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THE INTERACTION OF ESCHERICHIA COLI INITIATION FACTOR 3 WITH COLIPHAGE-MS2 RNA

by

Martin John Francis FOWLER

Department of Biochemistry
Imperial College of Science and Technology
London SW7 2AZ

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To the late Maria Szekely

(1925 - 1981)

ABSTRACT

The interaction of Escherichia coli Initiation Factor 3 (IF3) with coliphage-MS2 RNA has been investigated. IF3 protected MS2 RNA against pancreatic ribonuclease digestion to give a protected site that migrated as a distinct band upon polyacrylamide gel electrophoresis under non-denaturing conditions. Homochromatography fingerprinting of this material showed that it was comprised of several species of RNA of different length and nucleotide composition.

Nucleotide sequence analysis showed that the protected site consisted of three non-contiguous regions of MS2 RNA located close together in the primary sequence of the molecule: (1) Region I covered maximally residues 3344 - 3363; (2) Region II covered maximally residues 3367 - 3386; and (3) Region III covered residues 3427 - 3460 and possibly up to residue 3466. The protected site was particularly rich in G residues and was partly located in the most G-rich portion of the MS2 RNA molecule.

Mild heat denaturation of MS2 RNA prior to binding did not significantly alter the site specific binding of IF3; however, studies using partially heat denatured S_1 nuclease fragmented MS2 RNA suggested that additional weak and possibly non-specific binding sites on the RNA were produced by the partial denaturation process. E. coli ribosomal protein S1 reduced the total amount of MS2 RNA protected by IF3, but did not modify the regions of RNA protected as judged by gel electrophoresis.

Studies using two-dimensional gel electrophoresis of the protected fragment suggested that the individual regions of the protected site were strongly held together by secondary structure interactions.

Thus, the IF3 protected site in MS2 RNA appears to consist of three non-contiguous regions of RNA held together by strong secondary and possible tertiary structure interactions to form a single site. On the basis of these observations, a theoretical secondary structure model for the protected site is proposed.

CONTENTS

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ABBREVIATIONS USED THROUGHOUT THE TEXT

Standard abbreviations are used throughout the text. Additional non-standard abbreviations are as defined below.

Miscellaneous:

PA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PNK	polynucleotide kinase

Buffers:

Buffer	Defined in section	Buffer	Defined in section
TAE	3.1.2	PPS	3.3.4
TMS	3.2.2	PPS(50)	3.3.5
TMSG	3.2.3	TMM	3.3.5
TAM	3.2.4	SSC	3.4.2
TAMU	3.2.7	TMA	3.6.1
TS	3.3.1	BAP	3.7.2
KPS	3.3.2	ASZ	3.10

In the list of references:

BBA	Biochim. Biophys. Acta
BBRC	Biochem. Biophys. Res. Commun.
Nature NB	Nature New Biology
PNAS	Proc. Natl. Acad. Sci. (USA)

INTRODUCTION

One of the major problems in contemporary molecular biology concerns the interplay of two key macromolecular species, the proteins and the nucleic acids. Although there has been considerable work in this field, little of a detailed and fundamental nature is known about the type of interactions involved (e.g., Hélène and Lancelot, 1982) and the way that they contribute to the total organisation of biological systems. Indeed, there is no reason to suppose that there should be any general 'rules' for these interactions (Brimacombe, 1978) since, for example, an interaction between DNA and RNA polymerase (e.g., Sierbenlist et al., 1980) is unlikely to have very much in common with the interaction of a ribosomal protein and ribosomal RNA (e.g., Ungewickell et al., 1977). Whilst this may be the case, the characterisation of the various protein : nucleic acid interactions is an essential prerequisite for a detailed understanding of the different mechanisms by which these macromolecules can interact with their varying degrees of specificity.

In this work, the interaction of E. coli Initiation Factor IF3, a protein involved in the initiation of prokaryotic protein synthesis, with the RNA of coliphage MS2, has been investigated. The results from this study, in addition to characterising the interactions between these two molecular species, permits some insight into the interaction of the protein with 16S RNA of the 30S ribosomal subunit where IF3 is functionally active.

1.1 E. COLI INITIATION FACTOR 3

1.1.1 Discovery,physicochemical properties and multiple forms of IF3

In the middle 1960's, the discovery of translation initiation factors in Escherichia coli was independently reported by three groups of workers (Stanley et al., 1966; Revel and Gros, 1966, 1967; Eisenstadt and Brawerman, 1966). It was observed that the washing of ribosomes with 0.5M NH_4Cl caused a considerable decrease in their ability to synthesize polypeptides when natural mRNAs were used. Supplementing the ribosomes with the ribosomal wash restored the original activity. From the 0.5M wash, two initiation factors, IF1 and IF2, were separated and partly purified. Two years later, a third initiation factor, IF3, was isolated from the 1M NH_4Cl wash and was found to be essential for the specific translation of natural mRNA's (Iwasaki et al., 1968; Revel et al., 1968).

Since that time, the initiation factors of E. coli have been purified and their function in the reaction leading to the formation of active 70S initiation complexes extensively characterised (for recent reviews, see: Ochoa and Mazumder 1974; Revel, 1977; Grunberg-Manago and Gros, 1977; Grunberg-Manago et al., 1978; Gualerzi and Pon, 1981; Maitra et al., 1982). For the purpose of this review, suffice it to say that IF1 consists of a polypeptide chain of 71 amino acids (Pon et al., 1979) with an experimentally determined M_r of 8,900-9,400 (Dubnoff and Maitra, 1971a; Dubnoff et al., 1972a; Wahba and Miller, 1974; Lee-Huang et al., 1971; Hershey et al., 1977) and is involved, together with IF3, in the dissociation of 70S ribosomes (cf section 1.1.4 below) and also in the catalytic recycling of IF2 (Chae et al., 1969; Benne et al., 1972, 1973; Dubnoff et al., 1972b; Stringer et al., 1977). For IF2, two forms, IF2a (M_r = 90,000-118,000) and IF2b

(M_r = 82,000-90,000) have been described (Fakunding et al., 1972; Miller and Wahba, 1973; Krauss and Leder, 1975; Hershey et al., 1977) with the latter form probably reflecting an artefact of the isolation procedure due to limited proteolysis of IF2a (Hershey et al., 1977). The role of IF2 in initiation complex formation appears to be to stabilize the interaction of the formylated initiator t.RNA with the ribosome (Maitra et al., 1982).

IF3 has been purified to homogeneity by a number of workers (Iwasaki et al., 1968; Dubnoff and Maitra, 1971b; Wahba et al., 1969; Revel et al., 1970; Sabol et al., 1970; Lee-Huang and Ochoa, 1973, 1974; Sabol and Ochoa, 1974; Hershey et al., 1977) and the reported M_r values for the factor are in the range 21,000-23,500 (Table 1). The sedimentation constant of 2.2S (Dubnoff and Maitra, 1971b) agrees with the M_r estimates (Table 1) and the hydrodynamic properties suggest that the factor has a rather globular shape in solution (Gualerzi and Pon, 1981).

It has been reported (Lee-Huang and Ochoa, 1971, 1973), although not universally found (Dubnoff and Maitra, 1971b; Schiff et al., 1974; Hershey et al., 1977), that E. coli contains at least two forms of IF3 having similar M_r values, but with different isoelectric points, chromatographic behaviour and mRNA discrimination properties. IF3α (M_r 22,500) was reported to catalyse the translation of MS2 RNA preferentially to late bacteriophage T_4mRNA, whereas IF3β (M_r 21,000) had the inverse specificity. Suryanarayana and Subramanian (1977) found that 30S subunits from different strains of E. coli yielded at least two forms of IF3. The primary structures of these have been determined and found to differ only at the N-terminal regions, with one being 6 residues longer than the other (Brauer and Wittmann-Liebold, 1977). The longer form, IF3-1, consists of 181 amino acids and has an M_r deter-

TABLE 1

Molecular weight ratio determinations of E. coli IF3

Method	Species	M_r	Ref
Amino acid sequence:	IF3-1	20668	a
	IF3-s	19997	a
SDS-PAGE:	IF3α	23000-23500	b
	IF3β	21000-21500	b
	IF3	21000	c,d,e
		22200-22800	f
		22600	g
Sedimentation:	IF3	25000	e
Gel filtration:	IF3α	23000	b
	IF3β	21000	b
	IF3	32000	h

References:

a Brauer and Wittmann-Liebold (1977)

b Lee-Huang and ochoa (1973)

c Sabol et al. (1970)

d Dubnoff and Maitra (1971b)

e Dubnoff et al. (1972a)

f Hershey et al. (1977)

g Schiff et al. (1974)

h Gualerzi et al. (1971)

mined by chemical analysis of 20,668 and the shorter form, IF3-s, consists of 175 amino acids and has an M_r of 19,997. In addition, at least one minor form (IF3-1') has been found which differs from IF3-1 by the absence of the first amino acid residue, namely formyl-methionine (Brauer and Wittmann-Liebold, 1977). All three forms were found to be active in phage RNA directed protein synthesis and ribosome dissociation.

The relationship between IF3-1 and IF3-s and IF3α and IF3β is unclear. Whereas the isoelectric points of the larger IF3α and smaller IF3β were found to be 8.3 and 8.4 respectively (Lee-Huang and Ochoa, 1973), the higher molecular weight form IF3-1 was found to be more basic than the smaller IF3-s (Suranarayana and Subramanian, 1977). It has been reported that mild trypsin digestion of IF3-1 gives rise to IF3-s (Gualerzi and Pon, 1981) and it is likely that the latter form arises from proteolytic cleavage of the Arg 6-Val 7 peptide bond during purification.

Computer predictions of the secondary structure of IF3-1 based on the amino acid sequence (Brauer and Wittmann-Liebold, 1977) suggest that the α helix content is in the range 34-37%, β structure 6-8%, turns (or loops) 15-17% and random coil 40-43%. Experimentally determined values obtained from circular dichroism studies were reported to be in good agreement with these values (cited in Brauer and Wittman-Liebold, 1977).

1.1.2 IF3 levels in E. coli and expression of the infC gene

IF3 is present in an approximately equimolar amount with IF1 and IF2 and at a factor : ribosome molar ratio of 0.15-0.2 in cells growing in enriched medium (Howe et al., 1978; Howe and Hershey, 1981). Early studies suggested that IF3 activity in the cell was particularly unstable and was strikingly diminished in the stationary phase (Scheps and Revel, 1972) or upon chloramphenicol treatment (Young and Nakada

1971). However, when Minks et al. (1978) studied the ratios of IF3-1 and IF3-s during the growth cycle of E. coli, they found that the relative ratios of the two forms and the total amount of IF3 relative to the ribosome content were essentially the same in the stationary phase and in mid-log phase. Likewise, Howe and Hershey (1983) found that the IF3 : ribosome ratio was constant at different growth rates and that the IF3 level was approximately proportional to the growth rate. These results suggest that the IF3 gene expression is co-ordinated with those of the ribosomal components.

The gene for IF3 (infC) has been mapped at 38 min. on the E. coli genome and is not contiguous with ribosomal proteins or translation factors (Springer et al., 1979). The nucleotide sequence of a 1.26kb fragment containing the gene has been determined (Sacerdot et al., 1982) and studies have been made on transcription units around the gene (Springer et al., 1982). Expression of the infC gene is apparently not under 'autogenous regulation' as is the case for ribosomal protein and RNA synthesis (Nomura et al., 1982), since IF3 levels are proportional to infC dosage and IF3 added to a cell-free DNA dependent protein synthesis system does not specifically inhibit IF3 synthesis (Lestienne et al., 1982).

1.1.3 Nucleic acid binding properties of IF3

It has been known for some time that IF3 is endowed with a nucleic acid binding capacity (Sabol et al., 1970). Several studies have shown that this factor can bind to single- and double-stranded viral RNAs (Sabol et al., 1970; Kaempfer and Kaufmann, 1973; Jay et al., 1974a), hairpin loop structures (Wickstrom, 1974), synthetic polynucleotides (Wickstrom, 1974; Wickstrom et al., 1980) and DNA (Kaempfer and Kaufmann, 1973). IF3 binds to 16S and 23S ribosomal RNAs (Gualerzi and Pon, 1973) and there is indirect evidence to suggest that the factor binds to the 30S subunit directly via the 16S rRNA moiety rather than through the 30S proteins (Gualerzi and Pon, 1973; Pon and

Gualerzi, 1976) (cf section 1.1.5).

Wickstrom (1974) found that IF3 bound to RNA hairpin loops with 20 or less nucleotides when they contained the sequence A-U-G in an exposed configuration, but not when they were lacking the A-U-G sequence. Additionally, binding of the factor to the trinucleotide A-U-G was observed and it was suggested that IF3 was specifically recognising an A-U-G sequence in an exposed hairpin loop structure. Binding of IF3 to the double-stranded RNA of the phage ϕ6 was shown by Jay et al. (1974a), albeit with a lower apparent affinity than that for R17 RNA. The affinity of the factor for MS2 RNA was found to be less than that for the 30S ribosomal subunit (Vermeer et al., 1973a); however, this binding is sufficiently strong to protect specific fragments of MS2 RNA from RNase digestion (Johnson and Szekely, 1977, 1979) (cf section 1.3.7).

The stoichiometry of the binding of IF3 to the single-stranded polynucleotides poly (A), poly (C) and poly (U) was determined to average 14 ± 1 nucleotides per IF3 molecule (Wickstrom et al., 1980). This is in the same range as that of ribosomal protein S1 binding to poly (C) (15 nucleotides per S1) (Bear et al., 1976; Draper and Von Hippel, 1978) and also that of the lac repressor binding to non-operator DNA (12-15 base pairs per tetramer) (Butler et al., 1977; Revzin and Von Hippel, 1977; Kelsey et al., 1979).

1.1.4 IF3 activities in protein synthesis

IF3 appears to possess two activities in protein synthesis (for recent reviews see: Grunberg-Manago and Gros, 1977; Kurland, 1977; Grunberg-Manago et al., 1978; Bosch and Van der Hofstad, 1979; Gualerzi and Pon, 1981; Maitra et al., 1982). Firstly, the factor acts as a 'dissociation factor' displacing the equilibrium:

$$70S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} 50S + 30S$$

towards dissociation. Secondly, IF3 appears to be required for the efficient ribosomal binding of mRNA during initiation complex formation.

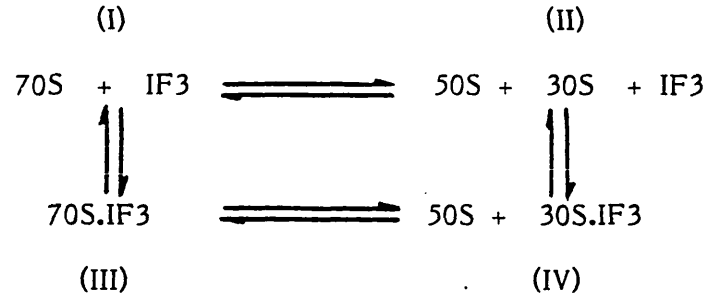
(i) 'Dissociation factor' activity

Two classes of 70S ribosomes can be discriminated by their differences in sensitivity to hydrostatic pressure during centrifugation (Hapke and Noll, 1975). Type-A ribosomes ('tight couples'), prepared by centrifugation in 5mM Mg^{2+}, are most active in protein synthesis and are resistant to hydrostatic pressure, whereas Type-B ribosomes ('loose couples') are more flexible and appear to undergo a conformational change that makes them unfit for reassociation (Van Digglen and Bosch, 1973; Noll et al., 1973; Debey et al., 1975). At physiological Mg^{2+} concentration (4-5mM), type-A ribosomes are nearly all associated (Debey et al., 1975; Noll and Noll, 1976) and, since each initiation event is preceded by dissociation of the ribosome, a requirement for protein factors is explained (Noll and Noll, 1976).

A study on ribosomal equilibrium by Godefroy-Colburn et al. (1975) showed that IF3, by binding to the 30S subunit, caused a decrease in the site constant K_2 for association, but had no effect on the dissociation rate constant K_1. In this 'anti-association' model, the IF3 dissociation action appears to be purely passive, with the factor binding to the 30S subunit which then becomes unfit for reassociation with the 50S subunit. A modulating effect of IF1 was found to be due to it greatly increasing the rate constant K_1 and K_2 of the reaction. Thus, IF1 increases the rate of dissociation of the 70S couples and IF3, by binding to the 30S subunit, prevents their reassociation, thereby displacing the equilibrium towards the dissociated state.

Such an 'anti-association' model is supported by a recent kinetic study (Chaires et al., 1981) and also by the observation that the affinity of IF3 for the 30S subunit is strong (see section 1.1.5) and that at physiological Mg^{2+} concentrations, IF3 does not bind significantly to Type-A 70% couples (Weiel et al., 1978). At high Mg^{2+} concentration (10mM), some interaction with 70S ribosomes may however occur (see section 1.1.6).

In principle, IF3 would act by either of the pathways: (I-III-IV or I-III-IV):



The 'anti-association' model described above would follow the pathway I → II → IV. However, the finding that IF3 may interact with the 70S couple, at least under certain ionic conditions, suggests that under some circumstances the alternative pathway I → III → IV may also apply (Chaires et al., 1982).

A completely different model for IF3 activity in dissociating 70S ribosomes has been proposed by Goss et al. (1980a). In this allosteric-type model, two conformations exist for the subunits and 70S ribosomes and the action of IF3, together with other effectors (Mg^{2+} and spermidine) is to shift the conformational equilibrium. However, doubt has been cast on the validity of this model, since the kinetic data upon which the model was based are in disagreement with those of Chaires et al. (1981) and may have resulted from some modification or inactivation of the subunits during dissociation of ribosomes by treatment in

the presence of 1 mM Mg^{2+} (Chaires et al., 1981).

(ii) Activity in the initiation of protein synthesis

In order to help to understand the activity of IF3 in the initiation of protein synthesis, the sequence of events leading to the formation of the 70S initiation complex (Fig. 1) is briefly summarised below (based on Maitra et al., (1982).

The first step in the formation of the 30S initiation complex involves the co-operative binding of three initiation factors to a 30S subunit to form a 30S.IF1.IF2.IF3 complex (Fig. 1) to which fMet-$tRNA_f^{Met}$ and mRNA specifically bind. The order of addition of these two species to the 30S complex is, however, uncertain with some evidence suggesting that fMet-$tRNA_f^{Met}$ and mRNA may bind independently of one another, whereas other data are compatible with the binding of mRNA preceding that of mMet-$tRNA^{Met}$. IF3 is released from the 30S ribosomes during the binding of fMet-$tRNA^{Met}$ and GTP. Finally, addition of the 50S subunit to the 30S initiation complex results in the formation of the 70S initiation complex, together with the hydrolysis of GTP by ribosome bound IF2 to GDP and Pi and their release, together with IF1 and IF2 from the ribosomal complex.

The results of early studies on the requirement of IF3 during initiation complex formation were somewhat confused and probably reflect to some extent the lack of a standard method for preparing ribosomes (thereby giving rise, for example, to different groups using preparations of ribosomes having different proportions of Type-A and Type-B ribosomes), as well as the degree of purity of the factor preparation. For example, some studies suggested that initiation complex formation in the presence of natural mRNAs required the presence of IF3, whereas synthetic did not, and it was proposed that the factor was probably involved in the direct recognition of some specific messenger starting signals on the mRNA (Iwasaki et al., 1968; Revel et al., 1970; Meier et al., 1973; Vermeer et al., 1973b). The isolation of two

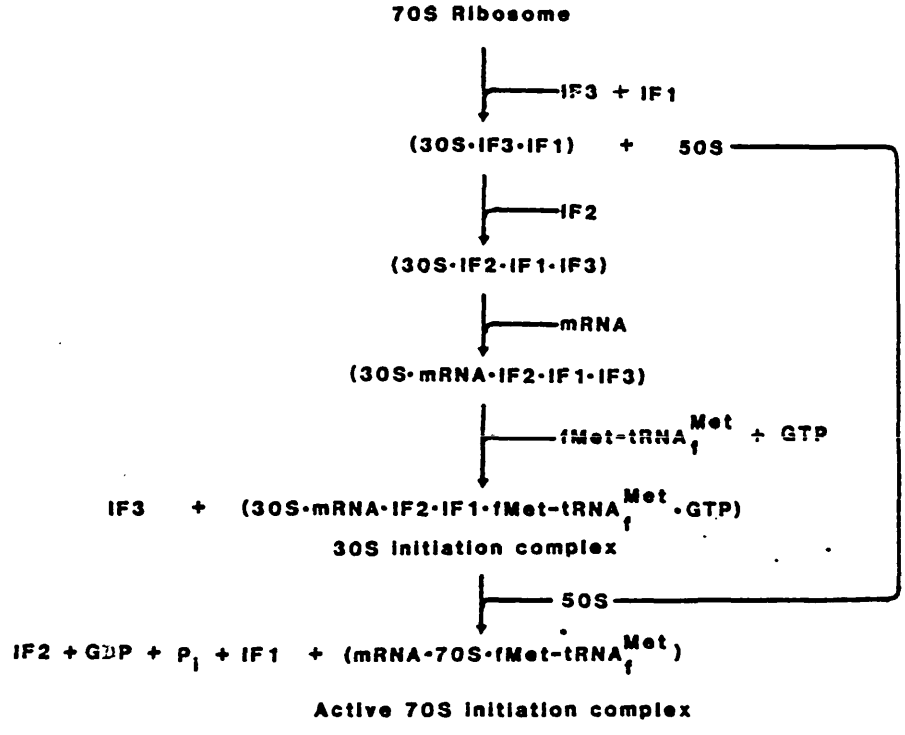


FIGURE 1 Sequence of reactions leading to the formation of an active 70S Initiation Complex. Modified from Maitra et al. (1982).

mRNA discriminating species of IF3 (see section 1.1.1) as well as the observation that different variants of IF3 directed ribosomes to different cistrons in a polycistronic message (Berissi et al., 1971) lent support to this hypothesis. Further purification of the IF3 variant, however, pointed to the existence of a more homogeneous species of IF3 in combination with other proteins, the interference factors, which had either a positive or negative influence on the binding of ribosomes to initiation sites (Groner et al., 1972). One of these factors was later shown to be the ribosomal protein S1 (Wahba et al., 1974).

Other early work suggested that IF3 was essential for initiation complex formation with both natural and synthetic mRNAs (Wahba et al., 1969; Miller and Wahba, 1973; Suttle et al., 1973; Bernal et al., 1974; Dondon et al., 1974; Sobura et al., 1977). Since many of these experiments were performed using 70S ribosomes, the stimulating activity of IF3 could have resulted from an effect on the 70S ribosome - subunit equilibrium, rather than from a specific influence on initiation complex formation. However, at high Mg^{2+} concentration, when IF3 is unable to promote the dissociation of 70S ribosomes, the factor was found to be able to stimulate the translation of synthetic messages (Schiff et al., 1974).

Recently, it has been observed that the requirement of IF3 in the translation of both natural and synthetic messages, is greatly dependent on the concentration of the messenger. Thus, the requirement for IF3 in the translation of synthetic polynucleotides can be made nearly absolute by reducing the concentration of the polynucleotide (Schiff et al., 1974; Gualerzi and Pon, 1981) and, conversely, the requirement for IF3 in the translation of natural mRNAs is not absolute (Benne and Pouwels, 1975; Zipori et al., 1978) and may be overcome by increasing

the concentration of mRNA.

These observations suggest that IF3, rather than being an essential component of the reaction leading to initiation complex formation, may be involved in facilitating an already inherent ability of the 30S subunit to form initiation complexes. The possibility that this function may involve facilitating the recognition by the 30S subunit of some aspect of natural initiation sites that are absent from synthetic mRNAs arises from the work of Steitz et al., 1977. These workers found that ribosomal recognition of the coat and replicase cistron initiation sites on R17 RNA were several-fold more dependent on the presence of initiation factors than was binding to the A-protein initiator region. Likewise, Hershey et al. (1977) found that initiation complex formation with R17 RNA was more dependent on IF3 than was complex formation with A-U-G or poly (A,G,U). A clue to the nature of these signals comes from the observation that the R17 factor requirement found by Steitz et al. (1977) is inversely proportional to the degree of complementarity of the Shine-Dalgarno interaction between the 3' end of the 16S-RNA moiety of the 30S subunit and a purine-rich sequence near the mRNA initiation codon. Such an interaction was proposed by Shine and Dalgarno (1974, 1975) to be important in the recognition by 30S subunits of initiation sites on natural mRNAs and has received experimental support from the demonstration that such annealing could indeed occur (Steitz and Jakes, 1975; Steitz and Steege, 1977). Further support for this hypothesis comes from the observation that oligonucleotides complementary to the 3' end of 16S-RNA could inhibit ribosomal interaction with mRNA, but not with A-U-G or poly-U (Taniguchi and Weissmann, 1978; Eckhardt and Lührmann, 1979), as well as the finding that reducing the complementarity of the Shine-Dalgarno interaction in

the case of the 0.3 gene mRNA of bacteriophage T7 caused a significant decrease in the efficiency of synthesis of gene 0.3 protein in infected cells (Dunn et al., 1978).

Since IF3 appears to exert its action in both the dissociation of 70S ribosomes and initiation complex formation through the 30S subunit, a consideration of the factor's interaction with the 30S subunit is essential before considering the molecular mechanisms involved in these activities.

1.1.5 Topography of the interaction of IF3 with 30S ribosomal subunits

IF3 binds to the 30S ribosomal subunit with high affinity at a stoichiometry of 1 : 1 (Vermeer et al., 1973a) and with a binding constant in the range of 0.5 - 4 x 10^7 M^{-1} (Subramanian and Davis, 1970; Sabol et al., 1973; Godefroy-Colburn et al., 1975; Weiel et al., 1978; Weiel and Hershey, 1981), but not to an extent to modify the hydrodynamic parameters of the subunit. Kinetic studies have shown that in vitro the rate constant for this association is in the region 1.7 x 10^4 to 4.0 x $10^8 m^{-1}s^{-1}$ (Goss et al., 1980b; Woolley and Box, 1979). Whilst excess IF3 has been shown to alter the conformation of the 30S subunit as measured by low angle X-ray diffraction and sedimentation studies (Paradies et al., 1974), equimolar IF3 does not (Beaudry et al., 1976). Likewise, Giri et al. (1979) have reported no detectable change in the hydrodynamic parameters of 30S subunits upon IF3 binding. However, some rearrangement of amino acid side chains may occur (Gualerzi et al., 1975).

Numerous studies have been made using protein-protein and protein-RNA crosslinking reagents, immunoelectron microscopy and chemical modifications of 30S subunits in order to investigate near neighbours of IF3 bound to the subunit (reviewed in Brimacombe et al.,

1976; Grunberg-Manago and Gros, 1977; Grunberg-Manago et al., 1978; Brimacombe, 1978; Gualerzi and Pon, 1981).

By far the most information concerning the 30S proteins at the binding site of IF3 has come from cross-linking studies using either electrophilic reagents (Hawley et al., 1974a; Van Duin et al., 1975; Heimark et al., 1976; Gualerzi and Pon, 1981; Chaires et al., 1982), direct photochemical cross-linking (Mackeen et al., 1980) or by the use of a part electrophilic and part photochemical reagent (Cooperman et al., 1981) (Table 2). Strong overlap is observed between the results obtained using the different cross-linking methods, although it should be noted that with the exception of the work of Cooperman et al. (1981) and Chaires et al. (1982), some proteins may have been cross-linked to the factor by a second protein since bifunctional reagents were used. Cooperman et al. (1981) showed that IF3 could be directly cross-linked to six proteins (S2, S3, S11, S12, S18 and S21) and Chaires et al. (1982), using a different reagent, demonstrated that direct cross-links could be made to two proteins (S9 or S11 and S12). Thus, at least these six proteins are in close proximity to the IF3 binding site.

Immunoelectron microscopy studies on the location of proteins within the 30S subunit, together with the IF3 cross-linking data, allowed Lake (Lake and Kahan, 1975; Lake, 1978, 1980) to infer that IF3 binds in a region that includes part of the platform and the upper one third (Fig. 2a). A closely analogous IF3 binding site has also been proposed by Stöffler and co-workers based on the Berlin model of the 30S subunit (Stöffler et al., 1980; Wittmann et al., 1980). With the exception of S3, antigenic determinants for all of the proteins found to be cross-linked to IF3 lie within the proposed IF3 binding site (Fig. 2b). Since S3 is thought to be an elongated protein (Wittmann et al., 1980), it is

TABLE 2 30S PROTEIN CROSS-LINKED T IF3*

S protein	FMN hν[a]	PNMB-IF3 hν[a]	PNMB hν[a]	Direct hν[b]	DMS[c,d,e]	PDM[e]	TDA[f]	2-IT[g]
1			+	+	+[c]	+		
2		++	+	+				
3		+++	++					
7				++			++	
9								+?
11		+	++	++	++[c] ++[d]	+++		+?
12	++	++	++	++	+[c] +[e]	++		+
13			+		+[c] +[d]			
18		++		++				
19					++[c] +[d]			
21		+		++		+		

For notes - see overleaf

TABLE 2 NOTES

Number of pluses indicate relative amounts of cross-linking where such comparisons can be made.

*	Modified from Cooperman et al. (1981)		
a	Cooperman et al. (1981)	-	all S proteins tested
b	Mackeen et al. (1980)	-	all S proteins tested
c	Heimark et al. (1976)	-	all S proteins tested except S17
d	Gualerzi and Pon (1981)	-	all S proteins tested
e	Hawley et al. (1974a)	-	only S12 tested
f	Van Duin et al. (1975)	-	only S7 identified, although other proteins are cross-linked
g	Chaires et al. (1982)	-	all S proteins tested except S1 and S17. S9 and S11 were poorly resolved and consequently which protein was cross-linked to IF3 was not identified.

Abbreviations:

FMN : flavin mononucleotide

PNBM: p-nitrobenzyl maleimide

DMS : dimethyl suberimidate

PDN : phenylene diazide

TDA : tartaryl diazide

2-IT : 2-iminothiolane

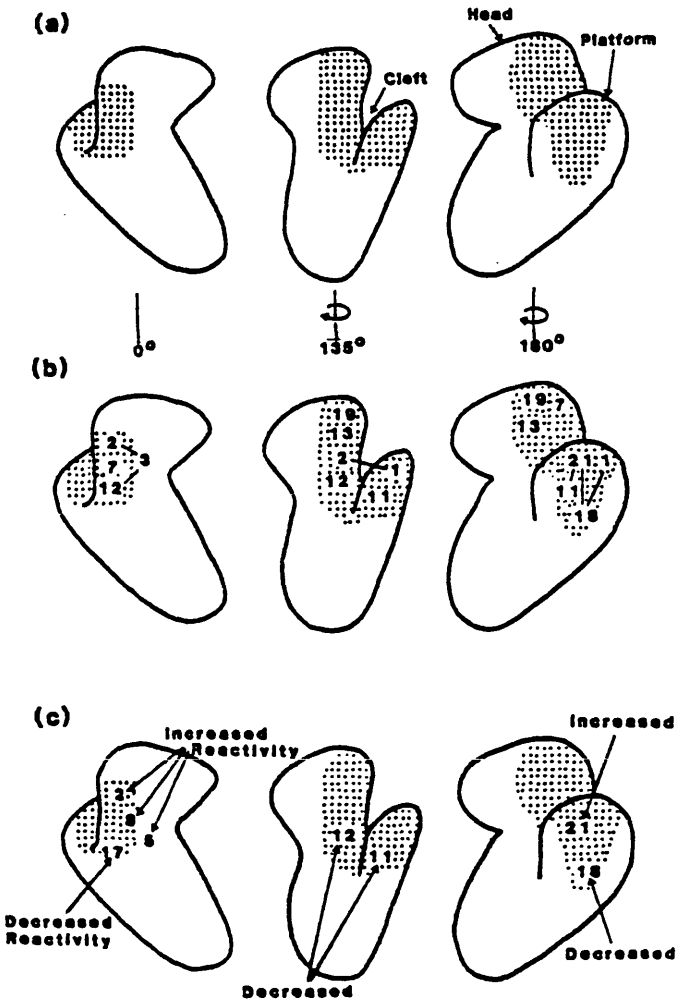


FIGURE 2 Topography of the interaction of IF3 with the 30S ribosomal subunit

(a) Localisation by immuno-electron microscopy. (After Lake and Kahan, 1975).

(b) Locations of antigenic determinants for the 30S proteins cross-linked to IF3. Solid lines are drawn between cross-linked 30S proteins. (After Cooperman et al., 1981).

(c) Locations of antigenic determinants for those 30S proteins with altered chemical modification properties in the presence and absence of IF3 (After Gualerzi and Pon, 1981).

possible that the portion that is cross-linked to IF3 is some distance from the antigenic determinant site. Support for this idea comes from the observation that S3 can be cross-linked with S2 and S12 (Sun et al., 1974; Traut et al., 1980), both of which are mapped within the IF3 binding region, both by cross-linking (Table 2) and by immunoelectron microscopy (Lake and Kahan, 1975; Lake, 1978).

Studies using chemical modification of proteins of 30S subunits, with and without bound IF3, (Ewald et al., 1976; Michalski et al., 1978) show that the proteins which acquire an increased reactivity towards modification following IF3 binding are located on the exterior surface primarily in the upper one third portion of the subunit, or on the subunit interface of the platform (Fig. 2c). Those proteins which are protected from modification are located in the cleft. These observations are in general agreement with those of the cross-linking and immunoelectron microscopy studies of the location of the IF3 binding site (Fig. 2). Gualerzi and Pon (1981) have proposed that IF3 may bridge the platform and upper one third portion of the subunit by inserting in the cleft.

Ribosomal proteins S1 and S21, both of which can be cross-linked to IF3 (Table 2), are in close proximity of the 3'-end of 16S rRNA (Czernilofsky et al., 1975) and the 3'-end of the RNA has been mapped to the same region of the subunit as that of IF3 (Politz and Glitz, 1977; Olson and Glitz, 1979).

The binding of IF3 with normal affinity to 30S subunits depleted of proteins S1, S2, S3, S9, S10, S14, S20 and S21 (Laughrea et al., 1978), as well as those lacking either S1 or S11, S12, S19, S21 and possibly S14 (Pon and Gualerzi, 1976), together with the observation that IF3 binding was inhibited by RNA ligands (Pon and Gualerzi, 1976),

suggests that the factor binds to the 30S subunit through the 16S rRNA moiety (Gualerzi and Pon, 1973; Pon and Gualerzi, 1976). Likewise, the observation by Weiel and Hershey (1981) that increasing the Mg^{2+} concentration decreased the IF3 : 30S association constant 2.5 fold, although in contradiction to earlier studies (Sabol and Ochoa, 1971; Vermeer et al., 1973c; Godefroy-Colburn et al., 1975) is consistent with the notion that the 16S rRNA is an important contributor to the stability of IF3 binding.

Van Duin et al. (1975) showed that IF3 became cross-linked to the 3'-terminus of 16S rRNA when 30S subunits were treated with periodate oxidation and borohydride reduction. However, doubt has been cast on the validity of this result with the observation that many of the 30S proteins can become cross-linked to the RNA by this method (Rinke and Brimacombe, 1978) and may reflect a high degree of flexibility of the 3'-end of the RNA. Laughrea et al. (1978) found that removal of the 3'-terminal 49 nucleotides of 16S rRNA did not prevent the binding of IF3, although up to 10% of the free energy of binding may be contributed by this region of the RNA.

IF3 has been shown to be photochemically cross-linked to 16S rRNA present in 30S subunits, either in the presence of photosensitizers (Cooperman et al., 1977) or by near-ultra violet irradiation alone (Mackeen et al., 1980). Pon et al. (1977) studied the photochemical and formaldehyde cross-linking of the factor to 16S RNA in intact 30S subuits and found that after limited RNase T_1 digestion of the subunits the factor was unequally linked to two sub-particles. The major sub-particle (80-90% of cross-linked IF3) represented the first 900 nucleotides from the 5'-end of the RNA, together with several ribosomal proteins (S4, S5, S6, S15, S18, S20 and S16 or S17). The minor sub-

particle (10-20% of the cross-linked factor) represented the remaining 3'-side of the RNA minus the last 150 nucleotides, together with several proteins (S7, S9, S10, S14 and S19). These studies indicate that, although some interaction of IF3 is seen with the 3'-portion of 16S rRNA, the main interaction takes place at the 5'-portion of the molecule.

