HETEROGENEITY IN THE METHYLATION AND 5′ TERMINI OF NOVIKOFF ASCITES HEPATOMA 5.8 S RIBOSOMAL RNA

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Received 22 May 1974

1. Introduction

Cytoplasmic ribosomes of eukaryotic cells contain two low molecular weight RNA species, 5 S and 5.8 S rRNA's [1–6]. Both have been isolated from the larger ribosomal subunit in equimolar ratios to the 28 S rRNA molecule; the 5.8 S species was found to be hydrogen bonded to the high molecular weight RNA component [2–6]. Labeling kinetics indicate that the 5.8 S rRNA as well as the two high molecular weight components are cleavage products of high molecular weight ribosomal RNA precursors [2] but its position in nucleolar RNA and the reactions involved in its processing are not yet defined.

The nucleotide sequence of yeast (*Saccharomyces cerevisiae*) 5.8 S rRNA has recently been determined by Rubin [7–8]. Preliminary studies have also been made on 5.8 S RNA of the broad bean [9] and in wheat embryo [10]. As an initial step in the determination of the primary nucleotide sequence in the 5.8 S rRNA from the Novikoff ascites hepatoma, alkaline digests and oligonucleotide fragments have been analyzed. Like the yeast RNA [8] the mammalian species exhibits sequence heterogeneity at its 5′ end but unlike yeast 5.8 S rRNA it also contains 2′-O-methylated nucleotides. One of these is only partially methylated resulting in some sequence heterogeneity within the RNA molecules. Oligonucleotides which contain methylated or modified nucleotides have been further analyzed and their sequences are reported here.

2. Methods

Novikoff hepatoma ascites cells were maintained in male Holtzman rats for six days and then labeled in vitro with [^{32}P]orthophosphate as described by Mauritzen et al. [11]. RNA was prepared from both whole cells or ribosomes. When preparing RNA from whole cells the 5–10 g cell pellet was directly suspended in 100 ml of 0.3% (w/v) sodium dodecyl sulphate – 0.14 M NaCl – 0.05 M sodium acetate (pH 5.1) and extracted with 100 ml of phenol solution [12] at 65°C. When preparing RNA from ribosomes, polysomes were prepared from labeled cells essentially as described by Rich [13] and the RNA was subsequently extracted with sodium dodecyl sulphate–phenol at 65°C. No significant differences were observed in subsequent pancreatic or T_1 RNase maps of 5.8 S rRNA prepared by these procedures. Low molecular weight RNA was separated on 10–40% sucrose density gradients and fractionated by one-dimensional electrophoresis on 10% polyacrylamide gel slabs [14] or by two-dimensional electrophoresis on 10–12% polyacrylamide gels [15]. The 5.8 S rRNA was detected by autoradiography and recovered from the gel by homogenization in water followed by high-speed centrifugation (30 000 *g*, 1 hr) and filtration through a Millipore (Millipore Corp., Bedford, Mass.) filter (0.45 μ).

Purified 5.8 S rRNA (specific activity, 10^6 dpm/μg) was precipitated at −20°C with 2 vol of ethanol

