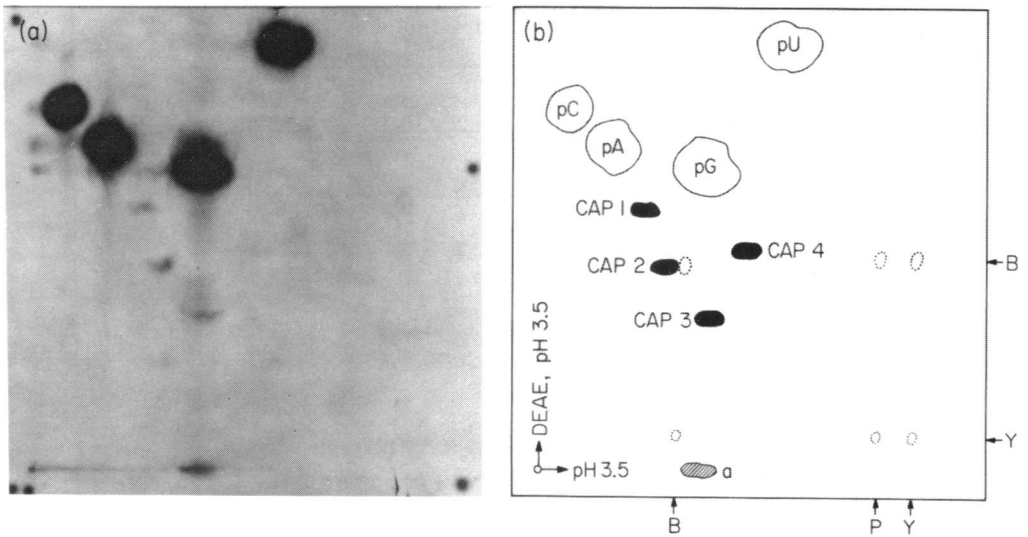


Figure 2. (a) Autoradiogram after two-dimensional electrophoresis of a complete nuclease P1 digest of *in vivo* long-term [ $^{32}\text{P}$ ]-labeled poly(A)-containing RNA from cytoplasmic free polysomes of HeLa cells. First dimension, ionophoresis on paper at pH 3.5 is from left to right. Second dimension, ionophoresis on DEAE paper at pH 3.5 is from bottom to top.

(b) Tracing of the autoradiogram in Figure 2a. Mononucleotide spots are marked by solid line and symbols. The black spots mark the positions of migration of the individual labeled "cap" dinucleotides, which coincide with authentic markers. For the significance of the cross-hatched spot see text. Dotted circles mark the positions of the dye markers. Letters on the margins denote the positions of the dye markers in the first and second dimension. (B = blue, Y = yellow, P = pink).

$m^7\text{GpppCm}$ ; cap 2:  $m^7\text{GpppAm} + m^7\text{Gpppm}^6\text{Am}$ ; cap 3:  $m^7\text{GpppGm}$ ; cap 4:  $m^7\text{GpppUm}$ ). Identification of the "cap" dinucleotides was carried out by comparison of their migration with that of synthetic authentic "cap" dinucleotides added as internal markers (a control experiment showed that  $m^7\text{GpppAm}$  and  $m^7\text{Gpppm}^6\text{Am}$  migrated to approximately the same position). The nature of spot "a" is discussed below. The total estimated proportion of phosphate in "cap" structures (0.1%, Table I) would correspond to one such structure per 3000 nucleotides, which is close to the average size of HeLa cell mRNA<sup>15,23</sup>; this indicates that the great majority of poly(A)-containing RNA molecules from cytoplasmic polysomes in HeLa cells contain a "cap" structure, in agreement with earlier observations<sup>24,25</sup>. Also the distribution of methylated nucleotides at the 5'-terminus which has been observed here is very close to that previously reported<sup>24,25</sup>. The analysis of the distribution of radioactivity among the four 5'-mononucleotides reveals a base composition very similar to that reported for HeLa cell poly(A)-containing mRNA<sup>26</sup> (Table I).

Table I. Distribution of Radioactivity among the P1 Digestion Products of Cytoplasmic Polysomal Poly(A)-Containing RNA from HeLa Cells

Digestion Products		Percent <sup>32</sup> P
	pU	22.8
	pG	22.6
	pA	31.3
	pC	23.2
cap 1	m <sup>7</sup> GpppCm	0.016
cap 2	m <sup>7</sup> GpppAm + m <sup>7</sup> Gpppm <sup>6</sup> Am	0.033
cap 3	m <sup>7</sup> GpppGm	0.043
cap 4	m <sup>7</sup> GpppUm	0.007

A total of about 135,000 cpm was counted in the identified spots.

