STUDIES ON THE CONFORMATION OF HeLa CELL 5·8S rRNA.

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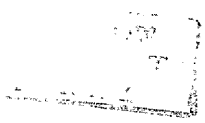

SUMMARY

The low molecular weight ribosomal RNA species, 5.8S rRNA, is found in the larger ribosomal subunit of eukaryotic cells; it occurs in equimolar ratios to the 28S rRNA molecule and is hydrogen-bonded to that component. The nucleotide sequences of 5.8S rRNA from several organisms have been determined and a secondary structure proposed, based on maximised base pairing. The aim of this work has been to investigate the conformation of HeLa cell 5.8S rRNA.

The approach to this investigation involved the use of the technique of chemical modification. The assumption behind such an approach is that a base residue which is not modified by a reagent specific for that base is inaccessible. Bases involved in double helical stems of the molecule are unreactive and the reaction will be diminished if a base is involved in maintaining the tertiary structure through stacking or base pairing. Only bases not involved in such interactions will be significantly modified by a reagent specific for that base. The correlation between the three dimensional structure of yeast $tRNA^{Phe}$ and the results obtained from chemical modification studies suggest that such studies carried out on 5.8S rRNA should provide useful data concerning the conformation. Two modifying reagents, were used in this study: sodium bisulphite, which reacts specifically with non-hydrogen bonded cytidine residues, converting them to uridine, and carbodiimide, which under the conditions used has high specificity for non-hydrogen bonded uridine residues. When these techniques are coupled with 'fingerprinting' procedures it is possible to investigate the structure of 5.8S rRNA.

When these studies were carried out on isolated 5.8S rRNA at 25°C, the reactivities obtained for the cytidine and uridine bases were compared with their positions in the secondary structure model (Nazar et al., 1975). Those pyrimidine bases proposed to be in loop regions exhibited the highest reactivity, whereas those which were internally located within helices exhibited little or no reactivity. A number of bases which were proposed to be associated with helical imperfections, or which formed 'closing' pairs at the ends of helices exhibited intermediate reactivity. At 37°C, the conformation was shown to be similar, although two of the helices (a and d) were partially destabilised. Helix (a) is proposed to contain several imperfections and helix (d) to possess a high AU base content. At 50°C, it was shown that most of the molecule had dissociated with only helices (c) and (e) remaining intact. The high stability of these helices can be attributed to their high GC base content.

These findings confirmed that 5.8S rRNA contains six loop regions, five designated helical regions, as well as some helical imperfections. This provided the basis for further studies on the interaction of 5.8S and 28S rRNA. This was accomplished by reacting sodium bisulphite with 28S rRNA at 25°C, removing the 5.8S rRNA component by heating, and then 'fingerprinting' it. By comparing the data from this experiment with those obtained from the modification of 'unbound' 5.8S rRNA, it was possible to detect changes in accessibility of individual cytidine residues to sodium bisulphite as a result of the 5.8S - 28S rRNA interaction. Although most of the cytidine residues reacted to the same extent in 'bound' 5.8S rRNA, two were shown to exhibit higher reactivity and

some others lower reactivity. These differences were confined to cytidine residues in loops I and V and helices (a) and (b) (see Chapter 4 for explanation of nomenclature). The cytidine residues in other regions of the molecule were reactive to the same extent in 'bound' and 'unbound' 5.8S rRNA, indicating that they are not directly involved in the interaction with 28S rRNA and remain conformationally unaltered. It has been suggested by Pace et al. (1977) that nucleotides from helix (a) form the junction with 28S rRNA. The results presented in this thesis support that idea.

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INTRODUCTION

The ribosome is the site of protein synthesis in both prokaryotic and eukaryotic cells. In each case it consists of two subunits of unequal size and is made up of about one-third protein and two-thirds RNA by weight. Despite a common function, there are major differences in ribosomes from the two sources, as shown in Table 1.1. These differences may be summed up: eukaryotic ribosomes are larger, the RNA has more secondary modifications, and they possess an extra low molecular RNA which is about 160 nucleotides long. This '5.8S' rRNA is not found in prokaryotes (Erdmann, 1976).

Nucleotide sequence homology has been demonstrated between rRNAs from a wide range of eukaryotes, but not between rRNAs from eukaryotes and E. coli (Sinclair and Brown, 1971). However, more detailed analysis has demonstrated the existence of sequence homologies at the 3' end of E.coli 16S rRNA and eukaryotic 18S rRNA (Alberty et al., 1978). Homologies are found between the ribosomal proteins of distantly related eukaryotes, when examined on two dimensional polyacrylamide gels (Delauney et al., 1973), but prokaryotic ribosomal proteins are fewer in number and immunologically unrelated to those of eukaryotes, with one exception (Wool and Stoffler, 1974).

The differences in ribosomes from eukaryotic and prokaryotic sources are puzzling since both carry out the same basic function in protein synthesis. If mammalian mRNA is to be successfully translated in prokaryotic systems, a detailed knowledge of the functional and structural aspects of both prokaryotic

Table 1.1

Two major classes of ribosome occur in nature, the 70S ribosomes of prokaryotes and the 80S ribosomes of eukaryotes. The main differences are given below.

	Small Subunit		Large Subunit	
	Mammalian	E. coli	Mammalian	E. coli
Sedimentation coefficient of subunit	40S	30S	60S	50S
Sedimentation coefficient of RNA	18S	16S	28S + 5.8S + 5S	23S + 5S
Approx. chain length of RNA (nucleotides)	2000	1600	5000 + 160 + 120	3200 + 120
Methyl groups per RNA	45 (mainly ribose)	14 (mainly base)	68 + 2 (mainly ribose) + zero	16 (mainly base) + zero
Proteins per subunit	30	21	40	34

The values obtained for distantly related eukaryotes are closer to those for mammals than E. coli, except that the chain length of 28S rRNA is shorter in lower eukaryotes (approx. 4000).

and eukaryotic ribosomes is essential. This thesis describes work carried out on the structural aspects of 5.8S rRNA, the ribosomal component which is found only in eukaryotes.

1.1 Ribosomal Genes

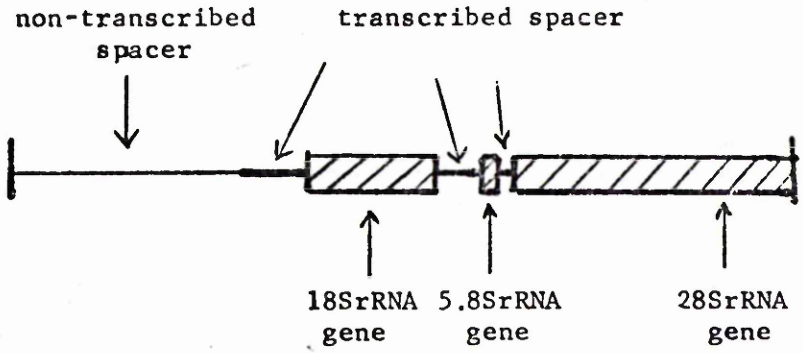
The primary transcript product of the genes coding for HeLa cell ribosomal RNA is a 45S precursor which contains the sequences of 18S, 28S and 5.8S rRNA (Maden, 1976). In all eukaryotes so far examined, these rRNA genes are located in the nucleolus in repeating units (for review see Reeder, 1974). There is an increase in the number of copies of these ribosomal cistrons during evolution which roughly parallels the increase in overall genomic content. Maximal values of multiplicity are found in the amphibian oocyte, where the number of ribosomal cistrons is increased to a few million per cell by a gene amplification process. In HeLa cell, the number is about 1200 (Birnsteil et al., 1971).

Xenopus Laevis

Xenopus laevis ribosomal DNA has provided the most accessible system for genetic analysis in eukaryotes because it can be prepared purely, due to its high G + C content relative to the bulk DNA (Wallace and Birnsteil, 1966; Sinclair and Brown, 1971). Investigations on this amphibian have shown that there are about 450 repeating units of rDNA clustered in the nucleolus organiser. Each of these units contains an 18S rRNA gene, an intervening transcribed spacer (which contains the 5.8S rRNA gene), a 28S rRNA gene and a larger spacer which is mostly non-transcribed (Brown and Weber, 1968; Wellauer

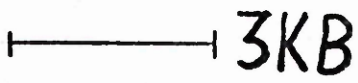
Fig. 1.1

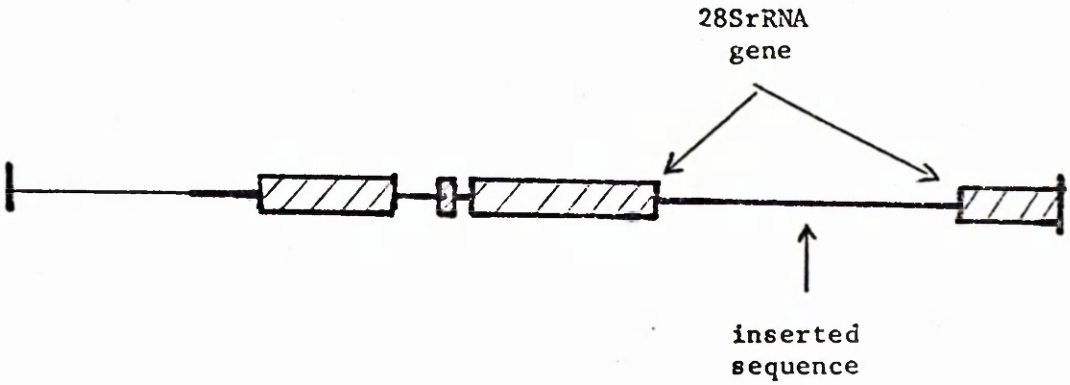
Ribosomal DNA repeat units.



(a) Xenopus laevis

Adopted from Speirs and Birnsteil (1974)





(b) Drosophila melanogaster

35% of the rDNA cistrons from the X chromosome are of this type. The other 65% plus those from the Y chromosome are similar to those of X. laevis.

Adapted from Pellegrine et al. (1977).

NOTES

1. Light lines represent non-transcribed spacer.
2. Dark linesrepresent transcribed spacer.
3. Areas in boxes represent regions whichcode for ribosomal RNA.

and David, 1974; Wellauer et al., 1974; Wellauer and Reeder, 1975). See Fig. 1.1(a).

In Xenopus laevis, the genes which code for 18S and 28S rRNAs are conserved during evolution, while the non-transcribed spacer sequences evolve rapidly (Brown et al., 1972). Although the rDNA spacers show length heterogeneity, they have not accumulated a large number of nucleotide substitutions. A large fraction of the spacer region of rDNA is composed of short subrepeats which constitute internally repetitous segments. It is the number of copies of these subrepeats which distinguish long spacers from short spacers (Wellauer et al., 1976).

Drosophila

In the other organism where the ribosomal DNA has been extensively characterised, Drosophila, the genes transcribed to yield 18S, 28S and 5.8S rRNAs are clustered in two nucleolus organisers; one in the X chromosome and one in the Y (Rittossa et al., 1966). The number of genes in each, though variable, is normally about 250 in the X chromosome and somewhat less in the Y (Spear, 1974). In Drosophila melanogaster cells, there are two major classes of rDNA repeat units. The smaller size is similar to that of Xenopus laevis, whilst the larger size has an additional segment of DNA inserted at a reproducible site within the 28S rRNA gene (White and Hogness, 1977; Wellauer and David, 1977; Pellegrini et al., 1977). See Fig. 1.1(b).

5.8S rRNA The portion of the genome coding for 5.8S rRNA is located within the transcribed spacer between the regions coding for 18S and 28s rRNAs

(Speirs and Birnsteil, 1974). This was unambiguously demonstrated by Pace and Walker (1977), who hybridised 5.8S rRNA to fragments generated from Xenopus laevis rDNA by the restriction endonuclease, EcoRI. Since the polarity of the 18S and 28S rRNA cistrons had already been established (Hackett and Sauerbier, 1975; David and Wellauer, 1976; Reeder et al., 1976) and the positions of the EcoRI cleavage sites had been mapped (Wellauer et al., 1974), it was possible by this method to determine unambiguously the position of the 5.8S rRNA cistron in its transcriptional unit. Using X. laevis rDNA fragments which had been cloned in E.coli , Boseley et al. (1978) were able to sequence the 5.8S rDNA gene and the area around it. The position in HeLa cell rDNA is probably similar, since the sequence coding for 5.8S rRNA has been located at an analogous site in the 45S primary gene product (Maden and Robertson, 1974).

5S rRNA The genes coding for 5S rRNA are not genetically linked to rDNA. In HeLa cell, 5S rRNA was shown to hybridise with DNA isolated from chromosomes of all size classes, indicating that 5S DNA is probably located on most of the chromosomes in this cell type (Aloni et al., 1971). The 5S DNA, like rDNA, consists of many tandemly repeated copies. In HeLa cell there are 2000 copies (Halten and Attardi, 1971).

1.2 Transcription

Eukaryotic rRNA is transcribed as a large precursor molecule which contains the sequences of 18S, 28S and 5.8S rRNA, as well as considerable stretches of transcribed spacer. In HeLa cell, this molecule contains 13000

nucleotides and sediments at 45S. The size of this ribosomal transcription unit increases considerably from lower to higher vertebrates (Loening et al., 1969; Perry et al., 1970; Maden, 1971). These species variations are due to differences in the lengths of the transcribed spacer regions and, to a lesser extent, the 28S rRNA sequence. The spacer sequences are removed during processing.

Polarity of transcription

In the large nucleolar precursor, the positions of the 18S and 28S rRNA sequences have been characterised (see Fig. 1.2). Two independent studies (Hacket and Sauerbien, 1975; Liau and Hurlbert, 1975) on the biosynthesis of 45S rRNA, both analogous to the well-known experiment of Dintzis (1961), strongly suggested that the 18S rRNA sequence is nearer the 5' end of the molecule than is the 28S rRNA sequence. This assignment of polarity was unambiguously demonstrated by Dawid and Wellauer (1976) using a combination of electron microscopy and exonuclease digestion of ribosomal DNA plasmids.

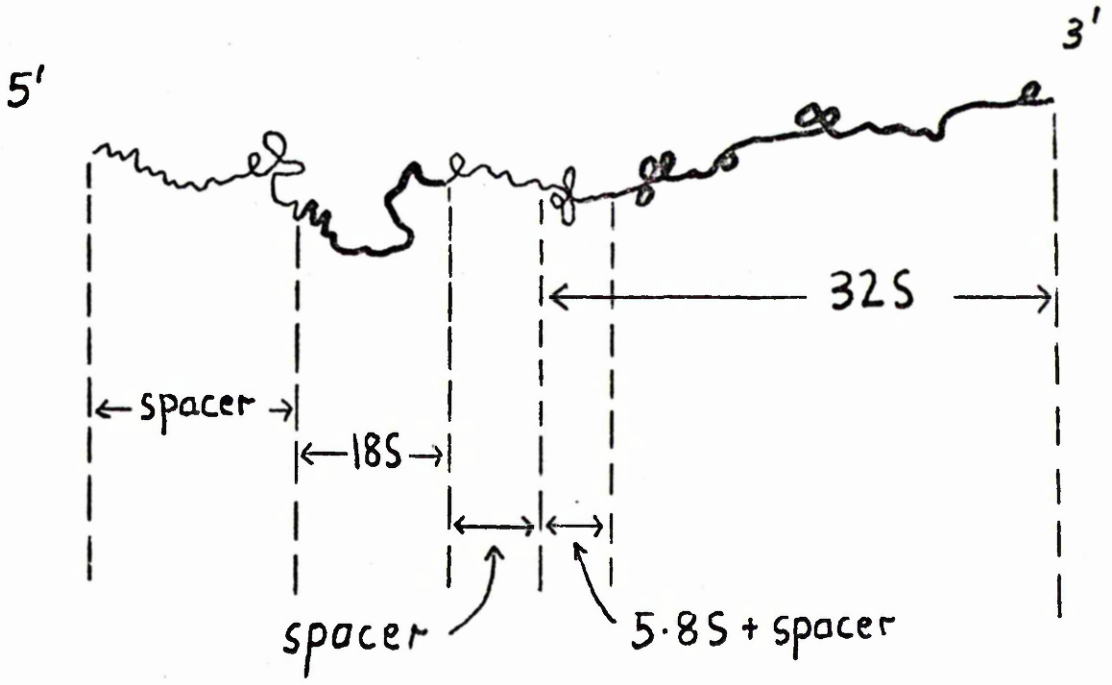
The 5.8S rRNA sequence within the 45S precursor has been shown to be between the 18S and 28S rRNA sequences. 'Fingerprinting' analyses made by Maden and Robertson (1974) and Nazar et al. (1975) demonstrated that it is contained within the 32S part of the ribosomal precursor RNA and therefore located near the 28S rRNA sequence. As mentioned previously, this is analogous with the positioning of the sequences coding for 5.8S rRNA within the ribosomal cistrons.

1.3 Ribosomal RNA Maturation

The major steps in the maturation of rRNA involve the specific elimination

Fig. 1.2

Topographical map of HeLa cell 45SrRNA



Taken from Maden (1976).

NOTES

1. Light lines represent spacer regions.
2. Dark lines represent rRNA sequences as indicated.

of the transcribed spacer regions from the 45S precursor RNA to yield 18S and 28S rRNA. Elimination of these transcribed spacers occurs in several stages within the nucleus (see Fig. 1.3). This process was established by following the kinetics of labelling cellular RNA with radioactive uridine (Scherrer and Darnell, 1962; Scherrer, Latham and Darnell, 1963). Further studies by Weinberg et al. (1967) and Weinberg and Penman (1970) enabled the maturation pathway to be proposed as in Fig. 1.3. The longest lived intermediate is the 32S rRNA which contains the 28S and 5.8S ribosomal RNA sequences.

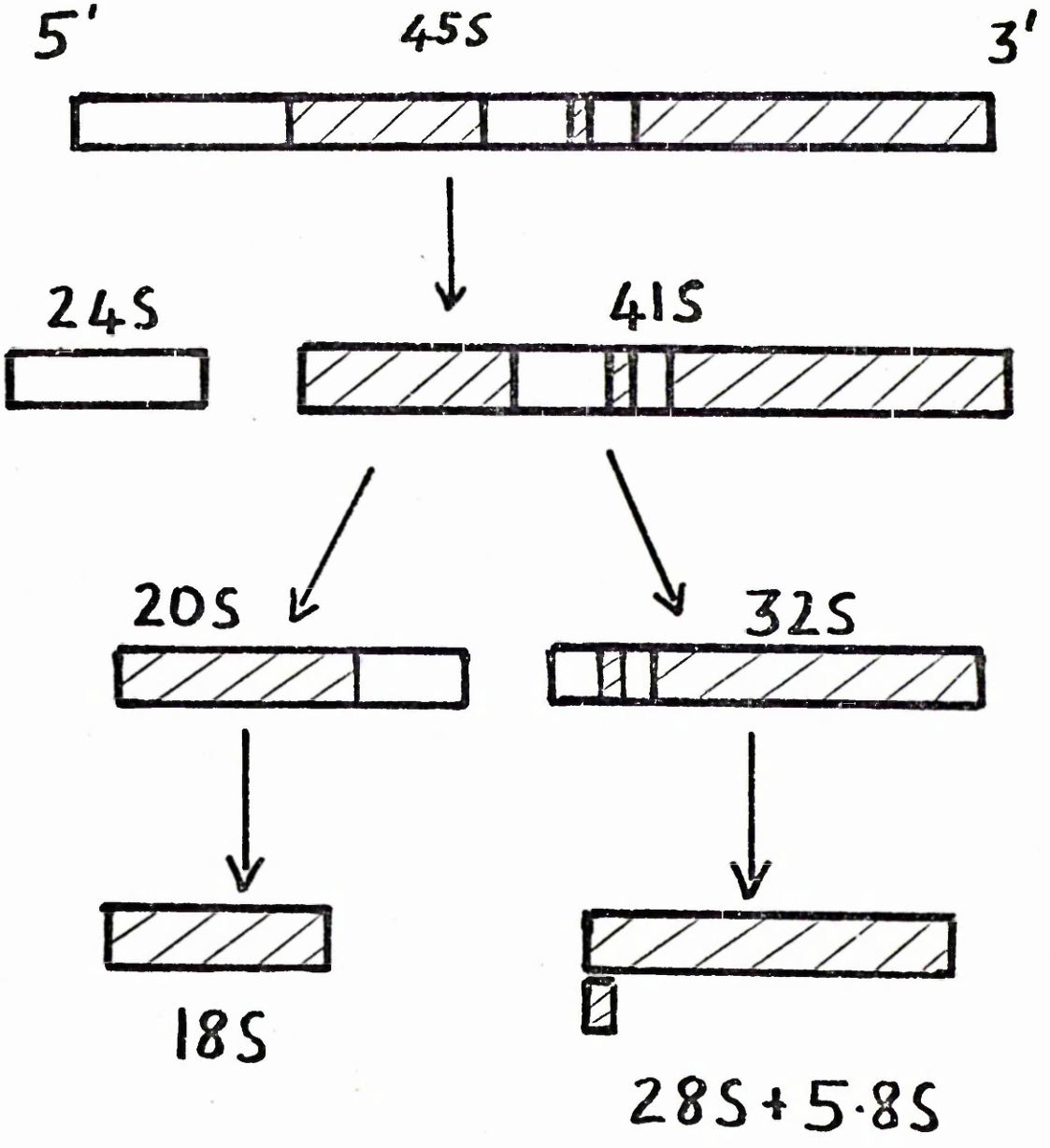
In E. coli, ribonuclease III, which is specific for double stranded RNA regions, plays a central role in RNA processing. It has been suggested that a similar type of nuclease may be involved in 45S rRNA processing in eukaryotes (Nikolaev et al., 1974).

RNA - Protein Interactions during Ribosomal Maturation

Production of mature ribosomes involves numerous protein interactions with the RNA precursors. These proteins can be subdivided functionally into: (1) ribosomal proteins, (2) proteins involved with the excision of the transcribed spacer, and (3) those proteins which produce the secondary modifications (methylations and pseudouridylations). 45S and 32S rRNA can be extracted from nucleoli in ribonucleoprotein particles (RNPs) which sediment at 80S and 55S (Warner and Soiero, 1967). These particles contain most of the proteins of the large subunit, as well as 5S rRNA. Attempts to identify the small subunit proteins within the 80S particles have proved inconclusive (Shepherd

Fig. 1.3

Maturation of HeLa cell 45S precursor rRNA.



Adapted from Hadjiolov and Nikolaev (1976).

NOTES

1. Maturation of rRNA occurs in several stages as indicated.
2. Various precursor molecules have been isolated (as shown above) allowing the maturation pathway to be resolved.
3. Shaded areas represent rRNA sequences.
4. Unshaded areas represent transcribed spacer regions.

and Maden, 1972). The non-ribosomal proteins on the RNPs are removed before leaving the nucleus and are continually recycled (Kumar and Warner, 1972).

1.4 Secondary Modifications

In addition to the excision of the transcribed spacer regions, the other major step in rRNA maturation is the secondary modification of residues in the 18S, 28S and 5.8S rRNA sequences. In HeLa cell 45S rRNA, there are some 200 secondary modification sites (see Table 1.2). These sites are all in the ribosomal sequences of the precursor (Maden, 1977). There are two types of secondary modification.

Methylation

Methylation is essential for ribosome maturation in HeLa cells (Vaughan et al., 1967). The vast majority (90%) of methyl groups are of one chemical type, namely 2'-O-ribose methylation, and occur at the level of the 45S precursor. All methyl groups are conserved during processing. In addition six late methylations occur on the bases of 18S rRNA, taking place during the last processing steps leading to mature rRNA. These findings have been fully documented by Maden and Salim (1974).

All methylation sites possess different primary sequences. It is therefore unlikely that it is simply the respective sequences that are recognised for modification, since it would require that there be a different enzyme for every modification site. Rather, it is likely that these methylations are carried out by one, or at the most, a few different enzymes which must recognise some common feature. Since this common feature is not one of primary sequence,

it must be one of conformation. Studies have been carried out on the conformation of methylation sites in HeLa cell ribosomal RNA, as determined by their accessibility to sodium bisulphite and nuclease-S_1 (Goddard and Maden, 1976; Khan and Maden, 1978). These showed that methylation sites have a range of different conformations. Since it has been demonstrated that the methylation process occurs on nascent chains (Greenberg and Penman, 1966; Liau and Hurlbert, 1975), it may be that sites that are accessible to the methylating enzyme system during polynucleotide chain growth become buried by secondary or tertiary interactions during ribosome maturation. It is interesting to note that the four sites of late methylation are all sensitive to nuclease-S_1 digestion and therefore must be in exposed, single-stranded regions within 18S rRNA.

Methylated nucleotide sequences are highly conserved in vertebrate ribosomal RNAs. For example, there is 95% homology between the methylated nucleotide sequences of rRNA from two distantly related vertebrates such as man and Xenopus laevis (Khan et al., 1978). Ribosomal RNA from non-vertebrates such as yeast (Klootwijk and Plant, 1973, 1974) and Drosophila (Maden and Tartof, 1974) possess fewer methyl groups (yeast contains half as many as HeLa cell). Nevertheless, some remarkable secondary modification homologies have been conserved. For example, the late methylated sequence in 18S rRNA, G-$^{6}_{2}$mA-$^{6}_{2}$mA-C-U-Gp, has been identified in bacteria, yeast and mammalian species (Hadjiolov and Nikolaev, 1976).

The functions of the methylated sequences are unknown. However, since those regions of precursor rRNA which do not contain methylated sequences are

excised during processing, this suggests that methylation may play a role in rRNA maturation.

Pseudouridylation

The other major type of modified nucleotide present in eukaryotic rRNA is pseudouridine. Pseudouridine is present in eukaryotic 18S, 28S and 5.8S rRNAs in considerable numbers (Amaldi and Attardi, 1968; Klootwijk and Planta, 1974; Hughes and Maden, 1978) (see Table 1.2). Observations made by Robertson and Maden (1973) strongly suggest that within precursor rRNA, pseudouridines are restricted to the ribosomal RNA sequences. In HeLa cell, pseudouridine is formed largely or entirely at the level of precursor rRNA in the nucleolus (Jeanteur et al., 1968; Maden and Forbes, 1972). The possible role of pseudouridine in the structure and processing of rRNA remains to be elucidated.

Table 1.2 Approximate numbers of pseudouridines and methyl groups in HeLa cell rRNA.

	Pseudouridine	Methyl groups	
		2'-O-Methyl	Base
18S	37	38	7
28S	60	63	5
5.8S	2	1.2	-
Total rRNA	100	101	12

Adapted from Maden et al. (1977).

1.5 Ribosomal RNA

28S and 18S rRNA

The HeLa cell transcription product of the ribosomal genes ultimately yields two large rRNA species which have sedimentation coefficients of 18S and 28S. These molecules are characterised by an increase of their molecular weights in evolution (Table 1.3). The G + C content of rRNA also markedly increases, particularly in 28S rRNA, with values up to 68% (Attardi and Amaldi, 1970). In prokaryotes, the analogous molecules sediment at 16S and 23S.

Inter-species sequence homologies

There is sequence homology between rRNA species from a wide range of eukaryotes. In nucleic acid hybridisation experiments, Sinclair and Brown (1971) showed that without exception, the DNA of all eukaryotes analysed had some molecular homology with X. laevis rDNA. No homology was found between X. laevis rRNA and the DNA of prokaryotes. Similarly, extensive homologies between the methylated nucleotide sequences in several vertebrate rRNAs were detected by Khan et al. (1978).

In all eukaryotes so far examined the 3'-terminal sequence of 18S rRNA is G-A-U-C-A-U-U-A. This terminal oligonucleotide is in a highly exposed conformation (Vass and Maden, 1978). The 3' ends of E.coli 16S (Ehresmann et al., 1975), yeast 17S (de Jonge et al., 1977) and rat 18S rRNAs (Alberty et al., 1978) have been sequenced and shown to exhibit extensive homology. In particular, the sequence G-m^6_2A-m^6_2A-Cp, which is universal in eukaryotes,

Table 1.3 Molecular Weights of the Large Ribosomal RNAs

Species	Molecular Weight x 10^{-6}	
	Large Subunit	Small Subunit
E. coli	1.07	0.56
Yeast	1.30	0.72
Drosophila	1.40	0.73
X. laevis	1.51	0.70
Chick	1.58	0.70
Mouse	1.71	0.70
Human (HeLa)	1.75	0.70

Adapted from Loening (1968).

is found near the 3' end of E. coli 16S, yeast 17S and rat 18S rRNAs.

The 3' end of 16S rRNA in E. coli is complementary to a sequence found near the initiation codon of prokaryotic mRNA, allowing the formation of a complex between the two RNAs. This is assumed to play an important role in the initiation of protein synthesis (Steitz and Jakes, 1975). Differences between prokaryotic and eukaryotic sequences in this region might give rise to difficulty in the expression of eukaryotic genes in bacterial cells.

5.8S rRNA

The low molecular weight rRNA species, known as 5.8S rRNA ($\sim$ 160 nucleotides) is found in the large ribosomal subunits of eukaryotic cells (Pene et al., 1968; King and Gould, 1970). It is encoded by the major ribosomal DNA (Speirs and Birnsteil, 1974; Walker and Pace, 1977; Boseley et al., 1978) and is co-transcribed with 18S and 28S rRNA within the ribosomal precursor RNA (Maden and Robertson, 1974; Nazar et al., 1975). When eukaryotic 28S rRNA is briefly heated or otherwise denatured, this small non-covalently attached molecule is released.

Nucleotide sequences of 5.8S rRNA have now been determined for several diverse eukaryotic species: yeast (Rubin, 1973), rat hepatoma (Nazar et al., 1975), mouse (Hampe et al., 1976), turtle (Nazar and Roy, 1976), rainbow trout (Nazar and Roy, 1977), chick and HeLa (Khan and Maden, 1977) and Xenopus (Ford and Mathieson, 1978). The most striking feature observed in these sequences is the high degree of inter-species homology (see Table 1.4). The data reveal that 5.8S rRNA exhibits heterogeneity at its 5' end, although there is no evidence for the existence of sequence heterogeneities within the

Table 1.4 5.8S rRNA Sequence Homologies

- as compared to the sequence of HeLa cell 5.8S rRNA.

Yeast	75%
Rainbow Trout	95%
Xenopus Laevis	97%
Chick	99%
Turtle	> 99%
Rat	> 99%
Mouse	> 99%
HeLa	100%

5.8S molecules from the same species.

As with the larger rRNA species, 5.8S rRNA undergoes secondary modification at the level of precursor ribosomal RNA. Mammalian 5.8S rRNA contains two methylation sites, both of which are on ribose residues. One of these sites is fractionally methylated to an extent which varies between species. Two pseudouridylic acid residues are also present in mammalian 5.8S rRNA.

A highly conserved RNA sequence, such as is found with 5.8S rRNA, suggests a rigorous structural requirement for its biological function. Although its function is unknown, it is assumed that the common role of all 5.8S rRNAs in ribosome function will result in the existence of a general structure. Such a structure, based on maximised base-pairing has been proposed (Nazar et al., 1975). It is consistent with all of the presently known sequences. This structure was recently supported by comparative studies on the conformation using physical techniques (Van et al., 1977). Studies by Khan and Maden (1976) using S_1-nuclease as a probe have also elucidated various features of the structure. However, evidence on the conformation of individual residues within the structure remains to be settled.

5S rRNA

The other small ribonucleic acid associated with the large ribosomal subunit is 5S rRNA ($\sim$ 120 nucleotides). Unlike 5.8S rRNA, it contains no modified bases and is found in both prokaryotic and eukaryotic organisms. 5S rRNA from both groups differ significantly in a number of ways: their sequences are quite different; eukaryotic 5S rRNA seem to be primary transcript products,

whereas prokaryotic 5S rRNAs are derived from precursor molecules; reconstitution experiments show that different prokaryotic 5S rRNAs can be reconstituted into B. stearothermophilus 50S subunits to yield active ribosomes, whereas eukaryotic 5S rRNA cannot. These differences indicate that 5S rRNA may have different functions in prokaryotes and eukaryotes (for review, see Erdmann, 1976).

1.6 Aims of Project

As with the larger rRNA species, 5.8S rRNA is transcribed within the 45S rRNA precursor, contains modified bases and possesses secondary structure. Its smaller size has allowed this molecule to be extensively characterised and sequenced in several species. This makes it a useful model to study to gain an insight into the structure and function of ribosomal RNA in general.

The aim of this work is to investigate the conformation of HeLa cell 5.8S rRNA using chemical reagents as tools to probe the structure. The assumption behind such chemical modification studies is that a base, which is not modified by a reagent specific for that base is inaccessible for one or more reasons. Bases involved in helical regions of the molecule will be unreactive, and it is also assumed that the reaction will be diminished if a base is involved in maintaining tertiary structure through stacking or hydrogen-bonding. Only bases that are not involved in such interactions will be significantly modified by a reagent specific for that base.

The three dimensional structure of yeast $tRNA^{Phe}$ has been determined by X-ray crystallographic analysis (Robertus et al., 1974a). When this was

compared to results obtained using chemical modifying reagents (Robertus et al., 1974b), the correlation between the exposed regions of the model and the regions of chemical reactivity were everywhere consistent. It was on the basis of this finding that the approach to this project was initiated.

In searching for an effective probe, several factors have to be taken into account. The chosen reagent must not disrupt the structure of the RNA, it should be highly specific towards one type of base and should exhibit high selectivity towards bases in single stranded regions. Two reagents which possess these properties and have been used successfully in conformation analysis of tRNAs are sodium bisulphite (Goddard and Schulman, 1973; Lowdon and Goddard, 1976) and carbodiimide (Rhodes, 1975). Sodium bisulphite has also been used to study the conformation of methylated bases within 28S rRNA (Goddard and Maden, 1976). Under the conditions used in this study, sodium bisulphite reacts specifically with cytidine residues and carbodiimide reacts specifically with uridine residues.

When the technique of chemical modification is coupled with standard 'fingerprinting' procedures, the reactivities of individual cytidine or uridine residues within HeLa cell 5.8S rRNA can be determined. The 'fingerprinting' system used was essentially that of Sanger et al. (1965), Brownlee and Sanger (1967) and Fellner (1969), with minor modifications. In this system, the RNA is enzymically digested and the oligonucleotide products separated in two dimensions by electrophoretic chromatography. The two enzymes most commonly used to digest the RNA are T_1 ribonuclease (which cleaves specifically after

Gp) and pancreatic ribonuclease (which cleaves specifically after Cp and Up).

Sodium Bisulphite Modification

Sodium bisulphite reacts with non-hydrogen bonded pyrimidine residues. Of the resulting adducts, 5-6 dihydrocytidine-6-sulphonate undergoes easy deamination to give the uridine analogue. This may be converted to uridine by adduct removal at a slightly elevated pH. The reaction sequence illustrated in Fig. 1.4 constitutes a mild method of bringing about the Cp $\longrightarrow$ Up conversion (for review see Hayatsu, 1976).

Using this technique it is possible to identify non-hydrogen bonded cytidine residues within the 5.8S rRNA sequence. The site and extent of modification can be obtained by comparing sequence data from 'fingerprints' of modified and unmodified 5.8S rRNA. Those oligonucleotides containing reactive (i.e. exposed) cytidine residues will be present in reduced molar yields. This can be verified by the appearance of the corresponding uridine-containing oligonucleotide in another part of the 'fingerprint'.

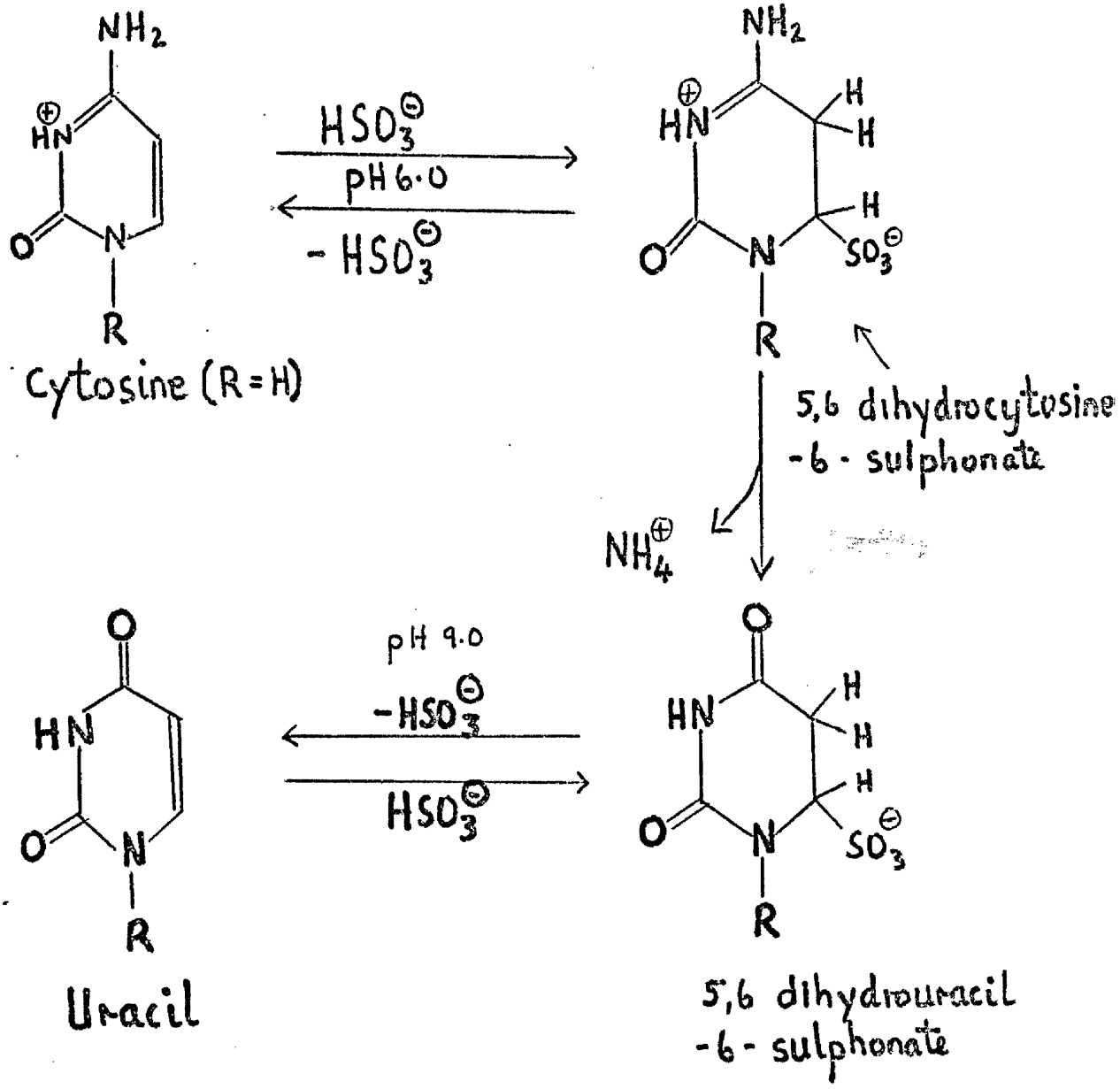
Carbodiimide Modification

Water-soluble carbodiimide forms an adduct with the N^3 and N^1 position of uracil and guanine residues respectively (see Fig. 1.5) (Brownlee, 1972). This reaction is greatly inhibited if the residue occurs in the hydrogen-bonded regions of the molecule. Therefore, as with sodium bisulphite, carbodiimide is a useful probe of secondary structure in a molecule such as 5.8S rRNA.

Brownlee et al. (1968) found that it is possible to carry out the reaction so that only the more accessible uridine residues are modified and that there

Fig. 1.4

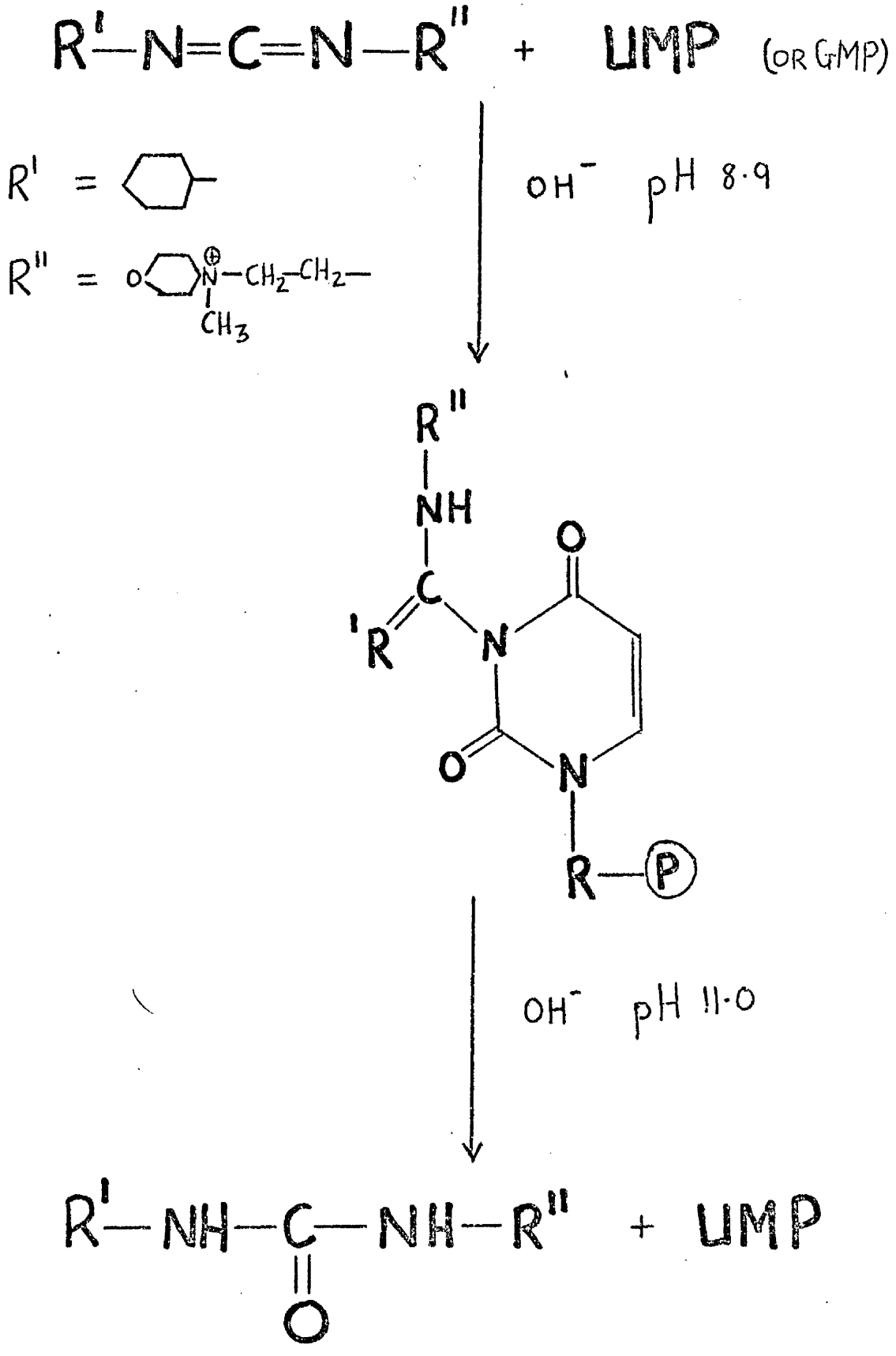
Bisulphite-catalysed deamination of cytosine based on Hayatsu (1976).



See Methods section for further details.

Fig. 1.5

The reaction of a water-soluble carbodiimide derivative with uridylic acid (Brownlee, 1972).



is only a slight reaction with guanine residues. This considerably simplifies the interpretation of data. Pseudouridine also reacts with carbodiimide, although the process is more complicated (Ho and Gilham, 1971).

'Fingerprinting' of modified and unmodified 5.8S rRNA allows the determination of reactivities by observed changes in molar yields of oligonucleotide fragments. In the first dimension of the 'fingerprint', the oligonucleotides containing modified residues travel more slowly due to their extra positive charge at pH 3.5. Their mobility in the second dimension is considerably greater than that of the corresponding unmodified oligonucleotides and is consistent with the addition of two positive charges at pH 1.9. This change in mobility of the oligonucleotides containing modified uridine residues allows interpretation from the 'fingerprint' of the extent and site of the reaction.

Carbodiimide has an added advantage. The modified uridine residue confers resistance on the adjacent phosphodiester bond to cleavage with pancreatic ribonuclease (Lee et al., 1965). After carbodiimide treated 5.8S rRNA has been digested with pancreatic ribonuclease and fractionated in two dimensions, it is possible to detect some non-uridine containing oligonucleotides present in low molar yields. This is caused by uridine residues immediately preceding these oligonucleotides in the sequence reacting with carbodiimide. The pancreatic ribonuclease can no longer hydrolyse the phosphodiester bond between the 'blocked' uridine and the non-uridine containing oligonucleotide sequence. This results in the formation of a new pancreatic ribonuclease product and the concomitant reduction in the molar yield of the non-uridine containing oligonucleotide. This blocked group may be removed by mild alkali (pH 11)

under conditions which do not cause cleavage of phosphodiester bonds (Brownlee, 1972).

MATERIALS AND METHODS

2.1 Materials and Solutions

(a) Chemicals

All chemicals used were AnalaR reagents from B.D.H. Ltd., Poole, Dorset, with the exception of those listed below.

1-Cyclohexyl 1-3-(2 morpholinoethyl)-carbodiimide	Ralph N. Emmanuel Ltd., Wembley, England.
^{32}P-orthophosphate (^{32}Pi., 10mCi/m mol) carrier free (in soln. of dil. HCl)	Radiochemical Centre, Amersham.
Sodium Bisulphite (Sodium Metabisulphite)	Sigma Chemical Co., St. Louis, U.S.A.
xylene cyanol FF	G.T. Gurr Ltd., London

(b) Enzymes

T_1 Ribonuclease (crystalline) (made by Sankyo Co. Ltd., Japan))	Calbiochem Ltd., Hereford, England.
Pancreatic Ribonuclease (chromatographically homogeneous)	Calbiochem Ltd., Hereford, England.
Trypsin	Difco Laboratories, Michigan, U.S.A.

(c) Materials for 'fingerprinting' analysis

Cellulose Acetate electrophoresis strips	Oxoid Ltd., London.
Whatman DE81 paper (46 cm x 50 m)	H. Reeve-Angel & Co. Ltd., London.
Whatman No. 52 and 3MM paper	H. Reeve-Angel & Co. Ltd., London.

Kodirex KD 54T (35 x 43 cm) X-ray film	Kodak Ltd.
DX-80 Developer	
FX-40 X-ray liquid fixer	
Triton X-100	Koch-Light Laboratories Ltd., Colnbrooke, England.
Toluene	
2,5,diphenyloxagole (PPO)	

(d) Materials for cell growth

Calf serum	Bio-Cult Laboratories Ltd., Paisley, Scotland.
Foetal calf serum	
Amino Acids	
Vitamins	
Penicillin	Glaxo Pharmaceuticals, London.
Streptomycin	

(e) Solutions

Balanced Salts Solution (BSS: Earle, 1943)

BSS contained 0.116 M NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1 mM $Na\,H_2PO_4$, 1.8 mM $CaCl_2$ and 0.002% (W/U) phenol red. The pH of the solution was adjusted to 7.0 with 5.6% (W/V) $NaHCO_3$.

Trypsin/Versene

Versene 4 parts, trypsin/citrate 1 part (W/U).

Versene

0.6 mM EDTA in PBS (A).

Phosphate Buffered Saline (A) (PBS (A)).

0.17 M NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4 and 2.4 mM KH_2PO_4.

RSB (Reticulocyte Salt Buffer)

0.01 m NaCl, 0.0015 M $MgCl_2$, 0.01 M tris-HCl, pH 7.4.