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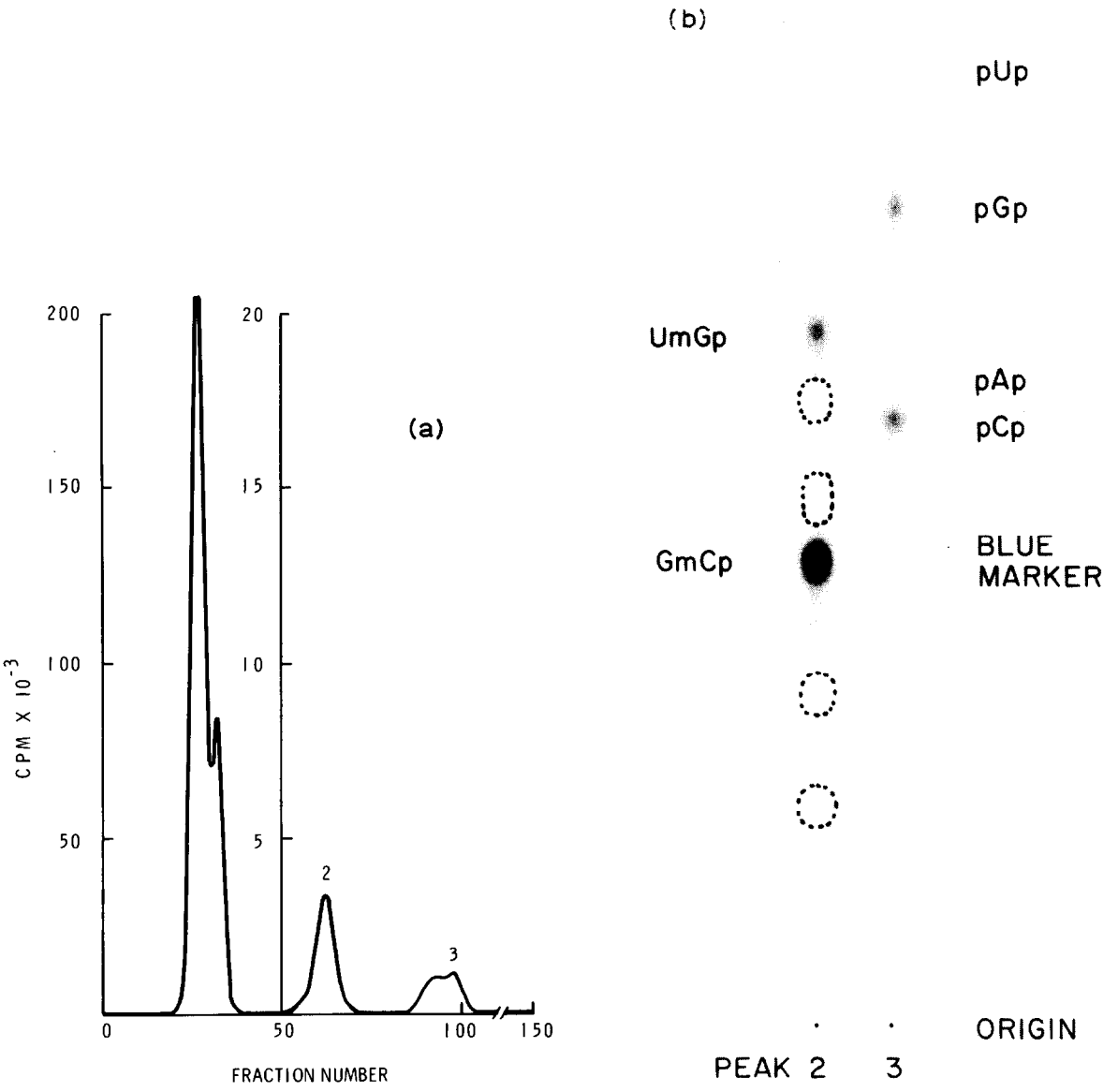

Fig. 1. Analyses of ^{32}P-labeled Novikoff hepatoma 5.8 S rRNA after alkaline digestion. (a) DEAE-Sephadex A-25 column chromatography of ^{32}P-labeled fragments at neutral pH. 5.8 S rRNA was digested with 0.3 N NaOH for 72 hr at 37°C. The digest was applied to a 0.7 × 20 cm column and eluted with a 600 ml gradient of 0.0 to 0.2 M NaCl in 7 M urea and 0.05 M Tris–HCl. pH 7.5. 4 ml fractions were collected every 10 min. (b) Separation of chromatographic fractions by electrophoresis on Whatman 3 MM paper at pH 3.5. Pooled fractions were desalted on 0.5 × 1 cm columns of DEAE-Sephadex A-25, eluted with 30% TEA-carbonate [16,18], dried, dissolved in water and spotted for electrophoresis [16]. The broken circles indicate the positions of mononucleotide markers as observed with UV light. The approximate positions of the origin and the blue dye marker are also given in the margin.