The nature of the regions of IF3 that are involved in the interaction with the 30S subunit have also been investigated. Bruhns and Gualerzi (1980) studied the effect of iodination of the factor upon its biological activity and its binding to 30S subunits. Three Tyr residues and one His residue were found to be modified and of these, Tyr-109 was found to be important in the binding of the factor to the subunit. Based on studies of the binding of model oligo-peptides to nucleic acids (Hélène, 1977; Mayer et al., 1979; Hélène and Lancelot, 1982), Bruhns and Gualerzi (1980) proposed that the region of IF3 including Tyr-109 and Lys-112 has the potential information to select single-stranded RNA and to recognise guanine residues both in single- and double-stranded configurations. A hydrophobic region of the factor also appears to be important in binding to the 30S subunit (Box et al., 1981; Pon et al., 1982). The single Cys residue of the factor was found not to be located within the ribosomal binding site (Pon et al., 1982).

1.1.6 The interaction of IF3 with 50S subunits and 70S ribosomes

IF3 has been reported not to bind to the 50S ribosomal subunit (Gualerzi and Pon, 1973; Pon and Gualerzi, 1976). However, when labelled IF3 was added to 50S subunits and followed by periodate oxidation, labelled IF3 was found to be associated with the 23S rRNA moiety (Van Duin et al., 1976). This complex was located in the immediate vicinity of the 3'-terminus of the 23S RNA but, by analogy

with the case of 16s rRNA (Rinke and Brimacombe, 1978), this may merely reflect the high reactivity of the 3'-end of this molecule in this reaction.

Largely from experiments using sucrose gradient centrifugation, it has been shown that IF3 does not appear to interact with the 70S ribosome (Sabol and Ochoa, 1971; Pon et al., 1972; Thibault et al., 1972; Sabol et al., 1973). However, in contrast to these observations, IF3 alters the electrophoretic mobility of 70S ribosomes (Talens et al., 1970), as well as their reactivity to lactoperoxidase (Michalski et al., 1978). IF3 has been shown to be cross-linked to 70S ribosomes at 18mM Mg^{2+} (Hawley et al., 1974b) and an association of the factor with 70S ribosomes following glutaraldehyde fixation has been demonstrated (Vermeer et al., 1973a). Spectroscopic studies suggest that at 10mM Mg^{2+}, IF3 interacts with 70S ribosomes and alters their conformation (Chaires et al., 1982).

Chaires et al. (1982), using the cross-linking agent, 2-iminothiolane, found that at 10mM Mg^{2+} concentration, IF3 could be cross-linked to proteins L2, L5 and L17 of purified 50S subunits and likewise, Schwartz (cited in Chaires et al., 1982) found that the factor could be photochemically cross-linked to proteins L2, L4, L27 and L33. Immunoelectron microscopy has suggested that proteins L2, L5, L10 and L27 are located at the interface of the 50S and 30S subunits (Cover et al., 1981) and cross-links can be formed between several of the 50S and 30S proteins in the vicinity of the IF3 binding sites on these subunits, namely, L2-S11, L5-S12, L5-S13 and L5-S19 (Cover et al., 1981).

These results suggest that under certain circumstances, the 70S ribosome may have a well-defined site to accommodate bound IF3. However, the biological significance of this is uncertain, since the re-

sults of these studies at high Mg^{2+} concentration may not necessarily be generalised to lower Mg^{2+} concentrations at which the factor is more functionally active. As we have already seen (section 1.1.4), it is possible that at high Mg^{2+} concentration, a pathway involving the binding of IF3 to the 70S couple may apply to the mechanism of dissociation of the 70S ribosomes by IF3. Such a pathway may be permitted by a conformational change of the ribosome induced by the high Mg^{2+} concentration (Noll and Noll, 1976).

1.1.7 Mechanism of action of IF3

The observation that IF3 could be cross-linked to the 3'-end terminus of 16S and 23S rRNAs (Van Duin et al., 1975, 1976) suggests that the ends of these RNAs were both near to IF3 and to each other. This led to the hypothesis that in the 70S couple, the 3'-end of the 16S rRNA moiety of the 30S subunit formed complementary base pairs with the 3'-end of the 23S rRNA moiety of the 50S subunit, thereby stabilising the 70S couple (Van Duin et al., 1976). This proposal is supported by the reasonably complementary nature of the 3'-terminal sequences of the two RNAs (Fig. 3a) and by the finding of differences in nuclease susceptibility of 16S rRNA in 30S, as opposed to 70S ribosomal particles (Chapman and Noller, 1977; Santer and Shane, 1977).

To account for both the 'anti-association' factor activity of IF3 and its activity in the initiation of protein synthesis (section 1.1.4), a model of IF3 action has been proposed whereby upon dissociation of the 70S couple, the base pair existing between the 16S and 23S rRNAs are replaced by self-complementary base pairs in both RNAs (Fig. 3b) and that the 30S conformation is stabilised by the presence of bound IF3 (Van Duin et al., 1976; Kurland, 1977; Grunberg-Manago and Gros, 1977; Grunberg-Manago et al., 1978). By binding to the 30S subunit

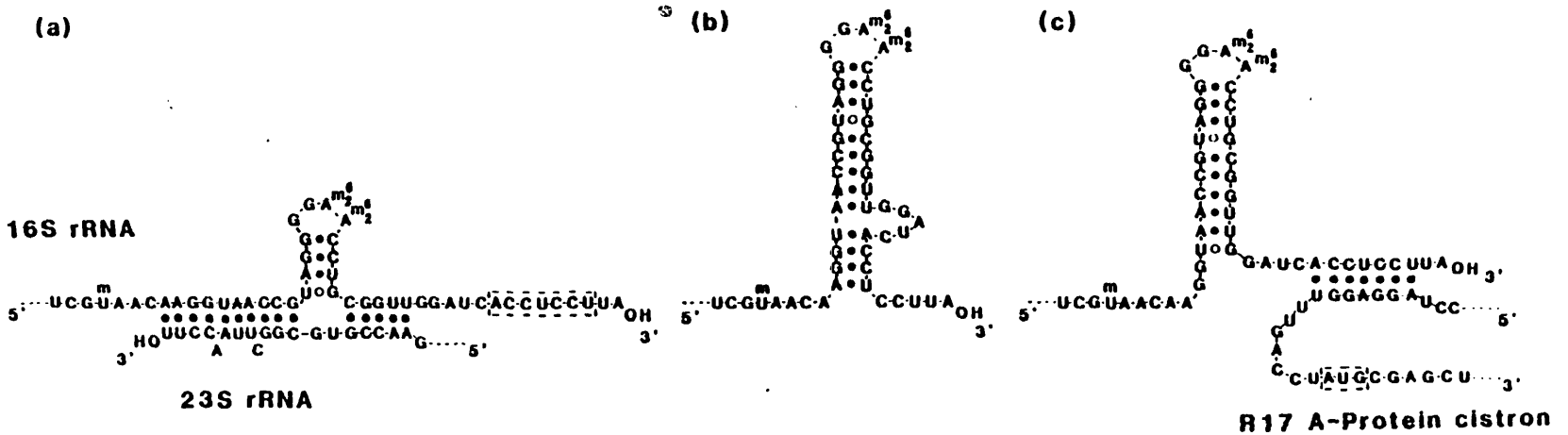


FIGURE 3 Base-pairing postulated to occur between the 3'-terminus 16S rRNA and 23S on mRNA. (a) Base-pairing scheme proposed between the 3'-termini of 16S and 23S rRNAs. The 'Shine-Dalgardo' sequence in 16S rRNA (see text) is boxed. After Van Duin et al. (1976). 23S rRNA sequence taken from Brosius et al. (1980). (b) Proposed intra-molecular base-pairing of the 3'-terminus of 16S rRNA present in free 30S subunits. After Steitz and Jakes (1975). (c) Base-pairing scheme of the 'Shine-Dalgardo' sequence of the 3'-end of 16S rRNA and the phage R17 A-protein cistron. After Steitz and Jakes (1975).

in a region near to the 3'-end of 16S rRNA (section 1.1.5), IF3 can act as an 'anti-association' factor by preventing the interaction of the 16S and 23S rRNAs. Furthermore, by binding in this region, IF3 may facilitate the further transition of complementary nucleotide interactions from the self-complementary interactions of the 16S rRNA (Fig. 3b) to the Shine-Dalgarno interaction between 16S rRNA and mRNA (Fig. 3c). According to this model, initiation of protein synthesis may occur more efficiently at cistrons having greater complementary pairing in the Shine-Dalgarno interaction (cf Steitz et al., 1977; section 1.1.4). Those mRNA initiation sites showing poor complementarity would bind weakly and be more dependent on IF3, or other stabilising factors.

An alternative model for the mechanism of action of IF3 in initiation complex formation has been proposed by Gualerzi and Pon (1981). In this model, IF3 binds to two domains on the 30S subunit and may thus bridge two regions on the subunit (i.e., the platform and the upper one third - see section 1.1.5) by inserting in the cleft. Binding and 'unbinding' at one or both of these sites could cause a small conformational change which would convert a 30S pre-initiation complex containing non-interacting mRNA and fMet tRNA into a 30S initiation complex in which the codon-anticodon interaction has taken place.

Of these two models, the former appears to be probably more correct since it can readily account for the two, at a first glance apparently unrelated, activities of the factor. It is, of course, possible that the mechanism by which IF3 facilitates the transition of the complementary nucleotide interactions shown in Fig. 3 may involve small conformational changes of the 30S subunit, perhaps involving a mechanism similar to that proposed by Gualerzi and Pon (1981). The characterization of the interaction of IF3 with 16S rRNA may help

to distinguish between the validity of these two models of action. In view of the fact that the regions of 16S rRNA that interact with IF3 have been, as yet, poorly characterized (see section 1.1.5), the characterization of the interaction of IF3 with MS2 RNA, which is the subject of this work, may add to our understanding of the interaction of the factor with the 30S subunit and, ultimately, its mechanism of action.

1.2 COLIPHAGE MS2 AND ITS RNA GENOME

1.2.1 Physical properties of coliphage MS2

Coliphage MS2, isolated by J. Clark from a sewer in Berkley, California (USA), (cited in Davis et al., 1961; Weissmann, 1974), is a member of the Group I family of RNA coliphages, which include R17, f2, M12, M2 and R23 in their number (Fiers, 1979). MS2 phage has a sedimentation constant, $S_{20,w}$, of 78.5-81S and a molecular weight ratio of 3.6 - 3.87 x 10^6 (Strauss and Sinsheimer, 1963) and has an isoelectric point of 3.9 (Overby et al., 1966a).

An MS2 virion consists of a single 3569-nucleotide-long RNA molecule, a shell composed of 180 copies of a coat protein and one molecule of a maturation, or A, protein (Fiers, 1979). Each particle additionally contains nearly 1000 molecules of spermidine, which is sufficient to neutralize a large proportion of the negative charge on the viral RNA (Fukuma and Cohen, 1975). The diameter of the particle is about 2 6nm with the protein shell being 2-3nm thick, as determined by X-ray scattering (Zipper et al., 1971) and by electron microscopy (Crowther et al., 1975). Studies by neutron scattering have suggested the presence of a central hole with a radius of 6nm (Jacrot

et al., 1977). The isocosahedral shell has a T = 3 surface lattice, probably consisting of coat protein molecules associated as dimers, which form rings of five about the 5-fold axes and rings of six about the 3-fold (quasi 6-fold) axes (Crowther et al., 1975). A superficially similar, but actually different, bonding scheme has been proposed by Dunker and Paranchych (1975). The earlier capsid model proposed by Vasquez (1966), involving 32 morphological subunits, is not compatible with the more recent, higher-resolution data.

The physical properties of the RNA molecules of the Group I coliphages have been recently reviewed by Fiers (1979). The exact value of the molecular weight ratio of MS2 RNA has been determined from the complete nucleotide sequence to be 1.23×10^6 for the Na^+ salt (Fiers et al., 1976; Fiers, 1979). M_r values for MS2 and R17 RNAs determined by a variety of other methods are in the range $1.0 \times 1.4 \times 10^6$ (Table 3) and the variations reflect the various uncertainties inherent in the techniques used for these determinations.

The relatively high sedimentation constant of MS2 and R17 RNAs ($S_{20,w}$, 16.5S (10mM NaCl), 26-27S (0.1M NaCl) - Gesteland and Boedtker, 1964; Mitra et al., 1963) suggests that these are exceptionally compact molecules. This is reflected in the radius of gyration of MS2 RNA being 16nm in 0.2M NaCl (Strauss and Sinsheimer, 1963) and of R17 RNA being 18.3 - 19.6nm in 0.1M NaCl and 48.5nm in 10mM NaCl (Gesteland and Boedtker, 1964). Small-angle X-ray scattering studies of MS2 RNA at ionic strength 0.1 showed that the RNA appears to be a flat, elongated coil of about 62nm diameter and with ratios of about 2 : 1 : 0.5 for the mean radii of gyration in the three directions of space (Zipper et al., 1975).

Optical studies have revealed a high degree of secondary struct-

TABLE 3

Molecular weight ratio determinations of MS2 and R17 RNAs

Method	Phage	M_r	Reference
Complete nucleotide sequence:	MS2	1.23×10^6	Fiers et al. (1976), Fiers (1979)
Light scattering:	MS2	$1.05 - 1.15 \times 10^6$	Strauss and Sinsheimer (1963)
	MS2	1.0×10^6	Overby et al. (1966b)
	MS2	1.15×10^6	Slegers et al. (1973)
	R17	$1.1 \pm 0.1 \times 10^6$	Gesteland and Boedtker (1964)
Sedimentation viscosity:	MS2	$1.09 - 1.35 \times 10^6$	Slegers et al. (1973)
	R17	$1.0 - 1.4 \times 10^6$	Mitra et al. (1963)
Gel electrophoresis:	MS2	1.23×10^6	Reijnders et al. (1973)
	R17	1.3×10^6	Boedtker (1971)
Purine tract analysis:	MS2	$1.0 - 1.12 \times 10^6$	Fiers et al. (1965)
	R17	$1.1 \pm 0.1 \times 10^6$	Sinha et al. (1965)

ure in the Group I phage RNAs. In 0.1M phosphate buffer, the hypochromicity amounts to 21 - 23% and the T_m is 58°C and the latter is further enhanced by the addition of Mg^{2+} (Gesteland and Boedtker, 1964; Boedtker, 1967; Slegers and Fiers, 1972). Mitra et al. (1963) estimated that approximately 76% of the nucleotides are in a helical configuration. By comparing the hypochromicity of R17 RNA before and after reaction with HCHO (which prevents hydrogen bonding, but not base stacking), Boedtker (1967) estimated that 73% ± 5% of the bases were hydrogen bonded. Isenberg et al. (1971), using infra-red spectroscopy of the deuterated RNA of coliphage μ2 in H_2O buffer, determined the helicity to be 63% ± 5%; however, as noted by Fiers (1979), this may represent a lower estimate since some denaturation may have occurred during their sample preparation. Recently, Thomas et al. (1976), using laser-Raman spectroscopy, have shown that approximately 85% of the bases of MS2 RNA are either paired or stacked, both inside the virion and in the isolated viral RNA. Such a degree of secondary structure is quantitatively very similar to that found in tRNA (Thomas and Hartman, 1973). Secondary structure models based on the known nucleotide sequence of MS2 RNA are discussed in section 1.2.4.

1.2.2 Genetic organisation of the RNA genome

Mainly because of the small size of their genomes, the genetics of MS2 and other members of the Group I coliphages have been investigated in depth (for a review, see Horiuchi, 1975). Complementation and physiological analyses of amber and temperature sensitive mutants of these phages established the existence of three cistrons by the middle 1960s (Horiuchi, 1975) corresponding to the RNA replicase (the product of gene 1 or C), a maturation protein (gene 2 or A) and a coat protein (gene 3 or B). Since recombination among RNA phages

has never been observed, the order of the cistrons had to be determined by nucleotide sequence analysis and for R17, as for the other members of this group, this order is:

5'-gene 2 (maturation protein)-gene 3(coat)-gene 1(replicase)-3' (Jeppesen et al., 1970) (Fig. 4a).

In the course of the search for opal (U-G-A) mutants of f2 phage, a mutant, Op3, was isolated which did not lyse a non-permissive (Su^-) cell, but did lyse any of three independently isolated U-G-A suppressor strains (Model et al., 1979). In the Su^- host, the Op3 mutant produced a normal burst of phage which could be released by lysis in vitro. No differences were found between the particles made in the Su^+ and Su^- hosts with regard to infectivity or their physical properties. These properties required that the Op3 mutant be placed in a new complementation group and suggested the existence of a fourth gene. Beremand and Blumenthal (1979) identified a 75 amino acid polypeptide (L protein) following f2 infection of E. coli cells and which was absent in the Op3 mutant. Likewise, the binding of mammalian ribosomes to MS2 RNA together with translation in vitro yielded a 75 amino acid polypeptide coded for in the +1 reading frame (Fig. 4b), initiating at an AUG triplet within the coat protein gene and terminating at a UAA codon in the replicase gene (Atkins et al., 1979). The binding of wheat germ ribosomes to fragmented R17 RNA was found by Kozak (1980) to be at a similar site to that of mammalian ribosomes (Atkins et al., 1979).

1.2.3 Primary structure of MS2 RNA

The brilliant elucidation of the entire nucleotide sequence of MS2 RNA by Fiers and co-workers (Min Jou et al., 1972; Fiers et al., 1975, 1976), as corrected by Iserentant et al. (1980), made this the first

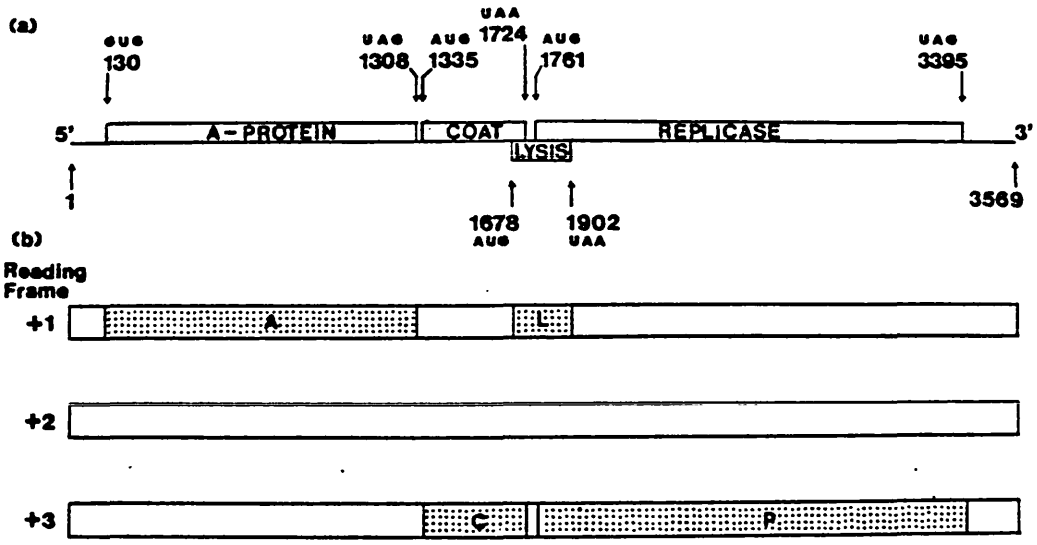


FIGURE 4 Genetic and reading frame map of MS2.

(a) Genetic map. The nucleotides are numbered from the 5' to the 3' end and the positions of some important signals are indicated (Fiers, 1979; Beremand and Blumenthal, 1979)(the initiation codon is considered part of the genes and the termination codon part of the untranslated region).

(b) Reading frame map of MS2. Reading frame +1 begins with nucleotide 1 at the 5' end, reading frame +2 with nucleotide 2 and reading frame +3 with nucleotide 3 (after Beremand and Blumenthal, 1979).

life-form for which the entire primary chemical structure has been determined. The MS2 RNA genome is some 3569 nucleotides long and starts from the 5' end with a 129 nucleotide-long untranslated sequence (Fig. 4). There next follows the A-protein gene of 1179 nucleotides, starting with a G-U-G codon and ending with a U-A-G codon and read in the +1 reading frame (Fig. 4b). This is a protein of 393 amino acids with a molecular weight of 38,000-42,000 and is present in one copy per virion (Nathans et al., 1966; Steitz, 1968; Remaut and Fiers, 1972). The protein is required for correct encapsidation of the viral RNA since infection of non-permissive cells with amber A-protein mutants of f2 and R17 leads to the production of non-infectious particles having a normal shell of coat proteins, but from which part of the RNA may 'dangle' out (Lodish et al., 1965; Argetsinger and Gussin, 1966; Heisenberg, 1967). Selective removal of the A-protein by high salt treatment of virions correlates closely with loss of viability (Verbraeken and Fiers, 1972).

Following the A-protein termination codon is an intercistronic region of 26 nucleotides, followed by the coat protein gene of 390 nucleotides, starting with an A-U-G codon, ending with a U-A-A codon and read in the +3 reading frame (Fig. 4b). This protein is 129 amino acids long and has a molecular weight of approximately 14,000 (Weber and Konisberg, 1975) and is notable for its absence of histidine residues. The most obvious function of the coat protein is that of encapsulation of the viral RNA. A less obvious function is that of regulating the translation of the replicase cistron by binding at the intercistronic region preceding this cistron and thereby preventing ribosome attachment (cf sections 1.2.5 and 1.3.3) (Bernardi and Spahr, 1972; Berzin et al., 1978).

There next follows an 'intercistronic' region of 36 nucleotides (however, this is not a true intercistronic region since it contains the information coding the lysis gene described below), followed by the replicase gene. This is some 1655 nucleotides long, starting with an A-U-G codon, terminating with a U-A-G codon and read in the +3 reading frame (Fig. 4b). Of the Group I phages, the f2 coded protein has been the most studied and has a molecular weight of 65,000 (Federoff and Zinder, 1971) and, based on the MS2 RNA sequence, consists of 544 amino acids. The active replicase complex consists of four polypeptides: α, β, γ and δ, of which β is specified by the replicase gene, while the remaining three are coded for by the E. coli host and appear to be the same as those found in the Group III RNA phage Qβ (Federoff, 1975). Protein α has been identified as ribosomal protein S1 (Wahba et al., 1974) and γ and δ as the elongation factors EF-Tu and EF-Ts respectively (Blumenthal et al., 1972). An additional protein factor from the host may be required for efficient replication of the Group I phages (Federoff, 1975), although this has yet to be characterised. The whole replicase complex is responsible for the autocatalytic replication of the viral RNA via a double-stranded replicative intermediate (see Weissmann, 1974).

Following the replicase gene is a 174 nucleotide-long untranslated segment forming the 3'-terminal portion of the molecule.

Starting some nine-tenths of the way through the coat protein gene with an A-U-G codon and read in the +1 reading frame is the 228 nucleotide-long lysis protein gene (Fig. 4b) which spans the intercistronic region between the coat and replicase genes and terminates with a U-A-A codon within the latter gene (Beremand and Blumenthal, 1979; Atkins et al., 1979). Initiation of protein synthesis at this gene

requires a frame-shift of ribosomes reading the upstream coat protein mRNA with the out-of-phase fraction of ribosomes terminating at either one or two nonsense codons just preceding the lysis cistron and being followed by initiation at the A-U-G codon of the lysis protein gene (Kastelein et al., 1982). The gene thus overlaps the coat protein and replicase genes, both of which are read in the +3 reading frame. Overlapping genes have also been found in the DNA bacteriophages Ø x 174 and G4 (Barrell et al., 1976; Sanger et al., 1977; Shaw et al., 1978; Godson et al., 1978) as well as in the two mammalian viruses, SV40 (Fiers et al., 1978; Reddy et al., 1978) and polyoma (Soeda et al., 1979, 1980).

A fifth phage-specific protein of approximately 25,000 daltons has been reported to be produced in f2 infected E. coli (Beremand and Blumenthal, 1979) and may be the product of contiguous segments of both the coat and lysis genes arising from a shift in reading frame during protein synthesis.

Of the 3569 nucleotides of MS2 RNA, some 91% are used for the coding of five polypeptides with a total size of approximately 150,000 daltons. Of the three possible reading frames, only two (+1 and +3) are utilised for coding these proteins and thus information is extremely efficiently stored in this group of small viruses.

1.2.4 Secondary structure of MS2 RNA

Several secondary structure models have been proposed for the nucleotide sequence of MS2 RNA (Min Jou et al., 1972; Vandenberghe et al., 1975; Fiers et al., 1975, 1976; Fiers, 1979; Iserentant and Fiers, 1979; Iserentant et al., 1980). These models have been based on: (1) the isolation of specific RNA complexes (DeWachter and Fiers, 1972), (2) on studies of limited RNase digestion and chemical

modification studies (Fiers et al., 1975, 1976; Iserentant and Fiers, 1979), (3) theoretical predictions of the stability of proposed structures using the methods of Tinoco et al. (1971, 1973) and (4) pure conjecture. No studies have so far been made using a direct experimental approach such as that of Ross and Brimacombe (1979), which has been used in the case of E. coli 16S rRNA (Glotz and Brimacombe, 1980). Secondary structure studies of MS2 and f2 RNAs using electron microscopy have also been made (Jacobson, 1976; Jacobson and Spahr, 1977; Edlind and Bassel, 1977). These studies give information on possible long-range interactions within the RNA molecules; however, as yet there is no evidence that the loop structures seen in the electron microscope are related to those which might occur in solution or within the bacterial cell.

The most greatly characterised portions of the secondary structure of MS2 RNA are those of the 5'- and 3'-terminal regions. The two early tentative models for the 5'-region based on RNase T_1 and CM-RNase cleavage points and estimates of thermodynamic stability (DeWachter et al., 1971a; Fiers et al., 1975) have been refined and a secondary structure model based on the chemical modification by methoxyamine and kethoxal on an isolated 5'-terminal fragment of 117 nucleotides has been proposed (Fig. 5a) (Iserentant and Fiers, 1979). Since the model was based on an isolated RNA fragment, it has to be assumed that the secondary structure in the fragment will largely be the same as the corresponding domain in the macro-molecule. Of course, in the total viral RNA molecule, long range interactions may be present, as suggested by electron microscopy studies (Jacobson, 1976; Jacobson and Spahr, 1977; Edlind and Bassel, 1977). The proposed model of this fragment is validated by it being in complete agreement

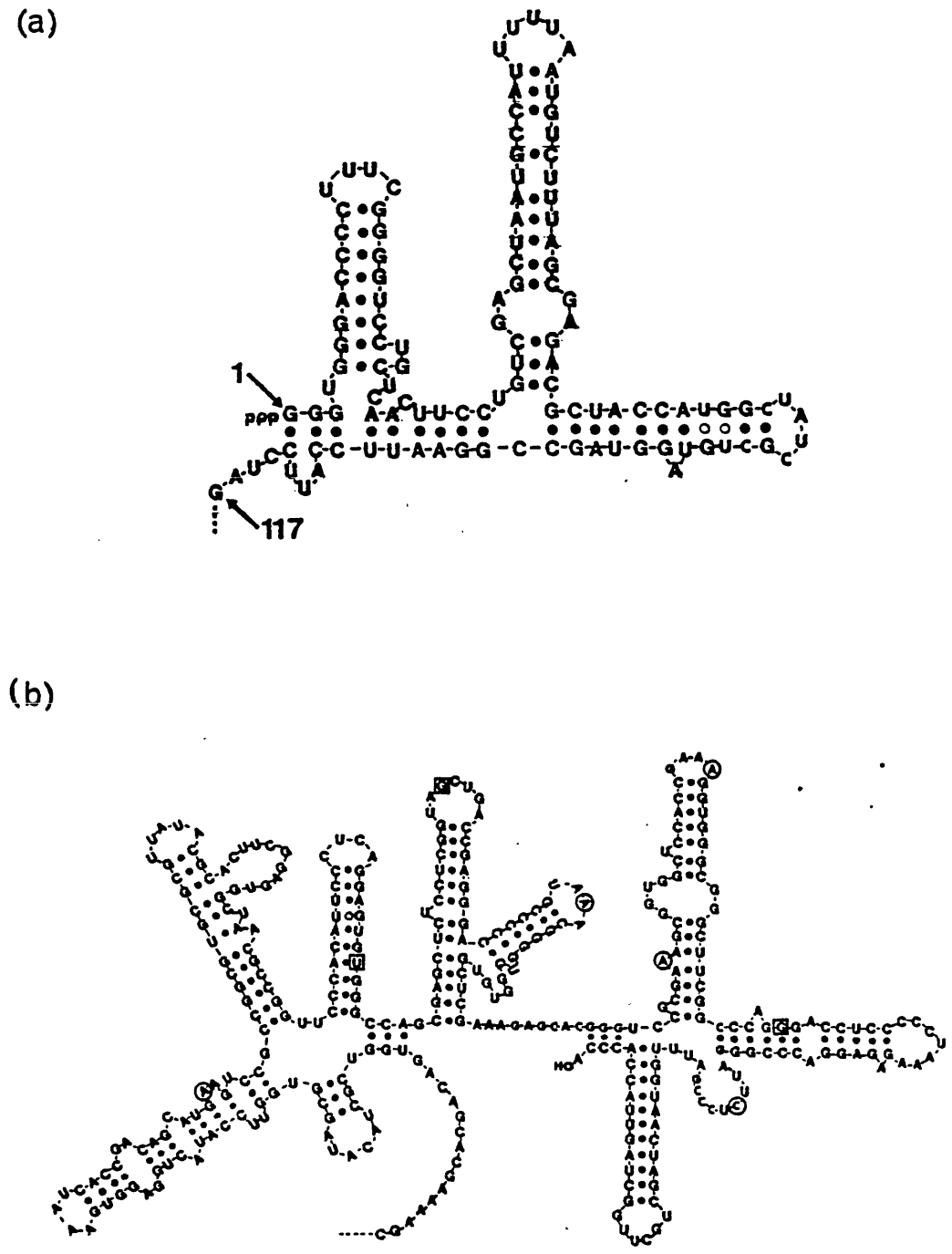


FIGURE 5 Secondary structure models for the 5'- and 3'-termini of MS2 RNA

(a) Secondary structure model for 5'-terminus (after Iserentant and Fiers, 1979).

(b) Secondary structure model for 3'-terminus. Positions of mutation are indicated by circles and silent mutation and reversions are indicated by squares. After Iserentant et al. (1980).

with the partial enzymatic digestion data for complete MS2 RNA (Fiers et al., 1975) and consists of three hairpin-loops with some 72% of the total nucleotides being base paired (Fig. 5a).

For as far as the untranslated 5'-terminal nucleotide sequences have been determined, MS2, R17 and f2 all have an identical nucleotide sequence (Adams and Cory, 1970; Ling, 1971; DeWachter et al., 1971b) and it is clear that the severe resistance against variation implies an important biological role. One possibility is that of an involvement in the control of expression of the A-protein cistron whereby the secondary structure masks the ribosome binding site for this gene (Robertson and Lodish, 1970; Iserentant and Fiers, 1979) (Section 1.2.5). An alternative possibility is that the secondary structure is important for recognition by the replicase complex, since a similar model for the secondary structure can be constructed for the 3'-end of the complementary (minus) strand (Iserentant and Fiers, 1979).

In contrast to the 5'-terminal sequence, the secondary structure of the 3'-terminal region has not been fully characterised. Early models were based on RNase cleavage points and estimates of thermodynamic stability of proposed structures (Vandenberghe et al., 1975; Fiers et al., 1976). The latest model (Iserentant et al., 1980) (Fig. 5b), covering the last 349 nucleotides of the sequence and containing a correction to the primary sequence, consists of nine hairpin-loops with mutations obtained in the study by treatment with nitrous acid, occurring in single-stranded regions and reversions occurring in both single-stranded and base paired regions. Some 57% of the nucleotides in this model are base paired, which is somewhat lower than the estimates of 73-85% for the overall molecule (Mitra et al., 1963; Boedtker, 1967; Thomas et al., 1976). The partial enzymatic digestion data for this

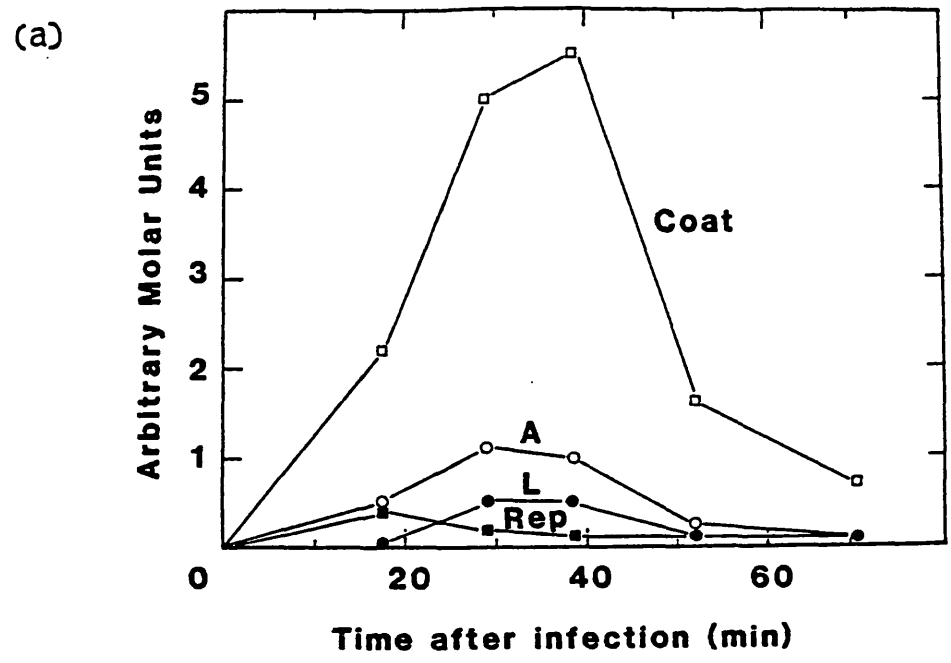
region of the molecule (Fiers et al., 1976) are in poorer agreement with this model than is the case for the 5'-region model.

The nucleotide sequences for the 3'-terminal portions of several of the Group I coliphages (MS2, R17, JP501, FR1, BO1) have been compared (Inokuchi et al., 1982) and found to differ by only 3.3% over more than 200 residues. Like the 5'-terminal portion of the molecule, this high degree of conservation of nucleotide sequence would suggest that this region has an important biological function, possibly in the initiation process of RNA replication (Fiers, 1979).

1.2.5 Translation of MS2 RNA

The expression of the viral genes of the Group I RNA phages is finely controlled, both in their relative amounts and times of synthesis throughout the short (40-60 min.) infectious cycle (for reviews see: Kozak and Nathans, 1972; Robertson, 1975; Weissmann, 1974; Lodish, 1975; Fiers, 1979). The molar ratio of synthesis of coat protein to A-protein to replicase β subunit is approximately 20 : 2 : 1 in the cases of MS2 and f2 (Nathans et al., 1966; Viñuela et al., 1967; Fromageot and Zinder, 1968) and synthesis of the f2 lysis protein exceeds that of the replicase subunit (Beremand and Blumenthal, 1979).

Analysis of the kinetics of the synthesis of phage proteins in E. coli (Oeschger and Nathans, 1966; Nathans et al., 1969; Beremand and Blumenthal, 1979) (Fig. 6a), shows that the replicase subunit is the first detectable phage protein, appearing between 5 and 15 minutes after infection whereupon its rate of synthesis increases until about 25 minutes after infection and then diminishes. Synthesis of the A-protein starts later than that of the replicase and continues later in the infectious cycle.