LETS (Lithium-EDTA-tris-sodium dodecyl sulphate buffer)

0.1 M LiCl, 0.01 M EDTA, 0.01 M tris-HCl, 0.2% SDS, pH 7.4.

Electrophoresis dye mixture

1% xylene cyanol F.F., 2% orange G, 1% acid fuschin.

(f) Scintillants

Scintillation counting of non-aqueous samples was carried out in scintillant prepared by dissolving 5 g of 2,5 diphenyl oxazole (PPO) in one litre of toluene. 10 ml of this solution was used to count each sample.
Counting of aqueous samples was carried out in scintillant prepared by dissolving 5 g PPO and 0.5 g p-Bis (0-methyl styryl) benzene (Bis-mSB) in 650 ml of toluene and 350 ml Triton X-100.

(g) Cells and Growth Media

Cells HeLa cells (Gey et al., 1952) grown in a monolayer culture, were used in this study.

Media The cells were grown in slightly modified Eagle's MEM (Eagle, 1959) with the addition of calf serum to 10% (V/V), penicillin (100 units/ml) and streptomycin sulphate (100 µg/ml). The constituents of the medium are shown in Table 2.1.

Before labelling with ^{32}Pi, cells were grown in medium containing one

Table 2.1 Eagle's Minimum Essential Medium (modified)

Amino Acids	mg/litre	Vitamins	mg/litre
L-arginine HCl	126.4	D-Ca pantothenate	2.0
L-cysteine	24.0	Choline chloride	2.0
L-glutamine	292.3	Folic acid	2.0
L-histidine HCl	41.9	i-Inositol	4.0
L-isoleucine	52.5	Nicotinamide	2.0
L-leucine	52.5	Pyridoxal HCl	2.0
L-lysine HCl	73.1	Riboflavine	0.2
L-methionine	14.9	Thiamine HCl	2.0
L-phenylalanine	33.0		
L-threonine	47.6		
L-tryptophan	10.2		
L-tyrosine	36.2		
L-valine	46.9		

Inorganic Salts and other components

	mg/litre		per litre
$CaCl_2$	200	Penicillin	10^5 units
D-glucose	4500	Streptomycin	10^5 µg
$MgSO_4$	98		
KCl	400		
NaCl	6800		
$NaH_2PO_4.2H_2O$	140		
Phenol Red (Na)	10		

tenth the concentration of phosphate (1.0×10^{-4}M) of that used in the medium described above.

2.2 Growth and Radioactive Labelling of Tissue Culture Cells.

(a) Routine Maintenance of Cells

HeLa cells were maintained routinely as monolayers in a rotating 'burler' bottle (80 oz. clear-glass Winchester). 25×10^6 cells were seeded into 180 ml of medium and grown at 37°C for 3-4 days in a 5% CO_2 atmosphere. When the cells were at a density of $150\text{-}200 \times 10^6$ per burler, they were harvested (see below).

(b) Radioactive labelling

To prepare ^{32}P labelled RNA, 25×10^6 cells were seeded into a burler and grown for 48 hr at 37°C as previously described. The medium was then poured off and replaced with 50 ml of medium containing one-tenth the normal concentration of phosphate. Lowering the amount of unlabelled phosphate present during labelling increased the uptake of radioactive phosphate into the cells.

10 mCi of ^{32}Pi was added to each burler and the cells were allowed to grow at 37°C for a further 18 hours before harvesting.

2.3 Preparation of rRNA from Tissue Culture Cells

Quantities of reagents given in this section refer to operations on one burler containing 150×10^6 cells.

(a) Cell harvesting

The growth medium was poured off and the cells were washed twice with

20 ml of trypsin/versene which had been prewarmed to 37°C. After the second washing a few ml of trypsin/versene were left in the bottle and the cells were incubated for 10 min at 37°C. During this time the cells were seen to dislodge from the glass. When all the cells had become detached, they were taken up in 40 ml of ice-cold balanced salts solution containing 10% (V/V) serum to neutralize tryptic activity, and transferred to a 50 ml tube on ice. The cells were pelleted by centrifugation at 2,000 rpm in a MSE Mistral 4L centrifuge at 4°C. The cell pellet was washed twice by resuspension in 10 ml balanced salts solution followed by centrifugation as before.

(b) Cell Fractionation

All operations were carried out at 4°C. The washed cell pellet was thoroughly drained, vigorously resuspended by vortexing in 4 ml hypotonic RSB solution and the cells allowed to swell for 10 - 15 min. The cell suspension was homogenized with 15 strokes of a stainless steel Dounce homogenizer (clearance 0.003"). The homogenate was transferred to a 15 ml Corex centrifuge tube and centrifuged at 2,000 rpm for 2 min. After centrifugation the supernatant fluid (containing the cytoplasm) was carefully removed from the pelleted material using a Pasteur pipette. This pelleted material was washed by resuspension in a further 2 ml RSB and centrifuged as before. The supernatant fluid from this washing was removed and added to that already collected.

(c) Purification of RNA

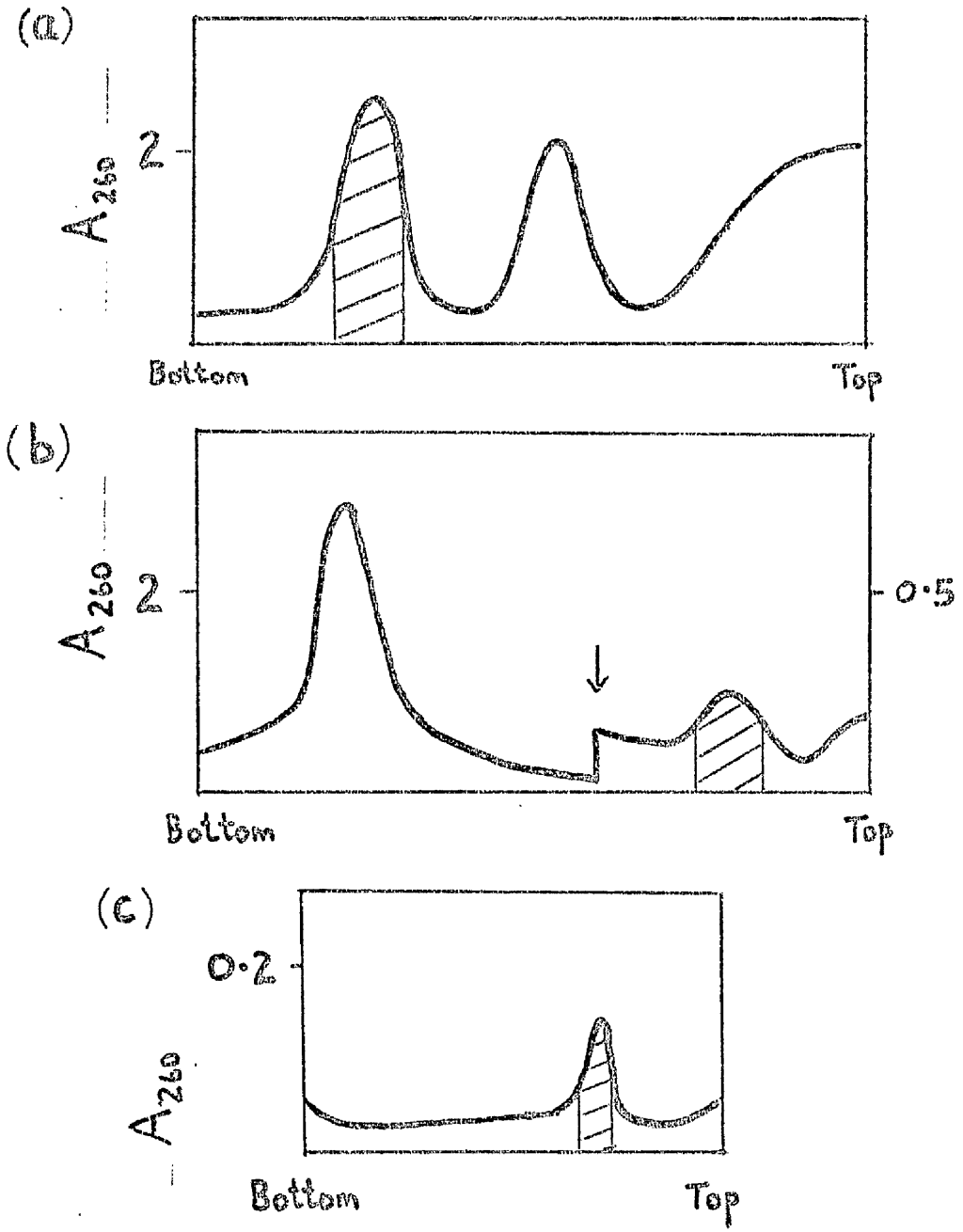
The pooled cytoplasmic supernatant was brought to room temperature

and a one-tenth volume of 5% SDS solution added. An equal volume of water-saturated phenol was added and the solution vortexed continuously for 3 min. The mixture was then separated by centrifugation for 15 min at 3500 rpm at 20°C. The aqueous (upper) layer was removed and the RNA precipitated by the addition of one-ninth volume of 1.7 M NaCl and 2.5 volumes of ice-cold ethanol. After standing for 3 hours at -20°C, the RNA precipitate was collected by centrifugation at 3500 rpm for 30 min, drained and dissolved in 1 ml LETS and then layered onto a 37 ml, 10% - 25% sucrose/LETS gradient in the large buckets of the SW27 rotor. The gradients were centrifuged at 22,000 rpm for 17 hours at 20°C in a Beckman L250 centrifuge. The solution was then fractionated by pumping through the 0.5 cm flow cell of a Gilford recording spectrometer while continuously monitoring the absorbance at 260 nm (Fig. 2.1(a)). Fractions corresponding to 28S and 18S rRNA were collected and precipitated with ethanol as described above.

(d) Isolation and Purification of 5.8S rRNA

The precipitated 28S rRNA was dissolved in 1 ml of LETS, heated in a sealed tube at 60°C, incubated in a waterbath at 60°C for 5 min and rapidly cooled by immersion in an ice-water mixture. The sample was layered onto a 37 ml 10% - 25% sucrose/LETS gradient in the large buckets of the SW27 rotor and centrifuged as described above. After fractionation, peaks corresponding to 28S and 5.8S rRNA could be identified (Fig. 2.1 (b)). By increasing the sensitivity of the Gilford recording spectrometer fourfold (as indicated by arrow), the peak corresponding to 5.8S rRNA could be magnified relative to the 28S rRNA peak.

Fig 2.1.



O. D. profiles of rRNA after sucrose gradient centrifugation in the preparation and purification of 5·8SrRNA. RNA in shaded regions was collected.
(a) Cytoplasmic RNA, 10-25% sucrose-LETS. (37 ml).
(b) Heat treated 28S RNA 10-25% sucrose-LETS (37 ml).
(c) Repurification of 5·8SrRNA, 10-25% sucrose-LETS (17 ml).

The 5.8S rRNA was precipitated by adding 2.5 volumes of ethanol and leaving at -20°C overnight. Further purification of 5.8S rRNA was achieved by a second centrifugation on a sucrose gradient. The 5.8S rRNA was precipitated, dissolved in 0.5 ml LETS and layered onto a 17 ml 10% - 25% sucrose/LETS gradient in the small buckets of the SW27 rotor and centrifuged at 20°C for 24 hours at 25,000 rpm. The sample was then fractionated as before (Fig. 2.1 (c)).

The 5.8S rRNA was precipitated with ethanol to remove sucrose and SDS, dissolved in water and its specific activity determined. The RNA concentration was established by measurement of absorbance, assuming that RNA, at a concentration of 40 μg/ml has an absorbance at 260 nm of 1.0, for a 1 cm pathlength. Radioactivity was assayed by liquid scintillation counting of 10 μl aliquots in 10 ml triton/toluene scintillant. RNA samples were stored in 0.15 M NaCl in 2.5 volumes of ethanol at -20°C until required for use. 150×10^6 cells generally yielded about 2 - 3 μg of 5.8S rRNA containing about 8×10^5 cpm/μg.

2.4 Chemical Modification of RNA

(a) Sodium Bisulphite

5.8S rRNA was precipitated in ethanol by centrifugation and the pellet drained. The sample was dissolved in 3 M sodium bisulphite pH 6.0, 10 mM $MgCl_2$ (total volume 2 - 3 ml), and incubated at the desired temperature. At the appropriate time, bisulphite was removed by dialysis against 0.15 M NaCl, 50 mM tris-HCl, pH 7.5, and then against 0.15 M NaCl, 20 mM tris-HCl, pH 7.5 for 2 hours each at 4°C. Removal of bisulphite 'adducts' was then effected by dialysis against 0.1 M tris-HCl, pH 9.0 at 37°C for 9-10 hours.

The RNA was neutralised by dialysis against 20 mM tris-HCl, pH 7.0, then 5 mM Tris-HCl, pH 7.0 for 2 hours each at 4°C. Finally the samples were dialysed exhaustively against water at 4°C to remove salt. Throughout this long process, sterile glassware and sterile solutions were used. The RNA solution was divided into samples ready for 'fingerprinting'. The amount of RNA in each sample was made up to 20 µg with cold 18S rRNA 'carrier'; generally there were $2 - 4 \times 10^5$ cpm in each sample due to the ^{32}P labelled 5.8S rRNA.

(b) Carbodiimide

Carbodiimide modification was based on the method of Brownlee et al. (1968) in which the reaction with guanine residues is slight. Purified ^{32}P labelled 5.8S rRNA was precipitated from ethanol by centrifugation and dissolved in 1 ml of distilled water. The RNA solution was divided into aliquots of 1 µg; 20 µg of cold 18S rRNA 'carrier' was added to each sample. These samples were then lyophilised prior to reaction with carbodiimide. The reaction mixture (total volume of 50 µl) contained the RNA with 10 mg/ml carbodiimide dissolved in 0.02 M tris/HCl, 10 mM $MgCl_2$ at pH 8.9 . This was incubated in a sealed tube for the desired time, at the desired temperature. The reaction was halted by adding one-fifth volume of 0.85 M NaCl and 2.5 volumes of cold ethanol and placing at -20°C. The RNA was precipitated twice with ethanol, then lyophilised prior to 'fingerprinting'.

2.5 RNA 'fingerprinting' technique

The techniques of RNA 'fingerprinting' were primarily those of Sanger et al.

(1965), Brownlee and Sanger (1967) and Fellner (1969), and have been comprehensively described by Brownlee (1972).

(a) Enzymic Digestion of RNA

The two enzymes used for RNA digestion in conjunction with the 'fingerprinting' technique were T_1 ribonuclease and Pancreatic ribonuclease.

T_1 ribonuclease

Digestion of RNA with T_1 ribonuclease yields oligonucleotides with a guanylic acid at the 3' terminus. Sanger et al. (1965) described the oligonucleotide "graticules" obtained by this type of fractionation (See Fig. 2.2 and 2.3). The mobility of oligonucleotides is influenced particularly by the number of uridylic acid residues present. Increasing content of uridylic acid causes reduced mobility in the second dimension of the 'fingerprint'. All oligonucleotides in the same graticule contain the same number of uridylic acid residues.

Under the conditions which were used (see below), electrophoresis for 16 hours in the second dimension gives a good separation of oligonucleotides which contain one or no uridylic acid residues, but is less satisfactory for other graticules. Better resolution of oligonucleotides in the two, three and four "U graticules" was achieved by extending electrophoresis in the second dimension to 40 - 60 hours. These separations are termed "Long T_1" fingerprints.

Samples were prepared for digestion by lyophilisation in siliconised test tubes. Digestion with T_1 ribonuclease was carried out, unless otherwise stated, with an enzyme to substrate ratio of one to ten in 0.02 M tris-HCl, 0.002 M

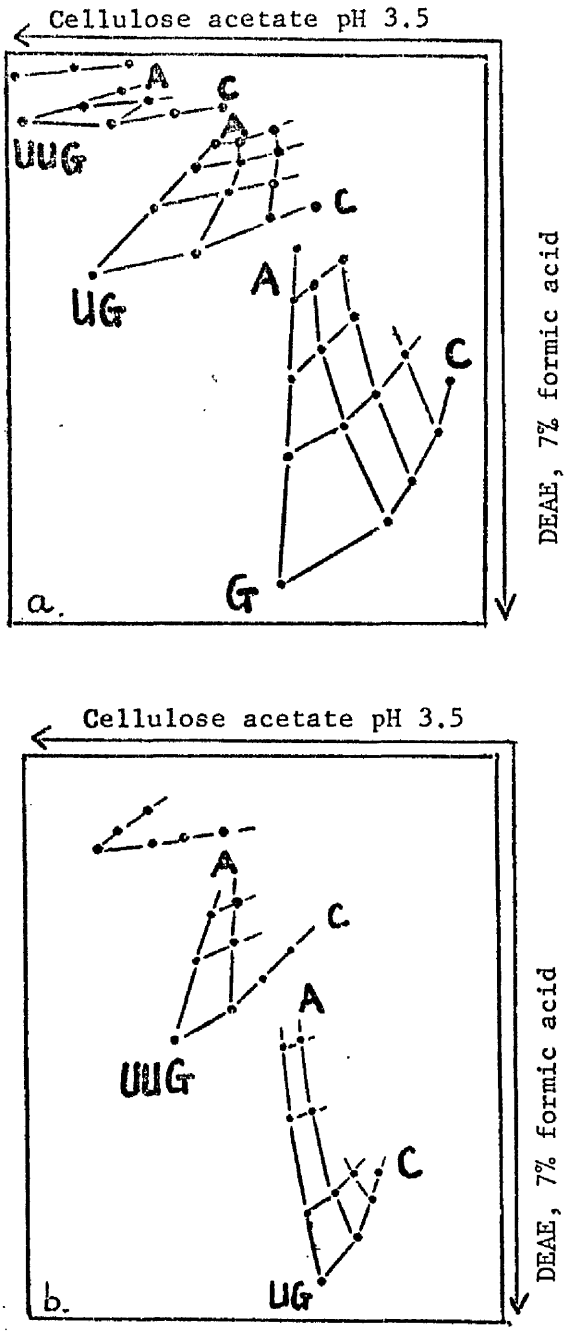
FIG 2.1

TWO-DIMENSIONAL FRACTIONATION OF OLIGONUCLEOTIDES AFTER T_1 RIBONUCLEASE DIGESTION.

NOTES.

1. All oligonucleotides in the same graticule contain the same number of uridine residues (zero,one,or two as shown)
2. Each oligonucleotide contains one guanine residue.
3. The number of adenine or cytidine residues in oligonucleotides(these are represented by dots) can be determined by counting from Gp,U-Gp, U-U-Gp.

Fig. 2.2



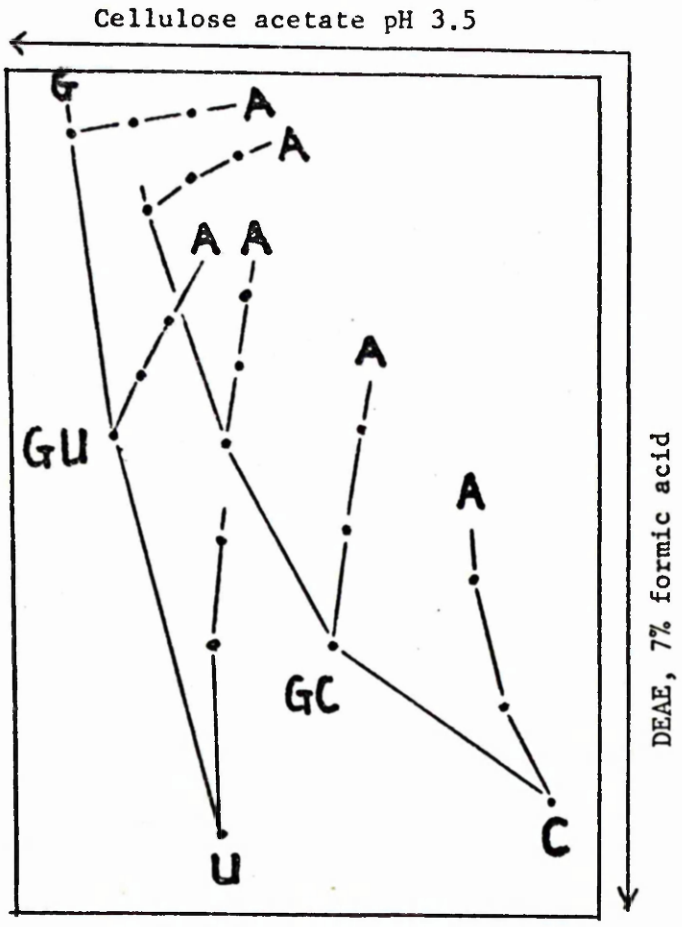
Two-dimensional fractionation of oligonucleotides after T_1 ribonuclease digestion.

(a) Standard T_1 ribonuclease fingerprint.

(b) 'Long' T_1 ribonuclease fingerprint.

Adapted from Brownlee (1972).

Fig. 2.3



Fractionation of oligonucleotides after pancreatic ribonuclease digestion.

FIG 2.3

TWO -DIMENSIONAL FRACTIONATION OF OLIGONUCLEOTIDES AFTER PANCREATIC RIBONUCLEASE DIGESTION

NOTES.

1. There are two graticules;one contains oligonucleotides terminated by Up,the other contains oligonucleotides terminated by Cp.
2. The number of adenine or guanine residues in an oligonucleotide can be determined by counting from Up or Cp.

EDTA, pH 7.4 for 30— 35 minutes.

Pancreatic Ribonuclease

Pancreatic ribonuclease cleaves RNA to yield oligonucleotides with either uridylic acid or cytidylic acid at their 3' terminus. Two dimensional separation of the products of this type of digestion gives 'fingerprints' with two different sets of graticules; one set being oligonucleotides terminated by uridylic acid and the other set being oligonucleotides terminated by cytidylic acid (Fig. 2.3). Digestion with pancreatic ribonuclease was achieved by use of identical conditions to those already described for T_1 ribonuclease.

All digestions were carried out in a volume of 5 µl contained in the tip of a drawn-out capillary at 37°C in a humidified oven. These were applied directly to the fractionating system.

(b) Two Dimensional Fractionation

The products of enzymic digestion of RNA were separated by two dimensional electrophoresis. Separation in the first dimension was carried out on cellulose acetate at pH 3.5

Cellulose acetate strips (2.5 x 95 cm) were wetted with 7 M urea, pH 3.5 buffer (5% acetic acid, 7 M urea adjusted to pH 3.5 with pyridine) and excess buffer was dried off from an area about 10 cm from one end. The RNA digest was applied as a small discrete spot to this dried area in the centre of the strip. Marker dye was applied on either side of the sample spot. The remainder of the strip was blotted and quickly placed over a rack in the electrophoresis tank with the end of the strip nearest to the sample dipping into the cathode compartment. Electrophoresis was carried out at 4.8 KVwith the cellulose acetate drawing very

little current.

For standard T_1 ribonuclease digests the first dimension was generally run for 3 hours and in the case of 'long T_1' ribonuclease separation, it was run for 4 - $4\frac{1}{2}$ hours. In the case of pancreatic ribonuclease digests, the first dimension was run for 2 - $2\frac{1}{2}$ hours.

On completion of the first dimension, electrophoresis strips were removed from the tank and excess white spirit was then allowed to drip off. The fractionated oligonucleotides were blotted onto a sheet of DEAE cellulose paper (DE 81, 43 x 94 cm) at a distance of 10 cm from one end with a pad of 5 strips of Whatman 3 MM paper soaked in water. After allowing 20 min for transferance of oligonucleotides, the wetted area of the DEAE sheet was dipped in methylated spirit for about 2 min. This process removes urea which is transferred from the cellulose acetate strip along with the oligonucleotides.

For standard T_1 ribonuclease digests, the transfer was carried out with the blue marker dye on the cellulose acetate strip 4" in from the left-hand edge of the DEAE paper. For 'long T_1' ribonuclease digests, the transfer was carried out with the blue dye 1" from the left-hand edge. Pancreatic ribonuclease separations were transferred with the original point of digest application about 1" from the left hand edge of the sheet.

For the second dimension of electrophoresis, marker dye was applied and the half of the paper containing the oligonucleotides was carefully wetted with 7% formic acid, allowing diffusion across the origin. The sheet was draped over an electrophoresis rack and the other half wetted. The rack was carefully

lowered into the electrophoresis tank and electrophoresis carried out at 1.0 - 1.4 KV. Electrophoresis was continued until the blue dye had travelled about 40% of the length of the paper for standard T_1 ribonuclease and pancreatic ribonuclease digests (16 - 18 h). For long separations of T_1 ribonuclease digests, electrophoresis was continued until the blue dye had travelled the entire length of the paper (at least 40 h).

After electrophoresis, the paper was thoroughly dried in an oven until no smell of formic acid remained.

(c) Autoradiography

The DEAE paper was marked with ink containing ^{35}S-sulphate to identify and permit alignment of paper and film and then fixed to sheets of Kodirex X-ray film by adhesive tape. Autoradiography was carried out in lead lined folders, kept in a light-tight cupboard. After exposure, X-ray films were developed in DX-80 developer and fixed in FX-40 fixer. Generally there was enough radioactive material on the 'fingerprints' to give satisfactory autoradiograms with an overnight exposure.

2.6 Recovery of Oligonucleotides from Fingerprints

(a) Excision of Oligonucleotides

Autoradiographs were accurately aligned with the corresponding DEAE sheets using the marking made with ^{35}S containing ink. The positions of the oligonucleotide "spots" were marked with pencil, numbered and the spots excised from the sheet using a scalpel.

For quantitation of radioactivity in each spot, the excised pieces of

DEAE paper were placed in scintillation vials with 10 ml of PPO/toluene and subjected to liquid scintillation counting in a Packard Tri Carb Scintillation counter.

(b) Elution of Oligonucleotides

Oligonucleotides are bound ionically to DEAE paper and it is necessary to neutralise the charge on the DEAE in order to elute them. This was done by eluting with a 30% aqueous solution of triethylaminecarbonate, pH 10. Elution from Whatman 52 paper was done with water as there are no ionic charges to neutralise in this case. Elution was continued until 0.2 - 0.4 ml had been collected on a polyvinyl chloride sheet. Eluates were evaporated to dryness in an oven at 60°C.

Triethylamine carbonate was made by adding pieces of solid CO_2 to a 30% solution of aqueous redistilled triethylamine until the solution became clear. The pH was checked to be within the range pH 9.9 - 10.1.

2.7 Analysis of Oligonucleotides and RNA

(a) Complete Hydrolysis of Oligonucleotides and RNA

For base composition analysis, oligonucleotides and whole RNA were completely hydrolysed by alkali digestion (Brownlee, 1972, pp. 76-77). The RNA or oligonucleotides were taken up in 10 µl of 0.2 M NaOH, sealed into drawn out capillary tubes and incubated at 37°C for 16 - 18 hours. The hydrolysate was applied to Whatman No. 52 paper and then subjected to electrophoresis at pH 3.5 for 40 min at 4.8 KV. Products were detected by autoradiography and quantified by liquid scintillation counting.

(b) Digestion of Oligonucleotides with Pancreatic Ribonuclease

It was analytically useful to digest oligonucleotide products of T_1 ribonuclease digestion with pancreatic ribonuclease, especially where these products contained adenylate residues. Samples were digested in 10 µl of 0.01 M tris-HCl, 0.001 M EDTA, pH 7.4, containing 0.2 mg/ml pancreatic ribonuclease. Digestions were carried out for 30 min at 37°C. Separation of products was achieved by electrophoresis on Whatman No. 52 paper at pH 3.5 for 40 min at 4.8 KV. Products were identified by their mobility (Brownlee, 1972) or by further analysis of their nucleotide composition.

(c) Digestion of oligonucleotides with T_1 Ribonuclease

It was also analytically useful to digest oligonucleotide products of pancreatic ribonuclease digestion with T_1 ribonuclease. The digestion conditions and the separation of products were the same as for pancreatic ribonuclease.

2.8 The Reaction of Sodium Bisulphite with the 5.8S - 28S rRNA Complex

The aim of this procedure was to investigate the extent of the reaction of sodium bisulphite with 5.8S rRNA while it was bound to the 28S rRNA. The procedure generally follows that described for the reaction of sodium bisulphite with 'unbound' 5.8S rRNA with some alterations.

(a) Reaction with Sodium Bisulphite

^{32}P-labelled 28S rRNA (50 - 100 µg) which had been isolated on a sucrose gradient as previously described, was dissolved in 3 M sodium bisulphite, pH 6.0, 10 mM $MgCl_2$ (total volume 2 - 3 ml). This was first incubated at 25°C for 24 hours in a sealed test tube and then the first two dialyses were carried out

as before to remove bisulphite. This process permitted adduct formation and deamination of 'bound' 5.8S rRNA under conditions identical to those used for 'unbound' 5.8S rRNA.

(b) Isolation and Purification of Bisulphite Modified 5.8S rRNA

The solution was removed from the dialysis tubing, 2.5 volumes of cold ethanol added and the RNA left to precipitate at -20°C. When the 'bisulphite-modified' 28S rRNA had been recovered, it was applied to a 37 ml 10% - 25% sucrose/LETS gradient in the large buckets of the SW27 rotor. Centrifugation and recovery of the RNA from the gradient were carried out as usual. The rationale behind this step was as follows:

(i) It removed any 5.8S rRNA which had been detached from the 28S rRNA, (ii) It removed any breakdown products of 28S rRNA caused by the reaction. It should be noted at this point that there was no sign that either of these two processes had occurred.

When the repurified 'bisulphite-modified' 28S rRNA had been precipitated from ethanol it was dissolved in 1 ml of LETS and heated to 60°C for 5 mins in a sealed tube. After rapid cooling, the solution was layered onto a sucrose gradient and the 5.8S RNA species recovered as previously described. The treated 5.8S rRNA was found to contain some impurities. By re-running the 5.8S rRNA on another sucrose gradient it is possible to obtain a pure sample.

When this 'bisulphite-modified' 5.8S rRNA had been precipitated, it was dissolved in a solution containing 0.15 M NaCl, 20 mM tris-HCl, pH 7.5

(2 ml), and dialysed against 0.1 M tris-HCl, pH 9.0 at 37°C for 9 - 10 hours. This dialysis treatment had been shown to cause a partial breakdown of 28S rRNA. Therefore if the 5.8S rRNA was to be recovered in a pure form, it had to be removed from 28S rRNA before this stage. After this treatment, the RNA was neutralised and then exhaustively dialysed against water to remove salt. The 'treated' 5.8S rRNA was then lyophilised and 'fingerprinted' as described above.

The Existence within HeLa Cell 5.8S rRNA of a region Partially Resistant to Ribonuclease Digestion

3.1 Enzymic Digestion of 5.8S rRNA

The main part of this thesis involves the comparison of 'fingerprints' of modified and unmodified 5.8S rRNA. As a necessary prerequisite to this, the conditions and extent of ribonuclease digestion had first to be thoroughly investigated and the oligonucleotides characterised. This latter task has also been performed by Nazar et al. (1976) and Khan and Maden (1977). As discussed in Chapter 2, HeLa cell 5.8S rRNA was normally digested at a 1 : 10 enzyme : substrate ratio for 30 min at 37°C. The two enzymes used were T_1 ribonuclease (which cleaves specifically after Gp in the nucleotide sequence) and pancreatic ribonuclease (which cleaves specifically after Up and Cp).

(a) Digestion with T_1 Ribonuclease

5.8S rRNA was digested as described and fractionated by high voltage electrophoresis on cellulose acetate (pH 3.5) and DEAE paper (7% formic acid). Those larger oligonucleotides which could not be sufficiently resolved after 17 hours in the second dimension were further separated by an extended run of 40 - 60 hours (Plates 3.1 and 3.2). Thirty different products could be detected as a result of T_1 ribonuclease digestion (Table 3.1). Two of these occurred as a result of heterogeneity at the 5' end (products T10a, pC-Gp and pGp) and another is product T8 (Um-Gp) which is due to the partial methylation of U-14 in the sequence (Fig. 3.1). Products T6, T16 and T22 persistently

'Fingerprints' of HeLa cell 5.8S rRNA

Plate 3.1 T_1 ribonuclease digest

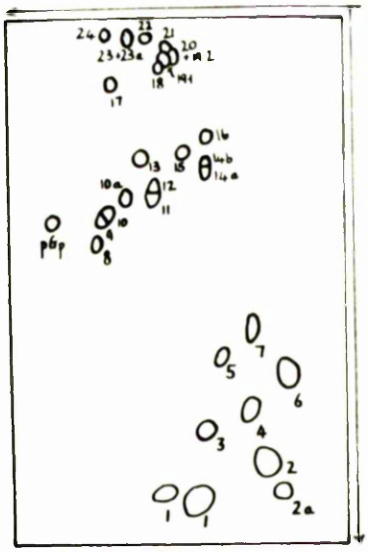
The sequences are identified by the standard numbering system (Table 3.1). HeLa cell 5.8S rRNA was digested as described in Chapter 2 (Methods section), i.e. 1 : 10 enzyme : substrate ratio, for 30 min at 37°C. It was then fractionated by high voltage electrophoresis on cellulose acetate (pH 3.5) and DEAE paper (7% formic acid) according to the general methods of Brownlee (1972). The first dimension was run for 3 h at 4.7 KV and the second dimension for 17 h at 1.2 KV.

Plate 3.2 T_1 ribonuclease digest, extended fractionation

The digestion and electrophoretic separation were carried out as described for Plate 3.1, except that the first dimension of electrophoresis was run for 4 h at 4.8 KV and the second dimension for 40 - 60 h at 1.2 KV. This allowed greater separation of the longer oligonucleotide fragments.

Plate 3.3 Pancreatic ribonuclease digest

The sequences are identified by the standard numbering system (Table 3.2). Digestion and fractionation were carried out as described in Chapter 2 (Methods section). Electrophoresis was carried out for 2.5 h at 4.7 KV in the first dimension and for 16 h at 1.1 KV in the second dimension.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23a
22
21
20
19-2
19-1
18
17
16
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14b
14a
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5
4
3
2
2a
1
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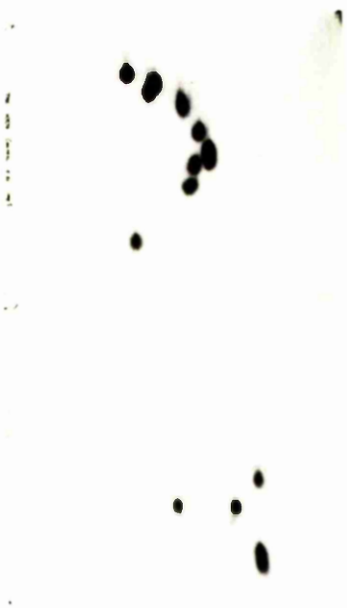

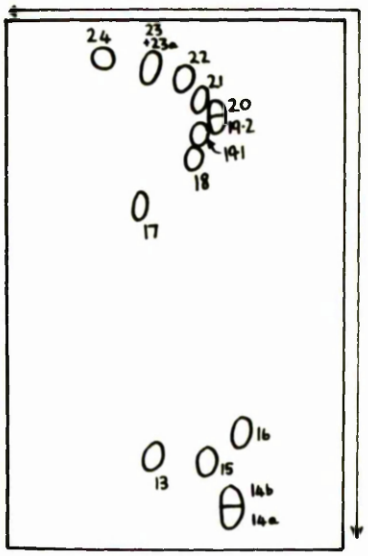
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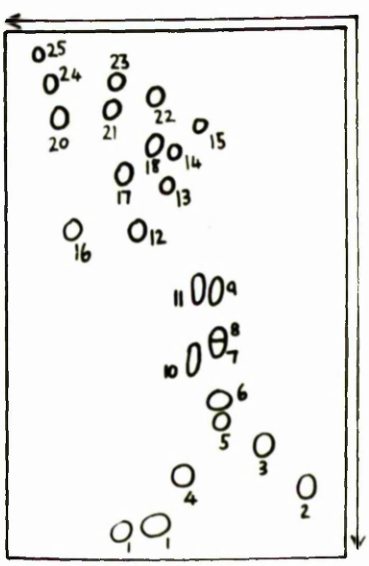
14b
14a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1

Table 3.1

In the sequence, hyphens designate internal residues and Gp signifies guanosine 3' phosphate. Um and Gm signify 2'-O-methyl U and 2'-O-methyl G. Numbers in parentheses under "molar yield" are the theoretical yields expected on the basis of nucleotide sequence (Nazar et al., 1976). The molar yields were calculated from the total counts obtained from all the oligonucleotide fragments and are the means of several determinations from independent experiments.

(a) Products T6, T16 and T22 persistently gave low yields. These products are all derived from nucleotides 116 - 137 in the sequence, which are believed to give rise to a partially enzyme resistant helix consisting entirely of GC pairs with an associated hairpin loop.

(b) Product T7 was also recovered in lower than theoretical yield. In this and related work, A-A-C-Gp and the methylated sequence A-Am-G-Gp were recovered in variable yield, apparently because this sequence transfers poorly from the first to the second dimension of the 'fingerprint'. (unpublished observations of M.S.N. Khan and B.E.H. Maden).

Table 3.1 Sequences and Molar Yields of T_1 Ribonuclease Products from HeLa Cell 5.8S rRNA

Spot No.	Sequence	Molar Yield		
T1	Gp	11.15	(10)	
T2	C-Gp	6.20	(6)	
T2a	C-U-U	0.91	(1)	
T3	A-Gp	2.36	(2)	
T4	C-A-Gp	1.04	(1)	
T5	A-A-Gp	1.01	(1)	
T6	C-C-C-C-Gp	0.45	(≤ 1?)	(a)
T7	A-A-C-Gp	1.44	(2)	(b)
T8	Um-Gp	0.21	(0.2)	
T9	U-Gp	1.99	(1.8)	
T10	Ψ-Gp	1.02	(1)	
T10a	pC-Gp	0.42	(0.4-0.5)	
T11	U-C-Gp	1.92	(2)	
T12	C-Ψ-Gp	1.03	(1)	
T13	A-U-Gp	1.15	(1)	
T14a	C-U-C-Gp	2.08	(1)	
T14b	C-C-U-Gp		(1)	
T15	C-U-A-Gp	1.06	(1)	
T16	C-U-A-C-Gp	0.67	(1?)	(a)
T17	U-C-U-Gp	1.10	(1)	
T18	C-A-C-U-U-Gp	0.91	(1)	
T19.1	A-U-C-A-U-C-Gp	0.87	(1)	
T19.2	A-U-C-A-C-U-C-Gp	1.82	(1)	
T20	A-C-A-C-U-U-C-Gp		(1)	
T21	A-C-A-C-A-U-U-Gp	0.87	(1)	
T22	U-U-C-C-U-C-C-C-Gp	0.80	(1)	(a)
T23	A-C-U-C-U-U-A-Gp	1.86	(1)	
T23a	A-A-U-U-Gm-C-A-Gp		(1)	
T24	A-A-U-U-A-A-U-Gp	0.84	(1)	

Table 3.2 Sequences and Molar Yields of Pancreatic Ribonuclease Products from HeLa Cell 5.8S rRNA.

The conventions are the same as for Table 3.2. The molar yields were calculated in a similar manner.

Spot No.	Sequence	Molar yield	
P1	Up + Ψp	19.00	(20)
P2	Cp	19.80	(19)
P3	A-Cp	5.68	(5)
P4	pCp	0.46	(0.4-0.5)
P5	Gm-Cp	0.92	(1)
P6	G-Cp	7.06	(8)
P7	G-A-Cp	4.72	(2)
P8	A-G-Cp		(3)
P9	G-A-A-Cp	0.98	(1)
P10	A-Up	2.47	(2)
P11	A-A-Up	1.06	(1)
P12	G-G-Cp	1.91	(2)
P13	G-A-G-Cp	0.97	(1)
P14	A-G-G-A-Cp	0.98	(1)
P15	G-A-A-G-A-A-Cp	0.77	(1)
P16	G-Up + G-Ψp	5.45	(5)
P17	G-A-Up	2.23	(2)
P18	G-G-A-Up	1.12	(1)
P20	G-G-Up	0.95	(0.8) (a)
P21	G-G-A-Up	0.83	(0.8) (a)
P22	G-A-G-A-A-Up	0.94	(1)
P23	G-G-G-G-Cp	0.45	(≤ 1) (b)
P24	G-G-G-Up	0.76	(1) (b)
P25	G-G-Um-G-G-A-Up	0.20	(0.2) (a)

(a) G-G-Up and G-G-A-Up represent the unmethylated form of the sequence G-G-Um-G-G-A-Up

(b) These products were always recovered in low yield, presumably for similar reasons to those for the low recovery of T6, T16 and T22.

gave low yields. These products are all derived from nucleotides 116 - 138, which are believed to give rise to a helix consisting entirely of GC pairs, with an associated hairpin loop (Nazar et al., 1975).

(b) Digestion with Pancreatic Ribonuclease

5.8S rRNA was digested and fractionated by high voltage electrophoresis (Plate 3.3). These conditions resulted in good resolution of all 24 products of pancreatic ribonuclease digestion on the 'fingerprint' (Table 3.2). Products P23 and P24 persistently gave low yields similar to those found in T_1 ribonuclease 'fingerprints' for products T6, T16 and T22 (Table 3.1). It was noted that all five of these products are encompassed by nucleotides 116 - 138 in the sequence (Fig. 3.1). As mentioned above, it is thought that these nucleotides form a GC rich helix and associated hairpin loop as part of 5.8S rRNA secondary structure. The high stability of such a helix may be associated with partial resistance to T_1 ribonuclease and pancreatic ribonuclease digestion.

3.2 Effect of Varying 5.8S rRNA Digestion Conditions

It is possible that the low yields obtained for products T6, T16, T22, P23 and P24 is due to the stability of a helix composed entirely of GC pairs. If this is the case, digestion of 5.8S rRNA under less severe digestion conditions should lead to a further reduction in the yield of these products relative to other oligonucleotides. Consequently, more severe digestion conditions should lead to an increased yield in these products relative to other oligonucleotides.

To test this possibility, T_1 ribonuclease digestion of 5.8S rRNA was

carried out under various conditions (Table 3.3). Under the least severe digest conditions (1 : 50 enzyme : substrate ratio), there was a dramatic decrease in the yield of products from this helical region. No significant decrease in other oligonucleotides was detected. In an attempt to increase the yield of the products derived from this helical region, more rigorous digest conditions were used.

(a) 1 : 5 enzyme : substrate ratio.

(b) incubation of digestion mixture at 100°C for 5 min prior to incubation at 37°C for 30 min.

(c) incubation of digestion mixture for 80 min at 37°C instead of the usual 30 min.

As is evident from Table 3.3, these methods failed to produce any significant increase in the molar yields of the oligonucleotides encompassed by nucleotides 116 - 138.

A similar approach was adopted when investigating pancreatic ribonuclease digestion. Prior to incubation at 37°C, the enzyme digestion mixture was heated to 100°C for 5 min. The yield of P23 (G-G-G-G-Cp) remained unchanged at 0.45. However, the molar yield of the other pancreatic ribonuclease product from this region, P24 (G-G-G-Up) was increased from 0.76 to 0.98.

3.3 Investigation of Undigested Fragments located in T_1 Ribonuclease and Pancreatic Ribonuclease 'Fingerprints"

Table 3.3 Molar Yields of T_1 Ribonuclease Products of HeLa Cell 5.8S rRNA under Various Digestion Conditions

Spot No.	Enzyme : Substrate Ratios					
	1 : 5	1 : 10	(b) 1 : 10 'heated'	(a) 1 : 10 (80 min)	1 : 25	1 : 50
T1	10.33	11.15	10.28	12.74	10.38	8.13
T2	5.83	6.20	5.62	5.48	5.33	4.79
T6	0.42	0.45	0.50	0.61	0.35	0.19
T16	0.62	0.67	0.57	0.73	0.45	0.21
T22	not resolved	0.80	0.89	0.85	0.47	0.18
(c) T13	1.02	1.15	0.98	1.03	1.09	1.02
(c) T3	1.96	2.36	2.07	2.13	2.15	2.17

(a) Unless otherwise stated all digestions were carried out for 30 min at 37°C. In 1:10 (80 min), the digestion was carried out for 80 min at a 1:10 enzyme: substrate ratio.

(b) In 1:10 'heated', the digestion mixture, including the enzyme, was incubated at 100°C for 5 min prior to incubation for 30 min at 37°C.

(c) Products T3 and T13 are included for comparison.

(d) The molar yields were calculated in the same way as in Table 3.1.

(a) T_1 Ribonuclease 'Fingerprints'

In some 'fingerprints', a partial digestion product was recovered which migrated rapidly in the first dimension of electrophoresis, but remained bound to the origin during the second dimension. When this product was eluted, redigested with T_1 ribonuclease and rerun on DEAE paper (7% formic acid), products with the mobilities of T6, T16 and T22 were obtained together with Gp and C-Gp.

(b) Pancreatic Ribonuclease 'Fingerprints'

A fragment of variable yield was detected in pancreatic ribonuclease 'fingerprints' immediately above the position of P23 (Plate 3.3). When this fragment was eluted, it was digested with alkali as described in Methods section, 2.7. Its nucleotide composition was shown to be: 6% Up, 66% Gp, 2% Ap and 26% Cp. Clearly this fragment originates from a GC rich region of the 5.8S rRNA molecule.

3.4 Conclusions

This series of experiments has demonstrated that within HeLa cell 5.8S rRNA there exists a GC rich region which exhibits partial resistance to ribonuclease digestion. The nucleotide sequence stretching from 114 - 138 is very GC rich (Fig. 3.1). The existence of complementary sequences within this region led Nazar et al. (1975) to propose that the secondary structure of this region took the form of a helix consisting entirely of GC pairs and an associated hairpin loop (Fig. 3.2). The data presented here supports the

Fig. 3·1

Nucleotide sequence of He La cell 5·8S rRNA.

- based on Nazar et al., (1976) and the evidence in tables 3·1 and 3·2. The positions of the oligonucleotides produced by digestion with pancreatic ribonuclease (P) or T_1 ribonuclease (T) are shown. Those indicated with heavy lines occur once per molecule. The first nucleotide, pCp, is present in approx. 40% of the molecules in He La cell 5·8S rRNA and U14 bears a 2'-O-methyl group in approx. 20% of the molecules.