(2% potassium acetate) and digested completely by alkali, pancreatic ribonuclease or T_1 ribonuclease [16]. The alkaline digests were analyzed by chromatography on DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Uppsala) at pH 7.5 [17] followed by electrophoresis on Whatman 3 MM paper at pH 3.5 [16]. The primary structure of RNA fragments obtained by ribonuclease digestion was analyzed using the fingerprinting methods of Sanger and Brownlee [16] and related procedures [18]. Alkali resistant dinucleotides were identified by electrophoresis on Whatman 3 MM paper at pH 3.5 and on DEAE-paper at pH 1.9 before and after digestion with purified Worthington venom phosphodiesterase [19] or alkaline phosphatase [18]. Pseudouridine was identified by electrophoresis on Whatman 3 MM paper and by ascending chromatography [18] on cellulose thin layers (Eastman Kodak Co., Rochester, N.Y.) in propan-2-ol (680 ml)–HCl (176 ml)–H_2O (to 1 liter).

3. Results and discussion

3.1. *Analysis of alkaline digestion products*

Fig. 1 shows the distribution of radioactivity in fragments of 5.8 S rRNA after digestion with 0.3 N NaOH for 72 hr at 37°C. Three major peaks were observed on DEAE-Sephadex A25 at pH 7.5 (fig. 1a) with −2, −3 and −4 charges, respectively. Further analysis by electrophoresis at pH 3.5 indicated that the first peak contained only mononucleotides, the second contained alkali-stable dinucleotides and the third contained the 5′-nucleotide termini.

Two alkali resistant components with a −3 charge were isolated from peak 2 by electrophoresis at pH 3.5 (fig. 1b). Alkaline phosphatase released 50% of the radioactivity as inorganic phosphate from each component confirming that these were dinucleotides containing 2′-O-methylated nucleotides. On electrophoresis on DEAE-paper at pH 1.9, the slower moving component ran slight behind a Gp marker and the faster component ran slightly behind the blue marker. Complete digestion with venom phosphodiesterase released only pC from the slower moving component and pG from the faster moving component. Therefore, on the basis of charge at neutral pH, electrophoretic mobilities at pH 3.5 [20] or 1.9 [18] and in 7% formic acid (fig. 2) and the venom phosphodiesterase digestion products these components were identified as Gm–Cp and Um–Gp.

Peak 3 was also separated into two major components by electrophoresis at pH 3.5 (fig. 1b). After complete digestion of these components with alkaline phosphatase, only inorganic phosphate was found indicating that these were 5′-terminal nucleoside diphosphates. On the basis of electrophoretic mobility at pH 3.5 the faster and slower moving components corresponded to pGp and pCp respectively [18]. After electrophoresis on DEAE-paper at pH 1.9 the slower moving component ran with Gp and the faster component trailed the blue marker also corresponding to pCp and pGp respectively. Furthermore very brief alkaline phosphatase digestion (0.1 mg/ml of phosphatase for 15 sec, 25°C) of the slower and faster moving components produced traces of the corresponding mononucleotides. Therefore, on the basis of charge at neutral pH, electrophoretic mobilities and the alkaline phosphatase digestion products, these termini are pCp and pGp.

The chain length reported for 5.8 S rRNAs in various species ranges from 158–200 nucleotides [1,7,10]. Peak 3 (fig. 1b) contained 1.1% (average of two determinations) of the total radioactivity indicating that Novikoff hepatoma 5.8 S rRNA has a chain length of approximately 180 nucleotides. For yeast 5.8 S rRNA, Rubin [8] reported that there are three 5′ termini; pAp was the terminus in 90% of the molecules. The present study indicates that such heterogeneity in termini also exists in mammalian cells. Approximately 51% of the molecules have pGp at their 5′ termini while 41% begin with pCp. The nucleoside diphosphates pAp and pUp were also present (fig. 1b), each containing about 4% of the total radioactivity in peak 3. Until the complete sequence is known it is not possible to determine if these minor components originate from 5.8 S rRNA or minor contaminants. However, in view of its extensive purification, its migration as a single spot on two-dimensional gels [15], its apparently homogeneous 3′ terminal U_{OH} [6] and the molar yield of fragments containing Gm–Cp, it is clear that this 5.8 S RNA contains at least two major 5′ termini. As in yeast 5.8 S RNA [8], the terminal heterogeneity indicates either that there is sequence heterogeneity in the high-molecular weight rRNA precursor or that

