Studies on Ribosomal RNA and Ribosomal Precursor RNA in Mammalian Cells

by

Mohammad Salim, M.Sc.

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CONTENTS

ABBREVIATIONS

rRNA	Ribosomal RNA
rpre RNA	Ribosomal precursor RNA
DNase	Deoxyribonuclease
RNase	Ribonuclease
SDS	Sodium dodecyl sulphate
EDTA	Disodium salt of ethylene diamine tetra acetic acid
S	Sedimentation coefficient
PDE	Phosphodiesterase
VPDE	Venom phosphodiesterase
dpm	Disintegrations per minutes

SUMMARY

HeLa cell rRNA and its nucleolar precursors were examined by techniques for RNA fingerprinting and sequence analysis, using RNA which had been labelled *in vivo* with ^{14}C methyl methionine or ^{32}P phosphate. The following observations were made:-

1. Fingerprints obtained by digesting methyl labelled 18S RNA with T_1 ribonuclease or with a combination of T_1 ribonuclease plus alkaline phosphatase showed about 40 spots.
2. Fingerprints obtained by digesting 28S methyl labelled RNA in the same way showed about 54 spots.
3. The fingerprints of 28S RNA were widely different from those of 18S RNA, there being only 9 spots which are common to both RNA species.
4. 32S fingerprints were almost identical to 28S fingerprints in all respects.
5. The fingerprints of 45S RNA contain most of the 18S spots as well as all the 28S characteristic spots.
6. A close examination of 45S RNA showed that there are two 18S spots which are not present in 45S RNA and one 18S spot which is only weakly labelled in 45S RNA. These 18S products were found to contain methylated bases. They represent secondary

methylation of the RNA. All of the other methylated products characterized in this study contain 2'-O'methyl ribose groups. This suggested that most if not all of the early methylation of the precursor molecule is 2'-O-methylation while the secondary methylation which occurs late in the maturation process is base methylation.

7. A wide variety of sequences have been found to be methylated. Most of these sequences are present only once in the RNA molecule.
8. The methylation patterns of rRNA from three different mammalian species (man, HeLa; C13, hamster and L929 cells, mouse) were compared and found to show remarkable similarities.

SECTION 1

INTRODUCTION

Ribosomes are universal constituents of both prokaryotic and eukaryotic cells. They consist of two subunits, each containing RNA and proteins. Ribosomal RNA in mammalian cells (and probably in all eukaryotic cells) is believed to be synthesized in the nucleolus in the form of a high molecular weight RNA molecule, which then undergoes a series of specific cleavage to generate mature rRNA. This study was concerned with the structural analysis of mammalian cell rRNA and proof of its relationship to its presumed nucleolar precursors. In view of the availability of recent review articles (Maden 1968, 1971; Darnell, 1968; Perry, 1969; Spirin and Gavrilova, 1969; Nomura, 1970; Attardi and Amaldi, 1970; Burdon, 1971) covering various aspects of ribosome structure and formation, this introductory account will deal only with those aspects of rRNA and ribosome structure which are relevent to the present study.

1. 1. Ribosomes

50 - 63% of the mass of ribosomes consists of rRNA, the difference being made up of protein. A large amount of experimental evidence suggests the existence of two classes of ribosomes in living cells. One class, with a sedimentation coefficient of about 70S, is found in bacteria and in organelles (mitochondria & chloroplasts) of eukaryotic cells. Another class, with a sedimentation coefficient of approximately 80S, is found on the

endoplasmic reticulum or as free ribosomes in the cytoplasm of eukaryotic cells. All ribosomes consist of two dissimilar subunits, one small and another large, which dissociate reversibly during protein synthesis. The 70S ribosome contains 30S and 50S subunits. The 80S ribosome contains 40S and 60S subunits. The small subunit contains a 16 - 18S RNA molecule and a varity of proteins. The large subunit contains one large 23 - 28S (Kurland, 1960) and one small 5S RNA molecule and a large number of proteins. The ribosomes of many eukaryotic cells have a 7S RNA species attached by hydrogen bounds to the RNA of larger subunits (Pene, Knight and Darnell, 1968).

The high RNA content of ribosomes and other theoretical considerations led Crick (1968) to suggest that primitive ribosomes consisted entirely of RNA. They would then have reached their present level of complexity due to evolutionary development. Even in the ribosomes of higher animals RNA is believed to play an important structural role. Spectroscopic evidence from E. coli and reticulocyte rRNA suggests the presence of multiple short double helical regions (Cox, 1966). Hypochromicity and O.R.D. spectra of isolated rRNA and of intact ribosomes are similar (Schlessinger, 1960; Cotter, McPhie and Gratzer, 1967) suggesting that conformational properties inherent in rRNA are important determinants of ribosome structure. Dye binding experiments indicate that a large proportion of the

RNA is present on the surface of the ribosomes (Cotter, McPhie and Gratzer, 1967). This RNA might be involved in interaction with transfer RNAs, messenger RNA and other components of protein synthesis.

1. 2. High Molecular Weight rRNA

rRNA of different organisms differs not only in size but also in base composition. Moreover high molecular weight rRNA of various organisms are methylated to varying extents. In E. coli 16S RNA contains 22 methyl groups and 23S RNA has 27.5 methyl groups. (Fellner and Sanger, 1968). Animal and plant cell RNA shows a higher level of methylation (Brown & Attardi, 1965; Hudson, Gray and Lane, 1965; Vaughan et al., 1967; Lane and Tamaoki, 1969). The small rRNA component (16 - 18S) in E. coli as well as in animal cells is methylated to a greater extent than the larger rRNA (23 - 28S) (Brown and Attardi, 1965; Vaughan et al., 1967; Zimmerman and Holler, 1967; Burdon, 1966). Methylation can occur either on the base or on the ribose moiety (2'-OH group). The latter results in the resistance to alkaline hydrolysis of the phosphodiester bond between the 3'-hydroxyl group of that nucleotide and the 5'-hydroxyl group of neighbouring nucleotide (Brown and Todd, 1955). In E. coli rRNA more than 80% of the methyl groups are present on the bases (Fellner and Sanger, 1968). But in L. cells and HeLa cells 80 - 90% of the methyl groups have been reported as being on the ribose moieties (Brown and

Attardi, 1965; Vaughan et al., 1967; Lane and Tamaoki, 1969). However, Iwanami and Brown (1968) suggested that a higher value for 2'-O-methyl ribose may have been obtained due to losses of some unstable methylated bases during manipulations.

1. 3. Biosynthesis of High Molecular Weight rRNA

Nucleoli were first suspected to be the site of rRNA synthesis in animal cells (sea urchin, Drosophila cells and others) on the basis of cytochemical observations (Capersson and Schultz, 1940; Schultz, Capersson and Aquilonius, 1940; Brachet, 1942). Experiments using autoradiography then showed that most of the RNA of the cytoplasm was synthesized in the nucleus (Amano and Leblond, 1960; Sisken and Kinosita, 1961; Goldstein and Eastwood, 1966). Some ribonuclease sensitive granules similar to large ribosomal subunits were also found in the nucleolus by electron microscopy (Jacob and Sirlin, 1963; Karasaki, 1965; Holtzman, Smith and Penman, 1966). Parallel autoradiographic and sedimentation experiments were performed by Perry (1962) on tissue culture of L strain fibroblasts. He has shown that rRNA is synthesized in the nucleolus, using actinomycin D which blocks selectively the synthesis of nucleolar RNA. Combined use of electron microscopy and autoradiography then localized the fibrillar region of the nucleolus as the site of RNA synthesis (Firket and Granboulan, 1963). More recently kinetic studies on the labelling of the nucleoli have indicated that initially

labelling takes place in the fibrillar element of the nucleolus and apparently there is a transfer in a relatively short time of the isotope to the granular element (Unuma, Arendell and Busch, 1968). Finally genetic evidence for the association of rRNA production with nucleolar function came from the demonstration that anucleolate mutants of Xenopus laevis lack the ability to synthesize rRNA (Brown and Gurdon, 1964). These mutants also lack the ribosomal DNA. (Brown and Weber, 1968).

The discovery of a large RNA molecule sedimenting at 45S initiated the biochemical analysis of rRNA production in the mammalian nucleolus. Improvement of cell fractionation to yield uncontaminated nucleoli, nucleoplasm and cytoplasm provided new evidence for the synthesis of rRNA in the nucleolus (Penman, Smith and Holtzman, 1966). Several experiments which demonstrated the synthesis of different types of RNA in the nucleus or nucleolus may be summarized as follows. When HeLa cells are exposed for a short time to labelled RNA precursors such as uridine, radioactivity first appears in the nucleus in two types of rapidly sedimenting RNA. One class is characterized by its heterogeneous sedimentation (20 - 80S) in sucrose gradients (Warner et al., 1966) and a rapid turnover rate. This class is termed heterogeneous nuclear RNA. The other class of rapidly labelled nuclear RNA constitutes a homogeneous fraction. In HeLa cells it appears on sucrose

gradients as a peak emerging over a background of heterogeneous RNA. This peak has a sedimentation coefficient of approximately 45S (Sherrer and Darnell, 1962; Sherrer, Latham and Darnell, 1963) and is found only in the nucleolus (Penman, Smith and Holtzman, 1966). After about 20 minutes of labelling radioactivity begins to appear in two more species of RNA, 32S RNA which is also confined to the nucleolus, and 18S rRNA which passes rapidly to the cytoplasm. Finally after about 50 minutes of labelling in HeLa cells 28S RNA became labelled first in the nucleoplasm and shortly afterwards in cytoplasm (Penman, 1966; Penman, Smith and Holtzman, 1966). If after a short pulse with an RNA precursor actinomycin D is added to block further RNA synthesis, label disappears from 45S RNA and appears in the 32S and 18S components and (to a small extent) in 28S RNA (Girard, Penman and Darnell, 1964). It was also established that 45S and 32S remain structurally intact after heating and other types of denaturation, thus strongly suggesting that the polynucleotide chains are covalently continuous (Jeanteur, Amaldi and Attardi, 1968). These observations suggested that 45S RNA yields by cleavage 18S and 32S RNA, the latter is then converted to 28S rRNA.

Additional evidence that 45S RNA is the precursor to both rRNAs was obtained from the fact that rRNA and presumed precursors are methylated. Methylation of 45S RNA

takes place either during or immediately after synthesis (Greenberg and Penman, 1966; Zimmerman and Holler, 1967). If methyl labelled methionine is added to a culture of HeLa cells, label appears almost immediately in nucleolar 45S RNA. Exactly the same flow of radioactivity into 32S, 18S and 28S is observed with methyl label as with uridine either on continuous labelling or during an actinomycin chase (Greenberg and Penman, 1966; Zimmerman and Holler, 1967).

The relative extent of methylation was found to differ in 18S and 28S rRNA (Brown and Attardi, 1965; Iwanami and Brown, 1968). The distribution of methyl groups between the various alkali resistant dinucleotides of rRNA and nucleolar RNA was examined by Wagner, Penman and Ingram (1967). The 2'-O-methylation pattern of 32S rpre RNA was found to be identical to that of 28S rRNA, and that of 45S rpre RNA identical to that of an equimolar mixture of 28S and 18S rRNA. These findings provide further chemical evidence that 45S RNA contains both rRNA sequences and that 32S RNA contains the 28S sequences.

It was found by polyacrylamide gel electrophoresis that conversion of 45S RNA to rRNA involves some short lived intermediates which are present in very small amounts (Weinberg et al., 1967). These were termed 41S and 20S. In addition a small amount of 28S RNA can normally be detected in the nucleolus, proving that the 32S → 28S

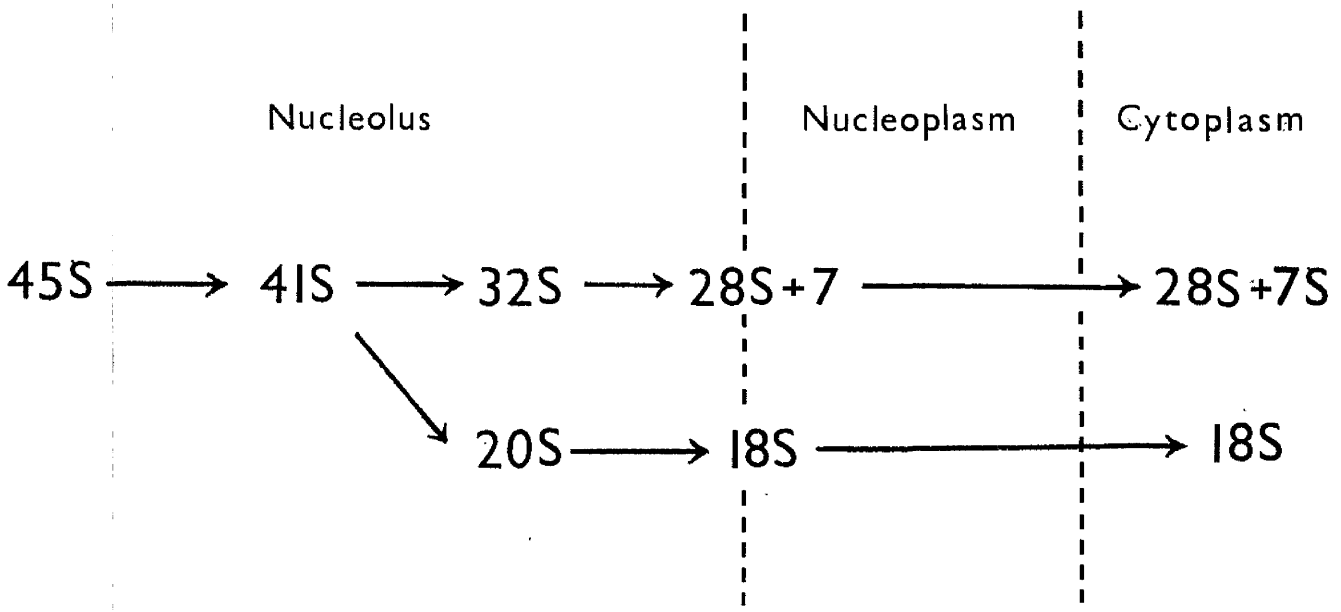


Fig. I.I Proposed maturation pathway for rRNA in HeLa cell nucleolus (modified from Weinberg and Penman, 1970)

transition takes place in this organelle. Kinetics of labelling of 41S and 20S indicated that they are true intermediates in rRNA production. After infection of HeLa cells with poliovirus, which interferes with normal nucleolar processing (Weinberg et al., 1967), some of the RNA species (41, 28, 20 & 18S) were found to accumulate in nucleolus. An analysis of their estimated molecular weights based on electrophoretic mobilities together with the levels of methylation of various intermediates indicated that 41S RNA contains approximately the same number of methyl groups as 45S and therefore contains both 18S and 28S material (Weinberg and Penman, 1970). 20S RNA was found to contain same number of methyl groups as 18S RNA and 32S to contain same number as 28S RNA (Weinberg and Penman, 1970). Figure 1.1. shows the scheme for rRNA maturation in HeLa cell which was inferred from these experiments.

1.4. Non-ribosomal Material

Determination of radioactive methyl to polynucleotide ratios (Weinberg et al., 1967; Weinberg and Penman, 1970) showed that the level of methylation increases approximately twofold during the conversion of 45S to 28S and 18S RNAs even though there is little or no addition of methyl groups during the maturation process. Determination of alkali resistant dinucleotide contents of these RNAs gave corresponding results (Vaughan et al., 1967). Taken together

Table 1.1 Molecular Weights of rpre RNA and rRNA

	45S	32S	28S	18S	Reference
Molecular weight x 10^{-6} calculated from					
(a) Sedimentation velocity	4.6	2.2	-	-	(1)
(b) Sedimentation equilibrium	4-4.5	2.4	1.9	0.71	(2)
(c) Acrylamide gel electrophoresis	4.1	2.1	(1.65)*	0.65	(3)

References:

(1) Amaldi and Attardi (1968); Jeanteur, Amaldi and Attardi (1968)

(2) McConkey and Hopkins (1969)

(3) Weinberg and Penman (1970)

* Value of Petermann and Pavlovec (1966) used for calibration.

these observations suggested that a considerable amount of non-methylated material is eliminated during processing of 45S to rRNA. In agreement with this conclusion hybridization experiments suggested that about 50% of the length of 45S RNA and 30% of 32S are represented by non-ribosomal sequences (Jeanteur, Amaldi and Attardi, 1968; Jeanteur and Attardi, 1969).

The base compositions of rRNA and its precursors have been determined with highly purified RNA (Amaldi and Attardi , 1968; Jeanteur, Amaldi and Attardi, 1968; Willems et al., 1968). The results showed that precursor RNA (45S and 32S) have significantly higher G + C content than 28S or 18S RNA. This implies that the non-ribosomal material in 45S and 32S in very high in G + C content.

Molecular weight of HeLa rRNA and rpre RNA have now been estimated by three methods, including sedimentation equilibrium and sedimentation velocity as well as electrophoretic mobility in polyacrylamide gels. The results from these methods are in reasonably good agreement (Table 1.1) with each other and also with the amounts of non-ribosomal material in rpre RNA estimated by hybridization and methylation data.

Summarizing all the above findings, HeLa cells 45S RNA contains approximately 50% of its length as non-ribosomal material and 32S RNA contains 30% of non-ribosomal material. This non-ribosomal material in HeLa cell rpre RNA is believed

to be of high G + C content and lacks methyl groups. It is eliminated during the maturation process.

1. 5 Low Molecular Weight rRNA

1. 5a 5S rRNA

The larger ribosomal subunit from bacterial (Elson, 1961; Rosset & Monir, 1963; Comb et al., 1965; Galibert et al., 1966) animal (Galibert et al., 1965; Brown and Littna, 1966; Bachvaroff and Tongur, 1966; Knight and Darnell, 1967) and plant cells (Chakravorty, 1969; Li and Fox, 1969) contain a low molecular weight rRNA component. This RNA is found to sediment at 5S. Its base composition is quite different from that of high molecular weight rRNA. In all cells examined so far, except yeast it has a high G + C content (Rosset, Monier & Julien, 1964; Comb & Zehavi-Willner, 1967; Hatlen, Amaldi and Attardi, 1969; Forget & Weissman, 1969; Marcot-Queiroz et al., 1965) and lacks pseudouridine (Hatlen, Amaldi and Attardi, 1969). Another feature which distinguishes it from high molecular weight rRNA is the absence of methylated nucleotides (Schleich and Goldstein, 1966; Galibert et al., 1965; Hatlen, Amaldi and Attardi, 1969). Since in eukaryotic cells 45S rpre RNA loses about 50% of its polynucleotide sequences during 45S → 18S + 28S RNA transition, which are G + C rich, and non-methylated, it was suggested that 5S RNA might be derived from 45S rpre RNA.

It was found however that in HeLa cells newly labelled 5S RNA appears in cytoplasmic ribosomes more slowly than

newly formed 28S RNA. This suggests a nuclear pool of 5S RNA (Knight and Darnell, 1967). Moreover actinomycin D which completely blocks the formation of rpre RNA at low concentration was found to cause relatively little inhibition of most nuclear RNA synthesis (Perry, 1962; Penman, Vesco and Penman, 1968). Under these conditions the synthesis of 5S RNA is apparently not inhibited (Perry and Kelley, 1968). Furthermore major part of the 5S molecule in HeLa cells has ppGp- and pppGp- at the 5'-end (Hatlen, Amaldi and Attardi, 1969). This would restrict any possibility of derivation of 5S RNA from the 45S rpre RNA to one copy per molecule. But a large excess of 5S was found in the cells. These observations in HeLa cells indicated separate synthesis of 5S RNA. In agreement with this conclusion anucleote mutants of Xenopus leavis which lack the genes for high molecular weight rRNA were found to possess DNA complementary to 5S RNA (Brown and Weber, 1968). It has also been found that during oogenesis in amphibian the genes for 18S and 28S RNA are amplified but not those of 5S RNA (Brown and Dawid, 1968). Furthermore DNA complementary to 5S RNA in Xenopus leavis displays a buoyant density lower than the DNA which contains the genes for 18S and 28S RNA (Brown and Weber, 1968). These observations indicate that in eukaryotes 5S RNA is synthesized independently of 45S rpre RNA and from extranucleolar sites.

The sequence of 5S RNA from KB (human) tumor cells

(Forget and Weissman, 1967, 1969) E. coli (Brownlee, Sanger and Barrell, 1967, 1968) and a mouse cell line (Williamson and Brownlee, 1969) have been determined. Two sequences of 5S RNA are present in E. coli, differing in position 13 where either A or G may occur. An interesting finding is that the sequence of mouse and human (KB tumour cells) 5S RNA are identical. However a partial sequence analysis of HeLa 5S RNA yielded a trinucleotide (ApGpCp) which was absent from KB tumour cell 5S RNA (Hatlen, Amaldi and Attardi, 1969). The presence of this trinucleotide, together with the reproducibly much lower than expected molar yield of larger oligonucleotides (Hatlen, Amaldi and Attardi, 1969) suggested the occurrence of alternate sequences at various sites in the 5S molecule of human cells.

1. 5b 7S RNA

The larger ribosomal subunit of eukaryotic cells contains a small 7S RNA molecule which separates from 28S rRNA by treatments which disrupt hydrogen bonds.(Pene, Knight and Darnell, 1968). Weinberg and Penman (1969) who further characterized it as 5.7S RNA have referred it to as 28SA RNA (28S associated). It is found in a wide variety of eukaryotes (Sy & McCarty, 1969; Burdon & Clason, 1969; King & Gould, 1970; Uden & Warner, 1972). It does not contain methylated nucleoside and is about 130 nucleotide long. It was found associated in molar amounts not only with cytoplasmic 28S RNA but also with nucleoplasmic 28S RNA

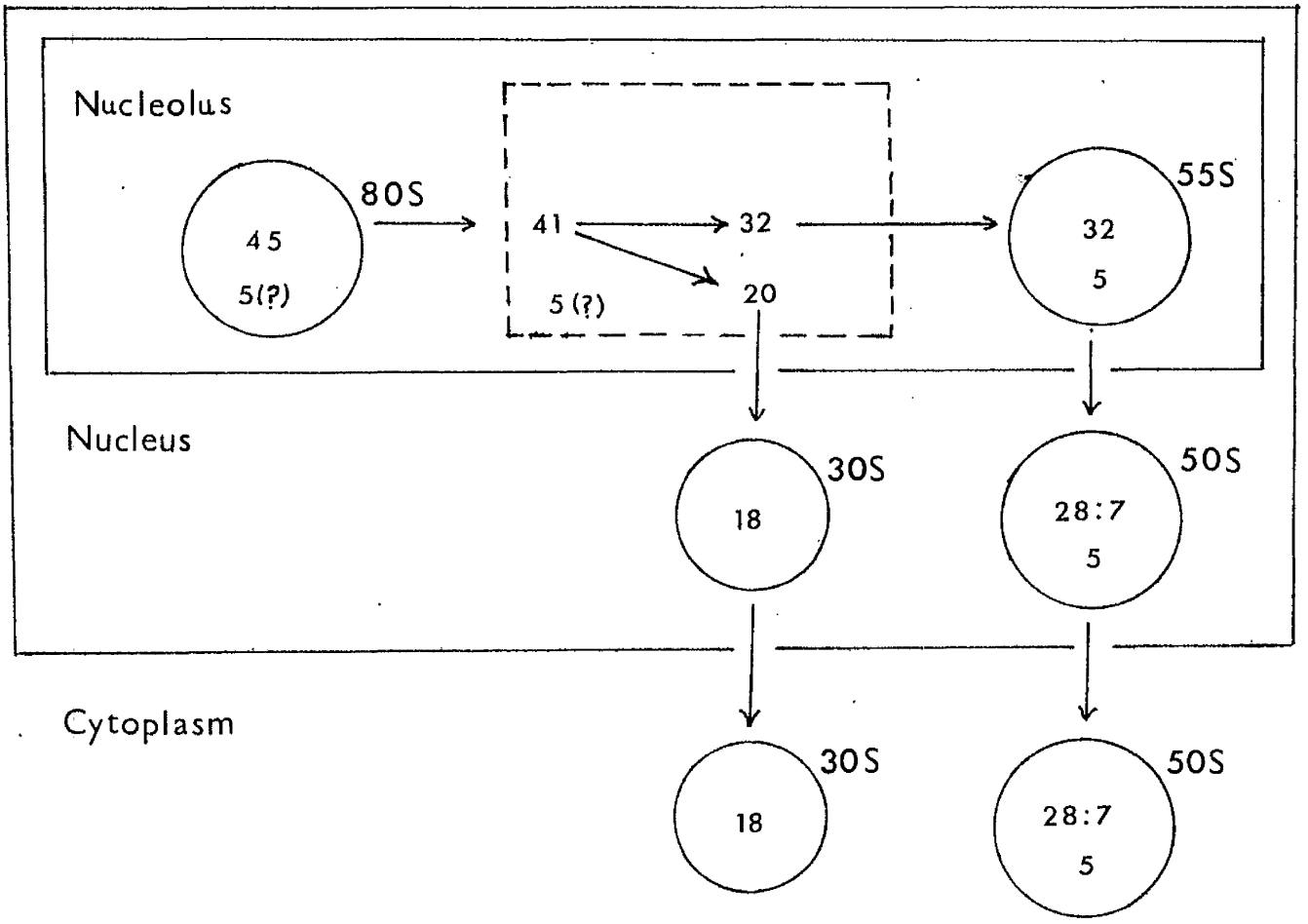


Fig. 1.2 Schematic representation of ribosomal formation in HeLa cells. The numbers outside the particles represent sedimentation constants of the particles. The numbers inside the particles represent the sedimentation constants of the RNA species which they contain. Note that the 30S particle is found in the nucleoplasm only in very small amounts (adapted from Maden, 1971).

derived from nucleolar 50S ribosomal precursor particles (Pene, Knight and Darnell, 1968). 7S RNA is not released from 32S RNA after heating or other types of denaturation implying that it is covalently linked within the 32S molecule. Kinetics of labelling of this RNA are identical to those of 28S rRNA, suggesting that it is generated in 32S → 28S conversion.