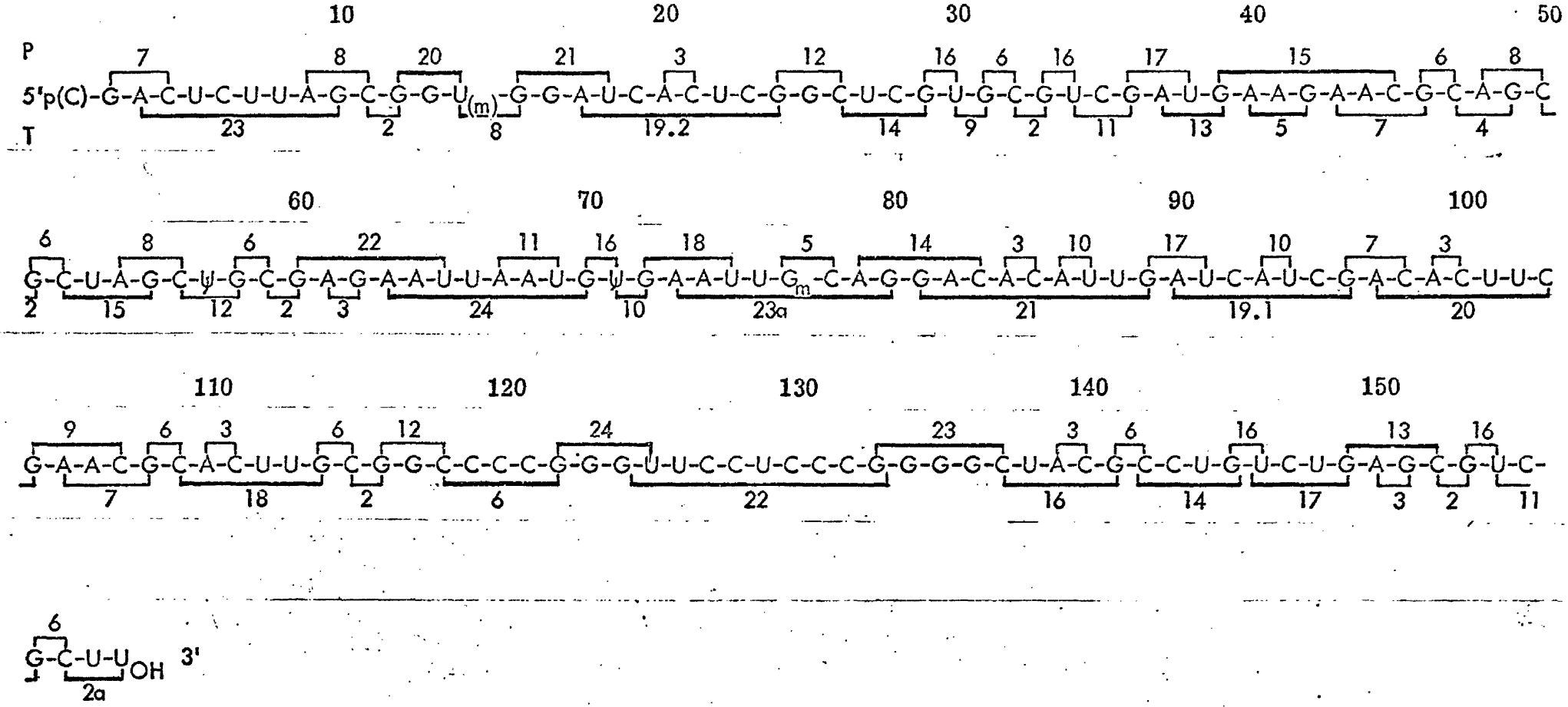
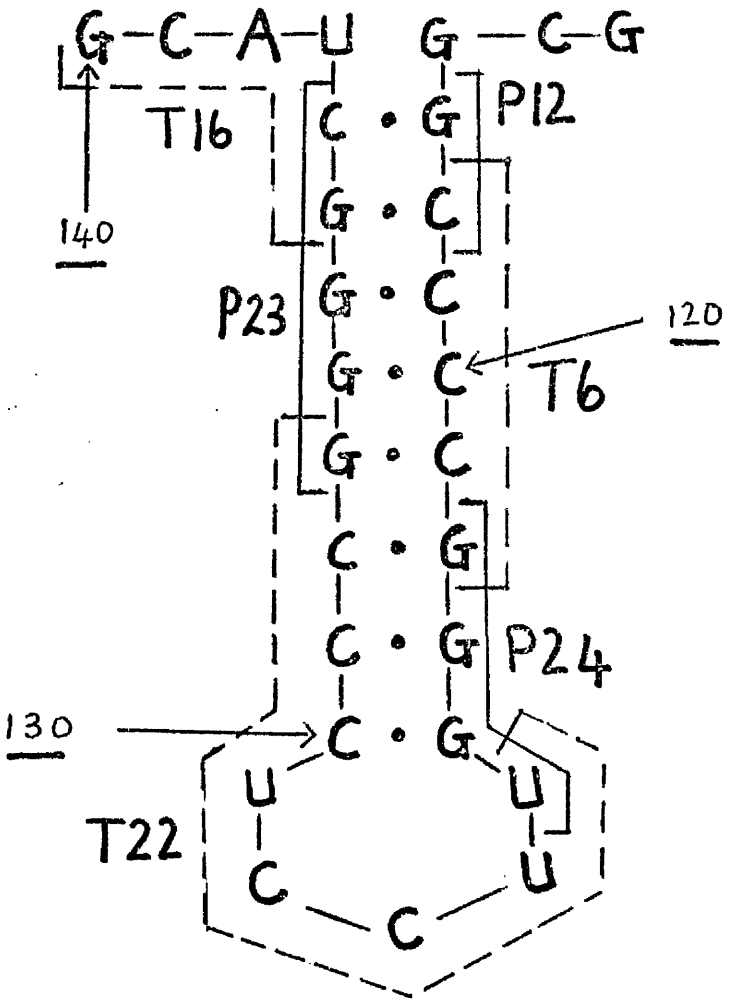

10
20
30
40
50
P
5'p(C)-G-A-C-U-C-U-U-A-G-C-G-G-U(m)-G-G-A-U-C-A-C-U-C-G-G-C-U-C-G-U-G-C-G-U-C-G-A-U-G-A-A-G-A-A-C-G-C-A-G-C
T
60
70
80
90
100
G-C-U-A-G-C-ψ-G-C-G-A-G-A-A-U-U-A-A-U-G-ψ-G-A-A-U-U-Gm-C-A-G-G-A-C-A-C-A-U-U-G-A-U-C-A-U-C-G-A-C-A-C-U-U-C
110
120
130
140
150
G-A-A-C-G-C-A-C-U-U-G-C-G-G-C-C-C-C-G-G-G-U-U-C-C-U-C-C-C-G-G-G-G-C-U-A-C-G-C-C-U-G-U-C-U-G-A-G-C-G-U-C-
6
G-C-U-U OH 3'
2a

Fig. 3.2

Proposed secondary structure of the nucleotide sequence 116-138.



Notes

1. Numbers represent positions of nucleotides in the sequence.
2. Broken lines represent T_1 ribonuclease fragments.
3. Solid lines represent pancreatic ribonuclease fragments.

existence of a stable helical region such as this.

The particularly low yield of product T6, C-C-C-C-Gp, may be due to both G117 and G112 being within the helix, whereas with products T16 and T22, only one of the bounding guanine residues in each case is within the helix. The lower yield of the pancreatic ribonuclease product P23 as compared to P24 can be explained in an analogous manner. In P23, G-G-G-G-Cp, both C137 and C132 are within the helix, whereas with P24, G-G-G-Up, only C121 is within the helix. U125, adjacent to the pancreatic cleavage point at the 5' end of this oligonucleotide, occurs within the loop.

3.5 The nucleotide sequence of HeLa cell 5.8S rRNA

The nucleotide sequence of HeLa cell 5.8S rRNA was determined by Nazar et al. (1976) (Fig. 3.1), after partial enzyme digestion and the subsequent sequence analysis of the oligonucleotide fragments. A similar approach was adopted by Ford and Mathieson (1978) when they sequenced Xenopus 5.8S rRNA.

Another approach to sequencing 5.8S rRNA was used by Boseley et al. (1978). They located the 5.8S rDNA gene within cloned rDNA fragments from Xenopus and subjected it to sequence analysis. This indicated that the sequence was identical to that proposed by Ford and Mathieson, apart from a GC deletion at positions 51 and 52 (Fig. 3.1). The presence of this GC dinucleotide within the sequence would have given rise to a Hha I/Hae II restriction site; this was not found. This finding suggests that the published Xenopus 5.8S rRNA sequence is incorrect, although it is possible that the sequenced gene could be a rare variant or that the deletion could have arisen as an artefact of gene cloning.

The published sequences of HeLa cell and Xenopus 5.8S rRNAs are identical in this region and so it is possible that such a deletion may arise in HeLa cell 5.8S rRNA.

As can be seen in Fig. 3.1, the nucleotides in question (C50 and G51) are located in the T_1 ribonuclease product, T2 (C-Gp) and the pancreatic ribonuclease product, P6 (G-Cp). Since both these dinucleotides are present in high molar yields on 'fingerprints' (Tables 3.1 and 3.2) it is not possible to resolve the sequence ambiguity from these data. In this thesis the numbering of the 5.8S rRNA nucleotide sequence will assume the presence of G51 and C52. Further discussion concerning this problem will be made as the need arises.

The reaction of sodium bisulphite with HeLa cell 5.8S rRNA

4.1 Kinetics of reaction at different temperatures

[^{32}P]-labelled HeLa cell 5.8S rRNA was reacted with 3M sodium bisulphite in the presence of 10 mM $MgCl_2$ at 25°C, 37°C and 50°C as indicated (Table 4.1). After reaction and completion of dialysis procedure (see Chapter 2.3), the RNA samples were lyophilised and then digested with 0.2N NaOH for 18 h. The nucleotides were separated by electrophoresis (4.8 KV for 40 min) at pH 3.5 on Whatmann No. 52 paper (Brownlee, 1972).

The reaction results in the conversion of cytidine residues to uridine residues. Therefore it is possible to calculate the rate and extent of the reaction at the three temperatures by measurements of the decreased cytidine content and the concomitant increased uridine content of 5.8S rRNA after various times of reaction. The conversion of cytidine to uridine was calculated from the distribution of ^{32}P label within the nucleotide monophosphates after alkaline digestion (Table 4.1).

Fig. 4.1 shows the extent and rate of the reaction of sodium bisulphite with 5.8S rRNA at different temperatures. At 37°C and 25°C a plateau of no further cytidine reaction had not been reached after 24 h and 72 h respectively. However, by extrapolating the curves it is possible to obtain estimates of the (%) cytidine not available for reaction at the different temperatures. These are:

25°C - 35% (approx. 15 cytidine residues)

37°C - 25% (approx. 11 cytidine residues)

50°C - 14% (approx. 6 cytidine residues)

Fig. 4.2 is drawn on semi-logarithmic graph paper and is a plot of (%)

Table 4.1 Reaction of sodium bisulphite with 5.8S rRNA at different temperatures

Reaction time (h)	Total cytidine content (%)		
	25°C	37°C	50°C
0	27.7	27.3	27.3
2	27.2	22.9	19.2
6	25.1	19.2	10.7
12	23.8	14.1	7.3
24	19.5	9.4	4.5
48	14.5	-	-
72	11.4	-	-

Reaction time (h)	Total uridine content (%)		
	25°C	37°C	50°C
0	23.5	23.6	23.6
2	24.7	27.4	32.6
6	27.0	32.9	43.5
12	28.6	35.6	44.2
24	32.8	39.1	48.6
48	37.9	-	-
72	40.3	-	-

Footnotes

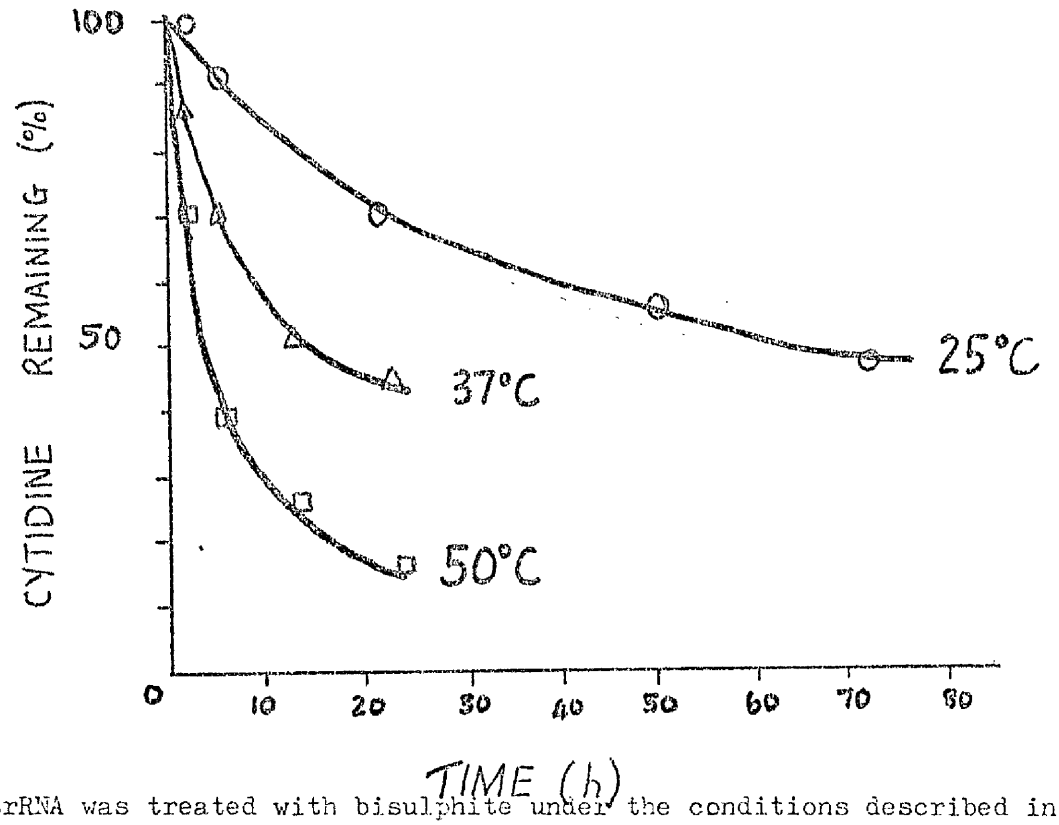
(a) Each operation was performed in triplicate.

(b) The (%) content of the other two nucleotides was very constant. The average (%) content of adenine and guanine was 20.2% and 28.7% respectively. These values are accurate to $\pm$ 2%.

(c) For any reaction time, at any temperature, the (%) Cp loss should balance the (%) Up gain. e.g.

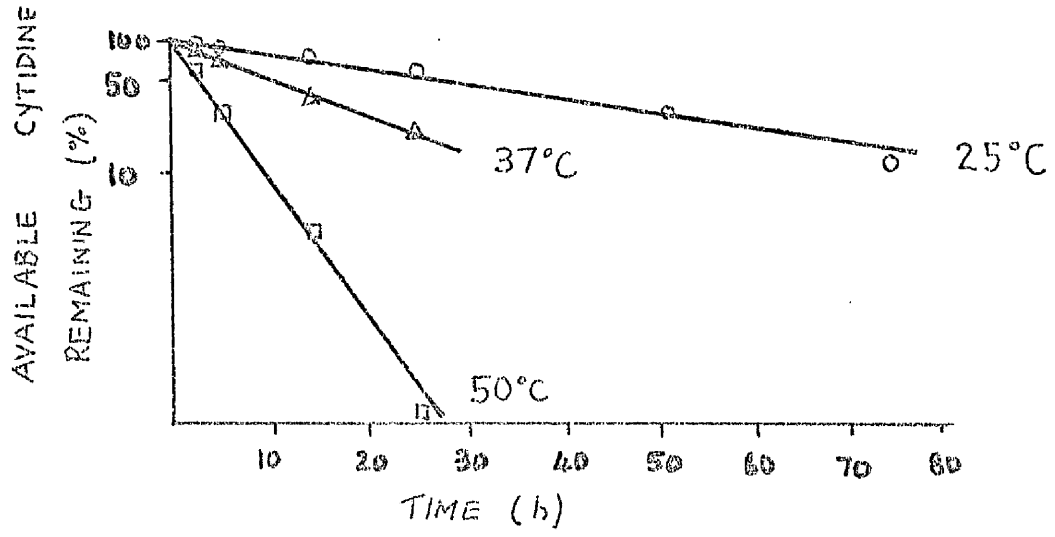
25°C - time zero	Cp+Up = 51.2%
25°C - time 24 h	Cp+Up = 52.3%
37°C - time 24 h	Cp+Up = 48.5%
50°C - time 24 h	Cp+Up = 53.1%

Fig. 4·1. Time course of reactions of cytidine in 5·8SrRNA with sodium bisulphite at 25°C, 37°C and 50°C.



5·8SrRNA was treated with bisulphite under the conditions described in the text, and triplicate samples removed at the times indicated. Adducts were removed as described in Methods Section. Hydrolysis was carried out with alkali (Brownlee 1972), and the products were separated by electrophoresis at pH3·5 on Whatman 52 paper.

Fig. 4·2 Semi-logarithmic plot of (%) available cytidine remaining in 5·8SrRNA after various times of bisulphite reaction at 25°C, 37°C and 50°C.



available cytidine remaining against time at the different temperatures. The results obtained are consistent with pseudo-first order rate kinetics. The times of half reaction can be read directly off this graph. These are:

$25^{\circ}C$ - 25 h

$37^{\circ}C$ - 9 h

$50^{\circ}C$ - 3.5 h

More detailed analysis is required to obtain estimates of the reactivities of individual cytidines. The present data formed the basis for the choice of conditions in which these further experiments were performed.

4.2 Reaction of sodium bisulphite with 5.8S rRNA at $25^{\circ}C$.

(a) Choice of conditions

On the basis of experience gained during studies with other RNA species (Goddard & Schulman, 1972; Lowdon & Goddard, 1976; Goddard & Maden, 1976), the reaction of 5.8S rRNA with sodium bisulphite was studied using 3 M bisulphite, pH 6.0, at $25^{\circ}C$ with 10 mM $MgCl_2$ present. Fig. 4.1 shows the time course of reaction of the total cytidine complement of 5.8S rRNA under these conditions. From this it is clear that not all of the 46 cytidine residues in HeLa cell 5.8S rRNA have reacted. Although a plateau of no further cytidine reaction has not been reached, even after 72 h, extrapolation of the curve suggests that approximately 30 - 35% of the total cytidines are completely unreactive (a more accurate estimate of the number of unreactive cytidine residues and of their positions is obtained by further sequence analyses reported below).

If we assume that 65% of the total cytidine residues are available for reaction,

then the kinetics are, as previously stated, pseudo-first order, 50% of the available cytidine residues having reacted in 25 h. It seemed likely that the plot in Fig. 4.1 would represent an average of many different reactivities of individual cytidine residues in 5.8S rRNA. On the basis of this experiment, and earlier experience with tRNA (Lowden & Goddard, 1976), a 24 h reaction period was chosen for examining the relative reactivities of most of the residues within 5.8S rRNA. Although this single reaction period does not permit direct measurement of initial rates, it offers the advantage that the more reactive cytidines undergo extensive reaction during the period and are therefore easily identified, and that relatively low levels of reactivity of other cytidines are also detectable.

(b) Interpretation of data from 'fingerprints'

Plate 4.1 shows 'fingerprints' of T_1 ribonuclease digests of HeLa cell 5.8S rRNA, untreated and after reaction with sodium bisulphite. Plate 4.2 shows 'fingerprints' of T_1 ribonuclease digests with long separations in both dimensions, in which the more slowly migrating products of Plate 4.1 are better resolved. Plate 4.3 shows 'fingerprints' of pancreatic ribonuclease digests. In the 'fingerprints' obtained after the reaction with bisulphite, many cytidine-containing oligonucleotides are present in reduced yield compared with controls. The reduction in the yields of these products provided the primary data for estimating the reactivities of the various cytidine residues. These are summarised in Tables 4.2 and 4.4 and described in more detail below. In addition, since the end result of the reaction of sodium bisulphite with cytidine is deamination to uridine,

it was often possible to identify the respective uridine-containing conversion products. In favourable cases, the original cytidine-containing oligonucleotide gave rise to a qualitatively new uridine-containing product which could be characterised and molar yield determined.

In other cases, cytidine-containing products (such as G-G-Cp) were converted to uridine-containing products (G-G-Up), which were already present in 'fingerprints' of control samples, but the amounts of these products were augmented after sodium bisulphite treatment. Last, for several longer T_1 ribonuclease oligonucleotides containing multiple cytidine residues, it was not possible to resolve the various conversion products and to determine the separate reactivities of the individual cytidines, although the reactivities of the oligonucleotides as a whole were readily determined from their extents of diminution in the 'fingerprints' after sodium bisulphite treatment. The means by which the reactivities were calculated are explained more fully in Appendix I.

In view of the difficulties inherent in obtaining precise quantification from 'fingerprinting' methods, the numerical reactivity of values in Tables 4.2 and 4.4 should be regarded as approximate. Such estimates are sufficient for the conclusions which we wish to draw.

(c) Reactivity of individual cytidine residues

The data in Tables 4.2, 4.3, 4.4 and 4.5 permit estimates to be obtained for the reactivities of most of the cytidine residues within 5.8S rRNA. It is

'Fingerprints' of HeLa cell 5.8S rRNA before (left) and after (centre) reaction with 3 M sodium bisulphite, pH 6.0, for 24 h at 25°C.

Plate 4.1 T_1 ribonuclease digest

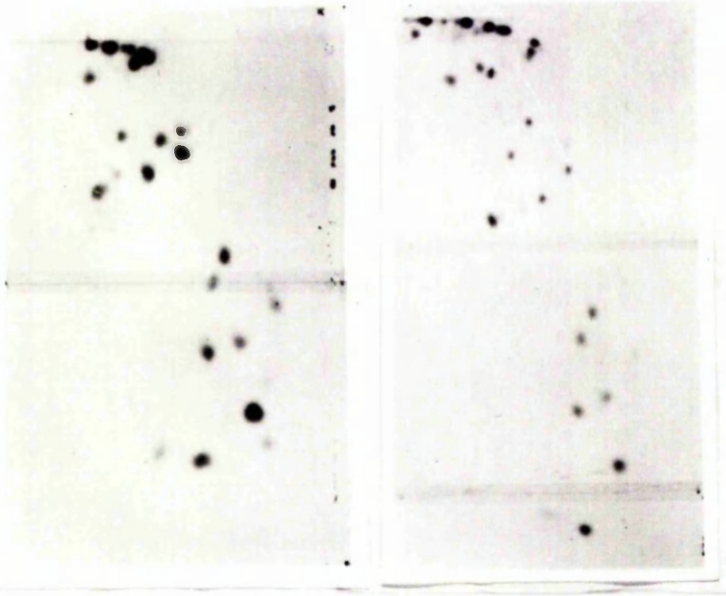
The key (right) identifies new sequences (shaded circles) which arise as a result of the reaction of sodium bisulphite; diminished cytidine-containing sequences (broken circles) and unreactive sequences (closed circles) are identified by the standard numbering scheme (text and Table 4.2). Electrophoretic separation was performed in the first dimension on cellulose acetate, pH 3.5, at 4.7 KV for 3 - 3.5 h and in the second dimension on DEAE paper, 7% formic acid, at 1.2 KV for 17 h.

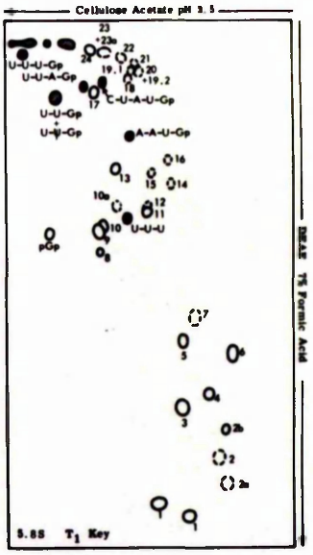
Plate 4.2 T_1 ribonuclease digest, extended fractionation

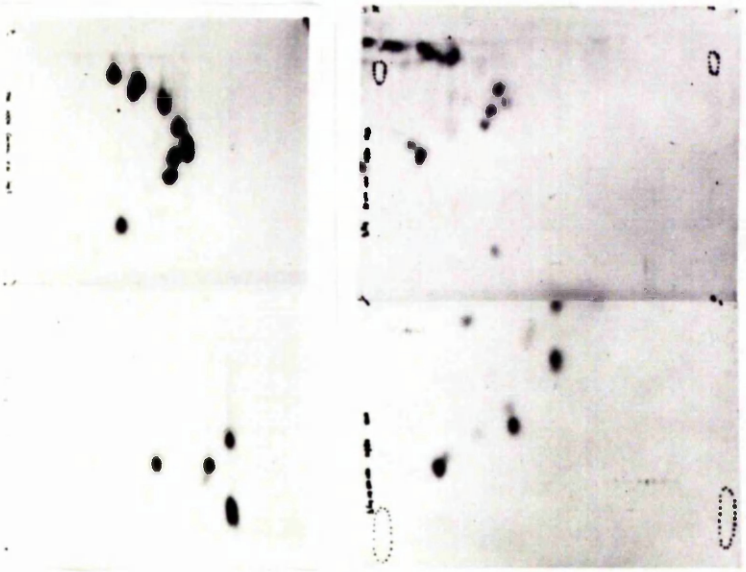
The key (right) identifies new, diminished and unreacted sequences as in Plate 4.1. Electrophoresis was carried out at 4.7 KV for 4 h in the first dimension and at 1.2 KV for 40 - 60 h in the second dimension.

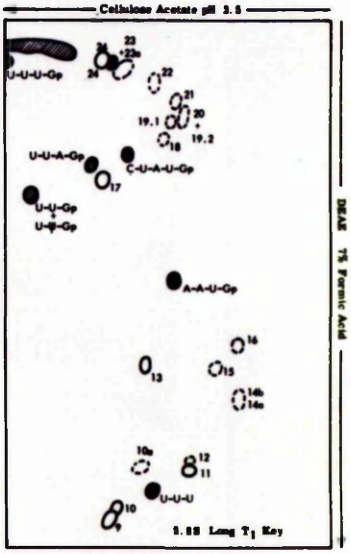
Plate 4.3 Pancreatic ribonuclease digest

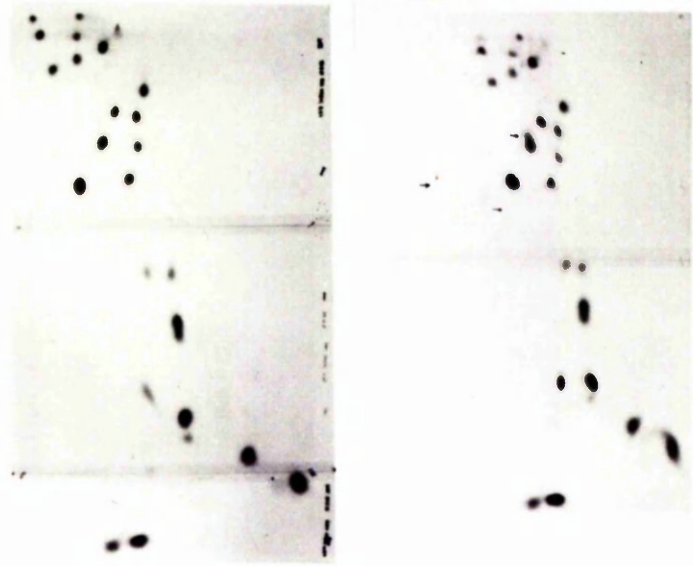
The key (right) identifies new, diminished and unreacted sequences as in Plate 4.1, with the numbering of spots as in Table 4.4. Electrophoresis was carried out at 4.7 KV for 2.5 h in the first dimension and at 1.1 KV for 16 h in the second dimension.




Cellulose Acetate pH 3.5
DEAE TS Formic Acid
5.8S T1 Key




Cellulose Acetate pH 3.5
DEAE TS Formic Acid
5.8S Long T1 Key



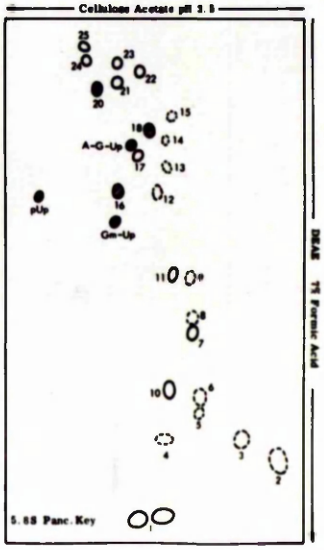

Cellulose Acetate pH 3.5
DEAE TS Formic Acid
5.8S Panc. Key

Table 4.2 Sequences and molar yields of T_1 ribonuclease products from HeLa cell 5.8S rRNA, and the extent of reaction of cytidine-containing oligonucleotides after treatment of 5.8S rRNA with sodium bisulphite.

The conventions are as in Table 3.1 The positions of the cytidine residues in the nucleotide sequence are indicated. The bisulphite reaction was carried out for 24 h at 25°C in 3 M bisulphite, pH 6.0, in the presence of 10 mM $MgCl_2$. The molar yields after bisulphite treatment were calculated assuming that the sum of A-A-Gp (T5), A-U-Gp (T13) and A-A-U-U-A-A-U-Gp (T24) was three, and are the means of several determinations from independent experiments. These three oligonucleotides were generally obtained in reliable yield; they neither react with bisulphite, nor gain material from the conversion of other cytidine-containing products to uridine-containing products.

Footnotes

(a) The gain in U-Gp after the bisulphite reaction (1.68 moles) is derived from reaction of cytidine in C-Gp (1.89 moles decrease).

(b) These two products were fairly well separated after prolonged electrophoresis in the second dimension of the 'fingerprints'. The reactivity is predominantly in the more rapidly migrating product, C-U-C-Gp.

(c) The yield of product T17 is unreliable after bisulphite treatment due to the partial conversion of C-U-C-Gp to (U_2,C) Gp, which then co-migrates with T17.

(d) In 'fingerprints' in which these two products were resolved it was found that product T19.2 was the more reactive of the two. However, T20 is still extensively reactive.

(e) These two products were only partly resolved in 'fingerprints'. Product T23a can be independently inferred to be about 40% reactive from the behaviour of the corresponding pancreatic ribonuclease product, P5. Products T23 and T23a appeared to be diminished to approximately the same extent in those 'fingerprints' in which they could be distinguished. Since there was a 49% overall decrease in material in products T23 and T23a, it can be concluded that T23 is approximately 50% reactive.

Table 4.2

Spot No.	Sequence	Cytidine residue(s)	Control	Bisulphite treated	% reactivity	
T1	Gp	-	11.15	11.12	-	
T2	C-Gp	11,32,50,115 152	6.20	4.31	30%	
T2a	C-U-U	157	0.91	0.32	65%	
T3	A-Gp	-	2.36	2.59	-	
T4	C-A-Gp	47	1.04	0.95	9%	
T5	A-A-Gp	-	1.01	1.04	-	
T6	C-C-C-C-Gp	118-121	0.45	0.48	zero	
T7	A-A-C-Gp	45, 107	1.44	0.91	37%	
T8	Um-Gp	-	0.21	0.16	-	
T9	U-Gp	-	1.99	3.67	—	(a)
T10	ψ-Gp	-	1.02	1.17	-	
T10a	pC-Gp	1	0.42	0.16	62%	
	pGp	-	0.12	0.07	-	
T11	U-C-Gp	35, 155	1.92	1.64	15%	
T12	C-ψ-Gp	56	1.03	0.54	48%	
T13	A-U-Gp	-	1.15	1.12	-	
T14a	C-U-C-Gp	26, 28	2.08	0.96	54%	(b)
T14b	C-C-U-Gp	142, 143				
T15	C-U-A-Gp	52	1.06	0.31	71%	
T16	C-U-A-C-Gp	137, 140	0.67	0.44	34%	
T17	U-C-U-Gp	147	1.10	0.99	10%	(c)
T18	C-A-C-U-U-Gp	109, 111	0.91	0.42	54%	
T19.1	A-U-C-A-U-C-Gp	92, 95	0.87	0.58	33%	
T19.2	A-U-C-A-C-U-C-Gp	19,21,23	1.82	0.28	85%	(d)
T20	A-C-A-C-U-U-C-Gp	98, 100, 103				
T21	A-C-A-C-A-U-U-Gp	83, 85	0.87	0.55	37%	
T22	U-U-C-C-U-C-C-C-Gp	127, 128, 130-132	0.80	0.11	86%	
T23	A-C-U-C-U-U-A-Gp	4,6	1.86	0.95	49%	(e)
T23a	A-A-U-U-G_m-C-A-Gp	78				
T24	A-A-U-U-A-A-U-Gp	-	0.84	0.84	-	

Table 4.3 Sequences and molar yields of uridine containing products of the sodium bisulphite reaction; T_1 ribonuclease products

The conventions are as for Table 3.1. In the examples shown the uridine-containing products of bisulphite reaction migrate to distinctive positions in the 'fingerprint' and can therefore readily be quantified. The (%) reactivity signifies the amount of newly appeared uridine-containing product relative to the amount of the corresponding product in the 'control fingerprints' as described in Appendix I.

Spot No.	Sequence	Molar yield	% reactivity	derived from	
T43	pU-Gp	0.26	62%	T10a	(a)
T10b	U-U-U	0.56	62%	T2a	
T36	A-A-U-Gp	0.72	50%	T7	
T41	U-U-G+ U-Ψ-Gp	0.84	28%	T11,T12	(b)
T40	U-U-A-Gp	0.68	64%	T15	
T42	C-U-A-U-Gp	0.18	28%	T16	
T39	U-U-U-Gp	0.95	-	T14,T17	

Footnotes

(a) This product, T43, migrates quickly in the first dimension and is not detectable on 'fingerprints' unless the first dimension is run for less than 3 h at 4.7 KV.

(b) Chromatographic analysis of T41 (Hughes et al., 1976) showed that the ratio of Ψp/Up + Ψp is 0.45. Therefore 90% of this product arises from the deamination of C-Ψ-Gp (T12). This points to the reactivity of C56 in C-Ψ-Gp being approximately 50%, which is similar to that derived from the diminution of product T10.

Table 4.4 Sequence and molar yields of pancreatic ribonuclease products from HeLa cell 5.8S rRNA and extent of reaction of cytidine-containing products after treatment with sodium bisulphite.

Conventions and reaction conditions are as in Table 4.2. The molar yields after sodium bisulphite treatment were calculated on the distribution of ^{32}P between pairs of products representing C → U conversions, such as G-A-A-Cp plus G-A-A-Up. The values shown are the means of those obtained by the various methods for quantification.

Footnotes

(a) After reaction with bisulphite, A-G-Up appeared in the pancreatic ribonuclease'fingerprints' in an amount equivalent to 54% of the original quantity of A-G-Cp. There was no significant increase in the molar yield of P17, G-A-Up, the potential reaction product of P7, G-A-Cp.

Table 4.4

Spot No.	Sequence	Cytidine residues(s)	Control	Bisulphite treated	% reactivity
P1	Up + Ψp	-	19.00	25.01	-
P2	Cp	-	19.80	16.59	-
P3	A-Cp	21,85,100, 111,140	5.68	4.70	18%
P4	pCp	1	0.46	0.21	54%
P5	G_m-Cp	78	0.92	0.53	42%
P6	G-Cp	32,47,52,59, 109,115,142,157	7.06	6.29	11%
P7	G-A-Cp	4,98	4.72	1.92	zero
P8	A-G-Cp	11,50,56		1.05	63%
P9	G-A-A-Cp	107	0.98	0.70	29%
P10	A-Up	-	2.47	3.99	-
P11	A-A-Up	-	1.06	1.16	-
P12	G-G-Cp	26,118	1.91	1.69	12%
P13	G-A-G-Cp	152	0.97	0.69	29%
P14	A-G-G-A-Cp	83	0.98	0.62	37%
P15	G-A-A-G-A-A-Cp	45	0.77	0.67	13%
P16	G-Up + G-Up	-	5.45	6.72	-
P17	G-A-Up	-	2.23	2.29	-
P18	G-A-A-Up	-	1.12	1.36	-
P20	G-G-Up	-	0.95	1.29	-
P21	G-G-A-Up	-	0.83	1.29	-
P22	G-A-G-A-A-Up	-	0.94	0.90	-
P23	G-G-G-G-Cp	137	0.45	0.48	zero
P24	G-G-G-Up	-	0.76	0.73	-
P25	G-G-U_m-G-G-A-Up	-	0.20	0.15	-

Table 4.5 Sequences and molar yields of uridine-containing products of the sodium bisulphite reaction; pancreatic ribonuclease products.

The conventions are as for Table 4.2. In the examples shown, the uridine-containing products of bisulphite reaction migrate to distinctive positions in the 'fingerprint' and can be readily quantified. The (%) reactivity signifies the amount of newly appeared uridine-containing product relative to the molar yield in the 'control fingerprints' of the oligonucleotide from which it was derived.

Spot No.	Sequence	Molar yield	% reactivity	derived from
16a	G_m-Up	0.32	35%	P5
16b	pUp	0.26	56%	P4
17a	A-G-Up	1.52	54%	P8

Table 4.6 Correlated data on the reactivities of individual cytidine residues

In the examples shown, the uridine-containing products of the bisulphite reaction (shown in parentheses) migrate to distinctive positions in the 'fingerprints' and can therefore readily be quantified. The (%) reaction signifies the loss of the cytidine-containing product, or the amount of newly appeared uridine containing product relative to the amount of the corresponding cytidine-containing product in 'control fingerprints'. (see appendix I)

Cytidine residue	Product	% Reaction	Mean
11	T10a pC-Gp	62%	58%
	(pU-Gp)	62%	
	P4 pCp	54%	
	(pUp)	56%	
52	T15 C-U-A-Gp	71%	68%
	(U-U-A-Gp)	64%	
56	T12 C-Ψ-G	48%	50%
	(U-Ψ-G)	52%	
78	P5 G_m-Cp	42%	39%
	(G_m-Up)	35%	
140	T16 C-U-A-C-Gp	34%	31%
	(C-U-A-U-Gp)	28%	
157	T2a C-U-U	65%	64%
	(U-U-U)	62%	

convenient to distinguish between "direct evidence", where in most cases the data were obtained from oligonucleotides that occur once per 5.8S rRNA molecule and contain a single cytidine residue, and "indirect evidence", where the estimates were derived more circuitously.

Direct evidence

C1 The 5' terminus of 5.8S rRNA, pC-Gp, was recovered in low yield (0.4 - 0.5) in control samples due to terminal heterogeniety (Nazar et al., 1975, 1976). There was considerable further diminution after bisulphite treatment, both of pC-Gp in T_1 ribonuclease digests and of pCp in pancreatic ribonuclease digests. The combined data of the reactivity of C1 are summarised in Table 4.6. The data show that this cytidine has reacted to the extent of approximately 58% after 24 h treatment at 25°C.

C45 This is the next nucleotide in the sequence for which direct evidence is available from an unique oligonucleotide containing a single cytidine residue. Product P15, G-A-A-G-A-A-Cp, shows 13% diminution after bisulphite treatment. No uridine containing the derivative product could be detected. Therefore C45 is fairly unreactive.

C47 The quantity of C-A-Gp (T_4) was only slightly diminished after reaction, and no U-A-Gp appeared. Thus C47 shows little or no reactivity.

C52 This clearly reacts extensively (approximately 70%), judged by both the diminution of C-U-A-Gp (T_{15}) and the appearance of a derivative product U-U-A-Gp in approximately equivalent yield (see Table 4.3).

C56 C-Ψ-Gp (T_{12}) can be fully resolved from U-C-Gp (T_{11}) in long separations of T_1 ribonuclease digests (Plate 4.2). After reaction of 5.8S rRNA

with sodium bisulphite, product T12 was diminished by approximately 48% and a new product of composition $(U + \Psi)_2\ G_1$ (T41) appeared. Chromatographic analysis of the latter (Hughes et al., 1976) showed that the ratio of $\Psi p/(\Psi p + Up)$ in the new spot to be 0.45. This points to the reactivity of C56 being 52%. By taking the mean of these two values, the reactivity of C56 in C-Ψ-Gp was approximately 50%.

C78 This is adjacent to the methylated nucleotide, $G_m 77$ and the data in Table 4.6 indicate that C78 is approximately 40% reactive.

C83 shows 37% reactivity judged by diminution of the pancreatic ribonuclease product A-G-G-A-Cp (P14).

C107 exhibits 27% reactivity judged by the 29% decrease in P9 (G-A-A-Cp) and the increase of 25%, relative to P9, shown by product P18(G-A-A-Up).

C118-121 These four residues occur in the T_1 ribonuclease product, C-C-C-C-Gp (T6). As discussed in Chapter 3, this product was recovered in low yield from 'control' digests. The yield was not further diminished after bisulphite treatment, indicating that all four cytidine residues are unreactive.

C137 This was recovered within product P23, G-G-G-G-Cp, for which similar comments apply as to the product T6 above. From the fact that the recovery of this product was not further diminished after reaction of 5.8S rRNA with bisulphite, it is concluded that C137 is unreactive.

C152 This is significantly reactive towards sodium bisulphite (29%) judged by the diminution of pancreatic ribonuclease product, G-A-G-Cp (P13).

C157 This cytidine residue, close to the 3' terminus, shows considerable reactivity (64%), judged by both the diminution of product T2a, C-U-U, and

the appearance of a corresponding derivative product, U-U-U (Table 4.6).

There are four further cytidine residues that can be directly inferred to be fairly unreactive although they do not occur within unique oligonucleotides:

C4, C98 These are located within the pancreatic ribonuclease product, G-A-Cp (P7) which occurs twice per molecule. There is little or no diminution of G-A-Cp after bisulphite treatment and little or no augmentation of the potential reaction product, G-A-Up (Table 4.4). It follows that C4 and C98 are both unreactive.

C35, C155 Similarly, the T_1 ribonuclease product U-C-Gp (T11), which contains two cytidines is diminished only slightly (15%). The molar yield of the potential reaction product U-U-Gp (T41, Table 4.3) suggests only 5% reactivity. It can therefore be concluded that C35 and C155 exhibit little (10%) if any reactivity.

Indirect evidence

It was possible to infer less directly the reactivities of several further cytidine residues by combining data from other digestion products with the "direct evidence" described above.

C6 The T_1 ribonuclease product, T23, which contains C4 and C6 was diminished by approximately 50% after reaction of 5.8S rRNA with bisulphite (see footnote (e) to Table 4.2). Since C4 is largely unreactive (see above), C6 must be moderately reactive (approximately 50%).

C11, C50, C56 The pancreatic ribonuclease product, A-G-Cp (P8) occurs

three times per molecule. The product is diminished by 63% after the reaction with bisulphite, and a new product, A-G-Up (P17a), appeared in an amount equivalent to 54% of the original A-G-Cp. Therefore, the three cytidines represented by A-G-Cp show, on average, 50 - 60% reactivity. C56 was shown to be 50% reactive, as discussed under "direct evidence" above. Therefore C11 and C50 must be on average 55 - 60% reactive. These two nucleotides will be reconsidered when the secondary structure of 5.8S rRNA is discussed.

C26, C118 The pancreatic ribonuclease product, G-G-Cp (P12) occurs twice per molecule, containing C26 and C118. There is slight diminution of approximately 0.2 in the molar yield of G-G-Cp after reaction with sodium bisulphite and G-G-Up is correspondingly augmented (Table 4.4). C118 is completely unreactive, as discussed (product T6, C-C-C-C-Gp). Therefore C26 shows significant reactivity (approximately 25%).

C26, C28; C142, C143 These cytidine residues, of which C26 has just been considered, are encompassed by the isomeric T_1 ribonuclease products C-U-C-Gp and C-C-U-Gp (T14a and T14b). These tend to overlap in 'fingerprints', but are fairly well resolved after prolonged separation in the second dimension (Plate 4.2). About 50% of the total material disappeared after sodium bisulphite treatment (Table 4.2), and this was largely due to the disappearance of material from C-U-C-Gp, the lower component in the 'fingerprint' (Plate 4.2). A 70% reactivity for this product, together with the approximate 25% reactivity of C26 already discussed, indicates that C28 is considerably reactive (approximately 60%). Residues C142 and C143 in C-C-U-Gp appear to be less reactive with a combined reactivity of approximately 30%.

C85 This is recovered together with C83 in product T21. This oligonucleotide shows approximately 37% reactivity. Since C83 also shows 37% reactivity (product P14), C85 must be unreactive.

C140 A converse argument to the foregoing applies. C140 is recovered with C137 in product T16. Since C137 is unreactive (product P23, above), the reactivity of product T16 (34%, Table 4.2) must be due to C140. Moreover, a unique reaction product, C-U-A-U-Gp was recovered in 28% yield (Table 4.3). It can therefore be concluded that C140 is 31% reactive.

C32, C59 and C115 Finally under the heading "indirect evidence", we can deduce that C32, C59 and C115 are largely or completely unreactive. The pancreatic ribonuclease product G-Cp (P6) represents eight different cytidines: C32, 47, 52, 59, 109, 115, 142 and 157. Of these, C52 and C157 show more than 50% reactivity each, as deduced from the T_1 ribonuclease products T15 and T2a ("direct evidence", above). Since the overall reactivity of G-Cp is rather low, as indicated by the fairly small difference in molar recovery between "control" and "bisulphite-treated" samples (Table 4.4), the remaining six cytidines must be for the most part fairly unreactive. Evidence for the lack of reactivity of C142 is given above, and for C47 under "direct evidence", product T4.

Corroborative evidence for the lack of reactivity of C32, C59 and C115 can be derived indirectly from the T_1 ribonuclease product T_2 (C-Gp; Table 4.2). This occurs six times per molecule (C11, 32, 50, 59, 115 and 152). Of these, C11 and C50 are reactive and C152 is slightly reactive ("direct evidence", above). These three cytidines account for most or all of the reactivity of T_2 (Table 4.2).

Therefore C32, C59 and C115 are inferred to be largely or completely unreactive, in agreement with the inference from the pancreatic ribonuclease 'fingerprint'.

Oligonucleotides with multiple cytidine residues

For five T_1 ribonuclease products with multiple cytidine residues, it is possible to estimate the reactivities of the oligonucleotides but not to estimate the reactivities of the individual cytidines. Three of these oligonucleotides, T19.2 (especially), T20 and T22 are highly reactive, whereas the other two, T18 and T19.1, are moderately reactive. Consideration of products T18 and T19.1 will be deferred until discussing the probable secondary structure.

With regard to product T20, A-C-A-C-U-U-C-Gp, one of the cytidines, C98, is unreactive, as inferred from pancreatic ribonuclease product P7. Therefore, the high reactivity of product T20 must be due to C100 and/or C103. The results for T19.2 or T22 could arise in various ways. One cytidine per oligonucleotide might account for all the reactivity, or the reactivity could be distributed, equally or otherwise, between two or more cytidine residues. For example, if two cytidines reacted equally and the recovery of the oligonucleotide is decreased by 84%, each cytidine must have reacted to 60%. The amount of each cytidine remaining unreacted would then be 40%, and hence the total amount of oligonucleotide remaining unreacted would be 40% x 40% = 16%. At any rate, the results for these oligonucleotides signify the presence of one or more exposed cytidines per oligonucleotide.

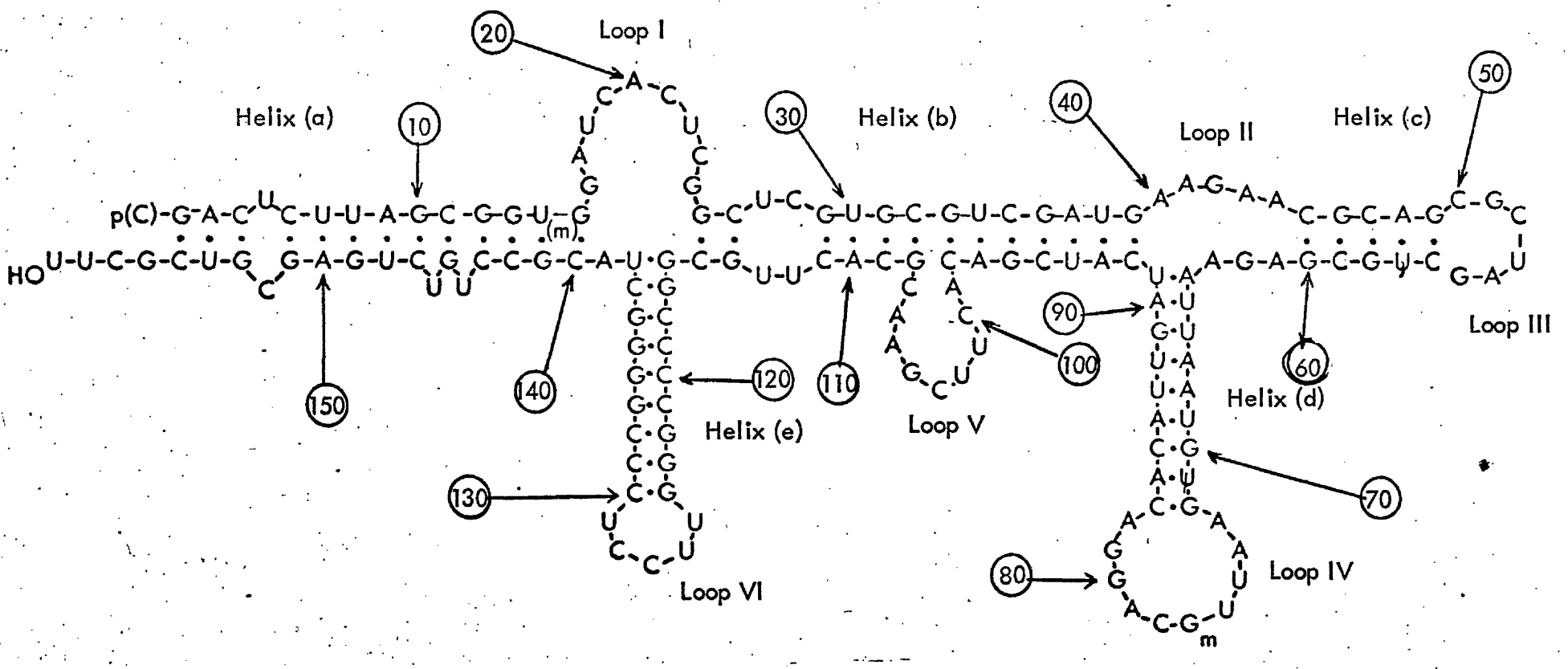
(d) Discussion

Relation of findings to conformation of 5.8S rRNA

The results described above provide either direct or indirect estimates of

Fig. 4.3 Secondary structure model for HeLa cell 5.8S rRNA based on that of Nazar et al. (1975).