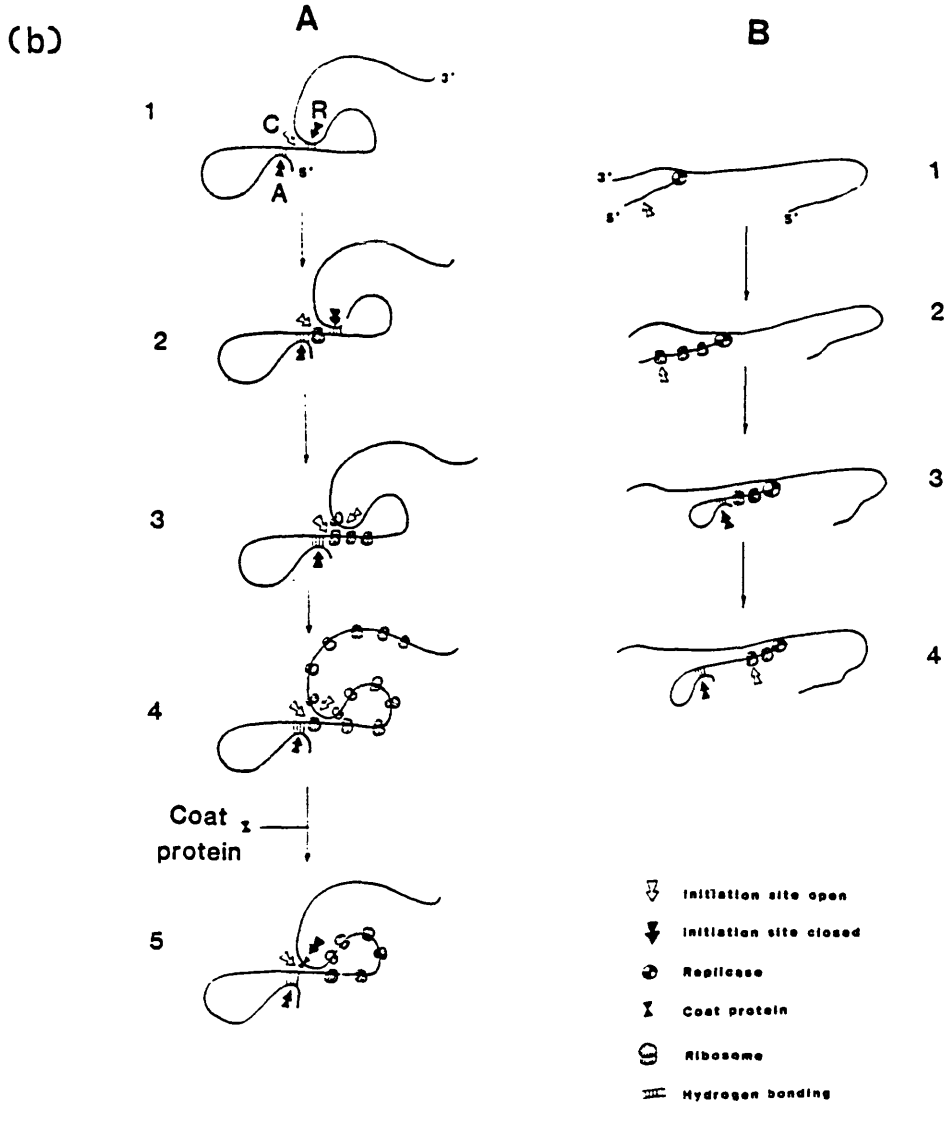


FIGURE 6 Translation of coliphage RNA

(a) Time-course of f2-coded polypeptide synthesis (after Beremand and Blumenthal, 1979)

cont'd overleaf...

(b) Model of translation on mature and nascent phage RNA

A: Translation on mature RNA (1). Only the coat protein initiation site is accessible to ribosomes (2). As the coat cistron is translated, ribosomes can attach at the replicase cistron (3) giving rise to a polysome on which the coat and replicase, but not the maturation cistron, are translated (4). During later stages of the infective cycle, coat protein accumulates in the cell and binds to the RNA so as to block initiation at the replicase cistron (5).

B: Translation of nascent RNA. The viral replicase initiates synthesis of a plus strand at the 3'-end of a minus strand (1). When the ribosome binding site of the A-protein has been formed, ribosomes attach and begin translation of this cistron (2). As plus strand synthesis progresses, the plus strand assumes a secondary structure which prevents access of ribosomes to the A cistron (3). At this point, initiation of protein synthesis is now possible only at the coat cistron (4), as in the case of mature RNA (part A). (After Weissmann, 1974).

Coat protein synthesis begins *before* that of the replicase and continues to be made throughout most of the phage growth cycle. The lysis protein appears late in the infection after the replicase starts to decline and its synthesis continues during the next 10 minutes and then gradually decreases during the latter stages of infection. With the determination of the complete nucleotide sequence of MS2 RNA (Fiers et al., 1976) the possible molecular processes involved in this regulation could be proposed and are briefly described below.

When ribosomes are added to intact R17 RNA, they bind only to the coat protein initiator region whereas, when partially degraded RNA is used, binding occurs also to the A-protein and replicase subunit initiation sites (Steitz, 1969). Likewise, partial denaturation of intact viral RNA was found to increase ribosome initiation at the A-protein and replicase sites (Lodish, 1970, 1971, 1975) and an estimate of the relative intrinsic efficiencies of the ribosome binding sites was found to be: A > Replicase > Coat (Steitz, 1973). Thus, for intact viral RNA, the A-protein and replicase subunit ribosome binding sites are blocked; possibly by some aspect of the secondary structure of the RNA.

Robertson and Lodish (1970) proposed that the A-protein is synthesized only on nascent strands and that when the RNA chain grows longer, a three-dimensional folding occurs which effectively shuts off further expression (Fig. 6b). The secondary structure model for the A-protein cistron includes an interaction between a region just before the G-U-G initiator triplet and a segment located at about two-thirds distance (Fiers et al., 1975). However, this model for autocontrol is not yet strongly supported by direct experimental data.

Likewise, the initiator region of the replicase cistron may also be hidden by the conformation of the RNA (Fig. 6b). Characterisation of suppressor-sensitive amber mutants revealed a class of mutants in the coat gene which gave only poor and delayed complementation (Gussin, 1966). These polar mutants interfered with the expression of the replicase gene and were subsequently identified as being in position 6 of the coat gene for R17, f2 and MS2 (reviewed in Horiuchi, 1975). Amber mutations at positions 50, 54 or 70 are not polar and do not interfere with the expression of the replicase cistron (see Horiuchi, 1975). Thus, translation of the coat gene is required for the expression of the replicase gene. A possible molecular basis for the polarity effect stems from the secondary structure model of the coat protein gene by Min Jou et al. (1972) in which a base-paired region can be formed between residues .1409-1433 (localised at the beginning of the coat protein gene, but distal from position 6) and residues 1738-1769 (encompassing the replicase gene ribosome binding site). When ribosomes translate the coat gene, they therefore open this base-paired structure and thus make the initiator region of the replicase gene accessible to ribosomes.

Additional regulation of replicase synthesis has been proposed by translational repression by the coat protein (Gussin, 1966). The protein binds in the region of the initiating A-U-G of the replicase gene (section 1.3.3), thus preventing its further functioning (Fig. 6b). How this molecule is again removed is unclear, since the infecting parental RNA molecule must be free from such a repressor when it enters the cell.

Translational repression by the replicase may occur in the Group III phages in order to clear the template of ribosomes prior to replica-

tion and a similar process may also occur in the Group I phages (Fig. 7). In the case of Qβ RNA, the replicase is known to be able to reduce the binding of ribosomes to the viral RNA (Kolakofsky and Weissmann, 1971a, 1971b). The Qβ replicase binds to a region around the coat protein cistron initiation site (Weber et al., 1972) (section 1.3.4), thus preventing further initiation at this site. Since the template is complete, the A-protein gene initiation site is already closed by secondary structure. Ribosomes thus translate the coat and replicase genes and, once the first segment of the coat gene has been translated by the last ribosome translating this gene, the polarity effect comes into effect and the replicase gene initiator region becomes closed. The remaining ribosomes on the replicase gene complete translation and then the RNA template is clear of ribosomes and is ready for RNA replication.

1.2.6 Stability of the genomes of the Group I coliphages

Before moving on to consider proteins interacting with MS2 RNA, the stability of the RNA sequence of MS2 as a repository of genetic information should be considered. The early work of Zinder and Cooper (1964) showed that some f2 mutations had a reversion frequency of 10^{-3} and thus, if this drift rate of back mutation were indicative of a general drift, and if no counter selection against variability existed, this would suggest that there would be considerable variability in individual genomes of MS2. Under these circumstances, the determination of the location of any protein binding site by sequence analysis of one strain of MS2, followed by comparison with the total nucleotide sequence of another strain, as is the case of the work presented in this thesis, would prove difficult. Fortunately, studies comparing the nucleotide sequences of several Group I phages has shown that these

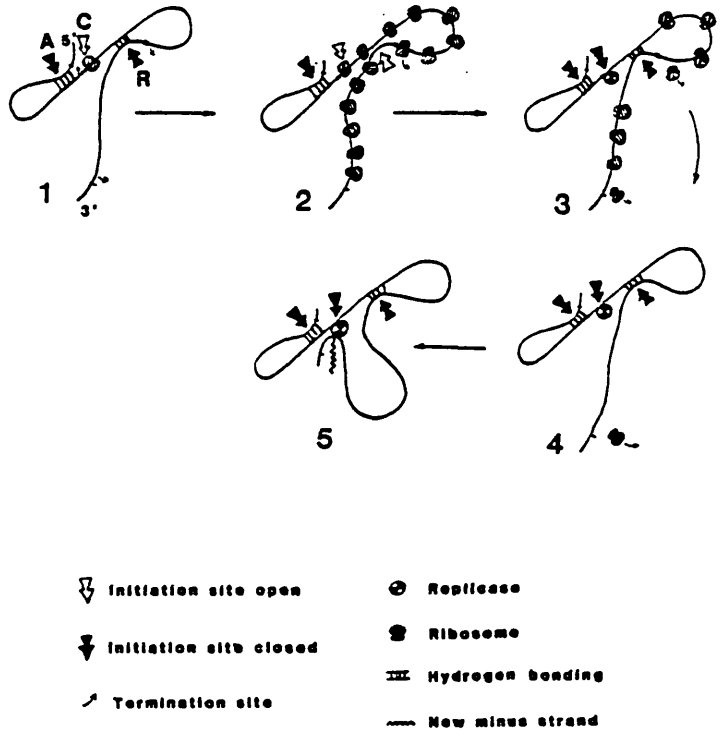


FIGURE 7 Transition of phage RNA from polysome to replicating complex.

(1) Ribosomes attach to the RNA at the coat initiation site. The initiation site of the replicase cistron is unavailable because of the secondary structure of the RNA. (2) Translation of the coat cistron ensues and the initiation site of the replicase cistron is exposed. The replicase cistron is translated. (3) When replicase becomes available, it attaches to the initiation site of the coat protein and blocks attachment of ribosomes in this position. The RNA refolds, preventing initiation at the replicase cistron. (4) The RNA is cleared of ribosomes. (5) Replicase can now attach to the 3'-terminus and initiate synthesis of the minus strand. The A cistron initiation site is at all times inaccessible to ribosomes because of the secondary structure of the mature RNA (cf Fig. 6). (After Kolakofsky and Weissmann, 1971b).

genomes are well conserved.

Min Jou and Fiers (1976) compared the nucleotide sequences of MS2, R17 and f2 phage RNAs and estimated the overall degree of variation to be 3.9% for MS2 versus R17, 3.4% for MS2 versus f2 and 3.7% for R17 versus f2. These results are somewhat higher than those found earlier by Robertson and Jeppesen (1972); however, larger regions of the RNA sequences were studied by Min Jou and Fiers (1976), rather than just RNase T_1 oligonucleotides, which would tend to underestimate the extent of variability. Variation in the untranslated regions was found to be more constrained than in the translated regions as had already been found by several authors (DeWachter et al., 1971a; Sanger, 1971; Adams et al., 1972; Robertson and Jeppesen, 1972) and, as noted above, the sequences of the first 74 untranslated nucleotides of the 5'-end of MS2, R17 and f2 are identical (Adams and Cory, 1970; Ling, 1971; DeWachter et al., 1971a). Likewise, the comparison of the nucleotide sequences of approximately 250 nucleotides from the 3'-ends of MS2, JP501, BO1 and FR1 (Inokuchi et al., 1982) showed variability between 0.9 and 2.3%. Earlier, the nucleotide sequences of the 3'-terminal 51 nucleotides of R17 RNA had been shown to be identical to that of MS2 RNA (Cory et al., 1972) and in this respect the sequences of up to 40 residues from the 3'-end of the RNAs of the various Group I phages studied by Inokuchi et al. (1982) were found to be completely conserved.

Domingo et al. (1978) studied the RNase T_1 oligonucleotides obtained from several clones of the Group III phage Qβ and estimated that each viable phage in a multiply passaged population differed in one or two positions from the 'average' sequence of the parental population. A similar slight heterogeneity in the sequences of individual isolates of MS2 were seen by Min Jou et al., (1972) and also in sequences of RNA

derived from the 'Cambridge' and 'Ghent' isolates of R17 (Min Jou and Fiers, 1976). Domingo et al. (1978) proposed that a Qβ phage population is in a dynamic equilibrium, with viable mutants arising at a high rate on the one hand (Batschelet et al., 1976; Domingo et al., 1976) and being strongly selected against on the other hand. A similar situation may apply to MS2 and other members of the Group I phages and, thus, the genomes of these phages, like that of Qβ , cannot be described as a defined unique structure, but rather as a weighted average of a large number of slightly different individual sequences. However, these differences would not appear to preclude the determination of a nucleotide sequence, such as a protein binding site, on one isolate and then comparison with the complete nucleotide sequence determined from a different isolate of the same phage.

Finally, since the nucleotide sequences of MS2, R17 and f2 RNAs vary independently and thus the variation observed between a given pair of phages is not a subset of the variation present in another pair (Robertson and Jeppesen, 1972; Min Jou and Fiers, 1976), these phages are not of linear descent, but may have evolved from a common ancestor.

1.3 PROTEINS INTERACTING WITH MS2 RNA

Several proteins, or complexes of proteins, have been found to interact with the RNA of the Group I coliphages: ribosomes (both prokaryotic and eukaryotic), the viral A- and coat proteins, the Qβ phage host factor, ribosomal protein S1, the viral replicase complex and Initiation Factor IF3. the characteristics of the interactions of these proteins on MS2 RNA (and also the other Group I phage RNAs, mainly

R17) are discussed below.

1.3.1 Ribosomes

Takanami et al. (1965), using an RNase protection technique, were the first to show that a single prokaryotic ribosome binds to an f2 RNA molecule. Using an improved technique for isolating ribosome binding sites, followed by nucleotide sequence analysis, Steitz (1969) showed that ribosomes could protect three sites on R17 RNA, each approximately 35 nucleotides long and containing an A-U-G triplet at about the middle. These binding sites corresponded to the start of the coat, A-protein and replicase genes. The three ribosome binding sites on MS2 RNA were later found to be very similar to those of R17 RNA both in terms of their size and nucleotide sequences (Table 4) (Min Jou et al., 1972; Volckaert and Fiers, 1973). One major difference, however, was that the MS2 A-protein gene was found to start with a G-U-G initiation codon. This initiation codon is functionally equivalent to A-U-G and, likewise, the repressor gene i of the E. coli lac operon has been shown to be initiated by a G-U-G codon (Steege, 1977).

Mammalian ribosomes bind to MS2 RNA and the region that is most thoroughly protected against RNase digestion contains a central A-U-G triplet in the +1 reading frame and is located within the coat protein gene (Table 4). This protected region covers the initiation codon of the lysis gene. A similar binding site was observed for wheat germ ribosomes on MS2 RNA (Kozak, 1980).

In order to attempt to understand the mechanisms by which ribosomes recognise functional initiation sites on MS2 RNA, one has to remember that ribosomes are complexes of both proteins and RNA molecules (e.g., Brimacombe et al., 1976; Brimacombe, 1978; Wittmann, 1982) and that these components, either singly or in combination, may

TABLE 4 Ribosome binding sites on MS2 RNA

		Ref
E. coli ribosomes:	HO A U U C C U C C A C U . . . . . 3' terminus of E. coli 16S rRNA	
A protein gene:	C A U U C C U A G G A G G U U U G A C C U G U G C G A G C U U U U U A G U G	(a)
Coat gene:	C C U C A A C C G G A G U U U G A A G C A U G G C U U C C C A A C U U U U A C U	(b)
Replicase gene:	A A A C A U G A G G A U U A C C C A U G U C G A A G A C A A C A A A A G	(b)
Mammalian ribosomes:		
Lysis gene:	U C U C C U A A A A G A U G G A A A C C C G	(c)

References:

(a) Volckaert and Fiers (1973)

(b) Min Jou et al. (1972)

(c) Atkins et al. (1979)

Note:

The underlined segment is complementary to part of the 3'-terminal sequence of E. coli 16S rRNA shown at the top of the table. Dots indicate a G - U base pair and the gap in the underlining of the coat gene implies a looped out base. Initiation codons are boxed.

contribute to the specificity of the interaction. Indeed, the way in which 16S rRNA moiety of the 30S subunit of prokaryotic ribosomes appears to play an important role in determining the specificity of this interaction has already been described (section 1.1.4).

The A-protein, coat and replicase gene ribosome binding sites on MS2 RNA all contain sequences complementary to 16S rRNA before the initiation codon (Table 4), whereas the lysis gene binding site for mammalian ribosomes does not. However, E. coli ribosomes do not directly initiate at this site, since translation depends on a frame-shift during translation of the overlapping coat gene (Kastelein et al., 1982) and, in this respect, a Shine-Dalgarno region seems to be dispensable for a translational restart (Napoli et al., 1981). Furthermore, the role of a possible Shine-Dalgarno interaction in the mechanism of initiation site selection by eukaryotic ribosomes is somewhat in doubt (Kozak, 1978).

The Shine-Dalgarno interaction does not, however, totally explain the selection of initiation sites by E. coli ribosomes since: (i) MS2 RNA, as well as other mRNAs, contains many regions with potential initiation codons preceded by a sequence complementary to 16S rRNA and yet do not function as initiation sites (Fiers et al., 1976; Fiers, 1979); and (ii) Sprague et al. (1977) found that 16S rRNA from B. Stearothermophilus and from E. coli have an identical nucleotide sequence near the 3' terminus and yet preferentially recognise different initiation signals in R17 RNA.

We have already seen that secondary structure interactions may be involved in regulating initiation at the MS2 RNA A-protein and replicase initiation sites (section 1.2.5). From studies of the cro gene of bacteriophage λ, Iserentant and Fiers (1980) have proposed that initi-

ation of translation involves the interaction of an activated 30S subunit with the 5'-terminal region of an mRNA folded in a specific secondary structure.

Of the protein components of the initiation system, IF3 and the ribosomal proteins S1 and S12 are probable candidates for influencing the selection of initiation sites by ribosomes. The influence of IF3 has been described earlier (sections 1.1.4 and 1.1.7). Dahlberg and Dahlberg (1975) found that S1 reversibly binds to the colicin E3 fragment of 16S rRNA, which represents the last 49 nucleotides of the molecule and suggested that S1 may open up a double-stranded structure in this region (cf Fig. 3b) to facilitate annealing with mRNA. However, some doubt has been cast on this mechanism with the observation that the last 49 nucleotides at the end of the 16S rRNA are not essential for the high affinity binding of S1 to 30S subunits (Laughrea and Moore, 1978). Additionally, proton magnetic resonance studies of the secondary structure of the colicin E3 fragment suggest that S1 protein may not be required to open up this double-stranded structure (Baan et al., 1977).

Heterologous reconstitution experiments of 30S subunits implicated the protein moiety of the subunit to confer cistron specificity (Goldberg and Steitz, 1974). Specifically, the protein S12 was found by Held et al. (1974) to be involved and, although the mechanism by which such specificity was conferred was uncertain, an involvement of the 16S rRNA moiety was proposed.

Thus, from the above, it is apparent that the specific interaction of the 30S subunit with mRNA appears to include an RNA : RNA interaction and possibly protein : RNA interactions in order to determine the high degree specificity. The interactions described below are

purely protein : RNA interactions, with the degree of specificity being conferred by the protein moiety.

1.3.2 Viral A-protein

The binding of the A-protein to the Group I phage RNAs has been implied for some time by the finding that the A-protein penetrates the host cell, together with the phage RNA (Paranchych et al., 1970; Kozak and Nathans, 1971). Leipold and Hofschneider (1975, 1976) isolated and characterised an RNA-A-protein complex with a 1 : 1 molar ratio isolated from coliphage M12; however, the site of interaction on the RNA was not investigated. Later, Leipold (1977) isolated a similar complex from MS2 and suggested that the spermidine present in the virions (Fukuma and Cohen, 1975) may play a role in maintaining a stable configuration of the infecting RNA-A-protein complexes and protect them from cell associated nucleases. Recently, Leipold et al. (cited in Fiers, 1979) found that under appropriate conditions a number of hairpins derived from a few quite separate regions of MS2 RNA could be protected by the A-protein from ribonuclease digestion. It would appear from their studies that the A-protein interacts with several different sites on the macromolecule and may function in phage assembly by bringing these scattered regions together in a more compact structure (Fiers, 1979).

1.3.3 Viral coat protein

Bernardi and Spahr (1972) found that the R17 coat protein binds to R17 RNA at the replicase cistron initiation site and thereby prevents ribosomes from initiating the synthesis of the replicase protein. The binding was stable enough to protect a part of the RNA from RNase T_1 digestion, which was characterised both in respect to its primary and secondary structures (Bernardi and Spahr, 1972; Gralla et

al., 1974). The 59 nucleotide-long protected sequence corresponds to residues 1708-1766 in the total MS2 RNA sequence (Fiers et al., 1976). In a similar way, Weber (1976) isolated several specific fragments derived from the replicase cistron initiation site of Qβ RNA.

Berzin et al. (1978) digested an MS2 RNA coat protein complex with RNase T_1 and isolated two protected fragments, the shorter of which was identical to the 59 nucleotide-long site isolated from R17 RNA, but with two U to C base transitions. The larger fragment had the same sequence, but was extended by an additional 44 nucleotides at the 3'-end and was evidently generated by a larger amount of coat protein bound since the relative yield increased at higher coat protein-to-RNA input ratios.

Spahr et al. (1969) observed that only one molecule of coat protein was tightly bound to R17 RNA, whereas other workers have observed that, in the case of f2 RNA, repression was due to a co-operative binding of free coat protein dimers to the RNA, thus forming a hexameric protein cluster on the RNA (Zagórska et al., 1975; Chroboczek and Zagórski, 1975). Berzin et al. (1978) estimated that the complex of coat protein with the shorter fragment of MS2 RNA contained a two-fold excess of the protein, whereas the sedimentation data of Gralla et al. (1974) suggests an equimolar binding of R17 coat protein to R17 RNA. Apparently, the interaction of the phage RNA with coat protein does not possess a definite stoichiometry and varies depending upon the conditions of complex formation and coat protein concentration.

The proposed secondary structure of the larger of the coat protein protected regions on MS2 RNA consists of three hairpin loops (Fig. 8) (Berzin et al., 1978). The hairpin loop structures (a) and (b) have

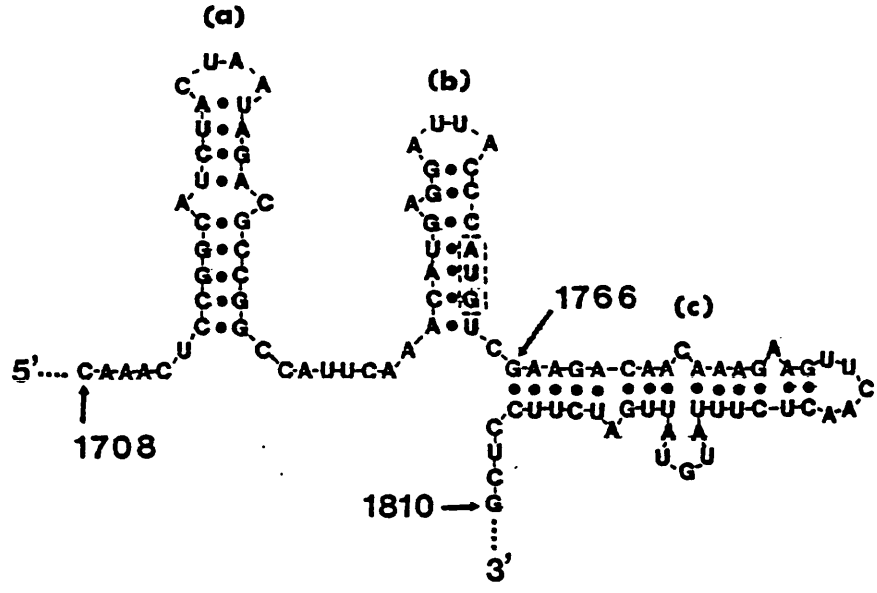


FIGURE 8 Possible secondary structure model for the coat protein binding site on MS2 RNA (residues 1708 - 1810). After Berzin et al. (1978).

received experimental support from analysis of the corresponding structure in R17 RNA, both by melting kinetics (Gralla et al., 1974) and by NMR measurements (Hilbers et al., 1974). Additional support for these structures has recently been obtained from nuclease digestion studies of the protected fragment (Carey et al., 1983). This model is considerably different from that proposed by Fiers et al. (1976) which involves a 'long distance' interaction at this site. It is, of course, possible that for this region of the molecule alternative conformation may exist and that conformational rearrangement may even be linked to biological function, as is the probable case with 16S rRNA (Wittmann, 1982).

There are many reasons to presume that the necessary information required for the recognition and association with the coat protein is encoded in the hairpin loop (b) of this structure (Fig. 8) and probably in some aspect of the secondary structure. Gralla et al. (1974) demonstrated that R17 RNA was recognised while it assumed a stable secondary structure, that the rate of melting of helix (b) was slowed in the RNA-protein complex and that RNase T_1 digestion of the R17 RNA-coat protein complex sometimes yielded only hairpin (b) in the protected fragment, but never only hairpin (a). Both Steitz (1974) and Jansone et al. (1979) showed that the fragment covering mainly hairpin loop (b) of R17 RNA alone was sufficient for recognition by the coat protein. Likewise, Berzin et al. (1978) demonstrated that a fragment containing only loop (a) of the MS2 RNA protected site was completely inactive in binding the coat protein. Recently, Carey et al. (1983) found that the region of the 59-mer fragment that was essential for protein binding extended from about position 1746 to 1761 and encompassed the four residues in hairpin loop (b), the upper part of the stem region and the bulged A residue at position 1751 (Fig. 8). Thus, the protein appears to be recognising some aspect of the RNA secondary structure in

hairpin (b), rather than a single-stranded region. It has been estimated that most of the free energy for binding results from non-electrostatic interaction between the protein and the RNA, although ionic contacts may also contribute to the interaction (Carey and Uhlenbeck, 1983).

1.3.4 Qβ 'phage host factor

A heat-stable host factor protein is required in the Group III Qβ 'phage replication reaction when the viral (plus) strand is the template (Franze de Fernandez et al., 1972). This factor, a hexamer of identical subunits of approximately 12000 daltons (Franze de Fernandez et al., 1972), is probably of ribosomal origin (Carmichael et al., 1975) and binds and protects from RNase T_1 digestion two A-rich oligonucleotides of Qβ RNA. One oligonucleotide is located at residues -63 to -38 from the 3'-end of the molecule and the other located within the replicase cistron (Senear and Steitz, 1976; Billeter, 1978). The tight binding of the factor has been proposed to result from the binding of an aggregate of circular spatial orientation which can interact simultaneously with several low affinity sites on the RNA (de Haseth and Uhlenbeck, 1980a, 1980b).

Although it is doubtful whether this factor is involved in the replication of the Group I phages (Federoff and Zinder, 1973), it can nevertheless bind to a single region of R17 RNA. Senear and Steitz (1976) found that a 21 nucleotide-long segment of R17 RNA was protected against RNase digestion and, undoubtedly, this corresponds to residues 2350-2370 of the MS2 RNA sequence (Fiers et al. 1976) and is located in the middle of the replicase cistron. This interaction presumably has no biological meaning and, since the protected region contains approximately 60% A residues, the interaction is probably reflecting the

affinity of this factor for A residue (Carmichael et al., 1975).

1.3.5 Ribosomal protein S1

Ribosomal protein S1 binds and protected two regions of Qβ RNA from RNAse digestion (Senear and Steitz, 1976; Goelz and Steitz, 1977). One of the fragments is 21 nucleotides long and is derived from the intercistronic region preceding the coat protein gene. The other is 26 nucleotides long and is derived from residues -63 to -38 from the 3'-terminus and is the same as that protected by the host factor (see above section 1.3.4). Both oligonucleotides are rich in pyrimidine residues in agreement with the observation that isolated S1 protein has an affinity for pyrimidine rich sequences (Tal et al., 1972; Miller and Wahba, 1974).

In contrast to Qβ RNA, S1 protein binds to a large number of sites in R17 RNA (Senear and Steitz, 1976) and protects fragments in the size range 40 to 100 nucleotides from RNase T_1 digestion. Fingerprint analysis of the protected regions gave a complex pattern, suggesting that binding was to a large number of sites. In binding to MS2 RNA., as well as to synthetic and natural single-stranded polynucleotides, S1 protein has been shown to disrupt the secondary structure present in these molecules (Szer et al., 1976, 1977; Bear et al., 1976; Thomas et al., 1978).

1.3.6 Viral replicase complex

By the very nature of the mode of replication of the Group I coliphages (Weissman, 1974), the replicase complex must interact with the viral RNA. Although it has been known for some time that the replicases of the RNA coliphages are strongly complexed with endogenous template RNA (August et al., 1963; Weissman et al., 1963), little is known about the nature of the interaction of the replicase complex with the Group I phage RNA templates. What is known is

that this interaction is highly specific, since neither Qβ RNA nor reovirus RNA and E. coli cellular RNA are supported as templates for the f2 replicase complex (Fedoroff, 1975). Likewise, MS2 replicase accepts only MS2 RNA as template and not Qβ RNA and vice versa (Haruna and Spiegelman, 1965a).

To attempt to understand the nature of the replicase-RNA interaction in the Group I phages, it is necessary to consider the case of the related Group III phage Qβ , since the Qβ replicase is the only coliphage RNA-dependent RNA polymerase that has been obtained in sufficient quantity and purity to carry out binding and protection studies. As in the case of the Group I replicases, the Qβ replicase is composed of four non-identical subunits: ribosomal protein S1, the phage-coded replicase subunit and the protein synthesis elongation factors, EF-Tu and EF-Ts (Kamen, 1970; Kondo et al., 1970; Blumenthal et al., 1972; Wahba et al., 1974). The complex binds avidly and specifically to Qβ RNA (August et al., 1968) and this binding was found to involve the overall conformation of the RNA, rather than the 3'-end, since partially degraded Qβ RNA would no longer sustain initiation of RNA synthesis (Haruna and Spiegelman, 1965b). Weber et al. (1972) isolated several fragments of Qβ RNA arising from the same region of the molecule that were protected by the replicase against RNase T_1 digestion. The largest fragment was about 100 nucleotides long and sequence analysis showed that the 3'-end of the protected region overlapped the ribosome binding site of the coat protein cistron. This interaction may have an important role, not only in replication, but also in the regulation of translation since, as we have seen in section 1.2.5, the replicase appears to prevent ribosomes from initiating translation of the coat protein (Kolakofsky and Weiss-

mann, 1971a, b). This repressor activity agrees well with the specificity of this replicase-template interaction.

Under different binding conditions (lower temperature and in the presence of Mg^{2+}) the replicase binds to another region of Qβ RNA located within the replicase gene (Weber et al., 1974). This site consists of three non-overlapping sequences of lengths 164, 160 and 21 nucleotides respectively (Meyer et al., 1975) and may play an important role in forming the initiation complex for replication. The binding of the host factor to two sites in Qβ RNA (Senear and Steitz, 1976) (section 1.3.4) may function by bringing the 3'-end of the RNA template into an appropriate position to the replicase complex bound within the replicase cistron.

Using a different approach, Vollenweider et al. (1976) showed by electron microscopy of Qβ RNA-replicase complexes that the replicase interacts with the two sites on the viral RNA. These sites presumably correspond to the two sites described above.

There are, however, a number of differences between the details of the mode of replication in the Group I and III phages which suggest that a different mechanism of template recognition and interaction may apply in the Group I phages. Firstly, it was noted above that the interaction between replicase and template is very specific with, for example, Qβ RNA being unable to be utilised as a template for the MS2 replicase (Haruna and Spiegelman, 1965a). This would suggest that the structures being recognised by the different replicase complexes are somewhat different. Secondly, the Qβ replication reaction requires the presence of a host factor for copying the plus strand RNA (Carmichael et al., 1975), whereas this protein is apparently not required for efficient replication of the Group I phages (Feder-

off, 1975). However, an as yet unidentified host derived protein may be required for efficient replication of this group of phages (Federoff, 1975). Thirdly, S1 protein, the α component of both the Group I and III replicase complexes, bind to two specific sites on Qβ RNA (Goelz and Steitz, 1977) (cf section 1.3.5), one of which may be involved in the initiation of replication, whereas in R17 RNA this protein binds to a large number of sites. It is possible, however, that a site near the 3'-end of the RNA homologous to that of Qβ RNA is, indeed, present in the S1 protein protected regions and is important for the initiation of replication.

1.3.7 Initiation Factor IF3

During the course of the purification of E. coli IF3, Sabol et al. (1970) demonstrated that this factor could bind to MS2 RNA. Jay et al. (1974a) showed that the binding of IF3 to R17 RNA was saturable with a molar ratio of about 50 : 1 (IF3 : R17 RNA) being required to retain the maximum amount (75%) of the RNA in the nitrocellulose filtration assay. The factor exhibited a greater apparent affinity for R17 RNA than for ϕ6 double-stranded RNA. Leffler and Szer (1974) further showed that IF3 purified from both E. coli and Caulobacter crescentus could protect a region of MS2 RNA against RNase digestion. To determine whether IF3 was recognising a specific site (or sites) on MS2 RNA, Johnson and Szekely (1977) isolated and characterised the site that was protected from RNase digestion by this factor. Some 0.5% to 1% of the RNA was resistant to RNase A digestion and, when isolated, this protected site migrated as a single homogeneous band in a non-denaturing gel. The minimum size of this fragment determined from the extent of protection was determined to be 18-36 nucleotides and the fragment migrated in the gel with a mobility similar to that

of the 30 nucleotide-long 2S rRNA from Drosophila melanogaster (Jordan et al., 1976).

Sequence analysis of the protected fragment by paper fingerprinting of RNase T_1 and RNase A digests gave simple patterns suggesting that the binding was not random, but sequence specific. Five of the oligonucleotides found to be present in the RNase A digests (Table 5) are unique to the sequence of MS2 RNA (Fiers et al., 1976; Iserentant et al., 1980): P9 (G-A-A-A-G-C) at positions 3455-3460; P11 (A-G-G-G-A-C) at positions 3496-3501; P12 (G-A-A-A-G-A-G-C) at positions 3437-3444; P15 (A-G-G-A-G-U) at residues 3367-3372; and P16 (G-A-A-A-G-G-U) at residues 3473-3479 (Fig. 9), although oligonucleotide P15 was not recognised as such by Johnson and Szekely (1977). The remainder of the RNase A and all of the RNase T_1 digestion products had no unique sequences and therefore could not be located unambiguously in the MS2 RNA sequence; however, the majority of the oligonucleotides could be located in the last 143 residues of the MS2 RNA sequence (Fig. 9). The finding that two pyrimidine-rich sequences present in this portion of the RNA sequence (residues 3466-3472 and 3488-3495) were not found in the fingerprints of the protected site, together with the finding that several RNase A and RNase T_1 digestion products originated from more distant parts of the sequence suggested that the protected site was non-contiguous with protected regions being separated by non-protected regions. Homochromatography fingerprinting of native protected material that migrated as a single band upon gel electrophoresis showed that the protected site did, in fact, comprise several stretches of RNA of different length and base composition (Johnson and Szekely, 1979). These RNA stretches were presumably held together by secondary and tertiary interactions in the

TABLE 5

Main digestion products identified in Pancreatic RNase and RNase T_1 fingerprints of the IF3 protected site in MS2 RNA. (After Johnson and Szekely, 1977)

RNase T_1 fragments				Pancreatic RNase fragments			
T_1.	G	T_8.	UG	P_0.	C□	P_9.	(G,AAAG)C*
T_2.	CG	T_{9a}.	UCCG	P_1.	U	P_{10}.	GGGC
T_3.	AG	T_{9b}.	CUCG	P_2.	AC	P_{11}.	(G,G,AG)AC*
T_4.	CCG	T_{10}.	UCUCG	P_3.	GC	P_{12}.	(G,AG,AAG)C*
T_{5a}	CACG	T_{11}.	▽$(U_{3/4},C_{4/5},AC,AU)$	P_4.	AU	P_{13}.	GGU
T_{5b}.	CCAG			P_5.	AGC	P_{14}.	GGGU
T_6.	AACG			P_6.	GU	P_{15}.	(G,AG,AG)U*
T_7.	AAAG			P_7.	GGC	P_{16}.	(G,G,AAAG)U*
				P_8.	(AG,G)C		

□ Not found in fingerprints of IF3 protected site by Johnson and Szekely (1977) since mobility was slow in the first dimension and therefore was not transferred to the second dimension (cf Sanger et al., 1965).

* Unique oligonucleotides occurring only once in the MS2 RNA sequence (Fiers et al., 1976; Iserentant et al., 1980).

▽ Revised composition (B. Johnson pers. commun.).