1.6 Assembly of Ribosomes

Ribonucleoprotein particles containing rpre RNA have been extracted from the nucleoli of various cultured mammalian cells (Tamaoki and Mueller, 1965; Tamaoki, 1966; Yoskikawa-Fukada, 1967). Two types of particles were isolated from nucleoli of HeLa cells, sedimenting in EDTA at 80S and 55S (Warner and Soeiro, 1967). The 55S particles contain 32S and most of the proteins associated with 28S RNA in the 50S cytoplasmic ribosome, but lack one protein which associate with 50S particle in the cytoplasm. On the other hand 80S particles which are present in much smaller amounts than 55S are found to contain 45S RNA and some 32S RNA (Warner and Soeiro, 1967). More recently (Shepard and Maden, 1972) these 80S nucleolar particles from HeLa cells have been shown to contain small amounts of peptides characteristic to the small ribosomal subunit (30S) as well as to the large subunit (50S). These observations indicate that 80S nucleolar particles are the common precursor to both ribosomal subunits. Large ribosomal subunits (50S) are then produced via the 55S

intermediate particles (figure 1.2).

It is evident that coordination between rpre RNA and ribosomal structural protein synthesis is necessary for efficient production of ribosomes. In fact such coordination was revealed by the following observations. Complete and instantanious cessation of protein synthesis by cyclohexamide (Willems, Penman and Penman, 1969; Craig and Perry, 1970) or 70 to 80% inhibition during valine starvation (Maden et al., 1969) resulted in a balanced slowdown of the synthesis of 45S RNA and its conversion to 32S RNA. Similarly a gradual inhibition of protein synthesis over a 1.5 hours induced by short term incubation of HeLa cells in hypertonic medium (Robbins, Pederson and Klein, 1970) resulted in a reduced rate of 45S RNA processing. The protein content of 80S and 55S particles containing 45S and 32S RNA respectively, were also decreased (Pederson and Kumar, 1971). It was shown that after inhibition of RNA synthesis by the drug camptothecin, the amount of ribosomal structural protein is also decreased in the nucleolus whereas other nucleolar proteins remain present in near normal amounts (Wu, Kumar and Warner, 1971).

These results from cultured cells point to an apparent feedback control between ongoing rate of protein synthesis in the cytoplasm and the intranucleolar steps in rRNA processing and ribosome assembly.

1.7 Outline of Present Study

The present study was undertaken to further examine the

process of rRNA formation in mammalian cells and to carry out a partial sequence analysis of rRNA. HeLa cells were selected in view of the amount of previous information available concerning ribosome formation in these cells and also because of ease of radioactive labelling of these cells. A comparative study of rRNA was also done using other cell lines.

The introduction of oligonucleotide fingerprinting techniques (Sanger, Brownlee and Barrell, 1965) greatly helped the analysis of these RNA species. The high molecular weight rRNAs are large molecules and therefore yield a large number of oligonucleotides on digestion with endonucleases. It was desirable to label selectively some of these oligonucleotides in the RNA so that simpler fingerprints could be obtained. As already mentioned these RNAs contain a number of methyl groups. These can be selectively labelled by growing cells in the presence of methyl labelled methionine, and the resulting methyl labelled RNA may be fingerprinted. Fingerprints prepared in this way were much simpler than fingerprints of ^{32}P labelled RNA and showed the following.

(a) The methylation pattern of 18S and 28S RNAs are quite different from each other. There were a few common spots and a large number of unique spots (Their absolute frequencies have been determined and a number of them have been sequenced in this study).

(b) 45S RNA contains the great majority of methylated

sequences characteristic of both 18S and 28S RNA but lacks three spots which are unique to 18S RNA.

(c) 32S RNA contains only 28S material.

(d) All the methylated oligonucleotides analyzed in this study contain only 2'-O-methyl ribose except the three oligonucleotides found in 18S RNA but not in 45S RNA. These three products contain methylated bases and represent secondary methylation.

(e) A comparative study of the rRNA showed remarkable similarities and few dissimilarities in the methylation patterns of three different mammalian rRNA species.

SECTION 2

MATERIALS & METHODS

MATERIALS

1. Tissue Culture Material

HeLa cells (monolayer and suspension) BHK-21/C13 and L cells were used. Eagle's medium and filtered calf serum were purchased from Flow Laboratories, Irvine, Scotland. Sabouraud medium and brain heart infusion broth used to check the contamination by yeast and bacteria were purchased from Oxoid Ltd., London. Difco Laboratories, Detroit, Michigan provided trypsin, agar and PPLO broth for production of PPLO agar plates and tryptose phosphate broth. Streptomycin/ Penicillin were purchased from Vestric Ltd., Glasgow.

2. Composition of Media & Standard Solutions

(a) Eagle's medium

This was prepared by the method of Busby, House and McDonald (1964). It contains Penicillin (100 units/ml), Streptomycin (100 ug/ml), 0.2 ug/ml of an antimycotic agent, n-butyl, p-hydroxy benzoate and 0.002% (w/v) phenol red.

(b) Buffered saline solution (BSS)

This consisted of 1mM $MgSO_4$, 1mM NaH_2PO_4, 1.8mM $CaCl_2$, 5.4mM KCl, 0.116M NaCl and 0.002% (w/v) phenol red and was adjusted to pH 7.0 by the addition of 8.4% (w/v) $NaHCO_3$.

(c) Trypsin/citrate

Trypsin was made up as a 0.25% (w/v) solution in citrate buffer which consisted of 10.5mM NaCl, 1mM sodium citrate and 0.002% (w/v) phenol red adjusted to pH 7.8 using NaOH.

(d) Reticulocyte standard buffer (RSB)

This consisted of 0.01M NaCl, 0.01M tris and 0.0015M $MgCl_2$. It was adjusted to pH 7.4 with HCl (Penman, 1966).

(e) High ionic strength buffer (HSB)

This buffer contained 0.5M NaCl, 0.05M $MgCl_2$ and 0.01M tris. It was adjusted to pH 7.4 with HCl.

(f) Lithium, EDTA, tris, SDS buffer (LETS)

This consists of 0.1M lithium chloride, 1mM EDTA, 0.01M tris, 0.5% SDS. It is adjusted to pH 7.4 with HCl.

3. Chemicals

Dinucleotides monophosphates were purchased from Calbiochem Ltd., London. Actinomycin D was also supplied by Calbiochem Ltd. in vials containing 200 ug. Adenosine and guanosine were obtained from Koch-Light Lab., Colnbrook, England. Methylated purines were purchased from Sigma Chemical Co. Ltd., London.

4. Radio-chemicals

L - [Me - ^{14}C] methionine (58Ci/Mole), L - [Me - ^{3}H] methionine (100 - 500 Ci/Mole) and ^{32}P orthophosphate (90 - 120 Ci/mg P) were purchased from Radiochemical Centre, Amersham, Bucks., England. ^{14}C methionine was obtained as solid, sealed in vacuo in ampoules, to prevent oxidation. ^{3}H Methionine was supplied in solution and ^{32}P orthophosphate in dilute HCl solution.

5. Enzymes

T_1 Ribonuclease (crystalline) made by Sankyo Co. Ltd., Tokyo, Japan was purchased from Calbiochem. Bacterial alkaline phosphatase (electrophoretically purified), venom phosphodiesterase and spleen phosphodiesterase were purchased from Worthington Biochemical Corporation. Pancreatic ribonuclease (chromatographically homogeneous) was purchased from Worthington Biochemical Corporation.

6. Material for Electrophoresis & Chromatography

Cellulose acetate strips measuring 2.5 cm by 85 cm. were cut from sheets of this material supplied by Oxoid Ltd., Southwark Bridge Rd., London. Whatman chromatography paper DE81 (DEAE-cellulose paper) was obtained from McCulloch Brothers, Glasgow in rolls (46 cm x 50 m) and was cut to the required size. Whatman 52, 3MM and No. 1 papers were purchased from Reeve-Angel Scientific Ltd., London. Xylene cyanol FF, orange G and acid fuchsin were purchased from G.T. Gurr Ltd., London.

7. Material for Autoradiography

Kodirex X ray film 35 x 43 cm., D-19 Developer powder and Kodafix solution were purchased from Kodak Ltd.

8. Scintillation Fluids

Toluene scintillation fluid was prepared by dissolving 5 g of 2,5 diphenyl oxazole in one litre of analar toluene. Dioxan based scintillation fluid was constituted by 7 g of 2,5 diphenyl oxazole dissolved with 100 g naphthalene in one litre of scintillation grade dioxan.

METHODS

1. Cell Culture Systems

Cells growing in monolayers were used in most of the experiments. However a few of the earliest experiments were conducted with S3 suspension HeLa cells.

(a) Suspension cells

HeLa S3 cells were grown in suspension. They were routinely cultured in 250 ml bottles containing a small magnet mounted on a glass rod or hanging free. This magnet spins and keeps the cells in suspension. Usually 150 ml of medium was placed in the bottle with 20×10^4 cells/ml. The cells were allowed to grow for one or two days when they were again diluted to the previous concentration. Air inside the bottle was replaced by an atmosphere of 5% CO_2 in air. A cotton stopper was used for the bottle to permit aeration.

(b) Monolayer cells

HeLa BHK/C13 and L cells were cultured as monolayers in rotating 80 oz. winchester bottles according to the method of House and Wildy (1965). 180 ml of Eagle's medium (Busby et al, 1964) containing 7% calf serum were used per winchester. The buffer capacity of the medium was maintained by replacing the air with an atmosphere of 5% CO_2 in air. The winchesters were seeded with 2×10^7 cells and were incubated at 37°C for 2 - 3 days. The cells were removed from the glass with trypsin citrate solution. They were suspended in Eagle's medium and were dispersed in appropriate concentration into

Roux or further winchester bottles. Although the medium contains antibiotics, cell lines were tested for contamination by micro organisms as follows. The cultures were examined for fungi andyeasts using Sabourauds medium. Bacterial contamination was checked with blood agar plates. Contamination by pleuropneumonia like organisms (PPLO) was monitored by using PPLO agar plates.

All glassware used for tissue culture methods was soaked overnight in chloros, washed, rinsed in distilled water and sterilized by autoclaving.

2. Incorporation of Radio-isotopes

Most of the RNA of the cells is present in the cytoplasm and the nucleoli contain 3 to 5% of the total cellular RNA. Therefore in order to obtain high specific activity ribosomal RNA it is necessary to label a small number of cells for a fairly long time. But for nucleolar RNA labelling, a large number of cells must be labelled to obtain sufficient RNA. High specific activity nucleolar RNA could be obtained by labelling for a relatively short time as the transit time for nucleolar RNA is much shorter than the life time of ribosomal RNA. Incorporation of radio-methionine was carried out in medium which was free of unlabelled methionine to obtain high specific activity ribosomal RNA. About 4.5 million cells were labelled for 36 - 40 hours ($1\frac{1}{2}$ generations) with 100 μCi of ^{14}C methyl methionine (about 1.7 uMoles = $\frac{1}{2}$ normal conc.) in 40 ml of

medium. Purine nucleosides (3×10^{-5}M) were added to the medium to suppress the uptake of label into the purine ring system. It was found that this does not completely block purine labelling. In later experiments 10mM sodium formate was also added. This completely stopped ring labelling. This concentration of formate does not seem to cause any harm to the cells as they grow and divide in 20mM or even in 25 mM formate. For nucleolar RNA labelling about 40 - 50 million cells were labelled with 100μCi of ^{14}C methyl methionine. They were allowed to grow for 2 hours in 30 ml of methionine-free medium containing purine nucleosides and formate.

To prepare high specific activity ^{32}P labelled RNA the cells were first labelled with 5mCi of ^{32}P orthophosphate in normal medium. However RNA obtained in this way was of low specific activity. It was necessary to prepare a high specific activity RNA for rapid manipulations. Therefore the concentration of phosphate in the labelling medium was decreased so as to increase the specific activity of radiophosphate in the medium, which in turn will lead to the production of high specific activity RNA. Different phosphate concentrations were tried and 1/6 normal phosphate concentration (14 mg phosphate/L) was found to be most suitable for 24 hours labelling.

3. Preparation of Ribosomal RNA

The cells were harvested using trypsin citrate solution

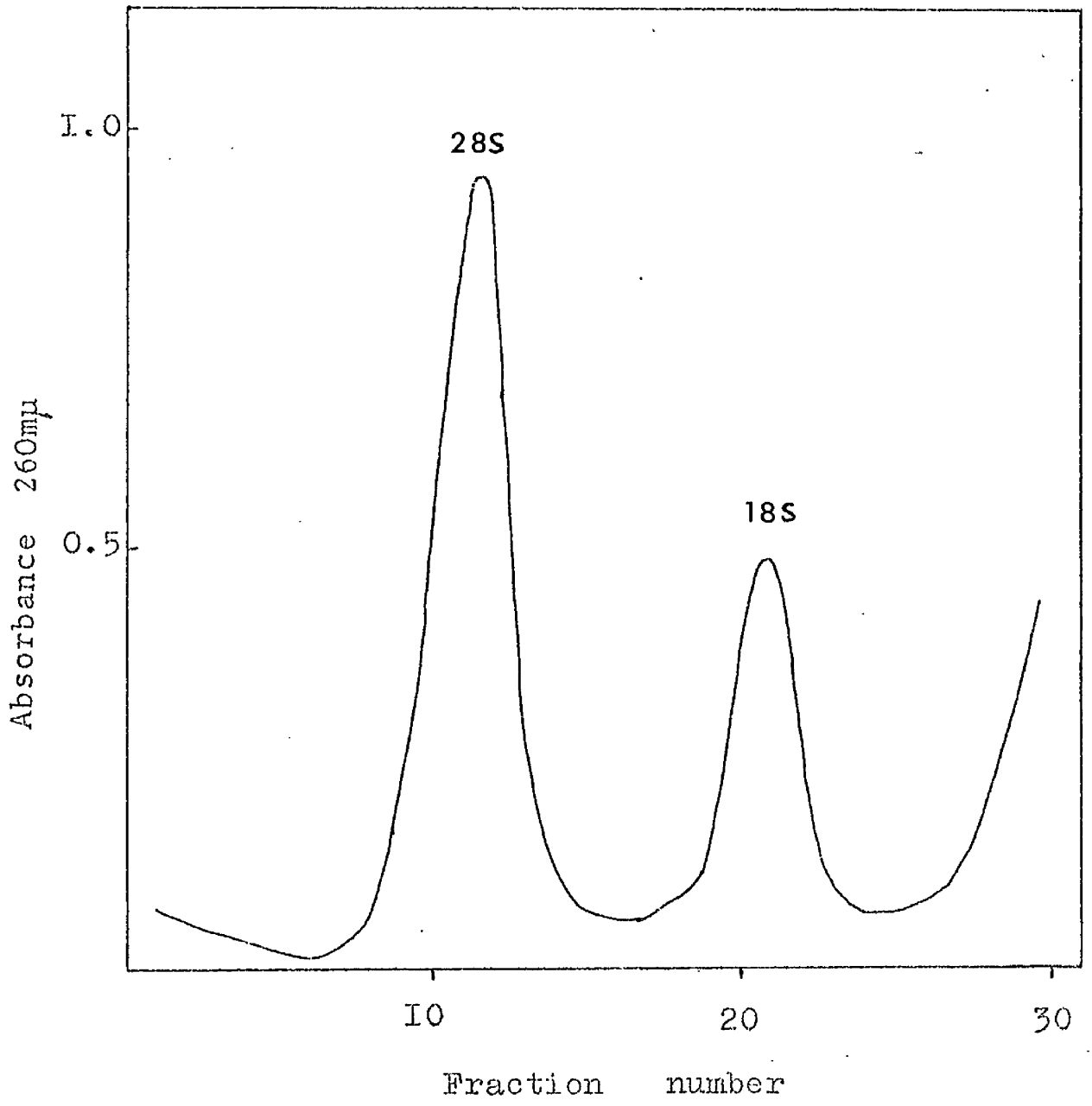


Fig. 2.1 Fractionation of 18S and 28S RNA by centrifugation through 15 - 30% sucrose gradient.

to detach them from the glass surface. They were then washed twice in cold BSS and resuspended in 2 ml of the hypotonic buffer RSB. The cells were allowed to swell for about 10 minutes and then broken with a stainless steel ball homogenizer. The homogenate was then centrifuged and cytoplasm was separated from nuclei. (Penman, 1966). In ^{14}C methyl labelling experiments the cytoplasm was made 1% to SDS and was layered on 15 to 30% sucrose gradient in LETS. It was centrifuged for 17 hours at 26,000 rev./min. in SW27. After centrifugation the gradient was pumped through a Gilford spectrophotometer to record the optical density. 18S and 28S RNA peak fractions were pooled. RNA was precipitated by the addition of ethanol. It was found by labelling the cells with ^{35}S methionine (as shown in figure 2.2) that some of the protein containing methionine moved into the gradient in 18S and 28S peaks. It was necessary to remove this small amount of protein. The ethanol-precipitated RNA was therefore extracted with cold phenol and then twice reprecipitated with ethanol. In case of ^{32}P labelling experiments the cytoplasm was first extracted with cold phenol, precipitated once with ethanol and then layered on 15 - 30% sucrose gradient in LETS.

4. Preparation of Preribosomal RNA

HeLa cells (about 5×10^7 cells) were harvested using trypsin citrate solution. They were washed twice with cold BSS and resuspended in 2 ml of hypotonic buffer RSB. The cells were allowed to swell for 10 minutes. They were

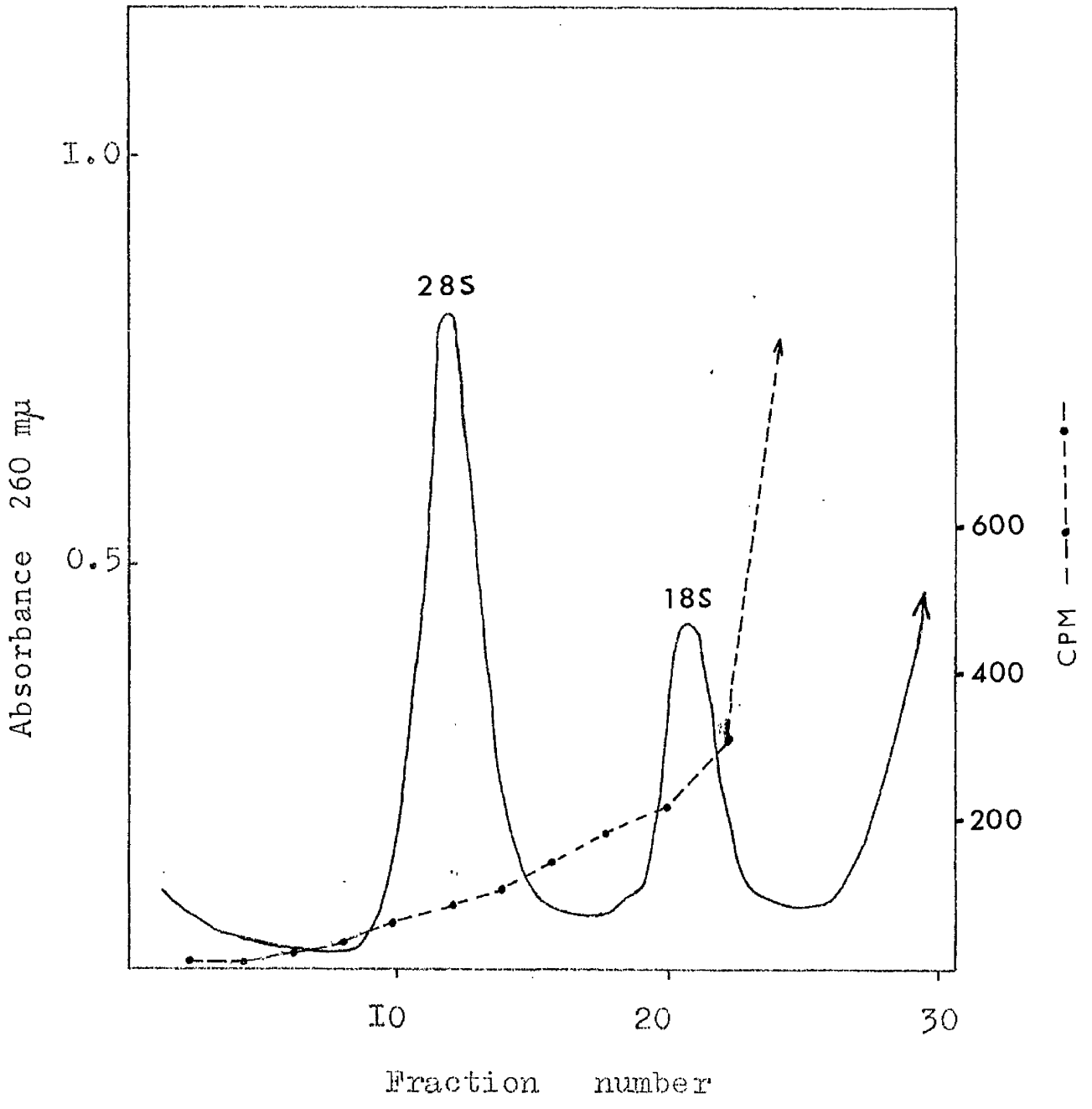


Fig. 2.2 Fractionation of 18S and 28S RNA from ^{35}S methionine labelled HeLa cells by centrifugation through 15 - 30% sucrose gradient.

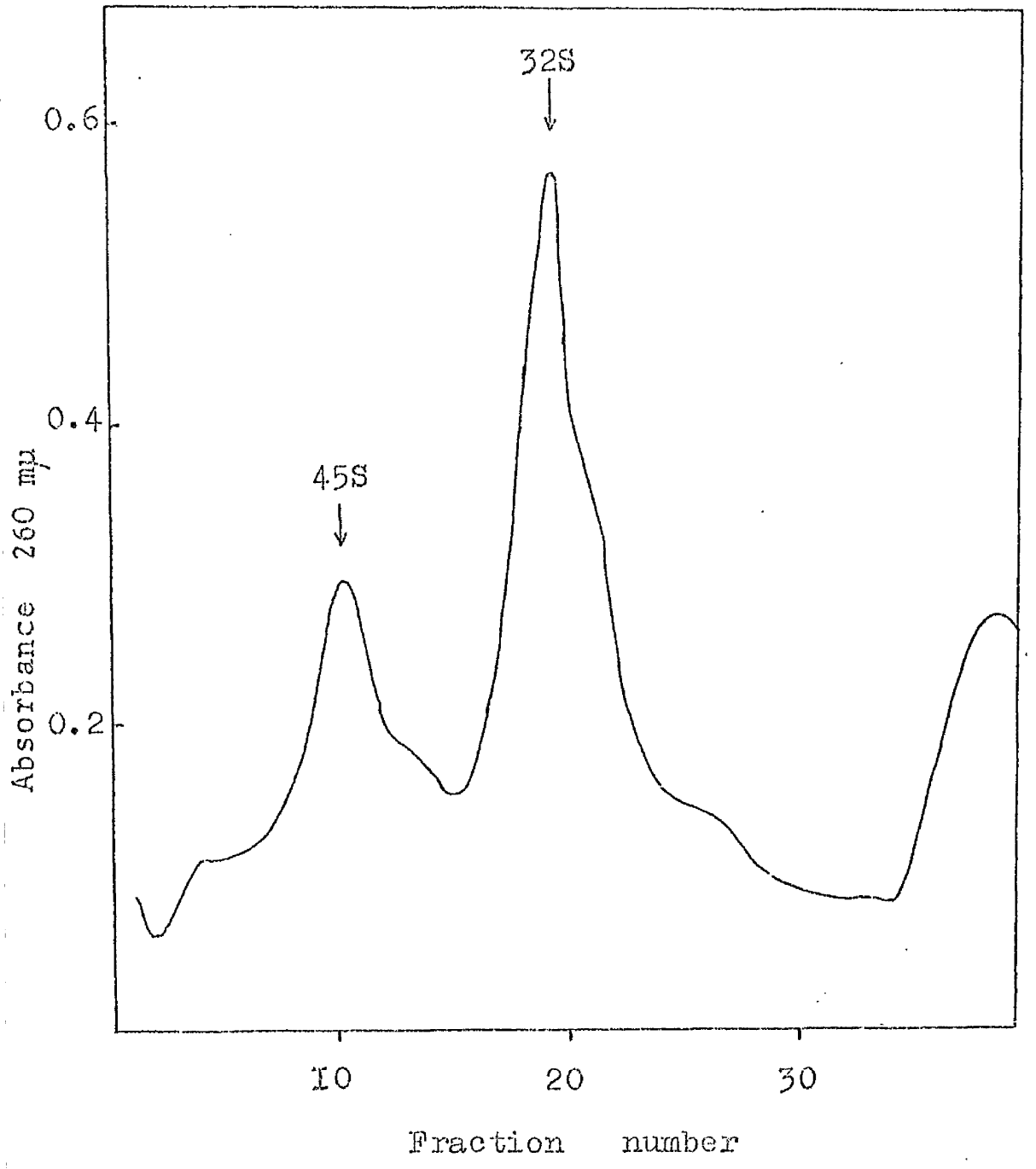


Figure 2.3 Fractionation of nucleolar RNA by centrifugation through 15 - 30% sucrose gradient.

homogenized in a stainless steel ball homogenizer. The nuclei were removed by centrifugation and again resuspended in 2 ml of RSB by vigorous pipetting. They were once more centrifuged and resuspended in 2 ml of RSB and 0.3 ml of a mixture of two detergents (one part of a 10% w/w solution of sodium deoxycholate and two parts of a 10% w/w solution of tween 40) was added. The nuclear suspension was shaken in a vortex mixer for 3 seconds. The mixture was centrifuged and pure nuclei were obtained. At this stage the nuclei can not be readily dispersed, since they show a strong tendency to aggregate. Two ml of HSB containing about 100 µg of DNase (RNase free) were added. The nuclei were resuspended with the aid of a pasteur pipette. The mixture was very viscous at this stage. It was then kept in a water bath at 37°C with continuous agitation till it was no longer viscous and all the DNA had been digested (Penman, 1966). The mixture was then layered on 15 to 30% sucrose gradient in HSB and centrifuged for 15 minutes at 22,000 rev./min. A pellet of pure nucleoli was obtained. It was dissolved in LETS and was layered on 15 to 30% sucrose gradients in LETS. The gradient was centrifuged in SW25 at 21,000 rev./min. for 16 hours. After centrifugation the gradient was pumped through a Gilford spectrophotometer and an optical density tracing was obtained. The peak fractions were pooled. They were extracted with cold phenol and precipitated twice with ethanol. It has been found by fingerprinting that 32S peak