Lines between nucleotides indicate phosphodiester bonds. Dots indicate the base pairs proposed by Nazar et al. (1975), except that their pairs C1.G156 and C50.G55 have been deleted (see text). The proposed loops are numbered according to Khan and Maden (1976) and the helices according to the text. The arrows indicate the positions of the nucleotides in the sequence.


Loop I
Helix (a)
Helix (b)
Loop II
Helix (c)
Loop III
Helix (d)
Helix (e)
Loop V
Loop VI
Loop IV
p(C)-G-A-C-U-C-U-U-A-G-C-G-G-U-G
HO-U-U-C-G-C-U-G
G-A-G-U-C-G-C-C-G-C-A-U-G-C-G-U-U-C-A-C-G
C-A-G-C-U-A-C
A-G-A-G-A-A-C-G-C-A-G-C-G-C
G-A-U
A-G-A-G-C-G-U-C
U-A
G-U
U-A
A-U
C-G
A-U
C-G
A-C-G-A-A
G-G-A-U
A-C-G-U
m
(m)
20
10
30
40
50
150
140
120
110
100
90
60
130
70
80

the reactivities of the great majority of the 46 cytidine residues in the nucleotide sequence of HeLa cell 5.8S rRNA. A wide range of reactivities were encountered, indicating that several cytidines occupy exposed locations within 'unbound' 5.8S rRNA, whereas others are protected.

The main conclusion is that the data fit well with the general features of the secondary structure model proposed for mammalian 5.8S rRNA by Nazar et al. (1975). The general model is shown in Fig. 4.3. In the orientation depicted, it contains a long, interrupted horizontal helical region, two downward projecting helical regions, six single-stranded loops which are numbers I to VI (as in Khan & Maden, 1976) and several other helix imperfections. The model (Fig. 4.3) presents several characteristic features with which the data can be compared.

Loops Each of the proposed loops contains one or more reactive cytidines except for loop II, which contains no cytidine residues. Loop I contains the highly reactive cytidine(s) encompassed by product T19.2. As discussed above, it was not possible to determine whether the observed reactivity is due to one, two or all three cytidines (C19, C21 and C23), but it can be concluded that at least one of them is reactive. Loop III contains the most highly reactive single cytidine residue for which direct evidence is available, namely C52 (Table 4.2). Loop IV contains a moderately reactive cytidine, C78, next to a 2'-O-methyl guanosine. Loop V contains two distinctive oligonucleotides, T20 and P9. As discussed, T20 possesses three cytidines, C98, C100 and C103 of which C98 is unreactive. This nucleotide is located in a helical region in the model. C100 and C103, either or both of which give rise to the observed reactivity of product T20, are both situated in the loop. C107 is on the edge of

the loop and is also significantly reactive (product P9). The pentanucleotide, loop VI, contains part of the highly reactive oligonucleotide T22. It was not possible to distinguish experimentally which cytidines in this product are reactive. However, in view of other evidence on the stability of the associated helix (see below, and Chapter 3), the high reactivity of product T22 is attributed mainly or entirely to cytidine residues 127 and 128 of the loop.

Helical regions All cytidines that are internally located within proposed helical regions, and for which reactivity estimates have been derived, are largely or completely unreactive. This is true in helix (a) for C4, C142, C143 and C155; in helix (b) for C32, C35 and C98, in helix (c) for C47 and C59 and in helix (d) for the only internally located cytidine in this helix, C85. Helix (e) contains the completely unreactive cytidines 118-121 and 137. As noted in Chapter 3, this region of the molecule was evidently incompletely digested under conditions which gave rise to complete digestion of the rest of the molecule. Both observations are in accord with the prediction that the sequence between nucleotides 116 and 137 should give rise to a highly stable GC helix with a short hairpin loop.

Ends of helical regions In contrast to cytidines that are internally located within helices, several of those that form GC pairs at the ends of helical regions in the model display some reactivity.

The 5' terminal pCp is clearly reactive (see Table 4.2, 4.4), although it is potentially in a position to form a base pair with G156. C26, at a corner between loop I and a bulge in helix (b) also exhibits reactivity(25%, product P12, Table 4.4). C45 at the left hand end of helix (c) is also slightly reactive

although it is a borderline case (13%). C83, at the bottom of helix (d) is clearly reactive (37%, product P14). C140, which has the potential for pairing with G15 at the right hand of helix (a), is also reactive (31%, see Table 4.4). It seems reasonable to infer from these findings that cytidines in "closing" GC pairs will frequently be more reactive towards sodium bisulphite than internally located cytidines, probably because pairs in these positions equilibrate to various extents between open and closed states. Further deductions can be made if we tentatively accept this inference.

A corner occurs at the transition from helix (d) to helix (b). This corner is encompassed by product T19.1, containing C92 and C95. The oligonucleotide shows 33% reactivity (Table 4.2). We can now attribute the reactivity of product T19.1 to C92, which forms a "closing" GC pair in helix (b) and so propose that the internally located C95 is unreactive. Similarly, it is possible to propose that most of the reactivity of product T18 is due to C111, which forms a GC pair next to a helix imperfection, and that C109, in an internal position, is largely unreactive. It is similarly possible that a small amount of the reactivity of product T22 is due to the terminal position of C130, although most of the reactivity of this oligonucleotide is likely to be due to the loop nucleotides C127 and C128 as already discussed.

It also becomes possible to suggest an explanation for the data on C11, C50 and C56. It has been shown that product P8, A-G-Cp, which represents three cytidines is at least 55% reactive. It has been shown, independently, that C56 is 50% reactive (product T12, Table 4.2). It follows that C11 and C50 must be, on average, 55 - 60% reactive. It is possible to suggest an

explanation as follows.

The pair G49. C56 is the terminal pair of helix (b). C50 and G55, which appear to possess the potential for base pairing are in fact unpaired. C11 forms a somewhat labile pair with G145 in helix (a), the pair being destabilized by the unpaired uridines 144 and 146 on either side of G145. (Thus C50 might be > 70% reactive, this being a comparable order of reactivity to C52 in loop III, whereas C11 might be 30 - 40% reactive, this being a comparable order of reactivity to that encountered in several of the "closing" GC pairs already discussed.)

The inference that C11. G145 is a weak pair seems reasonable in view of the adjacent unpaired uridines. By contrast there is no theoretical precedent for the conclusion that C50 fails to pair with G55. Gralla and Crothers (1973) concluded that a hairpin loop with four nucleotides can be closed by a GC pair. However, the data of Nazar et al. (1975) reveal that the phosphodiester bond between G55 and C56 is cleaved by T_1 ribonuclease at low concentration. This, like the data presented here, would be consistent with the presence of G55 and hence C50, in the loop. Possibly the nature of the nucleotides in the loop is important in determining the minimum loop size, for example by influencing stacking interactions. The model compounds of Gralla and Crothers (1973) contained only cytidines in the loop, whereas loop III contains all four different bases. This might favour stacking at points within the loop and thereby account for a length of six rather than four nucleotides.

There is, however, an alternative explanation to this apparent high reactivity of C50. As explained in Chapter 3, Boseley et al. (1978) determined the sequence

of a cloned fragment of Xenopus laevis DNA which included the gene for 5.8S rRNA. This sequence revealed a GC 'deletion' from the nucleotide sequence in the region of loop III. One possible explanation of the discrepancy between this sequence and the one determined from enzyme digestion of Xenopus 5.8S rRNA (Ford & Mathieson, 1978) is that the RNA contains the sequence A-G-C-U and not A-G-C-G-C-U in the region of loop III; i.e. there is a 'deletion' of a CG (or GC) between residue A48 and U53. If this 'deletion' occurs in HeLa cell 5.8S rRNA, then the data obtained for C50 and C52 would in fact have originated from the same source. It had been assumed that C50 was at least 60% reactive from the diminution of A-G-Cp (P8, Table 4.4) and that C52 was about 70% reactive (T15, Table 4.2). A GC deletion from loop III would mean that these reactivities refer to the same cytidine residue.

The net result of this deletion would be that loop III contains four nucleotides (instead of six): C50, U53, A54 and G55 (G51 and C52 being absent). The closing pair at the end of helix (c) would be made up from G49. C56, with C56 being about 50% reactive.

Helix imperfections In addition to the major loops, the model in Fig. 4.3 contains some helix imperfections which have until now been mentioned only in passing. Helix (b) contains a symmetrical bulge near the left hand end. The data suggest that C28, within the bulge and C26 which forms a "closing" CG pair to the left of the bulge both exhibit reactivity. In helix (a) the situation is more complex as there are two sites with imperfections. It was suggested above that C11 forms a labile pair with G145, between two 'looped-out' uridines. It is not possible to comment reliably on the reactivity of C147 to

the left of U146 (see footnote c, Table 4.2). The left hand imperfection in the proposed helix (a) consists of a mismatched pair, U5. C152. The latter nucleotide C152 is about 30% reactive (product P13; Table 4.2). C6, adjacent to U5, also appears to be reactive, although C4 showed little or no reactivity, perhaps surprising in view of other data in this section.

3' terminus C157, occupying an unpaired position close to the 3' terminus of 5.8S rRNA, shows considerable reactivity towards sodium bisulphite.

In summary, the observed or inferred reactivities of all the cytidines, except for a few for which estimates were not obtained, fit well with the features of the secondary structure model of Nazar et al. (1975)with two qualifications; cytidines in CG pairs at the end of helices are appreciably reactive, and loop III may contain six nucleotides rather than four.

4.3 A time-course study of the reaction of sodium bisulphite with HeLa cell 5.8S rRNA at 25°C.

The estimates for the reactivity of cytidine residues within 5.8S rRNA reported in section 4.2 have been made on the basis of the extent of the reaction of those residues after 24 hours. Such estimates are only valid if it can be established that the rate constant for the reaction of individual cytidine residues remains the same throughout the time of the reaction. If, on reaction with bisulphite, a conformational change occurs such that certain cytidine residues become more accessible, this would show itself in kinetics deviating from those expected for a pseudo-first order reaction.

A rigorous investigation of the kinetics of reaction of every cytidine residue would have proved prohibitively complex. However, an investigation of the

reaction kinetics of a few representative residues which could be analysed relatively easily would serve to indicate whether a general slow conformational change,of the type described above, was occurring. It may also be possible to elucidate further some of the problems and tentative conclusions reported in section 4.2 .

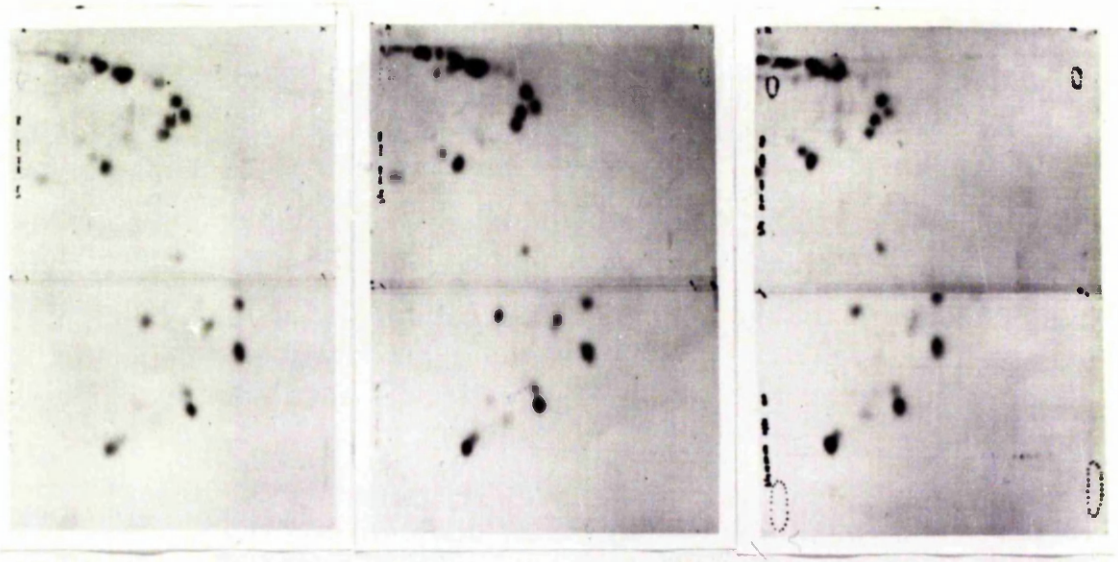
As before, the reaction of 5.8S rRNA with bisulphite was studied using 3 M sodium bisulphite, pH 6.0, at 25°C in the presence of 10 mM $MgCl_2$. It was found to be satisfactory to proceed with this investigation using 'fingerprints' of T_1 ribonuclease digests of HeLa cell 5.8S rRNA which had been run for 40 hours at 1.2 KV in the second dimension (Plate 4.4). This procedure had two advantages. It enabled the larger oligonucleotides, which contain some of the more rapidly reacting cytidines, to be better resolved . Secondly, although some of the smaller digestion products which contain reactive residues would be run off the bottom of the 'fingerprint' (e.g. product T2a, C-U-U), their reactivities could be followed by the appearance of the analogous uridine-containing products in the upper portion of the 'fingerprint' (in this case U-U-U, product 10b).

Rate of reactivity of individual cytidine residues

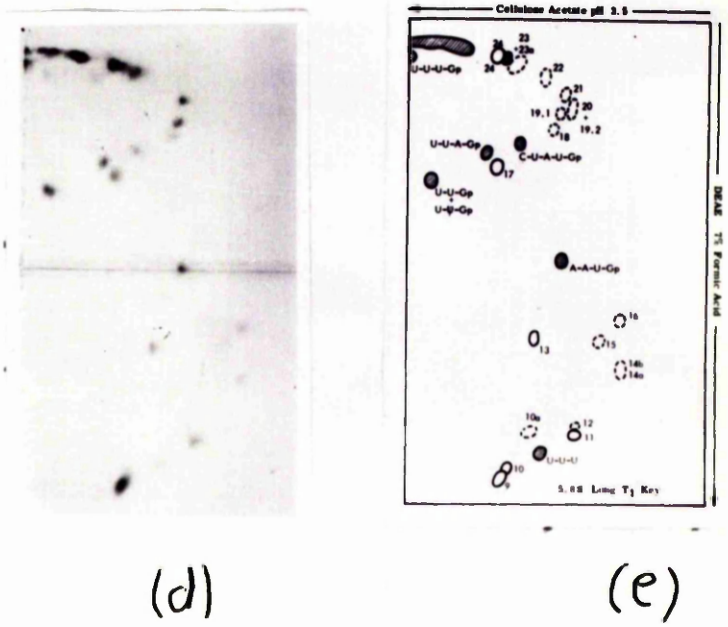
Plate 4.4 shows 'fingerprints' of T_1 ribonuclease digests of HeLa cell 5.8S rRNA which were run for 40 hours at 1.2 KV in the second dimension. The RNA had been reacted with sodium bisulphite for various times: (a) 6 h, (b) 12 h, (c) 24 h, (d) 42 h. In these 'fingerprints' many cytidine-containing oligonucleotides are present in progressively smaller molar yields as the reaction with sodium bisulphite proceeds. The data is summarised in Table 4.7. In addition,

Plate 4.4 'Long T' Fingerprints of HeLa cell 5.8S rRNA after reaction with 3 M sodium bisulphite, pH 6.0, at 25°C for (a) 6 h, (b) 12 h, (c) 24 h and (d) 42 h.

The key (e) identifies new sequences (shaded circles) which arise as a result of the reaction with sodium bisulphite, diminished cytidine-containing sequences (broken circles), and unreactive sequences (closed circles). These are identified by the standard numbering scheme (see Table 3.1). Electrophoretic separation was carried out in the first dimension on cellulose acetate, pH 3.5, at 4.7 KV for 3 - 3.5 h and in the second dimension on DEAE paper, 7% formic acid, at 1.2 KV for 40 h.



(a) (b) (c)



(d) (e)

Table 4.7 Molar yields of T_1 ribonuclease products of HeLa cell 5.8S rRNA after reaction with sodium bisulphite for various times as indicated, at 25°C.

The sequences can be identified by the standard numbering system from Table 3.1. The bisulphite reaction was carried out at 25°C in 3 M sodium bisulphite in the presence of 10 mM $MgCl_2$. When the experiment was carried out in the absence of $MgCl_2$ at 25°C, no significant differences in the molar yields were obtained. The molar yields after bisulphite treatment were calculated assuming that the sum of the molar yields of A-U-Gp (T13) and A-A-U-U-A-A-U-Gp (T24) was 2.0. These two oligonucleotides neither react with sodium bisulphite nor gain material from the conversion of other cytidine-containing products to uridine-containing products.

Footnotes

(a) The increase in T9, U-Gp, after bisulphite reaction is due to the conversion of T2, C-Gp, its uridine-containing derivative. The increase in the yield after 42 h treatment of T10, Ψ-Gp, is probably due to a 'spill-over' of counts from spot T9, since the two products are not always completely separated.

(b) Separate molar yields for T14a and T14b, T19.2 and T20 have only been given when these closely migrating pairs of products have been sufficiently resolved.

(c) The transient increase in T17, U-C-U-Gp, after 6 h and 12 h is probably due to the conversion of T14b, C-U-C-Gp to (U_2,C) Gp which then co-migrates with T17.

Table 4.7

Spot No.	Molar yields after reaction with sodium bisulphite					
	zero	6 h	12 h	24 h	42 h	
T9	1.99	2.50	2.67	3.67	4.31	(a)
T10	1.02	0.99	0.81	1.17	1.48	(a)
T10a	0.42	0.35	0.25	0.16	0.07	
T11	1.92	1.75	1.55	1.64	1.02	
T12	1.03	0.91	0.55	0.54	0.23	
T13	1.15	1.10	1.00	1.12	0.91	
T14a	2.08	1.01	0.72	0.96	0.40	(b)
T14b		0.75	0.48			(b)
T15	1.06	0.60	0.51	0.31	0.12	
T16	0.67	0.73	0.41	0.44	0.25	
T17	1.10	1.40	1.40	0.99	1.13	(c)
T18	0.91	0.71	0.63	0.42	0.25	
T19.1	0.87	0.60	0.64	0.58	0.37	
T19.2	1.82	0.21	0.14	0.28	0.05	(b)
T20		0.55	0.48			(b)
T21	0.87	0.85	0.70	0.55	0.35	
T22	0.80	0.43	0.15	0.11	0.03	
T23	1.86	1.49	1.38	0.95	0.77	
T23a						
T24	0.85	0.90	1.00	0.84	1.09	

Table 4.8 Molar yields of uridine-containing T_1 ribonuclease digest products of HeLa cell 5.8S rRNA after reaction with sodium bisulphite for various times, as indicated at 25°C.

The conventions are the same as for Table 4.7. In the examples shown, the uridine-containing products of bisulphite reaction migrate to distinctive positions in the 'fingerprint' and can therefore be readily quantified.

Spot No.	Molar yields after reaction with sodium bisulphite					
	zero	6 h	12 h	24 h	42 h	
T10b (U-U-U)	nil	0.29	0.45	0.56	0.66	(a)
T36 (A-A-U-Gp)	nil	0.49	0.51	0.72	0.81	
T41 (U-U-Gp+U-ψ-Gp)	nil	0.61	0.76	0.84	1.68	
T40 (U-U-A-Gp)	nil	0.44	0.54	0.68	0.91	
T42 (C-U-A-U-Gp)	nil	0.18	0.27	0.18	0.47	
T39 (U-U-U-Gp)	nil	0.20	0.47	0.95	1.66	

Footnotes

(a) This product, T10b, is the result of the reaction of the 3' terminal group C-U-U (T2a) with sodium bisulphite. It is the only indication in these 'long T_1' ribonuclease 'fingerprints' of the rate of reactivity of C157.

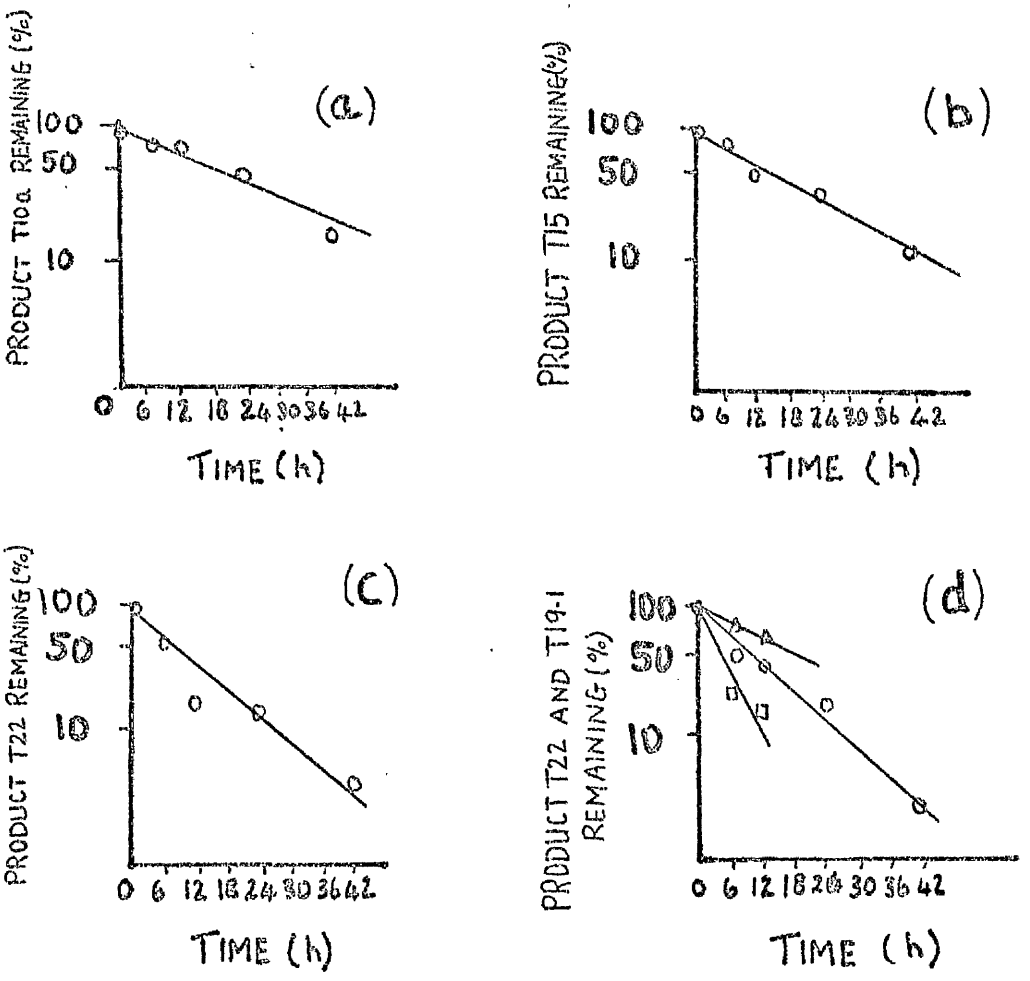
it is possible to observe the progressive appearance of the respective uridine-containing conversion products of several oligonucleotides. These products were characterised and their molar yields determined (Table 4.8).

The data in Table 4.7 provides a basis for analysis of the kinetics of reaction of several oligonucleotides. By drawing a semi-logarithmic plot of the percentage oligonucleotide remaining after various times of bisulphite reaction, it is possible to obtain the order and time of half reaction for some of the more reactive oligonucleotides (Fig. 4.4 a-d). The data in Fig. 4.4 show that the more rapidly reacting cytidines react with approximately pseudo-first order rate kinetics. These cytidines, at least, are accessible from the beginning of the reaction, and their reactivity does not depend upon a progressive change of conformation of the molecule as the reaction proceeds. The kinetics of the slower reacting cytidines appear to be more complex, reflecting their positions in labile GC 'closing' pairs. However, all cytidines which exhibit even slight reactivity are available from the beginning of the reaction, and do not become available at some later stage due to conformational changes.

From the graphs (Fig. 4.4 a-d), it is possible to obtain estimates of the times of half reaction for some of the more reactive oligonucleotides.

Spot No.	Sequence	Time of half reaction (hours)
T10a	pC-Gp	18 h
T15	C-U-A-Gp	13 h
T22	U-U-C-C-U-C-C-C-Gp	7 h
T19.2	A-U-C-A-C-U-C-Gp	3 h
T20	A-C-A-C-U-U-C-Gp	11 h

Fig. 4·4 Kinetics of reaction of cytidine residues in 5·8S rRNA with sodium bisulphite.



The reaction of 5·8S rRNA with sodium bisulphite was carried out for various time intervals as described in the text. After adduct removal the RNA was subjected to digestion with T_1 ribonucleus followed by "fingerprinting" analysis. The extents of reaction shown by the oligonucleotides after various times were determined (a) Product T10a; (b) Product T15; (c) Product T22; (d) Products T19·2(□) and T20(△), and T19·2 and T20(○).

The data obtained on the reaction kinetics of oligonucleotides from T_1 ribonuclease 'fingerprints' (Tables 4.7 and 4.8) indicate that there are a wide range of reactivities. The most reactive oligonucleotide is T19.2, A-U-C-A-C-U-C-Gp, which encompasses C19, C21 and C23. It has a time of half reaction of approximately 3 hours; however it was not possible to obtain data on the individual cytidines within this digest product. The most reactive cytidine for which direct evidence could be obtained is C52 within T15, C-U-A-Gp, which has a time of half reaction of 13 hours.

The results described in this Chapter have recently appeared as a published paper in the Biochemical Journal (Kelly et al., 1978).

5.1 The reaction of carbodiimide with 5.8S rRNA at 25°C

(a) Choice of conditions

Water soluble carbodiimide forms an adduct with the N^3 and N^1 positions of uracil and guanine respectively (Gilham, 1962). The blocked uracil then confers resistance on the adjacent phosphodiester bond to cleavage with pancreatic ribonuclease. This reagent 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide (referred to henceforth simply as carbodiimide) can be used to partially modify intact 5.8S rRNA. It was found to be convenient to follow the general method of Brownlee et al. (1972), in which only the more accessible uridine residues are modified. Under the conditions used there is only a small reaction of guanine residues, which would have considerably complicated the picture had it occurred more extensively. The reaction with pseudouridine is more complex, but almost twice as fast as with uridine (Ho & Gilham, 1971).

^{32}P-labelled HeLa cell 5.8S rRNA was reacted with 10 mg/ml (24 mM) carbodiimide in 0.02 M Tris-HCl, pH 8.9, in the presence of 10 mM $MgCl_2$. At 25°C, an incubation period of 16 h was found to be suitable.

(b) Interpretation of data from 'fingerprints'

Plate 5.1 shows 'fingerprints' of T_1 ribonuclease digests of HeLa cell 5.8S rRNA, untreated and after reaction with carbodiimide. Plate 5.2 shows 'fingerprints' of T_1 ribonuclease digests with long separations in both dimensions, in which the products at the top of the 'fingerprints' in Plate 5.1 are better resolved. Plate 5.3 shows 'fingerprints' of pancreatic ribonuclease digests. In 'fingerprints' obtained after carbodiimide treatment, many uridine-containing oligonucleotides

are present in reduced yields compared with the control. The reduction in yields of these products provides the primary data for estimating the reactivities of various uridines as summarised in Tables 5.1 and 5.2.

The data in Tables 5.1 and 5.2 permit estimates to be obtained for the reactivities of most of the uridine residues within 5.8S rRNA. A similar strategy to that used for sodium bisulphite modification was found to be convenient for interpreting the data. Namely, it is possible to distinguish between "direct evidence", where in most cases the data were obtained from unique oligonucleotides within the sequence which contain a single uridine residue and "indirect evidence" where the estimates were derived more circuitously.

Oligonucleotides containing carbodiimide modified uridines have more positive charge than the corresponding unmodified oligonucleotide. Consequently, in the first dimension (pH 3.5), they migrate to the anode with reduced mobility or they migrate slowly to the cathode, so that in Plates 5.1 to 5.3 products arising from carbodiimide reaction are found to the right of the unmodified oligonucleotide from which they are derived. The increased charge on these reacted oligonucleotides causes their mobility in the second dimension of electrophoresis to be considerably greater than that of the corresponding unmodified oligonucleotides.

Preliminary investigations indicated that there were in T_1 and pancreatic ribonuclease 'fingerprints' a large number of new oligonucleotides arising from carbodiimide reaction and that many of these occurred in low molar yields. Although a full analysis and characterisation of these products would have

'Fingerprints' of HeLa cell 5.8S rRNA before (left) and after (centre) reaction with carbodiimide (10 mg/ml), pH 8.9, for 16 h at 25°C.

Plate 5.1 T_1 ribonuclease digest

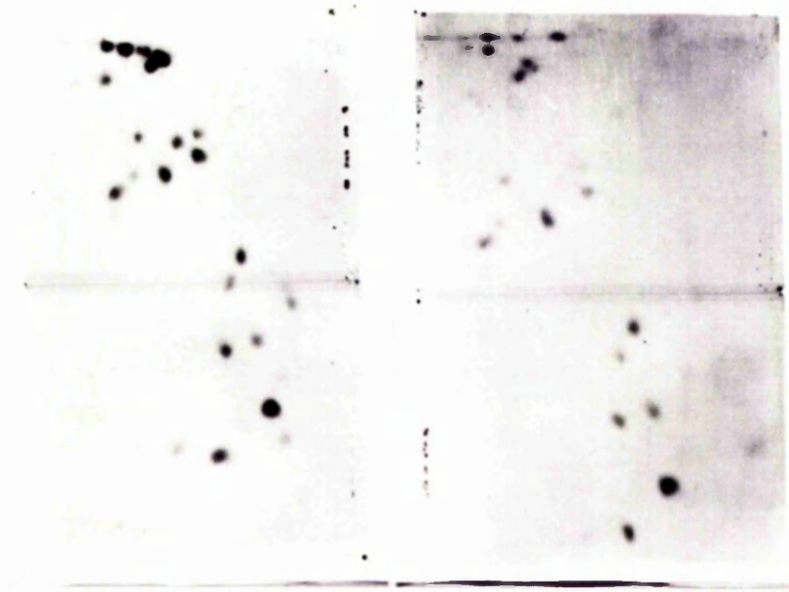
The key (right) identifies diminished (broken circles) and unchanged (closed circles) sequences after the reaction with carbodiimide. These sequences are identified by the standard numbering scheme (Table 5.1). Electrophoretic separation was carried out in the first dimension on cellulose acetate, pH 3.5, at 4.7 KV for 3 - 3.5 h and in the second dimension on DEAE paper, 7% formic acid, at 1.2 KV for 17 h.

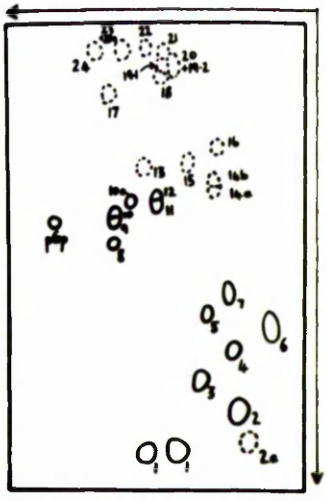
Plate 5.2 T_1 ribonuclease digest, extended fractionation

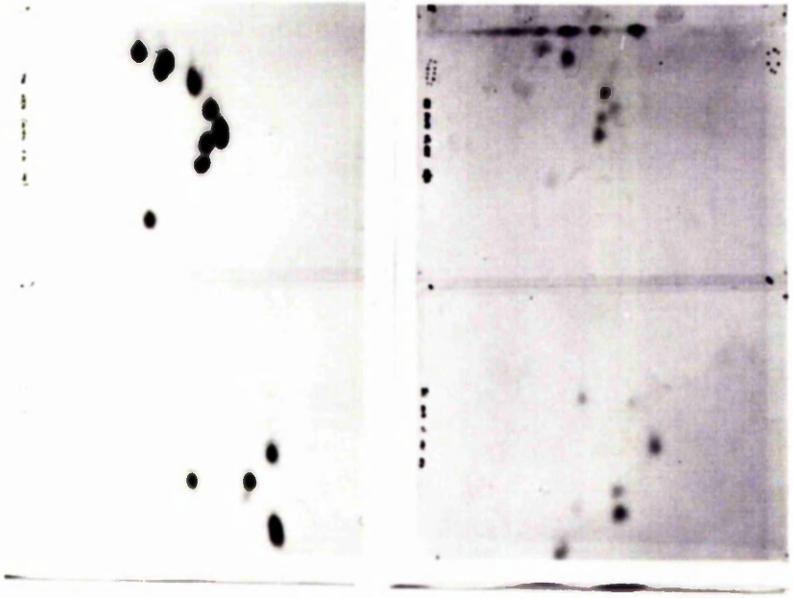
The key (right) identifies reacted and unreacted sequences as in Plate 5.1. Electrophoresis was carried out at 4.7 KV for 4 h in the first dimension and at 1.2 KV for 40 - 60 h in the second dimension.

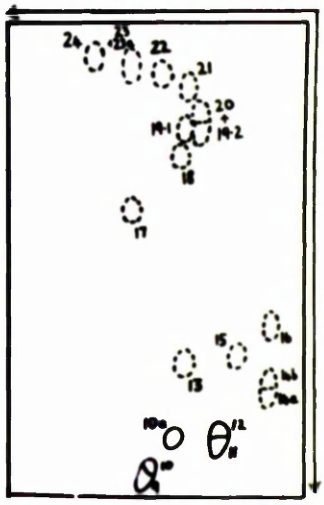
Plate 5.3 Pancreatic ribonuclease digest

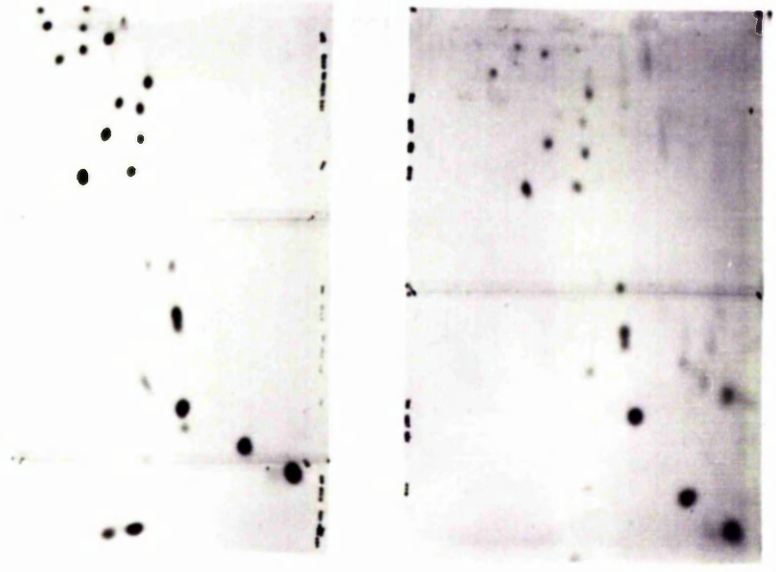
The key (right) identifies diminished and unreacted sequences as in Plate 5.1 with the numbering of spots as in Table 5.2. Electrophoresis was carried out at 4.7 KV for 2.5 h in the first dimension and at 1.1 KV for 16 h in the second dimension .











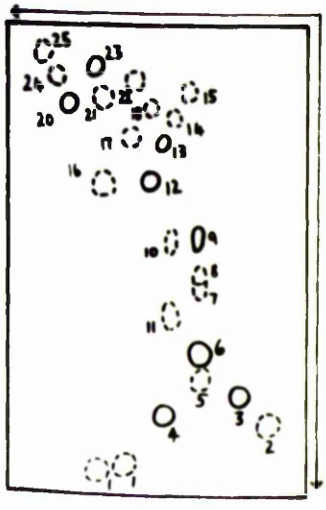


Table 5.1 Sequences and molar yields of T_1 ribonuclease products from HeLa cell 5.8S rRNA and the extent of reaction of uridine-containing oligonucleotides after treatment with carbodiimide at 25°C.

The conventions are as in Table 3.1 The positions of the uridine residues within the nucleotide sequence are indicated. The carbodiimide reaction was carried out for 16 h at 25°C in 10 mg/ml carbodiimide, in the presence of 10 mM $MgCl_2$, pH 8.9. The molar yields after carbodiimide treatment were calculated assuming that the yield of T4, C-A-Gp, was 1.00. The molar yields are the means of three determinations from independent experiments.

Footnotes

(a) The molar yield of Gp was slightly variable. It is unlikely that the decreased molar yield is significant since no such decrease was noticed when the reaction was carried out at 37°C and 50°C.

(b) See footnote (c) to Table 5.2.

Table 5.1

Spot No.	Sequence	Uridine residue(s)	Molar yields		% reactivity
			Control	Carbodi-imide treated	
T1	Gp	-	11.15	9.44	-
T2	C-Gp	-	6.20	6.18	-
T2a	C-U-U	158, 159	0.91	0.22	76%
T3	A-Gp	-	2.36	2.81	-
T4	C-A-Gp	-	1.04	1.00	-
T5	A-A-Gp	-	1.01	0.96	-
T6	C-C-C-C-Gp	-	0.45	0.33	-
T7	A-A-C-Gp	-	1.44	1.34	-
T8	U_m-Gp	14	0.21	0.19	10%
T9	U-Gp	14,30	1.99	2.06	zero
T10	ψ-Gp	71	1.02	1.12	zero
T10a	pC-Gp	-	0.42	0.42	-
	pGp	-	0.12	0.17	-
T11	U-C-Gp	34, 154	1.92	1.80	6%
T12	C-ψ-Gp	57	1.03	1.13	zero
T13	A-U-Gp	38	1.15	0.85	25%
T14a	C-U-C-Gp	27	2.08	0.92	12%
T14b	C-C-U-Gp	144		0.33	68%
T15	C-U-A-Gp	53	1.06	0.32	70%
T16	C-U-A-C-Gp	138	0.67	0.21	69%
T17	U-C-U-Gp	146, 148	1.10	0.24	78%
T18	C-A-C-U-U-Gp	112, 113	0.91	0.69	24%
T19.1	A-U-C-A-U-C-Gp	91,94	0.87	0.40	54%
T19.2	A-U-C-A-C-U-C-Gp	18,22	1.82	0.31	83%
T20	A-C-A-C-U-U-C-Gp	101, 102			
T21	A-C-A-C-A-U-U-Gp	87,88	0.87	0.54	38%
T22	U-U-C-C-U-C-C-C-Gp	125, 126, 129	0.80	0.03	96%
T23	A-C-U-C-U-U-A-Gp	5,7,8	1.86	0.61	67%
T23a	A-A-U-U-G_m-C-A-Gp	75,76			
T24	A-A-U-U-A-A-U-Gp	65,66,68	0.84	0.37	56%

Table 5.2 Sequences and molar yields of pancreatic ribonuclease products from HeLa cell 5.8S rRNA and extent of reaction of uridine-containing products after treatment with carbodiimide at 25°C.

The conventions are as in Table 3.1 The reaction conditions are the same as for Table 5.1. The molar yields were calculated assuming that the yield of product P9, G-A-A-Cp, was 1.00. This product gave a consistent yield at all temperatures at which the carbodiimide reaction was carried out. The values shown are the mean of those obtained in three independent experiments.

Footnotes

(a) A reduction in the yield of Cp results when a cytidine residue precedes a uridine in the nucleotide sequence which has reacted with carbodiimide. When this occurs, pancreatic ribonuclease cannot cleave the phosphodiester between them, resulting in the formation of a new digestion product which then migrates to a different part of the 'fingerprint'.

(b) Product P5, G_m-Cp, is reduced by 84% after reaction. This signifies that U76, which immediately precedes G_m-Cp in the nucleotide sequence is very reactive. Since the phosphodiester bond between U76 and G_m77 becomes resistant to pancreatic ribonuclease hydrolysis, the yield of G_m-Cp is greatly reduced.

(c) Product P13 is not significantly reduced by carbodiimide treatment. This means that U148 which immediately precedes this oligonucleotide in the nucleotide sequence has little or no reactivity. Therefore the reduction in the yield of product T17, U-C-U-Gp, after carbodiimide treatment (75%) must be due entirely to U146.

(d) This product is reduced by 0.3 M after reaction with carbodiimide. It is immediately preceded by a reactive uridine (U38) in the nucleotide sequence (product T13, Table 5.1) which reduces the yield of the T_1 ribonuclease digest product in which it occurs by the same amount. The decrease in product P15 is therefore due to the inability of pancreatic ribonuclease to cleave the phosphodiester bond between U38 and G39 when U38 has reacted with carbodiimide.

Table 5.2

Spot No.	Sequence	Uridine residue(s)	Molar yields		% reactivity
			Control	Carbodi-imide treated	
P1	Up + Ψp	-	19.00	11.94	-
P2	Cp	-	19.80	16.53	-
P3	A-Cp	-	5.68	6.27	-
P4	pCp	-	0.46	0.54	-
P5	G_m-Cp	-	0.92	0.15	-
P6	G-Cp	-	7.06	7.33	-
P7	G-A-Cp	-	4.72	1.78	-
P8	A-G-Cp	-		2.32	-
P9	G-A-A-Cp	-	0.98	1.00	-
P10	A-Up	87,94	2.47	2.15	13%
P11	A-A-Up	69	1.06	0.66	38%
P12	G-G-Cp	-	1.91	1.50	-
P13	G-A-G-Cp	-	0.97	0.90	-
P14	A-G-G-A-Cp	-	0.98	0.61	-
P15	G-A-A-G-A-A-Cp	-	0.77	0.47	-
P16	G-Up + G - Ψp	30,34,71,146 154	5.45	4.73	13%
P17	G-A-Up	38,91	2.23	1.71	23%
P18	G-A-A-Up	75	1.12	0.43	62%
P20	G-G-Up	14	0.95	0.93	2%
P21	G-G-A-Up	18	0.83	0.26	69%
P22	G-A-G-A-A-Up	65	0.94	0.55	41%
P23	G-G-G-G-Cp	-	0.45	0.34	-
P24	G-G-G-Up	125	0.76	0.12	84%
P25	G-G-U_m-G-G-A-Up	24,28	0.20	0.05	75%

permitted refinement of the results presented in this section, it would have proved very complex. It was therefore decided to limit the investigation to a study of the effect of carbodiimide treatment on the molar yields of oligonucleotides obtained from T_1 and pancreatic ribonuclease digestion of 5.8S rRNA.

(c) Reactivities of individual uridine residues

Direct evidence

U14 In HeLa cell 5.8s rRNA this base is partially methylated with 20% of the molecules having 2'-O-methyl uridine. Neither form shows any reactivity after carbodiimide treatment. Product T8, U_m-Gp, exhibits no significant decrease and product T9, U-Gp, which is present in 1.8 molar is non-reactive (Table 5.1). Product P20, G-G-Up, which also encompasses this nucleotide, shows no reactivity.

U18 When U14 is in its methylated form, U18 is contained within product P25, G-G-U_m-G-G-A-Up, which is diminished by 75% after carbodiimide treatment. When U14 is in its non-methylated form, it is no longer resistant to pancreatic ribonuclease hydrolysis and the oligonucleotide fragment G-G-A-Up (P21) contains U18. This product is reduced by 69% after the carbodiimide reaction (Table 5.2). Since these two reactivities are almost identical, all the reactivity of product P25 must be due to U18 with U14 being non-reactive, as concluded above.

U27 This is the next nucleotide in the sequence for which direct evidence is available from an unique oligonucleotide containing a single uridine residue. Product T14a, C-U-C-Gp, shows 12% diminution after carbodiimide treatment

(Table 5.1). Therefore U27 exhibits little or no reactivity.

U38 The quantity of A-U-Gp (T13) was reduced after reaction by the extent of 25% (Table 5.1). This base is therefore moderately reactive.

U53 This uridine residue is clearly extensively reactive as judged by the reduction (approximately 70%) of product T15, C-U-A-Gp, after reaction with carbodiimide (Table 5.1).

Ψ57 Product T12, C-Ψ-Gp (Table 5.1) can be resolved on T_1 ribonuclease 'fingerprints' which have been run for at least 40 h in the second dimension (Plate 5.2). This oligonucleotide fragment had not diminished at 16 h carbodiimide treatment at 25°C, indicating that Ψ57 is non-reactive.

U65 This base can be located in product P22, G-A-G-A-A-Up after pancreatic ribonuclease digestion. It is moderately reactive towards carbodiimide, being reduced by about 40% after the reaction.

Ψ71 After long separation (40 - 60 h) in the second dimension, the T_1 ribonuclease product containing this nucleotide T10, Ψ-Gp, can be resolved on a 'fingerprint' from T9, U-Gp (Plate 5.2). It is completely non-reactive.

U75 This uridine residue is recovered with product P18, G-A-A-Up (Table 5.2), which is reduced by 62% after reaction.

U125 Product P24, G-G-G-Up, is diminished by approximately 85% after treatment with carbodiimide (Table 5.2). U125 is the most reactive uridine in the nucleotide sequence of 5.8S rRNA for which direct evidence is available.

U138 This residue is encompassed by product T16, C-U-A-C-Gp (Table 5.1), which is reduced by about 70% after reaction with carbodiimide.

U144 In long separations of T_1 ribonuclease 'fingerprints' T14b, C-C-U-Gp, can be resolved from its isomer, T14a (Plate 5.2). This product exhibits 68% reactivity.

There are four uridine residues that can be directly inferred to be unreactive, although they do not occur within unique oligonucleotides:

U14, U30 These occur within the T_1 ribonuclease product T9, U-Gp, which occurs twice per molecule. There is little or no diminution of U-Gp after carbodiimide treatment.

U34, U154 Similarly, the T_1 ribonuclease product, U-C-Gp (T11), which also occurs twice per 5.8S rRNA molecule, is not significantly diminished after reaction with carbodiimide. It can be concluded that U34 and U154 are unreactive.

Indirect evidence

It was possible to infer less directly the reactivities of several uridine residues by combining data from other digestion products with the "direct evidence" above.

U5, U7, U8, U75 and U76 These five uridine residues are found in the T_1 ribonuclease products T23 and T23a (Table 5.1) which cannot be properly resolved from each other even on 'fingerprints' where separation in the second dimension has been extended (Plate 5.2). Each of these oligonucleotides are on average 67% reactive. Within product T23a, U75 has a reactivity of 62% (see "direct evidence" and Table 5.2), and U76 has a reactivity of 84% (Footnote (b), Table 5.2). This will cause product T23a to be reduced by 94% (amount of T23a remaining unreacted is 38% (U75) x 16% (U76) = 6%). Therefore since T23 and

T23a have an average reactivity of 67%, it can be concluded that T23 is about 40% reactive.