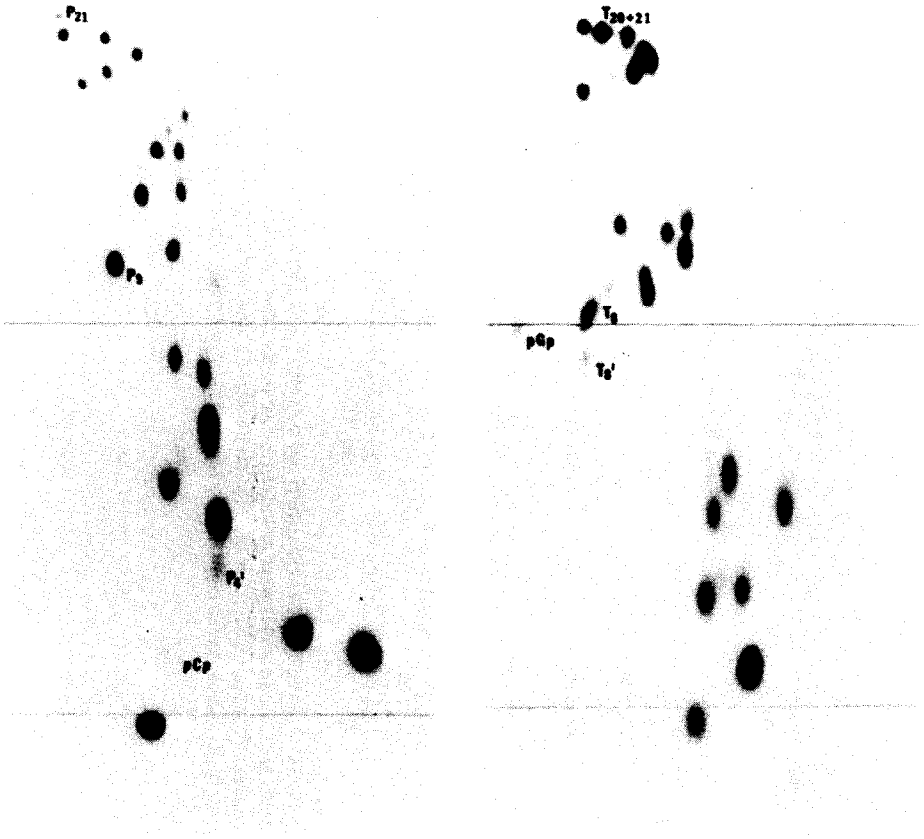

Fig. 2. Autoradiographs of two-dimensional fractionations of pancreatic (left) and T_1 (right) RNase digests of ^{32}P-labeled Novikoff hepatoma 5.8 S rRNA. Electrophoresis was from right to left on cellulose-acetate, at pH 3.5, and from top to bottom on DEAE-paper, in 7% formic acid. The 5′-termini and spots containing alkali-stable dinucleotides or pseudouridylic acid are labeled to correspond to the text.

there are alternative modes of splitting this precursor. In the latter case there may be several potential cleavage points for an endonuclease or the trimming of the molecule by an exonuclease may be somewhat relaxed. Heterogeneity in the 5′ nucleoside diphosphate termini also indicates that 5.8 S rRNA is an internal fragment of the 45 S nucleolar precursor produced by a 5′ nuclease.