For the analysis of mitochondrial poly(A)-containing RNA three to four times as much radioactivity was used as in the case of cytoplasmic RNA in order to have comparable amounts of radioactivity associated with the discrete species in the two preparations. A typical fingerprint of the nuclease P1 digest of mitochondrial poly(A)-containing RNA is shown in Fig. 3. This fingerprint is strikingly different from that of cytoplasmic polysomal poly(A)-containing RNA. In addition to 5'-mononucleotides, there are five main spots present (marked 1 to 4, and "a"), none of which is in the position of "cap" dinucleotides. In order to improve the sensitivity of detection, we performed a double digestion of mitochondrial RNA first with nuclease P1, and then, after adjustment of pH and ion concentration, with alkaline phosphatase: all radioactivity was converted to inorganic monophosphate. Since we had applied about  $10^6$  cpm of the mitochondrial RNA digest, and exposure of the autoradiograph was long enough to allow detection of 100 cpm in a single spot, we can conclude that typical "cap" dinucleotides are not present in mitochondrial RNA at this level of detection.

Next we turned to the identification of spots 1 to 4 in the fingerprint. In the experiment described above, all spots had been completely digested by alkaline phosphatase with release of inorganic phosphate; therefore, they had to be phosphorylated mononucleosides. Sensitivity to alkaline phosphatase was confirmed by eluting each spot and redigesting it separately. Further evidence as to the identity of each spot came from the following results. Spot 2, which was present in variable amount in different samples (Table I), was converted by snake venom exonuclease to AMP and orthophosphate (Pi); therefore, it was presumably 5'-ADP



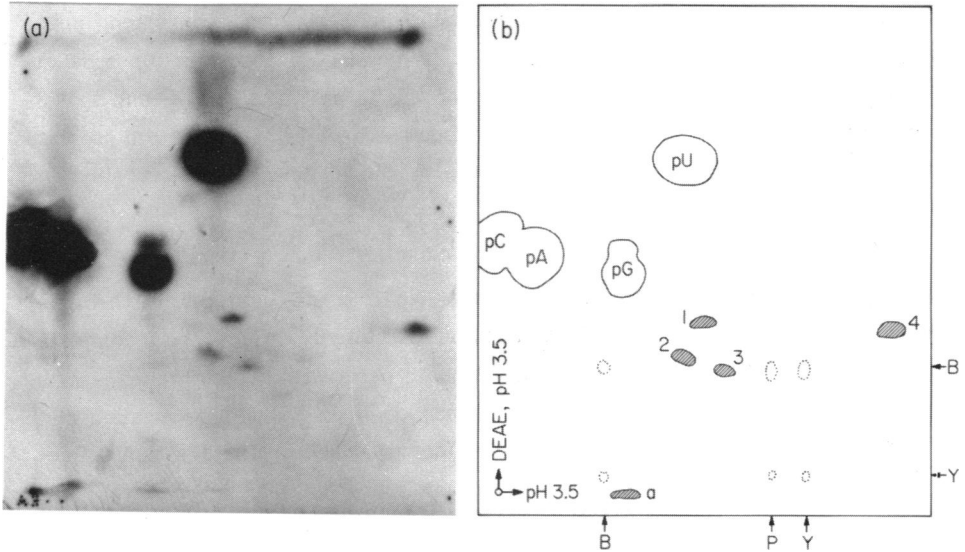


Figure 3. (a) Autoradiogram after two-dimensional electrophoresis of a complete nuclease P1 digest of mitochondrial poly(A)-containing RNA from HeLa cells labeled for 3 hours with [ $^{32}$ P]orthophosphate in the presence of 20  $\mu$ g/ml camptothecin.

(b) Tracing of the autoradiogram in Figure 3a. For significance of cross-hatched spots see text.