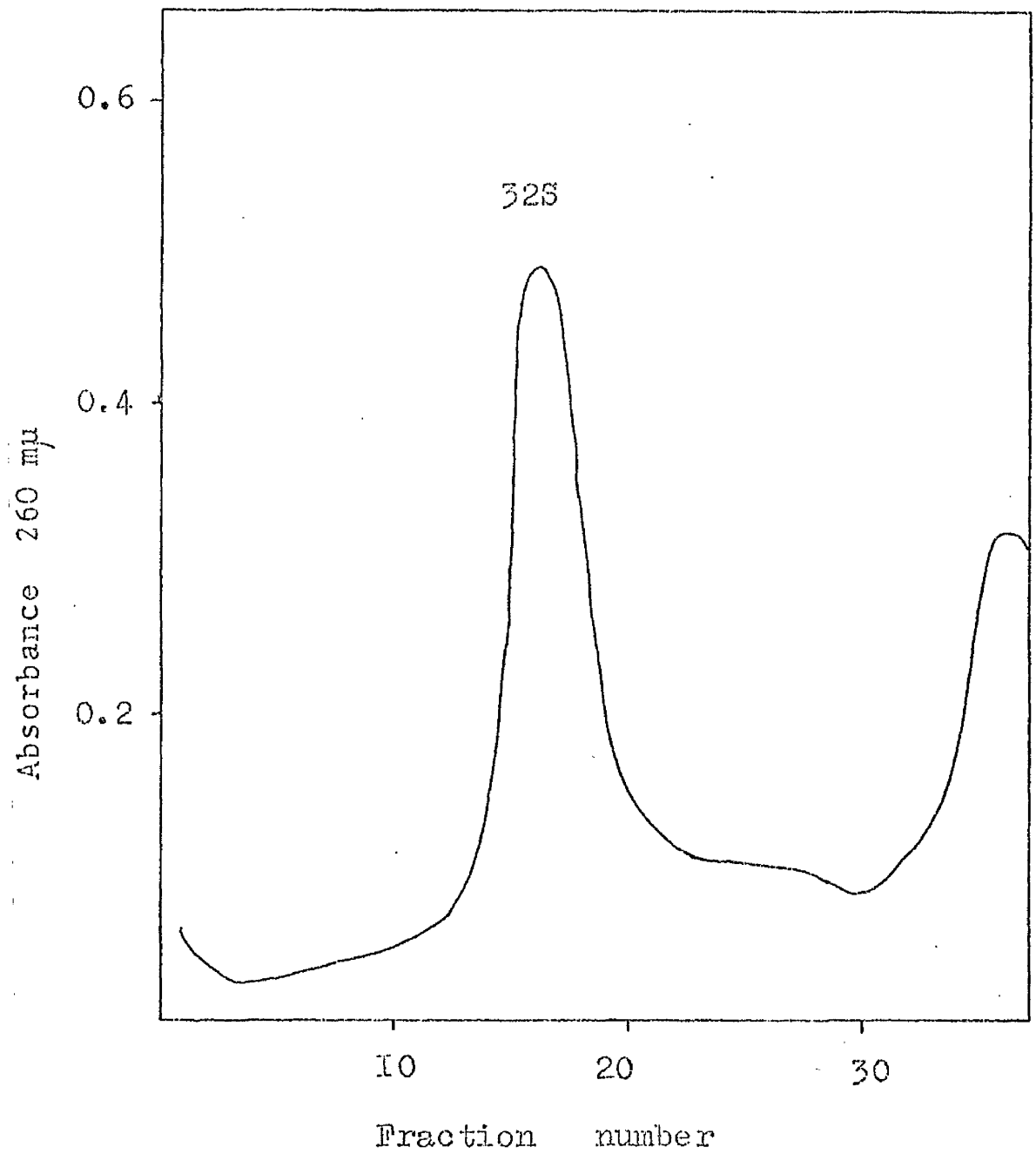


Figure 2.4 Fractionation of nucleolar RNA from actinomycin D treated HeLa cells by centrifugation through 15 - 30% sucrose gradient.

always contains very small amount of 18S material presumably due to the breakdown of some of the 45S RNA during manipulation. A preparation of 32S RNA containing no 45S breakdown products was obtained as follows. The cells were labelled for two hours with ^{14}C methyl methionine. Actinomycin was then added to 4 μg/ml and the cells were harvested after 45 minutes. The 45S RNA was therefore eliminated. The nucleoli were prepared as described above and layered on SDS gradient. Figure 2.4 shows the optical density tracing of such a gradient.

The purified RNAs were of the following specific activities.

^{14}C Methyl labelled RNA.

(a) 18S 2.5 - 3.3 x 10^3 dpm/μg

(b) 28S 1.6 - 2.3 x 10^3 dpm/μg

^{32}P labelled rRNA about 10^5 dpm/μg

5. Enzymic Digestion of RNA

50 to 60 μg quantities of RNA were lypholyzed in a siliconized tube. They were digested with T_1 ribonuclease to obtain oligonucleotides with a G residue at the 3′-terminus. An enzyme to substrate ratio of 1:20 was used. RNA was incubated for 30 minutes at 37°C with T_1 ribonuclease in about 0.05 ml of 0.01M tris buffer (pH 7.4) containing 0.002M EDTA (neutralized).

6. Two-dimensional Fractionation Procedure

The procedure described by Sanger et al (1965) was

used. Cellulose acetate strip was moistened with pH 3.5 buffer containing 7M urea. The point of application which was about 10 cm. from the cathode end of the strip was blotted and the digest was applied as a spot. Marker dye mixture was applied on both sides of the spot. The whole strip was blotted and quickly immersed in white spirit. It was then placed on the perspex supporting rack in the electrophoresis tank and was subjected to electrophoresis at 4.5 kilovolts. Most of the oligonucleotides ran between slowest pink and blue dye with the exception of some containing no uridylic acid residues which ran slower than blue. Electrophoresis was continued until the distance between slowest pink and blue was about 31 cm. The material from slowest pink to about 8 cm. behind the blue was then transferred to DEAE-paper in the following way. The cellulose acetate strip was held in air to drip off the extra spirit and was then placed on DEAE-paper sheet about 10 cm. from the cathode end. A pad of five strips of Whatman 3MM paper that had been soaked in water was then put on the cellulose acetate strips and a glass plate was placed on the top to press the strips. The water from the Whatman paper moved to DEAE-paper through cellulose acetate and carried the oligonucleotides which were then held by DEAE-paper due to its charge. The paper was dried and the urea was removed by ethanol. About half of the DEAE-paper was wetted with 7% formic acid. Marker dye mixture was applied and the

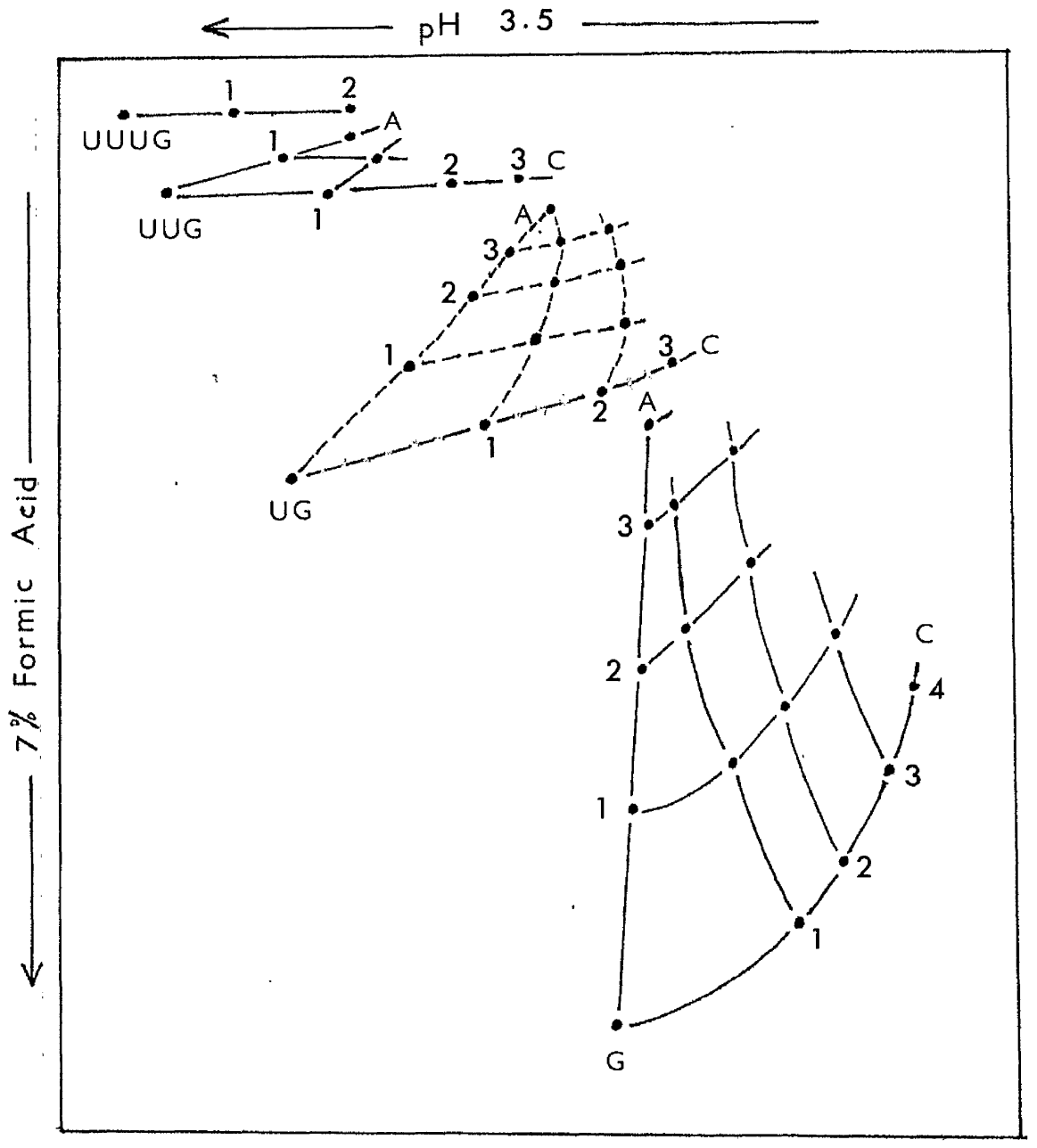


Figure 2.5 A diagrammatic representation of the fractionation of oligonucleotides from T_1 ribonuclease digest, according to their composition, as described by Sanger et al (1965).

paper was placed on a perspex rack. The rest of the paper was then wetted (If the whole of the paper is wetted in the first instance it is impossible to place it on the rack as it tears due to its fragility). It was then subjected to electrophoresis and was run at 1.3 kilovolts till the blue dye migrated about 40% of the length of the paper.

Uridylate (U)-rich oligonucleotides move rapidly in the first dimension but slowly in the second dimension. Therefore they separate into "graticules" containing no U residue, one U, two U residues and so on. Oligonucleotides containing more than three U residues are strongly bound by DEAE-paper and move very little or not at all in the second dimension. To improve the fractionation of such oligonucleotides Brownlee and Sanger (1967) digested the RNA with T_1 ribonuclease and bacterial alkaline phosphatase. The phosphatase removes the 3'-terminal phosphate group from the T_1 oligonucleotides thus lowers their net negative charge. Therefore all of the oligonucleotides move faster in the second dimension except those which do not contain uridylic acid residue. Another advantage of using dephosphorylated oligonucleotides is that they are easily digested with snake venom phosphodiesterase for sequence determination. For "T_1 plus phosphatase" digestion an enzyme to substrate ratio of 1:10 was used for T_1 ribonuclease and 1:5 for bacterial alkaline phosphatase. The RNA was originally digested for one hour in 0.01M tris at pH 8.5. But the digestion was

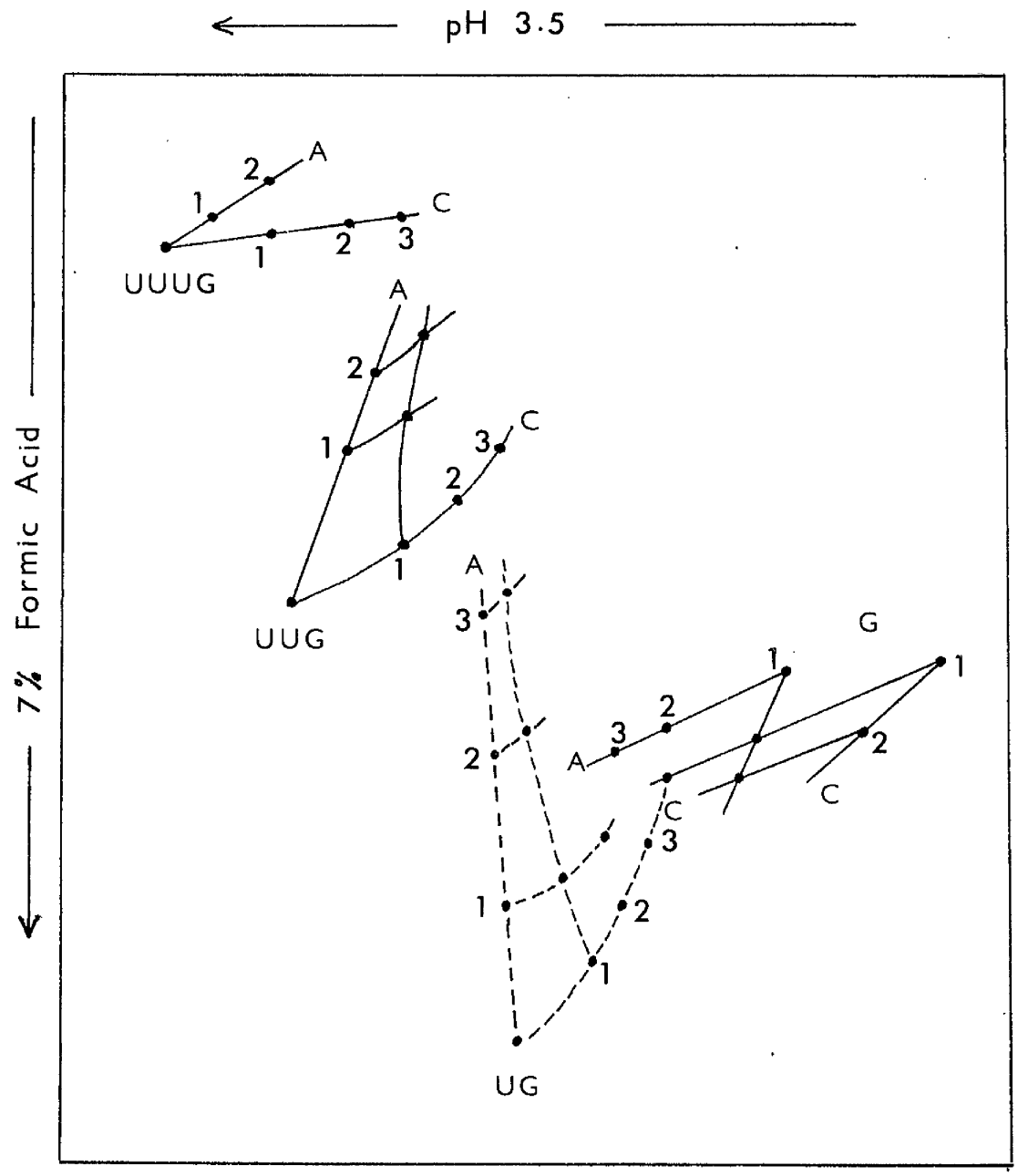


Figure 2.6 A diagram of the fractionation of dephosphorylated oligonucleotides from T_1 RNase plus alkaline phosphatase digest. (Adapted from Brownlee and Sanger, 1967).

found to be incomplete. This was found to be due to small amounts of ammonium sulphate present in alkaline phosphatase, decreasing the pH. Increasing the buffering capacity of the buffer to 0.02M tris resulted in complete digestion. The products were separated as in the case of T_1 digests except that the blue dye was allowed to migrate about 80% of the length of the paper in the second dimension. In doing so the dephosphorylated oligonucleotides containing no or one U residue migrate into the anode buffer.

The dye mixture which was used to follow the electrophoresis consisted of 2% xylene cyanol FF (blue), 4% orange G (yellow) and 2% acid fuchsin (pink).

7. Autoradiography

The papers were thoroughly dried and marked with a radioactive ink containing ^{35}S sulphate. This enabled the autoradiograph and paper to be properly aligned and thus the spots could be accurately cut out for subsequent manipulation. The papers were placed in contact with Kodirex X ray films in appropriate folders. Autoradiography of ^{14}C was carried out in one mm aluminium folders and of ^{32}P in 0.5 mm lead backed folders (Sanger et al, 1965).

8. Triethylamine Carbonate Solution 30% (v/v)

30 ml of triethylamine was mixed with 70 ml of distilled water. Solid CO_2 was added till the triethylamine phase disappeared.

9. Elution

Nucleotides are bound ionically to the DEAE-paper and cannot be eluted by water. A 30% aqueous solution of triethylamine carbonate was employed to elute the material. The eluate volume was 0.05 to 0.1 ml. This elute was placed as a spot on a polyvinyl chloride sheet stuck to a glass plate and was dried at 65°C. The nucleotides from Whatman paper (see below) were eluted by distilled water in drawn out capillaries and dried on polyvinyl chloride sheet.

10. Determination of Oligonucleotide Composition by Alkaline Hydrolysis

After elution and drying the oligonucleotides were dissolved in about 0.01 ml of 0.2N NaOH. They were taken into capillaries which were later sealed at both ends. After incubation for 16 hours at 37°C the hydrolysate was applied to Whatman 52 paper for electrophoresis at pH 3.5. It was allowed to run for about one hour at 4.5 kilovolts. The four mononucleotides were well separated on this system and the composition of each digest could be determined either by scintillation counting or by visual inspection of the autoradiograph. (Nearly all of the methylated products in this study also yielded on alkali resistant dinucleotide. See results.)

11. Enzymic Digestion of Oligonucleotides

The oligonucleotides obtained from the two dimensional fractionation procedure were eluted by triethylamine carbamate. Their sequence was determined by degrading them with

different nucleases. Microtechniques described by Sanger et al (1965) were used. The material was dissolved in 0.01 ml of digestion mixture and was then taken into a small capillary. For long incubations the capillary was sealed to avoid evaporation. The digestion products were separated on Whatman 52 or on DEAE-paper. These products were frequently eluted and hydrolysed with alkali to determine their composition. The digestion conditions for different enzymes were as follows.

(a) Pancreatic ribonuclease

In order to achieve complete hydrolysis of oligonucleotides by this enzyme the samples were treated with 2 µg of pancreatic ribonuclease in 0.001M EDTA, 0.01M tris buffer pH 7.4. The solution was taken up in a capillary and was incubated for 30 minutes at 37°C. After incubation the samples were mixed with 0.002 ml of 0.5N HCl and again incubated for one hour at 37°C to break down any cyclic phosphates. The products were then separated by electrophoresis on Whatman 52 paper at pH 3.5 (Sanger et al 1965). Many products can be recognized by their mobilities.

(b) Complete digestion with spleen phosphodiesterase

In this study this enzyme was used to digest alkali resistant dinucleotides which, however, were found to be somewhat resistant to the enzyme. The dinucleotide was dissolved in the following mixture: 0.1M ammonium acetate (pH 5.7), 0.002M EDTA, 0.05% Tween 80, 0.5 mg enzyme per ml.

The mixture was incubated at 37°C for 4 to 5 hours.

(c) Complete digestion with snake venom phosphodiesterase

The oligonucleotides were first treated with bacterial alkaline phosphatase to remove the terminal 3'-phosphate group as follows. The material was dissolved in 0.010 to 0.015 ml of 0.02M tris buffer (pH 8.5) containing 1 mg phosphatase/ml. After incubation for one hour at 37°C the products were separated on Whatman No.52 paper at pH 3.5. The bands were located by autoradiography, cut out and eluted with water. These dephosphorylated oligonucleotides were then treated with a mixture containing 0.1 mg venom phosphodiesterase/ml in 0.025M tris buffer (pH 8.5), 0.01M magnesium acetate. After incubation for two hours at 37°C the mononucleotides were separated by electrophoresis at pH 3.5 on Whatman No. 52 paper.

(d) Partial digestion with snake venom phosphodiesterase

The dephosphorylated oligonucleotide was dissolved in a mixture of 0.02 mg enzyme per ml in 0.02M tris buffer (pH 8.5), 0.01M MgCl. It was incubated at 37°C and the material was spotted on DEAE-paper after 10, 20 and 30 minutes. The products were separated by electrophoresis at pH 3.5.

12. Acid Hydrolysis & Chromatography

^{14}C Methyl labelled oligonucleotides or mononucleotides were digested with acid to liberate purine bases and pyrimidine nucleotides. The sample was dissolved in 0.01 ml of N HCl and was incubated for one hour at 100°C in a sealed

capillary. It was then applied to Whatman No. 1 paper for descending chromatography in the following systems:

(a) 1-butanol 86%, NH_3 5%, water 9% (Zimmerman, 1968);

(b) isopropanol 68%, conc. HCl 17.4%, water 14.6% (Wyatt, 1951).

(c) isopropanol 70%, conc. NH_3 1%, water 29% (Markham and Smith, 1952).

SECTION 3

RESULTS

3. 1 Relationship Between rRNA and rpre RNA by Fingerprinting of Methyl Labelled RNA

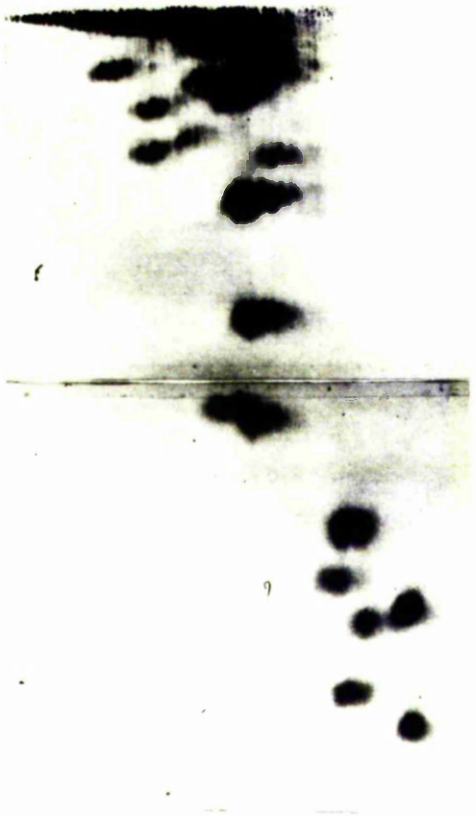
In the following pages ribosomal RNA from HeLa cells is compared with preribosomal 45S and 32S RNA on the basis of their methylation patterns. The first set of fingerprints (figure 3.1) was obtained from suspension HeLa Cells. The next two sets of fingerprints which are technically of better quality were obtained from monolayer HeLa Cells (figure 3.3 & 3.5).

3. 1a Fingerprints from Suspension HeLa Cells

Suspension HeLa cells were labelled with ^{14}C methyl methionine in the presence of unlabelled adenosine and guanosine ($3 \times 10^{-5}M$) to suppress uptake of label into the purine ring system. The autoradiographs of T_1 RNase digests of RNA are shown in figure 3.1. There is a fairly large number of spots and some unresolved material at the origin of the second dimension. The methylation pattern of 28S and 18S RNA show qualitative differences. The 28S fingerprint contains more spots and many of them are unique to 28S RNA. There are four spots which are present in the 18S fingerprint only (No. 8, 41, 42 & 43). Some of the spots are common to both of these RNA species. There are reproducible intensity differences between different spots in the same autoradiograph. For example spots 3 and 21 are always the densest ones in the 28S RNA fingerprint. In the 18S RNA fingerprint spot 30 is

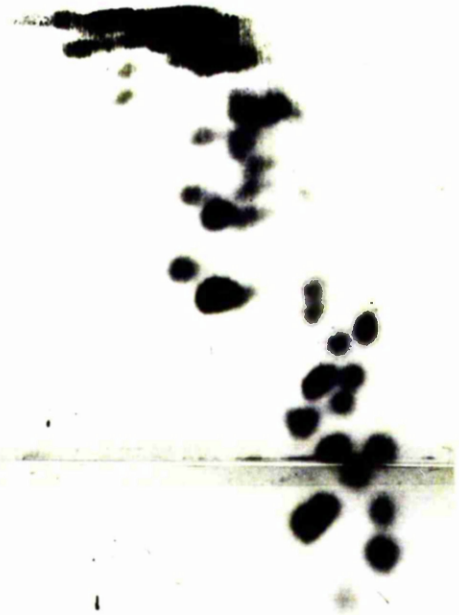
Fig. 3.1 Fingerprints of ^{14}C methyl labelled rRNA and rpre RNA from suspension HeLa cells

Suspension HeLa cells were labelled for 36 hours with ^{14}C methyl methionine. The cells were harvested and ribosomal and precursor (32S & 45S) RNAs were prepared. The purified RNA was digested with T_1 ribonuclease and fingerprinted as described in methods section. The first dimension (pH 3.5) is from right to left and the second dimension (7% formic acid) is from top to bottom. 18S and 28S fingerprints were run longer than 32S and 45S RNA. Radioactive spots were located by autoradiography.