Peak 2 (fig. 1b) contained about 1.6% of the total radioactivity. On the basis of four determinations (two by chromatography on columns, and two by electrophoresis on DEAE-paper, pH 1.9) the molar yield of Gm–Cp was found to be approximately 1 assuming a chain length of 180 nucleotides. The molar yield for Um–Gp, however, was consistently low, about 0.3–0.4 moles as was the yield for RNase digestion fragments containing Um–Gp (table 1). These results presumably reflect an under-methylation of the sequence G–G–U–G–G–A–Up. The corresponding non-methylated fragment G–G–Up has also been found in sub-molar amounts after pancreatic ribonuclease digestion.

Table 1
Distribution and sequences of methylated or modified oligonucleotides from ^{32}P-labeled Novikoff hepatoma 5.8 S rRNA

Spot	Nucleotide composition	Molar yield	Pancreatic RNase digestion products	T_1 RNase digestion products	U_2 RNase digestion products	Spleen phosphodiesterase digestion products	Sequence
P4′	GmC	1.0					Gm–Cp
P9	6GU + Gψ	6.7	(See fig. 4)				6G–Up + G–ψp
P21	AG_3UmGU	0.3		UmG,3G,AU		AG_2UmGU,AGUmGU[a]	G–G–Um–G–G–A–Up
T8′	UmG	0.3					Um–Gp
T8	4UG + ψG	4.2	(See fig. 4)				3U–Gp + ψ–Gp
T20	A_3U_2GmCG	1.0	A_2U,AG,GmC,U		U_2GmCAG,U_2GmCA, A	U_2GmCAG,UGmCAG, GmCAG	A–A–U–U–Gm–C–A–Gp

After determination of radioactivity each spot was eluted and identified by its nucleotide composition and by further complete digestion with pancreatic or T_1 RNase or partial digestion with U_2 RNase or spleen phosphodiesterase [18]. Molar yields were averages for five or six determinations and were calculated assuming a 180 nucleotide chain length for 5.8 S rRNA: a) This fragment could not be further digested stepwise with spleen phosphodiesterase indicating that the Um–Gp is at the 5′ end; b) T_{20} was separated from T_{21} by prolonged electrophoresis on DEAE-paper in 7% formic acid. These were also easily separated after treatment with alkaline phosphatase.

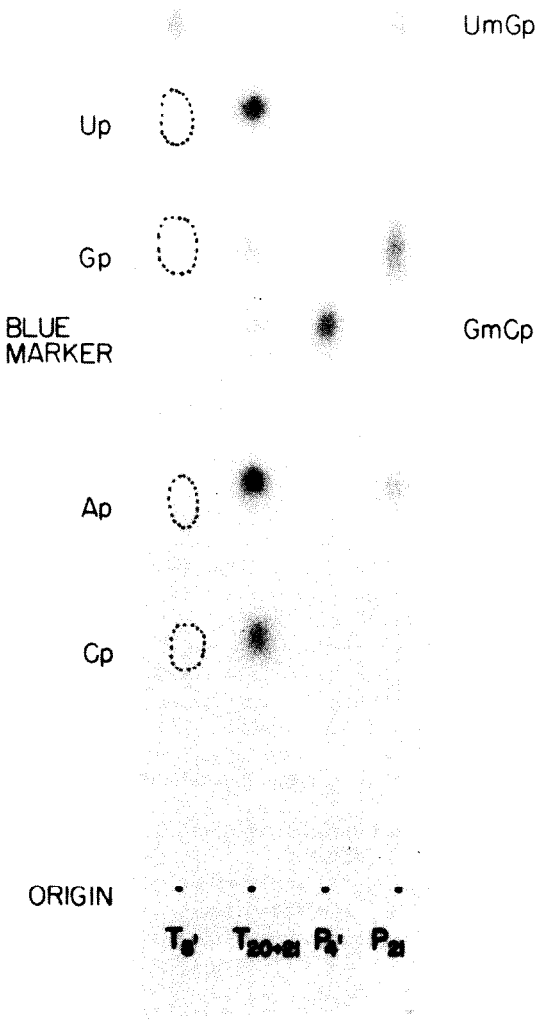

Fig. 3. Nucleotide analysis of oligonucleotides containing alkali-stable dinucleotides. ^{32}P-labeled oligonucleotides were eluted from DEAE-paper with 30% TEA-carbonate, digested with 0.3 N NaOH for 18 hr at 37°C and applied to Whatman 3 MM paper at pH 3.5. The broken circles indicate the positions of mononucleotide markers as observed with UV light. These are identified in the margin along with the approximate positions of the origin and the blue dye marker.