(ppA); its position corresponded in fact to the position of the ADP marker. In contrast, spots 1, 3, 4 were resistant to snake venom exonuclease, suggesting the presence of a phosphoryl group at the 3'-end. A clue to the identity of spots 1 and 4 came from redigestion with nuclease P1: upon redigestion, spot 1 was converted to CMP and Pi and spot 4 to UMP and Pi, presumably as a result of the 3'-nucleotidase (3'-phosphomonoesterase) activity of the nuclease P1<sup>21,22</sup>. The yield of these two spots was variable, and in some preparations they were completely missing. Therefore, we concluded that spot 1 is cytidine-5',3'-diphosphate and spot 4 uridine-5',3'-disphosphate, and that they probably resulted from cleavage of the RNA at some points by a contaminating pancreatic RNase-type activity. By contrast, spots 2 and 3 were completely resistant to redigestion by nuclease P1 and, therefore, they represented final products.

We were unable to determine conclusively the identity of the spot 3. It was completely resistant to further digestion with nuclease P1, snake venom exonuclease, nucleotide pyrophosphatase and spleen exonuclease. As stated above, it was completely digested by alkaline phosphatase with release of inorganic phosphate. It travelled close to the position of ADP, so it probably contained two phosphate groups in the arrangement pXp or Xpp. Some hint as to its identity came from redigestion of spot "a" with nuclease P1. This spot, which was found in both P1 and RNase digests of cytoplasmic and mitochondrial poly(A)-containing RNAs,

and whose yield was variable in different preparations, was converted, upon redigestion with nuclease P1, to spot 3, suggesting a precursor to product relationship. We suspect that spot "a" corresponds to poly(ADP-ribose) and spot 3 is phosphoribosyl-AMP; however, we do not have evidence for this at the present time. Poly(ADP-ribose) is known to occur in the nucleus<sup>27</sup> and, probably, in mitochondria<sup>28</sup> of eukaryotic cells.

The analysis of the distribution of radioactivity among the four 5'-mononucleotides shows a predominance of the label in pU and pA, with little radioactivity in pG (Table II). This distribution undoubtedly reflects the incomplete equilibration of the four nucleoside triphosphate pools within the organelles with the exogenous [<sup>32</sup>P]orthophosphate after 3 hour labeling; it should be recalled that the specific activities of the 5'-mononucleotides produced by P1 digestion reflect directly those of the corresponding precursor pools.

<u>Nuclease P1</u>		<u>RNases</u>	
Digestion Product	Percent <sup>32</sup> P	Digestion Product	Percent <sup>32</sup> P
pU	36.5 (32.9-42.9)	Up	28.5
pG	5.9 (2.8-9.0)	Gp	16.9
pA	40.0 (34.9-47.3)	Ap	28.0
pC	17.0 (8.7-23.2)	Cp	26.3
Spot 1 (pCp)	0.104 (0.047-0.168)	Spot 1 (pAp)	0.144
Spot 2 (ppA)	0.047 (0, 0.032, 0.110)	Spot 2	0.023
Spot 3	0.130 (0.020-0.322)	Spot 3	0.022
Spot 4 (pUp)	0.264 (0.067-0.573)	Spot "a"	0.076

The results of nuclease P1 digestion represent the averages and ranges (in parentheses) of the data from three experiments with different RNA preparations (from 427,000 to 558,000 cpm each).

## 2) Characterization of Mixed RNase Digests

The fingerprint of poly(A)-containing cytoplasmic polysomal RNA digested with a mixture of RNases is shown in Fig. 4. The most interesting feature is a field of 9 spots, shown as black areas in Fig. 4b, which represent tri- and tetranucleotide "caps," as recognized from their migration when compared with that reported for known "cap" structures<sup>18</sup>.

In comparison, the mixed RNase digest of mitochondrial poly(A)-containing RNA (Fig. 5) gave a simpler fingerprint. Here, there is no evidence of spots in the area where the "cap" structures are expected to migrate. The fingerprint shows one main spot, #1. This spot was completely digested by alkaline phosphatase, with release of Pi; it was resistant to spleen exonuclease, and it was converted to AMP and Pi by the nuclease P1: we therefore conclude that it is adenosine-3',5'-diphosphate. The amount of radioactivity associated with this spot was enough to account for one pAp per molecule of average size of about 1400 nucleotides (Table II). Very little radioactivity was recovered from spots 2 and 3 (Table II), so that they could not be analyzed in detail. Preliminary evidence suggests that spot 2 is pGp and spot 3 pUp. Spot "a" (which is also present in the RNase digest of cytoplasmic polysomal poly(A)-containing RNA (Fig. 4)) is presumably represented by the same material as spot "a" in the nuclease P1 digest, since it is converted by