18S

28S



45S

32S

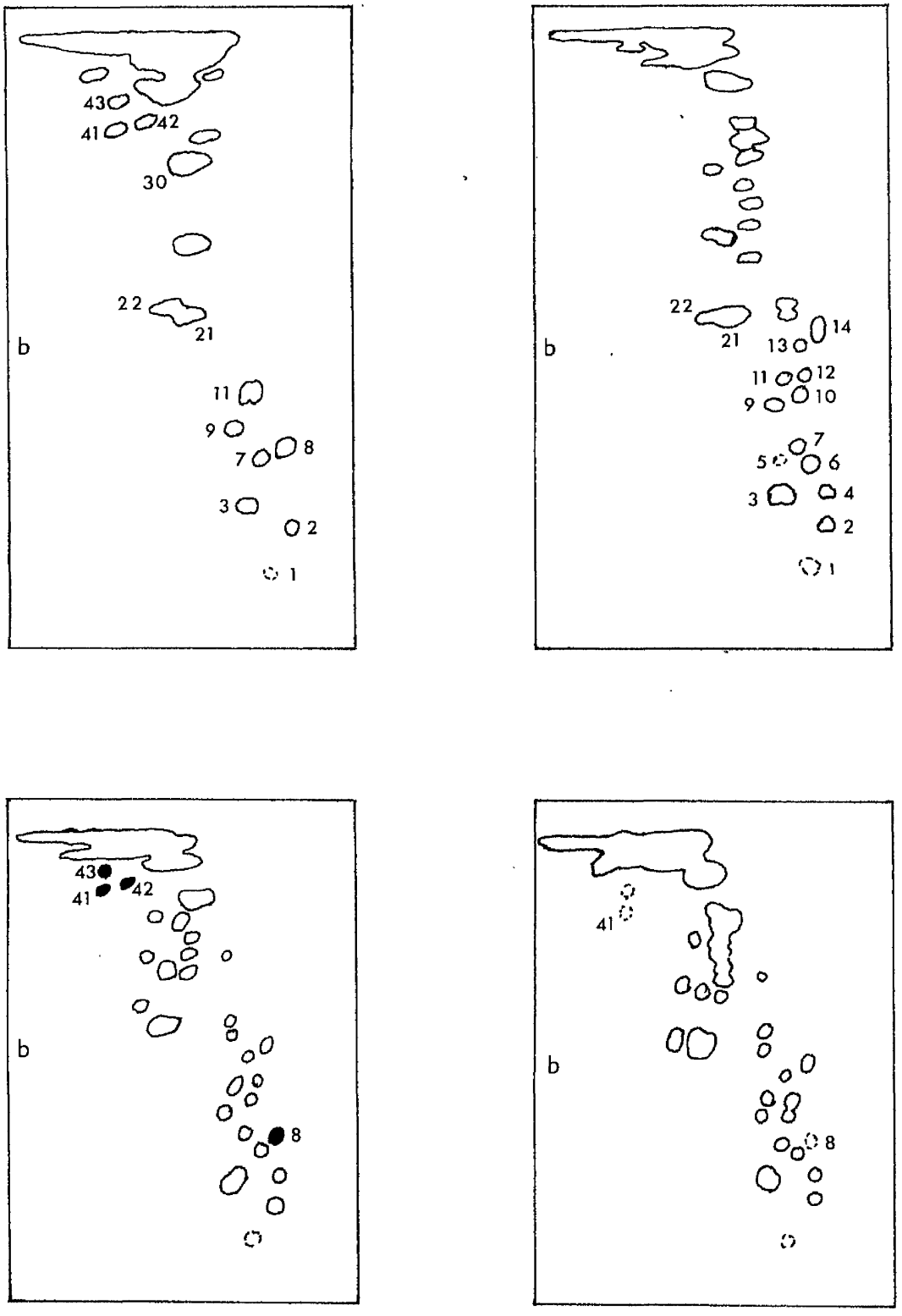


Fig. 3.2 Key to the fingerprints of HeLa cells rRNA and rpre RNA shown in figure 3.1. 18S RNA unique spots are marked black. The dotted circles represent faint spots. The position of blue dye is marked with b.

always densest. There are two faint spots in 28S (No. 1 & 5) and one (No. 1) in 18S RNA. They are heavily labelled when the cells are grown in the absence of purines. They correspond to G and AG in digests of ^{32}P labelled RNA and represent faint residual labelling of the purine ring system. They are completely eliminated when formate is added to the labelling medium (see below).

The fingerprint of 32S RNA is similar to that of 28S RNA. There is good qualitative and rough quantitative correspondence between all the well resolved spots in the two fingerprints. The 28S RNA fingerprint was run for a longer time as compared to 32S. Therefore the spots in 32S are not as well resolved as in the 28S RNA fingerprint. There are four very faintly labelled 18S spots in the 32S fingerprint (number 8, 41, 42 & 43) probably due to the breakdown of 45S RNA during extraction. They were completely eliminated from the later fingerprints. The fingerprint of 45S RNA contains all the spots present in 32S and 28S RNA. It also contains the four spots which are present in 18S only, and in fact resembles the fingerprints of 28S and 18S RNA superimposed.

3. 1b. Fingerprints from Monolayer HeLa Cells

Suspension cell growth medium contains about ten times more phosphate than the one used for monolayer cells. For a sequence analysis using ^{32}P it is necessary to use a medium containing low phosphate concentration in order to obtain high specific activity RNA. Therefore in all the later

experiments monolayer cells were used. First ^{14}C methyl labelled RNA fingerprints were prepared and then some of the methylated oligonucleotides were sequenced as described in the next section.

The ^{14}C methyl labelled RNA fingerprints were prepared from RNA which was first extracted with cold phenol to remove trace amounts of labelled protein. Therefore the origin in the second dimension is clearer in these fingerprints. Residual purine ring labelling was completely suppressed by the addition of formate as well as purine nucleosides to the growth medium. The fingerprints obtained are fairly similar to those obtained from suspension cells. When equal amounts of 18S and 28S RNA are mixed and fingerprinted, the resulting fingerprint is very similar to that of 45S RNA apart from four differences which are as follows. The 45S RNA fingerprint shows relatively faint labelling of spot 30 as compared to 18S + 28S RNA fingerprint. There are two spots (No. 34 & 49) which are present in 18S + 28S RNA fingerprint but are absent from 45S RNA. One spot (No. 38) is present in 45S RNA but is absent from 18 + 28S fingerprint. In order to make further comparison between ribosomal and preribosomal RNA, some of the corresponding spots from these fingerprints were digested with alkali. They were then run side by side at pH 3.5. Figure 3.4 shows that the same methylated products were obtained from the corresponding spots (digested in this study) of the fingerprint with the exception of spot 9.

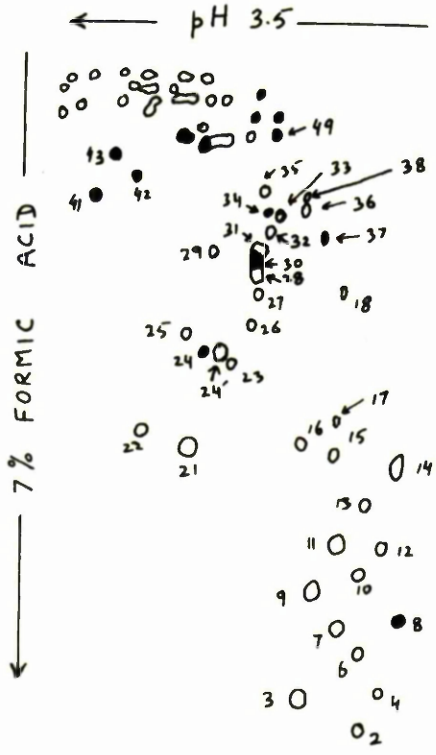
18S

28S

32S

45S

18S + 28S



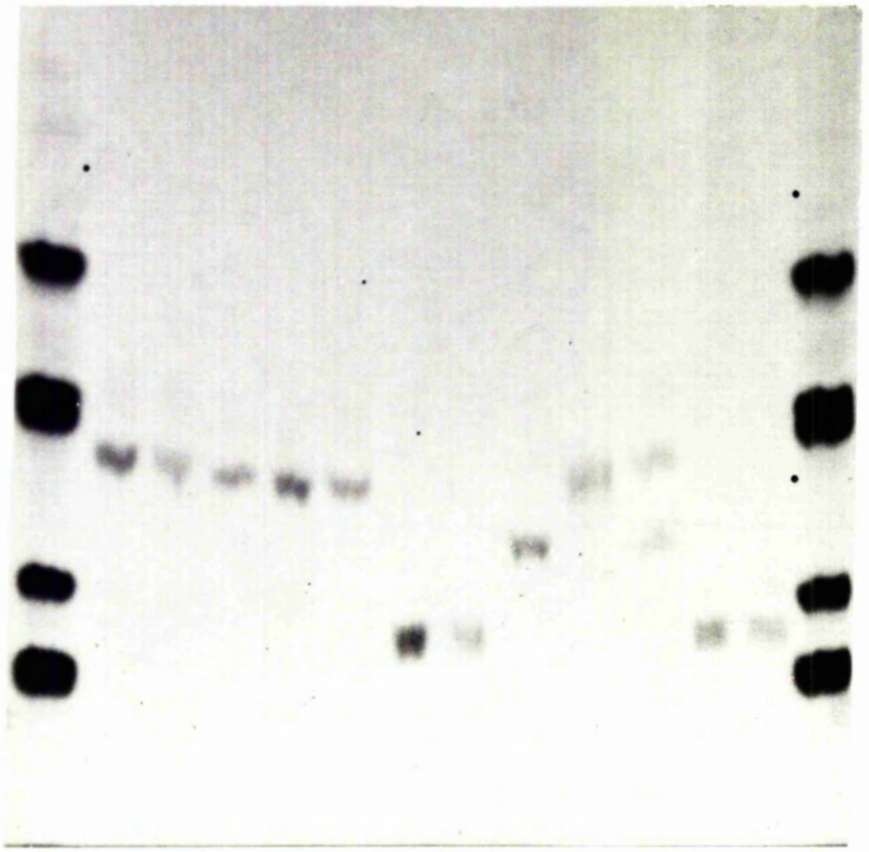
Key

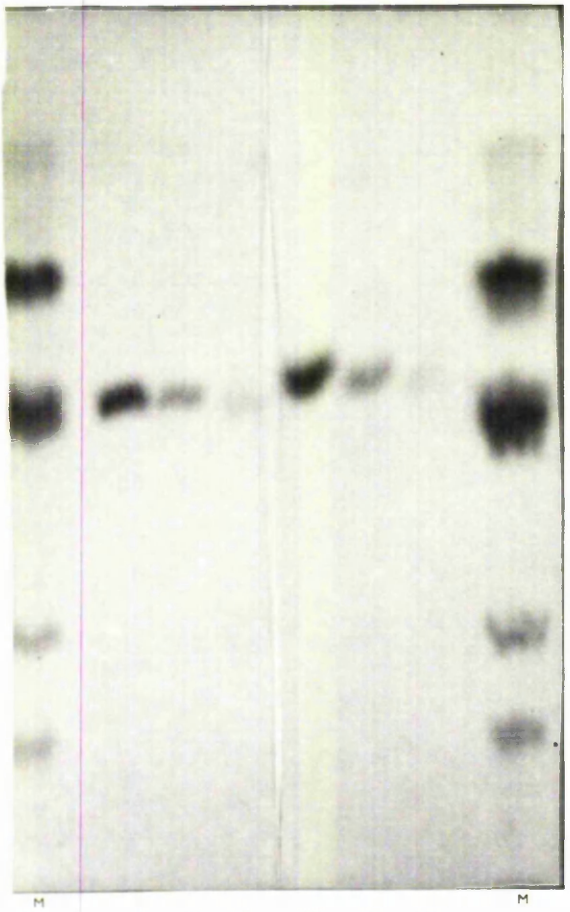
Fig. 3.3 Fingerprints of T_1 ribonuclease digested ^{14}C methyl labelled rRNA and rpre RNA from monolayer HeLa cells

Monolayer HeLa cells were labelled with ^{14}C methyl methionine. The cells were harvested and rRNA and rpre RNA (32S and 45S) were prepared (see methods section for details). The purified RNA was digested with T_1 ribonuclease and fingerprinted. Radioactive oligonucleotides were located by autoradiography.

This spot yielded AmA from 18S and AmG from 28S (characterized as described later). Both of these were present in the alkali digest of the corresponding spot from 45S RNA. Spot 9 in 32S contains AmG only.

The material which is present near the origin in T_1 digested RNA fingerprint is better fractionated using T_1 RNase together with alkaline phosphatase for digesting the labelled RNA. Figure 3.5 shows the autoradiographs of fingerprints made in this way. These fingerprints show longer methylated sequences and therefore a relatively large number of unique sequences are present which can be recognized by comparison with 18 + 28S fingerprint. About 40 methylated spots are obtained from 18S and 54 from 28S RNA fingerprints made by using both the methods of digesting the RNA. The 18S RNA fingerprint yields 12 or possibly 13 methylated spots in the "two U graticule". All of them are unique to 18S. On the other hand 28S RNA yields 5 spots in the "two U graticule". All of these spots are unique to 28S RNA. There are 7 spots present in the "three U graticule" of 18S RNA fingerprint. One of these spots is easily identified as 18S unique spot (No. 72). There are many spots present in 28S RNA fingerprint in this region. Three of them are easily recognized as 28S unique spots (No. 62, 63 & 65). There is still some material near the origin which probably contains even more U residues. Some of these spots are also characteristic to 18S or 28S RNA. For example spots


M
M
RNA
28 45
18 28 45
18 45
18 28 45
28 45
Spot
6
7
8
9
10


M
M
RNA
28 32 45
28 32 45
Spot
23
26

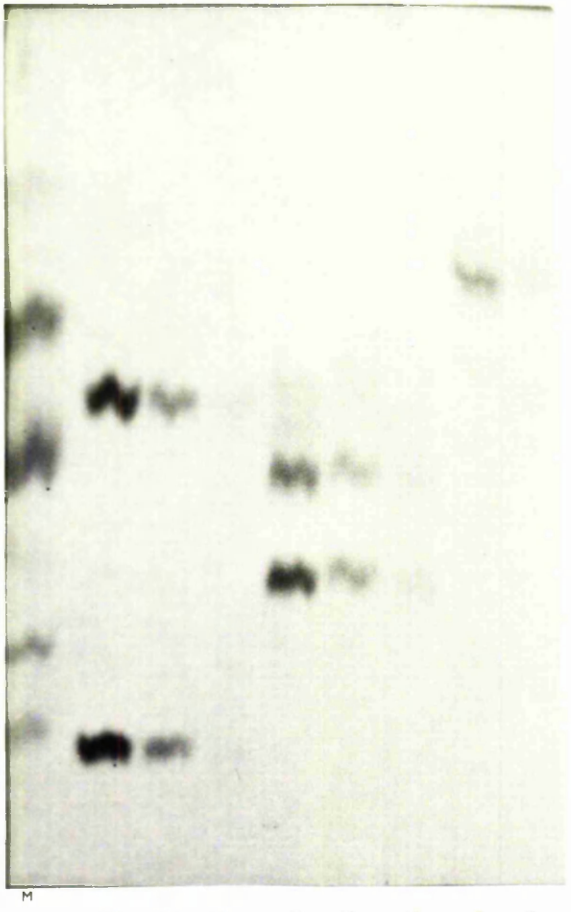

M
28 32 45
28 32 45
18 45
33
36
42

Fig. 3.4 Alkaline hydrolysis of ^{14}C methyl labelled oligonucleotides from rRNA and rpre RNA of HeLa cells

The oligonucleotides from ^{14}C methyl labelled fingerprints of 18S, 28S, 32S and 45S RNA were hydrolyzed with alkali. The products were separated at pH 3.5 on Whatman 52 paper. Radioactive products were located by autoradiography.

Fig. 3.5 Fingerprints of T_1 ribonuclease plus phosphatase digested ^{14}C methyl labelled rRNA and rpre RNA from monolayer HeLa cells

Monolayer HeLa cells were labelled with ^{14}C methyl methionine. Ribosomal RNA and ribosomal precursor RNA were prepared (see methods). The purified RNA was digested with a mixture containing T_1 ribonuclease and alkaline phosphatase. The products were separated by the two dimension fractionation procedure. B marks the position of blue dye in the second dimension. 32S fingerprint was run longer than others, therefore the blue dye has migrated into the buffer compartment. The radioactive oligonucleotides were located by autoradiography. In the key, some of the 28S unique spots are marked black.

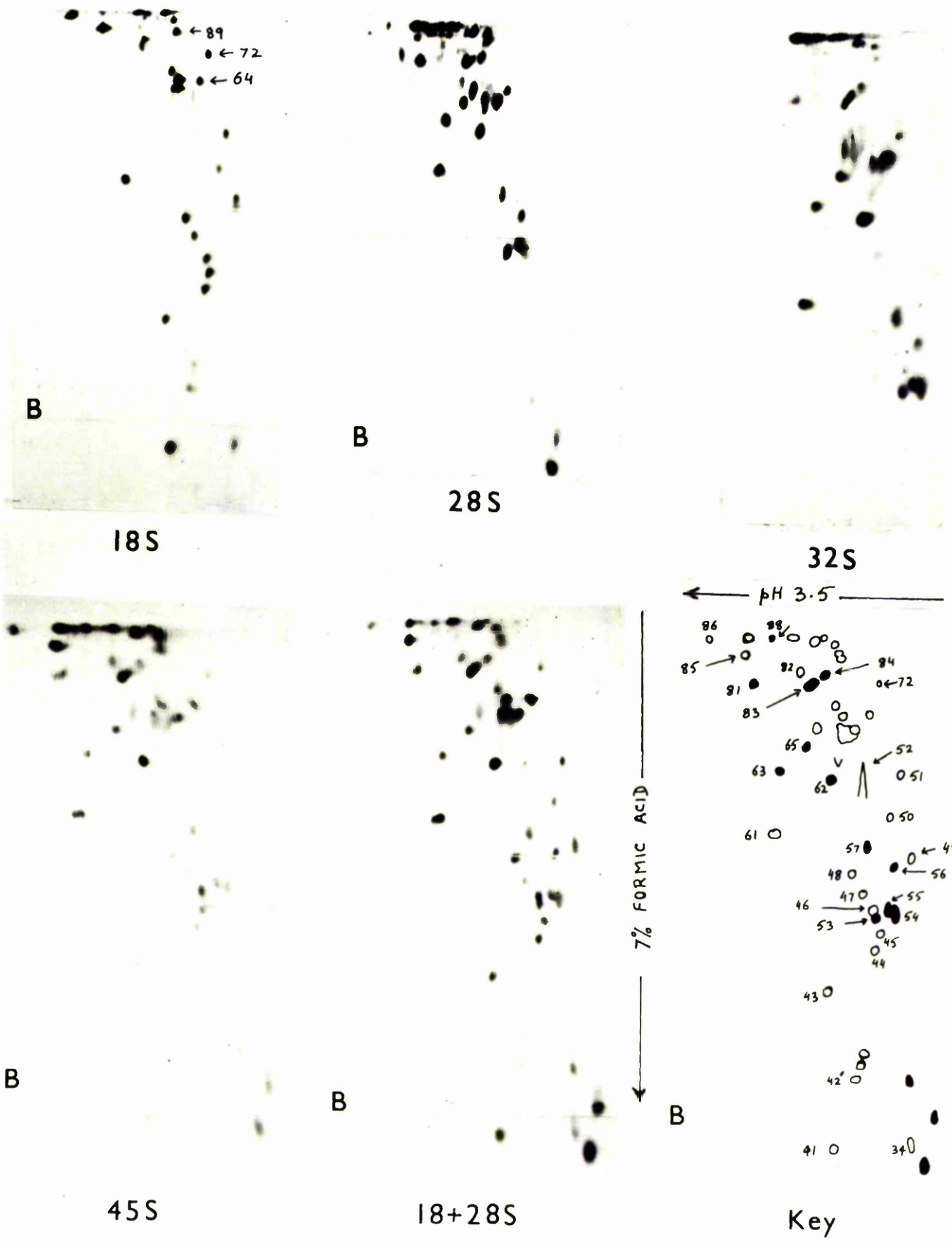

89
72
64
B
18S
B
28S
32S
pH 3.5
7% FORMIC ACID
86
85
81
88
82
84
72
83
65
52
63
62
51
50
61
57
49
48
56
47
55
46
54
53
45
44
43
42
41
34
B
45S
B
18+28S
B
Key

Table 3.1 Differences between 18S & 28S RNA

"Graticule"	18S RNA unique spots	28S RNA unique spots	Spots common to both 18S & 28S RNA
No U	8	4,6,10,12-18	2,3,7,9,11
One U	24,30,34,37	23,25,26-29 31-33,35,36	21,22,24'
Two U	41-52	53-57	
Three U	72	62,63,65 and others	61
>Three U	82,85,86	81,83,84,88	

81, 83, 84 and 88 are present in 28S fingerprint only, while 18S fingerprint has three easily recognizable unique spots (No. 82, 85 & 86).

32S RNA fingerprint made by using T_1 RNase and alkaline phosphatase is very similar to 28S RNA. The 45S RNA fingerprint is very similar to 18S + 28S RNA fingerprint except that it does not contain spots 49 and 34.

The following conclusions could be drawn from these RNA fingerprints.

1. 28S RNA is very different from 18S RNA
2. 32S RNA is apparently identical to 28S RNA
3. 45S RNA is similar to 18 + 28S RNA but consistantly lacks spots 34, 49 and have an extra spot. (No. 38) Spot 30 is weakly labelled.

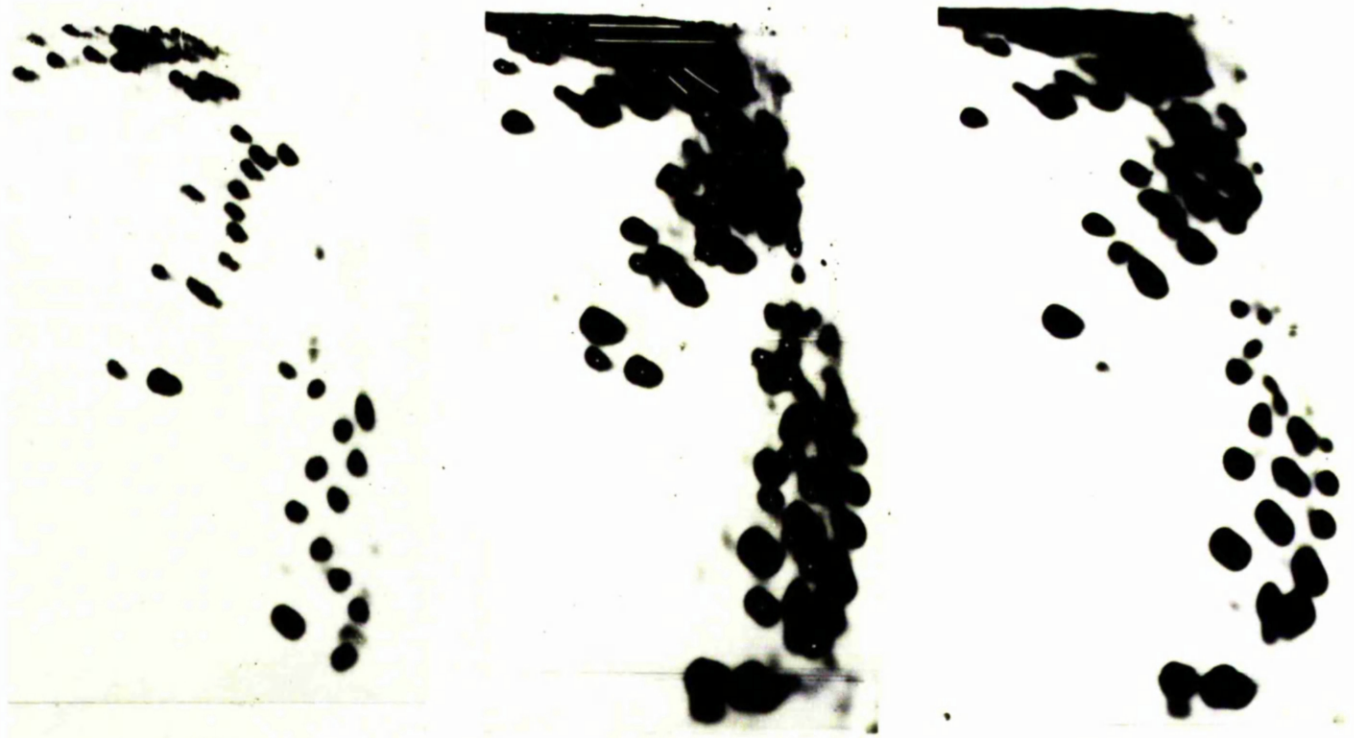
3. 2. Identification of Methylated Oligonucleotides

The methylated oligonucleotides in *E. coli* rRNA were identified by fingerprinting ^{32}P and ^{3}H labelled RNA and then counting the resulting oligonucleotides in a scintillation counter (Fellner & Sanger, 1968). However in the present study this method was not very successful for the following reasons. There were a lot more methylated spots in HeLa cell rRNA (about 40 in 18S and 54 in 28S) than in *E. coli* rRNA. The latter contained 8 methylated spots in 18S and 14 in 28S rRNA. Also the highest specific activity ^{3}H methyl labelled RNA prepared in this study did not contain sufficient activity for all methylated spots to be clearly identified

Fig. 3.6 Identification of methylated oligonucleotides obtained from T_1 ribonuclease digested 18S and 28S RNA from HeLa cells

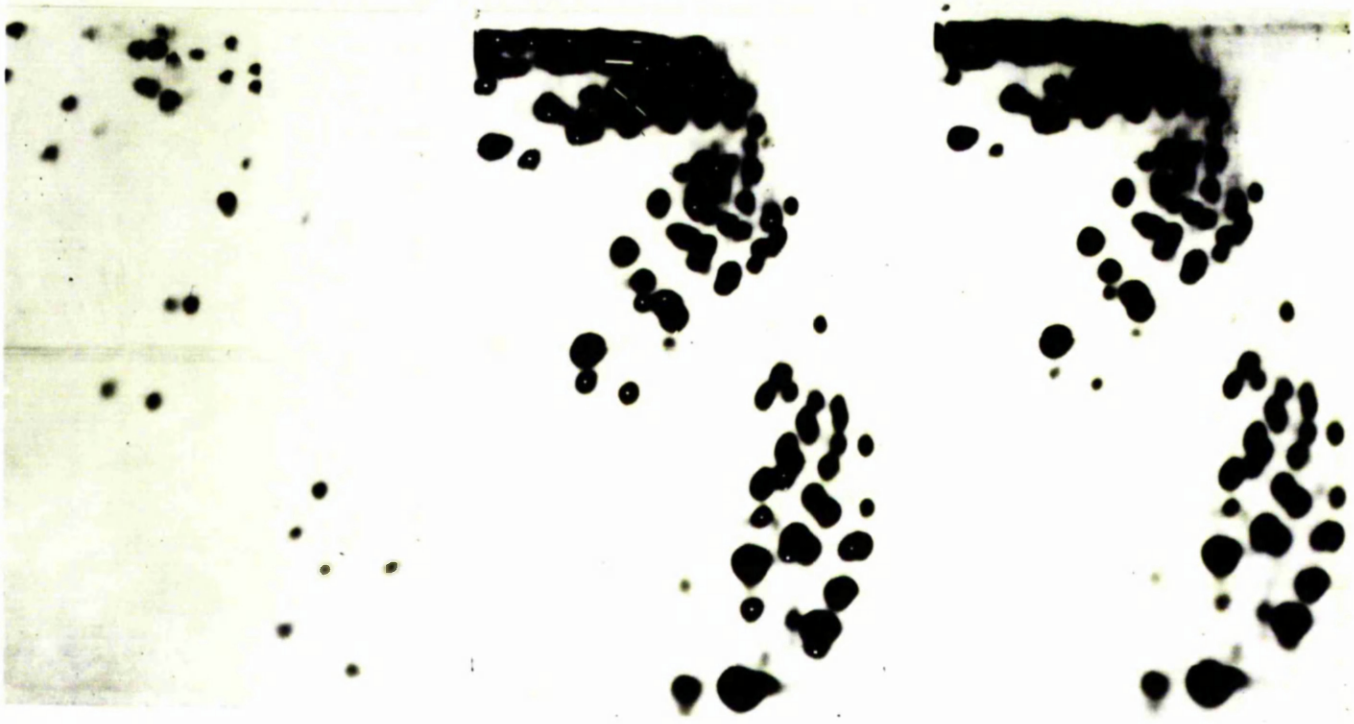
^{32}P and ^{14}C methyl labelled RNA were prepared separately from monolayer HeLa cells. About 180×10^3 dpm of ^{32}P-RNA and 90×10^3 dpm of ^{14}C-RNA were mixed. The mixture was digested with T_1 ribonuclease and fingerprinted. The first dimension (pH 3.5) is from right to left and second dimension (7% formic acid) from top to bottom.