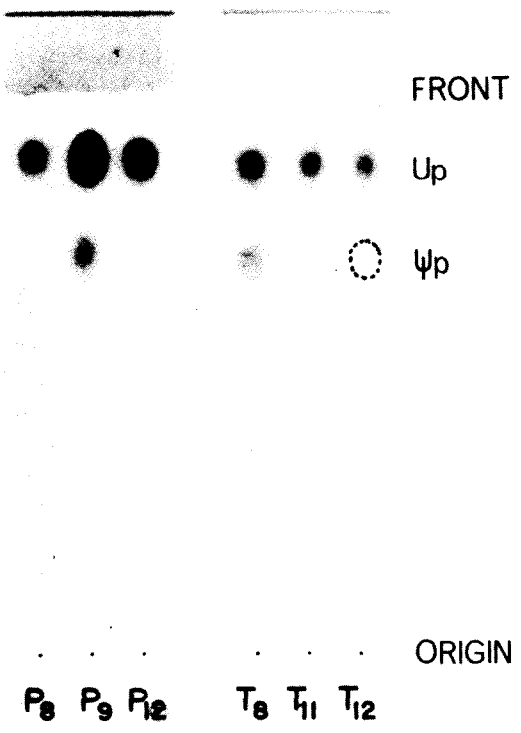

Fig. 4. Analysis of oligonucleotides containing pseudouridylic acid. ^{32}P-labeled oligonucleotides were eluted from DEAE-paper with 30% TEA-carbonate, digested with 0.3 N NaOH for 18 hr at 37°C and the nucleotides were fractionated by electrophoresis on Whatman 3 MM paper at pH 3.5. Spots corresponding to uridylic acid were eluted with water and further fractionated by chromatography on cellulose thin layers with propan-2-01–HCl–water [18]. The broken circles corresponds to a pseudouridylic marker as observed with UV light. The approximate positions of the origins and the solvent fronts are also given in the margin.

3.2. *Analysis of pancreatic and T_1 RNase digestion products*

Fig. 2 shows autoradiographs of complete pancreatic and T_1 ribonuclease fingerprints of hepatoma 5.8 S rRNA. The pancreatic ribonuclease fingerprint (fig. 2a) contained 21 major spots. Nucleotide composition analyses by alkali digestion and electrophoresis on Whatman No. 3 MM paper at pH 3.5 indicated that spot 4′ contained Gm–Cp and spot 21 contained Um–Gp (fig. 3). The T_1-ribonuclease fingerprint (fig. 2b) contained 22 major spots and nucleotide analyses indicated that spot 8′ contained Um–Gp and spot 20 contained Gm–Cp (fig. 3).

An earlier search for base modified nucleotides in hepatoma low molecular weight RNAs by Reddy et al. [21] indicated that 5.8 S rRNA did not contain base methylated nucleotides but contained a single pseudouridine residue. When the fingerprints were analyzed for pseudouridine a single residue (table 1) was detected in spot 9 of the pancreatic RNase map and in spot 8 of the T_1-RNase map (fig. 4).