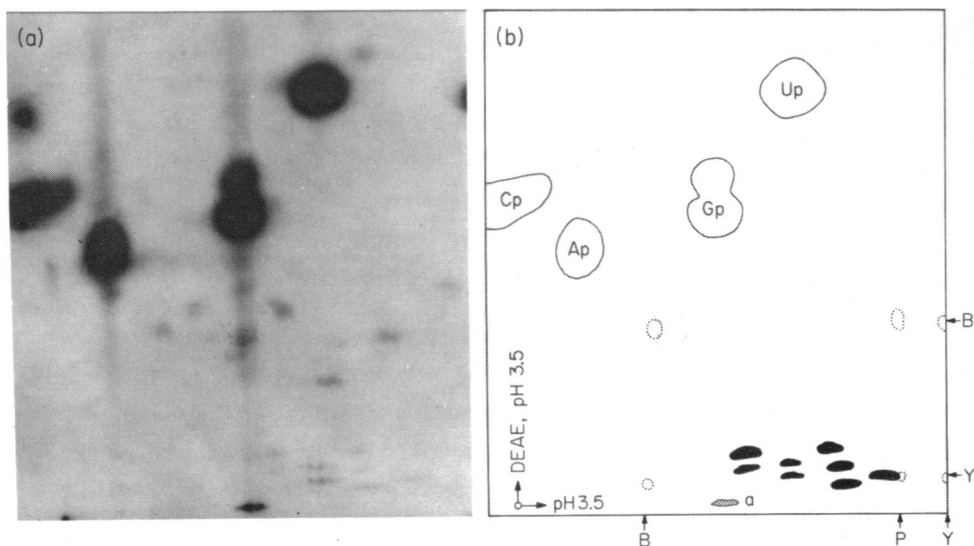


Figure 4. (a) Autoradiogram after two-dimensional electrophoresis of a complete digest by an RNase mixture of poly(A)-containing RNA from cytoplasmic free polysomes of HeLa cells labeled for 3 hours with [<sup>32</sup>P]orthophosphate in the absence of inhibitors.

(b) Tracing of the autoradiogram in Figure 4a. The black spots represent the positions of migration of presumptive "cap" trinucleotides and tetranucleotides (see text).

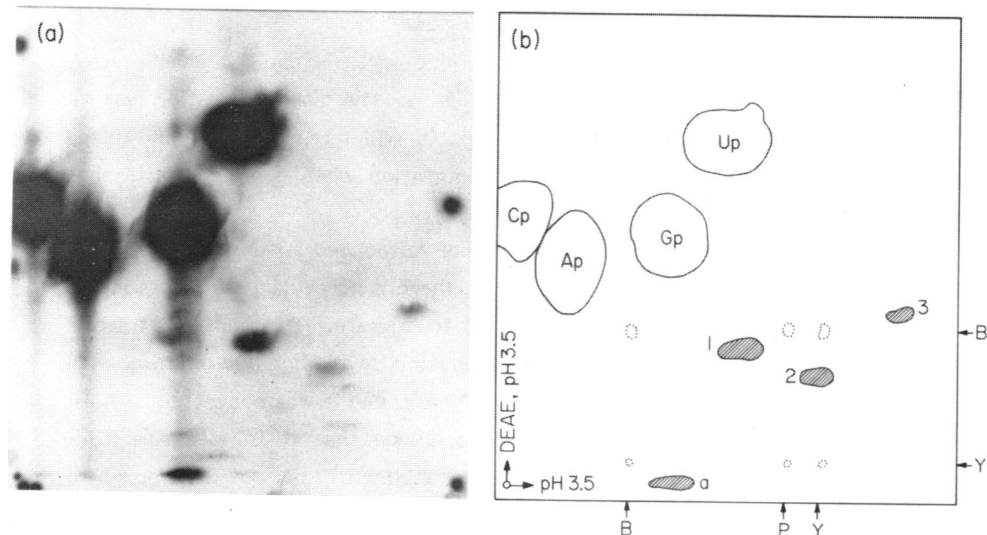


Figure 5. (a) Autoradiogram after two-dimensional electrophoresis of a complete digest by an RNase mixture of mitochondrial poly(A)-containing RNA from HeLa cells labeled for 3 hours with [ $^{32}\text{P}$ ]-orthophosphate in the presence of 20  $\mu\text{g}/\text{ml}$  camptothecin.