Autoradiographs were prepared and methylated oligonucleotides identified as described in the text. White dots on ^{14}C + ^{32}P autoradiographs indicate the position of methylated oligonucleotides. Some of the faintly labelled spots in 28S ^{14}C autoradiograph are due to ^{32}P which was not completely decayed. Gp is not shown in this autoradiograph.



^{14}C $^{14}C + ^{32}P$ ^{32}P

28S



^{14}C $^{14}C + ^{32}P$ ^{32}P

18S

in $^{32}P/^{3}H$ fingerprints. Some of the spots were weakly labelled with ^{3}H and heavily labelled with ^{32}P, and vice versa. It was therefore found more convenient to fingerprint ^{32}P and ^{14}C methyl labelled RNA together and then instead of counting all the resulting spots in a scintillation counter, to use a double autoradiographic procedure described below.

3. 2a. Preparation of ^{32}P and ^{14}C methyl labelled RNA

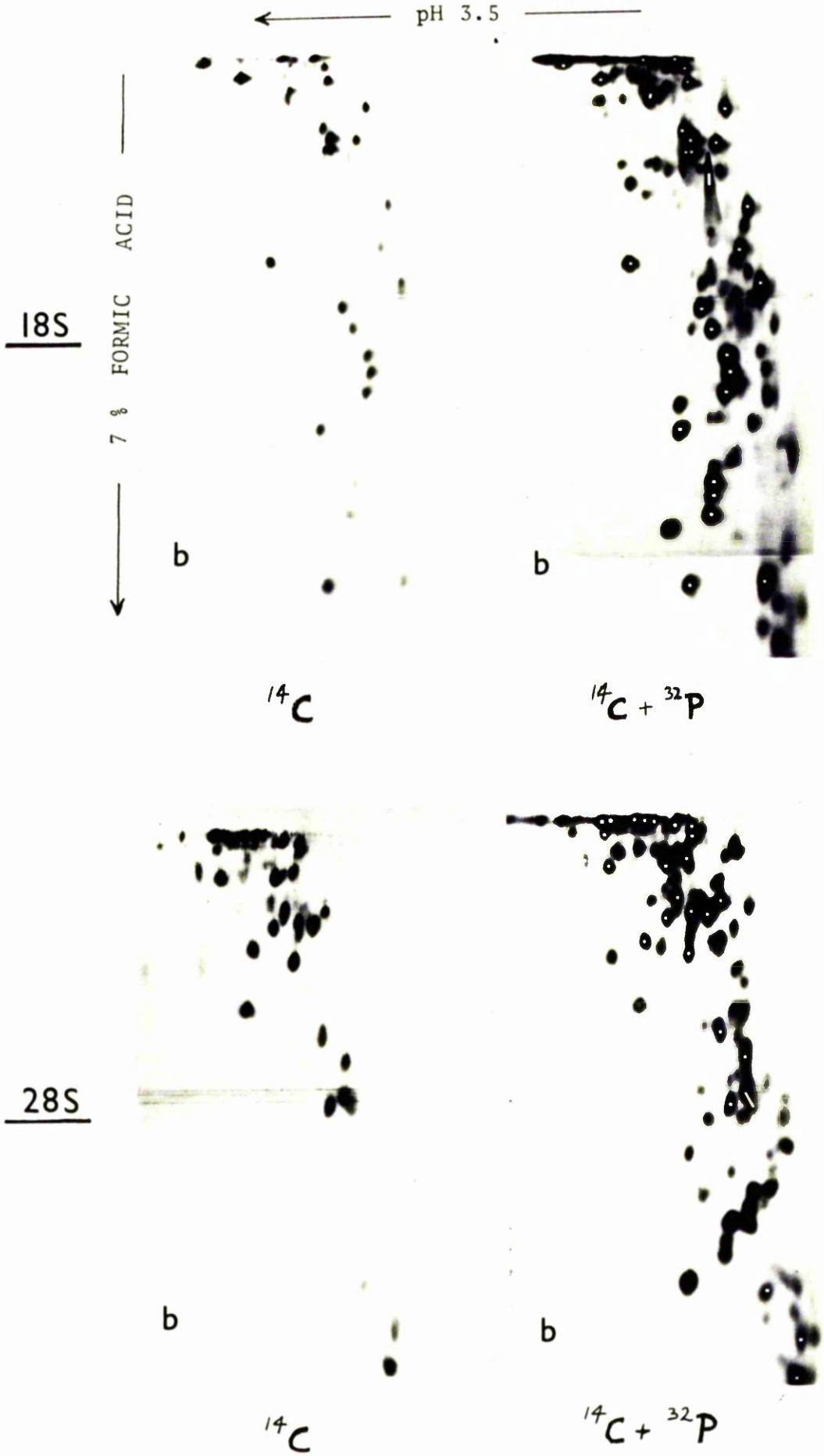
In order to control the amounts of ^{32}P and ^{14}C counts in a fingerprint, both ^{32}P and ^{14}C methyl labelled RNA were prepared separately as described in methods section. About 90×10^3 dpm of ^{14}C methyl labelled RNA were mixed with 180×10^3 dpm of ^{32}P labelled RNA.

3. 2b. Fingerprinting and identification

The mixture of ^{32}P and ^{14}C methyl labelled RNA was digested with T_1 ribonuclease and fingerprinted. The DEAE-paper was then placed in a folder with two Kodirex X ray films on the same side of the paper. The films were then developed after about three weeks. The film which was in direct contect with the DEAE-paper was affected by both ^{14}C and ^{32}P radiation, but the other film was affected only by ^{32}P radiation as ^{14}C emissions, being weak, were stopped by the first film. Comparison of the two films clearly revealed some of the methylated oligonucleotides, but others which were weakly labelled with ^{14}C and heavily labelled with ^{32}P were difficult to detect. To identify the remaining

Fig. 3.7 Identification of methylated oligonucleotides obtained from T_1 ribonuclease plus phosphatase digested 18S and 28S RNA

^{32}P and ^{14}C methyl labelled RNA were prepared separately from monolayer HeLa cells. About 90 x 10^3 dpm of ^{32}P-RNA and 180 x 10^3 dpm of ^{14}C-RNA were mixed. The mixture was digested with T_1 ribonuclease and alkaline phosphatase and fingerprinted. The autoradiographs were prepared and methylated oligonucleotides identified as described in the text. The position of blue dye is marked with b. White dots on the autoradiographs indicate the position of methylated oligonucleotides.


pH 3.5
7 % FORMIC ACID
18S
b
b
^{14}C
$^{14}C + ^{32}P$
28S
b
b
^{14}C
$^{14}C + ^{32}P$

methylated oligonucleotides the DEAE-paper was left for some months to allow the ^{32}P to decay completely. It was then again placed in a folder with Kodirex X ray film. A fingerprint showing only methylated oligonucleotides was obtained. When this fingerprint was superimposed on the double labelled fingerprint (or the ^{32}P labelled fingerprint), the position of all the methylated oligonucleotides was identified on the latter. In figure 3.6 white spots show the position of methylated oligonucleotides identified in this way.

In order to identify the position of methylated oligonucleotides containing three or more uridylic acid residues, doubly labelled RNA was digested with T_1 ribonuclease and bacterial alkaline phosphatase. Fingerprints were prepared and autoradiographs obtained as for T_1 digests. Figure 3.7 shows these fingerprints.

3. 3. Sequence Analysis of Methylated Oligonucleotides

Methylated oligonucleotides were located on a ^{32}P fingerprint with the help of the results obtained from double labelled experiments. This enabled these oligonucleotides to be excised, eluted and then subjected to sequence analysis. In most cases sequence analysis was conducted with ^{32}P labelled material only. In some cases where the methylated oligonucleotides were not obtained pure, double labelled material was used. However, information

derived from parallel work with ^{14}C methyl labelled oligonucleotides was very useful in the sequence analysis. In many cases analysis was simplified by knowledge of the alkali resistant dinucleotide present. The first step in the sequence analysis was the hydrolysis of oligonucleotides with alkali. This gave the base composition. In most of the oligonucleotides an alkali resistant dinucleotide was obtained due to the presence of a 2'-O-methyl ribose group. These alkali resistant dinucleotides and other methylated components were characterized as described below. For a trinucleotide knowledge of the products of alkaline hydrolysis including the structure of the alkali resistant dinucleotide was sufficient to determine the sequence. The longer sequences were determined by the use of a combination of further degradative procedures as described later.

3. 3a. Characterization of Methylated Components

The oligonucleotides from ^{14}C methyl labelled RNA fingerprints were digested with alkali. They were run at pH 3.5 on Whatman 52 paper. All the oligonucleotides digested in this study yielded only one methyl labelled product with the exception of spots 62 from 28S RNA and spots 30 and 85 from 18S RNA fingerprints. Most of the products have a different mobility from the standard mononucleotides. They were differentiated to some extent into mono- and dinucleotides by the treatment with alkaline phosphatase. All the mononucleotides after phosphatase

treatment yield nucleosides which are positively charged at pH 3.5 while all the dinucleoside monophosphates with the exception of CpC, CpA and ApC are negatively charged. Therefore if the product remains negatively charged after phosphatase treatment it cannot be a mononucleotide. All of the spots (examined in this study) digested with alkali changed their mobility on phosphatase treatment and most of them were negatively charged except for those listed in table 3.2 & 3.3.

The mononucleotides or alkali resistant dinucleotides were further characterized by using ^{32}P labelled RNA. Methylated oligonucleotides from a ^{32}P labelled RNA were digested with alkali. The methylated spots were identified with the aid of the previous alkali digest of ^{14}C methyl labelled oligonucleotides. They were eluted and divided into two parts. Half of the material was digested with spleen phosphodiesterase to give the composition. The other half was treated with alkaline phosphatase and was run at pH 3.5 on Whatman 52 paper. This gives a more definite identification of mono- verses dinucleotides, as mononucleotides will give only free phosphate, dinucleotides will yield equal radioactivity in the dephosphorylated product and free phosphate, and trinucleotides will yield twice as many counts in dephosphorylated product as in free phosphate. All the spots from 42 and onwards were obtained from T_1 RNase and phosphatase digested RNA, and therefore do not have a

Table 3.2 Identification of methylated products from alkali digest of 18S RNA oligonucleotides

Spot No.	Charge on dephosphorylated products (^{14}C)	Spleen hydrolysis (^{32}P)	VPDE hydrolysis of dephosphorylated products (^{32}P)	Structure of methylated products
2	-	C,G	G	CmG
3	-	A,G	G	AmG
7	-	C,G	G	CmG
8	+	C	C	CmC
9	-	A	A	AmA
11	+	A,C	C	AmC
21	-	G	G	GmG
22	-	U,G	G	UmG
24	-	A,G	G	AmG
24'	-	G	G	GmG
30.1	+			m_2^6A
30.2	-			di- or trinucleotide
30.3	-			-do-

Table 3.2 (continued)

Spot No.	Charge on dephosphorylated products (^{14}C)	Spleen hydrolysis (^{32}P)	VPDE hydrolysis dephosphorylated products (^{32}P)	Structure of methylated products
34	+			m A *
41	-	G,U	U	GmU
42	-	U	G	UmG*
43	-	U	U	UmU
44	-	U,C	C	UmC
45	-	C,U	U	CmU
46	-	A,U	U	AmU
47	-	U	G	UmG
48	-	A	A	AmA
49	+			m^1G m^2G
50	-	G	G	GmG
51	+	C	C	CmC

* Spot 42 onwards were obtained from T_1 plus phosphatase digestion therefore 3'-end nucleotide does not have a phosphate group and does not appear in spleen phosphodiesteras digestion.

* I-Methyl adenine is cconverted to 6-methyl adenine in the presence of alkali. Therefore specificity of methylation is not known.

Spot No.	Charge on dephosphorylated products (^{14}C)	Spleen hydrolysis (^{32}P)	VPDE hydrolysis dephosphorylated products (^{32}P)	Structure methylated products
61	-	G	G	GmG
64	-	G,C	C	GmC
72	-	A	A	AmA
82	-	A,U	U	AmU
89	-	A,U	U	AmU
85.1	-	U	G	UmG
85.2	-	U,C	C	UmC
86	-	U	U	UmU

phosphate at 3'-end of the oligonucleotide. Therefore if a dinucleotide is present at 3'-end of oligonucleotide, after digestion with alkali a dinucleoside monophosphate will be obtained which will not change its mobility by phosphatase treatment. For example the methylated products obtained by digestion of spot 42 and 47 with alkali did not change their mobility and gave only one nucleotide which was present at 5'-end, after digestion with spleen (table 3.2).

All the spots from 18S and 28S RNA which were treated with phosphatase after digestion with alkali, yielded equal activity in free phosphate and dephosphorylated product with the exception of those obtained from spot 30, 34 and 49 from 18S RNA and some which were present at 3'-end of the oligonucleotides obtained from T_1 ribonuclease and phosphatase digested RNA. It was therefore concluded that all the rest were dinucleotides. Three methylated spots were obtained from alkali digestion of spot 30 from 18S RNA. One of these had the same mobility as Ap and yielded only free phosphate after phosphatase treatment. It was therefore a methylated derivative of adenylic acid. This mononucleotide was digested with acid to give purine base which co-chromatographed with N^6-dimethylamino purine. It was therefore identified as N^6-dimethyl adenylic acid. The methylated spot from alkali digestion of spot 34 from 18S RNA also had the same mobility as Ap and yielded only free phosphate after phosphatase treatment. It was identified as 6-methyl adenylic acid as

Table 3.3 Identification of methylated products from alkali digest of 28S RNA oligonucleotides

Spot No.	Charge on dephosphorylated products (^{14}C)	Spleen hydrolysis (^{32}P)	VPDE hydrolysis of dephosphorylated products (^{32}P)	Structure of methylated products
2	-	C,G	G	CmG
3	-	A,G	G	AmG
4	-	C,G	G	CmG
6	-	A,G	G	AmG
7	-	C,G	G	CmG
9	-	A,G	G	AmG
10	+	C	C	CmC
11	+	A,C	C	AmC
12	+	C,A	A	CmA
13	+	C	C	CmC
15	-	A,G	G	AmG
16	-	G	G	GmG

Table 3.3 (continued)

Spot No.	Charge on dephosphorylated products (^{14}C)	Spleen hydrolysis (^{32}P)	VPDE hydrolysis of dephosphorylated products (^{32}P)	Structure of methylated products
21	-	G	G	GmG
22	-	U,G	G	UmG
23	-		U	CmU*
24'	-		G	GmG
25	-		U	AmU
26	-		A	UmA
29	-	U,G	G	UmG
61	-	G	G	GmG

* Some of these dinucleotides were characterized earlier therefore they were only characterized here by their mobilities and by digestion with phosphatase and venom phosphodiesterase.

after acid hydrolysis the base co-chromatographed with 6-methyl adenine in isopropanol ammonia system. Alkaline hydrolysis of spot 49 yielded mononucleotide G. After acid hydrolysis two bases were obtained which co-chromatographed with 1-methyl G and 2-methyl G in butanol ammonia system.

3. 3b. Digestion of oligonucleotides with endo- and exonucleases

Apart from some of the smallest oligonucleotides (di- and some trinucleotides) whose structures could be completely determined by procedures described above, oligonucleotides sequences were most frequently determined by partial digestion with venom phosphodiesterase under appropriate conditions. The enzyme digests inward from the 3'-OH terminus of an oligonucleotide and liberates nucleoside-5'-phosphates. The products obtained from such a partial digest were separated by electrophoresis on DEAE-paper at pH 3.5. Brownlee and Sanger (1967) examined the fractionation of venom phosphodiesterase digests by electrophoresis in this way. They noted that the removal of one nucleotide has a specific effect on the mobility of the product. The change in mobility depends on the nature of the nucleotide removed. This change in mobility was termed the M value and was defined as x/y, where y was the distance of migration of an oligonucleotide and x was the distance between it and its first degradation product. By determining the M values of

a series of degradation products it is frequently possible to elucidate a portion of the sequence of the original oligonucleotide. In this study M values were found to be useful in indicating the nature of some of the nucleotides released. But in most cases the products were then digested completely with venom phosphodiesterase and alkali to determine their composition. Pancreatic ribonuclease was also used to digest some of the oligonucleotides containing adenylic acid residues.

There were some methylated oligonucleotides which were not obtained pure from the two dimensional fractionation procedure. Nevertheless it was found possible to determine their sequences with the aid of two methods. In some cases ^{14}C methyl labelled material was mixed with ^{32}P labelled material and all the manipulations were carried out on this. Methylated products were identified due to the presence of ^{14}C. In other cases the presence of alkali resistant dinucleotides proved useful as described in individual cases.

3. 3c. Methylated oligonucleotides from 18S RNA

Spot 2

This product migrates slightly faster than CG in the second dimension. It was obtained pure from long second dimension runs.

Complete hydrolysis: Treatment of ^{14}C methyl or ^{32}P labelled material with alkali gave only one spot running slightly slower than Gp on electrophoresis at pH 3.5. Phosphatase

Table 3.4 Sequence of analysis of spots 2,3, & 7 from 18S RNA

Spot No.	Material	Hydrolysis: Alkaline	Spleen PDE	VPDE	Structure
	Complete hydrolysis				
2	^{32}P-oligonucleotide	CmG	(C,G)*	(G)	
	^{14}C-oligonucleotide	CmG			CmG
	Complete hydrolysis				
3	^{32}P-oligonucleotide	AmG	(A,G)	(G)	
	^{14}C-oligonucleotide	AmG			AmG
	Complete hydrolysis				
7	^{32}P-oligonucleotide	A			
		CmG	(C,G)	(G)	
		C**			
		G			
	^{14}C-oligonucleotide	CmG			ACmG

* In all the following tables nucleotides in paranthesis were obtained from alkali resistant dinucleotides.

**Small amount of C and G were obtained from contaminating spot.

treatment of ^{32}P labelled material yielded equal amounts of radioactivity in free phosphate and the dephosphorylated product. The compound was therefore a dinucleotide. Digestion with spleen phosphodiesterase yielded equal amounts of Cp and Gp. When the dephosphorylated product was treated with venom phosphodiesterase only one radioactive product pG, was obtained. The sequence of the original dinucleotide was therefore deduced to be CpGp. Since 2-O-methylation is known to confer resistance to alkali on the adjacent phosphodiester bond, it was further deduced that the structure of the original compound was 2-O-methyl CG abbreviated here as CmG.

Spot 3

This spot has a greater mobility than AG in the second dimension. Therefore it was obtained pure from the two dimensional fractionation procedure.

Complete hydrolysis: When ^{14}C methyl or ^{32}P labelled material was treated with alkali only one spot was obtained. After phosphatase treatment there were equal counts in free phosphate and dephosphorylated product. The spot contains Ap and Gp as shown by digestion with spleen phosphodiesterase. When dephosphorylated product was digested with venom phosphodiesterase pG was obtained. The sequence of the spot is therefore AmG.

Spot 7

This spot runs slightly faster than ACG and therefore

could be obtained in relatively pure from long runs.

Complete hydrolysis: Alkaline hydrolysis of ^{14}C methyl labelled material yielded only one spot which had slightly slower mobility than Gp. Alkaline hydrolysis of ^{32}P labelled material yielded Ap and one spot which runs slower than Gp. The latter spot was identified as CmG (table 3.2). The sequence of the spot is therefore ACmG.

Spot 8

This spot is unique to 18S RNA. It was found to co-fractionate with CCCG. Its dephosphorylated product does not separate from its non-methylated contaminant by electrophoresis at pH 3.5 on DEAE-paper.

Complete hydrolysis: Alkaline hydrolysis of ^{14}C methyl or ^{32}P labelled material liberated an alkali resistant dinucleotide identified as CmC (table 3.2). The base composition of the parent compound shows that it is a tetranucleotide (C_3, G). Complete hydrolysis of dephosphorylated ^{14}C methyl labelled oligonucleotide with venom phosphodiesterase yielded labelled nucleotide pC and nucleoside C. This indicates the presence of two alkali resistant dinucleotides, one at 5' end of the oligonucleotide and the other being internal. Thus two possible sequences could be present in the same spot namely CmCCG and CCmCG.

Partial hydrolysis: In order to confirm the above sequences the ^{32}P labelled oligonucleotide was dephosphorylated and

Table 3.5 Sequence analysis of spot 8 from 18S RNA

Materials	Hydrolysis			
	Alkaline	Spleen PDE	VPDE	Structure
Complete hydrolysis				
^{32}P-oligonucleotide	C_3			
	G			
	CmC*	(C)	(C)	
^{14}C-oligonucleotide	CmC		C	
			C (nucleoside)	
Partial hydrolysis				
VPDE products (^{32}P)				
(a)				CCmCG
				CmCCG
(b)	C			CC
(c)	C			CC
(d)	CmC (monophosphate)			CmC
(e)	C			CCC
(f)	C			
	CmC (monophosphate)			CCmC
	CmC (diphosphate)			CmCC
(g)	G			

* very small amount of CmC was obtained as compared to G.

then subjected to partial digestion with venom phosphodiesterase. Incubations were carried out for 10, 20 and 30 minutes at 37°C. The products were fractionated by electrophoresis on DEAE-paper at pH 3.5. The separation which was obtained is shown in figure 3.8. Methylated products were separated from the non-methylated ones. The original tetranucleotide is present at a. Product e contains non-methylated trinucleotide CpCpC, while product f is its methylated analogue. The product f on alkaline hydrolysis gave roughly equal amounts of alkali resistant dinucleoside diphosphate CmpCp and dinucleoside monophosphate CmpC. This shows that product f contains two isomers of the trinucleotide namely CmCC and CCmC. Product b and c were dinucleoside monophosphates (CpC) and product d contains its methylated analogue (CmpC). Therefore the original spot contains the two methylated sequences CCmCG and CmCCG.

Spot 9

This oligonucleotide was obtained completely pure from the fingerprint. It had relatively greater mobility than AAG in the second dimension.

Complete hydrolysis: Two products were obtained from the alkaline hydrolysis of ^{32}P labelled material. One was Gp and the other migrated between Gp and Ap. Alkaline hydrolysis of ^{14}C methyl labelled material yielded only one spot which also migrated between Gp and Ap. This was identified as AmA (table 3.2). The sequence was therefore

Fig. 3.8 Fractionation of VPDE partial products

Dephosphorylated ^{32}P labelled oligonucleotides were partially digested with venom phosphodiesterase (see text). The products were separated on DEAE-paper by electrophoresis at pH 3.5. The position of the blue marker is indicated by B.