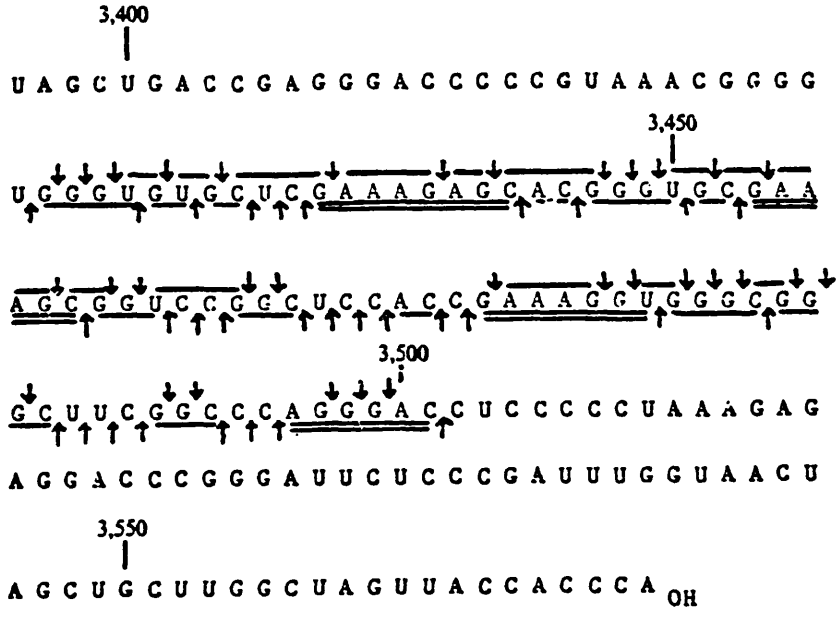


FIGURE 9 Extent of IF3 protected site on MS2 RNA as determined by Johnson and Szekely (1977).

The 3'-terminal untranslated sequence of MS2 RNA showing the locations of RNase T_1 and Pancreatic RNase digestion products (Table 5). RNase T_1 products are shown by a line above the sequence and pancreatic RNase products by a line below. The unique Pancreatic RNase products (in order from 5'-end: P_{12}, P_9, P_{16} and P_{11}) are indicated by a double underlining. Arrows indicate nuclease cleavage sites.

Note: The nucleotide sequence shown is that determined by Fiers et al. (1976). In the revised sequence by Iserentant et al. (1980), the RNase T_1 oligonucleotides U-C-C-G (residues 3461 - 3464 above) and U-G (residues 3450 - 3451) are interchanged.

non-denaturing gel and, in this respect, the protected site resembled the binding sites of several ribosomal proteins on 16S rRNA (Brimacombe et al., 1976; Brimacombe, 1978; Noller and Woer, 1981).

The determination of the nucleotide sequences of the IF3 protected regions together with the effects of various procedures known to disrupt RNA secondary and tertiary structures upon binding and protection by the factor, would support the validity of this model. Additionally, the knowledge of the sequences of the protected regions would allow the construction of possible secondary structure models of the protected site and give some insight into the nature of the interaction of the factor with the RNA.

1.4 SCOPE OF THIS THESIS

This thesis is a study of the interaction of E. coli Initiation Factor IF3 with coliphage MS2 RNA and, thus, forms a continuation of the work of Johnson and Szekely (1977, 1979) in this laboratory.

The nucleotide sequences of MS2 RNA protected by IF3 from pancreatic RNase digestions have been determined and the protected site located in the MS2 RNA primary sequence. Studies of the effects of various treatments of MS2 RNA known to affect the secondary and tertiary structures of the molecule on the binding and protection by IF3 have been investigated in order to determine the nature of these structures present at the IF3 protected site. A hypothetical secondary structure model of the protected site on the RNA that is consistent with these data is proposed. By comparison of the nature of the IF3 protected site on MS2 RNA with the nucleotide sequence and secondary structure model of 16S rRNA, some insight is permitted into the nature of the interaction of the factor with this moiety of the 30S subunit, where IF3 is functionally active.

MATERIALS

2.1 CHEMICALS

Glass distilled water was used throughout. All reagents, unless otherwise stated, were of analytical grade purchased from Hopkin & Williams, BDH Chemicals Ltd., The Sigma Chemical Co. or the Boehringer Corporation (London) Ltd. Tris(hydroxymethyl)methylamine was Trizma base from Sigma. Ribonuclease-free sucrose was from Bethesda Research Laboratories and electrophoresis dyes from Gurr Ltd. γ-(^{32}P)-ATP (3000 Ci/mmol), (^{32}P)-orthophosphate (carrier free) and L-(^{35}S)-methionine (1000 Ci/mmol) were obtained from the Radiochemical Centre, Amersham (U.K.).

2.2 COLUMN CHROMATOGRAPHY MEDIA

DE52 and P1J cellulose were obtained from Whatman Ltd., Sephadex G75 and G50(f) were from Pharmacia and hydroxylapatite was Bio-gel HTP from Bio-Rad Laboratories (U.S.A.).

2.3 PAPERS, FILTERS, THIN-LAYER PLATES AND X-RAY FILMS

Cellulose acetate was purchased as strips 3 x 55cm and 3 x 95cm from Schleicher & Schüll GmbH. 3MM papers were from Whatman in sheets of 46 x 57cm and DE81 paper from the same company in rolls of 46cm x 50m.

Nitrocellulose filters were from Millipore type HAWP 02500 (0.45μm). DEAE thin layer plates (Polygram Cell. 300 DEAE HR2/15, 20 x 40cm) were from Macherey & Nagel obtained through Camlab. Glass fibre filters were Whatman GF/C.

X-ray films (35 x 43cm) were Blue Brand, Kodirex and XH-1 brands of Kodak Ltd. X-ray cassettes were by Fujimex with Hanimex Mach II screens.

2.4 BIOLOGICAL MATERIALS

Ribonuclease T_1(EC 3.1.27.3)(3 x 10^5 units/mg) prepared by Sankyo Co. (Japan) from Aspergillus oryzae was supplied by Calbiochem. Bovine pancreatic ribonuclease A (EC 3.1.27.5)(3100 units/mg), ribonuclease-free Deoxyribonuclease I (EC 3.1.21.1)(2029 units/mg) code DPFF and bacterial alkaline phosphatase (EC 3.1.3.1)(40 units/mg) were obtained from Worthington Biochemical Corporation. Nuclease P1 (EC 3.1.30.1)(300 units/mg) Grade A from Penicillium citrinum was obtained from Calbiochem and was homogeneous on ultra-centrifugation and column chromatography. Nuclease S_1(EC 3.1.30.1)(10^5units/mg) from Aspergillus oryzae was obtained from Sigma. Hen egg white lysozyme (EC 3.2.1.17)(2200 units/mg) was obtained from Boehringer.

Coliphage MS2 RNA was the generous gift of Dr. B. Johnson, prepared according to the method of Strauss and Sinsheimer (1963) and migrated as a single band upon gel electrophoresis. E. coli $tRNA_f^{Met}$ was obtained from Boehringer. PcV-ds-RNAs from Penicillium chrysogenum virus were the generous gift of Dr. K.W. Buck.

Coliphage MS2 was obtained from Miles Laboratories. Bacteriophage T_4XF-1 infected E. coli B cells were from P.L. Biochemicals and E. coli MRE600 was grown in the Pilot Plant at Imperial College.

Purified E. coli Initiation Factors IF1 and IF2 and ribosomal RNAs were the generous gift of Dr. B. Johnson. Purified ribosomal protein S1 was the gift of Mr. R. Foreman, rabbit reticulocyte lysate ribosomal RNAs were from Dr. S. Legon and E. coli dialysed S_{100} was

the gift of Dr. J. Johnson.

2.5 INSTRUMENTS

The Powerpack for gel electrophoresis was a Shandon Southern Vokam SAE 2761. High voltage paper electrophoresis tanks were those described by Brownlee (1972) as 'hanging' and 'up-and-over' tanks. The fraction collector was an LKB type 7000. Spectrophotometers were Pye Unicam SP500 and Beckman DK2A and the liquid scintillation counter was a Beckman LS-2000B Liquid Scintillation System. Ultra-centrifuges were by Beckman and low speed centrifuges by MSE.

Variable pipettes were Pipetman P20 and P200 by Gilson and capillaries for incubation were drawn out from 100mm plain capillary tubes from Turner & Co. Ltd. Plastic conical tubes were by Eppendorf and were used with a mini-centrifuge model 5412 from the same company.

METHODS

3.1 MISCELLANEOUS

3.1.1 General

Glassware and Eppendorf tubes were siliconed prior to use using Repelcote (Hopkins & Williams Ltd.) and were autoclaved, as were pipette tips, for 20 mins at 15 p.s.i. Dialysis tubing was boiled before use in 10mM Na_2EDTA and then boiled in several changes of distilled water prior to storage at 4°C. Triethylamine was distilled and carbonated as described by Brownlee (1972). Phenol was distilled prior to use and buffers for high voltage paper electrophoresis were as described by Brownlee (1972). Scintillant contained in 5 litres of toluene: 30g Butyl-PBD [2-(4'-t-butylphenyl)-5-(4"-biphenylyl)-1,3,4-oxdiazole) and 250g napthalene (BDH)(Scales, 1967). Urea, in the form of a 10M solution in water, was deionised prior to use with Amberlite MB3 resin for 15 minutes.

3.1.2 Polyacrylamide gel electrophoresis of RNA

Polyacrylamide gels (20 x 20 x 0.4cm and 20 x 40 x 0.4cm) for RNA fractionation were prepared as described by Adams et al.(1969) using TAE running buffer (40mM Tris-OAc pH 8.2, 10mM Na_2EDTA). Electrophoresis was carried out at 4V cm^{-1} at 4°C.

Staining of unlabelled RNA species after fixing for 10 minutes in 1M HOAc was for 2 hours in Toluidine blue (0.2% in 0.4M NaOAc, pH 4.7) followed by extensive destaining with water (Szekely & Loviny, 1975).

RNA was electrophoretically eluted from crushed gel slices onto DEAE paper as described by Adams et al. (1969). Elution of DEAE paper was as described by Sanger et al. (1965).

Two dimensional 15% polyacrylamide gels were as described by Ross and Brimacombe (1979) using initially TAE buffer for the first dimension (20 x 20 x 0.2cm) and TAE buffer plus 8M urea for the second dimension (40 x 20 x 0.2cm). Later gels used the buffer system of Maxam and Gilbert (1977) in the second dimension and were run at approximately 40°C in this dimension.

Glyoxal denaturation of end-labelled protected fragments was as described by McMaster and Carmichael (1977).

3.1.3 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gels were prepared as described by Weber and Osborn (1969) using 10 x 0.7cm Plexiglass tubes. Electrophoresis was at 4mA gel^{-1} for 18 hours until the bromophenol blue tracking dye was near the bottom of the tube. After electrophoresis, the gels were stained for 3 hours in Coomassie Blue (1.25g in 454ml MeOH, 46ml 96% HOAc) and destained with successive changes of destain (7.5% HOAc, 5% MeOH).

3.1.4 Homochromatography fingerprinting

Two-dimensional homochromatography fingerprinting was as described by Brownlee (1972) after Brownlee and Sanger (1969). The first dimension was on a 3 x 55cm cellulose acetate strip in pH 3.5 electrophoresis buffer containing 7M urea at 4kV and was continued until the blue marker dye had migrated approximately 35cm from the origin. Transfer to the second dimension was by the method of Southern (1974) as modified by Volckaert et al. (1976). The second dimension was on a 20 x 40cm DEAE cellulose plate using either 3% or 5% homomix 'C' at 60°C (Brownlee, 1972).

Spots corresponding to ^{32}P oligonucleotides were eluted as described by Brownlee (1972) using an elution device connected to a

vacuum line to collect the cellulose followed by elution with 30% triethylamine carbonate, pH 10.

3.1.5 Autoradiography

Autoradiography of gels, papers and thin layer plates was direct using either Blue Brand or Kodirex X-ray films. Gels were wrapped in cling film and then, together with papers and thin layer plates, were mounted on lead-backed folders and exposed to two sheets of X-ray film at room temperature.

Autoradiography of 'wandering spots' was according to the method of Laskey and Mills (1977) using hypersensitized XH-1 film and Hanimex Mach II calcium tungstate intensifying screens at -70°C.

3.1.6 TCA precipitation

Labelled RNA was precipitated with 2.5ml of 5% TCA and 80µg of unlabelled MS2 RNA as carrier for 15 mins. at 0°C. The precipitates were filtered through Whatman GF/C filters and washed with three 10ml aliquots of ice cold 5% TCA and then with 10ml of ice cold 95% ethanol. The filters were dried and counted by liquid scintillation counting in the presence of 5ml of scintillant (section 3.1.1).

3.2 ISOLATION OF INITIATION FACTOR 3 FROM E. COLI

IF3 was prepared from 2kg lots of E. coli MRE 600 according to the method of Lee-Huang and Ochoa (1974) to step 4. Fractions containing IF3 were concentrated by elution from a second phosphocellulose column and then filtered through a Sephadex G75 column in the presence of 6M urea (Johnson and Szekely, 1979) to give a homogeneous preparation of IF3. A typical preparation is described below.

3.2.1 Growth of cells

E. coli MRE 600 was grown for 2 hours at 37°C in a 60 litre stainless steel fermenter in a medium, pH 7.0, containing 5g/l peptone (Oxoid), 7g/l yeast extract paste, 2g/l NaCl, 2.6g/l KH_2PO_4,4.6g/l NaH_2PO_4, 0.01% (v/v) polyglycol P-2000. The contents of the fermenter were then transferred to a 300 litre stainless steel fermenter and the growth continued for a further 1.5 hours in a medium containing 10g/l glucose monohydrate, 14g/l yeast extract paste, 2g/l NaCl, 2.6g/l KH_2PO_4, 4.6g/l $NaH_2PO_4.2H_2O$, 0.01% polyglycol P-2000, pH 7.0, until the O_2 uptake was 1.5% (mid log. phase - B. Johnson pers. commun.). The medium was then chilled and the cells harvested at 13 200 x g in a Sharples No.6 Super Centrifuge. The cell paste was then weighed and stored at -20°C until used. From a 300 litre fermentation, approximately 2 kg wet weight of cells was obtained. I thank Mr. G.T. Banks and the Pilot Plant Staff, Dept. of Biochemistry, Imperial College, for performing these fermentations.

3.2.2 Preparation of Ribosomes

The frozen cell paste was broken up into convenient sized pieces and 500g rapidly weighed out. The cells were thawed in 250ml of TMS buffer (20mM Tris HCl, pH 7.8, 10mM $Mg(OAc)_2$, 10mM 2-mercaptoethanol), centrifuged at 8 000 x g at 4°C and the pellet resuspended in a further 250ml of TMS buffer. Disruption of the cells was achieved by one passage through a pre-cooled APV Manton-Gaulin 15M-8BA homogeniser at 8 000 p.s.i. The viscous homogenate was cooled on ice and 1ml of 1mg/ml Deoxyribonuclease I added. The mixture was gently stirred at 4°C for 30 minutes, or until the viscosity had fallen sufficiently for ease of manipulation. Intact cells together with cell debris were removed by centrifugation at 4°C, first at 8 000 x g for 30

minutes and then at 30 000 x g for one hour.

Ribosomes were sedimented from this extract by centrifugation for 3 hours at 100 000 x g in a Beckman Type 60-Ti rotor at 4°C. The surface of each pellet was rinsed quickly with cold TMS buffer and the viscous pellets removed from each tube, pooled and stored at -20°C until used.

The above procedure was repeated three times for the remaining 1.5 kg of E. coli cells and the ribosomes pooled and stored at -20°C.

The pooled ribosomes were washed by resuspension overnight at 4°C in 200ml of TMS buffer and then pelleted by centrifugation for 3 hours at 100 000 x g at 4°C and resuspended in 100ml of TMS buffer at 4°C.

3.2.3 IM NH_4Cl wash and $(NH_4)_2SO_4$ fractionation

IF3 together with IF1 and IF2 were eluted from the ribosomes by stirring for 1.25 hours in TMS buffer containing 1M NH_4Cl (Lee-Huang and Ochoa, 1974) followed by centrifugation for 3 hours at 105 000 x g. A small aliquot of the 1M NH_4Cl washed ribosomes was subjected to a second 1M NH_4Cl wash, centrifuged, and the pellet suspended in TMAG buffer (10mM Tris HCl, pH 7.4, 5mM $Mg(OAc)_2$, 0.25M NH_4Cl, 50% glycerol) and stored at -20°C for use in the IF3 dependent ribosomal f(^{35}S)met-tRNA binding assay.

TMS buffer saturated with $(NH_4)_2SO_4$ was slowly added over 20 minutes with slow stirring at 4°C to the 1M NH_4Cl wash to give 35% saturation. The solution was stirred slowly for 30 minutes to allow the precipitate to form completely and then centrifuged at 20 000 x g for 20 minutes. Further NH_4Cl saturated TMS buffer was slowly added to the supernatant to give 45% saturation and the precipitate removed exactly as before. The procedure was repeated for the 45-55% and

55-75% fraction. The precipitate resulting from this final fraction (containing IF1 + IF3 - Lee-Huang and Ochoa, 1974) was resuspended in 10ml TMSG buffer (10mM Tris HCl pH 7.4, 1mM $Mg(OAc)_2$, 20mM 2-mercaptoethanol, 10% glycerol) and dialysed for 15 hours against 4 litres of TMSG buffer at 4°C with two changes of buffer. A 0-75% fraction was prepared containing total initiation factors, suspended in 5ml of TMSG buffer and dialysed together with the 55-75% fraction. Both dialysates were centrifuged for 20 minutes at 20 000 x g to remove any precipitate.

3.2.4 DEAE-cellulose column chromatography

The dialysate containing IF3 + IF1 (24ml) was applied to a 300ml bed volume DE52 column and washed with 750ml of TMSG buffer. Fractions (7.5ml) were collected from the start of loading the column. IF3 was eluted from the column with TMSG buffer containing 0.25M NH_4Cl with fractions collected every 7.5ml. The A_{280} of the fractions was determined (Fig.10) and fractions 101-150, containing the peak A_{280} eluted from the column, were rapidly frozen and stored at -20°C.

Assay of the fractions: IF3 activity in the column fractions was determined by the IF3-dependent ribosomal binding of f(^{35}S)met-tRNA$_f^{Met}$ in the presence of excess IF1 and IF2 with MS2 RNA as messenger (Lee-Huang and Ochoa, 1974). fmet-tRNA$_f^{Met}$ bound to the ribosome is retained on a cellulose nitrate filter upon filtration whereas unbound fmet-tRNA$_f^{Met}$ passes straight through (Nirenberg and Leder, 1964). Therefore, when f(^{35}S)met-tRNA$_f^{Met}$ is used together with a reaction mixture containing all the components required for its binding to ribosomes with the exception of IF3, the presence of this factor in the column fractions can be assayed by the amount of radioactivity retained on the filters.

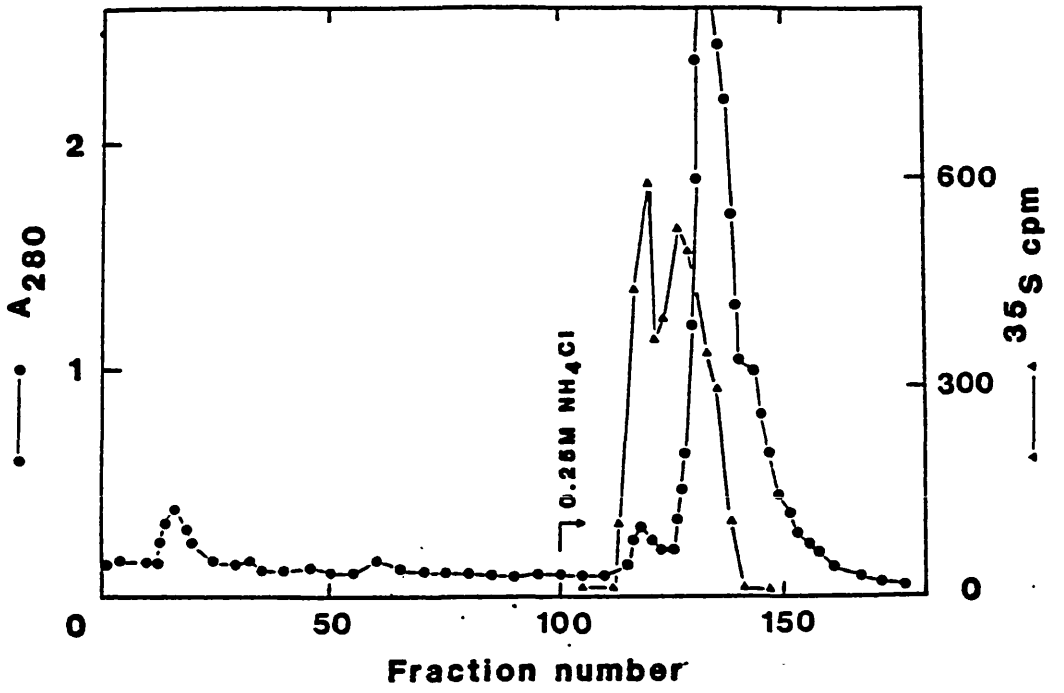


FIGURE 10 Purification of IF3 on DEAE cellulose.
Chromatography of 55 - 75% $(NH_4)_2SO_4$ fraction of 1M NH_4Cl ribosomal wash on DEAE cellulose. Fractions were collected every 7.5ml and assayed for A_{280} (●) and activity in the IF3 dependent ribosomal binding of f(^{35}S)met-tRNA$_f^{Met}$ assay (▲) after start of elution with 0.25M NH_4Cl.

Each assay was performed in a total volume of 25µl containing: 50mM Tris HCl pH 7.4, 5mM $Mg(OAc)_2$, 60mM NH_4Cl, 1mM dithiothreitol, 0.2mM GTP, 1µg purified IF1, 2.5µg purified IF2, 3 A_{260} units NH_4Cl washed E. coli ribosomes, 40µg MS2 RNA, 12 p moles f(^{35}S)met-$tRNA_f^{Met}$ and 5µl of selected column fractions. A blank assay containing 5µl of TMSG buffer was also prepared together with assays containing total initiation factors and known active purified IF3. The reaction mixtures were incubated for 20 minutes at 25°C in capillary tubes. The contents of each tube were then blown into individual siliconed glass tubes containing ice cold TAM buffer [50mM Tris HCl pH 7.4, 60mM NH_4Cl, 5mM $Mg(OAC)_2$] and slowly filtered through a nitrocellulose filter (Millipore HAWP 02500, 0.45µm) presoaked in TAM buffer and washed with 2 x 10ml aliquots of ice-cold TAM buffer. The filters were dried and counted in the presence of scintillation fluid (section 3.1) in a liquid scintillation counter (Fig 10). Fractions 115 to 129 inclusive containing IF3 activity were pooled for the next chromatographic step.

3.2.5 Large P11-cellulose column chromatography

Pooled fractions 115-129 from the DE52 column (100ml) were loaded onto a 58ml P11-cellulose column and washed with 500ml of TMSG buffer containing 0.25M NH_4Cl. Fractions (7.5ml) were collected from the start of the wash. IF3 was eluted with TMSG buffer containing 0.5M NH_4Cl and fractions collected in siliconed glass tubes. The A_{280} of the column fractions were determined (Fig.11) and fractions 71 to 120 stored at -20°C until the next chromatographic step.

Assay of the fractions: The presence of IF3 was assayed using the f(^{35}S)met-$tRNA_f^{Met}$ binding assay as described for the DE-52 cellulose column (Fig. 11). The total volume of each assay was 25µl and con-

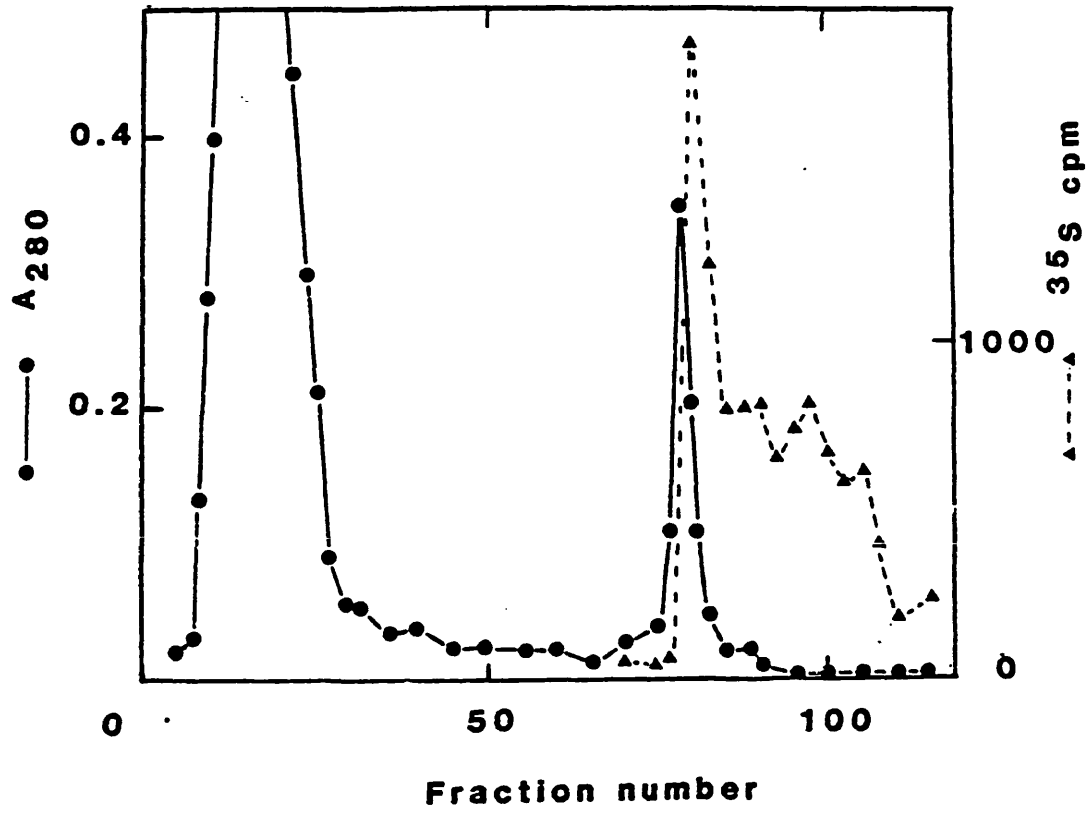


FIGURE 11 Purification of IF3 on P11-cellulose (I).
Chromatography of fractions from DEAE cellulose column on P11 cellulose. Fractions were collected every 7.5ml and assayed for A_{280} (●—●) and activity in the IF3 dependent ribosomal binding of f(^{35}S) Met-tRNA$_f^{Met}$ assay (▲—▲) during elution with 0.5M NH_4Cl.

tained 5µl of selected column fractions. Fractions 79-85 inclusive containing the peak IF3 activity were pooled for the next chromatographic step.

3.2.6 Small P11-cellulose column chromatography

The pooled fractions from the previous column were diluted with and equal volume of TMSG buffer to give a final concentration of 0.25M NH_4Cl. This solution (92ml) was loaded onto a 12ml bed volume P11 column over 4 hours and washed overnight with 450ml of TMSG buffer containing 0.25M NH_4Cl. IF3 was eluted from the column with TSMG buffer containing 0.5M NH_4Cl and fractions were collected in siliconed glass tubes, every 0.2ml being numbered from the start of elution. Fractions were stored frozen at -20°C until the next column step; the A_{280} of the individual fractions not being determined in order to reduce losses of IF3.

Assay of the fractions: The presence of IF3 was assayed using the f(^{35}S)met-tRNA$_f^{Met}$ binding assay as described previously. The reaction mixture (25µl) contained 1µl of each column fraction (Fig.12) and the peak IF3 activity was present in fractions 12-17. These fractions were pooled for the next chromatographic step.

3.2.7 Sephadex G75 column chromatography

Pooled fractions from the previous column (12ml) were vacuum dialysed against 3 x 1 litre volumes of TAMU* buffer for 18 hours at 4°C. At the end of this period the volume of the dialysate had usually reduced to 1ml and the dialysis tubing was rinsed with a further 1ml of TAMU buffer and added to the dialysate. This was carefully loaded onto a 190ml bed volume Sephadex G75 column and allowed to filter through the column. After the void volume (55ml), fractions were collected (2ml) and immediately dialysed overnight against 5 litres of

* 50mM Tris HCl pH 7.4, 0.25M NH_4Cl, 14mM 2-mercaptoethanol, 6M urea.

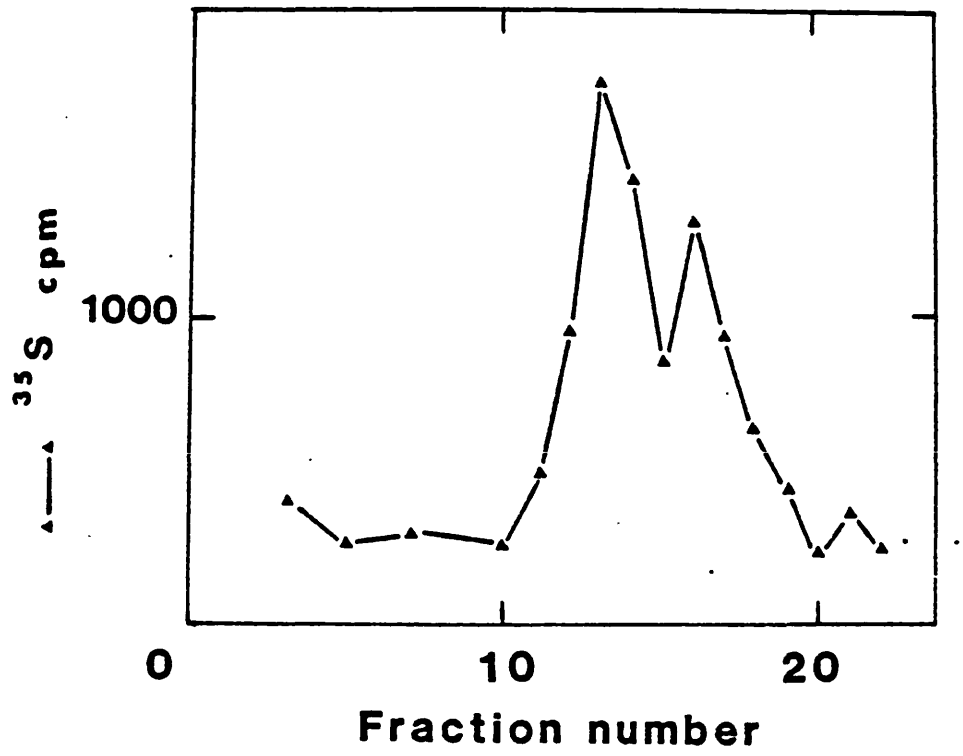


FIGURE 12 Purification of IF3 on P11-cellulose (II).
Chromatography of diluted fractions from large P11-cellulose column on small P11-cellulose column. Fractions were collected every 2.0ml from the start of elution with 0.5M NH_4Cl and assayed for activity in the IF3-dependent ribosomal binding of f(^{35}S)met-tRNA$^{Met}_f$ assay (▲——▲).

TMSG buffer containing 50mM NH_4Cl. The contents of each dialysis bag were emptied into small siliconed tubes and stored at -20°C.

Assay of the fractions: The presence of IF3 was assayed by SDS-polyacrylamide gel eletrophoresis as described in section 3.1.3 using 50µl of each fraction. (Result shown in section 4.1.1).

Fractions containing pure IF3 were pooled (volume 16ml) and concentrated by vacuum dialysis against TMSG buffer containing 50mM NH_4Cl at 4°C. A typical IF3 preparation gave a final yield of approximately 300µg of homogeneous IF3, determined by the method of McKnight (1977), from 2kg of E. coli cells.

3.2.8 Stability of the preparations

The preparations of IF3 at a concentration of 0.1mg/ml usually retained their MS2 RNA binding activity for up to six months. when stored at -20°C..

3.3 ISOLATION OF POLYNUCLEOTIDE KINASE

Polynucleotide kinase (EC 2.7.1.78) isolated from bacteriophage T_4 infected E. coli B cells was used for the in vitro 5'-end group labelling of RNA. The enzyme was isolated according to the method of Richardson (1971) as modifed by Anderton (1975). A typical preparation is described below.

3.3.1 Cell disruption

Approximately 25g of frozen bacteriophage T_4XF1 infected E. coli B cells were broken by grinding at 4°C with 2.5 times their weight of alumina and 10ml of TS buffer (50mM Tris HCl pH 7.4, 14mM 2-mercaptoethanol) for 30 minutes. A further 100ml of buffer was added and, after centrifugation to remove cell debris and alumina, the

A_{260} of the supernatant was adjusted to 105 with TS buffer (Fraction I).

3.3.2 Streptomycin sulphate precipitation

5% streptomycin sulphate was added dropwise with stirring at 0°C to Fraction I to give a 1% solution. After 30 minutes, the suspension was centrifuged at 20 000 x g and the supernatant discarded. The precipitate was resuspended in 20ml of KPS buffer (0.1M KPO_4, pH 7.5, 20mM 2-mercaptoethanol) and stirred slowly overnight at 4°C. A further 80ml of KPS buffer was then added to give 100ml of Fraction II.

3.3.3 Autolysis

Nucleic acid present in Fraction II was autolysed by the addition of 0.28ml of 1M $MgCl_2$ to activate endogeneous nucleases in the 100ml of Fraction II. Incubation was at 37°C and the A_{260} of the suspension and of the perchloric acid soluble fraction were determined at intervals. Autolysis was complete when the A_{260} of the acid soluble material was equal to or greater than 90% of the total A_{260}. The process typically took about 135 minutes (Fig.13). During autolysis a white precipitate formed; this was removed by centrifugation to give the supernatant - Fraction III.

3.3.4 $(NH_4)_2SO_4$ fractionation

Fraction III was made 10% in $(NH_4)_2SO_4$ by the gradual addition of 9.7g of the solid over 30 minutes with stirring at 4°C. After a further 20 minutes, the suspension was centrifuged and the precipitate discarded. 18.7g of $(NH_4)_2SO_4$ was added gradually as before to the supernatant. After centrifugation, the supernatant was discarded and the pellet dissolved in 20ml of PPS buffer (10mM $K.PO_4$ pH 7.5, 14mM 2-mercaptoethanol) and dialysed overnight against 2 litres of the same

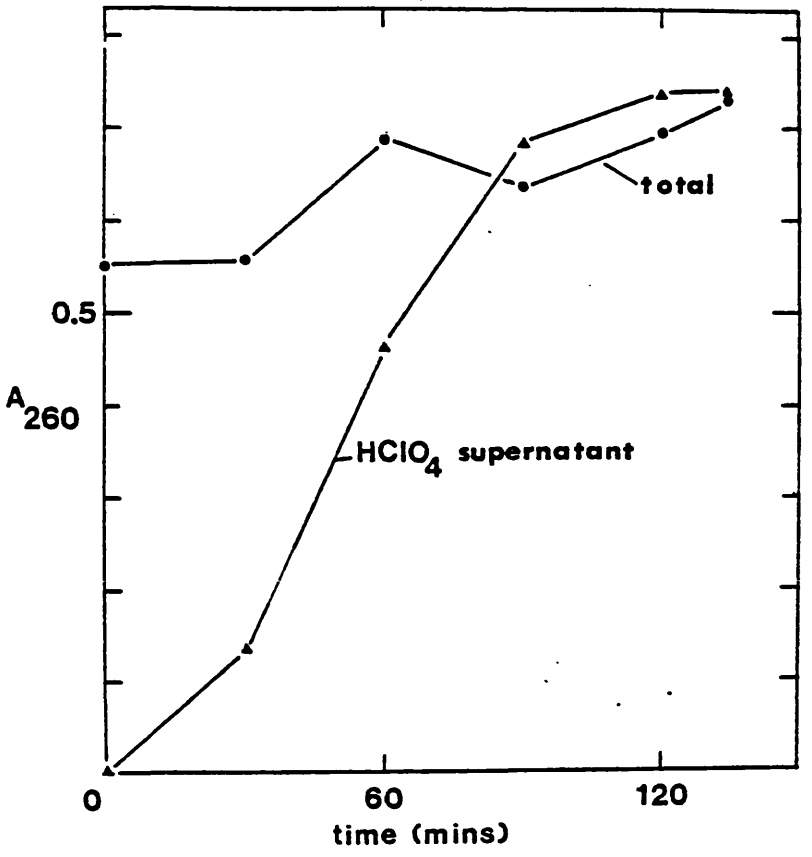


FIGURE 13 Time course of autolysis of Kinase Fraction II. Incubation was at 37°C and the A_{280} of the total suspension (●—●) and the perchloric acid soluble fraction (▲—▲) were determined at intervals as described in the text.

buffer at 4°C with two changes of buffer. This preparation was called Fraction IV.