(b) Tracing of the autoradiogram in Figure 5a. For significance of cross-hatched spots see text.

digestion with P1 to a spot corresponding in position to spot 3 of the P1 digest (Fig. 3). Poly(ADP-ribose) is known to be resistant to RNase digestion<sup>29</sup>. The distribution of radioactivity among the four 2',3'-mononucleotides produced by RNase digestion of mitochondrial poly(A)-containing RNA (Table II) reveals a base composition fairly similar to that previously observed, after alkali digestion, for the 30-min  $^{32}\text{P}$ -labeled total mitochondrial RNA<sup>30</sup>, with  $\sim 43\%$  GC, and A/G  $\sim 1.7$ .

## DISCUSSION

The main result reported in this work is the apparent absence of "cap" structures in mitochondrial poly(A)-containing RNA from HeLa cells. The data reported above have allowed us to place some limits to the significance of this negative result. In particular, the data of sequential digestion with P1 nuclease and alkaline phosphatase have indicated that any possible cap structures in the RNA analyzed here would involve altogether less than 0.04% of the phosphate residues. This figure has to be compared with the values of 0.2 to 0.3% expected for the proportion of phosphate residues in "cap" structures in RNA molecules of number average mol. wt. of 4 to 5  $\times 10^5$  daltons: the latter is the average size of the RNA studied here, as estimated from the densitometric profile. The comparable figures

derived from the literature for cytoplasmic mRNA from animal cells in culture, which has a larger average size<sup>15,23,31</sup>, are 0.1-0.2%. It can therefore be estimated that, in the RNA analyzed here, only one out of 5 to 10 molecules, at most, contained "cap" structures, and it seems likely that these structures were actually completely absent. This conclusion is subject to the proviso that, in the present experiments, the "caps" were not lost in the preparation of mitochondrial RNA, or that the use of camptothecin to block high mol. wt. nuclear RNA synthesis did not affect the capping of mitochondrial RNA. There is no evidence for such an effect of camptothecin in other systems.

"Cap" structures have not so far been detected in bacterial systems. In spite of the large number of investigations, the physiological role of these structures is still unknown. It seems likely that they are not essential for any of the steps of the translation process, although their presence apparently increases the rate of ribosome attachment to mRNA<sup>1</sup>. The apparent absence of such "cap" structures at the 5'-end of HeLa cell mitochondrial poly(A)-containing RNA has to be contrasted with the demonstrated presence in this RNA, at the 3'-end, of poly(A) stretches<sup>3,6</sup>: the latter represent another structural feature of uncertain physiological role, which is found in most eukaryotic mRNAs, and which is, in contrast, only rarely and very rudimentally present in bacterial mRNA<sup>32,33</sup>.

The present work has also provided some information concerning the nature of the main 5'-terminal groups in the mitochondrial poly(A)-containing RNA studied here. After P1 nuclease digestion, among the products different from 5'-mononucleotides, apart from pUp and pCp, which were present in variable amounts and were presumably due to contaminating pancreatic RNase-type activity, and from presumptive phosphoribosyl-AMP, a cleavage product of poly(ADP-ribose), only ppA was detected, although in variable amount. After mixed RNase digestion, the main product different from 2',3'-mononucleotides was pAp. There is an apparent discrepancy between the results obtained by P1 and by RNase digestion of the mitochondrial poly(A)-containing RNA. In fact, if the ppA observed after P1 digestion derived from the 5'-end of the molecule, one would have expected to find, after mixed RNase action, an equivalent amount of ppAp: this was not found. Any pAp produced by mixed RNase digestion from the 5'-ends would have not, by contrast, been recognized after P1 treatment, since it would have given rise to pA, thus becoming confused with the main products of the enzyme. It seems possible that both ppAp and pAp are 5'-terminal groups of the RNA investigated here, and that the ppAp became converted to pAp during RNase digestion due to some pyrophosphatase contamination of the enzyme, or that in this particular experiment the concentration of 5'-terminal ppA in the isolated RNA was low. Alternatively,

one cannot exclude that ppA derives from poly(ADP-ribose) as a result of P1 digestion. The effect of P1 on poly(ADP-ribose) is not known. In any case, it seems reasonable to conclude that pA is the main 5'-end of the mitochondrial poly(A)-containing RNA. Its amount is compatible with its being present at the 5'-end of the majority of the molecules of this RNA class. To what extent it represents a residue of the nucleoside triphosphate at the 5'-end of the primary transcription products, or, on the contrary, it results from physiological or artifactual cleavage of the primary transcripts cannot be said.

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