U8 can be shown to possess little or no reactivity as follows: product P8, A-G-Cp, is diminished by 0.6 moles after carbodiimide treatment. This is presumably due to a reactive uridine occurring immediately before A-G-Cp in the nucleotide sequence resulting in the intervening phosphodiester bond becoming resistant to pancreatic ribonuclease hydrolysis. A-G-Cp is preceded twice in the nucleotide sequence by uridine residues, U8 and U53. Since U53 has been shown to be approximately 70% reactive (product T15, Table 5.1), it alone must be responsible for the reduction in the molar yield of A-G-Cp. This means that U8 is completely unreactive.

The 40% reactivity of product T23 must be shared either equally or unequally between U5 and U7. It has not been possible to assign a reactivity directly to either one of these nucleotides.

U22 This nucleotide, together with U18, is found within T_1 ribonuclease digestion product T19.2 which migrates closely with product T20 on 'fingerprints'. It was not possible to completely resolve these products from each other on the carbodiimide treated 5.8S rRNA 'fingerprints' (Plate 5.2), but T19.2, the lower spot on the 'fingerprint', appeared to have diminished more. Since T19.2 and T20 have average reactivities of 83%, it is not unreasonable to assume that T19.2 has been reduced by over 90%. U18 can be directly shown to have a reactivity of approximately 70% (P21, Table 5.2). Therefore U22 may have a reactivity of the same order as this.

U65, U66, U69 These residues are all located within the T_1 ribonuclease digest

product, T24, A-A-U-U-A-A-U-Gp, which is reduced by 56% after carbodiimide treatment. Of these three uridines, U65 has been directly shown to be 40% reactive (P22, Table 5.2). Obtaining estimates for the reactivities of the other two residues is more complex.

Pancreatic ribonuclease product P11, A-A-Up, which includes U69, is reduced by 38% after reaction. U66 immediately precedes A-A-Up in the sequence and therefore the reduction in the molar yield of P11 after carbodiimide treatment could be caused by the reaction of U66 and/or U69 with carbodiimide. If U66 were to react with carbodiimide, it would result in the resistance of the adjacent phosphodiester bond (U66p A67) to pancreatic ribonuclease cleavage, therefore reducing the yield of A-A-Up. This means that U66 and U69 share, either equally or unequally, a total reactivity of 38%.

The combined reactivity of the pancreatic ribonuclease products within T24 (40% x 38%) result in a total reactivity of 63% for this oligonucleotide. This is comparable with the direct result (56%, Table 5.1).

U87, U88 The T_1 ribonuclease digestion product, T21, A-C-A-C-A-U-U-Gp, which encompasses these two nucleotides is diminished by 38% after carbodiimide treatment. It is not possible to allocate reactivities to each of these uridine residues, but is likely that each has a reactivity in the range 20 - 25% (see below).

U91, U94 These residues are located within the T_1 ribonuclease digestion product T19.1, A-U-C-A-U-C-Gp, which is reduced by 54% as a result of carbodiimide modification (Table 5.1). U94 can also be found in the pancreatic

ribonuclease digest product A-Up (P10), which occurs twice in the sequence of HeLa cell 5.8S rRNA. U87, the other uridine residue located in A-Up, was suggested to be about 20 - 25% reactive. This would account for most of the reactivity of A-Up, meaning that U94 was largely non-reactive. This being so, the reduction in the molar yield of T19.1 after reaction with carbodiimide must be due almost entirely to the reactivity of U91.

U112, U113 These nucleotides are contained within the T_1 ribonuclease product T18, C-A-C-U-U-Gp, which is reduced by 24% as a result of 16 h treatment with carbodiimide at 25°C (Table 5.1). U113 can be shown to be unreactive as follows. In the nucleotide sequence, U113 is followed by G-Cp (product P6 in a pancreatic ribonuclease 'fingerprint') which is not reduced after carbodiimide reaction. Had U113 reacted, the yield of G-Cp would have dropped due to the resistance of the adjacent phosphodiester bond to pancreatic ribonuclease hydrolysis. Therefore most of the reactivity of T18 must be due to U112.

U146, U148 The T_1 ribonuclease digest product T17, U-C-U-Gp, contains these two nucleotides. The high reactivity of this fragment (78%, Table 5.1) is due largely to U146. The pancreatic ribonuclease product, P13, G-A-G-Cp, which immediately follows U148 in the sequence, is not significantly reduced by carbodiimide treatment, meaning that U148 is unreactive (Footnote (c), Table 5.2).

Oligonucleotides with multiple uridine residues

For T_1 ribonuclease products containing multiple uridine residues, it is not always possible to estimate the reactivities of individual uridines, although the

reactivity of the oligonucleotide is known. The difficulties in allocating reactivities to individual uridines within T19.1, T19.2, T21, T23 and T24 have already been discussed above. They will be referred to again during the discussion of the possible secondary structure.

The T_1 ribonuclease digestion product T20, contains both U101 and U102 in the sequence A-C-A-C-U-U-C-Gp and migrates close to product T19.2 on 'fingerprints'. It was estimated that the average reactivity of T20 and T19.2 was 83% (Table 5.1). Oligonucleotide T19.2 appeared to have diminished more than T20 after carbodiimide treatment (Plate 5.2) and it was therefore allocated a reactivity of approximately 90%. This would mean that the reactivity of T20 was in the range 70 - 75%. There is no way of knowing, in the present circumstances, whether one uridine might account for all the reactivity, or whether the reactivity could be distributed equally or unequally between the two residues.

The problem is somewhat similar with product T22, U-U-C-C-U-C-C-Gp, which is over 95% reactive. The first nucleotide in the sequence U125, can be shown from the pancreatic ribonuclease data to possess about 85% reactivity (product P24, Table 5.2). Reactivities cannot be allocated directly to the other two uridines in this oligonucleotide (U126 and U129), although it is possible that their reactivities may be in the same range as U125.

3' terminus

The T_1 ribonuclease product T2a, C-U-U , which is found at the 3' end of HeLa cell 5.8S rRNA is reduced by 76% as a result of carbodiimide modification. It is not possible to allocate reactivities to either U158 or U159.

(d) Reactivity of guanine residues

The carbodiimide reaction was carried out under conditions in which the reactivity of guanine residues was slight, however there is evidence that two guanine residues may be reactive.

G80, G81 The pancreatic ribonuclease digest product P14, A-G-G-A-Cp, which encompasses G80 and G81 is reduced by 38% after the reaction with carbodiimide (Table 5.2). Two pieces of evidence suggest that these two residues occur in an exposed region of the molecule; limited ribonuclease digestion (Nazar et al., 1975) and sodium bisulphite modification (Chapter 4). It is therefore not improbable that both of these residues will have the same reactivity, i.e. about 20%.

(e) Relation of findings to the conformation of 5.8S rRNA

The results described provide either direct or indirect estimates of the reactivities of almost all the thirty three uridines and two pseudouridines in the nucleotide sequence of HeLa cell 5.8S rRNA. A wide range of reactivities (0 - 90%) was encountered, indicating that several uridines occupy exposed positions within 'unbound' 5.8S rRNA, whereas others are protected.

It will simplify the discussion to compare the data directly with the general features of the secondary structure model proposed for mammalian 5.8S rRNA proposed by Nazar et al. (1975), and supported by the sodium bisulphite data. This model (Fig. 5.1) presents several characteristics with which the carbodiimide data can be compared.

Loops Each loop region contains one or more reactive uridines except for loop II which contains no uridine residues. Loop I contains two highly reactive

uridines encompassed by product T19.2 (Fig. 5.1). Of these, U18 can be shown from the pancreatic ribonuclease digest data to be 73% reactive (Table 5.2) and more indirectly U22 can be shown to be about 70% reactive. Loop III contains one uridine (U53), which is very reactive (product T15, Table 5.1). Loop IV contains two reactive uridine residues, U75 and U76. These can be shown independently to have reactivities of 62% and 84% respectively (Table 5.2 and text). The two uridines from loop IV, U101 and U102, can be located within product T20, which is reduced by 70 - 75% after carbodiimide treatment (Table 5.1). It has not been possible to allocate reactivities to these uridines individually. Finally, loop VI contains three uridine residues which appear to be very reactive. U125 can be shown to be 85% reactive from the diminution of pancreatic ribonuclease product P24, G-G-G-Up (Table 5.2). Residue U125 occurs in the T_1 ribonuclease oligonucleotide T22, U-U-C-C-U-C-C-C-Gp, which also contains U126 and U129. The molar yield of this oligonucleotide is reduced by over 95% after carbodiimide treatment (Table 5.1). It is possible, regarding the proposed conformation of this loop (Fig. 5.1) that all three uridines have high reactivities, in the region of 80 - 90%, although it is not possible to distinguish between the reactivities of U126 and U129 or to accurately assess the extents of their reaction. It may be possible that U126 or U129 is involved in tertiary interactions and that the diminution of T22 can be accounted for by two highly reactive uridine residues.

Helical regions All uridine residues which are internally located within proposed helical regions and for which estimates of reactivity have been derived, are completely unreactive or significantly less reactive than those uridines in

loop regions. All internally located uridines in helix (a) for which data is available (U8, $U_{(m)}14$, U148 and U154) are completely unreactive. In helix (b) U30 and U34 are completely unreactive, although U38 exhibits moderate reactivity (25%, product T13, Table 5.1). However, Nazar et al. (1975) discovered that this residue is adjacent to a phosphodiester bond which is readily cleaved during partial enzyme hydrolysis. At this point the helical structure must be less stable. In helix (c), the only carbodiimide data available relates to Ψ57 which is completely non-reactive.

Helix (d) presents more difficult analytical problems, since several of the uridines within this helix are partially reactive. This helix is AU rich and contains only two GC pairs. Of the nucleotides with which carbodiimide specifically reacts, only Ψ71 is completely non-reactive. It is interesting to note that the A84.Ψ71 pair is located in the helix between two GC pairs. All AU base pairs similarly located in other helices within 5.8S rRNA are completely non-reactive. Only where this conformation does not arise are some uridines within helices subject to carbodiimide reaction.

Within helix (d), U66, U69, U87 and U88 are all approximately 20% reactive (see text) with U65 being about 40% reactive. It is not surprising to note that U92, which is proposed to form an A-U 'closing pair' at the end of helix (d), is the most reactive uridine within this helix (approximately 50%). A similar situation was found in studies of the reactivity of cytidine residues involved in forming G-C 'closing' pairs. The partial reactivity of the uridine residues within this helix reflects a looser conformation, which is probably attributable to the lack of the more stable G-C pairs within the helix.

Alternatively the labile nature of this helix could result from the high pH (8.9) at which the carbodiimide reaction was carried out.

At this point it is relevant to consider the existence of three modified uridine bases close to the end of helices. These are 2-O-methyl U14, Ψ57 and Ψ71. Each of these occur one base pair from the end of their respective helices (Fig. 5.1). In each case the 'closing' pair of the helix is G-C. Whether this is coincidence or whether these modified bases play a specific role in the structural conformation of 5.8S rRNA remains open to speculation.

Helix (e) contains no internal uridine residues, although the structural model proposed by Nazar et al. (1975) envisages a G-U base pair (involving G116. U138) at the end of this helix. The reactivity of U138 (70%, product T16, Table 5.1) demonstrates that this 'closing' pair is unlikely to exist. Therefore the 'closing' pair at this end of helix (e) must involve C137. G117.

Helix imperfections In addition to the major loops, the model in Fig. 5.1 contains some helix imperfections. Helix (b) contains a symmetrical bulge near the left hand end which contains three uridine residues. Surprisingly, all of these uridines, U36, U112 and U113, are only slightly reactive (20% or less). Either these nucleotides are sterically protected from carbodiimide reaction or they are involved in interactions which maintain the tertiary structure.

In helix (a), there are three uridines involved in helix imperfections. Of these U144 and U146 can be shown to have reactivities of around 70% (see text). Less directly, U5 can be shown to have a reactivity of up to 40%. All these results are compatible with the proposed conformation.

In summary, the observed or inferred reactivities of the uridines for which estimates were obtained generally fit well with the features of the secondary structure model of Nazar et al. (1975), with two qualifications:

(1) U138. G116 do not form the 'closing' pair at the end of helix (e). The closing pair therefore involves G117.C137.

(2) Helix (d) contains several A-U base pairs which exist in a more labile conformation than those in helices with a higher GC content.

5.2 Correlation of data from carbodiimide and sodium bisulphite modification of 5.8S rRNA at 25°C

By combining the data from the chemical modification of 5.8S rRNA at 25°C it is possible to characterise the conformation of most of the pyrimidine residues within the molecule (Fig. 5.1). The observations may be summarised as follows, with respect to the secondary structure model of Nazar et al. (1975):

(1) Nucleotides which exist in a single-stranded conformation within loop regions generally exhibit high reactivity towards chemical modifying reagents. Where a nucleotide which has been assigned to such a region exhibits moderate or low reactivity, it is likely to be involved in tertiary interactions.

(2) Uridines and cytidines which exist within helical regions generally exhibit little or no reactivity towards their respective modifying reagents.

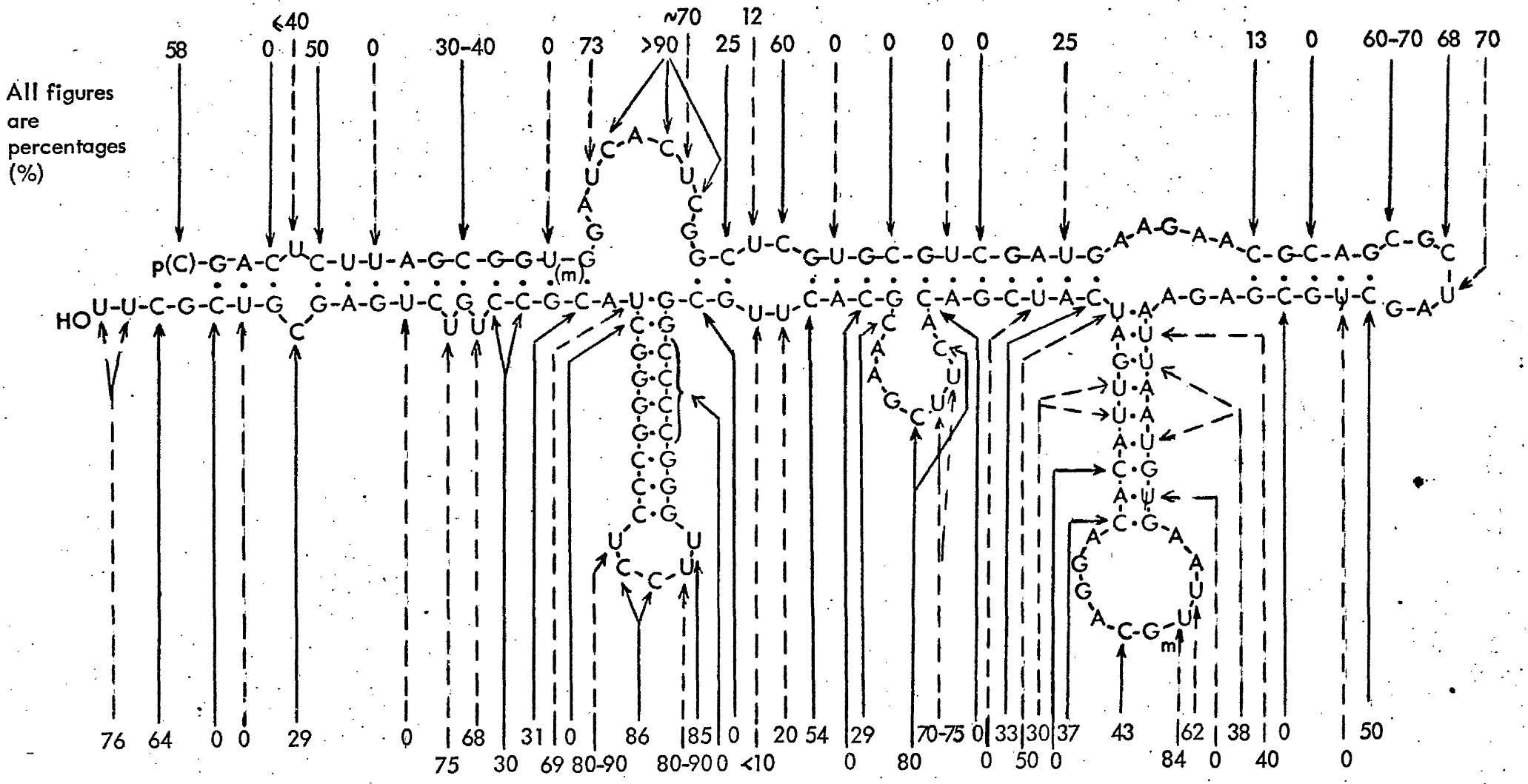
(3) Pyrimidines which form 'closing' pairs at the ends of helices are partially reactive.

(4) Pyrimidines involved in helical imperfections exhibit reactivity.

In Fig. 5.1, the reactivities of the pyrimidine bases towards their respective

Fig. 5.1 Secondary structure model of HeLa cell 5.8S rRNA based on that of Nazar et al. (1975) and correlated chemical modification data for 25°C.

Lines between nucleotides indicate phosphodiester bonds and dots represent base pairs as in Fig. 4.3. The helices and loops referred to in text are shown in Fig. 4.3. Unbroken lines indicate the percentage reactivity of cytidine residues towards sodium bisulphite after 24 h treatment at 25°C under the conditions described in Chapter 4. Broken lines indicate the percentage reactivity of uridine (and pseudouridine) residues towards carbodiimide after 16 h treatment at 25°C under the conditions described in Chapter 5.1. It should be noted that the percentage reactivities of cytidine residues are not directly comparable with those of uridine residues. Tied arrows, e.g. nucleotides 19, 21, 23, indicate that the cytidine or uridine residues were released within a single oligonucleotide in which the reactivities of the individual cytidine or uridine residues could not be distinguished from each other.


All figures are percentages (%)
58 ≤40 0 50 0 30-40 0 73 ≈70 >90 12 25 60 0 0 0 0 25 13 0 60-70 68 70
p(C)-G-A-C-U-C-U-U-A-G-C-G-G-U-G
(m)
HO U-U-C-G-C-U-G G-A-G-U-C G C-C-G-C-A-U-G-C-G U-U C-A-C-G C-A-G-C-U-A-C A-G-A-G-A-G-C-G-U-C G-A-U
76 64 0 0 29 0 68 31 0 86 85 0 20 54 29 70-75 0 33 30 37 43 62 38 0 50
75 30 69 80-90 80-90 0 <10 0 80 0 50 0 84 0 40 0

modifying reagents is indicated. It can be clearly seen that both sets of data are complementary. The relationship of this data to the conformation at different temperatures and during interaction with 28S rRNA will be discussed more fully in Chapter 7.

The conformation of 5.8S rRNA at 37°C and 50°C.

6.1 Reaction of sodium bisulphite and carbodiimide with 5.8S rRNA at 37°C.

Choice of conditions

The temperature at which HeLa cell 5.8S rRNA functions *in vivo* is 37°C. It is important to investigate the conformation at this temperature and to compare it with that at 25°C. So that results may be more easily compared, it was useful to carry out the reaction under conditions in which cytidines or uridines from exposed loop regions of the molecule react to the same extent at 25°C and 37°C. This would allow residues which became more exposed as a result of the higher temperature to be identified by their increased reactivity relative to residues which maintained an exposed conformation at both temperatures.

Fig. 4.1 shows the time-course of the reaction of the total cytidine compliment of 5.8S rRNA with sodium bisulphite at 37°C. Under these conditions not all of the 46 cytidines are available for reaction. At least 25% of the total cytidines residues (i.e. about 12 out of 46) in 5.8S rRNA are completely unreactive at this temperature. Assuming this, the kinetics are approximately pseudo-first order, with 50% of the available cytidine residues having reacted after 9 h (Fig. 4.2). This is similar to the stage reached after 24 h reaction at 25°C. On this basis, a 9 h reaction period was chosen for investigating the reaction of sodium bisulphite with cytidine residues within 5.8S rRNA.

A similar strategy was adopted for conformational studies using carbodiimide at 37°C. Rhodes (1977) has shown that the rate of reaction of carbodiimide with tRNA doubles every 10°C. When this information was considered together with experience gained from the bisulphite reaction, it was decided that a 6 h reaction

period at 37°C should result in a similar extent of modification as a 16 h reaction period at 25°C.

Apart from the changes described above, the reaction conditions for sodium bisulphite and carbodiimide modification were identical to those used at 25°C.

(a) Reaction of sodium bisulphite with 5.8S rRNA at 37°C

Reactivity of individual cytidine residues

Plate 6.1 shows 'fingerprints' of T_1 ribonuclease digests of 5.8S rRNA which have been treated with bisulphite for 9 h at 37°C. Electrophoresis was extended in both dimensions, to give better separation. Plate 6.2 shows 'fingerprints' of pancreatic ribonuclease digests which have been similarly treated. As with 'fingerprints' of 5.8S rRNA after treatment with bisulphite at 25°C, there is a reduction in the molar yields of several cytidine-containing oligonucleotides compared with the control values. Tables 6.1 and 6.3 summarise the molar yields of T_1 ribonuclease and pancreatic ribonuclease digest products obtained after reaction with sodium bisulphite for 9 h at 37°C. It was also possible to characterise several uridine conversion products (Tables 6.2 and 6.4). The molar yields were calculated as previously described.

The molar yields and extents of reaction described in Tables 6.1 and 6.3 permit comparison to be made of 5.8S rRNA conformation at 37°C with that at 25°C. As in Chapter 4, it is convenient to distinguish between 'direct evidence', where in most cases the data were obtained from unique oligonucleotides within the 5.8S rRNA sequence and which contain a single cytidine residue, and 'indirect evidence' where the estimates were derived more circuitously.

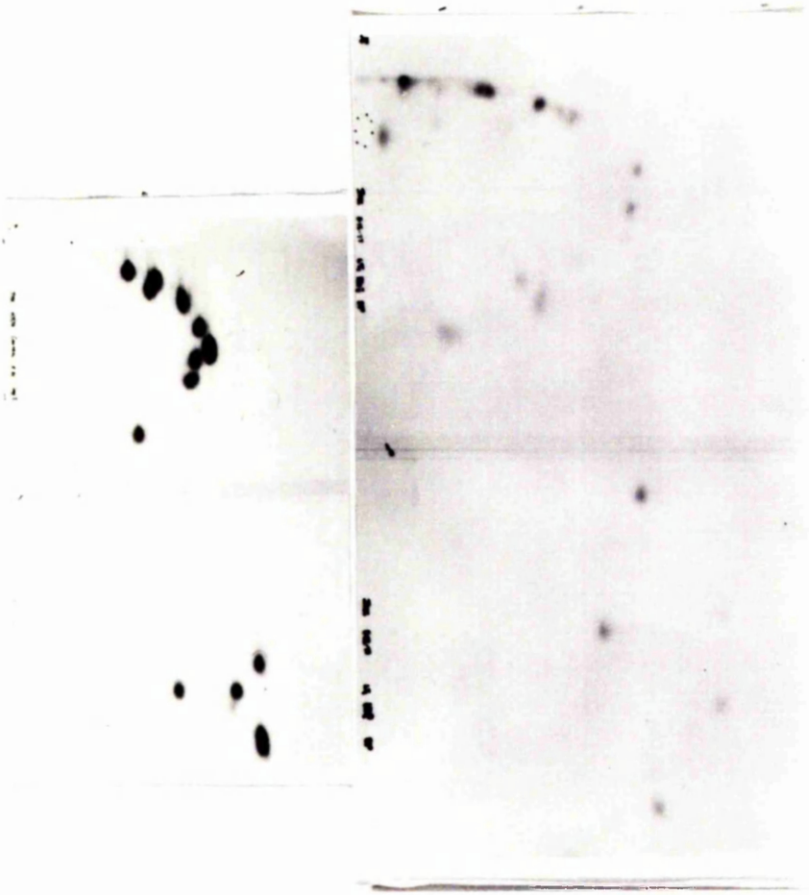
'Fingerprints' of HeLa cell 5.8S rRNA before (left) and after (centre) reaction with 3 M sodium bisulohite, pH 6.0, for 9 h at 37°C.

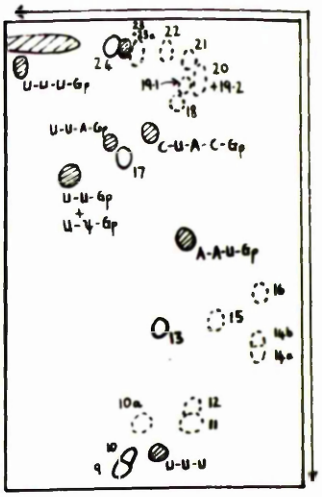
Plate 6.1 T_1 ribonuclease digest, extended fractionation

The key (right) identifies new sequences (shaded circles) which arise as a result of the reaction of sodium bisulphite; diminished cytidine-containing sequences (broken circles) and unreactive sequences (closed circles) are identified by the standard numbering scheme (see text and Table 6.1). Electrophoresis was carried out for 4 h at 4.7 KV in the first dimension on cellulose acetate, pH 3.5, and for 40 - 60 h at 1.2 KV in the second dimension on DEAE paper, 7% formic acid.

Plate 6.2 Pancreatic ribonuclease digest

The key (right) identifies new, diminished and unreacted sequences as in Plate 6.1, with the numbering of spots as in Table 6.3. Electrophoresis was carried out for 2.5 h at 4.7 KV in the first dimension and for 16 h at 1.1 KV in the second dimension.

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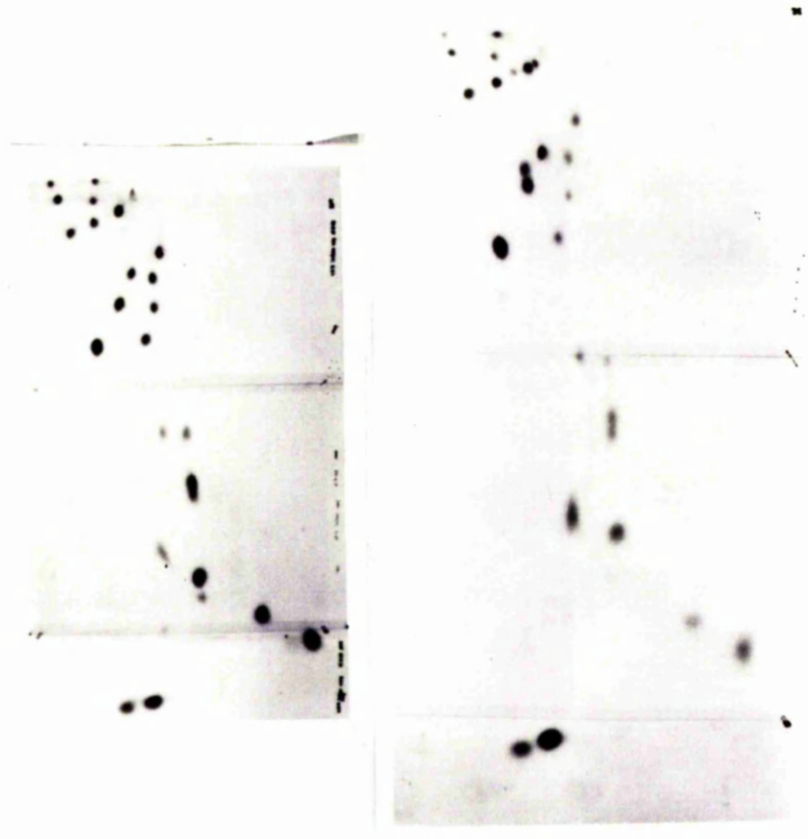
U-U-Gp
A-A-U-Gp
U-U-U



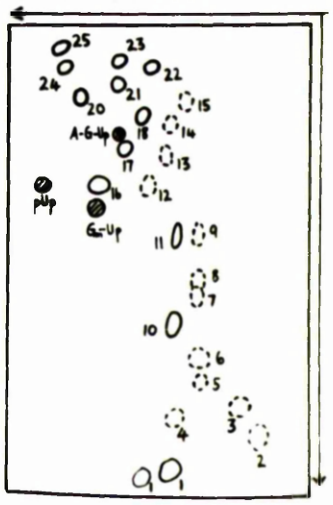

A-G-Up
pUp
G-Up

Table 6.1 Sequences and molar yields of T_1 ribonuclease products from HeLa cell 5.8S rRNA after treatment with sodium bisulphite at 37°C and extent of reaction of cytidine-containing products as compared to that at 25°C.

The conventions are as in Table 3.1. The positions of the cytidine residues within the nucleotide sequence are indicated. The reaction was carried out for 9 h at 37°C in the presence of 3 M sodium bisulphite, 10 mM $MgCl_2$ at pH 6.0. The molar yields were calculated as in Table 4.1.

Footnote

(a) These two products were only partly resolved. Product T23a can be independently inferrred to be about 50% reactive from the reactivity of the corresponding pancreatic ribonuclease product P5. Products T23 and T23a are diminished by almost 60% on T_1 ribonuclease 'fingerprints'. It can therefore be concluded that T23 is approximately 70% reactive.

Table 6.1

Spot No.	Sequence	Cytidine residue (s)	Molar yield	% Reactivity	% reactivity 25°C for 24 h
T1	Gp		12.36	-	-
T2	C-Gp	11,32,50,59, 115,152	3.04	51%	30%
T2a	C-U-U	157	0.22	76%	65%
T3	A-Gp	-	2.08	-	-
T4	C-A-Gp	47	0.91	12%	9%
T5	A-A-Gp	-	0.97	-	-
T6	C-C-C-C-Gp	118-121	0.45	zero	zero
T7	A-A-C-Gp	45,107	0.89	38%	37%
T8	U_m-Gp	-	0.17	-	-
T9	U-Gp	-	4.49	-	-
T10	ψ-Gp	-			
T10a	pC-Gp	1	0.10	76%	62%
	pGp	-	0.13	-	-
T11	U-C-Gp	35,155	0.98	49%	15%
T12	C-ψ-Gp	56	0.45	56%	48%
T13	A-U-Gp	-	1.10	-	-
T14a	C-U-C-Gp	26,28	0.40	81%	54%
T14b	C-C-U-Gp	142,143			
T15	C-U-A-Gp	52	0.23	78%	68%
T16	C-U-A-C-Gp	137,140	0.22	67%	34%
T17	U-C-U-Gp	147	0.97	12%	10%
T18	C-A-C-U-U-Gp	109,111	0.19	79%	54%
T19.1	A-U-C-A-U-C-Gp	92,95	0.43	51%	33%
T19.2	A-U-C-A-C-U-C-Gp	19,21,23	0.11	94%	85%
T20	A-C-A-C-U-U-C-Gp	98,100,103			
T21	A-C-A-C-A-U-U-Gp	83,85	0.37	57%	37%
T22	U-U-C-C-U-C-C-C-Gp	127,128, 130-132	0.09	89%	86%
T23	A-C-U-C-U-U-A-Gp	4,6	0.76	59%	49% (a)
T23a	A-A-U-U-G_m-C-A-Gp	78			
T24	A-A-U-U-A-A-U-Gp	-	0.93	-	-

Table 6.2 Sequences and molar yields of uridine-containing products of sodium bisulphite reaction at 37°C and extent of reactivity of the cytidine-containing derivative; T_1 ribonuclease products.

The conventions are as in Table 3.1 These products of bisulphite reaction migrate to distinctive positions in the 'fingerprint' and can be readily quantified. The '(%) reactivity' signifies the amount of newly appeared uridine-containing product relative to the amount of the cytidine-containing derivative in a 'control fingerprint'.

Spot No.	Sequence	Molar yield	(%) reactivity	derived from
T10c	U-A-Gp	0.03	3%	T4
T10b	U-U-U	0.64	70%	T2a
T36	A-A-U-Gp	1.02	71%	T7
T41	U-U-Gp+U-ψ-Gp	1.15	38%	T9, T10
T40	U-U-A-Gp	0.64	60%	T15
T42	C-U-A-U-Gp	0.37	55%	T16
T39	U-U-U-Gp	1.42	-	T14, T17

Table 6.3 Sequences and molar yields of pancreatic ribonuclease products from HeLa cell 5.8S rRNA after treatment with sodium bisulphite at 37°C and the extent of reaction of cytidine-containing products as compared to that at 25°C.

The conventions are as in Table 3.1. The positions of the cytidine residues within the nucleotide sequence are indicated. The reaction was carried out for 9 h at 37°C in the presence of 3 M sodium bisulphite, 10 mM $MgCl_2$ at pH 6.0. The molar yields were calculated as described previously (Table 4.3).

Table 6.3

Spot No.	Sequence	Cytidine residue(s)	Molar yieid	(%) reactivity	(%) reactivity 25°C for 24 h
P1	Up + ψp	-	29.19	-	-
P2	Cp	-	12.96	-	-
P3	A-Cp	21,85,100, 111,140	2.46	56%	18%
P4	pCp	1	0.08	83%	54%
P5	G_m-Cp	78	0.39	58%	42%
P6	G-Cp	32,47,52,59,109, 115,142,157	4.25	40%	11%
P7	G-A-Cp	4,98	1.23	35%	zero
P8	A-G-Cp	11,50,56	0.97	66%	63%
P9	G-A-A-Cp	107	0.41	58%	29%
P10	A-Up	-	5.60	-	-
P11	A-A-Up	-	1.16	-	-
P12	G-G-Cp	26,118	1.35	29%	12%
P13	G-A-G-Cp	152	0.54	44%	29%
P14	A-G-G-A-Cp	83	0.47	52%	37%
P15	G-A-A-G-A-A-Cp	45	0.47	39%	13%
P16	G-Up + G-ψp	-	8.55	-	-
P17	G-A-Up	-	2.74	-	-
P18	G-A-A-Up	-	1.68	-	-
P20	G-G-Up	-	1.61	-	-
P21	G-G-A-Up	-	1.28	-	-
P22	G-A-G-A-A-Up	-	0.93	-	-
P23	G-G-G-G-Cp	137	0.45	zero	zero
P24	G-G-G-Up	-	0.68	-	-
P25	G-G-U_m-G-G-A-Up	-	0.19	-	-

Table 6.4 Sequences and molar yields of uridine-containing products of sodium bisulphite reaction at 37°C and extent of reactivity of the cytidine-containing derivative; pancreatic ribonuclease products.

The conventions are as in Table 3.1. These products of bisulphite reaction migrate to distinctive positions in the 'fingerprint' and can be readily quantified. The ' (%) reactivity' signifies the amount of newly appeared uridine-containing product relative to the amount of the cytidine-containing derivative in the 'control fingerprint'.

Spot No.	Sequence	Molar yield	% reactivity	derived from
P16a	G_m-Up	0.38	41%	P5
P16b	pUp	0.62	100%	P4
P17a	A-G-Up	1.57	55%	P8

Table 6.5 Correlated data on the reactivities at 37°C of individual cytidine residues obtained from 'direct evidence'.

In the examples shown, the uridine-containing products of the bisulphite reaction (parentheses) migrate to distinctive positions and can be readily quantified. The ' (%) reaction' signifies the loss of the cytidine-containing product, or the amount of newly appeared uridine-containing product relative to the amount of the corresponding cytidine-containing product in 'control fingerprints'. For comparison purposes, the (%) reactivity at 25°C is also included.

Table 6.5

Cytidine Residue	Product	(%) Reaction	Mean	(%) Reaction at 25°C for 24h
1	T10a pC-Gp P4 pCp	76% 83%	80%	58%
45	P15 G-A-A-G-A-A-Cp	39%	39%	13%
47	T4 C-A-Gp (U-A-Gp)	12% 3%	8%	zero
52	T15 C-U-A-Gp (U-U-A-Gp)	88% 60%	74%	68%
56	T12 C-ψ-Gp	56%	56%	50%
78	P5 G_m-Gp (G_m-Up)	58% 41%	50%	39%
83	P14 A-G-G-A-Cp	52%	52%	37%
107	P9 G-A-A-Cp P18 (G-A-A-Up)	58% 57%	58%	25%
118-121	T6 C-C-C-C-Gp	zero	zero	zero
137	P23 G-G-G-G-Cp	zero	zero	zero
152	P13 G-A-G-Cp	44%	44%	29%
157	T2a C-U-U T10b U-U-U	76% 70%	73%	64%

Direct evidence

The reactivities have been summarised in Table 6.5.

Indirect evidence

C4, C98 These cytidine residues can be located within the pancreatic ribonuclease product, G-A-Cp (P7), which occurs twice per 5.8S rRNA molecule. There is approximately 35% diminution of G-A-Cp after bisulphite treatment and 27% augmentation of the potential reaction product G-A-Up (Table 6.3). It follows that C4 and C98 possess an average reactivity of approximately 30%.

C6 The T_1 ribonuclease digest product, T23, which contains C4 and C6 was diminished by about 70% after the reaction of 5.8S rRNA with sodium bisulphite (see footnote a, Table 6.1). C4 can be shown to be at least 30% reactive, meaning that the reactivity of C6 is probably in the range of 50 - 60%.

C11, C50 and C56 The pancreatic ribonuclease product, A-G-Cp (P8) which contains these cytidine residues, occurs three times per 5.8S rRNA molecule and is reduced by approximately 65% after reaction with bisulphite. A new product, A-G-Up (P17a, Table 6.4) appeared in an amount equivalent to 55% of the original A-G-Cp. Therefore these three cytidines have an average of 60% reactivity. C56 was shown to be 56% reactive, as demonstrated under 'direct evidence' (Table 6.5). Therefore, taken together, C11 and C52 are on average still about 60% reactive. If, as was suggested from the conformation at 25°C, C52 has a comparable reactivity to C54 within loop III (Table 6.1), i.e. around 75%, then C11 must have a reactivity of approximately 40 - 45%.

C26, C118 The pancreatic ribonuclease product, G-G-Cp (P12) which occurs twice in the sequence, encompasses residues C26 and C118. There is a 30% diminution of G-G-Cp after reaction with sodium bisulphite and G-G-Up is correspondingly augmented (leading to a net reactivity of 32%). Since C118 is completely unreactive, as shown in Table 6.1 (product T6, C-C-C-C-Gp), then C26 has a reactivity in the 60 - 65% range.

C26, C28; C142 and C143 These cytidine residues occur in the isomeric T_1 ribonuclease digestion products C-U-C-Gp and C-C-U-Gp (T14a and T14b). After sodium bisulphite treatment about 75% of the total material had disappeared (Table 6.1), this being mostly from C-U-C-Gp, the lower component on the fingerprint (Plate 6.1). Of the cytidines encompassed by this product, C26 has been shown to have a reactivity of 60 - 65% (discussed above) with C28 probably having a reactivity of at least 60% (as derived from data on conformation at 25°C in Chapter 4). This would lead to a reduction of approximately 90% in the molar yield of T14a after bisulphite treatment. Consequently, the two cytidines located in product T14b, C142 and C143, will have a combined reactivity of approximately 60%.

C35, C155 The T_1 ribonuclease product U-C-Gp (T11) is diminished by 50% after reaction with sodium bisulphite. The molar yield of the uridine-containing product(T41) indicates that the reactivity is about 35%. It can be concluded from this that C35 and C155 have an average reactivity of 40 - 45%.

C85 This residue is recovered together with C83 in product T21, which shows 57% reactivity. Since C83 has a reactivity of 52% (product P14), C85

must exhibit little or no reactivity.

C140 Here a converse argument applies. C140 is recovered with C137 in product T16. Because C137 is unreactive (product P23), the reactivity of product T16 must be due entirely to C140. Moreover, a reaction product C-U-A-U-Gp was recovered in a 55% yield (Table 6.2). It can be concluded from this that C140 is approximately 60% reactive.

Oligonucleotides with multiple cytidine residues

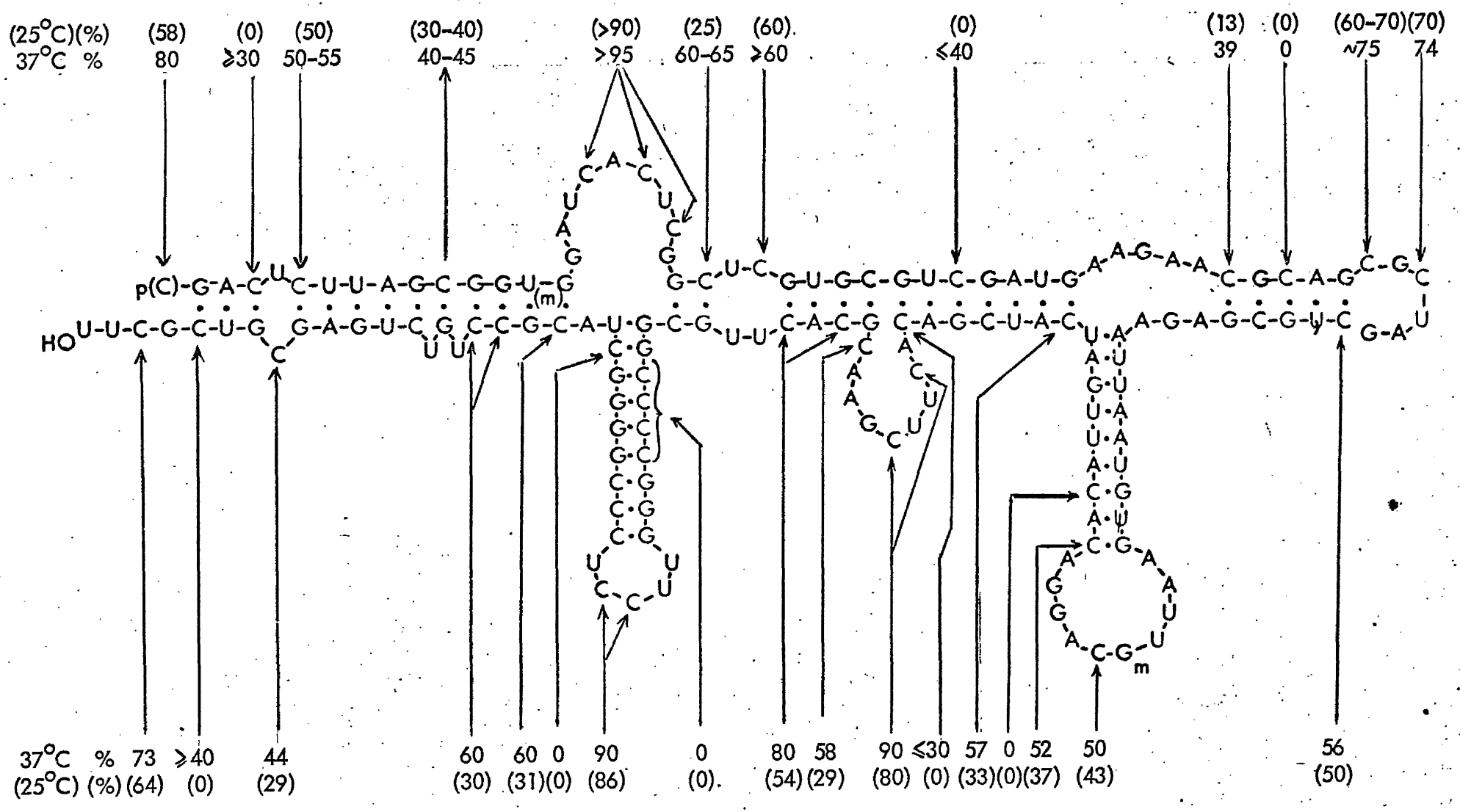
It is possible to obtain estimates of the reactivities for five T_1 ribonuclease products with multiple cytidine residues, but not to allocate reactivities to individual cytidines within these oligonucleotides. Four of the oligonucleotides T19.2, T20, T22 and T18 are highly reactive, whereas the other, T21, shows more moderate reactivity. With regard to product T20, one of the cytidines, C98, exhibits less than 30% reactivity, as inferred from the pancreatic ribonuclease product P7. Therefore most of the high reactivity of product T20 must be due to C100 and/or C103.

Relation of findings to conformation of 5.8S rRNA at 37°C

The results described provide either direct or inferred estimates of the reactivities of many of the cytidine residues within HeLa cell 5.8S rRNA. As was the case at 25°C, a wide range of reactivities was encountered, indicating the existence of different conformations of cytidine residues within 'unbound' 5.8S rRNA. It will simplify the discussion if we compare these findings directly with the secondary structure proposed to exist at 25°C (Fig. 4.3). This model presents several characteristic features with which the data can be compared.

Fig. 6.1 Reactivity at 37°C of the cytidine residues within the proposed secondary structure of HeLa cell 5.8S rRNA

Lines between nucleotides indicate phosphodiester bonds and dots represent base pairs as in Fig. 4.3. The arrows indicate the percentage reactivity of the cytidine residues towards sodium bisulphite after 9 h treatment at 37°C. The extent of reaction after 24 h treatment at 25°C is given in brackets. Tied arrows indicate combined reactivities of two or more cytidines as explained for Fig. 5.1.


(25°C)(%) (58) (0) (50) (30–40) (>90) (25) (60) (0) (13) (0) (60–70)(70)
37°C % 80 ≥30 50–55 40–45 >95 60–65 ≥60 ≤40 39 0 ~75 74
p(C)-G-A-C-U-C-U-U-A-G-C-G-G-U-G
HO-U-U-C-G-C-U-G G-A-G-U-C G C-C-G-C-A-U-G-C-G-U-U-C-A-C-G C-A-G-C-U-A-C-U-A-A-G-A-G-A-G-C-G-Ψ-C G-A-U
(m)
37°C % 73 ≥40 44 60 60 0 90 0 80 58 90 ≤30 57 0 52 50 56
(25°C) (%) (64) (0) (29) (30) (31) (0) (86) (0) (54) (29) (80) (0) (33) (0) (37) (43) (50)

Loops The extents of reaction of the oligonucleotides within the loop regions after 9 h bisulphite treatment at 37°C were similar to those found at 25°C when 5.8S rRNA was treated with bisulphite for 24 h. Loops I, III, IV, V and VI all contain highly reactive cytidine residues which are located within the T_1 ribonuclease digest products T19.2, T15, T23a, T20 and T22 respectively. This suggests that these cytidine residues exist in the same type of environment at 25°C and 37°C.

Helical regions In three of the helices internally located cytidines are unreactive. Helix (c) contains the unreactive C47, helix (d) has the unreactive C85 internally located and helix (e) contains the completely unreactive cytidines 118-121 and 137. Helices (a) and (b), however, present the more difficult analytical problems.

In helix (b), the only internally located cytidine residues for which data are available are C35 and C98. C35, together with C155 (in product T11, U-C-Gp) have average reactivities of 40 - 45%. In the proposed secondary structure model (Fig. 6.1), C155 is depicted as forming a GC 'closing' pair with G2 at the start of helix (a). At 37°C, under the conditions used, cytidine residues involved in such 'closing' pairs usually exhibit about 60% reactivity. This suggests that C35 is approximately 20% reactive.

C98 and C4, which are found within product P7, G-A-Cp, have average reactivities of 30%. Since both are adjacent to imperfections in their respective helices, it is likely that they are reactive to similar extents (i.e. about 30%). The information obtained on the reactivities of C98 and C35 confirm

the maintenance of helix (b) at 37°C, but suggest that it is less stable than at 25°C.

The reactivities of cytidine residues within helix (a) demonstrate that these residues are more available for reaction with bisulphite at 37°C than at 25°C (C4, C6, C11, C140, C142, C144, C153, C155 in Tables 6.1 and 6.3). It could be suggested from these data that the helix has in fact completely dissociated. However those cytidines which were shown to be involved in maintaining this helical structure at 25°C, all show less reactivity at 37°C than those cytidines which occur in loop or single-stranded regions of the 5.8S rRNA molecule. So although helix (a) retains its general conformation at 37°C, it is much less stable than at 25°C.