3.3.5 DEAE-cellulose column chromatography

Fraction IV (20ml) was applied to a 100ml bed volume DE52-cellulose column. After washing with PPS buffer, the enzyme was eluted with PPS(50) buffer (50mM KPO_4 buffer pH 7.5, 14mM 2-mercaptoethanol). Fractions were collected (10ml) and numbered when the PPS(50) buffer was applied to the column.

Assay of the fractions: The fractions were assayed according to the rapid semi-quantitative assay of Szekely and Sanger (1969). Each assay was performed in a total volume of 4µl containing: TMM buffer (10mM Tris HCl pH 8.1, 10mM $MgCl_2$, 14mM 2-mercaptoethanol), 5 nmoles CpU, 1 nmole γ-(^{32}P)-ATP and 2µl of each column fraction. Incubation was for 30 minutes at 37°C in capillary tubes. Richardson (1971) showed that phosphate anions inhibited the kinase activity by 50% at a concentration of 7mM and by up to 95% at 70mM. The final phosphate concentration in the assay was 25mM which meant that the enzyme was partially inhibited but, since the degree of inhibition in all the assays was the same, the assays were comparable without giving a maximal value for the kinase activity. An assay of 1µl of Fraction IV was performed at the same time, together with a control of 2µl of reaction mixture only. The samples were applied to a DE81 paper sheet, the sample line carefully wetted and then the whole sheet placed on a rack and wetted with electrophoresis buffer (0.5% pyridine - 5% acetic acid (v/v), pH 3.5) as described by Sanger et al. (1965). One small drop of dye mixture consisting of equal volumes of 1% Xylene Cyanol FF (Blue), 2% orange G (Yellow), 1% Acid Fuchsin (Pink) (Sanger et al., 1965) was applied at each end of the origin to follow

the progress of the electrophoresis. Electrophoresis was carried out at two kilovolts for one hour. The paper was allowed to dry, marked with radioactive ink and exposed against X-ray film.

Fig. 14 shows a one-dimensional run of such assays of DE52 column fractions. Fractions, 8, 9 and 10 contained the peak kinase activity as shown by the density of the labelled dinucleotide (^{32}pCpU) spots. These fractions were pooled to give kinase Fraction V.

3.3.6 Phosphocellulose column chromatography

Fraction V (30ml) was loaded onto a 13ml bed volume P11-cellulose column and washed with 10ml of PPS(50) buffer. The enzyme was eluted from the column with 20ml portions of PPS(50) containing KCl in increasing concentrations: 50mM, 0.1M and 0.25M. Fractions (2ml) were collected and numbered for collection when the 0.25M KCl buffer was applied. The active fractions were called Fraction VI.

Assay of the fractions: The reaction mixture was similar to that described previously, with a total volume of 6µl, of which 1µl was of each fraction, giving a final concentration of 8mM. Incubation and electrophoresis were exactly as described previously. Fractions 10-15 inclusive were pooled to give Fraction VI.

3.3.7 Hydroxylapatite column chromatography

Fraction VI (6ml) was diluted four-fold with SPP buffer (20mM KPO_4 pH 7.0., 14mM 2-mercaptoethanol) loaded onto a 5ml hydroxyl-apatite column and washed with 175ml of SPP buffer. The enzyme was eluted with successive 10ml batches of pH 7 buffer containing increasing concentrations of KPO_4: 0.1M, 0.2M, 0.3M and 0.5M, together with 14mM 2-mercaptoethanol. Approximately 2ml column fractions were collected. Since the elution of the enzyme from the hydroxyl-apatite is somewhat unpredictable (Anderton, 1975), fraction tubes were

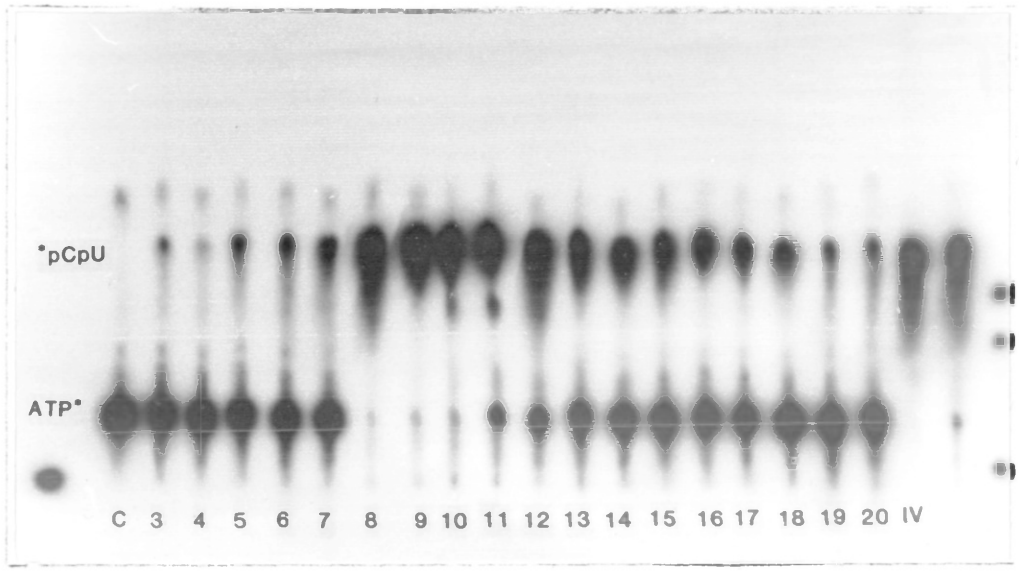


FIGURE 14 Purification of PNK on DEAE-cellulose.
Assay for PNK activity in fractions from DEAE-cellulose column as described in the text (section 3.3.5). C = control with no enzyme added; IV = Fraction IV PNK preparation.

numbered when the 0.1M buffer was applied and continued until the end of the 0.5M buffer.

Assay of the fractions: The column fractions had to be diluted in the reaction mixture for assaying since they were eluted at very high phosphate concentrations. Less ATP was used in the assays as the kinase was eluted in a total volume about five times greater than the volume loaded onto the column and was therefore dilute. The reaction mixture contained in a total volume of 20µl, TMM buffer (final concentration), 4 nmoles CpU and 0.2 nmoles γ-(^{32}P)-ATP. 2µl of each fraction were added, bringing about a 1:10 dilution of the phosphate concentration. This was still high however and thus the enzyme was under conditions of inhibition. Incubation, eletrophoresis and autoradiography were exactly as before.

Fractions 16-18 inclusive showed peak kinase activity with little contamination by phosphatase or nuclease. These fractions were pooled and concentrated by vacuum dialysis gainst 20mM KPO_4 pH 7.0, 14mM 2-mercaptoethanol, 25mM KCl. The final volume of Fraction VII enzyme was usually approximately 3ml.

An estimate of the enzyme concentration of Fraction VII was made by assaying serial dilutions of the preparation. Each reaction mixture (total volume 13µl) contained TMM buffer, 5 nmoles CpU, 1 nmole γ-(^{32}P)-ATP and an appropriate amount of the diluted enzyme. The concentration of phosphate anions in the assays was kept constant at 12mM. The result of the assay gave a minimum concentration, because of the phosphate anions, of 3 unit/µl. One unit represents the amount of enzyme required for the complete transfer of ^{32}P from 1 nmole of γ-(^{32}P)-ATP in 30 minutes at 37°C without any substrate specification (Anderton, 1975).

3.4 PREPARATION OF (^{32}P) LABELLED MS2 RNA

(^{32}P)-MS2 RNA was prepared as described by Brownlee (1972) except that the host was Escherichia coli C3000 and the growth medium contained per litre: 10g Bacto-peptone, 1g glucose, 5g NaCl, 20ml glycerol, 1g casamino acids (Difco), 1g $MgSO_4.7H_2O$. All solutions were autoclaved for 20 minutes at 15 p.s.i. and glassware baked at 180°C prior to use.

3.4.1 Revival of the 'phage

Bacteriophage MS2, stored as an E. coli C3000 lysate at -20°C, was revived by three successive passages through an E. coli C3000 host. A primary culture of E. coli C3000 was grown up overnight in 100ml of medium at 37°C. A 4% inoculation of this culture was then made into 100ml of medium supplemented with 0.25ml of 4mg/ml Thiamine hydrochloride (sterilized by filtration) and allowed to grow for 2.25 hours at 37°C. 0.2ml of 1M $CaCl_2$ was added together with 10ml of the 'phage lysate which had been stored at -20°C. After leaving for five minutes at room temperature without shaking to facilitate 'phage attachment, the incubation was continued on a shaker table at 37°C for a further 4.5 hours. After this time, 1.0ml of $CHCl_3$ was added overnight. A new primary culture was prepared and the above procedure repeated but infecting with 2.5ml of the previous day's lysate. The procedure was repeated for a third time, using 1ml of the previous day's lysate.

3.4.2 Growth of labelled 'phage and isolation of (^{32}P)-MS2 RNA

A primary overnight culture of E. coli C3000 was grown in 100ml of medium at 37°C. A 4% inoculation of this culture was then made into 100ml of medium in a Roux flask, supplemented with 0.25ml of 4mg/ml of Thiamine hydrochloride and allowed to grow for 2.5 hours at

37°C in a water bath with aeration through a sterile gas diffusion tube. The rest of the culture was left to grow at 37°C until required. The culture in the Roux flask was supplemented with 0.2ml of 1m-$CaCl_2$ and infected with 0.5ml of the previous day's 'phage lysate. The culture was left with slow aeration for ten minutes and then 10m Ci of (^{32}P)-orthophosphate (carrier free) was added and the culture allowed to grow for a further 4.5 hours at 37°C with strong aeration. Lysozyme (10mg), $CHCl_3$ (0.25ml) and 1.25ml of 0.2M Na_2EDTA (pH 7.8) were added and the aeration continued for a further 15 minutes at 37°C. $(NH_4)_2SO_4$ (45g) was added together with 50ml of the overnight culture and the mixture stored overnight at 4°C.

The lysate was centrifuged for 30 minutes at 20 000 x g and the supernatant discarded. The pellets were then resuspended and the tubes rinsed in a total volume of 15ml of SSC (0.15M NaCl, 0.015M trisodium citrate, pH 7.0). 0.4ml of 0.1M $MgCl_2$ together with 0.25mg of Deoxyribonuclease I was added and the mixture homogenized a few times using a homogenizer with a Teflon pestle. The mixture was left on ice for a total of 3 hours with homogenization every 30 minutes. The homogenate was centrifuged at 4°C for 30 minutes at 20 000 x g and the pellet of cell debris discarded. The supernatant was then centrifuged for 2 hours at 48 000 r.p.m. in a Beckman Spinco SW50.1 rotor at 4°C and the resulting supernatant discarded. The pellets were suspended in a total volume of 4ml of SSC with homogenization a few times to facilitate suspension. CsCl (2.6g) was added to the 4ml of homogenate and the solution centrifuged at 4°C for 40 hours at 25 000 r.p.m. in the SW50.1 rotor. After centrifugation, the virus could be seen as an opaque band approximately half-way down the gradient and was removed with a drawn-out Pasteur pipette and diluted to 6ml with

water.

An equal volume of water saturated phenol was added to the mixture together with 0.5ml of 10% sodium dodecyl sulphate and the emulsion mixed for 10 minutes on a whirlimix. The two phases were separated by centrifugation at 25°C. Both the aqueous phase and the phenol phase were re-extracted, centrifuged and the two aqueous phases combined. Last traces of phenol were removed by extraction with diethyl-ether. The solution was made 2% with respect to NaOAc, pH 5.4, and the RNA precipitated with ethanol at -20°C.

The precipitate was recovered by centrifugation, washed with ethanol, allowed to dry on ice and dissolved in 1ml of 2% NaOAc pH 5.4. The labelled RNA preparation was loaded onto a 10ml Sephadex G50(f) column and eluted with 2% NaOAc, pH 5.4. Fractions (0.5ml) were collected and 5µl aliquots spotted onto Whatman GF/C filters and counted in a liquid scintillation counter (Fig. 15). The majority of the radioactivity was eluted from the column in the void volume. Fractions 8 and 9 were pooled and precipitated with ethanol at -20°C. The precipitate was recovered by centrifugation, dried, resuspended in 0.25ml of sterile water and stored at -20°C until used. A typical preparation yielded 200µg of (^{32}P) MS2 RNA at a specific activity of 0.3Ci/g which migrated as a single band with little trailing upon electrophoresis on a 5% polyacrylamide gel.

3.5 PREPARATION OF f(^{35}S)Met-tRNA$_f^{Met}$

The reaction mixture (5ml) contained 0.5mg *E. coli* tRNA$_f^{Met}$, 40 nmoles (^{35}S) methionine (25Ci/mmole), 0.2mg pyruvate kinase (EC 2.7.1.40) (465 units/mg), 0.5ml dialysed S_{100}, 40mM Tris HCl pH 7.6, 6mM $MgCl_2$, 72mM NH_4Cl, 5mM dithiothreitol, 1mM ATP, 0.05µM CTP,

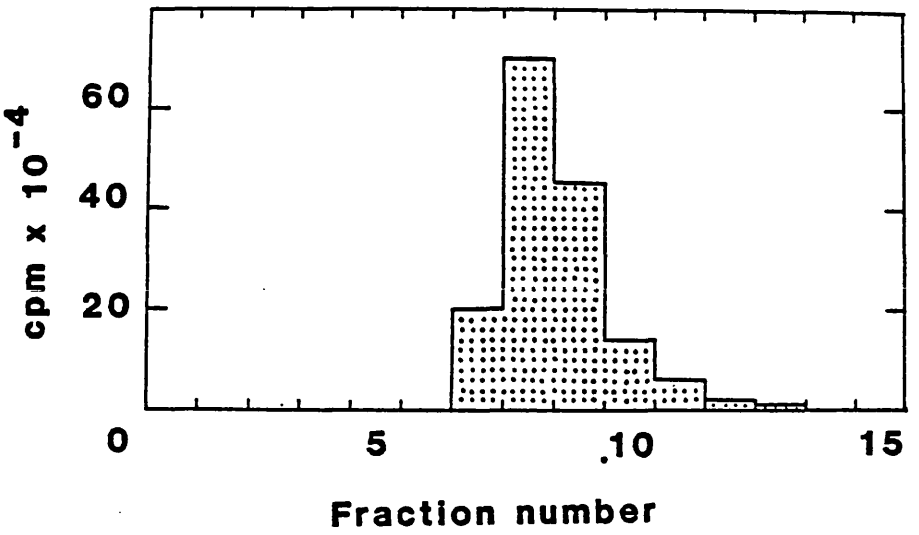


FIGURE 15 Purification of (^{32}P)MS2 RNA on Sephadex G50(f). Chromatography as described in the text (section 3.4.2). 5µl of each fraction (0.5ml) were spotted onto Whatman GF/C filters and counted in a liquid scintillation counter.

10mM phosphoenol pyruvate and 0.5mM leucovorine. Incubation was for 20 minutes at 37°C followed by two phenol extractions and the tRNA precipitated from the aqueous phase with ethanol at -20°C. After centrifugation to recover the precipitate, the tRNA was resuspended in 1ml of 2% NaOAc pH 5.4, 5mM DTT and reprecipitated with ethanol and the final precipitate dissolved in 5mM DTT and stored at -20°C.

The specific activity of the labelled product was usually in the region of 4.8Ci/mmole.

3.6 ISOLATION OF IF3 PROTECTED SITE IN MS2 RNA

3.6.1 Binding of IF3 to MS2 RNA

The nitrocellulose filtration technique of Jay et al. (1974a) as modified by Johnson and Szekely (1979) was routinely used to obtain saturation curves for the binding of IF3 to MS2 RNA. Each assay contained in a total volume of 40µl: 50mM Tris HCl pH 8.0, 50mM NH_4Cl, 1mM $Mg(OAc)_2$, 1mM GTP, 14mM 2-mercaptoethanol, 0.2µg (^{32}P) MS2 RNA and various amounts of IF3 (0-1.5µg). Incubation was for 12 minutes at 37°C and then the mixtures were diluted into 5ml of ice cold TMA buffer [50mM Tris HCl pH 8.0, 50mM NH_4Cl, 1mM $Mg(OAc)_2$] in siliconed glass tubes. Each aliquot was filtered through a nitrocellulose filter, presoaked in TMA buffer and washed with 30ml of TMA buffer. The filters were dried and counted in the presence of 5ml of scintillant (section 3.1.1).

3.6.2 Isolation of the protected site

For the isolation of the IF3 protected site, 20µg of MS2 RNA (either unlabelled or (^{32} p) in vivo labelled) was incubated with an amount of IF3 that gave maximum binding (typically 50-100µg) in a total volume of 4ml of the above binding buffer for 12 minutes at

37°C. Pancreatic RNase A (0.2μg) was added and the incubation continued for 30 minutes at 37°C. The mixture was then cooled to 4°C, divided into four aliquots and filtered as described above. The four filters were combined in a mixture of 5ml TMA buffer and 5ml TMA buffer saturated phenol and extracted for 15 minutes. The aqueous phase was re-extracted with phenol and followed by extraction with diethyl-ether. Carrier RNA (E. coli tRNA or Penicillium chrysogenum virus double stranded RNAs, 10μg) was added and the RNAs precipitated overnight with ethanol and followed by centrifugation for 3 hours at 30,000 r.p.m. in a SW50.1 rotor (Beckman) after which time the supernatant was discarded and the pellet dried.

3.7 5'-END LABELLING OF RNA

3.7.1 IF3 protected fragment

5'-end labelling of the IF3 protected fragment was by using polynucleotide kinase and γ-(^{32}P)-ATP (Szekely and Sanger, 1969). The mixture contained in 20μl of TMM buffer (section 3.3.5), IF3 protected MS2 RNA (very approximately 0.1 to 0.2μg), 1 unit polynucleotide kinase and 100μCi γ-(^{32}P)-ATP (2800 Ci mmole^{-1}). Incubation was for 30 minutes at 37°C followed by the addition of Na_2EDTA to 10mM and fractionation by 15% polyacrylamide gel electrophoresis (section 3.1.2).

3.7.2 Intact MS2 RNA and tRNA

For end-labelling of intact MS2 RNA or E. coli $tRNA_f^{Met}$, 10μg of either RNA was incubated with 0.7μg of bacterial alkaline phosphatase in 20μl of BAP buffer (0.1M Tris HCl pH 8.0, 50mM $MgCl_2$) for 60 minutes at 37°C and followed by phenol extraction and ethanol precipitation. End-labelling was by incubation of 5μg of RNA together with 20μCi of γ-(^{32}P)-ATP and 1 unit of polynucleotide kinase as described

above. The reaction mixture was then twice extracted with phenol and precipitated with ethanol.

3.7.3 RNase T_1 digests of MS2 RNA

End-labelling and total RNase T_1 digestion of MS2 RNA (2μg) in the same reaction mixture were as described by Szekely and Sanger (1969).

Partial RNase T_1 digestion of MS2 RNA was under the conditions described by Adams et al. (1969) with RNase T_1 to RNA (20μg) ratios of: 0, 1:40, 1:80, 1:400, 1:800 and 1:2400 (w/w) and followed by phenol extraction and ethanol precipitation. End group labelling of 1μg of the RNA was as described above, using 10μCi of γ-(^{32}P)-ATP and 1 unit of polynucleotide kinase.

3.8 WANDERING SPOT SEQUENCING

Conditions for partial nuclease P1 digestion of RNA for wandering spot sequencing were a slight modification of those of Richards et al. (1977). The mixture contained in 10μl of 50mM NH_4OAc pH 5.3, 5'-(^{32}P)-labelled RNA together with unlabelled RNA from the homochromatography (approximately 200μg) and nuclease P1 at a ratio of approximately 50ng per average sized spot. Incubation was at 20°C and aliquots were removed at 0, 5, 10 and 15 minute intervals, immediately frozen and then pooled and boiled in the presence of 5mM Na_2EDTA to inactivate the enzyme (Silberklang et al. (1977b). The mixture was then fractionated by two dimensional homochromatography fingerprinting (section 3.1.4) using 3% homomix 'C'.

3.9 THERMAL DENATURATION OF MS2 RNA

3.9.1 Thermal denaturation procedure

(^{32}P)-labelled MS2 RNA (4µg) was heated in 20µl of Tris HCl pH 8.0 (10-60mM as indicated) with or without $Mg(OAc)_2$ (2-10mM as indicated) in a sealed capillary tube for 5 minutes at 85°C. The tube was then immediately plunged into an ice-water mix and left for 5 minutes.

3.9.2 Thermal denaturation curves

MS2 RNA was diluted to a concentration of 14µg ml^{-1} with denaturation buffer described above. The solution was degassed on a water pump and then 2ml added to a stoppered quartz cuvette and weighed. This was then placed in the heated cuvette holder in the sample chamber of a Beckman DK2A Ratio Recording Spectrophotometer together with a similar cuvette containing denaturation buffer. The absorbance at 260nm was determined at 25°C and then the temperature increased by 4°C steps, allowing 5 minutes after first reaching the desired temperature to achieve equilibrium. At each temperature the A_{260} was determined.

After reaching 98°C, the sample was allowed to cool to room temperature and the cuvette was weighed to check for solvent loss. Generally, this was less than 1% and was ignored. If the loss was greater than 1% the determination was repeated. The A_{260} readings were corrected for the thermal expansion of water (Felsenfeld, 1971) and then plotted in the form of $A_t/A_{95°C}$ against temperature, t (Van Dieijen et al., 1976). The midpoint of the transition increase between 25°C and 95°C is defined as the T_m.

3.10 S_1 NUCLEASE DIGESTION OF MS2 RNA

Digestion of MS2 RNA with nuclease S_1 was under similar conditions to those of Rushizky et al., 1975). (^{32}P)-MS2 RNA (2.5µg) was digested with 0.1 to 0.825 units of nuclease S_1 for 45 minutes at 27°C in 20µl of ASZ buffer (20mM NaOAc pH 4.3, 250mM NaCl, 2mM $ZnSO_4$). The reaction was stopped by the addition of 5µl of 100mM Na_2EDTA pH 7.0.

3.11 HCHO DENATURATION OF IF3 PROTECTED SITE

Mild HCHO denaturation was as described by Lodish (1970). The mixture contained approximately 30ng of unlabelled IF3 protected MS2 RNA together with 0.4µg PcV-ds-RNAs in 5µl of neutralized 1M HCHO, 200mM NaCl, 9mM Na_2HPO_4, 1mM NaH_2PO_4 and was incubated for 11 minutes at 37°C and then chilled on ice. The RNA was twice precipitated with ethanol and then 5'-end labelled in 10µl of TMM buffer (section 3.3.5) containing 5µCi of γ-(^{32}P)-ATP and 0.1U polynucleotide kinase for 30 minutes at 37°C.

RESULTS

Studies have been made on the interaction of Escherichia coli Initiation Factor 3 (IF3) with coliphage MS2 RNA. The nucleotide sequences of the regions of MS2 RNA protected by the factor from pancreatic ribonuclease digestion have been determined and the protected site located in the primary sequence of MS2 RNA.

In order to investigate the RNA secondary and tertiary interaction involved at the protected site, the effects of partial heat denaturation treatment and the presence of ribosomal protein S1 upon IF3 binding and protection of MS2 RNA have been studied. Additionally, two-dimensional gel electrophoresis, under conditions used by other workers to determine the secondary structure interaction of E. coli 16S RNA (Ross and Brimacombe, 1979), was used in an attempt to directly investigate these interactions present in the isolated IF3 protected fragment. Finally, a search for possible sequence homology of the IF3 protected site with the 16S-RNA sequence was made.

4.1 ISOLATION OF HOMOGENEOUS IF3 AND ITS BINDING AND PROTECTION OF MS2 RNA

Homogeneous IF3, isolated from E. coli, was active in stimulating the binding of fMet-tRNA$_f^{Met}$ to ribosomes in an in vitro system and also capable of binding to the specific binding site on MS2 RNA. The IF3 protected RNA fragment was isolated from unlabelled MS2 RNA 5'-end labelled using polynucleotide kinase and γ-(^{32}P)-ATP. Homochromatography fingerprinting of the protected site was used to

determine the nature of the protected fragment which runs as a single band upon gel electrophoresis and yet contained pancreatic and RNase T_1 oligonucleotides from different parts of the 3'-terminal region of MS2 RNA (Johnson and Szekely, 1977; section 1.3.7).

4.1.1 Isolation of homogeneous IF3

IF3 was prepared according to the procedure of Lee-Huang and Ochoa (1974) to step 4. Fractions containing IF3 were concentrated by elution from a second phospho-cellulose column and this preparation was estimated by SDS-PAGE to be usually approximately 80% homogeneous (Fig. 16a). Since a 100 to 200 fold molar excess of IF3 was reported to be required for maximal binding of MS2 RNA (Johnson and Szekely, 1977, 1979), it was necessary to purify the preparations to homogeneity as judged by SDS-PAGE. This would reduce the possibility that binding and protection were due to the presence of a small amount of a contaminating protein having a high affinity for MS2 RNA.

The factor was purified to homogeneity by gel filtration in the presence of urea (Sabol and Ochoa, 1974; Johnson and Szekely, 1979). Whilst urea is a protein denaturing agent, Johnson and Szekely (1977, 1979) found that chromatography of IF3 in its presence did not alter the specificity of the binding of the factor to MS2 RNA. In a typical preparation, IF3 eluted from the G75 column soon after the void column as expected (Sabol and Ochoa, 1974; Johnson and Szekely, 1979) and fractions collected were immediately dialysed and assayed for the presence of IF3 by SDS-PAGE (Fig. 16b). Those fractions containing homogeneous IF3 were pooled and concentrated by vacuum dialysis.

The molecular weight ratios of the preparations of IF3 were estimated by SDS-PAGE together with protein standards of known molecular weight. After staining, the mobility of each protein relative to

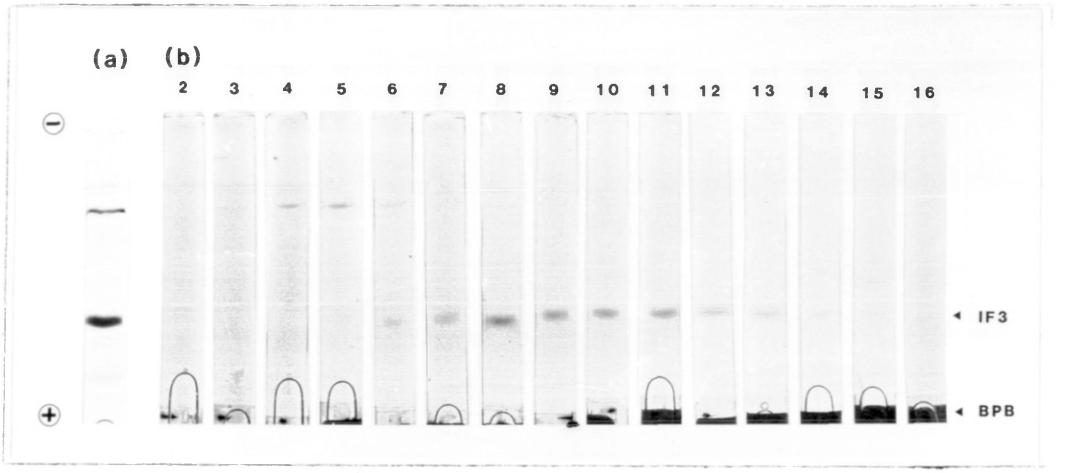


FIGURE 16 Purification of IF3 on Sephadex G75.
SDS-PAGE of various fractions from Sephadex G75 column electrophoresed and stained as described in section 3.1.3. (a) 50µl of material loaded onto the column. (b) 50µl of various fractions from G75 column.

the bromophenol blue marker was plotted against the $\log_{10}$ of the respective molecular weight to yield a smooth curve (Fig. 17) (Weber and Osborn, 1969). The molecular M_r determinations of the preparations of IF3 were in the region of 23 000 daltons $\pm$ 10% and agreed well with estimation by other workers (section 1.1.1).

To determine whether purified IF3 was still biochemically active after passage through the Sephadex column in the presence of urea, the IF3 preparations were assayed for their ability to stimulate the IF3-dependent ribosomal binding of f(^{35}S)Met-tRNA$_f^{Met}$ in the presence of excess IF1 and IF2 using MS2 RNA as messenger (Lee-Huang and Ochoa, 1974). The result of such an assay is shown in Fig. 18 where it can be seen that the purified IF3 promoted the binding of f(^{35}S)Met-tRNA$_f^{Met}$ to ribosomes and thus was functionally active in this respect. There was an approximately linear relationship between f(^{35}S) Met-tRNA$_f^{Met}$ binding and the amount of IF3 in the reaction until the binding reached a maximum. At very high concentration of IF3, an inhibition of the reaction occurred; a phenomenon also noted by Vermeer et al. (1973a).

From Fig. 18 an estimate can be made of the approximate specific activity of the IF3 preparation. Maximal binding of f(^{35}S)Met-tRNA$_f^{Met}$ (0.164 pmoles) by the ribosomes occurred at an input of 0.3µg of IF3. Taking the M_r of IF3 to be approximately 20 000 daltons (Table 1), this corresponds to a ratio of approximately 90 moles of IF3 required to be input into the reaction for every mole of f(^{35}S)Met-tRNA$_f^{Met}$ bound to the ribosome. Vermeer et al. (1973a) found that approximately 3 moles of IF3 were required for each mole of f(^{35}S) Met-tRNA$_f^{Met}$ bound. The discrepancy between these two results may be due to either a difference in the efficiency of the in vitro assay

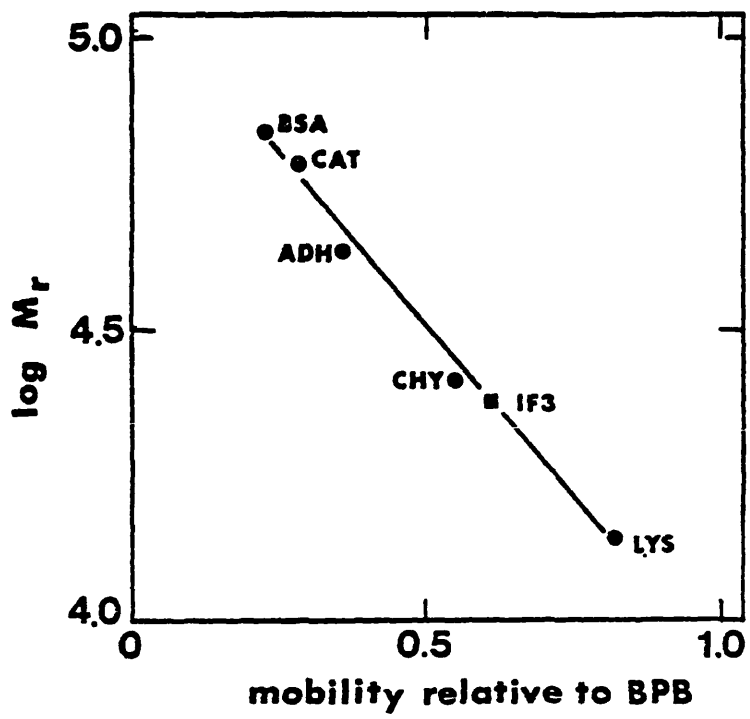


FIGURE 17 Molecular weight ratio determination of purified IF3. SDS-PAGE of various proteins of known molecular weight ratio, together with IF3, were plotted as mobility relative to bromophenol blue (BPB) marker vs. $\log_{10} M_r$. Protein standards together with their respective M_r values: BSA - bovine serum albumen (68 000), CAT - catalase (60 000), ADH - liver alcohol dehydrogenase (41 000), CHY - chymotrypsinogen (25 700) and LYS - lysozyme (14 300). (Weber and Osborn, 1969).

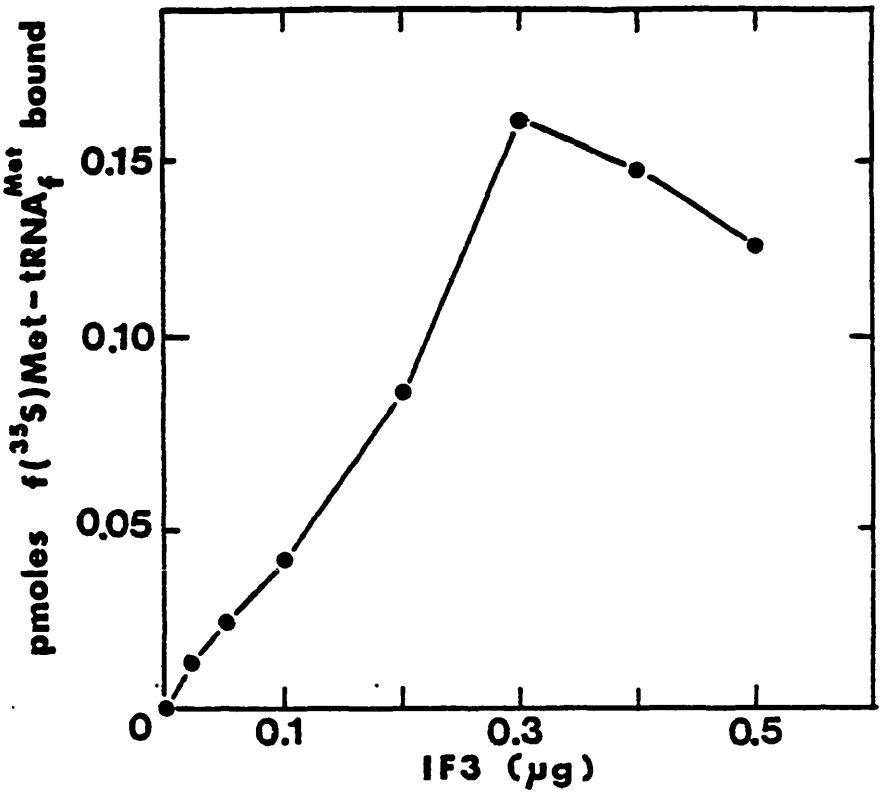


FIGURE 18 MS2 RNA directed ribosomal binding of f(^{35}S)met-tRNA$_f^{Met}$

Reaction mixtures of 25µl were incubated as described in methods in section 3.2.4 with varying amounts of IF3 and filtered over nitrocellulose filters. Blank values (without IF3) were subtracted.

systems or, more importantly, that after column chromatography in the presence of urea, a considerable amount (90%) of the IF3 had been irreversibly denatured and was no longer functionally active. In view of this possible high degree of denaturation, alternative methods for the isolation of homogeneous IF3 are discussed in section 5.1.1.

4.1.2 Binding of IF3 to (^{32}P)MS2 RNA

A modification (Johnson and Szekely, 1977, 1979) of the nitrocellulose filtration technique of Jay et al. (1974a) was used to assay the binding of IF3 to (^{32}P)MS2 RNA. In this technique, IF3 and (^{32}P) MS2 RNA were mixed and then filtered through a nitrocellulose filter. Any (^{32}P)MS2 RNA bound to IF3 was assumed to be retained on the filter and thus the amount of radioactivity remaining on the filter was a measure of the amount of MS2 RNA bound to IF3.