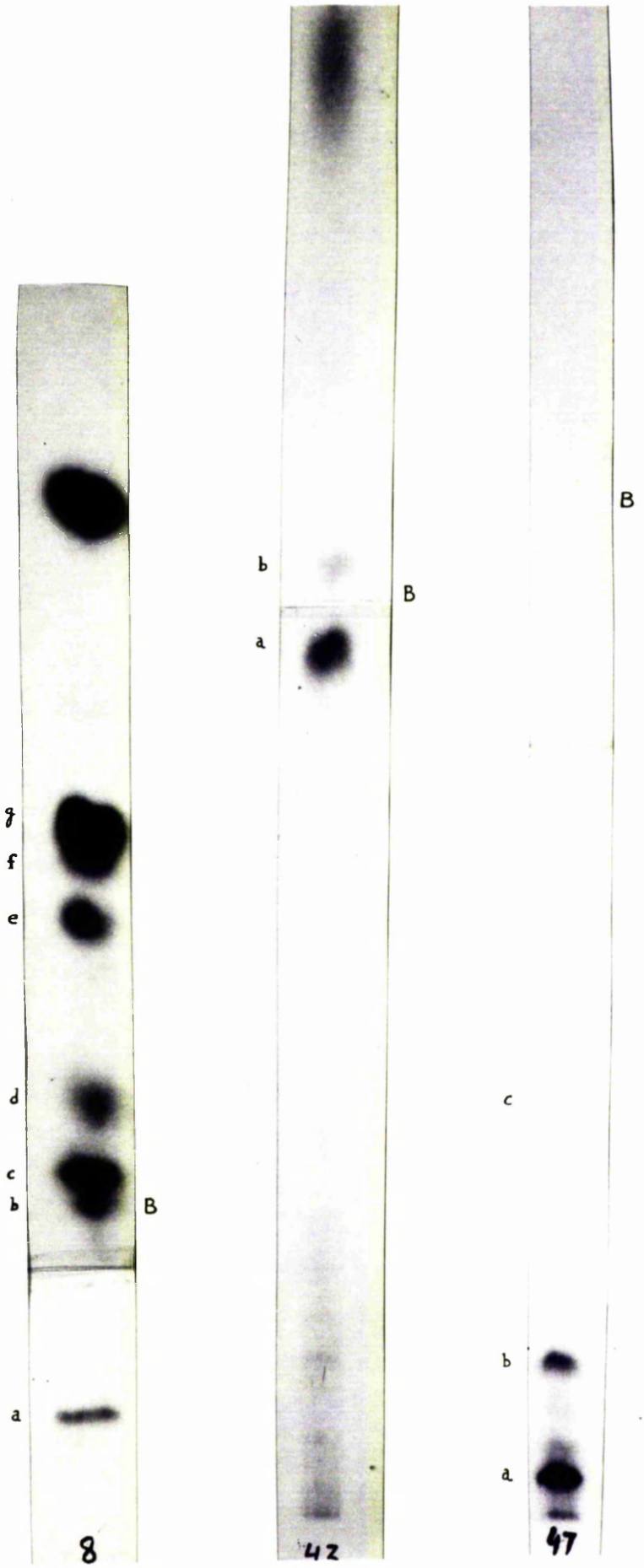

g
f
e
d
c
b
B
a
8
b
B
a
42
B
c
b
a
47

Table 3.6 Sequence analysis of spots 9 & 11 from 18S RNA

Spot No.	Material	Hydrolysis: Alkaline	Spleen PDE	VPDE	Structure
9	Complete hydrolysis				
	^{32}P-oligonucleotide	AmA	(A)	(A)	
		G			
	^{14}C-oligonucleotide	AmA			AmAG
11	Complete hydrolysis				
	^{32}P-oligonucleotide	A*			
		AmC	(A,C)	(C)	
		G			
		C			
	^{14}C-oligonucleotide	AmC			
	Pancreatic RNase products (^{32}P)				
	(a)	A			
		AmC			AAmC
	(b)	G			
					AAmCG

* More than one molar amounts of A and G, much less than one molar amount of C were obtained due to contaminating material.

identified as AmAG.

Spot 11

This spot has slightly slower mobility than AAG in both the dimension. In digests of ^{32}P labelled RNA it was contaminated with some non-methylated material.

Complete hydrolysis: Alkaline hydrolysis of ^{14}C methyl labelled material yielded one spot with slightly greater mobility than Ap. When ^{32}P labelled material was digested with alkali Ap, Cp, Gp and one spot which has slightly faster mobility than Ap were obtained. The last spot was found to be AmC as shown in table 3.2. Pancreatic ribonuclease digestion of ^{14}C methyl and ^{32}P double labelled material yielded AAC containing ^{14}C label and G with ^{32}P label only. The sequence of the oligonucleotide therefore is AAmCG. (The Cp in the alkali digest of the original spot was from contaminating non-methylated material).

Spot 21

This spot is obtained completely pure from the two dimensional fractionation procedure.

Complete hydrolysis: Treatment of ^{14}C methyl or ^{32}P labelled material with alkali gave only one spot running between Gp and Up. When ^{32}P labelled material was treated with phosphatase, the same number of counts were obtained in free phosphate and the dephosphorylated product. Therefore it is an alkali resistant dinucleotide. Only Gp was obtained on digestion with spleen. It is therefore GmG.

Table 3.7 Sequence analysis of spots 21 - 24' from 18S RNA

Spot No.	Material	Hydrolysis: Alkaline	Spleen PDE	VPDE	Structure
21	Complete hydrolysis				
	^{32}P-oligonucleotide	GmG	(G)	(G)	
	^{14}C-oligonucleotide	GmG			GmG
22	Complete hydrolysis				
	^{32}P-oligonucleotide	UmG	(U,G)	(G)	
	^{14}C-oligonucleotide	UmG			UmG
24	Complete hydrolysis				
	^{32}P-oligonucleotide	U			
		AmG	(A,G)	(G)	
	^{14}C-oligonucleotide	AmG			UAmG
24'	Complete hydrolysis				
	^{32}P-oligonucleotide	A			
		GmG	(G)	(G)	
	^{14}C-oligonucleotide	GmG			AGmG

Spot 22

This spot has slightly greater mobility than UG in the second dimension and was obtained completely pure from the fingerprint.

Complete hydrolysis: When ^{14}C methyl or ^{32}P labelled material was digested with alkali only one spot was obtained. This ran slightly faster than Up. Treatment with phosphatase showed it to be a dinucleotide. Spleen phosphodiesterase digestion gave Up and Gp. Complete venom phosphodiesterase digestion on dephosphorylated product gave pG. Therefore it is UmG.

Spot 24

This spot is unique to 18S RNA. It was obtained completely pure as it has greater mobility than UAG in the second dimension of the fractionation procedure.

Complete hydrolysis: Alkaline hydrolysis of ^{14}C methyl labelled material gave one spot running slightly slower than Gp. When ^{32}P labelled material was hydrolysed by alkali two products were obtained, Up and another running slower than Gp. This second product was identified as AmG (table 3.2). Therefore the sequence of spot 24 is UAmG.

Spot 24'

This spot has marginally slower mobility than CUG. Generally it was slightly contaminated after the two dimensional fractionation procedure. It was again subjected to two dimensional fractionation procedure to purify.

Complete hydrolysis: Alkaline hydrolysis of ^{14}C methyl labelled material yielded one spot running between Up and Gp. When ^{32}P labelled material was digested with alkali two products were obtained, one Ap and other running between Up and Gp. The latter was identified as GmG (table 3.2). Therefore the sequence was AGmG.

Spot 30

This spot is faintly labelled in 45S RNA fingerprint. It was obtained contaminated with non-methylated oligonucleotides from T_1 and phosphatase digested RNA fingerprint.

Complete hydrolysis: When ^{32}P labelled material from a T_1 plus phosphatase fingerprint was hydrolysed with alkali, A_3, C and U were obtained. G did not appear as it was dephosphorylated. Three spots were obtained on alkaline hydrolysis of ^{14}C methyl labelled material. One migrates with Ap and the other two have slightly slower mobilities than Gp. The spot which migrates with Ap was characterized as dimethyl A (section 3.3a). The other two spots which have slightly slower mobilities than Gp were not mononucleotides as ^{14}C methyl labelled material from these spots was negatively charged after phosphatase treatment. When dephosphorylated products from these spots (labelled with ^{14}C) were digested with venom phosphodiesterase, labelled nucleotide A as well as nucleoside A were obtained. The original spot therefore contains in addition to dimethyl A,

Table 3. 8 Sequence analysis of spots 41 & 42′ from 18S RNA

Spot No.	Material	Hydrolysis: Alkaline	Spleen PDE	VPDE	Structure
	Complete hydrolysis				
41	^{14}C-oligonucleotide	GmU			
	^{32}P-oligonucleotide from T_1 fingerprint	G			
		GmU	(G,U)	(U)	
	From T_1 + phosphate fingerprint	GmU			GmUG
42′	Complete hydrolysis				
	^{14}C-oligonucleotide	UmG			
	^{32}P-oligonucleotide	UmG	(U)	(G),U_2	
		U		G	
		C			CUUmG

a di- or trinucleotide.

Spot 34

This is a unique 18S RNA spot and is not present in 45S RNA. It was obtained contaminated with non-methylated oligonucleotide from T_1 plus phosphatase digested RNA fingerprint.

Complete hydrolysis: Alkaline hydrolysis of ^{14}C methyl labelled material gave one spot which had the same mobility as Ap at pH 3.5. This methylated product was identified as mono-methyl adenylic acid (table 3.2). Base composition of the spot 34 showed that it consists of monomethyl A, A_3, U, C and G.

Spot 41

This is a unique 18S RNA spot. It was obtained completely pure from the two dimensional fractionation procedure.

Complete hydrolysis: Alkaline hydrolysis of ^{14}C methyl labelled material gave one spot which ran slightly faster than Up. When ^{32}P labelled material obtained from T_1 digested RNA was hydrolyzed by alkali two products were obtained, Gp and another which had slightly greater mobility than Up. The latter product was identified as GmU (table 3.2). The sequence of this spot therefore is GmUG. This was further confirmed by the alkaline hydrolysis of spot 41 obtained from T_1 plus phosphatase digested RNA fingerprint. Hydrolysis with alkali yielded only GmU, as G which was present at 3'-end

of the trinucleotide was already dephosphorylated while preparing fingerprint.

Spot 42'

This spot is unique to 18S RNA. It was obtained pure from T_1 plus phosphatase digested RNA fingerprint.

Complete hydrolysis: When ^{14}C methyl labelled material from T_1 plus phosphatase digested RNA fingerprint was hydrolysed with alkali only one spot which migrates between Ap and Gp was obtained. Alkali digest of ^{32}P labelled material from T_1 plus phosphatase fingerprint gave rise to Up, Cp and one spot which ran between Ap and Gp at pH 3.5. The intensity of all the three spots was roughly the same. The latter spot was identified by spleen and venom phosphodiesterase as UmG (table 3.2). At this stage it is possible to define a sequence with one ambiguity (C,U) UmG. Venom phosphodiesterase digestion of ^{32}P labelled material resulted in one G residue and two U residues but no C. Therefore C will be present at 5'-end of the tetranucleotide. Therefore the sequence of spot 42' is CUUmG.

Spot 43

This is a unique 18S RNA oligonucleotide. It was obtained contaminated with small amounts of non-methylated (A, U_2) G from a T_1 plus phosphatase digested RNA fingerprint

Complete hydrolysis: Alkali digest of ^{14}C methyl labelled material from T_1 plus phosphatase digested RNA fingerprint yielded one spot running faster than Up. When ^{32}P labelled

Table 3.9 Sequence analysis of spot 43 from 18S RNA

Materials	Hydrolysis			
	Alkaline	Spleen PDE	VPDE	Structure
Complete hydrolysis				
^{14}C-oligonucleotide	UmU			
^{32}P-oligonucleotide	A			
	UmU (di-phosphate)	(U)	(U)	
	U (trace)			
Partial hydrolysis				
VPDE products (^{32}P)				
				AUmUG
(a)	A			
	UmU (monophosphate)			AUmU
(b)	A			
	U			

material was hydrolyzed with alkali, Ap, Up and the dinucleoside diphosphate UmU was obtained. The distribution of radioactivity between these products showed the original spot to be a tetranucleotide with a composition A, U_2, G.

Partial hydrolysis: ^{32}P labelled tetranucleotide was partially digested with venom phosphodiesterase. After the removal of pG the methylated trinucleotide was separated from non-methylated product. Spot a in figure 3.8 is the methylated trinucleotide while spot b is its non-methylated analogue. Spot a on alkaline hydrolysis gave Ap and dinucleoside monophosphate UmpU. Therefore the dinucleotide will be present at 3'-end of the trinucleotide. The sequence of spot 43 thus will be AUmUG.

Spot 47

This oligonucleotide is unique to 18S RNA. It was obtained completely pure from the two dimensional fractionation procedure.

Complete hydrolysis: Only one spot was obtained on alkaline hydrolysis of ^{14}C methyl labelled material which ran between Gp and Ap. Hydrolysis of ^{32}P labelled material with alkali gave A_2, C, U and an alkali resistant dinucleotide which ran between Gp and Ap. Phosphatase treatment of this alkali resistant dinucleotide did not change its mobility showing it to be a dinucleoside monophosphate which would be present

Table 3.10 Sequence analysis of spot 47 from 18S RNA

Material	Hydrolysis: Alkaline	Spleen PDE	VPDE	Structure
Complete Hydrolysis				
^{14}C-oligonucleotide	UmG			
^{32}P-oligonucleotide	A_2			
	U			
	C			
	UmG	(U)	(G)	
Pancreatic RNase products (^{32}P)				
(a)	A_2			
	C			AAC
(b)	U			
(c)	UmG			
Partial hydrolysis				
VPDE products (^{32}P)				
(a)				AACUUmG
(b)	A_2			AACUU
	C			
	U			
(c)	A_2			AACU
	C			

at 3'-end of the oligonucleotide. It was identified as UmG (table 3.2). Pancreatic ribonuclease digestion of ^{32}P labelled material gave AAC, U, UmG. These findings made it possible to define a sequence with one ambiguity (AAC, U) UmG.

Partial hydrolysis: ^{32}P labelled material was partially digested with venom phospho-diesterase for 10 and 20 minutes at 37°C. The products were separated on DEAE-paper at pH 3.5 (figure 3.8). Analysis of the partial digestion products was carried out with alkaline hydrolysis. Spot a is the original oligonucleotide. Spot b and c were obtained by the removal of pG and pU respectively. The analysis of these products is shown in table 3.10. The sequence of the oligonucleotide was elucidated as AACUUmG.

Spot 48

This spot is unique to 18S RNA. It was obtained pure from T_1 plus phosphatase digested RNA fingerprint.

Complete hydrolysis: When ^{14}C methyl labelled material was hydrolysed with alkali one spot which migrates between Ap and Gp was obtained. Alkaline hydrolysis of ^{32}P labelled material yielded U_2 and a spot which runs between Ap and Gp. The latter spot was identified as AmA (table 3.2). Therefore the partial sequence of spot 48 is (AmA, U_2) G.

Spot 61

The oligonucleotide was obtained completely pure from the two dimensional fractionation procedure.

Table 3.11 Sequence analysis of spots 48 & 61 from 18S RNA

Spot No.	Material	Hydrolysis: Alkaline	Spleen PDE	VPDE	Structure
	Complete hydrolysis				
48	^{14}C-oligonucleotide	AmA			
	^{32}P-oligonucleotide	U_2			
		AmA	(A)	(A)	(U_2,AmA)G
	Complete hydrolysis				
61	^{14}C-oligonucleotide	GmG			
	^{32}P-oligonucleotide from T_1 fingerprint	U_2			
		GmG(di-phosphate)	(G)	(G)	
	from T_1 + phosphatase fingerprint	U_2			
		GmG(monophosphate)			UUGmG

Complete hydrolysis: On alkaline hydrolysis of ^{14}C methyl labelled material from T_1 digested RNA fingerprint a spot which migrated between Gp and Up at pH 3.5 was obtained. When spot 61 from T_1 plus phosphatase digested RNA fingerprint was hydrolyzed with alkali, the mobility of ^{14}C spot was changed and it ran slightly faster than Ap. This shows that methylated fragment is at 3'-end of the oligonucleotide. When ^{32}P labelled material from T_1 plus phosphatase digested RNA fingerprint was hydrolyzed with alkali, Up and a spot with slightly greater mobility than Ap was obtained. The latter was identified as GmpG (table 3.2). There were twice as many counts of radioactivity in Up as in GmpG. Therefore the sequence of spot 61 is UUGmG.

3. 3d. Methylated oligonucleotides from 28S RNA

Spot 2

This spot has slightly greater mobility than CG. Therefore it was obtained pure from the fingerprint. Complete hydrolysis: Alkali digest of ^{14}C methyl labelled material gave a spot which had slightly slower mobility than Gp at pH 3.5. Venom phosphodiesterase digestion of ^{14}C methyl labelled material yielded nucleoside C. This shows that C is present at 5'-end of the molecule. When ^{32}P labelled material was digested with alkali only one spot was obtained which had slightly slower mobility than Gp. There were equal number of counts of radioactivity in

Table 3.12 Sequence analysis of spots 2-4 from 28S RNA

Spot No.	Material	Hydrolysis: Alkaline	Spleen PDE	VPDE	Structure
	Complete hydrolysis				
2	^{14}C-oligonucleotide	CmG		C(nucleoside)	
	^{32}P-oligonucleotide	CmG	(C,G)	(G)	CmG
	Complete hydrolysis				
3	^{14}C-oligonucleotide	AmG		A(nucleoside)	
	^{32}P-oligonucleotide	AmG	(A,G)	(G)	AmG
	Complete hydrolysis				
4	^{14}C-oligonucleotide	CmG		C	
	^{32}P-oligonucleotide	C_2*			
		CmG	(C,G)	(G)	CCmG
		G			

* About two molar amounts of C were obtained. Some of it arise from contaminating spots. Small amount of G was also obtained from contaminating spots.

free phosphate and dephosphorylated product after phosphatase treatment. Therefore it was a dinucleotide. Venom phosphodiesterase digestion of dephosphorylated product yielded pG. The original spot contains Cp and Gp as shown by digestion with spleen phosphodiesterase. The sequence of this alkali resistant dinucleotide therefore is CmG.

Spot 3

This spot had a greater mobility than AG in second dimension of the fractionation procedure and therefore was obtained pure.

Complete hydrolysis: When ^{14}C methyl labelled material was digested with alkali only one spot was obtained which had slightly slower mobility than Gp on electrophoresis at pH 3.5. Venom phosphodiesterase digestion of ^{14}C methyl labelled material gave nucleoside A. This shows that A is present at 5'-end. When ^{32}P labelled material was digested with alkali only one spot containing a dinucleotide was obtained. Spleen phosphodiesterase digestion of this dinucleotide yielded Ap and Gp while only pG was obtained by venom phosphodiesterase digestion of its dephosphorylated product. Therefore the sequence of spot 3 is AmG.

Spot 4

This spot lies between CG and CCG and so it is obtained slightly contaminated by both of these.

Complete hydrolysis: Alkali digest of ^{14}C methyl labelled

material yielded one spot which had slightly slower mobility than Gp. Venom phosphodiesterase digestion of dephosphorylated ^{14}C methyl labelled material yielded labelled mononucleotide C. Therefore this nucleotide is not present at 5'-end of the molecule. Alkali digests of ^{32}P labelled material yielded Cp and an alkali resistant dinucleotide which was identified as CmG (table 3.3). Small amounts of Gp were also present from contaminating spots. Therefore the sequence of spot is CCmG.

Spot 6

This spot had greater mobility than CAG and was obtained pure from the two dimensional fractionation procedure.

Complete hydrolysis: When ^{14}C methyl labelled material was hydrolysed by alkali, one spot with a slower mobility than Gp was obtained. Venom phosphodiesterase digestion of dephosphorylated ^{14}C methyl labelled oligonucleotide yielded labelled mononucleotide A showing that it was present internally in the oligonucleotide. Alkali digest of ^{32}P labelled material yielded Cp and an alkali resistant dinucleotide which had slightly slower mobility than Gp. This dinucleotide was found to be AmG (table 3.3). Therefore the sequence of the trinucleotide was CAmG.

Spot 7

This spot had slightly greater mobility than ACG in the second dimension of the fractionation procedure, but

Table 3.13 Sequence analysis of spots 6,7 & 9 from 28S RNA

Spot No.	Material	Hydrolysis: Alkaline	Spleen PDE	VPDE	Structure
	Complete hydrolysis				
6	^{14}C-oligonucleotide	AmG		A	
	^{32}P-oligonucleotide	C			
		AmG	(A,G)	(G)	CAmG
	Complete hydrolysis				
7	^{14}C-oligonucleotide	CmG		C	
	^{32}P-oligonucleotide	A			
		CmG	(C,G)	(G)	ACmG
		C*			
		G			
	Complete hydrolysis				
9	^{14}C-oligonucleotide	AmG		A	
	^{32}P-oligonucleotide	A			
		AmG	(A,G)	(G)	AAmG

* Small amounts of C and G were obtained from contaminating spot.

was not completely separated from it. Relatively pure material was obtained from long second dimensional run.

Complete hydrolysis: Alkali digest of ^{14}C methyl labelled material yielded only one spot which had slightly slower mobility than Gp. When the dephosphorylated oligonucleotide was digested with venom phosphodiesterase, labelled mononucleotide C was obtained. This showed that the mononucleotide was present internally in the oligonucleotide. When ^{32}P labelled material was digested with alkali Ap and an alkali resistant dinucleotide was obtained with small amounts of Cp and Gp from contaminating ACG. The alkali resistant dinucleotide was found to be CmG (table 3.3). The sequence of the trinucleotide was therefore ACmG.

Spot 9

The oligonucleotide was obtained completely pure from the fingerprint. It had slightly greater mobility than AAG in the second dimension of the fractionation procedure.

Complete hydrolysis: When ^{14}C methyl labelled material was hydrolyzed by alkali only one spot was obtained which had slightly slower mobility than Gp. Venom phosphodiesterase digestion of dephosphorylated ^{14}C methyl labelled oligonucleotide yielded labelled nucleotide A, showing that it was not present at 5'-end of the oligonucleotide. Alkali digest of ^{32}P labelled material yielded Ap and one dinucleotide which had slightly slower mobility than Gp. This dinucleotide was identified as AmG (table 3.3).

Table 3.14 Sequence analysis of spot 10 from 28S RNA

Material	Hydrolysis: Alkaline	Spleen PDE	VPDE	Structure
Complete hydrolysis				
^{14}C-oligonucleotide	CmC		C	
^{32}P-oligonucleotide	A			
	C_2			
	CmC*			
	G			
Partial hydrolysis				
VPDE products (^{14}C + ^{32}P)				
(a)	A			
	C_2			
(b)	A			
	CmC (diphosphate)			ACmCG
(c)	A			
	C			
(d)	A			
	CmC (monophosphate)			ACmC

* Present in less than one molar amount as compared to G.

The sequence of the trinucleotide therefore is AAmG.

Spot 10

The oligonucleotide was obtained heavily contaminated with (A, C_2) G.

Complete hydrolysis: Alkali digest of ^{14}C methyl labelled material yielded one spot which ran between Cp and Ap on electrophoresis at pH 3.5. Complete hydrolysis of dephosphorylated ^{14}C methyl labelled oligonucleotide with venom phosphodiesterase yielded labelled mononucleotide C, showing that it was present internally in the oligonucleotide. Alkali digestion of ^{32}P labelled material gave the dinucleotide CmC (which ran between Cp and Ap) together with Ap, Gp and Cp, the last arising from contaminating non-methylated material. At this stage it is possible to define the sequence as ACmCG. This was confirmed by partial hydrolysis as follows.

Partial hydrolysis: Dephosphorylated ^{14}C methyl and ^{32}P double labelled material was partially digested with venom phosphodiesterase and the products were separated on DEAE-paper at pH 3.5. Methylated tri- and tetranucleotides were separated from non-methylated products (Fig. 3.9). They were then digested with alkali to determine their sequence. Spot a is the non-methylated tetranucleotide. Spot b is the original dephosphorylated and methylated tetranucleotide which on hydrolysis with alkali yielded Ap and CmpCp (diphosphate). Spot d is the methylated

Fig. 3.9 Fractionation of VPDE partial products

A mixture of ^{32}P and ^{14}C methyl labelled dephosphorylated oligonucleotide was partially digested with venom phosphodiesterase (see text). The products were separated on DEAE-paper by electrophoresis at pH 3.5. The position of the blue marker is indicated by B.

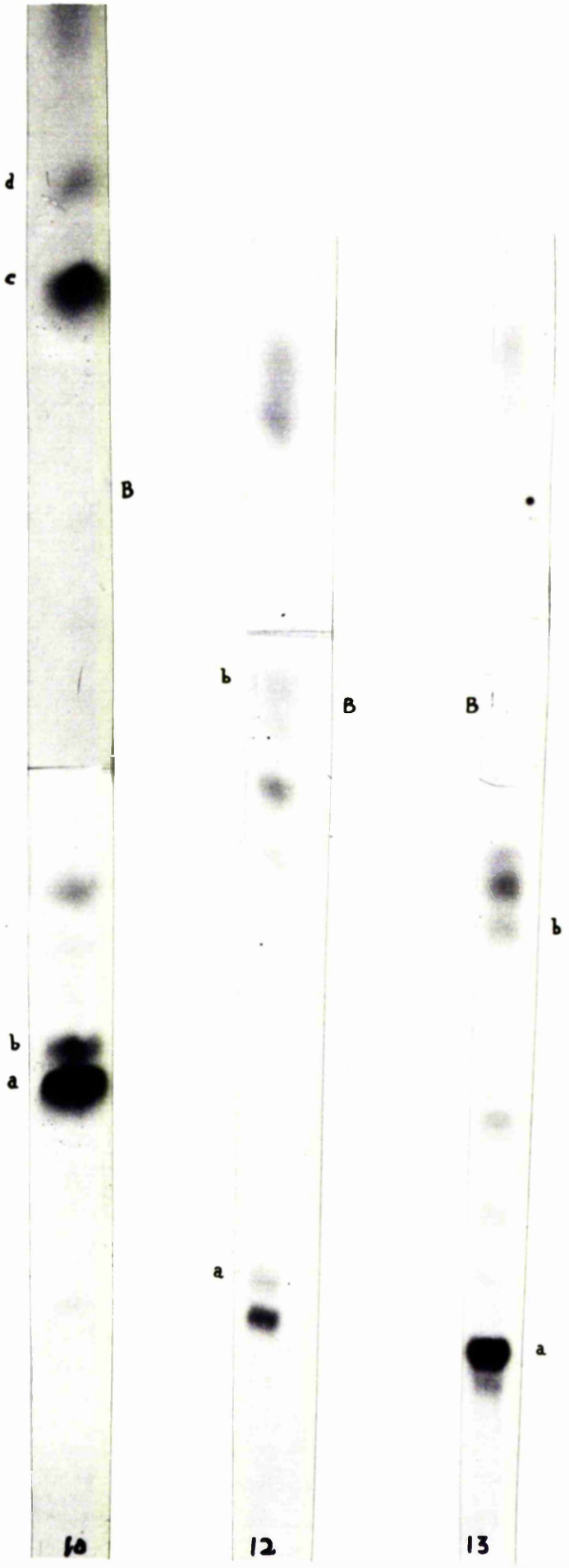

d
c
B
b
a
10
b
B
a
12
B
b
a
13

Table 3.15 Sequence analysis of spot 11 from 28S RNA

Material	Hydrolysis			
	Alkaline	Spleen PDE	VPDE	Structure
Complete hydrolysis				
^{14}C-oligonucleotide	AmC		A	
^{32}P-oligonucleotide	A			
	AmC	(A,C)	(C)	
	G			
Pancreatic RNase products (^{14}C + ^{32}P)				
(a)	A			
	AmC			AAmC
(b)	G			
				AAmCG

trinucleotide while spot c is the non-methylated one. When spot d was digested with alkali Ap and CmpC (monophosphate) was obtained. This showed the sequence of the trinucleotide as ACmC and of methylated tetranucleotide as ACmCG.

Spot 11

This spot was obtained completely pure from the two dimensional fractionation procedure.