End of helical regions Those cytidine residues which are involved in forming GC pairs at the end of helical regions are significantly more reactive at 37°C than at 25°C.

C1 has the potential to form a pair with G156 at the end of helix (a). The high reactivity (80%, Table 6.5) of the 5' terminal nucleotide toward bisulphite indicates that these bases are not involved in hydrogen bonding. This means that the 'closing' pair at the start of helix (a) must involve G2, C155. At 25°C, C155 exhibits little reactivity, but at 37°C this reactivity is more than 40%, typical of a cytidine residue involved in the formation of a 'closing' pair. All other cytidine residues thought to be involved in GC 'closing' pairs (C45, C83, C140, C156) are all significantly more reactive than at 25°C. It was suggested earlier that cytidine residues which form 'closing' pairs are frequently reactive towards bisulphite because GC pairs in these positions equilibrate to various

extents between 'open' and 'closed' states. It seems reasonable then that at higher temperatures, these pairs will exist more often in an 'open' state. This would account for the increase in reactivity of cytidine residues at the end of helical regions relative to those in loop regions at 37°C.

Helical imperfections

At 25°C, helix (b) contains a symmetrical bulge which has pairs G114.C26 and C115.G25 to its left hand side (Fig. 6.1). The existence at 37°C of these GC pairs must be subject to doubt when the high reactivity of C26 (product P12, Table 6.3) is considered. Presumably C115 is reactive to the same extent (60 - 65%), although this cannot be ascertained either directly or indirectly from the data. It is possible that at 37°C, loop I increases in size to include nucleotides 25 - 28.

As was stated above, the situation in helix (a) is more complex since there are two sites with helical imperfections. At 25°C, it was suggested that C11 forms a labile pair with G145 adjacent to the mismatched uridine residues U144 and U146. The data available on the reactivity of C11 (40 - 45%, see text) supports the continued existence of this labile pair at 37°C. The other imperfection within helix (a) consists of the mismatched pair U5.C152. The reactivity of C152 after bisulphite treatment (44%, Table 6.3) is consistent with the existence of a helix imperfection at this position.

3' terminus

C157 has a reactivity similar to that at 25°C (75%, Table 6.1) indicating that it maintains an unpaired conformation close to the 3' terminus of the 5.8S rRNA.

In summary, the sodium bisulphite modification data indicates that the conformation of 5.8S rRNA at 37°C is similar to that at 25°C, with two qualifications.

(1) At 37°C, helix (a) is somewhat unstable when compared with other helices in the structure. This may be of importance in the function of the molecule (see Chapters 7 and 8).

(2) The higher reactivities of cytidine residues involved in GC 'closing' pairs may mean that loop I is increased in size by four nucleotides.

(b) Reaction of carbodiimide with 5.8S rRNA at 37°C

Reactivity of individual uridine residues

Plate 6.3 shows 'fingerprints' of T_1 ribonuclease digests of HeLa cell 5.8S rRNA, untreated and after reaction with carbodiimide. Plate 6.4 shows 'fingerprints' of T_1 ribonuclease digests with long separations in both directions so that the products at the top of plate 6.3 could be better resolved. Plate 6.5 shows 'fingerprints' of pancreatic digests of 5.8S rRNA, untreated and after reaction with carbodiimide. There is a reduction in the molar yields of several uridine-containing oligonucleotides on these 'fingerprints' after reaction with carbodiimide. Tables 6.6 and 6.7 summarise the results of those molar yields obtained after carbodiimide treatment. The molar yields and extent of reaction of these oligonucleotides permits comparison of 5.8S rRNA conformation at 37°C to be made with that at 25°C.

The reactivity of several uridine residues could be determined directly because each occurs as the only uridine residue in an unique oligonucleotide.

'Fingerprints' of HeLa cell 5.8S rRNA before (left) and after (centre) reaction with carbodiimide (10 mg/ml), pH 8.9, for 6 h at 37°C

Plate 6.3 T_1 ribonuclease digest

The key (right) identifies diminished (broken circles) and unreactive (closed circles) sequences. These sequences are identified by the standard numbering system (Table 6.9). Electrophoretic separation was performed in the first dimension on cellulose acetate, pH 3.5, at 4.7 KV for 3 - 3.5 h and in the second dimension on DEAE paper, 7% formic acid, at 1.2 KV for 17 h.

Plate 6.4 T_1 ribonuclease digest, extended fractionation

The key (right) identifies diminished and unreacted sequences as in Plate 6.3. Electrophoresis was carried out at 4.7 KV for 4 h in the first dimension and at 1.2 KV for 40 - 60 h in the second dimension.

Plate 6.5 Pancreatic ribonuclease digest

The key (right) identifies diminished and unreacted sequences as in Plate 6.3 with the numbering of spots as in Table 6.10. Electrophoresis was at 4.7 KV for 2.5 h in the first dimension and at 1.1 KV for 16 h in the second dimension.

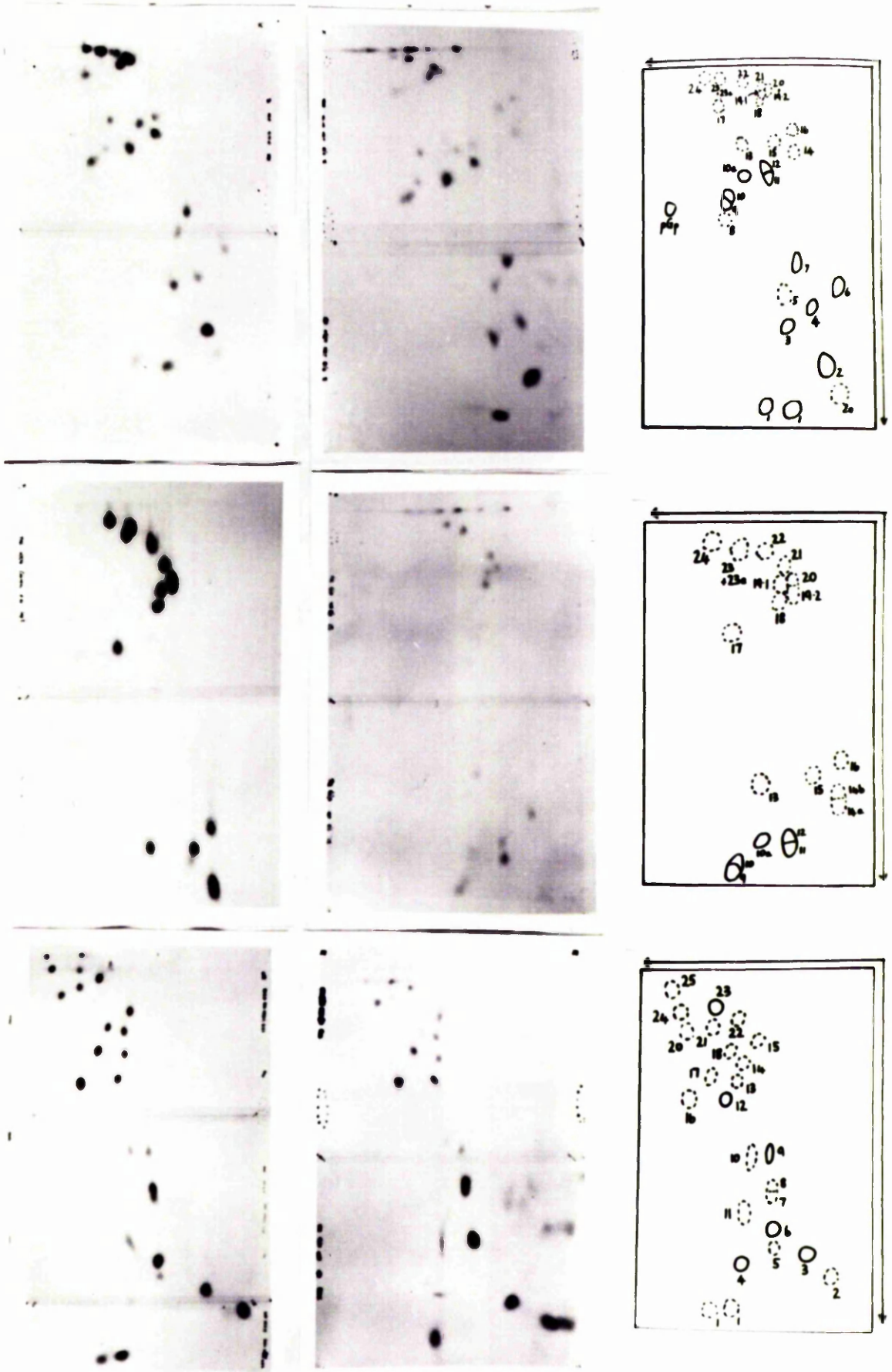


Table 6.6 Sequences and molar yields of T_1 ribonuclease digestion products from HeLa cell 5.8S rRNA and the extent of reaction of uridine-containing products after carbodiimide treatment at 37°C.

The conventions are as in Table 3.1. The positions of uridine residues within the nucleotide sequence are indicated. The carbodiimide reaction was carried out for 6 h at 37°C in 10 mg/ml carbodiimide, pH 8.9, in the presence of 10 mM $MgCl_2$. The molar yield was calculated assuming that product T4 C-A-Gp, was 1.00. The extent of reactivity at 25°C is included for comparison.

Footnote

(a) The lower yield of this product after carbodiimide treatment may be due to slight reactivity of G42, although the reduction is not highly significant.

Table 6.6

Spot No.	Sequence	Uridine residue(s)	Molar yield	(%) reactivity	(%) reactivity 16 h at 25°C	
T1	Gp	-	10.53	-	-	
T2	C-Gp	-	6.91	-	-	
T2a	C-U-U	158, 159	0.29	68%	76%	
T3	A-Gp	-	2.43	-	-	
T4	C-A-Gp	-	1.00	-	-	
T5	A-A-Gp	-	0.85	-	-	(a)
T6	C-C-C-C-Gp	-	0.35	-	-	
T7	A-A-C-Gp	-	1.47	-	-	
T8	U_m-Gp	14	0.15	29%	10%	
T9	U-Gp	14,30	1.91	4%	zero	
T10	Ψ-Gp	71	1.22	zero	zero	
T10a	pC-Gp	-	0.34	-	-	
	pGp	-	0.14	-	-	
T11	U-C-Gp	34, 154	2.10	zero	6%	
T12	C-Ψ-Gp	57	1.08	zero	zero	
T13	A-U-Gp	38	0.73	37%	25%	
T14a	C-U-C-Gp	27	0.83	20%	12%	
T14b	C-C-U-Gp	144	0.30	71%	68%	
T15	C-U-A-Gp	53	0.34	68%	70%	
T16	C-U-A-C-Gp	138	0.27	60%	69%	
T17	U-C-U-Gp	146, 148	0.20	82%	78%	
T18	C-A-C-U-U-Gp	112, 113	0.57	37%	24%	
T19.1	A-U-C-A-U-C-Gp	91,94	0.31	64%	54%	
T19.2	A-U-C-A-C-U-C-Gp	28,32	0.24	87%	83%	
T20	A-C-A-C-U-U-C-Gp	101, 102				
T21	A-C-A-C-A-U-U-Gp	87,88	0.34	61%	38%	
T22	U-U-C-C-U-C-C-C-Gp	125, 126, 129	0.04	95%	96%	
T23	A-C-U-C-U-U-A-Gp	5,7,8	0.44	76%	67%	
T23a	A-A-U-U-G_m-C-A-Gp	75,76				
T24	A-A-U-U-A-A-U-Gp	65,66,68	0.22	74%	56%	

Table 6.7 Sequences and molar yields of pancreatic ribonuclease digestion products from HeLa cell 5.8S rRNA and the extent of reaction of uridine-containing products after carbodiimide treatment at 37°C.

The conventions are as in Table 3.1. The reaction conditions are the same as for Table 6.6. The molar yields were calculated assuming that the yield of product P9, G-A-A-Cp, was 1.00. The extent of reactivity at 25°C is included for comparison.

Footnotes

(a) P5, G_m-Cp, is reduced by 83% after treatment. This reduction is related to the reactivity of U76 for the reasons described previously (footnote b, Table 5.2).

(b) P13, G-A-G-Cp, is diminished by 25% after the reaction. This is due to the reactivity of U146 and the resultant inhibition of pancreatic ribonuclease hydrolysis.

(c) The non-uridine containing product, P15, is reduced by 0.33 moles after carbodiimide treatment. This is presumably due to the reactivity of U38.

Table 6.7

Spot No.	Sequence	Uridine residue(s)	Molar yield	% reactivity	% reactivity 16 h at 25°C	
P1	Up+ψp	-	9.74	-	-	
P2	Cp	-	18.45	-	-	
P3	A-Cp	-	6.46	-	-	
P4	pCp	-	0.41	-	-	
P5	G_m-Cp	-	0.16	-	-	(a)
P6	G-Cp	-	7.45	-	-	
P7	G-A-Cp	-	1.75	-	-	
P8	A-G-Cp	-	2.06	-	-	
P9	G-A-A-Cp	-	1.00	-	-	
P10	A-Up	87,94	1.96	21%	13%	
P11	A-A-Up	69	0.46	57%	38%	
P12	G-G-Cp	-	1.81	-	-	
P13	G-A-G-Cp	-	0.73	-	-	(b)
P14	A-G-G-A-Cp	-	0.59	-	-	
P15	G-A-A-G-A-A-Cp	-	0.44	-	-	(c)
P16	G-Up+G-ψp	30,34,71, 146, 154	4.46	18%	13%	
P17	G-A-Up	38,91	1.66	26%	23%	
P18	G-A-A-Up	75	0.36	68%	62%	
P20	G-G-Up	14	0.86	9%	2%	
P21	G-G-A-Up	18	0.18	78%	69%	
P22	G-A-G-A-A-Up	65	0.36	62%	41%	
P23	G-G-G-G-Cp	-	0.40	-	-	
P24	G-G-G-Up	125	0.13	83%	84%	
P25	G-G-U_m-G-G-A-Up	24,28	0.06	70%	75%	

Table 6.8 Correlated data on the reactivities at 37°C of individual uridine residues obtained from direct evidence.

In the examples shown, the reactivities of specific uridines within the sequence of 5.8S rRNA can be determined directly. The '(%) reactivity' signifies the loss of the uridine-containing product relative to that obtained in control 'fingerprints'. For comparison purposes, the (%) reactivity at 25°C is also included.

Uridine residue	Product	(%) Reaction	Mean	(%) Reaction at 25°C for 16 h.
14	T8 U_m-G	29%	19%	zero
	P20 G-G-Up	9%		
18	P25 G-G-U_m-G-G-A-Up	70%	74%	73%
	P21 G-G-A-Up	78%		
27	T14a C-U-C-Gp	20%	20%	12%
38	T13 U-A-Gp	37%	37%	25%
53	T15 C-U-A-Gp	68%	68%	70%
57	T12 C-Ψ-Gp	zero	zero	zero
65	P22 G-A-G-A-A-Up	62%	62%	41%
71	T10 Ψ-Gp	zero	zero	zero
75	P18 G-A-A-Up	68%	68%	62%
125	P24 G-G-G-Up	83%	83%	84%
138	T16 C-U-A-C-Gp	60%	60%	69%
144	T14b C-C-U-Gp	71%	71%	68%
14,30	T9 U-Gp	zero	zero	6%
34,154	T11 U-C-Gp	zero	zero	zero

These are summarised in Table 6.8. Other uridines occur either in oligonucleotides which are present several times in the 5.8S rRNA nucleotide sequence or in oligonucleotides containing several uridine residues. The reactivities of many of these uridines towards carbodiimide can be determined indirectly.

Indirect evidence

U5, U7, U75 and U76 These four nucleotides can be located in the T_1 ribonuclease digest products T23 and T23a which are difficult to separate from each other, even with an extended separation in the second dimension (plate 6.4). They have an average reactivity after 6 h treatment of 76% (Table 6.6). Within product T23a, U75 has a reactivity of 68% (product P18, G-A-A-Up, Table 6.7) and U76 has a reactivity of 83% (footnote a, Table 6.7). This means that the total reactivity of product T23a is 94% (68% x 83%) and hence that of T23 must be 58%.

U8 At 37°C, U8 is slightly reactive. This was derived as follows. Pancreatic ribonuclease digestion product P8, A-G-Cp, is diminished by 0.8 moles after carbodiimide treatment, presumably due to a reactive uridine residue occurring immediately before this product in the sequence. A-G-Cp is preceded twice in the nucleotide sequence by uridine residues, U8 and U53. Residue U53 has been shown to be approximately 70% reactive (product T15, Table 6.6) and therefore most of the reduction in A-G-Cp after treatment must be due to the reactivity of this nucleotide. Therefore U8 has a maximum reactivity of 10%. This being so, the other two uridines encompassed by product T23,

U5 and U7, must have a combined reactivity of about 50%.

U22 This nucleotide, together with U18, is found in product T19.2 which migrates close to product T20 on T_1 ribonuclease 'fingerprints' (plate 6.4). Both of these oligonucleotides contain very reactive uridines, but product T19.2 the lower component on the T_1 ribonuclease fingerprint appears to have diminished more than T20. Since products T19.2 and T20 have an average reactivity of 85%, it seems probable that T19.2 has been reduced by approximately 95% (Table 6.6). U18 can be directly shown to have a reactivity of 78% (product P21, Table 6.7), therefore U22 must be at least as reactive.

U65, U66, U69 These three uridine residues are all found within the T_1 ribonuclease digestion product T24, A-A-U-U-A-A-U-Gp, which is reduced by 74% after reaction (Table 6.6). Of these uridines, U65 can be shown directly to be 62% reactive (pancreatic ribonuclease product P22, G-A-G-A-A-Up, Table 6.7). The other two uridines, U66 and U69 are responsible for the 57% reduction in the molar yield of product P11, A-A-Up. It is not possible to allocate individual reactivities to these two nucleotides.

The combined reactivities of the pancreatic ribonuclease products within T24 (62% x 57%) suggest a total reactivity of 83% for this T_1 ribonuclease digest product. This is of the same order as the reduction in the molar yield of T24 caused by the carbodiimide treatment.

U87, U88 T_1 ribonuclease digest product T21, which contains these two nucleotides, is reduced by approximately 60% after carbodiimide treatment (Table 6.6). It is not possible to specify which is the more reactive, although it is not improbable that they both have reactivities in the 35 - 40% range.

U91, U94 These uridine residues can be found in product T19.1, A-U-C-A-U-C-Gp, which is reduced by 64% after carbodiimide reaction (Table 6.6). U94 can also be located within the pancreatic ribonuclease digest product P10, A-Up, which also contains U87. A-Up is diminished by 21% after treatment, and since it has been suggested that U87 has a reactivity in the range 35 - 40%, then U94 must be largely unreactive. This being so, U91 can be adjudged to have reacted to the extent of about 60% after 6 h treatment at 37°C.

U112, U113 The T_1 ribonuclease digest product T18, C-A-C-U-U-Gp, which encompasses these two nucleotides, is reduced by 37% after carbodiimide treatment (Table 6.6). U113 can be demonstrated to be largely unreactive. Within the nucleotide sequence of HeLa cell 5.8S rRNA this uridine residue is immediately followed by G-Cp (product P6), which is not reduced after carbodiimide treatment. This indicates the lack of reactivity of U113. Therefore the reduction in the molar yield of product T18 must be due to U112.

U146, U148 These two uridine residues are contained within product T17, U-C-U-Gp. The high reactivity of this fragment (82%, Table 6.6) can be shown to be due mostly to U146. The pancreatic ribonuclease product P13, G-A-G-Cp, which immediately follows U148 in the sequence, is reduced by approximately 25% after carbodiimide treatment (footnote b, Table 6.7). Therefore U146 is about 75% reactive.

Oligonucleotides containing multiple uridine residues

It was possible to obtain estimates for the reactivities of two T_1 ribonuclease

digest products containing multiple uridine residues which have so far not been discussed. However, the reactivities of individual uridines within these fragments could not be specified.

Product T20, which contains U101 and U102, closely migrates with product T19.2 (plate 6.4). The average reactivity of both fragments was 87% (Table 6.6) with T19.2 being the more reactive. Product T20 probably has a reactivity of approximately 80%, although there is no way of knowing which of the two uridines it encompasses is the more reactive.

The problem is somewhat similar with product T22, U-U-C-C-U-C-C-C-Gp, which is 95% reactive. The first nucleotide in this sequence, U125, is about 80% reactive (product P24, Table 6.7). From the available data it is not possible to ascertain the individual reactivities of the other two uridines in product T22, U126 and U129.

3' terminus

The product T2a, C-U-U, which is found at the 3' end of HeLa cell 5.8S rRNA, is reduced by 68% after carbodiimide treatment. It is not possible to allocate reactivities to either U158 or U159.

Reactivity of guanine residues

Although the carbodiimide reaction was carried out under conditions in which the reactivity of guanine residues is slight, there is evidence that two guanine residues may be reactive.

G80, G81 These two nucleotides are encompassed by the pancreatic ribonuclease digest product P14, A-G-G-A-Cp, which is reduced by 40% after reaction with carbodiimide. At 25°C these two residues were assumed to occupy exposed positions within loop IV. This result indicates that at 37°C these two

guanine residues possess a similar conformation to that at 25°C.

Relation of findings to conformation of 5.8S rRNA at 37°C

These results provide direct or inferred estimates of the reactivities of almost all the uridine residues within 5.8S rRNA. A wide range of reactivities was encountered, indicating that the uridine residues within 'unbound' 5.8S rRNA exist in different conformations.

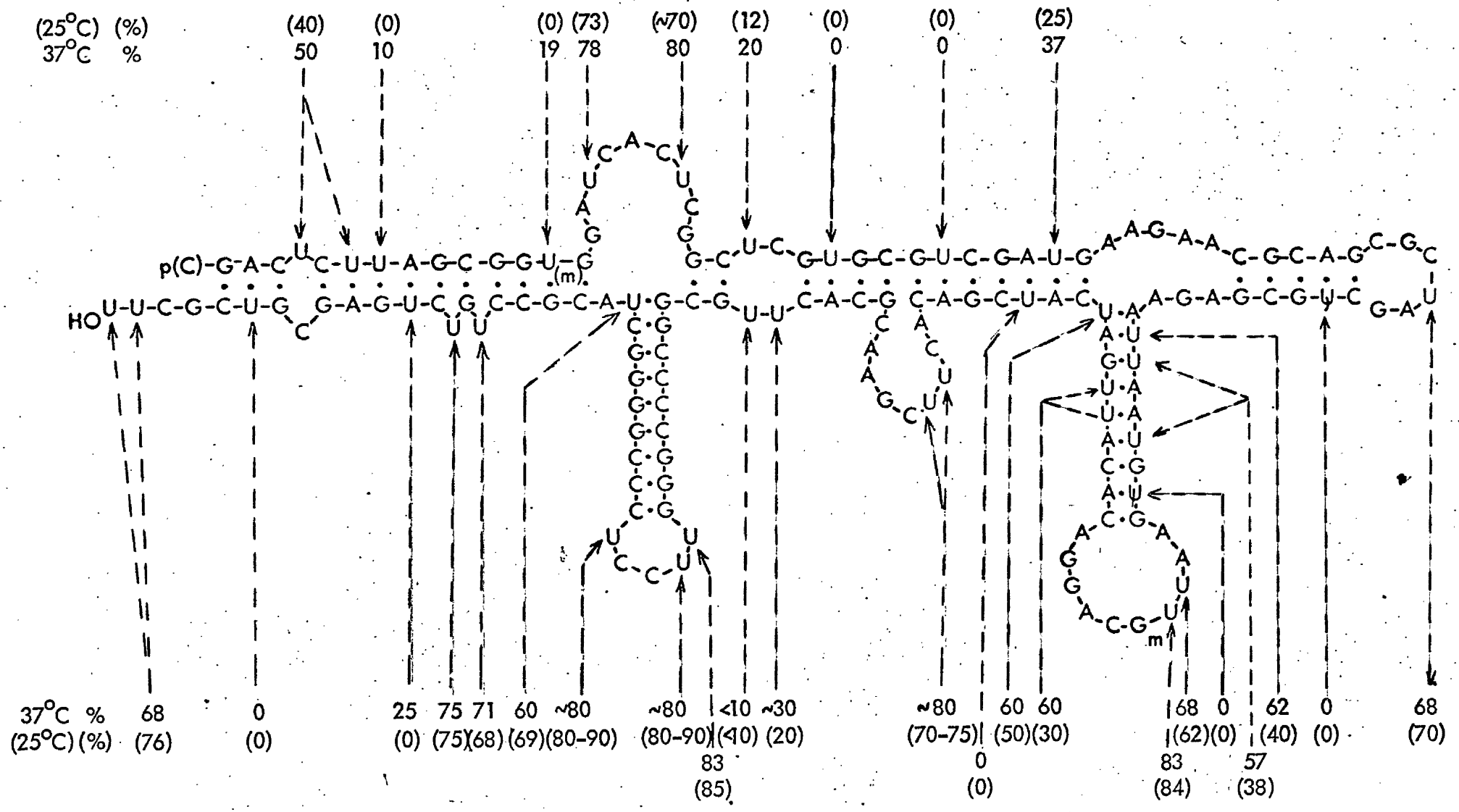
The data obtained from bisulphite modification at 25°C and 37°C and from carbodiimide modification at 25°C generally supported the secondary structure model of Nazar et al. (1975). The data from this experiment will be directly compared with this model.

Loops The reactivities of all uridine-containing oligonucleotides within the loop regions is similar to that found at 25°C when 5.8S rRNA had been treated for 16 h with carbodiimide. Loops I (U18, U22), III (U53), IV (U75, U76), V (U101, U102) and VI (U125, U126, U129) all contain at least one highly reactive uridine residue (see text, Tables 6.6 and 6.7). Therefore the conformation of the loop regions appears to be very similar at 37°C and 25°C.

Helical regions The data obtained from helical regions, unlike the loop regions, show some differences between 37°C and 25°C. At 37°C, the internally located uridines of helix (b), U30, U34 and U91, all exhibit negligible reactivity. However, U38, which was moderately reactive at 25°C (25%), showed a small, but significant, increase in reactivity at 37°C (38%, product T13, Table 6.6). In helix (c), the internally located Ψ57, remains completely unreactive at 37°C (product T2, Table 6.6). Helix (e) does not contain any internally located uridines and so cannot be investigated by this

Fig. 6.2 Reactivity at 37°C of the uridine residues within the proposed secondary structure of HeLa cell 5.8S rRNA

The arrows indicate the percentage reactivity of the uridine residues towards carbodiimide after 6 h treatment at 37°C. The extent of reaction after 16 h treatment at 25°C is given in brackets. Tied arrows indicate the combined reactivities of two uridines as explained for Fig. 5.1.


(25°C) (%)
37°C %
(40) 50
(0) 10
(0) 19
(73) 78
(~70) 80
(12) 20
(0) 0
(0) 0
(25) 37
p(C)-G-A-C-U-C-U-U-A-G-C-G-G-U-G
(m)
HO U-U-C-G-C-U-G G-A-G-U-C G C-C-G-C-A-U-G-C-G U-U C-A-C-G C-A-G-C-U-A-C
A-G-A-G-A-A-C-G-C-A-G-C-G-C
A-G-A-G-C-G-U-C G-A-U
37°C % 68 0 25 75 71 60 ~80 ~80 <10 ~30 ~80 60 60 68 0 62 0 68
(25°C) (%) (76) (0) (0) (75) (68) (69) (80-90) (80-90) (<10) (20) (70-75) (50) (30) (62) (0) (40) (0) (70)
83 (85)
0 (0)
83 (84)
57 (38)

chemical reagent. Helices (a) and (b) present more difficult analytical problems.

Helix (a) was shown by the sodium bisulphite data to be somewhat unstable at 37°C. This is corroborated by the carbodiimide data. U8, U14 and U148, all of which were unreactive at 25°C, show small but significant degrees of reactivity at 37°C. However, U154 remains unreactive (product T11, Table 6.6). This nucleotide is thought to form a base pair with A3, and has a GC base pair on each side in the helix. The resistance of uridine bases in such conformations to carbodiimide modification has already been noted at 25°C. The conclusions to be drawn from these data about the structure of helix (a) is similar to that drawn from the sodium bisulphite reactivity data, namely, that at 37°C helix (a) retains its secondary structure, but is less stable than at 25°C.

The results obtained for the reactivities of uridine residues within helix (d) show that these residues are more available for reaction at 37°C than at 25°C, with the exception of ψ71 (product T10, Table 5.3). U65, U66, U69, U87 and U88 all exhibited slight increases in reactivity (see text, Tables 6.6 and 6.7). It was noted at 25°C that most of the uridines within this helix were partially available for carbodiimide reaction. As expected, at the 37°C, this reactivity has increased. However, since the reactivities of uridines within this helix are significantly less than uridines in loop regions, it is probable that helix (d) retains its secondary structure, especially at the 'bottom' of the helix where two GC pairs exist.

The reactivity of uridine residues within helix (d) possibly reflects the

nucleotide composition of the helix. The 'top' of the helix, which contains the most labile A-U base pairs, contains no G-C base pairs. In contrast, at the 'bottom' of the helix, Ψ71, which is non-reactive, is located between the base pairs G70.C85 and G72.C83. In conclusion, it can be said that although helix (d) maintains its conformation at 37°C, it is less stable than at 25°C.

Helix imperfections

At 25°C, helix (b) contains a symmetrical bulge near the left hand end. This bulge contains three uridine residues U28, U112, U113, which were all less reactive at 25°C than might be expected from their positions in the secondary structure. A similar picture emerges at 37°C. These uridines, especially U28 and U113, are slightly more reactive at 37°C than at 25°C, but again this is less than might be expected from their location in the secondary structure proposed by Nazar et al. (1975). It is probable that these nucleotides are involved in maintaining the tertiary structure through base interactions and are therefore unavailable for reaction.

Helix (a) contains three uridine residues involved in helical imperfections. U146 and U148 which were both highly reactive at 25°C exhibit similar reactivity (about 70%) at 37°C. U5, which was moderately reactive at 25°C, is slightly more reactive at 37°C, perhaps reflecting the less stable nature of the helix at 37°C, as discussed above.

In summary, the carbodiimide modification data indicates that the structure of HeLa cell 5.8S rRNA at 37°C is generally similar to that at 25°C, with two qualifications.

(1) At 37°C, helix (a) is less stable than at 25°C. This is corroborated by the data from the sodium bisulphite reaction.

(2) At 37°C, the A-U rich half of helix (d) is much less stable than the 'bottom' half adjacent to loop IV, which contains two G-C pairs.

6.2 Reaction of sodium bisulphite and carbodiimide with 5.8S rRNA at 50°C

Choice of conditions

To investigate which are the more stable regions of its secondary structure, 5.8S rRNA was chemically modified with sodium bisulphite and carbodiimide at 50°C. At this temperature, it was assumed that the 5.8S rRNA would partially unfold. This would allow more cytidine and uridine residues to react with their specific modifying reagents than occurred at 25°C and 37°C. Those more stable regions of the molecule, which remained intact, would be detected by the lack of reactivity of pyrimidine bases.

5.8S rRNA was reacted with sodium bisulphite at 50°C for 24 h and then subjected to fingerprint analysis. By running this reaction to near completion, it should be possible to identify the most stable regions of the molecule by their total lack of reactivity. From the data in Fig. 4.2, it can be seen that these conditions are achieved by carrying out the reaction for 24 h at 50°C. The reaction was also investigated after 6 h, when it had reached approximately 75% completion. As before the reaction of 5.8S rRNA with bisulphite was carried out using 3 M sodium bisulphite, pH 6.0, in the presence of 10 mM $MgCl_2$.

A different approach was taken when the investigation was carried out using carbodiimide. So that the data could be compared more directly with that

obtained at 25°C and 37°C, a reaction time of 2 h was chosen. On the basis of the previous kinetic data, it was decided that 2 h carbodiimide treatment at 50°C would result in an equivalent amount of reactivity as had occurred after 16 h treatment at 25°C and 6 h treatment at 37°C. The reaction conditions were the same as those used at 25°C and 37°C.

(a) Reaction of sodium bisulphite with 5.8S rRNA at 50°C

Reactivity of individual cytidine residues

Plate 6.6 shows 'fingerprints' of T_1 ribonuclease digests of 5.8S rRNA which have been treated with bisulphite for 24 h and 6 h at 50°C. Plate 6.7 shows 'fingerprints' of T_1 ribonuclease digests of similarly treated 5.8S rRNA, where the second dimension of electrophoresis has been extended to at least 40 h. Plate 6.8 shows pancreatic ribonuclease 'fingerprints' of this bisulphite treated 5.8S rRNA.

After 24 h treatment at 50°C many of the cytidine-containing oligonucleotides have completely disappeared. Several uridine-containing derivatives of these oligonucleotides appear to be present in relatively high molar yields. Tables 6.9 and 6.11 summarise the molar yields of T_1 ribonuclease and pancreatic ribonuclease digestion products obtained after reaction with sodium bisulphite for 24 h and 6 h at 50°C. Several of the uridine-containing conversion products were also characterised (Tables 6.10 and 6.12). It is clear from these data that much of the 5.8S rRNA has denatured.

Relation of findings to the structure of 5.8S rRNA

Examination of the data presented in Tables 6.9 and 6.12 indicate that most of the 5.8S rRNA molecule has denatured. Helix (a) has certainly

'Fingerprints' of HeLa cell 5.8S rRNA after reaction with 3 M sodium bisulphite, pH 6.0, for 6 h (left) and 24 h (centre) at 50°C.

Plate 6.6 T_1 ribonuclease digest

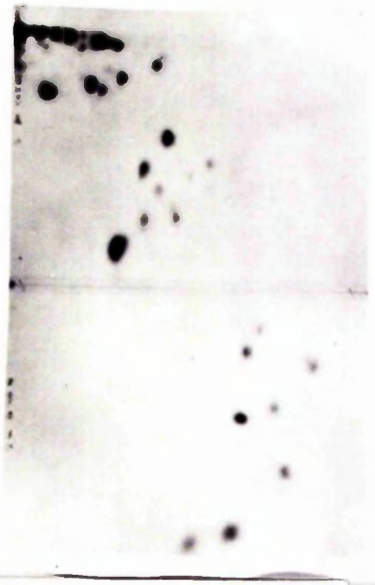
The key (right) identifies new, diminished and unreacted sequences as in Plate 6.1. Electrophoresis separation was carried out in the first dimension on cellulose, pH 3.5, at 4.7 KV for 3 - 3.5 h and in the second dimension on DEAE paper, 7% formic acid, at 1.2 KV for 17 h.

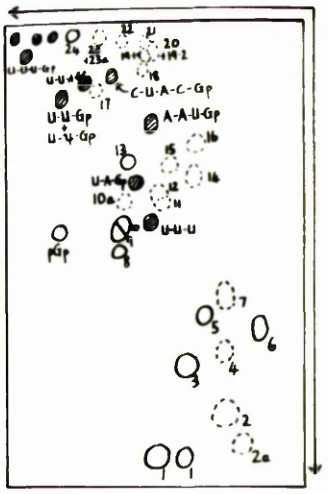
Plate 6.7 T_1 ribonuclease digest, extended fractionation

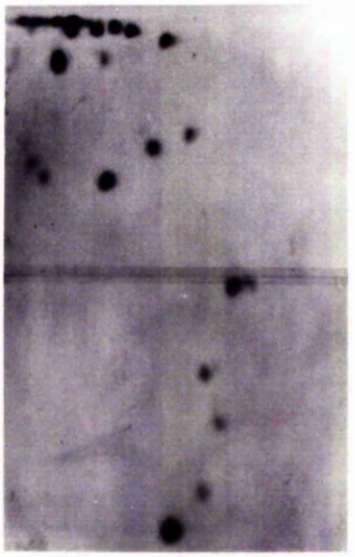
The key (right) identifies new, diminished and unreacted sequences as in Plate 6.1. Electrophoresis was carried out for 4 h at 4.7 KV in the first dimension and for 40 - 60 h at 1.2 KV in the second dimension.

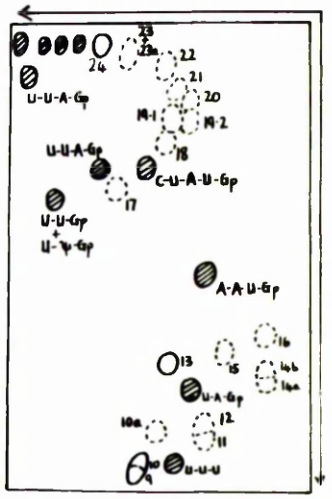
Plate 6.8 Pancreatic ribonuclease digest

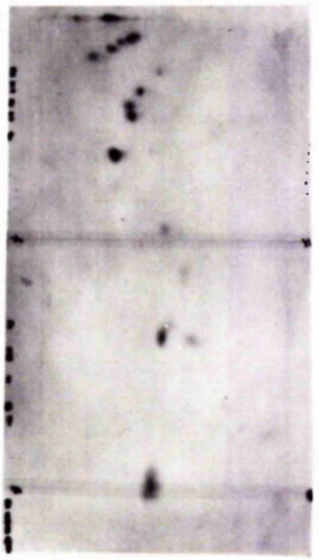
The key (right) identifies new, diminished and unreacted sequences as in Plate 6.1, with the numbering of spots as in Table 6.7. Electrophoresis was carried out for 2.5 h at 4.7 KV in the first dimension and for 16 h at 1.1 KV in the second dimension.

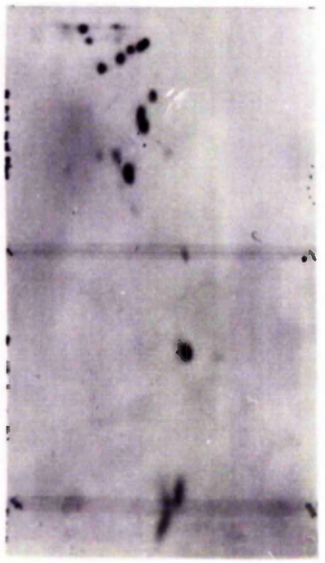



C-U-A-C-Gp
A-A-U-Gp
U-U-U




U-U-A-Gp
C-U-A-U-Gp
A-A-U-Gp
U-U-U





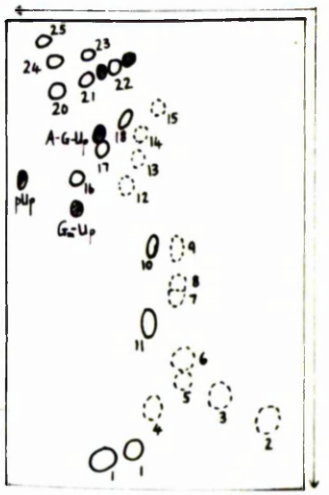

A-G-Up
pUp
G-Up

Table 6.9 Molar yields of T_1 ribonuclease digest products from HeLa cell 5.8S rRNA after treatment with sodium bisulphite at 50°C.

The conventions are the same as for Table 3.1. The bisulphite reaction was carried out for 6 h and 24 h at 50°C in 3 M sodium bisulphite, pH 6.0, in the presence of 10 mM $MgCl_2$. The molar yields were calculated as in Table 4.2.

Footnotes

(a) The high yield of U-C-U-Gp (T17), compared to other cytidine-containing oligonucleotides after 6 h reaction is due to two components - a residual amount of the original oligonucleotide, T17, which has not yet reacted; the conversion in each of T14a and T14b of one cytidine to uridine resulting in isomers of T17, in both cases (U_2, C)Gp. After 24 h these products will have either reacted, or reacted further, accounting for the large drop in the molar yield of T17 between 6 h and 24 h.

Table 6.9

Spot No.	Sequence	Control molar yield	Bisulphite treated at 50°C	
			6 h	24 h
T1	Gp	11.15 (10)	11.26	11.99
T2	C-Gp	6.20 (6)	2.05	0.74
T2a	C-U-U	0.91 (1)	0.09	zero
T3	A-Gp	2.36 (2)	2.33	2.46
T4	C-A-Gp	1.04 (1)	0.76	0.40
T5	A-A-Gp	1.01 (1)	1.00	1.02
T6	C-C-C-C-Gp	0.45 (≤1)	0.40	0.56
T7	A-A-C-Gp	1.44 (2)	0.42	0.03
T8	U_m-Gp	0.21 (0.2)	0.16	0.15
T9	U-Gp	1.99 (1.8)	6.20	8.93
T10	ψ-Gp	1.02 (1)		
T11	U-C-Gp	1.92 (2)	0.85	0.21
T12	C-ψ-Gp	1.03 (1)		
T10a	pC-Gp	0.42 (0.4-0.5)	0.03	zero
T13	A-U-G	1.15 (1)	1.09	1.13
T14a	C-U-C-Gp	2.08 (1)	0.16	0.03
T14b	C-C-U-Gp	(1)		
T15	C-U-A-Gp	1.06 (1)	0.09	zero
T16	C-U-A-C-Gp	0.67 (1?)	0.18	0.02
T17	U-C-U-Gp	1.10 (1)	0.73	0.05 (a)
T18	C-A-C-U-U-Gp	0.91 (1)	0.05	zero
T19.1	A-U-C-A-U-C-Gp	0.87 (1)	0.28	"
T19.2	A-U-C-A-C-U-C-Gp	1.82 (2)	0.03	"
T20	A-C-A-C-U-U-C-Gp			
T21	A-C-A-C-A-U-U-Gp	0.87 (1)	0.10	"
T22	U-U-C-C-U-C-C-C-Gp	0.80 (1)	0.02	"
T23	A-C-U-C-U-U-A-Gp	1.86 (2)	0.46	"
T23a	A-A-U-U-G_m-C-A-Gp			
T24	A-A-U-U-A-A-U-Gp	0.85 (1)	0.93	0.85

Table 6.10 Molar yields of uridine-containing products of the sodium bisulphite reaction with 5.8S rRNA at 50°C; T_1 ribonuclease products.

The conventions are the same as for Table 3.1. The sequences of these uridine-containing oligonucleotide products of T_1 ribonuclease digestion are given. The conditions of the bisulphite reaction are the same as in Table 6.9.

Spot No.	Derived from		Bisulphite treated at 50°C	
			6 h	24 h
T43 (pU-Gp)	T10a	pC-Gp	0.55	0.44
T10b (U-U-U)	T2a	C-U-U	0.75	0.81
T10c (U-A-Gp)	T4	C-A-Gp	0.29	0.64
T36 (A-A-U-Gp)	T7	A-A-C-Gp	1.30	1.64
T41 (U-U-Gp + U-Ψ-Gp)	T10 T 11	U-C-Gp C-Ψ-Gp	2.23	2.57
T40 (U-U-A-Gp)	T15	C-U-A-Gp	1.01	1.14
T42 (C-U-A-U-Gp)	T16	C-U-A-C-Gp	0.50	0.54 (a)
T39 (U-U-U-Gp)	T14a, T14b, T17	C-U-C-Gp C-C-U-Gp U-C-U-Gp	2.04	2.61

Footnote

(a) The molar yield of T42 indicates that C137 is almost completely non-reactive. The molar yield of T42, C-U-A-U-Gp, is almost the same as the initial yield of T16, C-U-A-C-Gp, the oligonucleotide from which it is derived. Since no U-U-A-U-Gp could be located on the 'fingerprints', C140 is reactive, whereas C137 is not. C137 is located within helix (e) which has been shown to be the most stable region of the molecule.

Table 6.11 Molar yields of pancreatic ribonuclease products from HeLa cell 5.8S rRNA after reaction with sodium bisulphite at 50°C.

The conventions are the same as for Table 3.1. The bisulphite reaction was carried out for 6 h and 24 h at 50°C in 3 M sodium bisulphite, pH 6.0, in the presence of 10 mM $MgCl_2$. The molar yields were calculated as in Table 4.4.

Table 6.11

Spot No.	Sequence	Control Molar yield	Bisulphite treated at 50°C 6 h	24 h
P1	Up + ψp	19.00 (20)	32.16	37.94
P2	Cp	19.80 (19)	5.37	2.92
P3	A-Cp	5.68 (5)	1.41	0.16
P4	pCp	0.46 (0.4-0.5)	0.05	zero
P5	G_m-Cp	0.92 (1)	0.36	0.05
P6	G-Cp	7.06 (8)	2.99	1.13
P7	G-A-Cp	4.72 (2)	0.60	0.62
P8	A-G-Cp	(3)	0.74	
P9	G-A-A-Cp	0.98 (1)	0.29	0.12
P10	A-Up	2.47 (2)	6.25	8.86
P11	A-A-Up	1.06 (1)	0.98	1.16
P12	G-G-Cp	1.91 (2)	0.41	0.33
P13	G-A-G-Cp	0.97 (1)	0.22	0.05
P14	A-G-G-A-Cp	0.98 (1)	0.34	0.02
P15	G-A-A-G-A-A-Cp	0.77 (1)	0.36	0.03
P16	G-Up + G-ψp	5.45 (5)	9.91	10.46
P17	G-A-Up	2.23 (2)	3.18	3.66
P18	G-A-A-Up	1.12 (1)	1.84	2.46
P20	G-G-Up	0.95 (1)	1.74	2.01
P21	G-G-A-Up	0.83 (1)	1.67	1.86
P22	G-A-G-A-A-Up	0.94 (1)	1.03	1.05
P23	G-G-G-G-Cp	0.45 (≤1?)	0.35	0.36
P24	G-G-G-Up	0.76 (1)	0.61	0.83
P25	G-G-U_m-G-G-A-Up	0.20 (0.2)	0.25	0.31

Table 6.12 Molar yields of uridine-containing products of sodium bisulphite reaction with 5.8S rRNA at 50°C; pancreatic ribonuclease products.

The conventions are the same as for Table 3.1. The sequences of these uridine-containing oligonucleotide products of pancreatic ribonuclease digestion are given. The conditions of the bisulphite reaction are the same as in Table 6.11.

Spot No.	Derived from	Bisulphite treated at 50°C	
		6 h	24 h
P16a (G_m-Up)	P5 G_m- Cp	0.57	0.81
P16b (pUp)	P4 pCp	0.54	0.51
P17a (A-G-Up)	P8 A-G-Cp	2.09	2.34

dissociated. The unique cytidine-containing oligonucleotides which it encompasses (T23, T16, T14b and P13; Tables 6.9 and 6.11) have all diminished by at least 95% after 24 h treatment at 50°C. The relatively high yield of product T17 after 6 h reaction can be readily explained (footnote a, Table 6.9). The dissociation of this helix is not surprising when its partial instability at 37°C is considered. A large contribution towards this instability is made by helical imperfections. The high reactivity of the unique cytidine-containing oligonucleotides from within helix (b) clearly demonstrates that this helix has also dissociated at 50°C. Products T14a, T19.1 and T18 (Table 6.9) have all but completely disappeared after 24 h treatment with sodium bisulphite at this temperature.

In contrast, at 50°C, helix (c) remains almost intact, as demonstrated by the low reactivity of product T4, C-A- Gp (Table 6.9). After 6 h treatment at 50°C, the molar yield of this oligonucleotide was only reduced to 0.76. Since 3 h treatment at 50°C is equivalent to 24 h treatment at 25°C (when the reactivities of cytidines within loops are compared), clearly C47 is not in a single stranded conformation at 50°C, and is involved in base interactions. The involvement of C59 in similar interactions, which maintain the structure of helix (c), may account for the significant amount of T2, C-Gp (Table 6.9) remaining after 24 h treatment. The high GC content of helix (c) probably accounts for its high stability compared to helices (a) and (b). At 50°C, helix (d) appears to have dissociated. The unique cytidine-containing oligonucleotides from within this helix (P14, T21; Tables 6.9 and 6.11) have

reactivities greater than 95% after 24 h reaction with bisulphite. Since this helix is composed largely of A-U pairs, it should be possible to confirm that the entire helix has dissociated from the carbodiimide data.