Various amounts of IF3 were bound to in vivo (^{32}P) labelled MS2 RNA at 37°C followed by filtration at 4°C and the binding curve plotted (Fig. 19). It can be seen that the binding was saturable with, in this case, maximum retention of MS2 RNA (72% of the total c.p.m. present) occurring when 0.6µg of IF3 was input into the reaction. At higher concentrations of IF3, a slight reduction in the amount of MS2 RNA bound was seen. Taking the M_r of IF3 to be approximately 20 000 daltons (Table 1) and that of MS2 RNA to be 1.2×10^6 (Table 3) this ratio corresponds to approximately 220 moles of IF3 being required to retain 1 mole of MS2 RNA on the filter. This ratio compares well with those determined previously which ranged from 100-250 moles of IF3 per mole of MS2 RNA (Johnson and Szekely, 1977, 1979; B. Johnson pers. commun.). It is, however, somewhat higher than the ratio of 50 : 1 (IF3 : MS2 RNA) obtained by Jay et al. (1974a) using the RNA from the closely related bacteriophage R17 and is probably

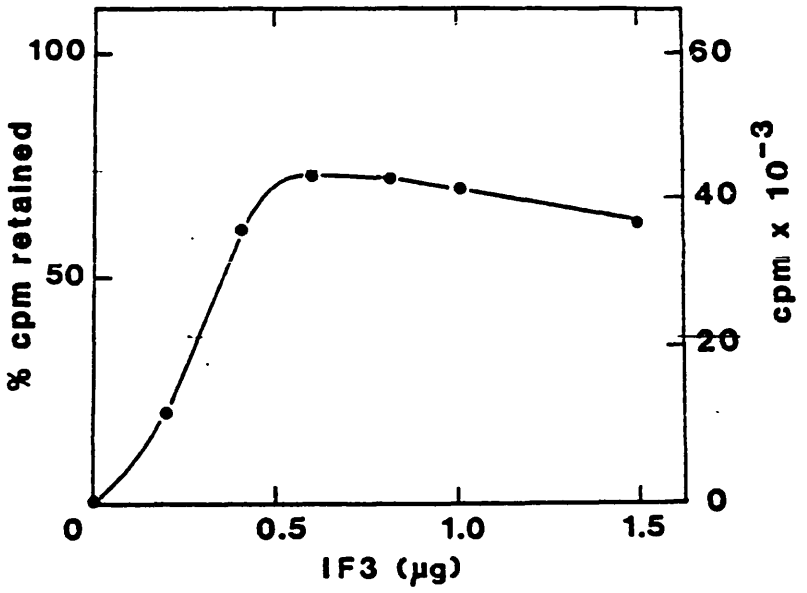


FIGURE 19 Binding of IF3 to *in vivo* (^{32}P) labelled MS2 RNA. Varying amounts of IF3 were incubated with (^{32}P)MS2 RNA (0.2µg, 60 500 cpm) as described in section 3.6.1, and filtered over nitrocellulose filters. A blank value (without IF3) of 350 cpm was subtracted.

due to the low specific activity of the IF3 preparations used in this work (see section 5.1.1).

4.1.3 Binding of IF3 to 5'-end labelled MS2 RNA

For use in the 'wandering spot' sequencing method that was chosen to be used for determining the nucleotide sequences of the IF3 protected regions of MS2 RNA, it was essential that the RNA to be sequenced was only labelled with (^{32}P) at either the 5' or 3' termini (Silberklang et al., 1977b), otherwise meaningless patterns of spots would be seen on the autoradiographs which would be uninterpretable. (^{32}P) in vivo labelled MS2 RNA could not therefore be used in the filtration assay to determine the ratios of the IF3 preparations that gave maximum retention of MS2 RNA on the filter. MS2 RNA labelled with either (^{14}C) autoradiography or (^{3}H), which would not be detected under the conditions of autoradiography (section 3.1.4), could have been used in the assay and for the preparation of the protected fragment. However, since neither of these labelled RNAs was readily available, the IF3 protected fragment was prepared from unlabelled MS2 RNA and the binding of IF3 assayed using the same RNA but in vitro labelled at the 5'-terminus with (^{32}P).

Polynucleotide kinase (EC 2.7.1.78) (abbreviated to PNK) isolated from bacteriophage T_4 infected E. coli cells catalyses the transfer of the γ-phosphate group of ATP to the 5'-hydroxyl terminus of polynucleotides, oligonucleotides and 3'-mononucleotides (Richardson, 1965) and can be used to end-label non-radioactive nucleic acids with (^{32}P) when used with γ-(^{32}P)-ATP (e.g. Szekely and Sanger, 1969).

To be suitable for use in labelling RNA, and especially for labelling the RNA species protected by IF3 for use in the 'wandering spot' sequencing method, the preparations of PNK had to be free of any con-

taminating RNase activity. The preparations were purified on hydroxylapatite as a final step to remove any nucleases (section 3.3.7) and therefore should have been free of RNase contamination. To check whether this was the case, and also to check the ability of the preparations to label RNA species of the size expected in the IF3 protected site (i.e., approximately 30 nucleotides long - Johnson and Szekely, 1977), unlabelled MS2 RNA was partially digested with RNase T_1 under conditions (Adams et al., 1969) which were known to produce discrete fragments in the case of the RNA of the related bacteriophage R17 (Robertson and Jeppesen, 1972). After phenol extraction to remove the RNase T_1 followed by ethanol precipitation, an aliquot of each partial digest was labelled with (^{32}P) using PNK and γ-(^{32}P)-ATP and then subjected to fractionation by PAGE together with the unlabelled partially digested RNAs. After autoradiography of the labelled material and staining of the unlabelled material, the band patterns in both the labelled and unlabelled digests were compared. Fig. 20 shows that no difference could be seen in the band patterns of both labelled and unlabelled digests, suggesting that this PNK preparation was free from RNase contamination. Had the preparation been contaminated with RNase, then different band patterns, or no bands at all, would have been expected in comparable labelled and unlabelled tracks. This experiment also demonstrates that the PNK labelled well all of the RNA species including those of the size expected in the protected site.

Van de Sande et al. (1973) have reported that PNK can be used to phosphorylate polynucleotides already having a phosphate group at their 5'-termini. However, to ensure efficient labelling of intact MS2 RNA, which has a phosphorylated 5'-terminus of the form pppGp... (De Wachter et al., 1968), the RNA was treated with bacterial alkaline

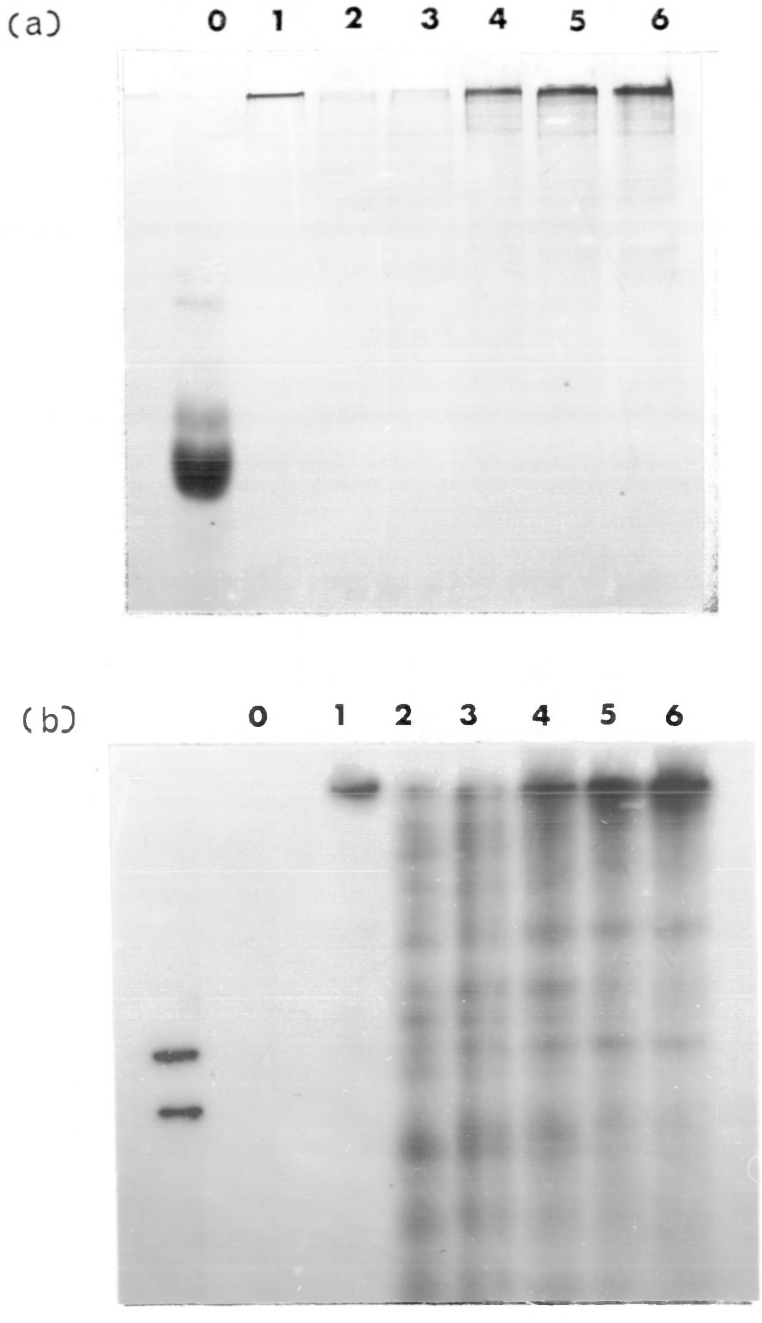


FIGURE 20 Assay of PNK preparation for ribonuclease activity. 12.5% PAGE of MS2 RNA partially digested with RNase T_1, as described in section 3.7.3, and labelling of an aliquot of each digest using PNK and γ-(^{32}P)-ATP. (a) stained gel of unlabelled RNA (19μg each track); (b) autoradiograph of PNK labelled RNA (1μg each track) Track O, unlabelled E. coli tRNAs (20μg), Track 1 undigested RNA, Tracks 2 - 6 MS2 RNA digested at RNase T_1: RNA ratios of 1 : 40, 1 : 80, 1 : 400, 1 : 800 and 1 : 2400 (w/w) respectively.

phosphatase (EC 3.1.3.1) to give 5'-hydroxyl termini. This material, after phenol extraction and ethanol precipitation, was 5'-end labelled using PNK and γ-(^{32}P)-ATP and an aliquot assayed for its intactness by PAGE together with E. coli and rabbit reticulocyte lysate ribosomal RNAs as size markers. The gel was stained and autoradiographed and it could be seen that the end-labelled MS2 RNA (Fig. 21b) was intact, migrating as a single band with a mobility similar to that of the unlabelled MS2 RNA (Fig. 21a). The mobility of MS2 RNA being similar to that of 23S E. coli rRNA is in agreement with the estimated molecular weights of both MS2 RNA and 23S rRNA, both being approximately the same (1.1×10^6 daltons) (Table 3; Kurland, 1960; Stanley and Bock, 1963).

To determine the maximal amount of IF3 required for maximal binding of the MS2 RNA preparation to be used for the preparation of the protected site, different amounts of IF3 were bound to 5'-(^{32}P)-labelled MS2 RNA and the binding assayed by the filtration technique. The binding curve, which is similar to that obtained for in vivo labelled MS2 RNA (Fig. 22) shows that the maximum retention of MS2 RNA on the filter (68%) occurred at an input of 1.0µg of IF3 into the reaction. The larger amount of IF3 required for maximal binding compared with in vivo labelled MS2 RNA (Fig. 19) reflects the fact that a different preparation of IF3 was used in the two experiments.

4.1.4 Isolation of unlabelled IF3 protected fragment

Since the RNA fragments for 'wandering spot' sequencing of the protected site were to be end-labelled after separation from the unprotected pancreatic RNase oligonucleotides, it was essential that a

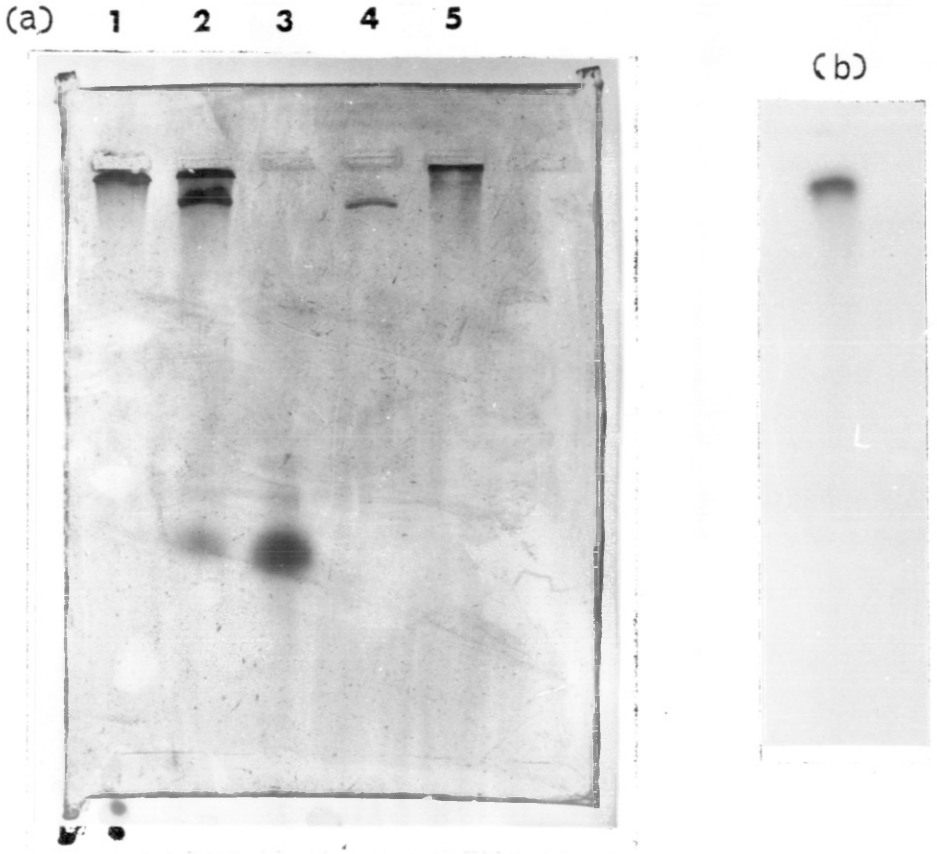


FIGURE 21 5% polyacrylamide gel electrophoresis of various RNAs. 5% polyacrylamide gel prepared and run as described in section 3.1.2. (a) stained gel: (1) MS2 RNA (20μg); (2) E. coli ribosomal RNAs (20μg); (3) E. coli tRNAs (20μg); (4) PcV-ds-RNAs (18μg); (5) rabbit reticulocyte lysate rRNAs (20μg).
(b) Autoradiograph of 5% PAGE of (^{32}P)-end labelled MS2 RNA (100 000 cpm, 0.15μg) (1 hour exposure).

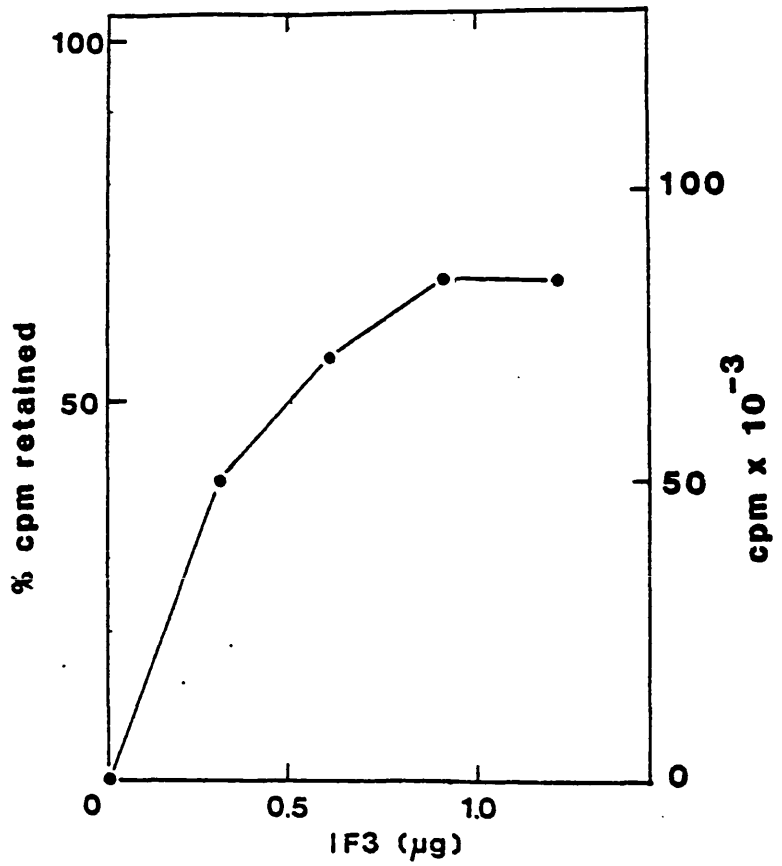


FIGURE 22 Binding of IF3 to 5'-end labelled MS2 RNA.
Varying amounts of IF3 were incubated with 5'-(^{32}P)-MS2 RNA (0.2µg, 125 000 cpm) as described in section 3.6.1 and filtered over nitrocellulose filters. Blank values without IF3 (225 cpm) were subtracted.

suitable carrier RNA was chosen to be used in the ethanol precipitation step of the isolation procedure. E. coli tRNAs, which were used by Johnson and Szekely (1977) as carrier for the in vivo labelled protected site, were thought unsuitable since, although they are relatively difficult to end-label with PNK, (Lillehaug and Kleppe, 1977) and therefore not expected to compete for the PNK and γ-(^{32}P)-ATP to a great extent, their size of 73-93 nucleotides (Rich and Raj Bhandary, 1976) would mean that they would migrate in the gel with a mobility close to that of the IF3 protected species. A mixture of three purified double-stranded RNAs from Penicillium chrysogenum virus (PcV) (Buck et al., 1971) were chosen for use as carrier for four main reasons. Firstly, they are of high molecular weight (2.18×10^6, 1.99×10^6 and 1.89×10^6 daltons; Wood and Bozarth, 1972) and thus would not be expected to enter the polyacrylamide gel used in the purification step. Secondly, double-stranded RNAs are relatively resistant to RNase digestion (Edy et al., 1976) and would thus be less likely than single stranded RNAs to be degraded by any contaminating RNases. Thirdly, double-stranded RNAs are relatively difficult to end-label using PNK (Szekely and Loviny, 1975) and would therefore not be expected to take up much of the (^{32}P) label when compared with the protected fragments. Finally, should any (^{32}P)-labelled digestion products be produced from these RNAs that have a mobility in the gel similar to that of the protected site, they would be immediately apparent as 'foreign' sequences, since the probability that they would have the same sequence to part of the MS2 RNA would be very small (very approximately 1 in 4^{20} for a 20 nucleotide long stretch, i.e., approximately 1 in 10^{12}).

Prior to use as carrier, PcV-ds-RNAs (the generous gift of Dr. K.W. Buck) were fractionated by PAGE to confirm their suitability for

use by being free from significant amounts of degradation products. Fig. 21 shows that the preparation used as carrier migrated as two bands with the two smaller species of similar molecular weight being poorly separated on the gel, and that the preparation was free from degradation products. The fast mobility of the PcV-ds-RNAs in the gel relative to the E. coli ribosomal RNA size markers (which have the molecular weights of: 23S, 1.1 x 10^6 ; 16S, 0.55 x 10^6 - Kurland, 1960; Stanley and Bock, 1963) probably reflects the double-stranded nature of the PcV RNAs.

In a typical preparation of the IF3 protected site, the factor was bound to unlabelled MS2 RNA at the ratio that gave maximum retention of end-labelled MS2 RNA in the filtration assay and then followed by digestion with pancreatic RNase as described in section 3.6.2. The protected RNA was separated from the digestion products by filtration and then extracted by phenol extraction, followed by ethanol precipitation with PcV-ds-RNAs as carrier.

4.1.5 5'-end labelling of IF3 protected fragment

Since the IF3 protected fragment had been prepared using pancreatic RNase which cleaves RNA to give 5'-OH and 3'-PO_4 termini (e.g., Barnard, 1969), the RNA species did not require treatment with alkaline phosphatase to provide suitable 5'-termini for the PNK. The protected RNA species were labelled with (^{32}P) using PNK and γ-(^{32}P)-ATP and then run directly on a non-denaturing gel at 4°C. Autoradiography of the gel (Fig. 23a for a typical example) gave a major band (I) with a mobility relative to the bromophenol blue tracking dye which was similar to the protected fragment isolated from in vivo

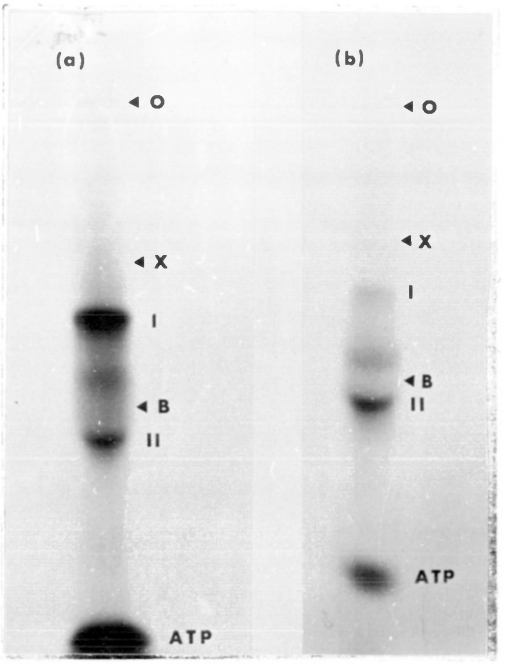


FIGURE 23 PAGE of 5' end labelled IF3 protected MS2 RNA. Autoradiograph of MS2 RNA protected fragments isolated from unlabelled MS2 RNA as described in section 3.6.2, end-labelled using PNK and γ-(^{32}P)-ATP and electrophoresed through a 15% gel. (a) Material labelled immediately after preparation of the protected site. (b) The same material as in (a), but which had been stored at -20°C for one month prior to labelling. X and B are the positions of the xylene cyanol FF and bromophenol blue tracking dyes. O marks the origin of each gel.

labelled MS2 RNA (Johnson and Szekely, 1977, 1979). This band was absent in control preparations made in the absence of IF3 and subsequently labelled using PNK. A minor band (II) migrating faster than band I was also found in some preparations of the protected fragment and its intensity varied from one preparation of the protected fragment to another. A spot corresponding to unincorporated γ-(^{32}P)-ATP could also be seen (Fig. 23a). Comparison of the band pattern upon gel electrophoresis of an aliquot of the same preparation of protected fragment as shown in Fig. 23a but which had been labelled using PNK after approximately one month's storage at -20°C is shown in Fig. 23b. Apart from the difference in overall mobility of the marker dyes and RNA bands caused by differences in the electrophoresis times, the relative intensities of band I and band II appeared to have changed upon storage; the amount of material in band II had increased relative to band I. Since the two gels represent the fractionation of an identical preparation of the protected site, which had been labelled using the same preparation of PNK, but which had been stored for a different length of time, band II would appear to consist of products derived from either band I or from the PcV-ds-RNAs and which had been produced during storage. B. Johnson (pers. commun.) working with in vivo labelled MS2 RNA also observed occasionally an additional band migrating in a position similar to band II in preparations of the protected site. An origin of band II from the PcV-ds-RNAs would therefore appear to be excluded.

In all of the preparations labelled with PNK and γ-(^{32}P)-ATP, incorporation of label was never found to be greater than 4-5% of that expected based on estimates of the size of the protected fragments and the amount of RNA protected. Whereas, in contrast, oligonucleotides

produced from a partial RNase T_1 digest of MS2 RNA were labelled well by the same preparations of PNK. To investigate whether the low uptake of (^{32}P) was due to some aspect of the secondary structure of the protected that was preventing labelling, the IF3 protected fragment was mildly denatured with formaldehyde, labelled with (^{32}P) using PNK, fractionated on PAGE and the band I cut out and Cerenkov counted (Table 6). From this table, it can be seen that the partial denaturation treatment had little effect on the uptake of (^{32}P) into the protected fragment and therefore either the treatment was too mild to have had any effect on the secondary structure of the site or there are other reasons for the poor uptake of label. Stronger denaturing conditions were not used in an attempt to increase the uptake of (^{32}P) for use in the sequencing studies since HCHO has been reported to cross-link RNA strands (Lodish, 1975). Although most of the HCHO molecules would be expected to dissociate from the adduct when the RNA was purified by ethanol precipitation (McGhee and von Hippel, 1975a,b), it was felt that the presence of any stable RNA cross-links (Lodish, 1975) would cause problems upon homochromatography fingerprinting and wandering spot sequence analysis of the protected site.

4.1.6 Two-dimensional homochromatography of IF3 protected fragments

The RNA species present in Band I from several preparations of the protected site were eluted from the gels and fractionated by two-dimensional homochromatography. Fig. 24 shows the patterns of spots from six of these preparations. Great heterogeneity in the patterns can be seen but also, to some extent, some similarity between the patterns is apparent. A similar heterogeneity in patterns was also found using

TABLE 6

Effect of formaldehyde denaturation of IF3 protected site prior to labelling

	^{v}cerenkov cpm in IF3 band
Native protected material	12733
Denatured protected material	12524
Native PcV-ds-RNA	258
Denatured PcV-ds-RNA	200
γ-(^{32}P)-ATP alone	213

IF3 protected material (approximately 32ng together with 0.4µg PcV-ds-RNA used as carrier) and PcV-ds-RNA alone (0.4µg) were partially denatured with formaldehyde and labelled using PNK and γ-(^{32}P)-ATP as described in section 3.11, electrophoresed through a 15% gel and autoradiographed for 3.5 hours. The main IF3 protected band was excised and the amount of (^{32}P) present was determined by ^{v}cerenkov counting. Controls of non-denatured material and γ-(^{32}P)-ATP run alone in the gel for background determination were also performed.

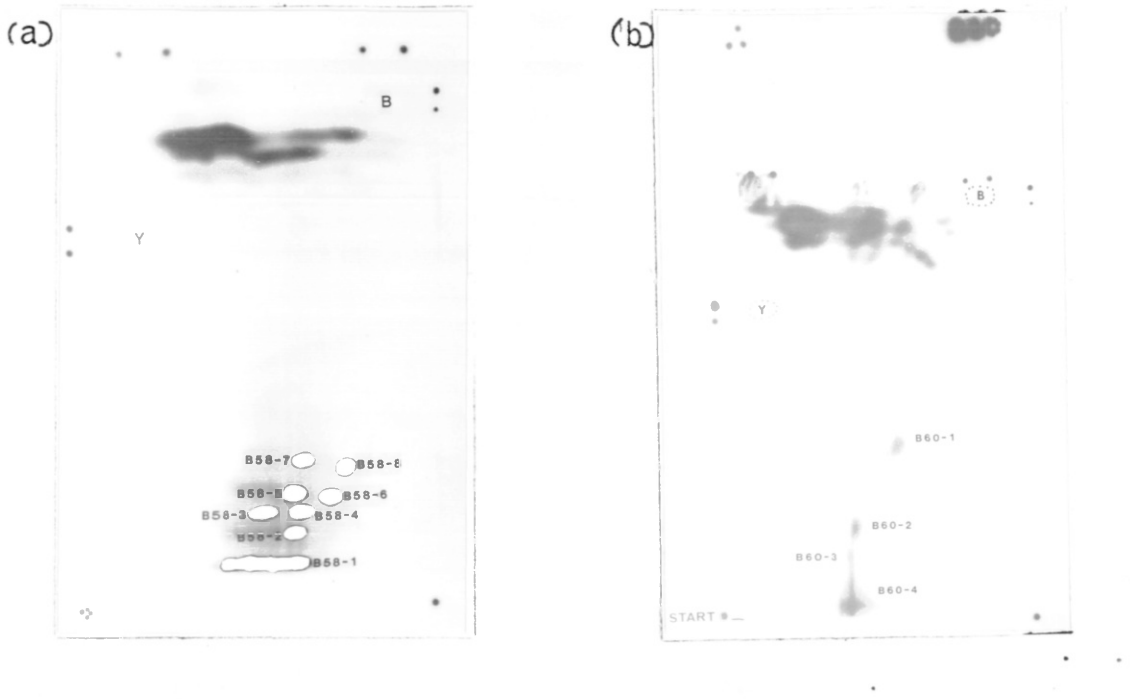
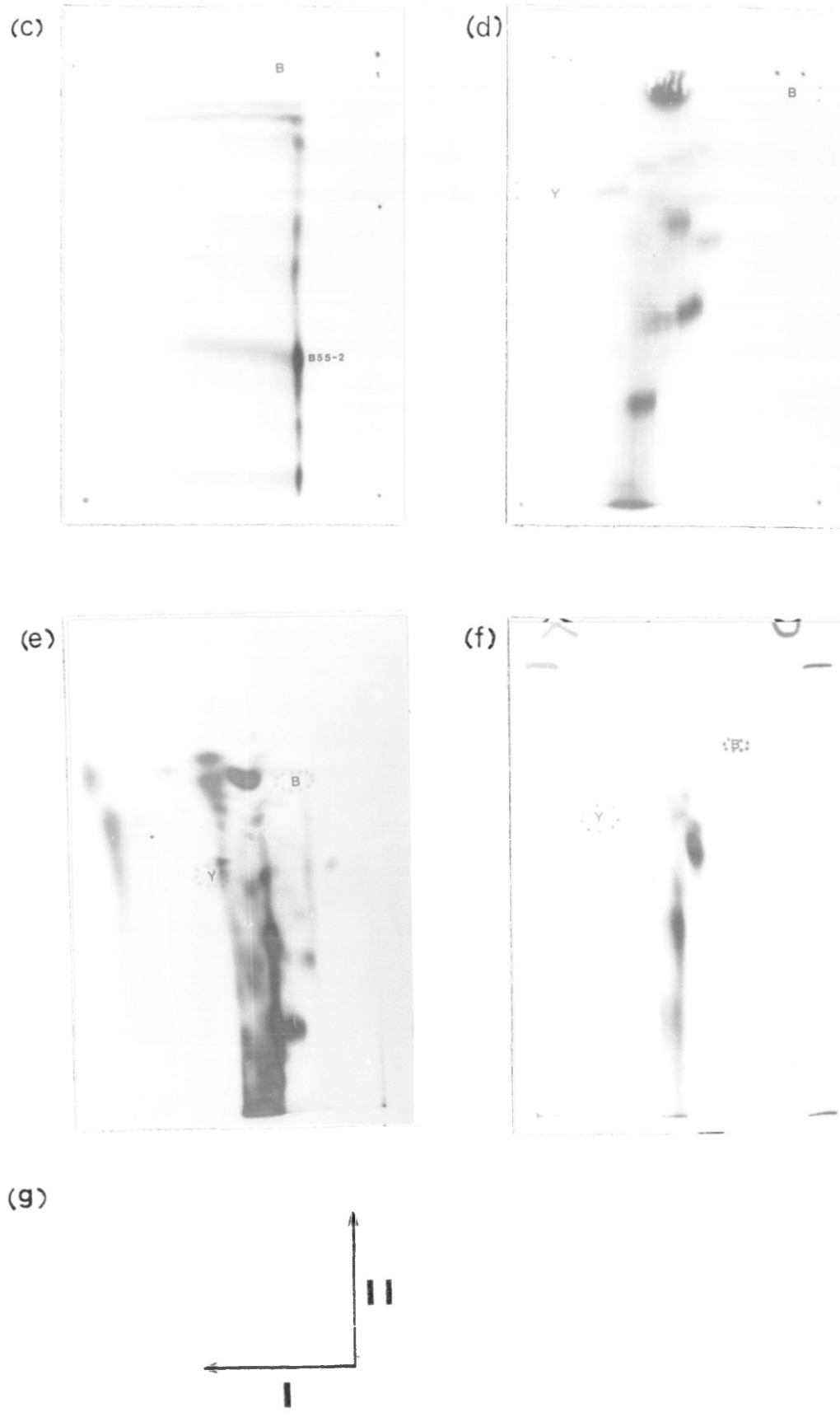


FIGURE 24 Two-dimensional homochromatography of 5'-end labelled IF3 protected MS2 RNA.

End-labelled RNA eluted from Band I from 6 preparations of the IF3 protected site as described in section 3.1.2 was fractionated by two-dimensional homochromatography fingerprinting (section 3.1.4) followed by autoradiography. (a) Preparation B58; (b) Preparation B60; (c) Preparation B55; (d) Preparation B62; (e) Preparation B53; (f) Preparation B54; (g) Directions of First (I) and Second (II) dimensions of the fractionation procedure. Note, preparation B55 (c) was poorly fractionated in the first dimension of the fingerprint. B and Y mark the positions of the Blue and Yellow dye markers.

FIGURE continued overleaf:

FIGURE 24 continued:



in vivo labelled protected material (B. Johnson pers. commun.).

The homochromatographs demonstrate that the material that migrates as a distinct band upon PAGE under non-denaturing conditions is composed of several species of RNA of different lengths. The RNA species are presumably held together by secondary and possibly tertiary interactions when running in the gel and these are destroyed in the strongly denaturing conditions of the homochromatography fingerprinting system. The possibility cannot be excluded, however, that the spots obtained upon homochromatography could be due to a single linear fragment which was degraded during the elution from the gel.

To test this latter possibility, end-labelled IF3 protected material was directly fractionated by homochromatography fingerprinting before and after being run on a non-denaturing polyacrylamide gel. Both Bands I and II were eluted from the gel and homochromatography fingerprinted. It is readily apparent from Fig. 25, that the pattern of spots obtained from the material that had been directly fractionated after labelling was similar to the sum total of the spots on the homochromatographs of material eluted from Bands I and II. This demonstrates that the material running as a distinct band upon PAGE is, in fact, comprised of several stretches of RNA, rather than just one linear stretch. It can also be seen that Band II consists mainly of short oligonucleotides in agreement with the hypothesis that it consists of degradation products of Band I.

The observation that the protected site comprises several stretches of RNA is further supported by the observation of Johnson and Szekely (1977) that several of the pancreatic RNase T_1 digestion products present in the fingerprints of the protected site originated from a more distant part of the MS2 RNA sequence to others present and

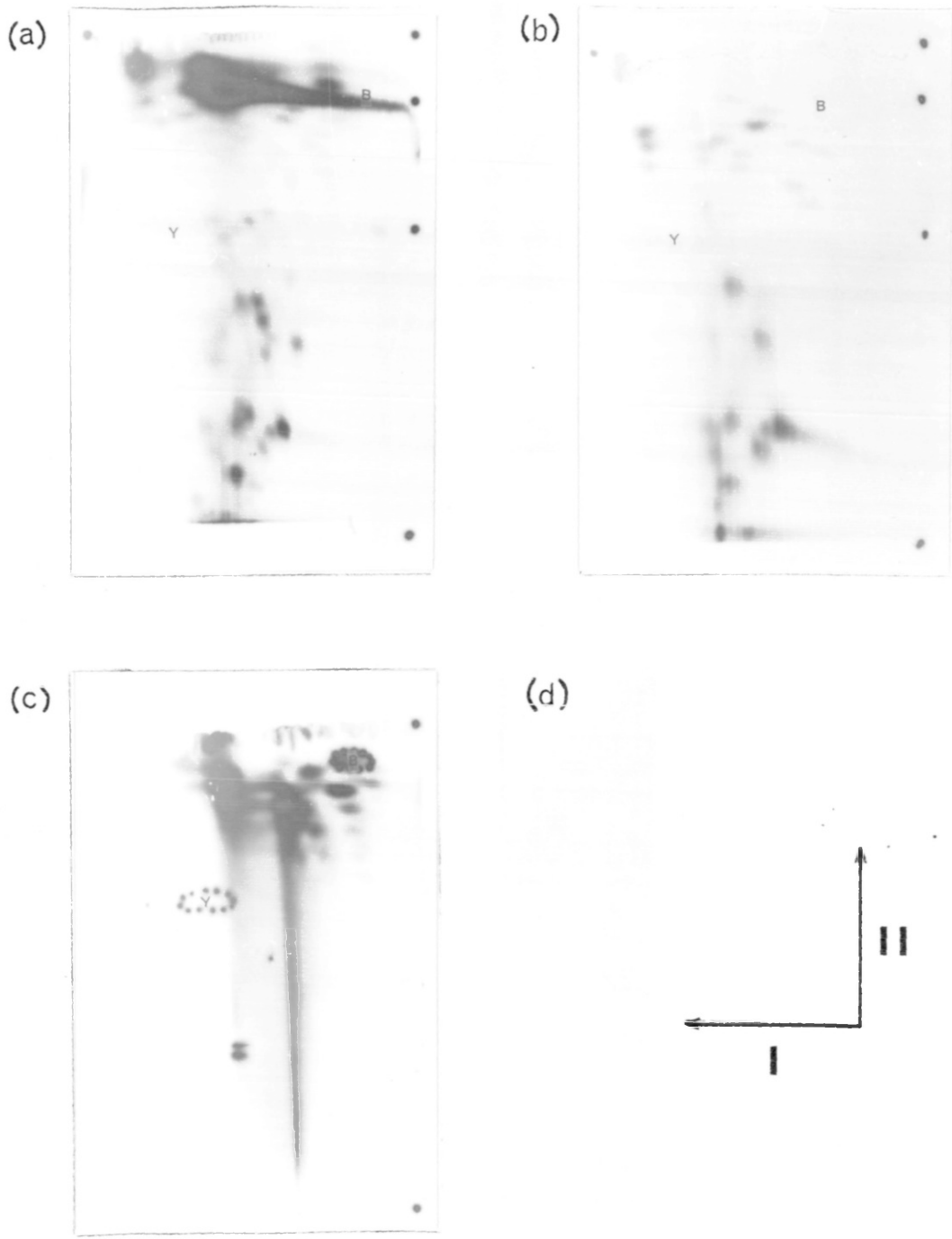


FIGURE 25 Two-dimensional homochromatography of 5'-end labelled IF3 protected MS2 RNA before and after PAGE purification.