The sequence information derived from further analysis of each spot is summarized in table 1. The alkali stable components appear in the sequences A–A–U–U–Gm–C–A–Gp and G–G–Um–G–G–A–Up respectively and the pseudouridine appears in the sequence Py–G–ψ–Gp. In preliminary partial digestion studies the fully methylated Gm–Cp fragment in Novikoff hepatoma 5.8 S RNA appears to be present in the end of a looped region [7] while the partially methylated Um–Gp component appears in a more highly hydrogen bonded region. Since methylation is not present in primitive eukaryotes such as yeast [7] its presence in higher forms such as wheat embryo [10] and rat is probably of evolutionary significance. Partial methylation may modulate the activity of 5.8 S rRNA. Although present in yeast the pseudouridine is contained in a different sequence, A–A–ψ–Up instead of Py–G–ψ–Gp. Whether it is present in a comparable region of the complete 5.8 S rRNA molecule will not be clear until the complete sequence is deduced.

Although labeling kinetics indicate a precursor product relationship between 45 S nucleolar RNA and 5.8 S rRNA, chemical evidence for this relationship is not available nor is the location of 5.8 S RNA within the 45 S precursor. The three modified sequences obtained in this study; A–A–U–U–Gm–C–A–Gp, G–G–Um–G–G–A–Up and Py–G–ψ–Gp will be useful markers for such chemical studies.

Acknowledgements

The authors wish to thank Mrs Rose K. Busch for supplying the Novikoff hepatoma bearing rats, Dr Yong C. Choi for the in vitro labeling of tumor cells and Mr Timothy W. Owens for technical assistance. These studies were supported by The Cancer Center Grant 10893-Pl.

References

[1] Forget, B. G. and Weissman, S. M. (1967) Nature 213, 878–882.
[2] Pene, J. J., Knight Jr., E. and Darnell Jr., J. E. (1968) J. Mol. Biol. 33, 609–623.
[3] Weinberg, R. A. and Penman, S. (1968) J. Mol. Biol. 38, 289–304.
[4] King, H. W. S. and Gould, H. (1970) J. Mol. Biol. 51, 687–702.
[5] Sy, J. and McCarty, K. S. (1970) Biochim. Biophys. Acta 199, 86–94.
[6] Prestayko, A. W., Tonato, M. and Busch, H. (1970) J. Mol. Biol. 47, 505–515.
[7] Rubin, G. M. (1973) J. Biol. Chem. 248, 3860–3875.
[8] Rubin, G. M. (1974) Eur. J. Biochem. 41, 197–202.
[9] Payne, P. I., Woledge, J. and Corey, M. J. (1973) FEBS Letters 35, 327–330.
[10] Azad, A. A. and Lane, B. G. (1973) Can. J. Biochem. 51, 606–612.
[11] Mauritzen, C. M., Choi, Y. C. and Busch, H. (1970 In: Methods in Cancer Research (Busch, H., ed.), Vol. VI, pp. 253–282, Academic Press, New York.
[12] Quagliarotti, G., Hidvegi, E., Wikman, J. and Busch, H. (1970) J. Biol. Chem. 245, 1962–1969.
[13] Rich, A. (1967) Methods Enzymol. 12, 481–491.
[14] Ro-Choi, T. S., Choi, Y. C., Savage, H. E. and Busch, H. (1973) In: Methods in Cancer Research (Busch, H., ed.), Vol. IX, pp. 71–153, Academic Press, New York.
[15] Reddy, R., Sitz, T. O., Ro-Choi, T. S. and Busch, H. (1974) Biochem. Biophys. Res. Commun. 56, 1017–1022.
[16] Sanger, F. and Brownlee, G. G. (1967) Methods Enzymol. 12, 368–381.
[17] Tener, G. M. (1967) Methods Enzymol. 12, 398–404.
[18] Brownlee, G. G. (1972) In: Laboratory Techniques in Biochemistry and Molecular Biology (Work, T. S. and Work, E., ed.), Vol. 3, pp. 1–265, North-Holland, Amsterdam, Oxford.
[19] Sulkowski, E. and Laskowski Jr., M. (1971) Biochim. Biophys. Acta 240, 443–447.
[20] Smith, J. D. (1967) Methods Enzymol. 12, 350–361.
[21] Reddy, R., Ro-Choi, T.-S., Henning, D., Shibata, H., Choi, Y. C. and Busch, H. (1972) J. Biol. Chem. 247, 7245–7250.