In contrast, helix (e) is the most stable region of the molecule. There is no diminution of product T6 (Table 6.9) which contains C118-121 and very little reduction in product P23 (Table 6.11) which contains C137. The non-reactivity of C137 is also demonstrated by the conversion of product T16 (C-U-A-C-Gp) to T42 (U-U-A-C-Gp) after the bisulphite reaction (footnote a, Table 6.10). Helix (e) is composed entirely of G-C pairs and as a result, its structure is maintained at 50°C. This result is in good agreement with the conclusions from Chapter 3, where the stability of helix (e) was demonstrated by its partial resistance to enzymic hydrolysis.

The results from these experiments suggest that at 50°C, three helical regions of 5.8S rRNA have dissociated. Unique cytidine-containing oligonucleotides from within these helices have reactivities comparable with those from loop regions. The two helices which remain, (c) and (e), are both GC rich. Helix (c) is partially labile at this temperature whereas helix (e) remains completely intact. Carbodiimide modification at this temperature should confirm these conclusions.

(b) Reaction of carbodiimide with 5.8S rRNA at 50°C

Reactivity of individual uridine residues.

Plate 6.9 shows 'fingerprints' of T_1 ribonuclease digests of 5.8S rRNA

'Fingerprints' of HeLa cell 5.8S rRNA before (left) and after (right) reaction with carbodiimide (10 mg/ml), pH 8.9, for 2 h at 50°C

Plate 6.9 T_1 ribonuclease digest

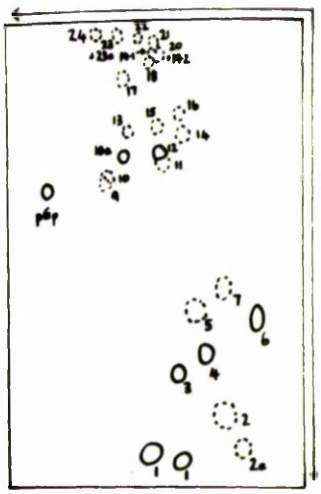
The key (right) identifies reacted (broken circles) and unreacted (closed circles) sequences. These sequences are identified by the standard numbering system (Table 6.11). Electrophoretic separation was performed in the first dimension on cellulose acetate, pH 3.5, at 4.7 KV for 3 - 3.5 h, and in the second dimension on DEAE paper, 7% formic acid, at 1.2 KV for 17 h.

Plate 6.10 T_1 ribonuclease digest, extended fractionation

The key (right) identifies diminished and unreacted sequences as in Plate 6.9. Electrophoresis was carried out at 4.7 KV for 4 h in the first dimension and at 1.2 KV for 40 - 60 h in the second dimension.

Plate 6.11. Pancreatic ribonuclease digest

The key (right) identifies diminished and unreacted sequences as in Plate 6.9 with the numbering of spots as in Table 6.12. Electrophoresis was carried out at 4.7 KV for 2.5 h in the first dimension and at 1.1 KV for 16 h in the second dimension.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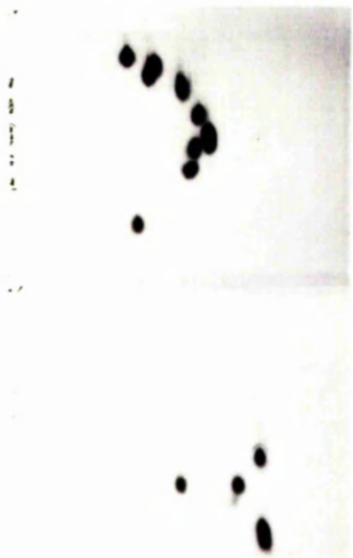

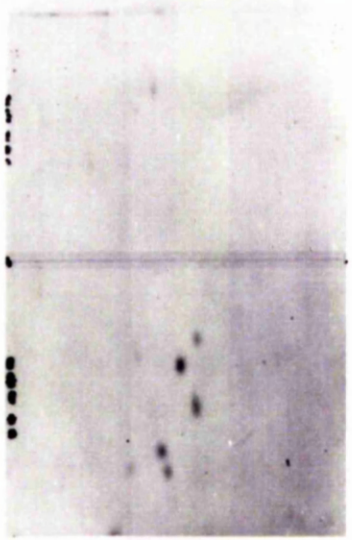

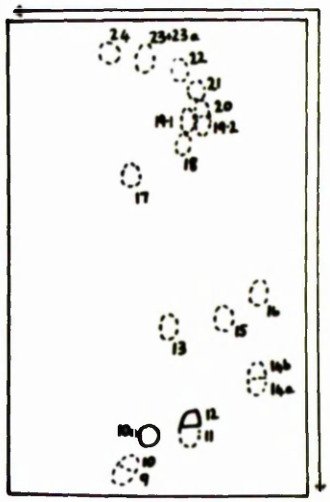
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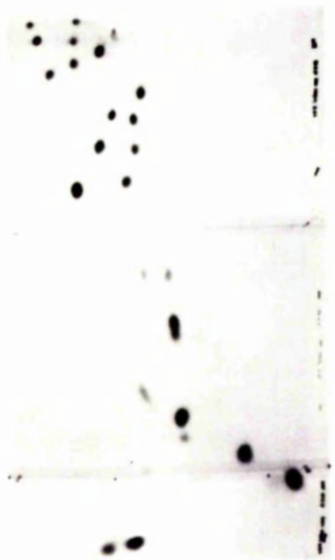

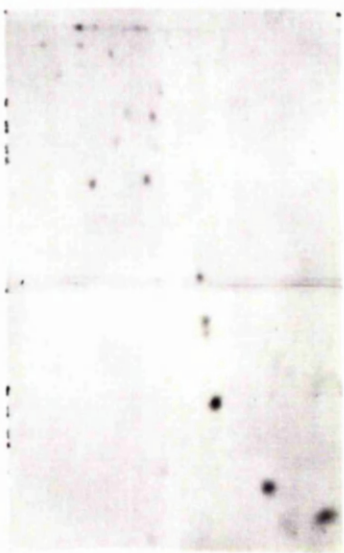

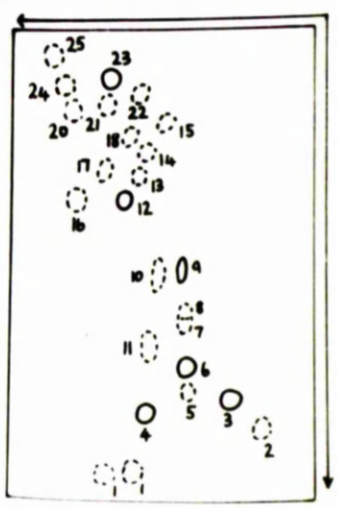
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Table 6.13 Sequences and molar yields of T_1 ribonuclease products from HeLa cell 5.8S rRNA, and the extent of reaction of uridine-containing oligonucleotides after treatment with carbodiimide at 50°C.

The conventions are as in Table 3.1. The positions of the uridine residues in the nucleotide sequence are indicated. The reaction was carried out for 2 h at 50°C in 10 mg/ml carbodiimide, pH 8.9, in the presence of 10 mM $MgCl_2$. The molar yields after carbodiimide treatment were calculated assuming that the yield of T4, C-A-Gp, was 1.00.

Footnotes

(a) There is a significant increase in the reactivity of product T2a relative to that found at 25°C and 37°C, under conditions which should yield a similar level of reactivity. This suggests that at least one of the uridine residues is more available at 50°C than at 25°C and 37°C.

(b) Product T5 is reduced by approximately 40% after treatment. This must be due to G42 being exposed and reacting with carbodiimide under these conditions.

(c) After reaction, there is an 0.45 M reduction in the yield of product T7, A-A-C-Gp. Most of this reduction can be attributed to the reactivity of G42 (see (b) above) which occurs immediately before A-A-C-Gp in the sequence of HeLa cell 5.8S rRNA and which would inhibit cleavage by T_1 ribonuclease at this point.

(d) It was not possible on this occasion to resolve products T9 and T10 and to allocate individual reactivities.

(e) Products T14b, T15 and T16 each contain one uridine (U144, U53 and U138 respectively) which were highly reactive at 37°C and 25°C. After reaction with carbodiimide at 50°C for 2 h they all reacted to similar extents, these being less than at 37°C and 25°C. Since these residues presumably still exist in single stranded regions at 50°C, the difference must be due to the shorter reaction time.

Table 6.13

Spot No.	Sequence	Uridine residue(s)	Molar yield	(%) reactivity	% reactivity 6h at 37°C	% reactivity 16h at 25°C
T1	Gp	-	12.01	-	-	-
T2	C-Gp	-	5.56	-	-	-
T2a	C-U-U	158, 159	0.03	97% (a)	68%	76%
T3	A-Gp	-	2.10	-	-	-
T4	C-A-Gp	-	1.00	-	-	-
T5	A-A-Gp	-	0.59	- (b)	-	-
T6	C-C-C-C-Gp	-	0.50	-	-	-
T7	A-A-C-Gp	-	0.99	-- (c)	-	-
T8	U_m-Gp	14	-	not recorded	29%	10%
T9	U-Gp	14,30	0.78	74% (d)	4%	zero
T10	Ψ-Gp	71				
T10a	pC-Gp	-	0.36	-	-	-
	pGp	-	0.61	-	-	-
T11	U-C-Gp	34, 154	0.69	64%	zero	6%
T12	C-Ψ-Gp	57	0.95	8%	zero	zero
T13	A-U-Gp	38	0.21	82%	37%	25%
T14a	C-U-C-Gp	27	0.30	71%	20%	12%
T14b	C-C-U-Gp	144	0.46	56% (e)	71%	68%
T15	C-U-A-Gp	53	0.63	41% (e)	68%	70%
T16	C-U-A-C-Gp	138	0.38	43% (e)	60%	69%
T17	U-C-U-Gp	146, 148	0.18	84%	82%	78%
T18	C-A-C-U-U-Gp	112, 113	0.08	91%	37%	24%
T19.1	A-U-C-A-U-C-Gp	91,94	0.05	95%	64%	54%
T19.2	A-U-C-A-C-U-C-Gp	28,32	0.15	92%	97%	83%
T20	A-C-A-C-U-U-C-Gp	101, 102				
T21	A-C-A-C-A-U-U-Gp	87,88	0.03	97%	61%	38%
T22	U-U-C-C-U-C-C-C-Gp	125, 126, 129	0.03	96%	95%	96%
T23	A-C-U-C-U-U-A-Gp	5,7,8	0.05	97%	76%	67%
T23a	A-A-U-U-G_m-C-A-Gp	75,76				
T24	A-A-U-U-A-A-U-Gp	65, 66, 68	0.02	98%	74%	56%

Table 6.14 Sequences and molar yields of pancreatic ribonuclease digest products from HeLa cell 5.8S rRNA, and the extent of reaction of uridine-containing products after treatment with carbodiimide at 50°C.

Conventions were as in Table 3.1. The conditions of the reaction were the same as for Table 6.13. The molar yields were calculated assuming that the yield of product P9, G-A-A-Cp, was 1.00.

Footnotes

(a) As was explained previously (Table 5.2, footnote b), product P5, G_m-Cp, is obtained in reduced molar yield after carbodiimide treatment because of the reactivity of U76.

(b) Product P13, G-A-G-Cp, is diminished by over 50% after reaction. This is a reflection of the conformation of U148 (Table 5.2, footnote c).

(c) The reduction in the yield of product P14, A-G-G-A-Cp, can only be due to the reactivity of G80 and/or G81, a total of 30%. These two nucleotides also exhibited slight reactivity at 25°C and 37°C.

(d) After treatment, product P15 is reduced by at least 65%; this reactivity is due entirely to U38 (Table 6.13, product T13).

Table 6.14

Spot No.	Sequence	Uridine residue	Molar yield	% reactivity	% reactivity 6 h at 37°C	% reactivity 16 h at 25°C
P1	Up + Ψp	-	7.61	-	-	-
P2	Cp	-	15.55	-	-	-
P3	A-Cp	-	6.00	-	-	-
P4	pCp	-	0.60	-	-	-
P5	G_m-Cp	-	0.29	- (a)	-	-
P6	G-Cp	-	6.91	-	-	-
P7	G-A-Cp	-	1.67	-	-	-
P8	A-G-Cp	-	1.41	-	-	-
P9	G-A-A-Cp	-	1.00	-	-	-
P10	A-Up	87,94	0.78	68%	21%	13%
P11	A-A-Up	69	0.15	86%	57%	38%
P12	G-G-Cp	-	1.70	-	-	-
P13	G-A-G-Cp	-	0.44	- (b)	-	-
P14	A-G-G-A-Cp	-	0.69	- (c)	-	-
P15	G-A-A-G-A-A-Cp	-	0.36	- (d)	-	-
P16	G-Up + G-Ψp	30,34,71, 146,154	2.15	61%	18%	13%
P17	G-A-Up	38,91	0.64	71%	26%	23%
P18	G-A-A-Up	75	0.51	54%	68%	62%
P20	G-G-Up	14	0.38	60%	9%	2%
P21	G-G-A-Up	18	0.20	76%	78%	69%
P22	G-A-G-A-A-Up	65	0.32	66%	62%	41%
P23	G-G-G-G-Cp	-	0.31	-	-	-
P24	G-G-G-Up	125	0.27	64%	83%	84%
P25	G-G-U_m-G-G-A-Up	24,28	0.07	65%	70%	75%

which have been treated with carbodiimide for 2 h at 50°C. Plate 6.10 shows 'fingerprints' of carbodiimide treated 5.8S rRNA, where the second dimension of electrophoresis has been run for over 40 h. Plate 6.11 shows pancreatic ribonuclease 'fingerprints' of treated 5.8S rRNA.

It can be observed from the 'fingerprints' that many uridine-containing oligonucleotides are present in reduced molar yields. On the right hand side of these 'fingerprints', several oligonucleotides which contain carbodiimide modified residues are located. Tables 6.13 and 6.14 summarise the molar yields of T_1 ribonuclease and pancreatic ribonuclease digestion products obtained from 5.8S rRNA after 2 h reaction with carbodiimide at 50°C. These data indicate that many more uridine bases are available for reaction than at 25°C and at 37°C.

Relation of findings to the structure of 5.8S rRNA

Analysis of the data obtained from the reaction of carbodiimide with uridine residues in 5.8S rRNA at 50°C is in good agreement with the conclusions reached from the reaction of sodium bisulphite with the cytidine residues at this temperature, i.e. most of the 5.8S rRNA molecule has denatured.

Helix (a) has clearly dissociated as judged by the molar yields of the uridine-containing oligonucleotides which it encompasses (Tables 6.13 and 6.14). Products T23, T14b, T17 and P13 (which indicates the reactivity of U148) are all highly reactive at 50°C, to an extent suggesting their existence as non-hydrogen bonded and largely unstacked bases. The dissociation of this helix at 50°C is not surprising considering that it has been previously demonstrated to be rather unstable at lower temperatures. The reactivity of the

uridine-containing oligonucleotides within helix (b) demonstrate that this helix has also largely dissociated at 50°C. ProductsT14a, T19.1, T13 and T18 (Table 6.13) have all extensively reacted after 2 h treatment with carbodiimide. The high reactivity of product T18, C-A-C-U-U-Gp, shows that the interactions in which U112 and/or U113 are involved in at 25°C and 37°C are not present at 50°C.

Helix (c) remained almost intact as judged by the lack of reactivity of Ψ57 within product C-Ψ-Gp, T12 (Table 6.13). After 2 h treatment at 50°C, the molar yield of this oligonucleotide was not significantly reduced. The high stability of this GC rich helix has already been demonstrated by its resistance to sodium bisulphite modification. The lack of reactivity of Ψ57 which exists as an A-Ψ base pair between two G-C base pairs, is further evidence for the continued existance of this helix at 50°C. Helix (d) has obviously dissociated at 50°C. The uridine-containing oligonucleotides within this helix, products P22, T24, P11 and T21 (Tables 5.6 and 5.7) all exhibit extensive reactivity after 2 h treatment with carbodiimide. This result is in good agreement with the sodium bisulphite data, and with the result obtained from the carbodiimide data at 25°C and 37°C. This suggested that helix (d) contains labile regions. Since helix (e) contains no uridine residues, no data are available on its conformation from carbodiimide treatment.

In conclusion, it can be stated that the results are in good agreement with those obtained using sodium bisulphite as a probe. Three helices (a, b, and d)

which were shown to have dissociated at 50°C using sodium bisulphite, can be shown to have dissociated when carbodiimide is used as a probe. Uridine residues located in the interior of these helices have reactivities comparable with those in loop regions. Helix (c) was shown to be stable at 50°C by both methods.

7.1 The reaction of sodium bisulphite with HeLa cell 5.8S rRNA bound to 28S rRNA

The experiments reported in Chapters 4 to 6 involved the reaction of chemical reagents with HeLa cell 5.8S rRNA free in solution. It is possible to question the relevance of the structure inferred from these findings to that occurring *in vivo*, in which 5.8S rRNA is hydrogen-bonded to 28S rRNA.

In an effort to solve this problem the following experiment was considered:- to react sodium bisulphite with 28S rRNA, remove 5.8S rRNA from the large molecule, after the usual preparations to 'fingerprint' it, and then to compare the data with those obtained from the reaction of sodium bisulphite with 'unbound' 5.8S rRNA. Changes in the reactivities of any cytidine residues as a result of interaction with 28S rRNA should give an insight into the structure of 5.8S rRNA in this 'bound' form. Although such an experiment seemed straightforward, several technical problems required solving before the experiment could be completed.

(a) Choice of conditions

For comparison with results already obtained from 'unbound' 5.8S rRNA, it was decided to carry out the reaction at 25°C for 24 h, using 3 M sodium bisulphite, pH 6.0, in the presence of 10 mM $MgCl_2$. Technical difficulties necessitated some changes in the procedure; however, the reaction conditions remained the same, enabling direct comparisons to be made. As explained in the Methods section, these procedural changes were necessary because 28S rRNA became partially degraded under the conditions in which deamination

occurred (dialysis at 37°C for 9 - 10 h in 0.1 M Tris-HCl, pH 9.0). To isolate a pure sample of treated 'bound' 5.8S rRNA, it was necessary to separate it from 28S rRNA before this dialysis step and then to proceed. The reaction of bisulphite with exposed cytidine residues takes place before this stage and the extent of such reactivity would in no way be affected by these changes in procedure. The changes are fully documented in the Methods section.

By carrying out the sodium bisulphite reaction under the same conditions as had been used previously with 'unbound' 5.8S rRNA, direct comparisons of the extent of reactivity of cytidine residues can be made. The differences in the reactivitity towards bisulphite of individual cytidine residues observed in the two states of 5.8S rRNA ('bound' and 'unbound') make it possible to establish whether the 5.8S rRNA exists in a different conformation when bound to 28S rRNA.

7.2 Differences in the extent of reactivity of cytidine residues within 'bound' and 'unbound' HeLa cell 5.8S rRNA.

The data in Tables 7.1, 7.2, 7.3 and 7.4 permit comparisons to be made between the reactivities towards sodium bisulphite of most of the cytidine residues within 'bound' and 'unbound' 5.8S rRNA. In most cases there were no significant differences between the results obtained. Where these differences do occur it is convenient to separate discussion of those oligonucleotides which contain a single cytidine residue from those which contain two or more cytidine residues.

'Fingerprints' of sodium bisulphite treated 'bound' (centre) and 'unbound' (left) HeLa cell 5.8S rRNA

Plate 7.1 T_1 ribonuclease digest

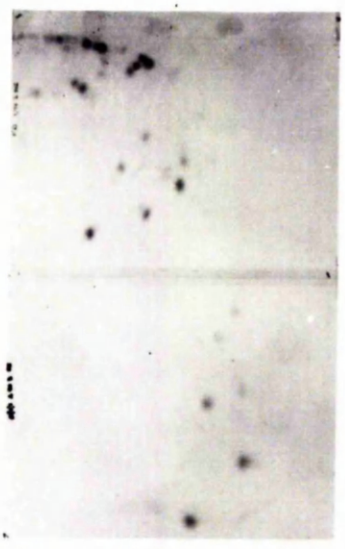
The key (right) refers to 'unbound' 5.8S rRNA and identifies new sequences which arise as a result of sodium bisulphite treatment (shaded circles), unreactive sequences (closed circles) and diminished cytidine-containing sequences (broken circles). The sequences are identified by the standard numbering scheme (Table 7.1). The reaction was carried out at 25°C for 24 h in the presence of 3 M sodium bisulphite, pH 6.0. Electrophoretic separation was performed in the first dimension on cellulose acetate, pH 3.5, at 4.7 KV for 3 - 3.5 h and in the second dimension on DEAE paper, 7% formic acid at 1.2 KV for 17 h.

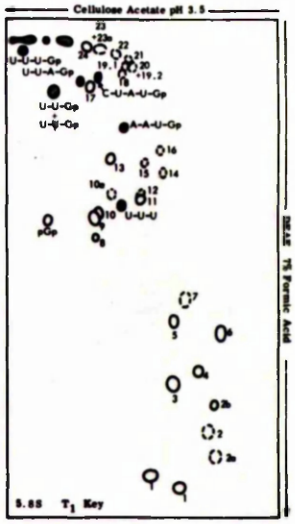
Plate 7.2 T_1 ribonuclease digest, extended fractionation

The key identifies new, diminished and unreacted sequences as in Plate 7.1. Electrophoresis was carried out at 4.7 KV for 4 h in the first dimension and at 1.2 KV for 40 - 60 h in the second dimension.

Plate 7.3 Pancreatic ribonuclease digest

The key identifies new, diminished and unreacted sequences as in Plate 7.1, with the numbering of spots as in Table 7.3. Electrophoresis was carried out at 4.7 KV for 2.5 h in the first dimension and at 1.1 KV for 16 h in the second dimension.

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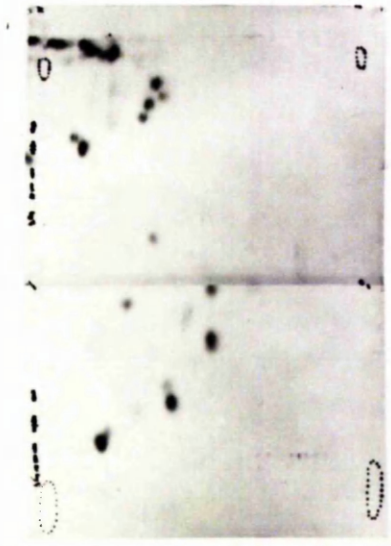
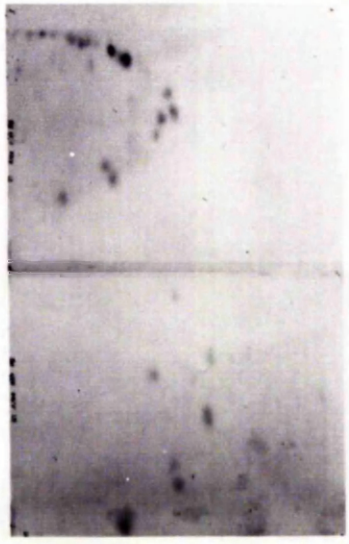
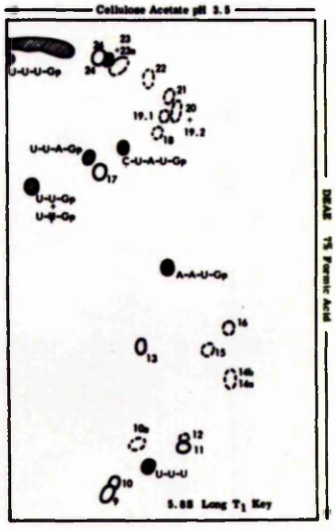
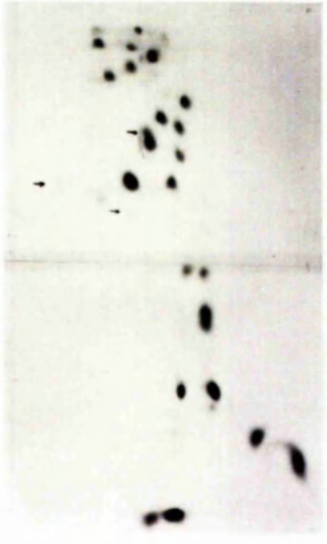
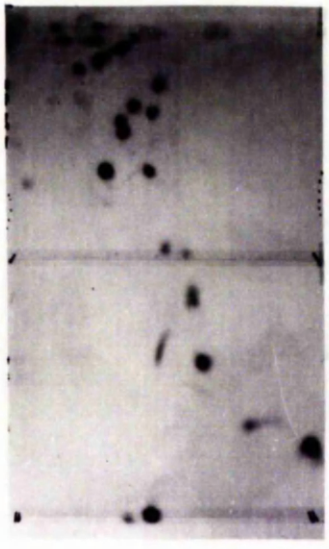
5.8S T1 Key






Cellulose Acetate pH 3.5
DEAE TS Formic Acid
5.8S Long T1 Key





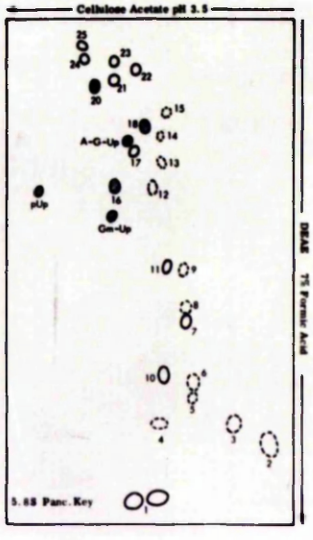

Cellulose Acetate pH 3.5
DEAE TS Formic Acid
5.8S Panc. Key

Table 7.1 Sequences and molar yields of T_1 ribonuclease products from 'bound' and 'unbound' HeLa cell 5.8S rRNA after treatment with sodium bisulphite at 25°C.

The conventions are as in Table 3.1. The bisulphite reaction was carried out for 24 h at 25°C in 3 M sodium bisulphite, pH 6.0, in the presence of 10 mM $MgCl_2$. The molar yields after bisulphite treatment were calculated assuming that the sum of A-A-Gp (T5), A-U-Gp (T13) and A-A-U-U-A-A-U-Gp (T24) was 3.0. These three oligonucleotides were generally obtained in reliable yield. The data presented for 5.8S rRNA chemically modified while bound to 28S rRNA is the mean of three independent experiments.

Footnotes

(a) The gain in U-Gp after bisulphite reaction is derived from the reaction of cytidine in C-Gp. There was no significant difference in the yield after treatment of 'bound' or 'unbound' 5.8S rRNA.

(b) These two products were fairly well separated after prolonged electrophoresis in the second dimension of 'fingerprints'. However they could not always be resolved sufficiently to allow the separate determination of their molar yields. This was possible in the case of treated 'bound' 5.8S rRNA.

(c) In 'fingerprints' where electrophoresis in the second dimension was extended, it was sometimes possible to resolve these two products sufficiently to obtain molar yields for each. This was possible in all experiments with treated 'bound' 5.8S rRNA.

Table 7.1

Spot No.	Sequence	Molar yields			
		Control	bisulphite treated 'unbound' 5.8S rRNA	bisulphite treated 'bound' 5.8S rRNA	
T1	Gp	11.15	11.12	12.47	
T2	C-Gp	6.20	4.31	4.36	
T2a	C-U-U	0.91	0.32	0.74	
T3	A-Gp	2.36	2.59	2.96	
T4	C-A-Gp	1.04	0.95	1.02	
T5	A-A-Gp	1.01	1.04	1.01	
T6	C-C-C-C-Gp	0.45	0.48	0.41	
T7	A-A-C-Gp	1.44	0.91	0.82	
T8	U_m-Gp	0.21	0.16	0.15	
T9	U-Gp	1.99	3.67	3.46	(a)
T10	Ψ-Gp	1.02	1.17	1.09	
T10a	pC-Gp	0.42	0.16	0.15	
	pGp	0.12	0.07	0.16	
T11	U-C-Gp	1.92	1.64	1.63	
T12	C-Ψ-Gp	1.03	0.54	0.50	
T13	A-U-Gp	1.15	1.12	1.15	
T14a	C-U-C-Gp	2.08	0.96	0.83	(b)
T14b	C-C-U-Gp			0.88	
T15	C-U-A-Gp	1.06	0.31	0.30	
T16	C-U-A-C-Gp	0.67	0.44	0.53	
T17	U-C-U-Gp	1.10	0.99	0.98	
T18	C-A-C-U-U-Gp	0.91	0.42	0.42	
T19.1	A-U-C-A-U-C-Gp	0.87	0.58	0.63	
T19.2	A-U-C-A-C-U-C-Gp	1.82	0.28	0.28	(c)
T20	A-C-A-C-U-U-C-Gp			0.59	(c)
T21	A-C-A-C-A-U-U-Gp	0.87	0.55	0.58	
T22	U-U-C-C-U-C-C-C-Gp	0.80	0.11	0.13	
T23	A-C-U-C-U-U-A-Gp	1.86	0.95	1.09	
T23a	A-A-U-U-G_m-C-A-Gp				
T24	A-A-U-U-A-A-U-Gp	0.84	0.84	0.84	

Table 7.2 Sequences and molar yields of new uridine-containing products arising from the sodium bisulphite reaction with 'bound' and 'unbound' 5.8S rRNA; T_1 ribonuclease digest.

The conventions and reaction conditions were as for Table 7.1. In the examples shown, the uridine-containing products of the bisulphite reaction migrate to distinctive positions in the 'fingerprints' and can therefore readily be analysed and quantified.

Spot No.	Sequence	Molar yields	
		bisulphite treated 'unbound' 5.8S rRNA	bisulphite treated 'bound' 5.8S rRNA
T10b	U-U-U	0.56	0.04
T36	A-A-U-Gp	0.72	0.64
T41	U-U-Gp+U-Ψ-Gp	0.84	0.98
T40	U-U-A-Gp	0.68	0.79
T42	C-U-A-U-Gp	0.18	0.27
T39	U-U-U-Gp	0.95	0.31

Table 7.3 Sequences and molar yields of pancreatic ribonuclease products from 'bound' and 'unbound' HeLa cell 5.8S rRNA after treatment with sodium bisulphite at 25°C.

The conventions and reaction conditions are as in Table 7.1. The molar yields of oligonucleotides after sodium bisulphite treatment were calculated from the distribution of ^{32}P between pairs of products representing C → U conversions, e.g. G-A-A-Cp plus G-A-A-Up. The values shown are the means of those obtained by various methods of quantification.

Table 7.3

Spot No.	Sequence	Molar yields		
		Control	bisulphite treated 'unbound' 5.8S rRNA	bisulphite treated 'bound' 5.8S rRNA
P1	Up + Ψp	19.00	25.01	23.42
P2	Cp	19.80	16.59	17.51
P3	A-Cp	5.68	4.70	3.86
P4	pCp	0.46	0.21	0.12
P5	G_m-Cp	0.92	0.53	0.34
P6	G-Cp	8.06	6.29	5.98
P7	G-A-Cp	4.72	1.92	1.78
P8	A-G-Cp		1.05	1.34
P9	G-A-A-Cp	0.98	0.70	0.49
P10	A-Up	2.47	3.99	3.78
P11	A-A-Up	1.06	1.16	1.05
P12	G-G-Cp	1.91	1.69	1.72
P13	G-A-G-Cp	0.97	0.69	0.13
P14	A-G-G-A-Cp	0.98	0.62	0.77
P15	G-A-A-G-A-A-Cp	0.77	0.67	0.58
P16	G-Up + G-Ψp	5.45	6.72	6.57
P17	G-A-Up	2.23	2.29	2.26
P18	G-A-A-Up	1.12	1.36	1.48
P20	G-G-Up	0.95	1.29	1.07
P21	G-G-A-Up	0.83	1.29	1.65
P22	G-A-G-A-A-Up	0.94	0.90	0.88
P23	G-G-G-G-Cp	0.45	0.48	0.47
P24	G-G-G-Up	0.76	0.73	0.74
P25	G-G-U_m-G-G-A-Up	0.20	0.15	0.25

Table 7.4 Sequences and molar yields of new uridine-containing products of the sodium bisulphite reaction with 'bound' and 'unbound' 5.8S rRNA; pancreatic ribonuclease digest.

The conventions and reaction conditions were as for Table 7.3.

Spot No.	Sequence	Molar yields	
		bisulphite treated 'unbound' 5.8S rRNA	bisulphite treated 'bound' 5.8S rRNA
P16a	G_m-Up	0.32	0.26
P16b	pUp	0.26	0.44
P17a	A-G-Up	1.52	1.93

(a) Differences in extent of reactivity of unique oligonucleotides containing single cytidine residues

C107 This cytidine residue is found within the unique oligonucleotide G-A-A-Cp, P9. When 'unbound' 5.8S rRNA was treated with sodium bisulphite, C107 exhibited 29% reactivity (Table 4.3). Treatment of 'bound' 5.8S rRNA results in a 51% decrease in the molar yield of G-A-A-Cp (Table 7.2) and a 40% increase in the molar yield of the derivative product G-A-A-Up, P18 (Table 7.2). This signifies a reactivity of approximately 45% for C107. From this it can be concluded that C107 is slightly more reactive as a result of 5.8S - 28S rRNA interaction.

C152 In the model of 5.8S rRNA previously discussed, this cytidine residue is thought to form a mismatched pair with U5 in helix (a). This inference is supported by the facts that product P13, G-A-G-Cp, which contains C152, is reduced by 29% after bisulphite treatment (Table 4.3), and that there is a concomitant rise in P21, G-G-A-Up. By contrast, when 5.8S rRNA is bound to 28S rRNA and subjected to similar sodium bisulphite treatment, there is a decrease of 0.84 moles (87%) in the yield of G-A-G-Cp and an increase of 0.82 moles in the yield of G-G-A-Up. This result demonstrates that C152 is more available for bisulphite reaction as a result of the interaction of 5.8S rRNA with 28S rRNA.

C157 When'unbound' 5.8S rRNA is treated with sodium bisulphite, this cytidine residue, which is near to the 3' terminus, shows considerable reactivity (64%) judged by both the diminution of product T2a, C-U-U , and the appearance

of a corresponding derivative product, U-U-U (Table 4.1 and 4.2). When 'bound' 5.8S rRNA is treated with bisulphite, product T2a, C-U-U, was reduced by only 19% and the derivative product, U-U-U, was found in very low yield (0.04 moles). Therefore when 5.8S rRNA interacts with 28S rRNA, C157 is much less reactive.

(b) Differences in extent of reactivity of unique oligonucleotides containing multiple cytidine residues

C19, C21, C23, C98, C100, C103 These cytidine residues are located within products T19.2 and T20 which migrate to similar positions on T_1 ribonuclease 'fingerprints'. These two products can often be resolved by extending the second dimension of electrophoresis to at least 40 h (Plate 7.2). Bisulphite treatment of 'unbound' 5.8S rRNA greatly reduced the molar yields of both these products (> 80%) indicating the presence of at least one highly reactive cytidine within each oligonucleotide. (In product T20, C98 can be inferred to be unreactive from the pancreatic ribonuclease product, P7. The reactivity of T20 must be due to C100 and/or C103.)

When 'bound' 5.8S rRNA is similarly reacted with bisulphite the molar yields of both of these oligonucleotides are significantly greater than those from 'unbound' 5.8S rRNA. T19.2 is recovered in a yield of 0.28 moles and T20 in a yield of 0.56 moles.

C26, C28, C142, C143 These residues are encompassed by the isomeric T_1 ribonuclease digestion products C-U-C-Gp (T14a) and C-C-U-Gp (T14b) which

tend to overlap in 'fingerprints', but are fairly well resolved after prolonged separation in the second dimension (see Plate 7.2). Reaction of sodium bisulphite with 'unbound' 5.8S rRNA results in the disappearance of about 50% of the total material from the 'fingerprint' (Table 4.1). For the reasons discussed (Chapter 4), C26 was attributed a reactivity of 26%, C28 approximately 60% and C142 and C143 a combined reactivity of about 30%. The data shown in Table 7.1 indicate that both of these oligonucleotides are less reactive when 5.8S rRNA is bound to 28S rRNA.

Product T14a occurs in a yield of 0.83 moles after treatment of 'bound' 5.8S rRNA (Table 7.1). Most of this reactivity can be attributed to C26 which can be shown to be about 15% reactive from the pancreatic ribonuclease data. This cytidine residue is found in product P12, G-G-Cp, which is reduced by 0.2 M after reaction; the uridine-containing conversion product G-G-Up (P20) exhibits a 0.12 M increase in molar yield. Therefore C28 can be inferred to be largely unreactive. Under the conditions prevailing during 5.8S - 28S rRNA interaction, C142 and C143 can also be shown to be largely unreactive since T14b occurs in a yield of 0.88 moles after bisulphite reaction.

7.3 Comparison of reactivities of cytidine residues from regions of 'bound' and 'unbound' 5.8S rRNA.

Having discussed differences in reactivities of cytidine residues, it is now possible to relate the findings to various structural aspects of 5.8S rRNA.

(a) Loop regions.

Loops III, IV and VI contain cytidine residues which exhibit approximately

the same reactivities in 'bound' and 'unbound' 5.8S rRNA. In loop III, C52 reacts by the same extent (about 70%) in both cases. Loop IV contains C78, which is next to a 2'-O-methyl guanine. In the unbound state, this cytidine is moderately reactive (about 43%) judged by the disappearance of product P5, G_m-Cp. When 5.8S rRNA interacts with 28S rRNA, this methylated product is reduced by over 60%. However, since this reduction in molar yield is not matched by a concomitant rise in the yield of the derivative product, G_m-Up (P16a, Table 7.4), there is probably no significant difference in the reactivity of C78 in 'bound' and'unbound' 5.8S rRNA. The pentanucleotide loop VI encompasses part of oligonucleotide T22 which contains C127 and C128. These appear to be highly reactive in both forms of 5.8S rRNA.

These results imply that the environments of the cytidine residues in loops III, IV and VI are not altered by the interaction of 5.8S rRNA with 28S rRNA. It can be inferred, tentatively, that these cytidine residues do not interact directly with 28S rRNA.

In contrast, the cytidine residues in loops I and V are less reactive in 'bound' than in 'unbound' 5.8S rRNA judged by the reactivity of oligonucleotides T19.2 and T20. Loop V also contains C107 which is slightly more reactive as a result of 5.8S - 28S rRNA interaction.

It may be inferred that the environments of some or all of the cytidine residues in these loops are altered by interaction with 28S rRNA. Such alteration may arise in either of two ways, direct interaction of one or more cytidine residues with 28S rRNA or internal rearrangement of the 5.8S rRNA secondary structure. One suggested internal rearrangement is described in Chapter 8.

(b) Helical regions

As in the loop regions, in the helical regions some cytidine residues exhibited similar reactivities in 'bound' and free 5.8S rRNA, whereas others exhibited altered reactivities. The cytidine residues within helices (c), (d) and (e) are reactive to the same extent in both 'bound' and 'unbound' 5.8S rRNA. This indicates that they are conformationally unchanged as a result of the interaction of 5.8S and 28S rRNAs. No significant difference in the reactivity was recorded for any cytidine residue within these helices. This is not the case for helices (a) and (b). In helix (b) most of the cytidine residues react to the same extent in 'bound' and 'unbound' 5.8S rRNA judged by the molar yields of the T_1 ribonuclease digest products T11, T19.1 and T18 (Table 7.1). The only differences, as already mentioned, involve the reactivities of C26 and C28 (product T14a). Within helix (a), differences were detected in the reactivities of at least three cytidine residues (C142 and/or C143, C152 and C157). However, the other cytidine residues within this helix, for which reactivities could be determined, exhibit no significant differences between 'bound' and'unbound' 5.8S rRNA. These included C1 (products T10a and P4), C4 and C6 (product T23), C140 (product T16) and C155 (product T11) (Tables 7.1 and 7.3). It can be inferred from these findings that helices (a) and (b) undergo conformational changes as a result of the 5.8S - 28S rRNA interaction .

It is worth noting at this point that interaction between 5.8S and 28S rRNA did not significantly stabilise labile G-C pairs at the ends of helical regions. At the ends of helix (c), the reactivities of C45 (product P15) and C56 (product T12) are not significantly altered and at the end of helix (d), the reactivity of

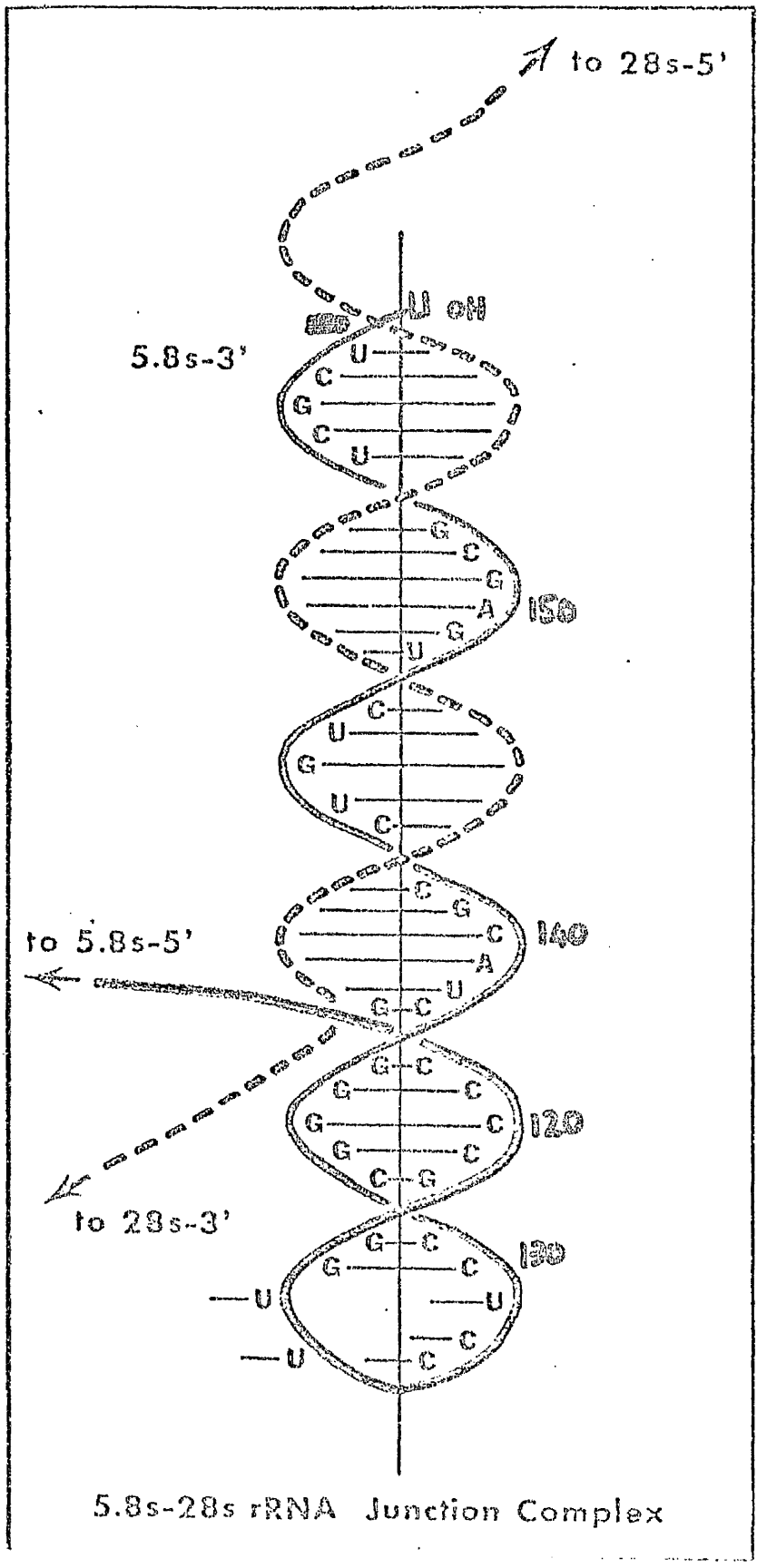
C83 (product T21) is unchanged. Similarly, at the end of helix (b), C92 (product T19.1) shows no difference in reactivity between 'bound' and 'unbound' 5.8S rRNA.

7.4 Relationship of findings to the conformation of 'bound' 5.8S rRNA

The results described above provide a basis for a comparison of the conformation of 'bound' 5.8S rRNA as compared to 'unbound'. Results previously obtained using sodium bisulphite and carbodiimide as probes of the conformation of 'unbound' 5.8S rRNA demonstrated that its structure is similar to that suggested by Nazar et al. (1975), with some minor modifications. On the basis of this model and of the reactivities of the cytidine residues within 'bound' 5.8S rRNA described above (section 7.1 - 7.3), it is possible to consider the nature of the interaction between 5.8S rRNA and 28S rRNA.

This problem has been considered recently by other workers. Pace et al. (1977), using mouse L-cells, proposed that nucleotides 139-159 in the sequence are involved in binding 5.8S rRNA to 28S rRNA. This suggestion followed the partial digestion of a reconstituted 5.8S - 28S rRNA complex which had been formed by annealing radioactively labelled 5.8S rRNA to unlabelled 28S rRNA from which 5.8S rRNA had been removed by heating and fractionation. A ribonuclease resistant fragment which was isolated was found to include 42 nucleotides from the 3' end of 5.8S rRNA; however, it was proposed that only the 20 or 21 nucleotides closest to the 3' end are associated by complementary base interaction with 28S rRNA. The other 21 - 22 nucleotides (116 - 138 in the sequence), which form helix (e) and loops VI in the 'unbound' 5.8S rRNA,

Fig. 7·1 The proposed 5·8S -28SrRNA Junction Complex (Pace et al., 1977)
The 3' - terminal 20-21 nucleotides of 5·8SrRNA are depicted as associated by complementarybase interaction with 28SrRNA. The 21-23 nucleotide intramolecular hairpin is aligned coaxially on the 5·8S-28SrRNA duplex. The numbers refer to the position of the respective nucleotide in the sequence of 5·8SrRNA.



were invisaged to maintain an intramolecular hairpin loop which stacks upon, and hence stabilises the RNA double helix formed by the 5.8S - 28S rRNA association (Fig. 7.1).

The 21 - 22 nucleotides which are proposed to interact with 28S rRNA to form the junction complex are located in helix (a) in 'unbound' 5.8S rRNA. The results presented in Tables 7.1 - 7.4 show that there are three oligonucleotides from this region which exhibit different reactivities towards sodium bisulphite in 'bound' and 'unbound' 5.8S rRNA. The 3' terminal oligonucleotide C-U-U, which contains C157, is highly reactive in 'unbound' 5.8S rRNA, but exhibits little reactivity during interaction with 28S rRNA. C142 and C143 which had a combined reactivity of 30% in 'unbound' 5.8S rRNA (product 14b, C-C-U-Gp), possess little, if any, reactivity in 'bound' 5.8S rRNA. In contrast, C152 (product P13, G-A-G-Cp) which was moderately reactive (29%) in the isolated 5.8S rRNA, is highly reactive (87%) in 'bound' 5.8S rRNA. This is consistent with an exposed conformation in which the cytidine residue is not hydrogen bonded to another base.