IF3 protected MS2 RNA was 5'-end labelled and an aliquot fractionated by PAGE in a 15% gel. After autoradiography, the RNA present in Bands I and II (cf Fig. 23) was eluted and fractionated by homochromatography fingerprinting together with some of the original material. (a) Original material not purified by PAGE. (b) Material from Band I. (c) Material from Band II. (d) Directions of first (I) and second (II) dimensions. B and Y are as described in Fig. 24.

that the unique digestion products in the intervening regions were not found in the fingerprints of the protected site. The stretches of RNA protected by IF3 therefore appear to be non-contiguous. Determination of the nucleotide sequences of the protected regions and comparison with the complete MS2 RNA sequence, as determined by Fiers et al. (1976), would therefore confirm that the protected site consisted of several non-contiguous stretches of RNA rather than one linear stretch.

4.2 STUDIES ON THE PRIMARY STRUCTURE OF MS2 RNA INVOLVED AT THE IF3 PROTECTED SITE

The nucleotide sequences of the IF3 protected MS2 RNA species were determined. Preliminary experiments to optimise the 'wandering spot' sequencing technique were undertaken, using RNase T_1 digestion products from MS2 RNA. Analysis by 'wandering spot' sequencing of the IF3 protected MS2 RNA, together with information from partial RNase T_1 digestion of in vivo labelled protected site by Dr. B. Johnson allowed the location of the protected site in the complete MS2 RNA sequence of Fiers et al. (1976) as corrected by Iserentant et al. (1980).

4.2.1 'Wandering spot' sequencing of RNA

The classical 'Sanger' techniques used by Johnson and Szekely (1977,1979) precluded the determination of the exact regions of MS2 RNA that were protected by IF3 and therefore one of the recent 'direct read-out' RNA sequencing techniques was required to be used for the sequencing task. Methods of this type, using either two-dimensional homochromatography of partial endonucleolytic digests of end-labelled RNA ('wandering spot' sequencing) (Silberklang et al., 1977a, 1977b) or polyacrylamide gel electrophoresis ('rapid gel' sequencing) of either partial endonucleolytic digests (Donis-Keller et al., 1977; Simoncsits et

al., 1977; Krupp and Cross, 1979) or partial specific chemical fragmentation (Peattie, 1979) of end-group labelled RNA have been described in the literature. Methods involving the primed synthesis of cDNA, using reverse transcriptase, followed by sequencing of the DNA product have also been described (Brownlee and Cartwright, 1977; McGeoch and Turnbull, 1978; Zimmern and Kaesberg, 1978).

Of these methods,the 'wandering spot' technique is perhaps better suited to sequencing RNAs of the size expected in the IF3 protected site (i.e., between 15 and 30 nucleotides long) than the 'rapid gel' methods and, therefore, the 'wandering spot' technique of Silberklang et al. (1977b), using 5'-end labelled RNA as modified by Richards et al. (1977) was chosen for this sequencing task.

This method involves the in vitro labelling with (^{32}P) of the RNA to be sequenced at the 5'-terminus using polynucleotide kinase and γ-(^{32}P)-ATP, followed by partial endonucleolytic digestion with nuclease P1 (EC 3.1.30.1) isolated from Penicillium citrinum (Fujimoto et al. 1974a) under conditions designed to give rise to digestion products of every possible length. This fungal enzyme, whilst not being entirely random in its selection of cleavage sites (Fujimoto et al., 1974a), cleaves RNA phosphodiester bonds to leave 3'-hydroxyl and 5'-phosphate ends (Silberklang et al., 1974b). An activity towards DNA of this enzyme has also been reported (Fujimoto et al., 1974b).

The partial digestion products are fractionated by two-dimensional homochromatography fingerprinting (Brownlee and Sanger, 1969). In the first dimension, electrophoresis at pH 3.5, oligonucleotides in a fluid subjected to a voltage gradient E move with a velocity

$$U = \frac{E.Q'}{K'}$$

(Smith, 1955) where Q' is the net charge of the molecules and K' is a constant depending on the size and shape of the molecule, representing the retarding effect of other ions in the solution on the charged molecule. In the case of an oligonucleotide, Q' is the algebraic sum of the charges of its component nucleotides at pH 3.5 and which, in turn, depend on the pK'_a values of the mononucleotides. Oligonucleotides of different composition will therefore migrate with different velocities in this dimension. In the second dimension, homochromatography on a DEAE thin layer plate, the nuclease P1 digested oligonucleotides, which bind tightly to the DEAE-cellulose, are displaced by a series of anions of different valency or affinity for the DEAE groups. Such a system is produced when a concentrated mixture of oligonucleotides is applied to the end of the DEAE chromatogram. The oligonucleotides saturate the DEAE groups and displace one another, producing a series of fronts. The smaller ones with lower valency or affinity are displaced by the larger ones and, therefore, move faster. The nuclease P1 digested oligonucleotides move with the different fronts and are fractionated according to their affinity for the DEAE groups or, in other words, their length (Brownlee, 1972).

Upon autoradiography of the homochromatograph, only those oligonucleotides containing the original labelled 5'-terminus are detected and thus a series of spots, the 'wandering spot', are seen corresponding to oligonucleotides differing by one nucleotide in length from each other. The longest oligonucleotide, the intact material, is represented by the spot nearest the start line of the second dimension. The next spot up corresponds to the intact RNA less the 3'-terminal mononucleotide and therefore migrates in a different position in the first dimension and faster in the second dimension. This applies to the remaining

oligonucleotides up to the 5'-terminal nucleotide. From the pattern of spots, the nucleotide sequence of the oligonucleotide can be determined by analysis of the mobility shifts between successive spots and comparison with those in the literature for both RNA (Gillum et al., 1975; Lockard and Raj Bhandary, 1976; Silberklang et al., 1977a, 1977b; Richards et al., 1977; Fuke and Busch, 1977; Rommelaere et al., 1979; Koper-Zwarthoff and Bol, 1979) and DNA (Sanger et al., 1973; Jay et al., 1974b; Tu et al., 1976).

A consensus pattern obtained from the above literature of the mobility shift of an oligonucleotide (n) either lengthened or shortened by a single nucleotide on homochromatography fingerprinting is shown in Fig. 26. Since there are differences in mobility shifts from one homochromatography system to another, and also to some extent within a single system, it was important to determine the mobility shifts in the actual system intended to be used prior to sequencing the IF3 protected site. Also shown in Fig. 26 are the relative d-values (the vertical distance between two spots) (Tu et al., 1976) for pyrimidine and purine nucleotides. The pyrimidine nucleotides have a d-value of approximately two thirds that of the purine nucleotides.

As nuclease P_1 is not entirely random in its selection of cleavage sites (Fujimoto et al., 1974a), certain internucleotide bonds may be cut less frequently than others; this can result in a final autoradiogram containing lighter and denser spots. Polyprimidine clusters, especially oligo-C stretches in single-stranded conformations, show up as a series of lighter spots (Silberklang et al., 1977b).

4.2.2 Partial digestion with nuclease P1 and trial 'wandering spot'

To determine the conditions of digestion that gave rise to an even distribution of partial digestion products, and also to perfect the

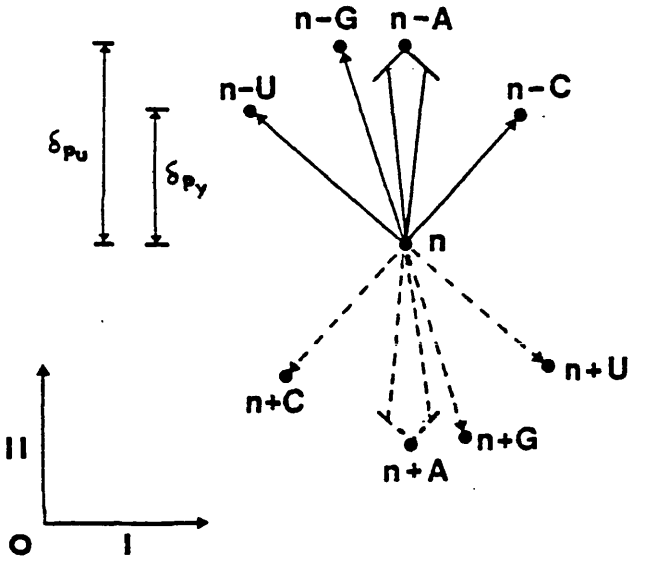


FIGURE 26 'Wandering spot' mobility shifts extracted from the literature.

Pattern of mobility shifts extracted from the literature (see text for references) of an oligonucleotide (n) either shortened or lengthened by a single nucleotide upon two-dimensional homochromatography. δ_{Pu} and δ_{Py} represent d-values (Tu et al., 1976) for removal of purine and pyrimidine nucleotides respectively. I and II are the directions of the first and second dimensions of the homochromatography fractionation procedure.

wandering spot technique, 5'-end labelled oligonucleotides were prepared by digesting unlabelled MS2 RNA with RNase T_1 and labelling the oligonucleotides with PNK and γ-(^{32}P)-ATP. The labelled digest was then fractionated by two-dimensional homochromatography and the autoradiography of the homochromatograph is shown in Fig. 27. Spots corresponding to large oligonucleotides (numbered T_2, T_7 and T_9 on Fig. 27) were eluted from the fingerprint for subsequent use. Fractions of two of these (T_2 and T_9) were digested with different amounts of nuclease P1 and aliquots of the reaction mixtures frozen at various times to be subsequently pooled and fractionated by one-dimensional homochromatography to determine the ratio of nuclease P1 to RNA that gave an even distribution of digestion products. The resulting autoradiogram (Fig. 28) shows that 12.5ng of nuclease P1 gave a good distribution of products in both the case of T_2 and T_9. This corresponds to a ratio of 50ng per spot from the homochromatograph and, since an average spot is estimated to contain very approximately 200µg of RNA (Barrell, 1971), although this can be expected to vary considerably depending on the size of the spot, the above ratio corresponds very approximately to 25ng per 100µg of RNA. This is within the same order of magnitude as that used by Richards et al., (1977) (10ng/100µg RNA), Lockard and RajBhandary, (1976) and Silberklang et al., (1977b) (both used 15ng per 100µg RNA). In later experiments with the IF3 protected site, the limited amount of radioactivity present in the oligonucleotides being sequenced precluded this type of experiment and, since the size of the spots on the homochromatographs varied a great deal, the amount of nuclease P1 used to obtain 'wandering spots' was either increased or decreased from this ratio, according to the relative areas of the protected fragment spots and the spots T_2 and T_9.

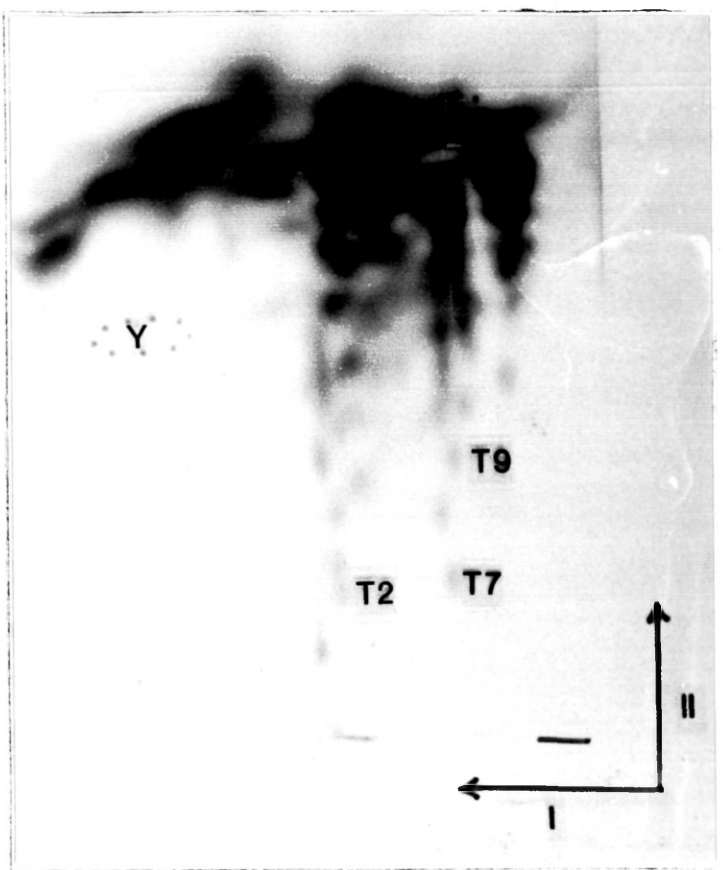


FIGURE 27 RNase T_1 homochromatography fingerprint of MS2 RNA. Autoradiograph of unlabelled MS2 RNA digested with RNase T_1, labelled using PNK and γ-(^{32}P)-ATP as described in section 3.7.3 and fractionated by two-dimensional homochromatography using 5% homomix 'C'. Oligonucleotides referred to in the text are identified. B and Y are the positions of the Blue and Yellow dye markers. I and II are the directions of the first and second dimensions of the fractionation.

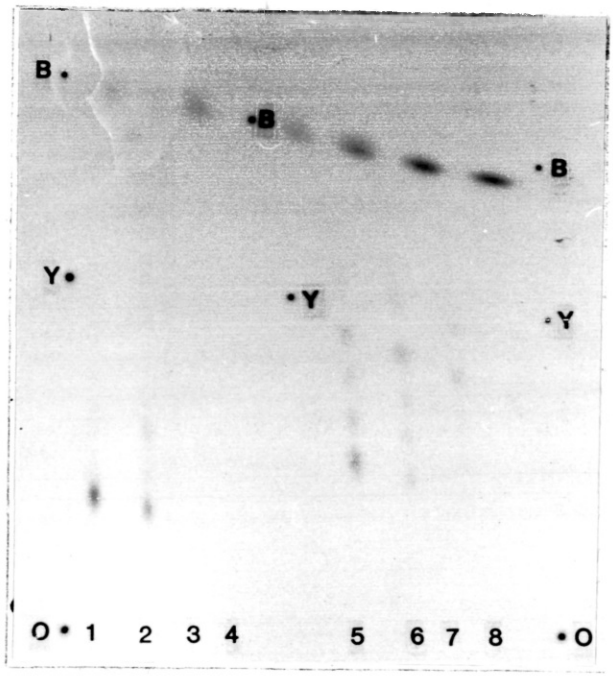


FIGURE 28 Partial nuclease P1 digestion of oligonucleotides T_2 and T_9

Autoradiography (2 days exposure) of one-dimensional homochromatography fractionation of partial nuclease P1 digests of oligonucleotides T_2 and T_9 eluted from the homochromatograph shown in Fig. 27, as described in section 3.1.4. 1-4, approximately 50µg of T_2 digested with 5, 12.5, 25 and 50ng respectively of nuclease P1 (section 3.8). 5-8, approximately 50µg of T_9 digested with 5, 12.5, 25 and 50ng of nuclease P1. B and Y are the positions of the Blue and Yellow dye markers. Fractionation using 5% homomix 'C' was from bottom to top.

To determine the mobility shifts upon loss or addition of a single nucleotide to a homologous series of oligonucleotides in the homochromatography system intended to be used in this work, spot T_7 material was partially digested with nuclease P1 and fractionated by homochromatography fingerprinting, using 3% homomix 'C' in the second dimension. The autoradiograph obtained from this fractionation is shown in Fig. 29, together with an interpretation of the mobility shifts. A large amount of the oligonucleotide has not been digested, probably due to sub-optimal digestion conditions, and can be seen as an intense spot near the origin of the second dimension.

Referring to Fig. 29, spot (1) runs slower than the blue marker and, since 5'-mononucleotides run with a mobility similar to that of the blue marker in this dimension, (Fig. 28 and Richards et al., 1977; Silberklang et al., 1977b) spot (1) probably represents an oligonucleotide rather than the 5'-terminal nucleotide. The d-value between spot (1) and the blue marker suggests that this oligonucleotide represents the 5'-terminus plus two or more additional nucleotides. The RNA species shorter than spot (1), represented by *p(N) (where *p represents the labelled 5'-phosphate), were not transferred from the first dimension since they were running slower than the blue marker in that dimension. Spot (1) is very close to the edge of the DEAE plate, as can be seen by the trailing down the edge, and this makes the mobility shift from spot (1) to spot (2) difficult to interpret. The proximity of the edge of the plate appears to have had the effect of pulling spot (1) towards the edge and also of reducing the d-value between spots (1) and (2). This edge effect was also observed in later wandering spots. From a comparison of the mobility shifts from spots (1) to (2) with the consensus shifts shown in Fig. 26, the change in position could be due to the addition of either a G or A residue: the addition of a C residue would result in a mobility shift in the opposite direction to that

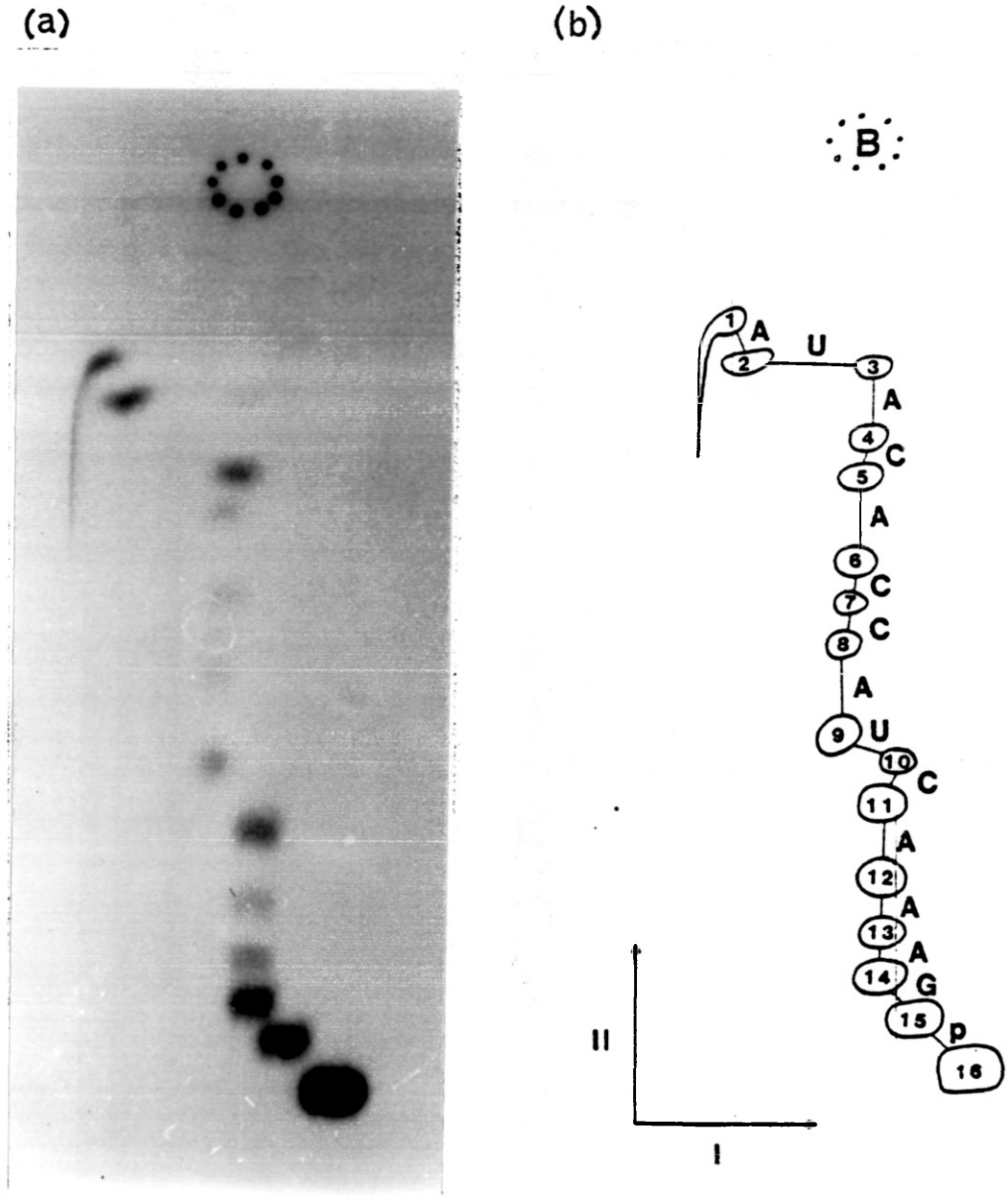


FIGURE 29 'Wandering spot' sequence analysis of oligonucleotide T_7 (a) Autoradiograph of two-dimensional fractionation of partial nuclease P1 digest (section 3.8) of oligonucleotide T_7 from Fig. 27. (b) Interpretation of mobility shifts - see text for details. B marks the position of the xylene cyanol FF dye marker and I and II are the directions of the first and second dimensions of the homochromatography fractionation. Exposure was for 7 days.

observed and the addition of a U residue is unlikely, in view of the shift between spots (2) and (3). Since the oligonucleotide T_7 has been prepared from a total RNase T_1 digest of MS2 RNA, no G residues, with the exception of the 3'-terminal residue, should be present in the oligonucleotide. Thus, the mobility shift from spot (1) to spot (2) is probably due to the addition of an A residue to spot (1) to give *p(N)-A (spot 2).

The low d-value, together with the strong shift to the right of spot (3) from spot (2), suggests that this represents the addition of a U residue to oligonucleotide (2) to give *p(N)-A-U. The high d-value of the change from spot (3) to (4), together with the near vertical direction of change, suggests that spot (4) represents the addition of an A residue to give *p(N)-A-U-A. The leftward mobility shift from spot (4) to (5) and the low d-value suggests that this is due to the addition of a C residue, to give *p(N)-A-U-A-C, and the change from spot (5) to spot (6), having a larger d-value and being nearly vertical, suggests the addition of an A residue to give *p (N)-A-U-A-C-A. Spots (6), (7) and (8) are in a straight line, angled slightly to the left, and each have a low d-value. They thus probably represent the addition of two C residues to oligonucleotide (6) to give *p(N)-A-U-A-C-A-C-C. The change from spot (8) to spot (9), based on the above mobilities, is indicative of the addition of an A residue, to give *p(N)-A-U-A-C-A-C-C-A and the shift from spot (9) to (10), which is a faint spot, is indicative of the addition of a U residue, to give *p(N)-A-U-A-C-A-C-C-A-U.

The mobility shift from spot (10) to (11) has a low d-value and is to the left, suggesting the addition of a C residue to (10) to give *p(N)-A-U-A-C-A-C-C-A-U-C. Spots (11), (12), (13) and (14) are all in a nearly vertical straight line, indicative of the addition of three

successive A residues to (11), to give oligonucleotide (14) of sequence *p(N)-A-U-A-C-A-C-C-A-U-C-A-A-A.

The mobility shift between spots (14) and (15) is indicative of the addition of either a U or G residue and the d-value of this shift suggests that a G residue is a probable candidate. If this is correct, then since oligonucleotide T_7 was prepared from a total RNase T_1 digest, this must represent the 3'-terminus of the oligonucleotide. However, an additional slower moving spot is present on the autoradiogram, suggesting an additional 3'-nucleotide. Since this oligonucleotide was prepared in the absence of alkaline phosphatase to remove the 3'-terminal phosphate group, this additional spot is probably due to the presence of a phosphate group at the 3'-terminus of oligonucleotide (16) and being removed by the 3'-phosphomonoesterase activity of nuclease P1 (Fujimoto et al., (1974a) to give spot (15). As the mobility of an oligonucleotide in the second dimension of the homochromatography system is essentially dependent upon the number of phosphate groups present in the oligonucleotide (Brownlee, 1972), the loss of the 3'-phosphate by the 3'-phosphomonoesterase activity of nuclease P1 will result in the appearance of a spot running faster than the 3'-phosphorylated oligonucleotide. A similar situation to this was observed by Lockard and RajBhandary (1976) when determining the sequences at the 5'-termini of α and β globin mRNA.

The nucleotide sequence of oligonucleotide T_7, as determined from the wandering spot, appears to be:

^{32}p(N)-A-U-A-C-A-C-C-A-U-C-A-A-A-Gp (sequence 1)

where (N) represents an unknown sequence of two, possibly three, nucleotides.

Comparison of the fingerprint of MS2 RNA shown in Fig. 27 with

an RNase T_1 fingerprint of *in vivo* labelled MS2 RNA published by Robertson and Jeppesen (1972) (Plate I) suggests that the oligonucleotide T_7 used for the wandering spot described above is in a similar position to oligonucleotide h'' shown on their fingerprint. The oligonucleotide h'' was reported by these workers to have the sequence:

C-A-A-A-U-A-C-A-C-C-A-U-C-A-A-A-Gp (sequence 2)

This sequence agrees with that determined from Fig. 29 for T_7 and shows that the nucleotide sequence of (N) consisted of the sequence *p-C-A-A.

Oligonucleotide T_7 (and h'') is located at position 1505 to 1521 in the complete nucleotide sequence of MS2 RNA (Fiers *et al.*, 1976) and is located in the coat protein gene.

Comparison of the mobility shifts seen in Fig. 29 with the mobility shifts obtained from the literature (Fig. 26) shows that there is good agreement between the systems, but that the G and U mobility shifts are slightly more flattened in Fig. 29.

4.2.3 Nucleotide sequences of IF3 protected fragments

The oligonucleotides from some of the homochromatographs of the protected site (Fig. 24) were eluted as described (section 3.1.4) and subjected to 'wandering spot' sequence determination. In many of the early attempts, the small amount of radioactivity present in the oligonucleotides precluded the determination of the nucleotide sequence except in isolated cases, since the wandering spot patterns that were obtained were very faint or invisible. In later experiments, protected sites B60 and B58 yielded oligonucleotides containing sufficient radioactivity for the 'wandering spots' to be visible and interpretable.

(i) 'Wandering spots' from protected fragment B60

The homochromatograph of protected site preparation B60 (Fig.

24) shows the presence of four large size spots (B60-1, -2, -3 and -4) corresponding to reasonably large oligonucleotides in view of their slow mobility on the second dimension. A number of small fragments running near to the top of the plate may have resulted from some breakdown of the protected fragments during isolation.

Oligonucleotides B60-1 to B60-4 were eluted from the homochromatograph and their nucleotide sequences determined by wandering spot sequencing.

Oligonucleotide B60-1 The 'wandering spot' from B60-1 is shown in Fig. 30, together with an interpretation of the mobility shifts. Using the same principles as those described for the trial wandering spot (section 4.2.2), the nucleotide sequence obtained from this 'wandering spot' was deduced to be:

*p(N)-C-G-C-C-G-G-U-U-C-C-C-A-C-A-Up (sequence·3)

where (N) represents an unknown sequence of two or three nucleotides that had not been transferred from the first dimension of the fractionation. The anomalous mobility shift of the 3'-terminal nucleotide (it has a very large d-value for a pyrimidine residue) is probably due to it representing the loss of the nucleotide diphosphate pUp, rather than the monophosphate pU. This is in contrast to that observed with the trial wandering spot (Fig. 29) where two spots corresponding to the loss of the 5'-phosphorylated nucleotide pG and the 3'-terminal phosphate group, released by the 3'-phosphomonoesterase activity of the enzyme, were seen. Preferential loss of this activity of nuclease P1 has been reported (Fujimoto et al., 1974a) and this is most likely to account for its loss of activity during storage between performing the trial wandering spot and the wandering spot of B60-1.

The above sequence, without the unknown sequence (N), occurs at

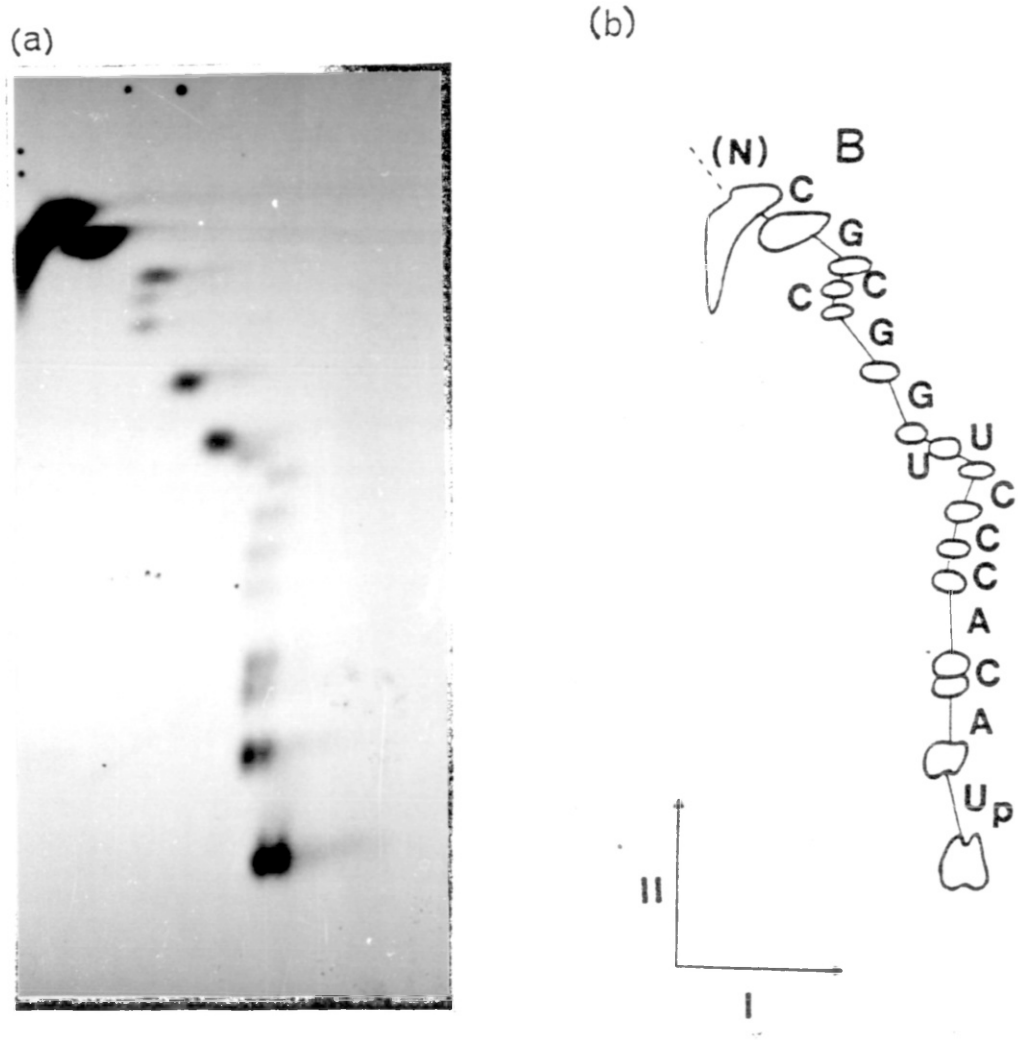


FIGURE 30 'Wandering spot' sequence determination of B60-1.
(A) Autoradiograph of fractionation of digest of B60-1 as in Fig. 29.
(b) Analysis of mobility shifts of 'wandering spot'. Autoradiography was for 14 days at -70°C. B, I and II are as described in Fig. 29.

position 3346-3360 in the complete nucleotide sequence of MS2 RNA as published by Fiers et al. (1976). This sequence is immediately preceded by the sequence G-C-U-A-A and, since the protected fragment was prepared using pancreatic RNase and only two or three of the partial digestion products appear not to have been transferred from the first dimension of the wandering spot, the sequence (N) is either U-A-A or A-A, depending upon the position of pancreatic RNase cutting. Since the RNase T_1 oligonucleotide A-A-C-G occurs in the fingerprint of the IF3 protected site (Table 5) and the oligonucleotide U-A-A-C-G does not, the sequence of (N) is most probably A-A and the sequence of B60-1:

*pA-A-C-G-C-C-G-U-U-C-C-C-A-C-A-Up (sequence 4)

This sequence is located at position 3344-3360 in the complete MS2 RNA sequence.

Oligonucleotide B60-2 The 'wandering spot' obtained from oligonucleotide B60-2 is shown in Fig. 31, together with its interpretation and the sequence deduced to be:

*p(N)-G-G-A-G-U-G-U-G-G-G-C-C-A-G-Cp (sequence 5)

where (N) represents the 5'-terminal residue, the nature of which cannot be determined from the mobility shifts. This sequence, less the 5'-(N) residue, occurs at positions 3368-3382 in the MS2 RNA sequence. It is preceded by the sequence U-C-C-C-U-A and, since the uppermost spot in Fig. 31 is running near to the blue marker and none of the possible RNase T_1 oligonucleotides that can be generated by cutting after any of the pyrimidine residues, with the exception of the 3'-U, are present in fingerprints of the protected site (Table 5), the sequence of oligonucleotide B60-2 is deduced to be:

*pA-G-G-A-G-U-G-U-G-G-G-C-C-A-G-Cp (sequence 6)

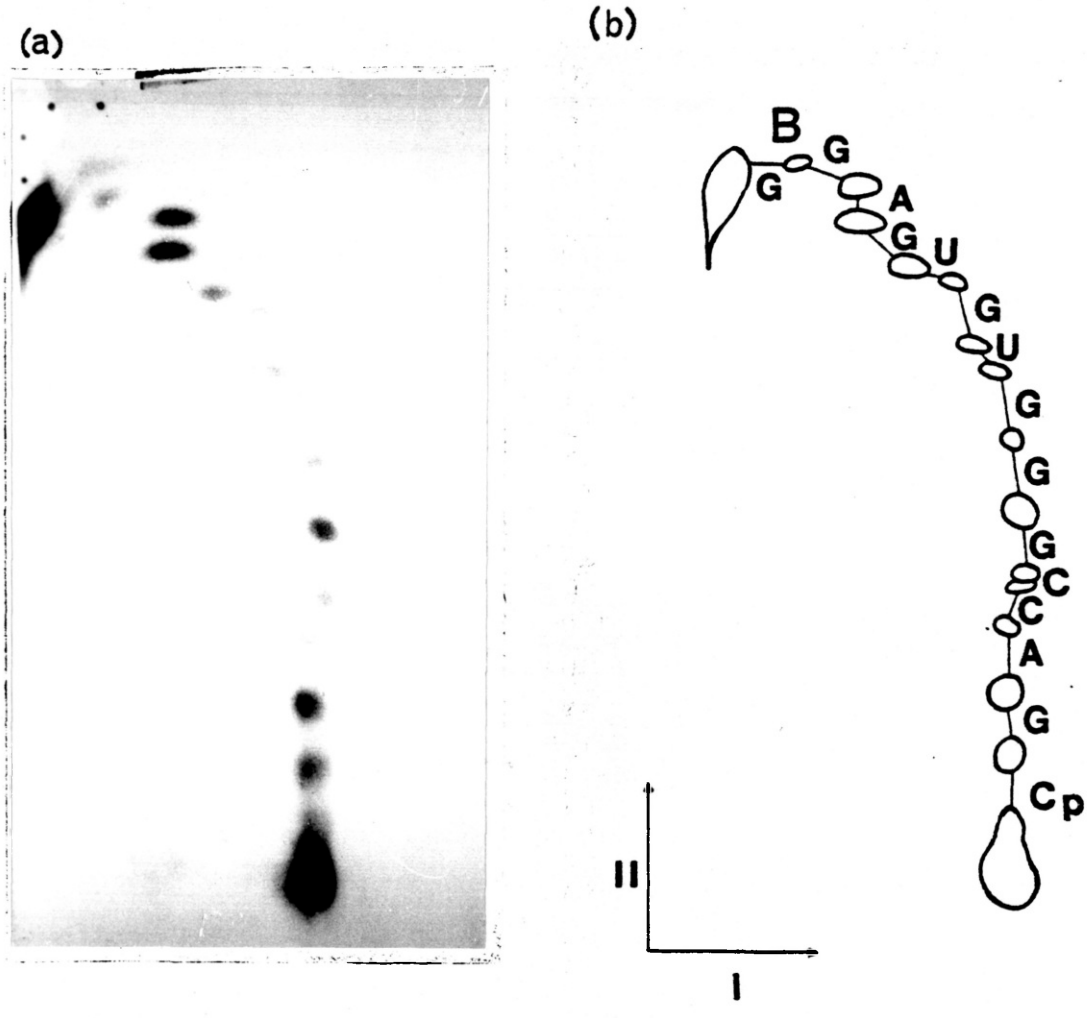


FIGURE 31 'Wandering spot' sequence analysis of B60-2.

(a) Autoradiograph of fractionation of digestion products.