Complete hydrolysis: Alkali digestion of ^{14}C methyl labelled material yielded only one spot with the same mobility as Ap. When the dephosphorylated, ^{14}C methyl labelled, oligonucleotide was digested with venom phosphodiesterase, labelled nucleotide A was obtained. This shows that it is not present at 5'-end of the oligonucleotide. Alkali digest of ^{32}P labelled material yielded Ap, Gp and a dinucleotide which was just resolved from Ap. The dinucleotide was identified as AmC (table 3.3). When ^{14}C methyl and ^{32}P double labelled material was digested with pancreatic ribonuclease, AAC containing ^{14}C counts and Gp containing only ^{32}P counts were obtained. These results suggest the sequence of spot 11 as AAmCG.

Spot 12

This oligonucleotide was obtained contaminated with a large amount of(A, C_3) G.

Complete hydrolysis: When ^{14}C methyl labelled material was digested with alkali only one spot was obtained which had

Table 3.16 Sequence analysis of spot 12 from 28S RNA

Material	Hydrolysis: Alkaline	Spleen PDE	VPDE	Structure
Complete hydrolysis				
^{14}C-oligonucleotide	CmA		C	
^{32}P-oligonucleotide	C_3			
	CmA*	(C,A)	(A)	
	G			
	A			
Partial hydrolysis				
VPDE products (^{14}C + ^{32}P)				
(a)				CCCmAG
(b)	C_2			
	CmA (monophosphate)			CCCmA

* Much less than one molar amount as compared to G.

same mobility as Ap. Complete venom phosphodiesterase digestion of dephosphorylated ^{14}C methyl labelled material yielded labelled nucleotide C. It showed that this nucleotide was present internally in the oligonucleotide. Alkali digest of ^{32}P labelled material yielded Cp, Ap, Gp and a dinucleotide which was just resolved from Ap. This alkali resistant dinucleotide was identified as CmA (table 3.3). The following partial sequence could be suggested at this stage (C_2, CmA) G.

Partial hydrolysis: ^{14}C methyl and ^{32}P double labelled material was digested with venom phosphodiesterase for 10, 20 and 30 minutes. The products were separated on DEAE-paper at pH 3.5 (Fig. 3.9). Methylated products were identified by counting and were digested with alkali. Spot a is the original methylated oligonucleotide. Product b is the methylated oligonucleotide which is obtained after removal of pG. Other products are non-methylated oligonucleotides. Alkali digestion of product b showed that it contains two cytidylic acid residues and one dinucleoside monophosphate which was identified as CmpA. The presence of dinucleoside monophosphate shows that it will be present as 3'-end of the tetranucleotide. Therefore the sequence of the tetranucleotide is CCCmA. Thus the original spot 12 possesses the following sequence, CCCmAG.

Spot 13

This oligonucleotide is obtained contaminated with

Table 3.17 Sequence analysis of spot 13 from 28S RNA

Material	Hydrolysis: Alkaline	Spleen	VPDE	Structure
Complete hydrolysis				
^{14}C-oligonucleotide	CmC		C(nucleoside)	
^{32}P-oligonucleotide	CmC*			
	C_2			
	A_2			
	G			
Partial hydrolysis				
VPDE products (^{14}C + ^{32}P)				
(a)				CmCAAG
(b)	A			
	CmC			CmCAA
(c)				

* Less than one molar amount was obtained due to the presence of large amount of contaminating material.

the non-methylated oligonucleotides from two dimensional fractionation procedure.

Complete hydrolysis: Alkali digestion of ^{14}C methyl labelled oligonucleotide yielded one spot which ran between Ap and Cp at pH 3.5. When dephosphorylated oligonucleotide was digested with venom phosphodiesterase, labelled nucleoside C was obtained, showing that it was present at 5'-end of the oligonucleotide. Alkali digestion of ^{32}P labelled material yielded Gp and an alkali resistant dinucleotide which was characterized as CmC (table 3.3). Roughly equal amounts of Ap and Cp containing twice as many counts of radioactivity as in Gp were also obtained. Therefore the base composition of spot 13 was C_2, A_2, G. These findings suggest the following sequence CmCAAG. This sequence was confirmed by partial hydrolysis.

Partial hydrolysis: Dephosphorylated ^{14}C methyl and ^{32}P labelled material was mixed and was digested with venom phosphodiesterase for 10 and 20 minutes at 37°C. The products were separated at pH 3.5 on DEAE-paper. Methylated products were separated from the non-methylated ones. Spot a is the original pentanucleotide. Product b is obtained after removal of pG. Other spots represent non methylated oligonucleotides. When product b was hydrolysed with alkali, dinucleoside diphosphate CmC and Ap were obtained. These results confirm the above sequence CmCAAG.

Spot 15

Table 3.18 Sequence analysis of spot 15 & 16 from 28S RNA

Spot No.	Material	Hydrolysis: Alkaline	Spleen PDE	VPDE	Structure
	Complete hydrolysis				
15	^{14}C-oligonucleotide	AmG			
	^{32}P-oligonucleotide	A_2			
		C			
		AmG	(A,G)	(G)	
	Pancreatic RNase products (^{14}C + ^{32}P)				
	(a)	A_2			
		C			AAC
	(b)	AmG			AmG
					AACAmG
	Complete hydrolysis				
16	^{14}C-oligonucleotide	GmG			
	^{32}P-oligonucleotide	C_2		C	
		GmG	(G)	(G),G_2	CCGmG

This oligonucleotide was obtained completely pure from the two dimensional fractionation procedure.

Complete hydrolysis: Alkaline hydrolysis of ^{14}C methyl labelled material yielded only one spot which migrated slightly slower than Gp at pH 3.5. When ^{32}P labelled material was digested with alkali C, A_2 and one dinucleotide running slower than Gp was obtained. The dinucleotide was found to be AmG (table 3.3). When ^{14}C methyl and ^{32}P double labelled material was digested with pancreatic ribonuclease AAC containing only ^{32}P counts and AmG containing both ^{14}C and ^{32}P counts were obtained. These findings show the sequence of spot 15 to be AACAmG.

Spot 16

This spot has slightly faster mobility than (A_3, C) G in the first dimension but slightly slower in the second. It was obtained completely pure from the two dimensional fractionation procedure.

Complete hydrolysis: When ^{14}C methyl labelled material was hydrolysed with alkali only one spot was obtained which migrated between Gp and Up at pH 3.5. Alkali digestion of ^{32}P labelled material yielded Cp and an alkali resistant dinucleotide which ran between Gp and Up. This dinucleotide was identified as GmG (table 3.3). The oligonucleotide contains two Cp residues as the number of counts in Cp were equal to the number in GmG. Therefore the sequence of the spot 16 is CCGmG.

Table 3.19 Sequence analysis of spots 21-24' from 28S RNA

Spot No.	Material	Hydrolysis: Alkaline	Spleen PDE	VPDE	Structure
	Complete hydrolysis				
21	^{14}C-oligonucleotide	GmG			
	^{32}P-oligonucleotide	GmG	(G)	(G)	GmG
	Complete hydrolysis				
22	^{14}C-oligonucleotide	UmG			
	^{32}P-oligonucleotide	UmG	(U,G)	(G)	UmG
	Complete hydrolysis				
23	^{14}C-oligonucleotide	CmU			
	^{32}P-oligonucleotide	CmU*		(U)	
		G			
		C			
		U			CmUG
	Complete hydrolysis				
24'	^{14}C-oligonucleotide	GmG			
	^{32}P-oligonucleotide	A			
		GmG		(G)	AGmG

* Very small amount as compared to G.

Spot 21

This dinucleotide was obtained completely pure from the two dimensional fractionation procedure.

Complete hydrolysis: Alkaline hydrolysis of ^{14}C methyl labelled material yielded one spot which ran between Gp and Up at pH 3.5. When ^{32}P labelled material was hydrolysed with alkali only one spot was obtained between Gp and Up. There were equal number of counts of radioactivity in free phosphate and dephosphorylated product after phosphatase treatment. This alkali resistant dinucleotide was identified as GmG (table 3.3).

Spot 22

This spot had slightly greater mobility than UG in the second dimension of the two dimensional fractionation procedure and therefore was obtained completely pure.

Complete hydrolysis: Only one spot was obtained on alkaline hydrolysis of ^{14}C methyl or ^{32}P labelled material. Phosphatase treatment of ^{32}P labelled material yielded equal number of counts of radioactivity in free phosphate and dephosphorylated product. This alkali resistant dinucleotide was identified as UmG (table 3.3).

Spot 23

This trinucleotide was obtained heavily contaminated with CUG and UCG.

Complete hydrolysis: Alkaline hydrolysis of ^{14}C methyl labelled material yielded one spot which had same mobility

as Gp. When ^{32}P labelled material was hydrolysed with alkali Cp, Up and Gp were obtained together with an alkali resistant dinucleotide which ran with Gp. This dinucleotide was identified as CmU (table 3.3). Therefore the sequence of spot 23 is CmUG.

Spot 24

This spot has a slightly slower mobility than CUG and was obtained contaminated with this non-methylated trinucleotide. It was purified by subjecting it again to two dimensional fractionation procedure.

Complete hydrolysis: Alkaline hydrolysis of ^{14}C methyl labelled material yielded one spot between Gp and Up. An alkali resistant dinucleotide GmG (table 3.3) and Ap were obtained from the alkaline hydrolysis of ^{32}P labelled material. The sequence of the spot therefore was AGmG.

Spot 25

The spot was obtained completely pure from the fingerprint as it migrated faster than AUG in the second dimension of the fractionation procedure.

Complete hydrolysis: When ^{14}C methyl labelled material was hydrolysed with alkali a spot which migrated between Gp and Up was obtained. Alkaline hydrolysis of ^{32}P labelled material yielded Gp and an alkali resistant dinucleotide AmU which ran between Gp and Up at pH 3.5 (table 3.3). The sequence of spot 25 therefore was AmUG.

Spot 26

Table 3.20 Sequence analysis of spots 25,26 & 29 from 28S RNA

Spot No.	Material	Hydrolysis: Alkaline	Spleen PDE	VPDE	Structure
	Complete hydrolysis				
25	^{14}C-oligonucleotide	AmU			
	^{32}P-oligonucleotide	AmU		(U)	
		G			AmUG
	Complete hydrolysis				
26	^{14}C-oligonucleotide	UmA			
	^{32}P-oligonucleotide	UmA		A	
		C		C	
		G		G	UmACG
	Complete hydrolysis				
29	^{14}C-oligonucleotide	UmG			
	^{32}P-oligonucleotide	A_2			
		UmG*	(U,G)		
		U			
		G			AAUmG

* Very small amount as compared to G.

This spot was obtained pure from the two dimensional fractional procedure.

Complete hydrolysis: Only one spot which migrated between Gp and Up was obtained on alkaline hydrolysis of ^{14}C methyl labelled material. When ^{32}P labelled material was hydrolysed with alkali Cp, Gp and an alkali resistant dinucleotide migrating between Up and Gp was obtained. The dinucleotide was identified as UmA (table 3.3). Complete venom phosphodiesterase digestion of dephosphorylated ^{32}P labelled material yielded pG, pC and pA, but pU was not present showing it to be on 5'-end of the tetranucleotide. The sequence of this tetranucleotide therefore is UmACG.

Spot 29

This spot was obtained heavily contaminated with (A_2, U) G.

Complete hydrolysis: Alkali digestion of ^{14}C methyl labelled material yielded one spot which had slightly greater mobility than Up at pH 3.5. When ^{32}P labelled material was hydrolysed with alkali Ap, Up, Gp and an alkali resistant dinucleotide UmG was obtained. As the base composition of spot 29 was A_2, U, G, therefore the sequence of the methylated tetranucleotide will be AAUmG.

Spot 61

This spot was obtained completely pure from the two dimensional fractionation procedure.

Complete hydrolysis: ^{14}C methyl labelled material yielded

Table 3.21 Sequence analysis of spot 61 from 28S RNA

Material	Hydrolysis			
	Alkaline	Spleen PDE	VPDE	Structure
Complete hydrolysis				
^{14}C-oligonucleotide	GmG			
^{32}P-oligonucleotide from T_1 fingerprint	U_2			
	GmG (di-phosphate)	(G)	(G)	
from T_1 + phosphatase fingerprint	U_2			
	GmG (monophosphate)			UUGmG

one spot on alkaline hydrolysis. When ^{32}P labelled material from T_1 digested RNA fingerprint was hydrolysed with alkali Up and an alkali resistant dinucleoside diphosphate was obtained. Alkaline hydrolysis of ^{32}P labelled material from T_1 plus phosphatase digested RNA fingerprint yielded Up and the alkali resistant dinucleoside monophosphate GmG (table 3.3). There were twice as many counts in Up as in GmG (monophosphate). Therefore the spot 61 contains two uridylate residues. The sequence of the tetranucleotide therefore is UUGmG.

Pseudouridine has been demonstrated in 18S and 28S RNA of HeLa cells (Amaldi and Attardi, 1968). This finding has been recently confirmed (Maden, personal communication). However in this study a distinction between pseudouridine and uridine was not made.

3.4 Quantitation of Ribosomal RNA Methylation

It was possible to obtain ^{14}C methyl labelled RNA fingerprints with radioactivities sufficient to provide data for quantitation. Relative molar ratios of the methylated sequences within the rRNAs were calculated from these fingerprints. In order to calculate the absolute frequencies of occurrence of these methylated sequences, in terms of moles of oligonucleotide/mole of rRNA, it was necessary to know the total number of methyl groups present in 18S and 28S RNAs. These quantities were estimated independently as described below.

3.3a Estimation of relative molar ratios of methylated oligonucleotides

^{14}C methyl labelled RNA fingerprints were prepared as described in methods section. The spots were excised and counted by scientillation counting. Since there is no reason to use any particular spot as a basis for calculating the molar ratios of other oligonucleotides, these ratios were expressed relative to an arbitrary value of unity for the mean of spots 41 and 43 in 18S RNA. In 28S RNA the mean of the counts from spots 4 to 13 was taken as unity. Table 3.22 shows the molar ratios of the 18S spots calculated in this way. Most of the spots are present in approximately equimolar amounts. Two 18S spots (37 & 42) are usually present in less than half these molar amounts. Two spots (8 & 11) are present in higher molar amounts. Spot 30 contains dimethyl A as well as methylated ribose and therefore shows a higher count. The molar ratios of methylated spots from 28S RNA are shown in table 3.23. 28S RNA like 18S RNA contains a large number of spots in approximately equimolar amounts. Two spots (17 & 18) are present in lower molar amounts. Spots 3 and 21 are the most densely labelled spots and they are present in about 4 and 6 times the average molar amounts respectively. There are three more spots which are also present in higher than average amounts, namely spots 14, 33 and 36.

Table 3.22 Molar ratios and absolute frequencies of methylated oligonucleotides from 18S RNA

Spot No.	I cpm	I ratios	II cpm	II ratios	III cpm	III ratios	IV** cpm	IV** ratios	V** cpm	V** ratios	Mean molar ratios	Absolute frequencie. nearest integer.
2	864	1.13	888	1.19	744	1.09					1.14	1
3	844	1.10	744	1.00	742	1.09					1.06	1
7	535	0.99	814	1.09	569	0.84					0.97	1
8	1175	1.54	1244	1.67	900	1.32					1.51	1.5
9	656	0.86	659	0.88	638	0.94					0.89	1
11	1126	1.48	1417	1.90	1228	1.80					1.72	1.5 - 2
21	760	1.00	578	0.78	902	1.32					1.03	1
22	854	1.12	559	0.75	643	0.94					0.94	1
24	750	0.98	456	0.61	567	0.83					0.81	1
24'	976	1.28	758	1.01	1136	1.67					1.32	1
30	2789	3.66	2329	3.12	2741	4.02	4280	3.63			3.60*	1
34	530	0.70	527	0.71	380	0.56	912	0.77			0.69	1

* It contains one dimethyl A and two other methylated components.

** Obtained from T_1 plus phosphatase fingerprint.

Table 3.22 continued.

Spot No.	I cpm	I ratios	II cpm	II ratios	III cpm	III ratios	IV cpm	IV ratios	V cpm	V ratios	Mean molar ratios	Absolute frequencies nearest integer.
37			445	0.60	290	0.43	252	0.21			0.41	0.5
41	754	0.99	799	1.07	724	1.06	1100	0.93	1507	0.99	1.01	1
42	428	0.56	208	0.28	200	0.29	539	0.46	1580	1.03	0.52	0.5
43	768	1.01	691	0.93	638	0.94	1260	1.07	1533	1.01	0.99	1
44							860	0.73	1608	1.05	0.89	1
45							1170	0.99	1563	1.03	1.01	1
46	3344	4.39	2145	4.49	2857	4.19	1090	0.92	1272	0.84	0.88	1
47							1105	0.94	1120	0.74	0.84	1
48							1125	0.95	1380	0.91	0.93	1
49			563	0.76	535	0.79	1170	0.99	1270	0.84	0.85	1
50			574	0.77	580	0.85	1000	0.85			0.82	1
51			434	0.58	435	0.64	900	0.76			0.66	1
52			408	0.55	480	0.70	1185	1.00	1375	0.95	0.80	1
61			819	1.10	731	1.07	1337	1.13	1570	1.03	1.08	1
72			453	0.61	536	0.79	970	0.82			0.74	1

Table 3.23 Molar ratios and absolute frequencies of methylated oligonucleotides from 28S RNA

Spot No.	I cpm	I ratios	II cpm	II ratios	mean molar ratios	absolute frequencies nearest integer
2	891	1.15	655	1.15	1.15	1
3	2811	3.63	2683	4.70	4.17	4
4	663	0.86	496	0.87	0.86	1
6	893	1.15	659	1.15	1.15	1
7	812	1.05	620	1.09	1.07	1
9	779	1.00	611	1.07	1.04	1
10	788	1.02	581	1.01	1.02	1
11	775	1.00	566	0.99	1.00	1
12	833	1.07	501	0.88	0.98	1
13	655	0.85	535	0.94	0.90	1
14	1186	1.53	767	1.34	1.44	1.5
15	661	0.85	496	0.87	0.86	1
16	501	0.65	491	0.86	0.76	1
17	244	0.29	225	0.39	0.34	0.5
18	255	0.33	302	0.53	0.43	0.5
21	4420	5.70	4024	7.04	6.37	6
22	827	1.07	665	1.16	1.12	1
23	) 1130	1.46	505	0.88		1
24			569	1.00		1
25	673	0.87	536	0.94	0.91	1
26	538	0.69	591	1.04	0.87	1
27	616	0.79	626	1.10	0.95	1
28	610	0.79	594	1.04	0.92	1
29	386	0.50	585	1.02	0.76	1
31	551	0.71	555	0.97	0.84	1
32	661	0.85	603	1.06	0.96	1
33	1654	2.13	1260	2.21	2.17	2
35	563	0.73	577	1.01	0.87	1
36	1032	1.33	1159	2.03	1.68	1.5 - 2

3.3b Absolute frequencies of methylated oligonucleotides in rRNA

The fact that many of the methylated oligonucleotides are recovered in approximately equal relative molar amounts suggests that these nucleotides may possibly occur once per molecule. To examine this possibility the total number of methyl groups in 18S RNA was first determined by the following isotopic ratio experiment (Fellner and Sanger, 1968). ^{14}C Methyl labelled and ^{32}P labelled RNAs were prepared separately. They were also counted separately in a scintillation counter with minimal spillover (about 3.5% of ^{32}P in ^{14}C and 1% of ^{14}C in ^{32}P). They were then mixed together in amounts yielding a roughly fourfold excess of ^{32}P-RNA over ^{14}C methyl labelled RNA. Care was taken to use amounts of isotopes (about 4×10^5 cpm of ^{32}P-RNA and 10^5 cpm of ^{14}C-RNA) sufficient to obtain accurate counts both of ^{32}P and ^{14}C in oligonucleotides. $^{32}P/^{14}C$ ratio of the mixed RNA was calculated. The mixture was digested with T_1 RNase and fingerprinted. Spots 3,9,21,22 and 41 which were obtained pure in the fingerprint were excised and eluted. (In the earlier experiments they were counted without elution, but it was found that the counting efficiency is different when the oligonucleotides are present on the DEAE-paper. Therefore the results obtained were incorrect.) They were counted in a scintillation counter. The number of methyl groups were calculated from the formula x.N/y, where x is

Table 3.24 Determination of the number of methyl groups in 18S RNA by $^{32}P/^{14}C$ ratio method

Material	^{32}P	^{14}C	$^{32}P/^{14}C$	$^{32}P/^{14}C$ per Me-nucleotide (x)	$\frac{x.N^*}{y}$
18S RNA	368.5×10^3	91.5×10^3	4.0264 = y		
Spot					
3 (AmpGp)	212	1002	0.2116	0.1058	53
9 (AmpApGp)	467	1360	0.3434	0.1145	57
21 (GmpGp)	285	1477	0.1930	0.0965	48
22 (UmpGp)	243	1342	0.1811	0.0905	45
41 (GmpUpGp)	479	1483	0.3230	0.1077	53
				Mean =	51

* N = number of nucleotides/RNA molecule, 18S RNA taken as 2000.

$^{32}P/^{14}C$ ratio per methylated nucleotide, N is the number of nucleotides/RNA molecule, y is $^{32}P/^{14}C$ ratio of the RNA. The results suggest that 18S RNA contains 51 methyl groups (table 3.24)

The number of methyl groups on 18S and 28S RNA was also calculated by Dr. B.E.H. Maden by a second method which is as follows. A large number of pure methylated oligonucleotides can be obtained by digesting RNA with T_1 plus pancreatic RNases, a procedure which yields smaller products than T_1 digestion. From the fraction of the total ^{32}P radioactivity in several of these pure methylated products together with knowledge of the sequences of the products and the total number of nucleotides in 18S and 28S RNA (2000 and 5000 respectively) the molar yields of these products were calculated. From this the number of methyl groups in this particular group of pure products (some of which were doubly methylated) was determined. The value obtained in this way was the total absolute number of these oligonucleotides. The total number of methyl groups in 28S and 18S rRNA could then be obtained from a quantitative analysis of ^{14}C methyl fingerprints obtained by the same digestion technique. The value for 18S RNA obtained in this way (48 methyl groups/mole) is in agreement with that obtained from $^{32}P/^{14}C$ ratio experiment. 28S RNA was found to contain 65 methyl groups as determined by this method.

On the basis of the absolute number of methyl groups

Table 3.25 · Absolute number of methyl groups in 18S and 28S RNA

	Spot No.	Methyl groups	Spot No.	Methyl groups
Number of spots containing one methyl group and of relative frequency = approx. 1	All except those listed below	36	All except those listed below	43
Spots containing >1 methyl group and of frequency = 1	30	3-4	5*spots	10
	85	2		
Spots multiply or fractionally represented	8	1.5-2	2	4
	11	2	21	6
	37	0.5	33	2
	42	0.5	36	1.5-2
			17	0.5
			18	0.5
Total number of methyl groups obtained		46.47		68
Total No. of methyl groups obtained from $^{32}P/^{14}C$ ratio expt.		51		
Total No. of methyl groups obtained from T_1 + pancreatic expt. (B.E.H. Maden, see text)		48		65

* Found in T_1 + pancreatic RNase digests.

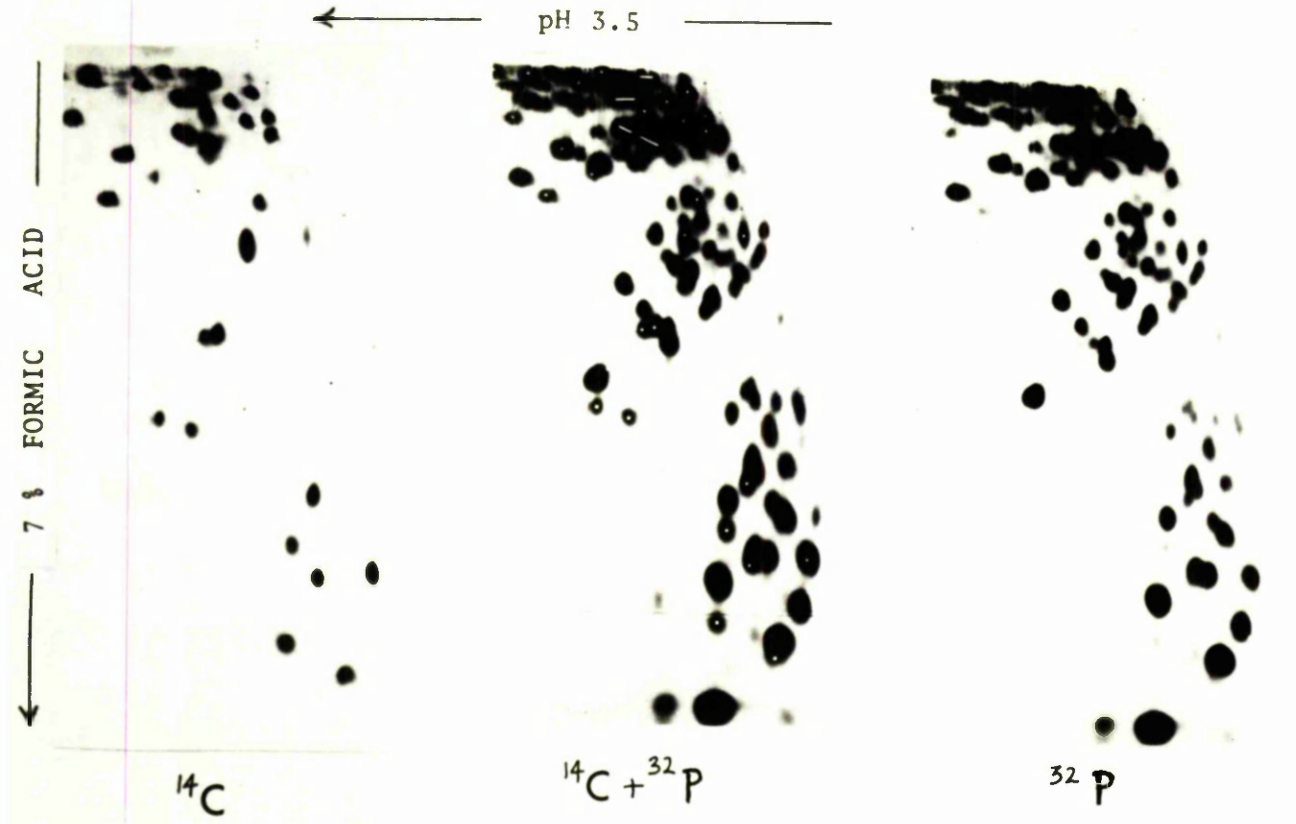
the frequencies of methylated oligonucleotides were calculated as follows. 18S RNA showed 40 methylated spots on T_1 and T_1 plus phosphatase fingerprints. Most of them contain one methyl group/oligonucleotide and are present in equimolar amounts. A few contain more than one methyl group or occur with higher than average frequency. If the spots containing one methyl group (and in approximately one relative molar frequency) are assumed to occur once and those containing two methyl groups or present in twice the relative molar amounts are taken as two methyl groups, a figure for total number of methyl groups can be obtained for both 18S and 28S RNA (table 3.25). The figures calculated in this way are very near to that obtained from $^{32}P/^{14}C$ ratio experiment or from T_1 plus pancreatic ribonuclease experiment. Therefore the assumption that the spots which are present in relative equimolar amounts occur once in the molecule is correct. The suggested frequencies of the spots is shown in table 3.22 and 3.23.