When these data are considered together with that of Pace et al. (1977), it is possible to consider more fully the nature of the junction complex. The model is based on the assumption that when 5.8S rRNA interacts with 28S rRNA, helix (a) dissociates and nucleotides 139 - 159 form this junction. The labile nature of helix (a) at 25°C and 37°C has been demonstrated by sodium bisulphite and carbodiimide modification studies (Chapters 4, 5 and 6). It is possible that helix (a) dissociates on interaction with 28S rRNA to form a more energetically stable conformation. This interaction is somewhat complicated. It leads to

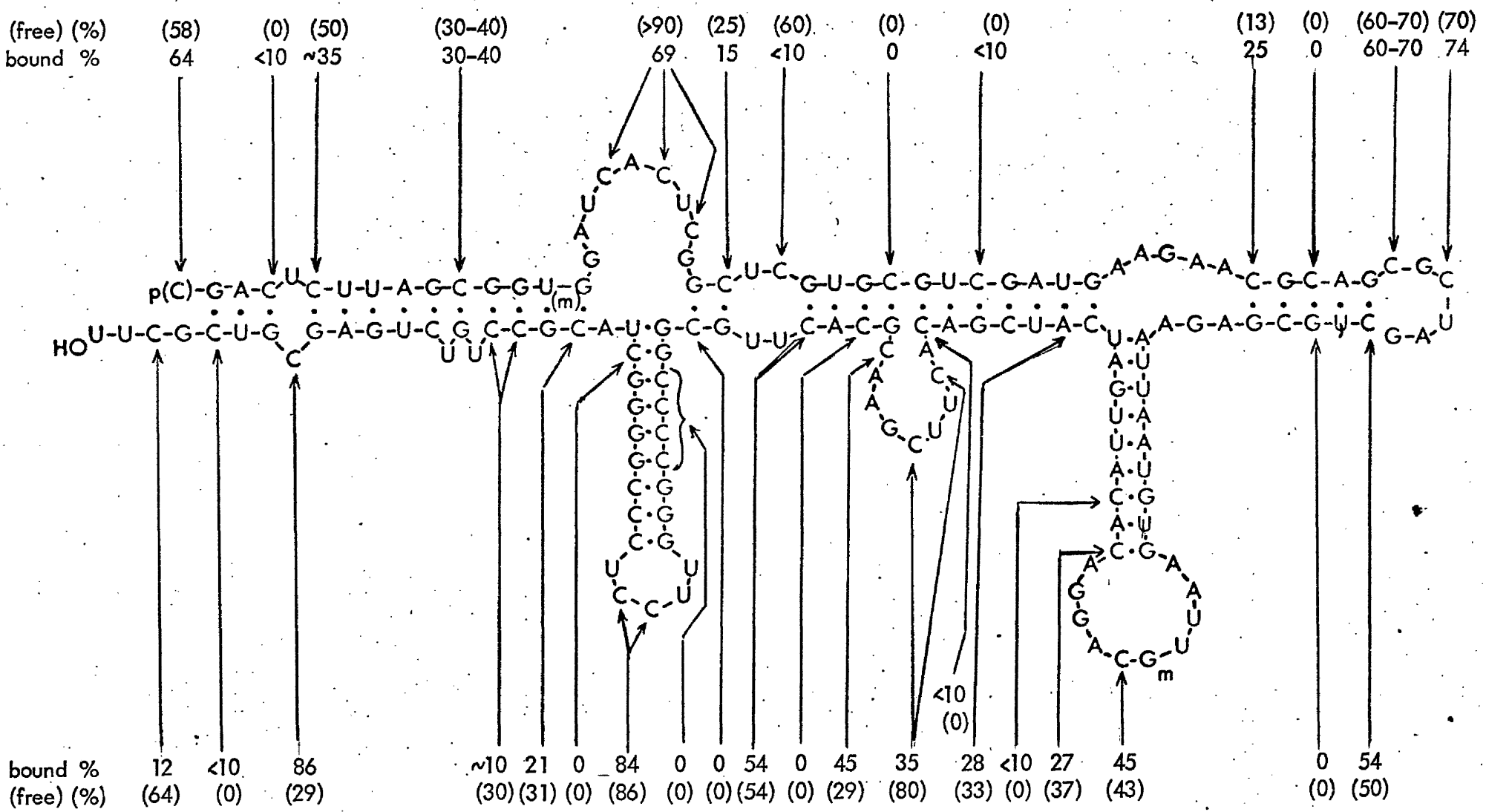
C157, C142 and C143 becoming more protected and to C152 becoming more exposed. There is no evidence that any other cytidine residue in the sequence 139 - 159 maintains an exposed conformation during interaction with 28S rRNA.

These results indicate that the conformation of the junction complex shown in Fig. 7.1 is a useful model on which to base further studies. However, the interactions involved in maintaining the structure are more complicated than shown in the model.

There are three other regions of the 5.8S rRNA molecule which contain cytidine residues that exhibit changes in reactivity as a result of complex formation with 28S rRNA. The T_1 ribonuclease product T20, A-C-A-C-U-U-C-Gp, which forms part of loop V, exhibits less reactivity as a result of interaction with 28S rRNA. In contrast the other cytidine residue in this loop, C107 (product P9, G-A-A-Cp), is slightly more reactive. These changes probably reflect small intramolecular rearrangements effecting the accessibility of the cytidine residues towards sodium bisulphite. The cytidine residues within loop I are located in T_1 ribonuclease product T19.2, A-U-C-A-C-U-C-Gp. At least one of these cytidine residues is less exposed as a result of interaction with 28S rRNA. Helix (b) maintains the same conformation in 'bound' and 'unbound' 5.8S rRNA over most of its length. The exception involves the region adjacent to loop I, where C26 and C28 were significantly reactive in 'unbound' 5.8S rRNA (25% and > 60% respectively). However, in 'bound' 5.8S rRNA, C26 can be shown to exhibit about 15% reactivity (product P12, Table 7.3) and C28 shown to be almost unreactive (product T14a, Table 7.1), thereby reflecting a significant change in the environment of these residues.

Fig. 7.2 Reactivity at 25°C of cytidine residues within 'bound' and 'unbound' 5.8S rRNA.

This model is used to compare the reactivities (%) of cytidine residues towards sodium bisulphite, after 24 h treatment at 25°C of 'bound' and free 5.8S rRNA. The arrows indicate the extent of reaction of cytidine residues within 'bound' 5.8S rRNA; the values given in brackets refer to the reactivities of cytidine residues within 'unbound' 5.8S rRNA.


(free) (%) (58) (0) (50) (30–40) (>90) (25) (60) (0) (0) (13) (0) (60–70) (70)
bound % 64 <10 ~35 30–40 69 15 <10 0 <10 25 0 60–70 74
HO
p(C)-G-A-C-U-C-U-U-A-G-C-G-G-U-G
(m)
U-U-C-G-C-U-G
G-A-G-U-C-G-C-C-G-C-A-U-G-C-G-U-U-C-A-C-G-C-A-G-C-U-A-C-U-A-A-G-A-G-A-G-C-G-U-C-G-A-U
bound % 12 <10 86 ~10 21 0 84 0 0 54 0 45 35 28 <10 27 45 0 54
(free) (%) (64) (0) (29) (30) (31) (0) (86) (0) (0) (54) (0) (29) (80) (33) (0) (37) (43) (0) (50)
<10
(0)

The results presented in Tables 7.1 - 7.4 also indicate that several regions of the 5.8S rRNA molecule are not involved directly in the interaction with 28S rRNA. As has already been discussed (Chapter 7.3), loops III, IV and V and helices (c), (d) and (e) maintain the same conformation in 'bound' and 'unbound' 5.8S rRNA as judged by the accessibility to sodium bisulphite of the cytidine residues they encompass (Fig. 7.2). It is therefore unlikely that they have a major role in the interaction between 28S and 5.8S rRNA.

There is one region of the 5.8S rRNA which has so far not been discussed as regards its conformation in 'bound' 5.8S rRNA; namely nucleotides 1 - 15 in the sequence. These are involved in helix (a) in 'unbound' 5.8S rRNA. Neither the data from Pace et al. (1977), nor from the experiments described in this chapter, provide conclusive evidence on the conformation of these nucleotides. Since it would be energetically unfavourable for this region to exist in a single-stranded conformation, there are two likely possibilities. Either some nucleotides from this region interact with 28S rRNA to further stabilise the complex or they interact with other regions of 5.8S rRNA. In 'bound' 5.8S rRNA, the four cytidines from this region have reactivities ranging from 0 - 65%. Clearly more information is required before it becomes useful to speculate further.

Discussion

8.1 The Validity of Techniques

The aim of this project was to investigate the conformation of HeLa cell 5.8S rRNA. This was carried out firstly using 5.8S rRNA which had been separated from 28S rRNA and then using the intact 5.8S - 28S rRNA complex. The approach adopted was to use the chemical reagents sodium bisulphite and carbodiimide as probes of the structure. Several criteria must be met if these chemical probes are to be effective tools for this investigation.

(a) The chemical reaction should not lead to breakage of the RNA chain.

(b) There should be no disruption of secondary or tertiary structure.

(c) The reaction should be easily followed analytically.

(d) To simplify analysis, the reagent should exhibit high selectivity to one type of base so that the pattern of modification can be more easily elucidated.

These criteria are met by both reagents used in this study.

(a) After chemical modification, the 5.8S rRNA could be shown to be intact by rerunning on a sucrose gradient or on a polyacrylamide gel.

(b) Investigation of the chemical reactivity of nucleotides within RNA molecules has proven to be an effective probe of conformation. This was shown convincingly for yeast $tRNA^{Phe}$ using carbodiimide (Robertus et al., 1974; Rhodes, 1975). The results from chemical modification studies on conformation corresponded with the X-ray crystallographic data.

(c) The reaction of both reagents can be easily followed analytically by coupling the technique with 'fingerprinting' procedures.

(d) Under the conditions used sodium bisulphite reacted specifically with cytidine residues and carbodiimide had a high specificity for uridine residues.

Using these two reagents it was possible to obtain estimates of the reactivities of most of the cytidine and uridine residues within HeLa cell 5.8S rRNA.

8.2 Secondary structure of 'unbound' 5.8S rRNA at 25°C

A detailed analysis of the results has already been made (Chapters 4 and 5). The main conclusion to be drawn is that at 25°C, the data fit the general features of the secondary structure model proposed for mammalian 5.8S rRNA by Nazar et al. (1975). (The relationship of the data to other possible models of 5.8S rRNA conformation is discussed later.) The reactivities observed for all the cytidines and uridines, except for the few for which estimates were not obtained, fit well with this model, with certain qualifications.

(a) Cytidine residues in G-C pairs at the end of helices are appreciably reactive. Similarly, the one A-U 'closing' pair at the end of helix (d) exhibits reactivity. Several cytidines forming G-C pairs adjacent to helix imperfections also exhibited some reactivity. By contrast, base pairs internally located within helices are resistant to chemical modification.

(b) The conformation of loop III remains unresolved. As was discussed in detail in Chapter 3, ambiguities in the nucleotide sequence of this area have arisen. If the sequence proposed by Nazar et al. (1975) is correct, the data in Chapter 4 suggest that loop III contains six nucleotides rather than four. However, if the sequence proposed by Boseley et al. (1978) is correct, then the reactivity data indicate that loop III contains four nucleotides.

(c) The uridine residue , U138, is highly reactive towards carbodiimide. This indicates that it is unlikely to be involved in forming a 'closing' pair with G116

as proposed in the model.

(d) Within helix (d), many of the uridine bases exhibit partial reactivity towards carbodiimide. It is interesting to note that Nazar et al. (1975) found no region of this helix which was subject to ribonuclease digestion at low concentration. One possible reason for the present finding might be the relative deficiency of G-C pairs in the helix. The 'loose' conformation of this helix, as reflected by reactivity with carbodiimide, may also be accentuated by the high pH (8.9) at which the reaction was carried out. Although the stability of nucleic acid helices is relatively insensitive to pH up to 9.0 (Bloomfield et al., 1974), it decreases above this pH. The reason being that at high pH the coil form of the nucleic acid conformation is ionised more readily than the helical form so that the helix-coil equilibrium is shifted toward the coil.

Comparison of data with S_1-nuclease sensitivity

A previous study of 'unbound' 5.8S rRNA using S_1-nuclease as a probe detected three exposed regions in the molecule, namely loop IV, loop VI and the 3' terminal group C-U-U. However other features described in Chapters 4 and 5 such as loops I, II, III and V and the helical imperfections, were not detected (Khan and Maden, 1976). There is a possible explanation for these findings.

The chemical reagents used are indicators of the conformation of individual nucleotides, whereas S_1-nuclease is specific for phosphodiester bonds. Phosphodiester bonds do not necessarily reflect the conformation of their adjacent bases and vice-versa. When tRNA was treated with S_1-nuclease (Rushizky et al., 1975), only the anticodon loop and the 3' end were specifically cleaved. Other

regions of the molecule which are accessible to chemical modifying reagents are not sensitive to cleavage by S_1-nuclease; such a situation probably occurs within 5.8S rRNA.

8.3 The secondary structure of 'unbound' 5.8S rRNA at 37°C and 50°C.

The results obtained on the conformation of 5.8S rRNA at 37°C and 50°C using sodium bisulphite and carbodiimide as probes have been discussed in Chapter 6. The question remaining unanswered concerns the relationship of these findings to the function of 5.8S rRNA.

At 37°C, the temperature at which the molecule functions in vivo, the conformation is similar to that at 25°C, except that helices (a) and (d) are less stable and loop I appears to be larger. The instability of helix (a) at this temperature may result from the presence of helical imperfections. This region has been proposed to be involved in the interaction with 28S rRNA and its unstable conformation may be important at some stage in this process.

At 50°C, the situation is different in that all but two of the helical regions (c and e) have completely dissociated. It may be concluded that those regions of the molecule that retain their conformation at 50°C almost certainly retain the same conformation in the intact ribosome. Hence it is possible to rule out these regions as sites for Watson-Crick base-pairing with 28S rRNA.

8.4 Alternative 5.8S rRNA structure

Recently several alternative structures of 5.8S rRNA have been published. Most are similar to Nazar et al. (1975), having only slight differences. However a 'cloverleaf' secondary structure for eukaryotic 5.8S rRNA has been proposed by Luoma and Marshall (1978) which differs extensively. This model was based on

data obtained from laser Raman studies on yeast 5.8S rRNA. By extending these results to rat hepatoma 5.8S rRNA, the authors were able to propose a secondary structure for mammalian 5.8S rRNA which differed significantly from that proposed by Nazar et al. (1975). However this new model is incompatible with the data presented in this thesis. With reference to Fig. 8.1, the reasons are given below.

(i) Inconsistences with sodium bisulphite data at 25°C.

(a) C28 was shown to exhibit 60% reactivity after 24 h treatment at 25°C. This means that it is unlikely to be located within a helix as shown in Fig. 8.1.

(b) C45 was found to be only 13% reactive after bisulphite modification. Unless it is involved in tertiary interactions, the reactivity of this residue is not consistent with its conformation as proposed in this model.

(c) C47 At 25°C and 37°C this cytidine was completely unreactive and at 50°C it showed only slight reactivity. Therefore, it is very improbable that C45 could exist within a loop region as shown in Fig. 8.1.

(d) C50 This cytidine residue has been demonstrated to be about 60% reactive after reaction with bisulphite. This is approximately double what would be expected from its location at the end of a helix as proposed in this model.

(e) C52 The product (T15) which contains this nucleotide is diminished by 66% after bisulphite treatment. This is consistent with a cytidine residue in an exposed loop region of the 5.8S rRNA molecule, unlike the conformation in Fig. 8.1.

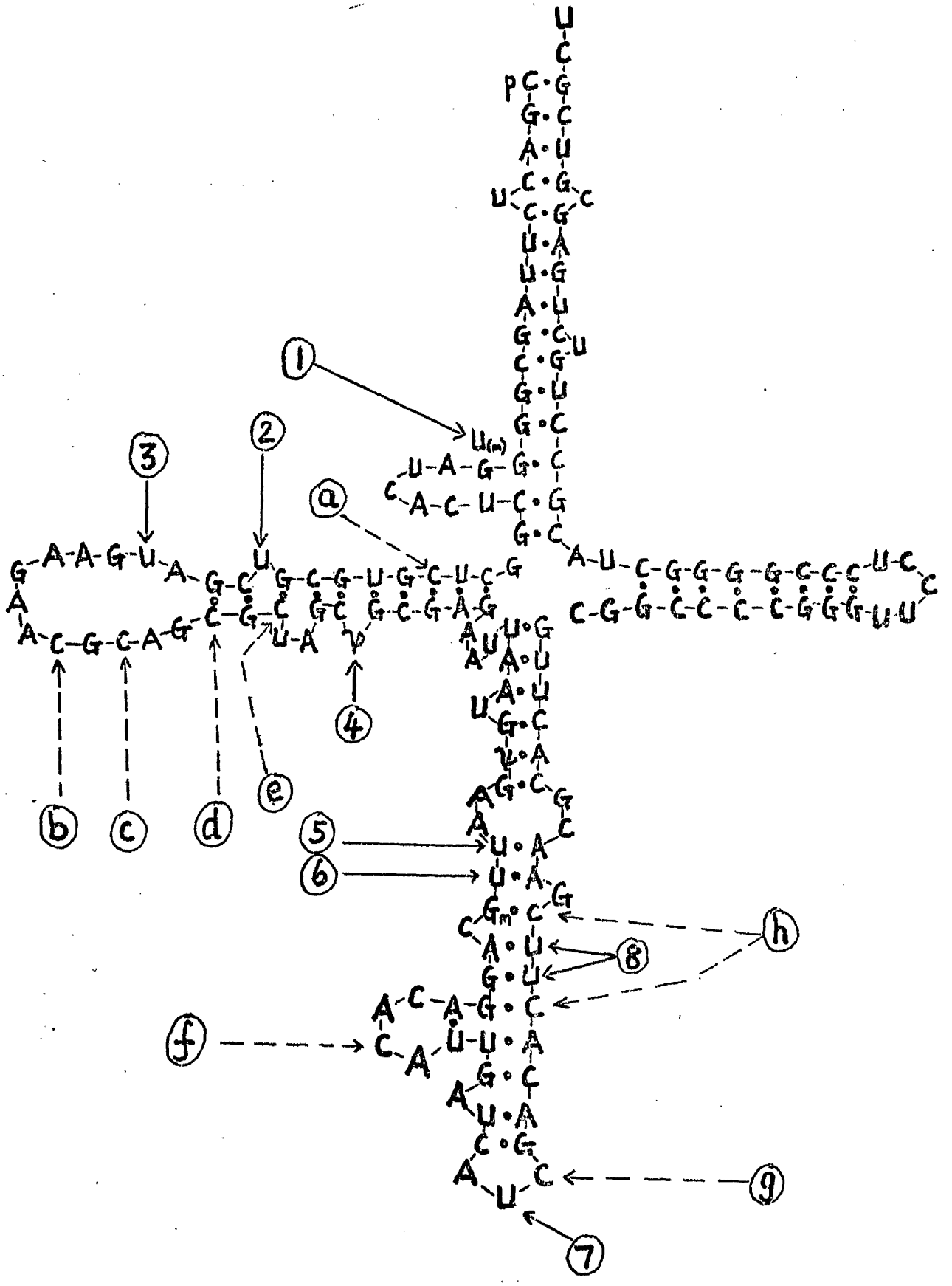
(f) C85 At 25°C, C83 was shown to be unavailable to react with bisulphite.

Fig. 8.1. A new cloverleaf secondary structure for rat hepatoma 5 8S rRNA

This model was recently proposed by Luoma and Marshall (1978). He La cell 5·8S rRNA differs from that of rat hepatoma only by the existence of an extra uridine residue at the 3' terminus. There are several features of this model which are incompatible with the chemical modification data presented in this thesis. These inconsistencies have been indicated as follows:-

(i) Sodium Bisulphite data. Cytidine residues whose reactivities are incosistent with their position within the structure are indicated by broken arrows. The letters (a-h) refer to the text.

(ii) Carbodiimide data. Uridine residues are similarly indicated by unbroken arrows. The numbers (1-8) refer to the text.


1
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Unless C85 is involved in tertiary interactions, the result cannot be compatible with this model.

(g) C95 This cytidine residue is non-reactive. In the model of Nazar et al. (1975), this residue is located within a helix, which is consistent with the data. However in this new model, C95 is assumed to be in a loop region. Considering the result, this seems unlikely.

(h) C100, C103 These two cytidine residues are responsible for the very high reactivity (> 80%) of product T20. The location of C100 and C103 within a helix, as proposed in this model is not consistent with this finding.

(ii) Inconsistencies with carbodiimide data at 25°C

(1) $U_{(m)}14$ This partially methylated uridine was shown to exhibit no significant reactivity after 16 h treatment with carbodiimide at 25°C. It is therefore unlikely that it exists in the conformation shown in Fig. 8.1.

(2) U34 was shown to be non-reactive and therefore probably exists internally located within a helix. The conformation suggested in this model, however, did not agree with this proposal.

(3) U38 After reaction with carbodiimide, this nucleotide was found to be 25% reactive. This is lower than expected for a uridine in a loop region as shown in Fig. 8.1 unless this uridine is involved in tertiary interactions.

(4) Ψ57 This nucleotide occurs in a very stable region of 5.8S rRNA, as demonstrated by its lack of reactivity at 50°C. This being so, the conformation of Ψ57 shown in this new model is probably incorrect.

(5) U75 was shown to be 62% reactive. A reactivity of this extent would be

unlikely if the secondary structure of 5.8S rRNA was similar to that shown in Fig. 8.1.

(6) U76 This uridine residue was shown to be very reactive (84%) after carbodiimide treatment. This result is inconsistent with the position of U76 within a helix as proposed in this model.

(7) U94 is unreactive towards bisulphite at 25°C. This indicates that it is unlikely to exist in a loop region as shown in Fig. 8.1.

(8) U101, U102 These two uridines have a combined reactivity of about 75%. Therefore it is improbable that they occur within a helix as suggested in this model.

As can be seen, several nucleotides which were proposed to be within helices exhibit high reactivity and several nucleotides which were proposed to be within loops have no reactivity. Although tertiary interactions can lessen the reactivity of nucleotides within loops, they do not reduce them to zero. In view of so many inconsistencies with the chemical modification data, it seems probable that this model is wrong.

Two other models for 5.8S rRNA conformation have been proposed recently (Ford and Mathieson, 1978). These models, for Xenopus 5.8S rRNA, were proposed after computer analysis of the nucleotide sequence using the rules of Tinoco et al. (1971) to find the most energetically favourable conformation. The most stable of these structures is similar to the model of Nazar et al. (1975) in many aspects except for the conformation of two areas of the nucleotide sequence, 16 - 43 and 92 - 107. The conformation of the first of these areas is not inconsistent with the chemical modification data; however the conformation

of the second area (92 - 107) is incompatible. Since the conformations of both these regions are interdependent, i.e. they must both exist together or not at all, the conclusion to be drawn is that this structure is not feasible, at least in HeLa cell 5.8S rRNA. Of all models so far considered, that of Nazar et al. (1975) is the most consistent with the chemical modification data.

8.5 The structure of 'bound' 5.8S rRNA

Although the rationale behind the experiment used in this investigation was straightforward, several technical problems were encountered; mainly problems of purification and isolation. A method of obtaining pure bisulphite-treated 'bound' 5.8S rRNA was developed using sucrose gradients. The possibility of using electrophoretic gels to purify this RNA was considered, but the sucrose gradients yielded material of adequate purity.

Pace et al. (1977) adopted another approach which might have proved useful in this study. The difficulties in purification arose due to 'nicks' in the 28 S rRNA producing fragments of a similar size to 5.8S rRNA. Since both 28S and 5.8S rRNAs were radioactively labelled, the resultant 'fingerprints' of treated, 'bound' 5.8S rRNA contained many impurities. This problem was solved by Pace et al. (1977). They isolated radioactively labelled 5.8S rRNA, and unlabelled '5.8S free' 28S rRNA and then reannealled the two RNA species. Any breakdown fragments from 28S rRNA would therefore not be seen on 'fingerprints'. This approach though, brings its own problems; namely the discovery of the most favourable reannealling conditions and the subsequent testing of the artificial complex to ensure the conformation is correct. These problems were successfully overcome by Pace et al. (1977). The artificial

complex was shown to exhibit the same temperature dependence of denaturation as the normal 28S - 5.8S rRNA complex.

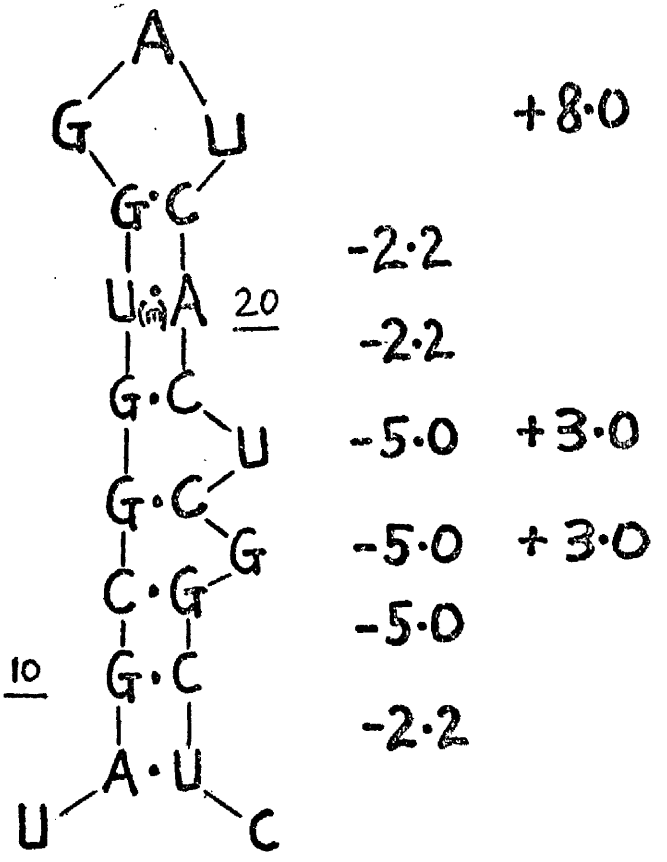
The results described in Chapter 7 provide data that are complementary to those of Pace et al. (1977). From both sets of data a model of the structure of 'bound' 5.8S rRNA can be proposed. The main feature of this model is the junction complex formed with 28S rRNA by nucleotides from the region 138 - 159 of the sequence. The techniques used in the chemical modification approach are sensitive enough to provide information on the conformation of individual nucleotides. Thus it was shown that at least one nucleotide (C152) from the sequence 138 - 159 is not directly involved in interactions maintaining this junction.

There are no conclusive data from which it is possible to directly infer the conformation of nucleotides 1 - 27 in the sequence. In 'unbound' 5.8S rRNA these nucleotides are located in helix (a) and loop 1, but in 'bound' 5.8S rRNA there is no such association. However, it is clear from the data that this region does not remain in a single-stranded conformation in 'bound' 5.8S rRNA (products T23, T19.2 and T14a, Table 7.1). This region must either be involved in further interactions with 28S rRNA or with other regions of the 5.8S rRNA molecule. One such possibility could be the formation of a helix and hairpin loop involving nucleotides 9 - 27 as shown in Fig. 8.2.

Tinoco et al. (1973) have devised rules for estimating the most stable structure of an RNA molecule from its sequence. Using these rules, it is possible to calculate the free energy of this structure as -7.6 Kcal. The existence of this helical structure within 'bound' 5.8S rRNA is therefore energetically feasible, although other conformations may be possible.

Fig. 8.2

A possible secondary structure of nucleotides 9-27 in 'bound' 5.8SrRNA.



This method takes account of contributions from sequence dependent base paired regions, helix imperfections and the hairpin loop to the free energy of this possible structure. ΔG (25°C) = -7.6KCal. The numbers unlined correspond to the positions in the nucleotide sequence. The numbers to the right of the helix correspond to the free energy (KCal) of various aspects of the structure.

Using these same rules, the free energy of helix (a) in 'unbound' 5.8S rRNA was calculated to be -15.8 KCal. Therefore within 'unbound' 5.8S rRNA this helix is an energetically more favourable structure than the helix shown in Fig. 8.2.

8.6 Possible relationships between the structure of 'bound' and 'unbound' 5.8S rRNA

There is evidence for the existence of intermediates in the maturation of 5.8S rRNA in both yeast and HeLa cell (Trapman et al., 1975; Khan and Maden, 1976). The 5.8S rRNA sequence is transcribed in the primary gene product before the 28S rRNA sequence. Therefore it might be expected that this 'pre-5.8S rRNA' sequence will assume a secondary structure before processing and attachment to 28S rRNA. Within this precursor, the 5.8S rRNA sequence may have a conformation similar to that proposed in this thesis, based on the model of Nazar et al., (1975). However, after association with 28S rRNA, the data when considered with those of Pace et al. (1977) indicate that the 5.8S rRNA alters conformation such that most of the nucleotides from 138 - 159 are involved in forming a junction with 28S rRNA.

It is now possible to summarise a hypothetical scheme for the maturation of 5.8S rRNA and its interaction with 28S rRNA. After transcription, the 5.8S rRNA sequence, probably within a precursor, exists in the conformation suggested for 'unbound' 5.8S rRNA with helix (a) intact. The association with 28S rRNA would be brought about by the unfolding of helix (a) and the formation of an energetically favourable junction complex with 28S rRNA by nucleotides from the region 138 - 159. The other nucleotides from helix (a) may also interact with

the 28S rRNA or other areas of the 5.8S rRNA molecule; for example, to form a helix and associated hairpin loop as shown in Fig. 8.2. These alterations in the structure of this region of 5.8S rRNA could result in slight changes in other regions of the molecule, such that nucleotides (for example, in loop V) become more or less accessible to the modifying reagent.

8.7 The function of 5.8S rRNA

The function of 5.8S rRNA is unknown. However, observations that the sequence is highly conserved suggest it is important to ribosome function. In all 5.8S rRNAs where the sequence is known, and in 5.8S rRNA from flowering plants, where partial sequence data are available, there are two sequences complementary to G-T-Ψ-Cp. This sequence is common to all tRNA molecules with the exception of eukaryotic initiator tRNA's where the sequence is A-U-C-Gp (Erdmann,1976). The complementary sequences in 5.8S rRNA are A-G-A-A-Cp at position 41 - 45 and Y-G-A-A-Cp at position 103 - 107. This finding has led to the suggestion that 5.8S rRNA is involved in the binding of tRNA to the ribosome (Erdmann, 1976).

In prokaryotes, this function has been attributed to 5S rRNA (Forget and Weismann, 1967). This rRNA species also contains a sequence complementary to the G-T-Ψ-Cp sequence located in loop IV of the cloverleaf structure of tRNA. There is considerable evidence supporting the binding of tRNA to the large subunit via interaction with 5S rRNA. For example, the oligonucleotide T-Ψ-C-Gp inhibits the non-enzymic binding of tRNAs to prokaryotic ribosomes (Shimuzi et al., 1970). More directly the T-Ψ-C-Gp 5S rRNA interaction was demonstrated with a specific 5S rRNA-protein complex, with interaction being destroyed by

N-oxidation of two adenosines in the 5S rRNA (Erdmann et al., 1973). It was suggested by Dube (1973) that in prokaryotes, one G-A-A-Cp sequence in 5S rRNA is used in P-site binding of initiator tRNA and the other G-A-A-Cp sequence is used in A-site binding. Brownlee et al. (1968) had shown that this sequence occurs twice in E. Coli 5S rRNA.

It has been assumed that prokaryotic and eukaryotic 5S rRNAs have identical functions; however, recently several observations have argued against this. A survey of sequence analysis shows that eukaryotic 5S rRNAs do not possess the sequence G-A-A-Cp at a specific site. Instead the conserved sequence Y-G-A-Up is located at a specific site (Barrel and Clark, 1974). This sequence is complementary to A-U-C-Gp, which is found in loop IV only in eukaryotic initiator tRNAs. There are also large differences in the sequences of eukaryotic and prokaryotic 5S rRNAs. These differences are such that eukaryotic 5S rRNAs cannot be successfully incorporated into bacterial ribosomes, whereas all prokaryotic 5S rRNAs so far tested can be incorporated (Nomura and Erdmann, 1970; Bellemore et al., 1973).

It seems possible that eukaryotic 5S rRNA plays a role in the initiation phase of protein synthesis by interacting with initiator tRNA. The question then arises as to which other rRNA the G-T-ψ-Cp sequence of amino acyl tRNA interacts at the ribosomal A-site. Since 5.8S rRNA contains two G-A-A-Cp sequences, it would seem a good candidate for this function in eukaryotic ribosomes, similar to that of 5S rRNA in prokaryotic cells. The presence of two G-A-A-Cp sequences in 5.8S rRNA raises the possibility that not only does it assist in binding aminoacyl-tRNA, but it could also assist in translocation.

If 5.8S rRNA indeed carries out these functions, it would be logical if the two relevant sequences occurred in exposed regions within 'bound' 5.8S rRNA. In HeLa cell 5.8S rRNA, the G-A-A-Cp sequences occur at positions 42 - 45 and 103 - 106 in the nucleotide chain. These are both in regions of the 5.8S rRNA molecule secondary structure not directly involved in association with 28S rRNA and they are both found in loop regions (loops II and V of 5.8S rRNA). In loop II, G42. A43 and A44 are located within the loop, whereas C46 forms a 'closing' pair with G60 at the start of helix (c). It is interesting to note that within the tRNA sequence, G-T-ψ-Cp, the guanine residue with which C46 would bind, is in an analogous position in the secondary structure of tRNA. All the nucleotides in the sequence 103 - 106 are located within loop V of 5.8S rRNA.

With knowledge of the conformation of these sequences, it is possible to confirm that 5.8S rRNA could perform the proposed roles within the ribosome without large structural alterations. Studies of 5.8S rRNA function are likely to yield more information when systems for the in vitro reconstruction of the eukaryotic large subunit are developed.

8.8 Relationship between 5.8S rRNA and 5S rRNA

Recently reconstitution experiments showed that two E. Coli 5S rRNA-binding proteins, L18 and L25, form a specific complex with yeast 5.8S rRNA, but not with yeast 5S rRNA. This yeast 5.8S rRNA-E. Coli protein complex was found to exhibit ATPase and GTPase activities that had been previously observed for the E. Coli 5S rRNA-protein complex (Wrede and Erdmann, 1977). This suggests that 5.8S rRNA has evolved from prokaryotic 5S rRNA and that these two RNAs perform a similar function in protein synthesis.

In prokaryotes, the genes coding for 5S, 16S, and 23S ribosomal RNA are linked in a polycistronic transcriptional unit (Pace, 1973). In eukaryotes, the analogous transcription unit contains the genes for 5.8S, 18S and 28S rRNA, whereas the 5S rRNA genes are generally found elsewhere in the genome. The analogous positions occupied by prokaryotic 5S rRNA genes and eukaryotic 5.8S rRNA genes in their respective genomes prompted Doolittle and Pace (1971) to speculate that these two RNA classes might have a common evolutionary origin.

If this was so, one would expect their sequences to bear some relationship to each other. Cedergen and Sankoff (1976) have demonstrated that no such relationship exists; there are no sequences in any 5S rRNA which show any relationship to any known 5.8S rRNA sequence. On this basis it can be suggested rRNA does not share a common ancestor with 5S rRNA and therefore did not arise by duplication of the 5S rRNA gene. One interesting possibility is that 5.8S rRNA evolved from the intercistronic region of the prokaryotic ribosomal genome.

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Appendix I.

The calculation of (%) reactivities of pyrimidine residues after chemical modification.

All the examples given refer to the reaction at 25°C.

(a) Sodium Bisulphite reaction

(i) Where an oligonucleotide is reduced, but no uridine-containing reaction product is characterised, e.g. product T22 (U-U-C-C-U-C-C-C-Gp).

$$(\%)\ \text{Reactivity} = \frac{0.80 - 0.11}{0.80} \times 100\% = 86\%$$

Molar yield of T22 before reaction = 0.80

" " " " after " = 0.11

(ii) Where an oligonucleotide is reduced and a new uridine-containing reaction product is isolated, e.g. T2a (C-U-U) and T10b (U-U-U).

The reactivity calculated from the reduction of the molar yield of T2a is calculated as for T22, i.e.

$$(\%)\ \text{Reactivity} = \frac{0.91 - 0.32}{0.91} \times 100\% = 65\%$$

The reactivity obtained from the molar yield of the reaction product (T10b) is calculated as follows:

$$(\%)\ \text{Reactivity} = \frac{0.56}{0.91} \times 100\% = 62\%$$

Molar yield of T2a (C-U-U) before reaction = 0.91

" " " " " " after " = 0.32

" " " T10b (U-U-U) " " = 0.56

Therefore, average reactivity - 63.5%

(iii) Where an oligonucleotide is reduced and the uridine-containing product reinforces the molar yield of another oligonucleotide already present in the 'fingerprint', e.g. P9 (G-A-A-Cp) and P18 (G-A-A-Up). The reactivity calculated from the reduction in the molar yield of P9 is calculated as for T22, i.e.

$$(\%)\ \text{Reactivity} = \frac{0.98 - 0.70}{0.98} \times 100\% = 29\%$$

The reactivity obtained from the increased molar yield of the reaction product (P18) is calculated as follows:

$$(\%)\ \text{Reactivity} = \frac{1.36 - 1.12}{0.98} \times 100\% = 25\%$$

Molar yield of P9 (G-A-A-Cp) before reaction = 0.98

" " " P9 " " after " = 0.70

" " "P18 (G-A-A-Up) before " = 1.12

" " "P18 " " after " = 1.36

Therefore, average reactivity = 27%

(b) Carbodiimide reaction

The (%) reactivities were calculated from the reduction in the molar yields of uridine-containing oligonucleotides in a manner similar to that explained for T22 in the sodium bisulphite reaction.

1. Why study 5.8S rRNA?

There are several reasons why structural/functional studies of 5.8S rRNA should interest those investigating the mechanism of protein synthesis or those engaged in the study of molecular genetics. In the following pages I will discuss 5.8S rRNA from both standpoints.

(a). 5.8S rRNA and the ribosome.

The functions of ribosomalRNA within the ribosome have yet to be elucidated. To gain an insight into some of the functions a structural study of the small 5.8S rRNA component was carried out using the technique of chemical modification. Together with results from other studies which involved enzymic (Khan and Maden, 1976, Nazar et al, 1975) and physical techniques (Van et al, 1977) it has been possible to confirm most of the features of the structural model proposed by Nazar et al. (1975) for isolated 5.8S rRNA. Such techniques are not always practical as tools for a similar investigation of the larger rRNAs. In future it may also be possible to further investigate the structure of isolated 5.8S rRNA using X-ray crystallographic analysis as has been done for tRNA (Robertus et al 1974). These studies should provide a basis for investigating the nature of the junction of 5.8S rRNA and 28S rRNA within the ribosome. Recent studies have shown that 5.8S rRNA can be removed from the large ribosomal subunit under similar conditions used to remove it from 28S rRNA(Giorgini and DeLucca, 1976; Nazar, 1978). It is therefore likely that 5.8S rRNA occurs near the surface of the ribosome and that its interactions with proteins are weak when compared to those with 28S rRNA. Under the conditions which 5.8S rRNA is released from the large subunit no other components are removed. This process may therefore be useful in 5.8S rRNA functional studies since it yields an intact large subunit less the 5.8S rRNA component. It should be possible to test these large subunits for such functions as poly(U)-directed phenylalanine synthesis or to assay for peptidyltransferase activity by the puromycin reaction (Cox and Greenwell, 1976). However, since no such experiments have been reported one must conclude that the conditions necessary for 5.8S rRNA removal irreversibly inactivate at least one ribosomal component. This problem may be tackled by other methods such as reconstitution experiments which have been used successfully with prokaryotic ribosomes, but the technical problems are likely to be immense. So far there have been few successful results reported from reconstitution experiments involving functional, intact eukaryotic ribosomes.

Although interactions between 5.8S rRNA and ribosomal proteins are probably weak, it has been demonstrated that 5.8S rRNA will specifically bind to various ribosomal proteins from the large subunit (Metspalu et al, 1978). Rat liver 5.8S rRNA was immobilised via 3^1-end ribose adipic acid - hydrazide - epoxy - activated Sepharose 6B and ribosomal proteins passed through the column. The bound proteins were identified on a two dimensional gel electrophoresis system and shown to be L5, L6, L7 and L18. Since some of these ribosomal proteins also bind 5S rRNA (L6, L7 and L18), the conclusion drawn was that

5S and 5.8S rRNA are in close proximity within a functional ribosome. It has also been demonstrated that yeast 5.8S rRNA will bind to E. Coli ribsomal proteins L18 and L25 (Wrede and Erdman, 1977). These proteins normally form a specific complex with E. Coli 5S rRNA, but have been shown not to interact with yeast 5S rRNA. This was taken to mean by the authors that 5.8S rRNA had evloved from prokaryotic 5S rRNA, that the two RNAs are related and have similar functions in eukaryotic protein synthesis. Therefore, the fact that rat liver 5.8S and 5S rRNAs bind to common ribosomal proteins may be more related to function than to their topography within the large subunit.

In summary, 5.8S rRNA is a useful model for the study of functional and structural aspects of rRNA. It is possible to investigate its interaction with other rRNAs and with ribosomal proteins and since it can be easily released from the large ribosomal subunit, it may be possible to investigate ribosome function in the absence of the 5.8S rRNA component.

(b). 5.8S rRNA and the ribosomal genes.

A mammalian ribosomal gene contains the sequences which code for 18S, 5.8S and 28S rRNA. These sequences are arranged within tandemly repeated units which in human DNA are approximately 20×10^6 daltons molecular weight (Arnheim & Southern, 1977). Hybridisation experiments have indicated that a human haploid genome contains about 300 of these repeat units (Jeanteur & Attardi, 1969) and that they are located in several chromosomes, numbers 13, 14, 15, 21 and 22 (Evans et al, 1974). However, different individuals may have different amounts of total rDNA, and have different amounts of rDNA in each of these chromosomes.

The DNA within ribosomal repeat units can be sub-divided into two categories; (1) the rRNA coding regions, and (2) the spacer regions. No sequence heterogeneities have been detected in ribosomal gene products from individuals of the same species. There is also a high degree of inter-species sequence homology within rRNA; for example, mammalian 5.8S rRNAs have identical sequences apart from the 5^1- nucleotide. In contrast, within the spacer regions, there is much greater heterogeneity both within and between species. The non-transcribed spacer within Xenopus laevis rDNA can vary from about 2.7KBP to about 9.0KBP (Wellaver et al, 1976). However, these spacers vary less in sequence than in the number of short repeat sequences (as few as 15 nucleotides) which they contain. Higher orders of periodicities of up to 750 nucleotides also occur (Botchan et al, 1977). Analyses of human and mouse ribosomal genes have demonstrated heterogeneity in the structure of the ribosmal repeat units within individuals (Arnheim & Southern, 1977).

It is clear that rRNAs have identical functions within mammalian ribosomes; it is therefore hardly surprising that the sequence of 5.8S rRNA has been highly conserved during evolution. A rRNA which contained a mutation deleterious to ribosome function would be rapidly eliminated by natural selection. However, within spacer regions, where sequence is probably less crucial, sequence changes or deletions will arise more rapidly simply because more mutations can be retained (Smith, 1973).

It would be true to say that one important factor in species differentiation other than the genes which an individual possesses, is the way in which they are controlled and expressed. Within the spacer regions there may be sequences which control the timing of gene expression, the co-ordination of that expression with the expression of other gene products (e.g. ribosomal proteins and rRNA) and the extent of gene expression. A factor which may be involved in 'driving' sequence changes in spacer regions, could be the use of chromosomal exchanges as a means of producing duplications and deletions (Smith, 1973). Duplications and deletions arising from unequal crossovers may provide a more rapid and common route of sequence divergence than single base mutations. Genetic exchanges between chromosomes involving ribosomal genes have been detected in mouse (Arnheim & Southern, 1977), Xenopus laevis (Wellaver et al, 1976) and Drosophila (Ritossa, 1973). Because of this higher rate of sequence changes, the spacer regions may play an important role in speciation and evolution.

The availability of restriction enzymes has enabled areas of the rDNA to become accessible to sequence analysis. The sequence of the 5.8S rRNA gene in Xenopus laevis and the regions to the 5^1 and 3^1 ends of the gene have recently been reported (Boseley et al, 1978). Further analytical studies of this kind should provide useful data on the control mechanisms of ribosomal genes. It may also shed light on an interesting problem; Is the 5.8S rRNA coding region genetically part of the 28S rRNA gene separated by an inserted sequence, or does it have separate genetic identity having evolved indenpendently? When further information is available on sequences at the 5^1-end of the E. Coli 23S rRNA gene it may be possible to detect some homologies with sequences found in 5.8S rRNA genes. A finding such as this would strongly indicate that the 5.8S rRNA gene is genetically part of the 28S rRNA gene and does not have a common evolutionary origin with the prokaryotic 5S rRNA gene.

In summary it is important to the molecular geneticist to gain information on the functions of 5.8S rRNA. This information should allow correlation with results obtained from sequence analysis of the spacer regions surrounding the 5.8S rRNA gene. Such a correlation would be important to an understanding of the control mechanisms within spacer regions. The 5.8S rRNA coding region is also a useful model for studying evolutionary change.

2. What has been learned from this study of 5.8S rRNA?

Before answering this question, it is necessary to discuss the limitations of the techniques used and how they effect the conclusions drawn. Results obtained from chemical modification experiments are limiting in that the data are only directly relevant to the conformation pertaining under the reaction conditions; in the case of the sodium bisulphite reaction this was 3M sodium bisulphite, 10mM $MgCl_2$, pH6.0. One must extrapolate from these data to gain an understanding of the structure in vivo. However it was shown that this chemical reaction does not cause 5.8S rRNA chain cleavage or disrupt secondary or tertiary structure in tRNA. When the results obtained from carbodiimidemodification (10mg/ml carbodiimide, 0.02M Tris-HCl, pH 8.9 in the presence of 10mM $MgCl_2$) were compared to those obtained from the sodium bisulphite reaction they were

found to be complimentary. This being so, it is possible to say with more confidence that the conformation being probed by the two reagents is similar to that found in VIVO.

As discussed earlier though, the question arises: How relevant is this structure to function? 5.8S rRNA exists as a specific complex with 28S rRNA in the ribosome and further interacts with ribosomal proteins. Some answers have been provided by the chemical modification studies carried out on the 28S-5.8SrRNA complex. It was found that within this complex, those regions of the 5.8S rRNA molecule which do not directly interact with 28S rRNA have similar conformations to that found in isolated 5.8S rRNA. This finding is of possible importance to the understanding of 5.8S rRNA function since it is unlikely that regions which directly interact with 28S rRNA play a major role in 5.8S rRNA function(s). One would expect regions of 5.8S rRNA which are directly involved in function to be free to interact with other components, be they proteins or RNA. For instance, it has been proposed that the sequence G-A-A-Cp which occurs twice in all 5.8S rRNAs so far sequenced may be involved in interaction with tRNA at some point in protein synthesis. Since it has been shown that in 5.8S rRNA, both these sequences are probably in exposed conformations within loop regions it is possible to infer that they would be available for interaction with tRNA. Further interpretation of 5.8S rRNA function from the results would be purely speculative.

Two separate but complimenatry approaches can be adopted for future structural-functional studies of 5.8S rRNA.

(1). Isolated 5.8S rRNA: The conformations of individual nucleotides within 5.8S rRNA could be further characterised by reaction with chemical reagents, e.g. Ketoxal, which reacts specifically with guanine residues. However, detailed information on the tertiary structure of 5.8S rRNA will require X-ray crystallographic analysis. This technique would need an intensive effort, not least in obtaining 5.8S rRNA crystals in which the molecules were packed within a well ordered lattice so that a diffraction pattern could be obtained.

(2). 5.8S rRNA within ribosomes: It is only within an intact ribosome that we can be certain that 5.8S rRNA attains its functional conformation. It may be possible to locate the exact position of 5.8S rRNA within the ribosome by crosslinking studies and to probe its structure by chemical modification. The results obtained from these studies will need close scrutiny and may be difficult to interpret due to the reaction conditions required, e.g. 3M sodium bisulphite, pH6.0. Intact, functional ribosomes do not willingly lend themselves to such treatment.

Another unsolved question is the positioning of the site within the 28S rRNA sequence of 5.8S rRNA interaction. A possible solution to this could be obtained by hybridising labelled 5.8S rRNA to cloned fragments of 28S rRNA genes. This approach could only be successful if the interaction involved a continuous sequence of nucleotides and was not dependent on the secondary structure of 28S rRNA.

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