3.5 Comparison of HeLa 18S & 28S RNA with C13 and L cells

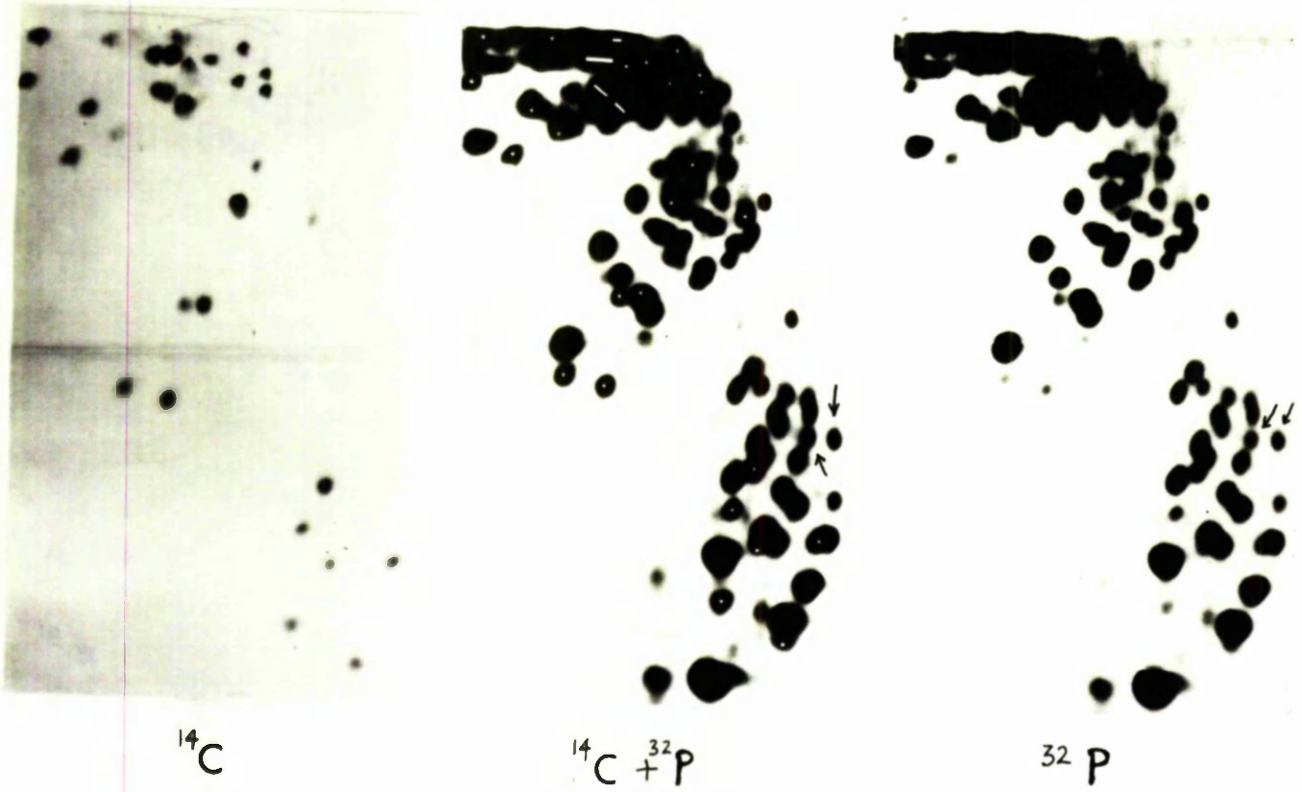
It was interesting to compare the methylation patterns of rRNA from different cells. Therefore fingerprints were prepared from C13 and L cell rRNA. The comparison with HeLa rRNA showed the following.

The patterns of methylated oligonucleotides containing no or one uridylic acid residue from 18S RNA of HeLa and C13 cells are identical. However two extra spots can be identified in "no U graticule" of HeLa cell ^{32}P fingerprints. Fingerprints showing longer methylated oligonucleotides containing two or more U residues are also very similar in both of these cells. There are a few differences which are indicated by arrows in the autoradiographs (figure 3.10 & 3.12)

Methyl labelled 28S RNA fingerprints from C13 cells prepared by digesting RNA with T_1 ribonuclease show a large number of well resolved spots. As for 18S RNA the pattern of these spots is identical to that obtained from HeLa cells. The HeLa cell ^{14}C methyl labelled RNA fingerprint shows a few faintly labelled spots. Some of these are ^{32}P spots as the autoradiograph was obtained from ^{14}C + ^{32}P fingerprint after most but not quite all of the ^{32}P had decayed. ^{32}P labelled RNA fingerprints obtained from both of these cells are similar, although a close examination reveals a few differences. The HeLa cell ^{14}C methyl labelled fingerprint contains an extra spot in the "two U graticule". Similarly



C 13



HeLa

Fig. 3.10 Comparison of oligonucleotides obtained from T_1 ribonuclease digest of 18S RNA from HeLa and BHK-21/C13 cells

^{32}P and ^{14}C methyl labelled RNA were prepared separately from HeLa as well as C13 cells. About 90 x 10^3 dpm of ^{14}C-RNA and 180 x 10^3 dpm of ^{32}P-RNA were mixed in each case. The mixture was digested with T_1 ribonuclease and fingerprinted. The fingerprints were placed in folders with two X ray films on the same side. The film which was in direct contact with the fingerprint was affected by both ^{14}C and ^{32}P, but the other film was affected only by ^{32}P radiation. Autoradiographs were developed and fingerprints were then left for some months to allow the ^{32}P to decay completely. They were then used to develop autoradiographs showing only ^{14}C labelled oligonucleotides. White spots in ^{14}C + ^{32}P autoradiographs show the position of these methylated oligonucleotides.

Fig. 3.11 Comparison of oligonucleotides obtained from T_1 ribonuclease digest of 28S RNA from HeLa and BHK-21/C13 cells

^{32}P and ^{14}C methyl labelled 28S RNA were prepared separately from HeLa and C13 cells. About 90 x 10^3 dpm of ^{14}C-RNA and 180 x 10^3 dpm of ^{32}P-RNA were mixed in each case. The mixture was digested with T_1 ribonuclease and fingerprinted. The first dimension (pH 3.5) is from right to left and second dimension (7% formic acid) from top to bottom. ^{32}P, ^{14}C + ^{32}P and ^{14}C autoradiographs were obtained as in the case of 18S RNA (figure 3.10). ^{32}P in HeLa ^{14}C fingerprint is not completely decayed. White spots in ^{14}C + ^{32}P autoradiographs show the position of methylated oligonucleotides.

^{14}C

$^{14}C + ^{32}P$

^{32}P

C 13 28S

^{14}C

$^{14}C + ^{32}P$

^{32}P

HeLa 28S

Fig. 3.12 Comparison of oligonucleotides obtained from T_1 RNase plus phosphatase digest of 18S RNA from HeLa and BHK-21/C13 cells

^{32}P and ^{14}C methyl labelled 18S RNA were prepared separately from HeLa and C13 cells. About 90 x 10^3 dpm of ^{14}C-RNA and 180 x 10^3 dpm of ^{32}P-RNA were mixed in each case. The mixture was digested with T_1 ribonuclease and alkaline phosphatase and fingerprinted. The first dimension (pH 3.5) is from right to left and second dimension (7% formic acid) from top to bottom. Oligonucleotides were located by autoradiography. Another autoradiograph was obtained from the same fingerprint after a few months when all the ^{32}P was decayed. White spots in ^{14}C + ^{32}P autoradiographs indicate the position of methylated oligonucleotides. The position of blue marker is indicated by b.

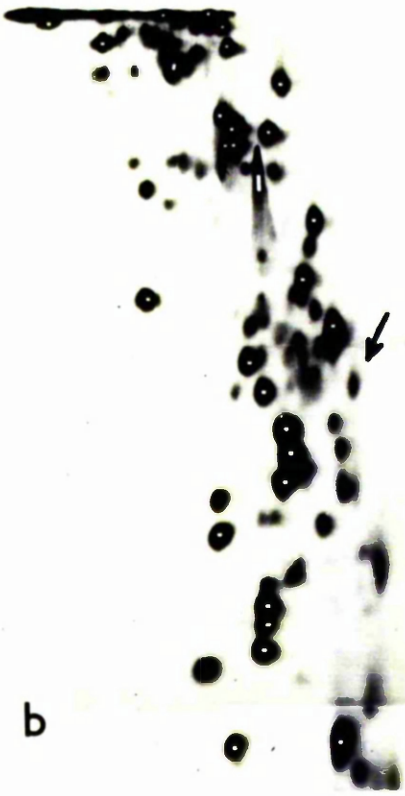
C 13

^{14}C

$^{14}C + {}^{32}P$

HeLa

^{14}C



$^{14}C + {}^{32}P$

Fig. 3.13 Comparison of oligonucleotides obtained from T_1 RNase and phosphatase digested 28S RNA from HeLa and BHK-21/C13 cells

^{32}P and ^{14}C methyl labelled 28S RNA were prepared separately from HeLa and C13 cells. About 90 x 10^3 dpm of ^{14}C-RNA and 180 x 10^3 dpm of ^{32}P-RNA were mixed in each case. The mixture was digested with T_1 ribonuclease and alkaline phosphatase and fingerprinted. The first dimension (pH 3.5) is from right to left and second dimension (7% formic acid) from top to bottom. Oligonucleotides were located by autoradiography. Another autoradiograph was obtained from the same fingerprint after a few months when all the ^{32}P was decayed. White spots in ^{14}C + ^{32}P autoradiographs indicate the position of methylated oligonucleotides. The position of blue marker dye is indicated by b.

C13

^{14}C

$^{14}C + ^{32}P$

HeLa

^{14}C

$^{14}C + ^{32}P$

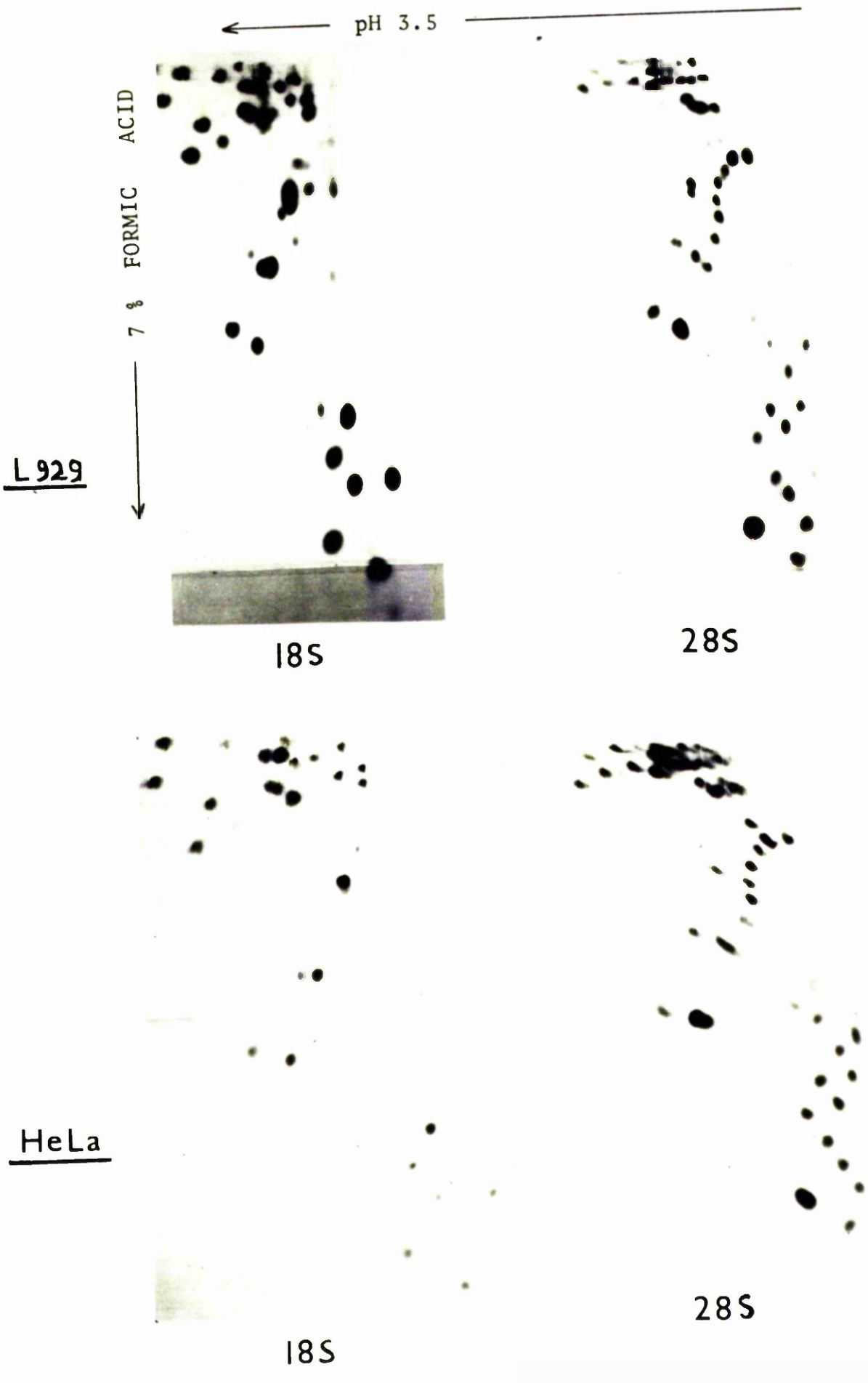
^{32}P fingerprints also show one or two differences in the "one U graticule". These differences are indicated in figure 3.11 . When 28S RNA from these cells is digested with T_1 ribonuclease plus alkaline phosphatase a few further differences were found in ^{14}C as well as in ^{14}C + ^{32}P labelled RNA fingerprints. Figure 3.13 shows these differences as indicated by arrows.

Fingerprints prepared by digesting 18S RNA from L cells with T_1 ribonuclease are also very similar to fingerprints of HeLa cell RNA. There are a few faintly labelled spots in L cells fingerprints but they are present in less than 0.2 molar amounts. The methylation pattern of L cell 28S RNA is basically similar to HeLa cells. However some extra spots are present in these fingerprints.

These results indicate that although there are a few differences in the methylated oligonucleotide patterns of the three different mammalian cells (HeLa, C13 and L) examined the patterns are nevertheless basically very similar.

Fig. 3.14 Comparison of HeLa and L929 cells rRNA

Monolayer HeLa and L929 cells were labelled with ^{14}C methyl methionine for 36 hours. The cells were harvested by trypsinisation. 18S and 28S RNA were prepared by sucrose gradient centrifugation (see methods). The purified RNA was digested with T_1 ribonuclease and fingerprinted. Radioactive oligonucleotides were located by autoradiography.


pH 3.5
7 % FORMIC ACID
L 929
18S
28S
HeLa
18S
28S

SECTION 4

DISCUSSION

4.1 Biosynthesis of high molecular weight rRNA in HeLa cells

As mentioned in the introduction the synthesis of rRNA in HeLa cells is believed to consist of one transcription and several post-transcription steps. First a single 45S RNA molecule is transcribed. This 45S RNA is present in 80S nucleolar ribonucleoprotein particles. Some nucleotides are removed from 45S RNA resulting in formation of 41S RNA, which is then cleaved to 32S and 20S nucleolar RNA. 32S RNA, which is present in 55S nucleolar particles, is then transformed to 28S nucleolar RNA and then to 28S rRNA. 20S nucleolar RNA gives rise to 18S rRNA. All these RNAs contain methyl groups. These methyl groups are incorporated into 45S RNA during or soon after transcription (Greenberg and Penman, 1966; Zimmerman and Holler, 1967). (A five minute label with methionine showed complete methylation of 45S RNA as detected by RNA fingerprinting; Maden unpublished results). As mentioned earlier the presence of 2'-O-methyl ribose substituents results in alkali resistance of the adjacent phosphodiester bonds. Analysis of these alkali resistant dinucleotides has shown that the methylation patterns of 18S and 28S RNA are significantly different (Wagner, Penman and Ingram, 1967). The present results are consistent with this finding but revealed some additional alkali resistant dinucleotides which were not reported earlier. For example 18S RNA also contains AmC and CmU, and 28S RNA

also contains AmC, UmA and UmG in addition to other dinucleotides reported by Wagner et al. Moreover methylation patterns of 18S and 28S RNAs are very different as revealed by fingerprinting. Thus it is possible to test whether a certain RNA molecule contains nucleotide sequences characteristic of one or both ribosomal species (18S and 28S). Fingerprints of the presumed precursors (32S and 45S) to rRNA were therefore prepared. A comparison of these fingerprints with rRNA fingerprints showed the following:-

The pattern of small as well as large methylated oligonucleotides from 32S nucleolar RNA is very similar to 28S RNA confirming that 28S rRNA is derived from 32S RNA.

The 45S fingerprint is similar to that of 18S plus 28S RNA in nearly all respects. Most of the 18S unique spots, including numbers 8, 24, 41, 42 and 43 are present as well as all 28S spots. Although little sequence analysis of nucleolar material has been carried out, all the di- and trinucleotides (for example number 2, CmG; 3, AmG; 9, AAmG + AmAG; 41, GmUG) can be characterized due to their distinctive mobilities and from the results of alkaline hydrolysis (Some of these nucleotides can also be identified in ^{32}P labelled 45S RNA fingerprints). There were some differences in detail between 45S and 18S + 28S fingerprints. These were as follows:-

(a) Spot 30 which is strongly labelled in 18S RNA fingerprints is only weakly labelled in 45S RNA.

(b) The 18S spot number 34 is not present in 45S RNA fingerprints.

(c) Spot 49 which is present in 18S RNA is absent from 45S RNA (The significance of a, b and c is discussed in section 4.2)

(d) 45S RNA fingerprints show an extra spot, number 38.

Apart from the above four differences 45S RNA fingerprints are virtually identical to those of 18S + 28S. These results clearly indicate three points.

1. 45S RNA contains the sequences of both 18S and 28S RNA.
2. Few methylation events take place at a later stage in the maturation of rRNA.
3. There is no methylated sequence of non-ribosomal type in 45S RNA with one possible exception (spot 38).

It was therefore concluded that 45S RNA is a common precursor to both high molecular weight ribosomal RNA species and that 32S RNA is the precursor to 28S rRNA. It was also concluded that a few late methylation events occur on 18S RNA and 45S RNA does not contain any methylated non-ribosomal sequences except spot 38.

4.2 Methylation of rpre RNA and rRNA in HeLa cells

As mentioned in the preceding section three spots could

be easily recognised in 18S RNA fingerprints, two of which are absent from 45S RNA and one is present in small amount. It was found earlier that one late methylation event takes place on 18S RNA (Zimmerman, 1968) and results in the formation of dimethyl A. In the present study the nature of these three spots of 18S RNA was investigated. It was found that all these spots contain a methylated base and one of the spots (30) contains dimethyl A. Two of these spots were absent in 41S and 18-20S nucleolar RNA fingerprint and one (spot 30) was weakly labelled (Maden, Salim and Summers, 1972). Spot 30 also contains 2'-O-methyl ribose in addition to dimethyl A. Therefore the small amount of radioactivity of spot 30 in these RNAs is probably due to 2'-O-methylation. These observations suggested that these three "late" methylation steps probably take place in the cytoplasm. Labelling the cells after addition of actinomycin D to block further RNA synthesis still results in labelling of these three spots in 18S RNA, confirming that labelling occurs late in the processing of rRNA (Maden, personal communication). In 28S RNA no secondary methylation was evident from the fingerprints shown in this thesis but labelling cells in the presence of actinomycin D also seems to result in late methylation of one spot (Maden, personal communication). The nature of this spot is still being investigated. All the other methylated oligonucleotides (which are methylated in 45S RNA and are present in 18S plus

28S RNA) sequenced in this study contain only 2'-O-methyl ribose. This raises the possibility that all primary methylation which takes place on 45S might be 2'-O-methylation whereas secondary methylation is base methylation. Similar results have recently been reported in yeast rRNA methylation (Retel, van den Bos and Planta, 1969).

Most of the large as well as small methylated sequences were found to be present only once in the molecule. A few of the sequences were present twice and two in relatively large amounts (AmG, 4 moles and GmG, 6 moles in 28S RNA). There are a few sequences which are present in about half molar amounts. This may be due to the heterogenity of rRNA or incomplete methylation or both.

The presence of a wide variety of methylated sequences might be taken to suggest that the primary structure of the RNA does not itself determine where methylation occurs. An alternate possibility is that secondary structure of 45S RNA determines the specificity of methylation.

4.3 Methylation of rRNA in different mammalian cells

Methylation patterns of rRNA from HeLa cells were compared with C13 and L cells. The following points were revealed by the comparison:-

1. There are some differences in ^{32}P fingerprints of 18S RNA from HeLa and C13 cells but the methylation patterns are similar.

2. Similarly ^{32}P labelled 28S RNA fingerprints from HeLa and C13 cells show some differences but methylation patterns are very similar.
3. L cells gave a fingerprint of 18S ^{14}C methyl labelled RNA which was basically similar to HeLa cells, but there were a few extra spots. These extra spots are very faint and are present in about 0.2 molar amounts.
4. The 28S RNA methylation pattern of L cells is also very similar to HeLa cells with a few minor differences.

These results therefore show very similar methylation patterns of rRNAs for three different mammalian species (man, mouse, hamster). This implies that the specificity of methylation was somehow conserved during the evolution of these species.

4.4 Topology of 45S RNA

There is little information available about the arrangement of 18S, 28S and non-ribosomal RNA sequences in 45S RNA. A few inferences can be drawn from recent knowledge of the intermediate products of processing of 45S RNA to rRNA. These are as follows.

The sequences of 28S and 18S rRNA are retained in the 41S molecule (Maden, Salim and Summers, 1972) therefore non-ribosomal material must be removed from one or other

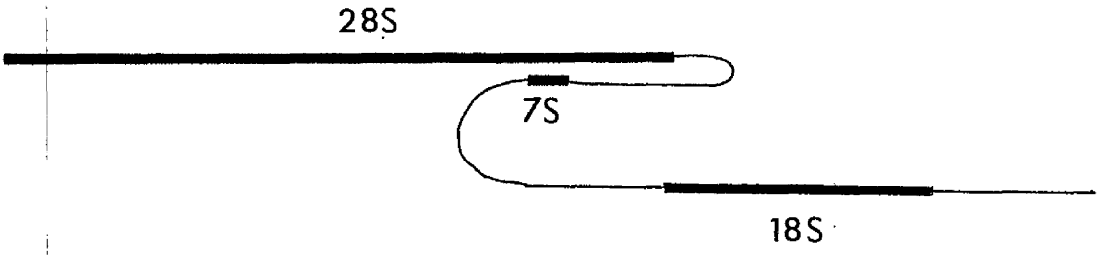


Fig. 4.I A model of 45S RNA

(or possibly both) ends of 45S RNA during conversion to 41S RNA.

The second inference is derived from the use of cordycepin (3' deoxyadenosine) which has been reported to cause premature termination of 45S RNA. This compound in suitable concentration appears to allow the synthesis of large fragments of rpre RNA which were reported to give rise to mature 18S rRNA but not 32S or 28S RNA in HeLa cells (Siev, Weinberg and Penman, 1969). This suggested that 18S sequences may be located towards the first synthesized 5'-end of 45S RNA. However, in Novikoff hepatoma cells the 5'-terminal of 28S and 45S was found to be mainly (80%) the dinucleoside triphosphate pCmpUp, suggesting that 28S RNA is present at the 5'-end of the 45S RNA (Choi and Busch, 1970). These experiments of end group determination are not in agreement with that of cordycepin.

A third point is that 7S RNA appears to be generated simultaneously with the 32S → 28S RNA transition (Pene Knight and Darnell, 1968). It could be inferred that 7S is linked to 28S through a loop of non-ribosomal RNA which is degraded during conversion of 32S to 28S RNA. Since non-ribosomal segments have not been detected, these segments must have been degraded in close conjection with the maturation steps of rRNA production (Weinberg and Penman, 1970). The above mentioned inferences suggest a model of 45S RNA which is shown in figure 4.1. Such a model requires

at least one endonuclease and possibly various exonuclease to convert 45S RNA to its final 18S, 28S and 7S products.

The relative arrangement of 18S and 28S rRNAs in 45S has not yet been settled. Useful data might be obtained about the topology of 45S RNA by the technique of sequence analysis used in this study together with the analysis of partial digests (to obtain large fragments) and end group determinations.

4.5 Conclusions

The following conclusions may be drawn from the present study.

1. Methylated oligonucleotide patterns from 18S and 28S RNAs are complex and very different from each other.
2. 45S RNA contains most 18S as well as all 28S methylated products. 32S RNA contains only 28S methylated products This shows the relationship between ribosomal precursor RNAs and rRNAs.
3. Sequence analysis confirmed the previous finding that rRNA of HeLa cells contains mainly 2'-O'methyl groups.
4. Three late methylation events occur on 18S RNA and result in the formation of methylated bases.
5. A wide variety of sequences have been found to be methylated. Most of these sequences are present only once in the RNA molecule.
6. Methylated oligonucleotide patterns from three different mammalian species examined in this study are very simila

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