(b) Analysis of mobility shifts of 'wandering spot'. Autoradiography was for 14 days at -70°C. B, I and II are as described in Fig. 29.

This sequence occurs at position 3367-3382 in the complete MS2 RNA sequence.

Oligonucleotide B60-3 Fig. 32 shows the 'wandering spot' pattern obtained from oligonucleotide B60-3, together with an intepretation of the mobility shifts. The sequence of this oligonucleotide deduced from the wandering spot is:

*p(N)-G-G-A-G-U-G-U-G-G-G-C-C-A-G-(X)p (sequence 7)

where (N) is the 5'-terminal nucleotide, the nature of which cannot be determined from the mobility shifts, but which can be deduced to be an A residue by arguments similar to those used for oligonucleotide B60-2. The known sequence occurs at position 3367-3381 and, since the next possible pyrimidine residues at which the pancreatic RNase could cut are located at positions 3382 and 3386, the nature of (X) may be either C or C-G-A-G-C. The latter possibility is supported by the observation that the oligonucleotide G-A-G-C occurs in the pancreatic RNase fingerprints of the protected site (Table 5) and that oligonucleotide B60-3 has a different mobility in the homochromatography system (Fig. 24) to that of B60-2, which has a similar sequence up to residue 3382 (Fig. 31).

Oligonucleotide B60-4 The 'wandering spot' pattern obtained from oligonucleotide B60-4, together with an interpretation of the mobility shifts, is shown in Fig. 33. The sequence of only 16 nucleotides can be deduced from this figure, since the lower spots are poorly resolved. The sequence is deduced to be:

*p(N)-G-G-U-G-U-G-C-U-C-G-A-A-A-G-A-G-(X) (sequence 8)

where (N), since it migrates at the level of the blue dye marker, is the 5'-terminal nucleotide and (X) an unknown sequence of nucleotides. The known sequence is located at residues 3428-3443 in the MS2 RNA

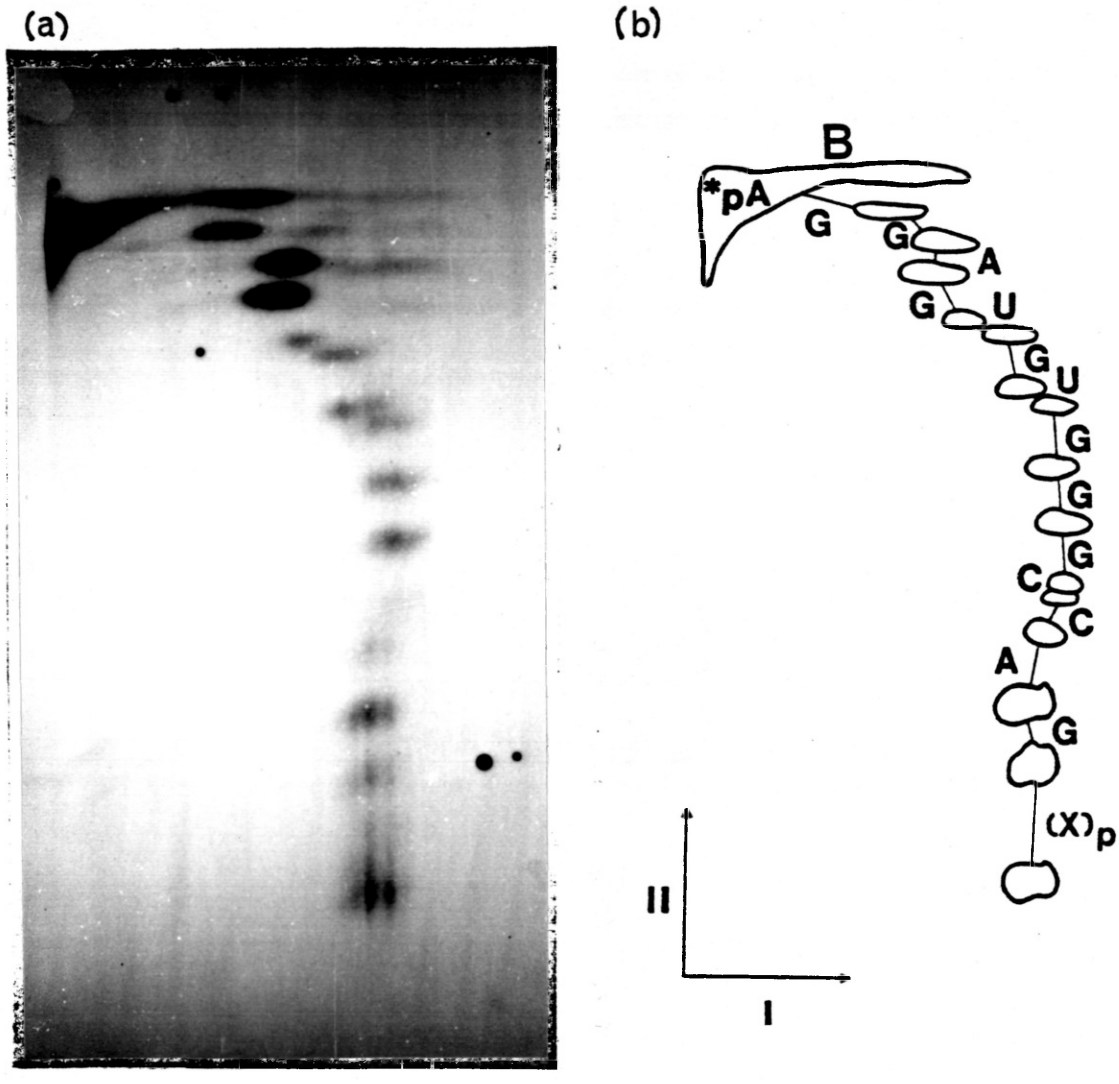


FIGURE 32 'Wandering spot' sequence analysis of B60-3.

(a) Autoradiograph of fractionation of digestion products.

(b) Analysis of mobility shifts of 'wandering spot'. Autoradiography was for 15 days at -70°C. B, I and II are as described in Fig. 29.

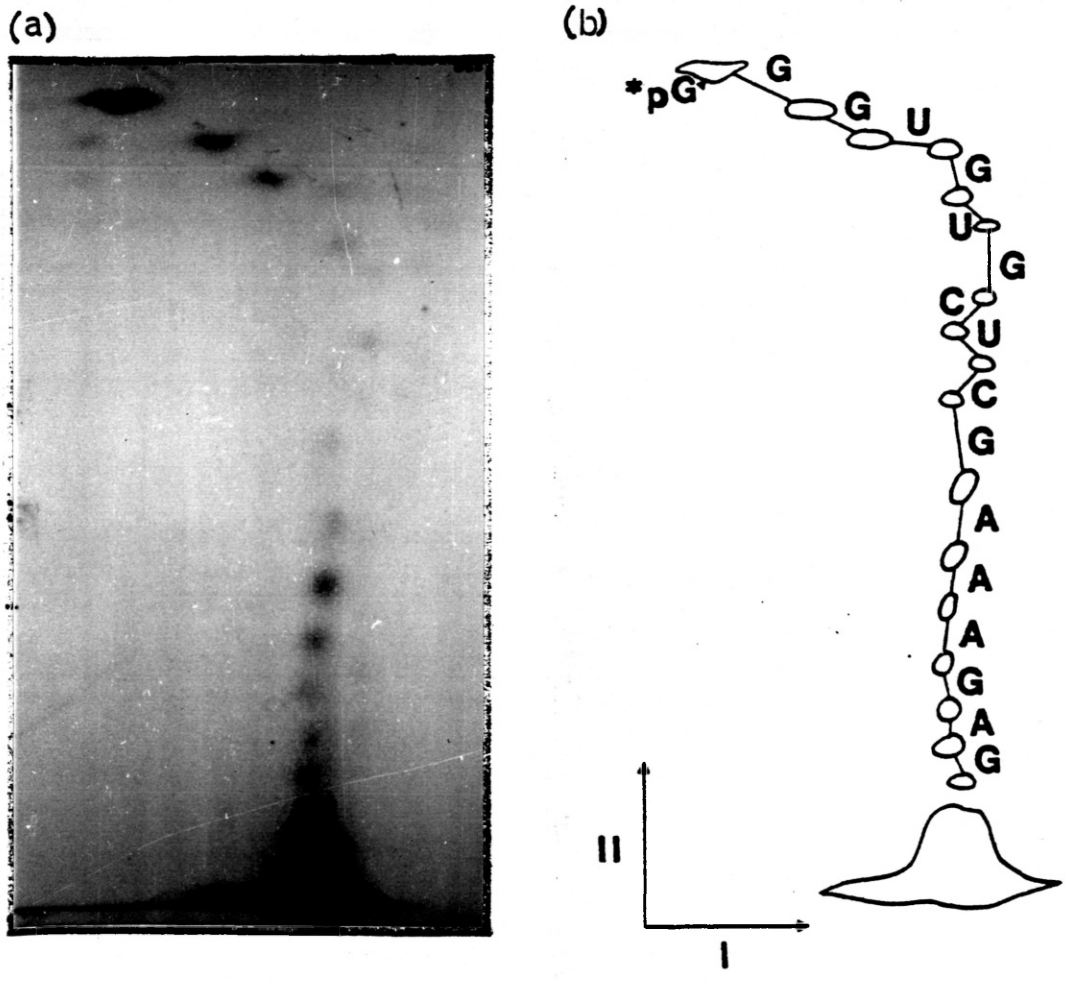


FIGURE 33 'Wandering spot' sequence analysis of B60-4.
(a) Autoradiograph of fractionation of digestion products.
(b) Analysis of mobility shifts of 'wandering spot'. Autoradiography was for 14 days at -70°C. I and II are as described in Fig. 29.

sequence and is preceded by the sequence A-C-G-G-G-U-G. Since the spot corresponding to (N) runs with a mobility similar to that of a mononucleotide and the pancreatic oligonucleotide G-G-G-G-U is not found in fingerprints of the protected site (Table 5), the nature of (N) is most probably a G residue with the pancreatic RNase cutting after the preceding U residue (number 3426).

The nature of the nucleotide sequence of (X) cannot be determined from the wandering spot; however, it must extend to the C residue at position 3444, since this would be the first position at which the pancreatic RNase could cut. Oligonucleotide B60-4 therefore covers the region 3427-3444 and beyond in the MS2 RNA sequence. This oligonucleotide would have been better suited to have been sequenced by one of the rapid gel sequencing techniques (e.g., Simoncits et al., 1977) or by 'wandering spot' sequencing of both 5'- and 3'-labelled material.

(ii) 'Wandering spots' from protected fragment B58

Oligonucleotides B58-1 to B58-8 inclusive were eluted from the homochromatograph of protected site preparation B58 shown in Fig. 24. Some streaking in the first dimension of this fractionation is apparent and the pattern of spots is more complex than that found for preparation B60 (Fig. 24), although some similarity to the homochromatograph published by Johnson and Szekely (1979) can be seen. Oligonucleotides B58-1 to B58-8, with the exception of B58-4 which was lost during elution, were subjected to wandering spot sequence analysis.

Oligonucleotide B58-1 The 'wandering spot' obtained from oligonucleotide B58-1 is shown in Fig. 34. The large amount of material remaining at the origin of the second dimension indicates that the digestion conditions were not optimal. The sequence of only 16 nucleotides can

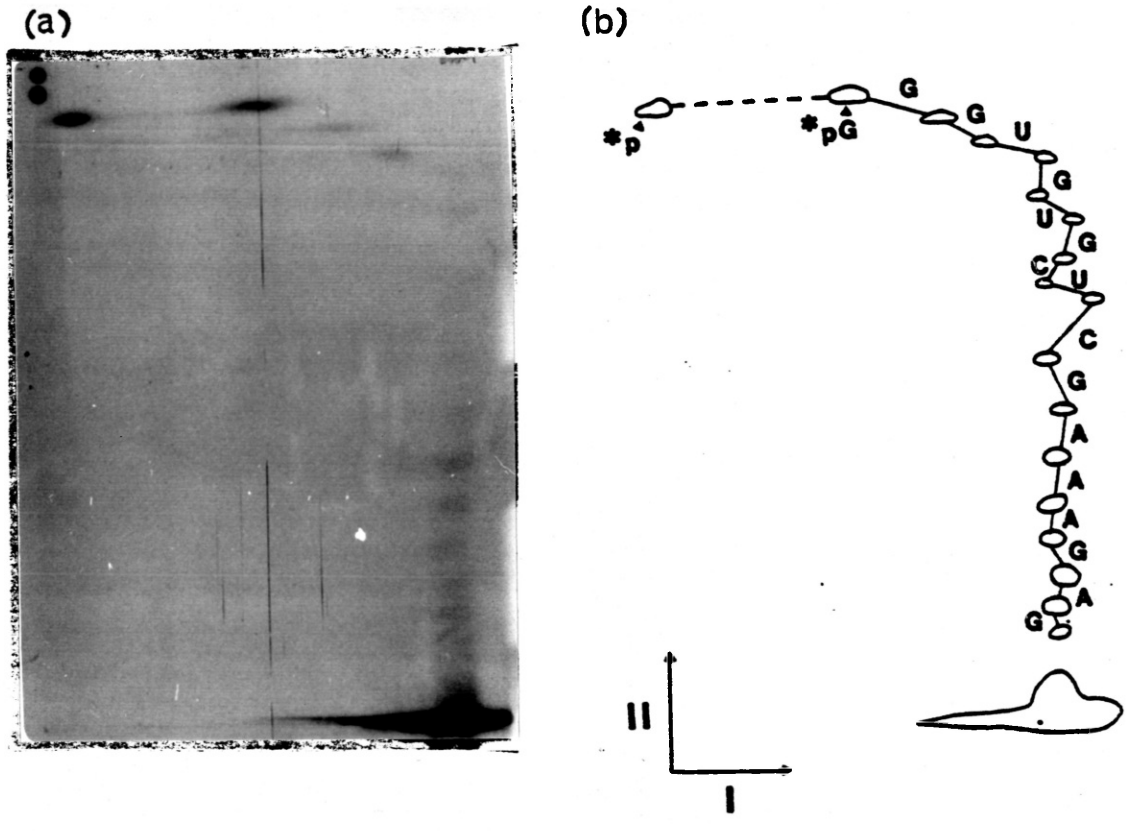


FIGURE 34 'Wandering spot' sequence analysis of B58-1.
(a) Autoradiography of fractionation of digestion products.
(b) Analysis of mobility shifts of 'wandering spot'. Autoradiography was for 21 days at -70°C. I and II are as described in Figure 29.

be determined from the mobility shifts and are deduced to be:

*pN-G-G-U-G-U-G-C-U-C-G-A-A-A-G-A-G-(X) (sequence 9)

where N is the 5'-terminal residue (since it runs with a mobility similar to that of the blue marker in the second dimension) and (X) is an unknown sequence the digestion products of which have poorly fractionated in the fingerprinting system.

This oligonucleotide has a similar sequence to that of B60-4, described previously, and by analogy with that oligonucleotide, the identity of N is most probably a G residue, giving the sequence:

*pG-G-G-U-G-U-G-C-U-C-G-A-A-A-G-A-G-(X) (sequence 10)

This sequence occurs at position 3427-3443 and beyond in the MS2 RNA sequence of Fiers et al. (1976).

Oligonucleotide B58-2 Fig. 35 shows the 'wandering spot' pattern obtained from oligonucleotide B58-2, together with an interpretation of the mobility shifts. The sequence of this oligonucleotide was deduced to be:

*p(N)-G-A-G-U-G-U-G-G-G-C-C-A-G-C-G-A-(X) (sequence 11)

where (N) represents the sequence of two or three nucleotides not transferred to the second dimension of the homochromatograph and (X) the 3'-terminal nucleotides, the spots of which were poorly resolved in the second dimension. The known oligonucleotides in this sequence are located at positions 3369-3384 in the complete MS2 RNA sequence. The sequence is similar to those of oligonucleotides B60-2 and B60-3, with the exception that this is longer at the 3'- end. By analogy with B60-2 and B60-3, the nature of (N) is A-G. The nucleotide sequence of complete MS2 RNA following the last identifiable residue in sequence 11 above is G-C-U-C-U-C-C-U-C-G (Fiers et al., 1976) and therefore the identity of sequence (X) is somewhat difficult to ascertain. The

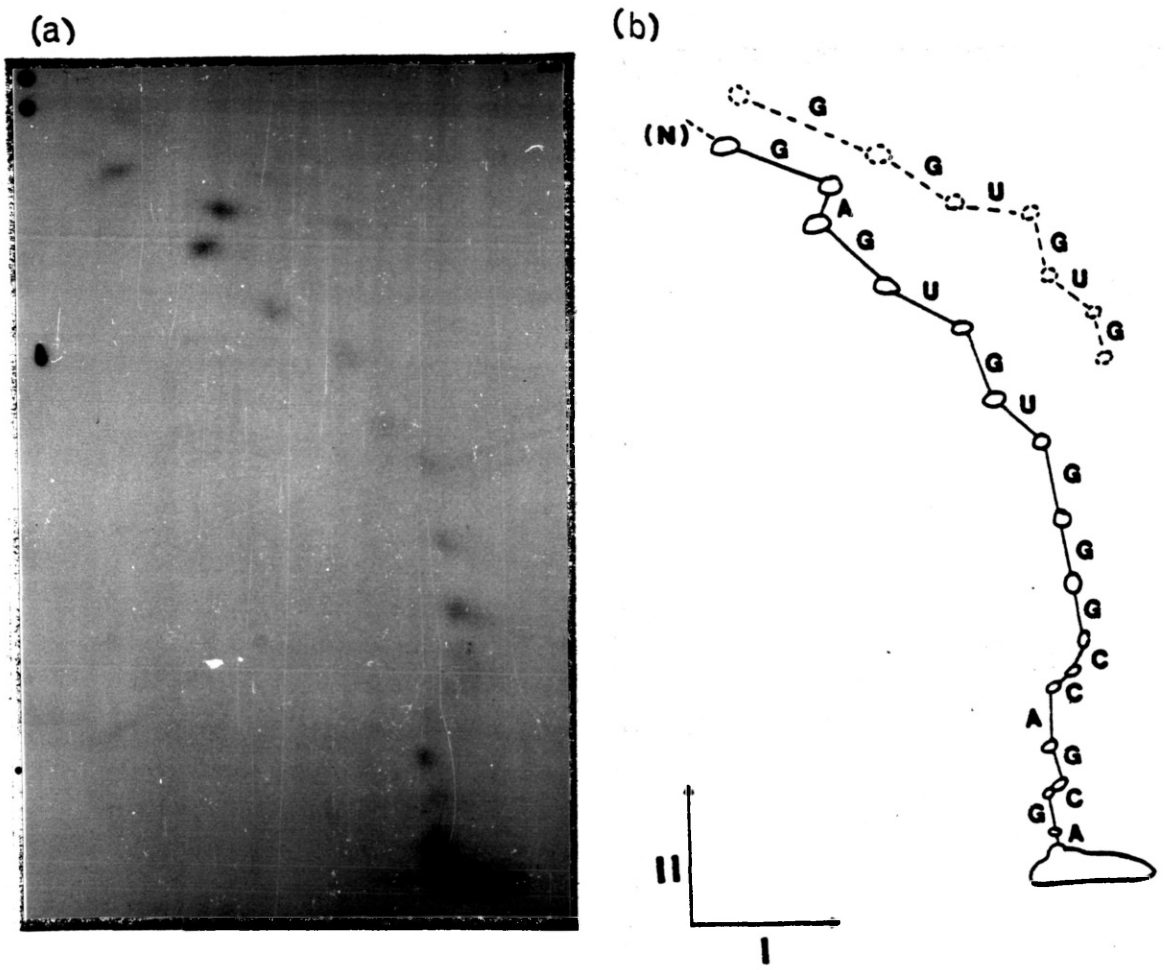


FIGURE 35 'Wandering spot' sequence analysis of B58-2.

(a) Autoradiograph of fractionation of digestion products.

(b) Analysis of mobility shifts of 'wandering spot'. Autoradiography was for 20 days at -70°C. I and II are as described in Fig. 29.

first position at which the pancreatic RNase could cut is after the second nucleotide of this sequence, i.e., after ...G-C; however, the RNase could also cut after any of the seven pyrimidine residues that follow this sequence. Thus, the exact 3'-terminus of this oligonucleotide is in doubt, but the minimum sequence of this fragment is:

*pA-G-G-A-G-U-G-U-G-G-G-C-C-A-G-C-G-A-G-C (sequence 12)

and covers the region 3367-3386 of the MS2 RNA molecule.

Also visible on Fig. 35 is a different wandering spot pattern resulting from contamination of this oligonucleotide with material from spot B58-1. The sequence represented by this wandering spot can be deduced to be:

*pG-G-G-U-G-U-G- ..... (sequence 13)

and represents the 5' portion of that spot.

Oligonucleotide B58-3 The 'wandering spot' pattern obtained from oligonucleotide B58-3 is shown in Fig. 36. This oligonucleotide spot was contaminated with another oligonucleotide and thus two wandering spot patterns can be discerned at the top of the plate. The sequence deduced from the main pattern is similar to that deduced for B60-2 and also to that of the 5' portion of B58-2 and is:

*pN-G-G-A-G-U-G-U-G-G-G-C-C-A-G-Cp (sequence 14)

The nature of N, the 5'-terminal nucleotide, by analogy with B60-2 and B58-2, is probably an A residue. This sequence covers the region 3367-3382 of the MS2 RNA sequence.

The partial sequence deduced for the oligonucleotide contaminating this spot is most likely to be *pN-G-G-(x) and, by comparison of the relative positions of the contaminating spots in both Figs. 35 and 36 with the main wandering spot, the contaminating material is probably derived from spot B58-1.

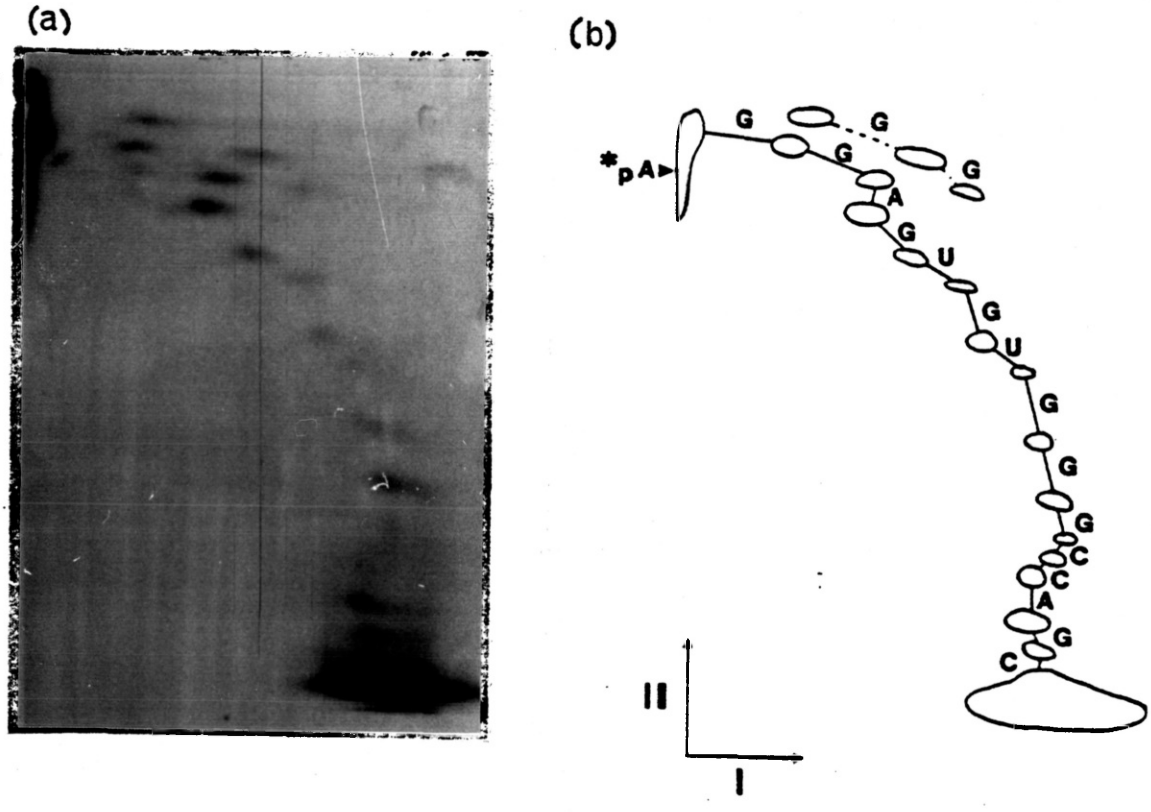


FIGURE 36 'Wandering spot' sequence analysis of B58-3.

(a) Autoradiograph of fractionation of digestion products.

(b) Analysis of mobility shifts of 'wandering spot'. Autoradiography was for 20 days at -70°C. I and II are as described in Fig. 29.

Oligonucleotide B58-5 The wandering spot pattern from spot B58-5 is shown in Fig. 37. It can be seen that this spot was contaminated with another nucleotide sequence or sequences and thus the mobility shifts from one spot to another are impossible to comprehend.

Oligonucleotide B58-6 Fig. 38 shows the wandering spot pattern obtained from oligonucleotide B58-6, together with an interpretation of the mobility shifts. The nucleotide sequence of this oligonucleotide was deduced to be:

*p(N)-C-G-C-C-G-G-U-U-C-C-C-A-C-A-U-C-C (sequence 15)

with (N) representing one or two spots not transferred from the first dimension of the fractionation procedure and the underlined 3'-terminal nucleotides being uncertain, since the spots are very faint on the autoradiograph. This sequence is similar to that found for oligonucleotide B60-1, but with the 3'-terminus lengthened by a possible three nucleotides. The nature of (N), using arguments similar to those used for B60-1, is probably A-A. This sequence would cover the position 3344-3363 in MS2 RNA if it terminated after the two C residues shown in sequence 15 above.

Oligonucleotides B58-7 and B58-8 The 'wandering spot' patterns obtained from spots B58-7 and B58-8 were very faint, with very few spots being visible. No sequence information could therefore be obtained.

(iii) 'Wandering spot' from protected fragment B55

Oligonucleotide B55-2 The wandering spot pattern obtained from oligonucleotide B55-2 from the homochromatograph shown in Fig. 24 is shown in Fig. 39, together with an interpretation of the mobility shifts. The sequence of part of this oligonucleotide was deduced to be:

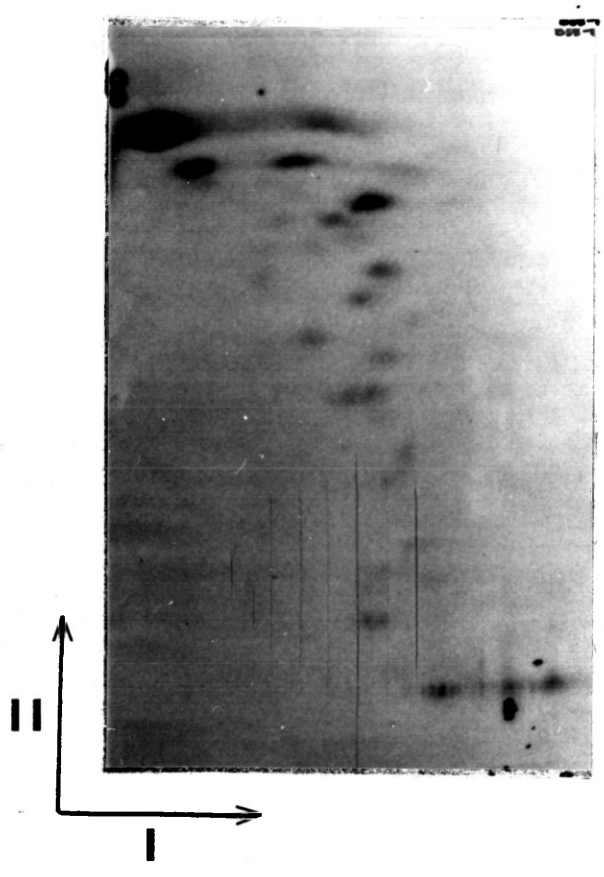


FIGURE 37 'Wandering spot' sequence analysis of B58-5.
Autoradiograph of fractionation of digestion products. I and II are as described in Fig. 29. Autoradiography was for 19 days at -70°C.

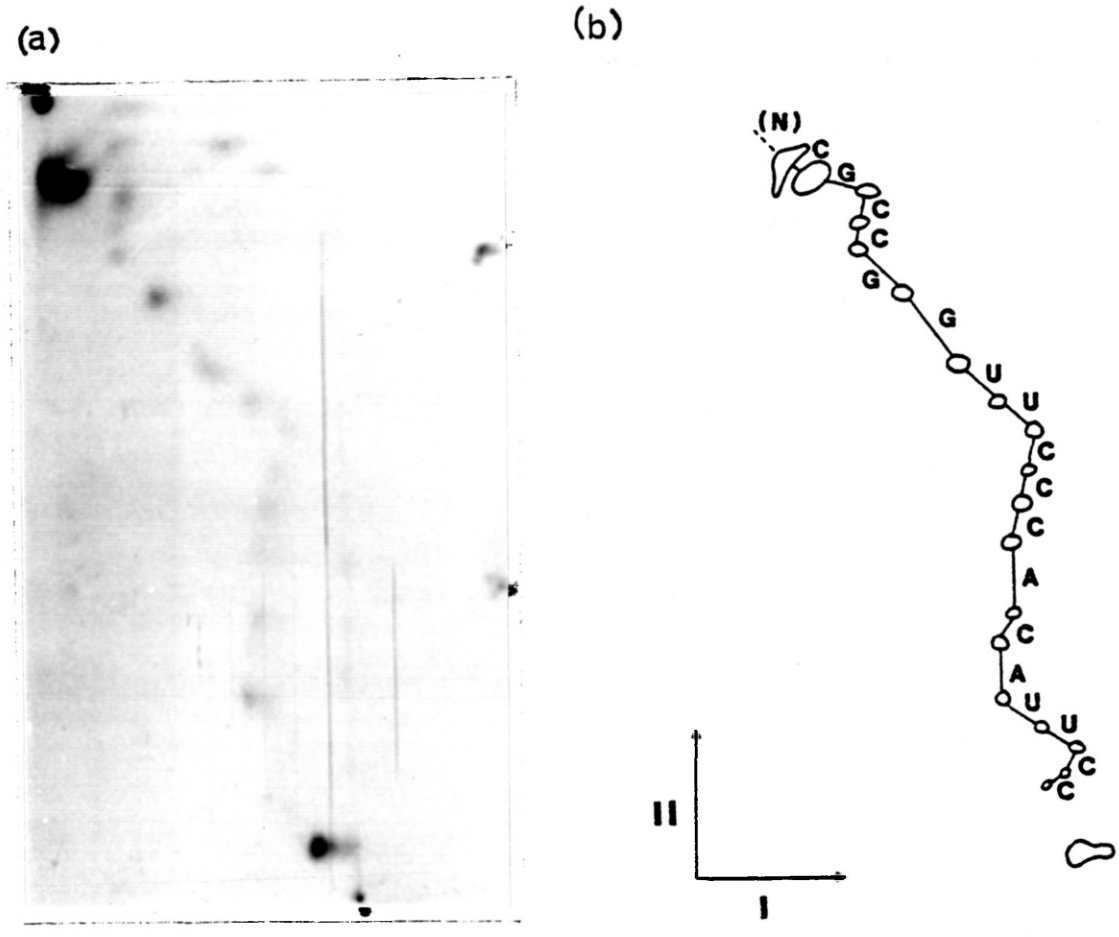


FIGURE 38 'Wandering spot' sequence analysis of B58-6.
(a) Autoradiograph of fractionation of digestion products.
(b) Analysis of mobility shifts of 'wandering spot'. Autoradiography was for 20 days at -70°C. I and II are as described in Fig. 29.

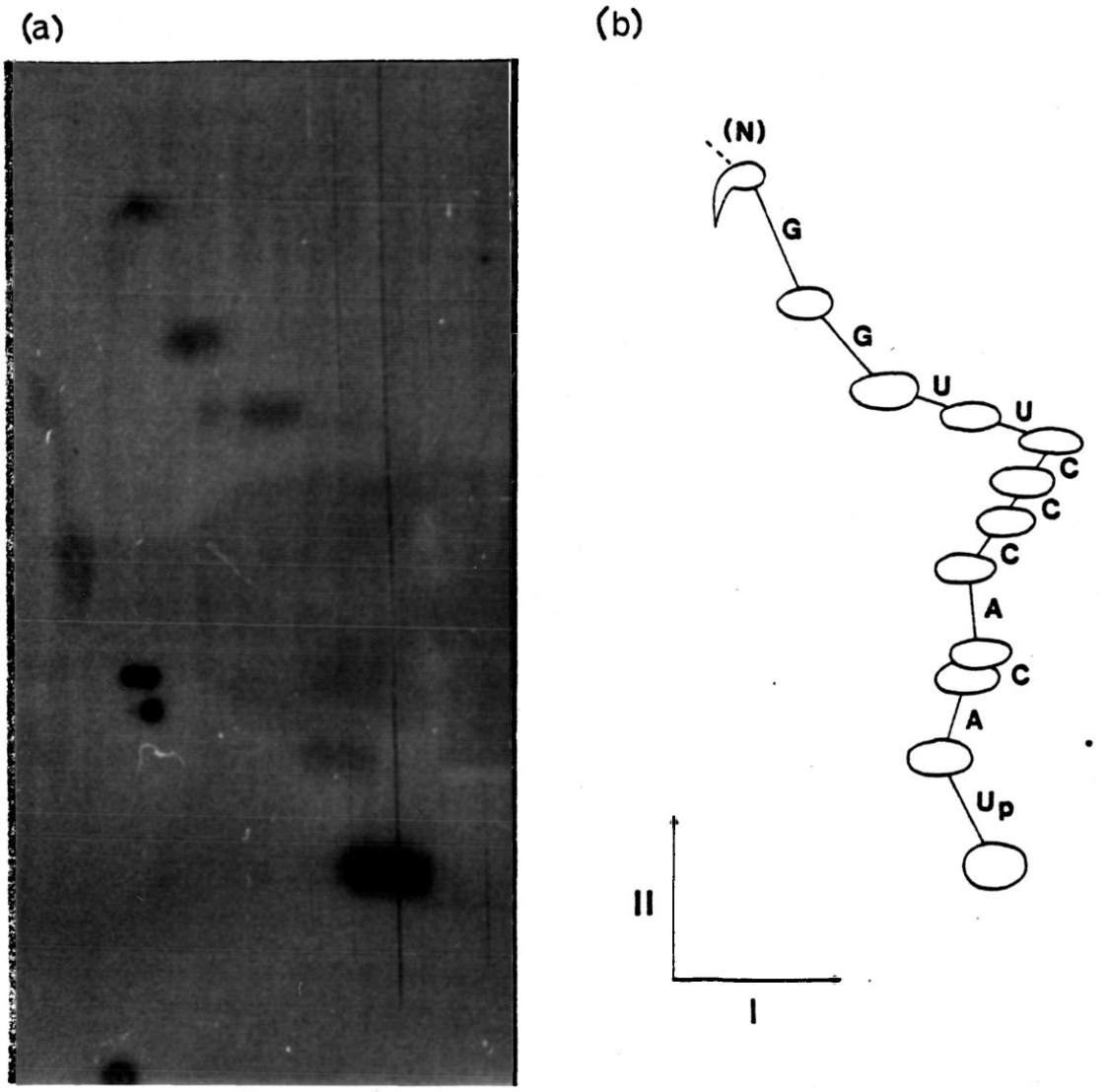


FIGURE 39 'Wandering spot' sequence analysis of B55-2.

(a) Autoradiograph of fractionation of digestion products.

(b) Analysis of mobility shifts of 'wandering spot'. Autoradiography was for 21 days at -70°C. I and II are as described in Fig. 29.

*p(N)-G-G-U-U-C-C-C-A-C-A-Up (sequence 16)

with the 5' partial digestion products (N) not being transferred from the first dimension of the fractionation procedure. The above sequence covers the region 3350-3360 in the complete MS2 RNA sequence.

4.2.4 Sequencing studies using in vivo labelled IF3 protected MS2 RNA

In this section, some of the unpublished data from Dr. B. Johnson working with in vivo (^{32}P) labelled IF3 protected MS2 RNA are presented which have a bearing on the elucidation of the nucleotide sequences present in the IF3 protected fragment. I thank Dr. B. Johnson for permission to reproduce these data.

The IF3 protected site on MS2 RNA, after PAGE purification, was partially digested with RNase T_1 and the digestion products fractionated by homochromatography fingerprinting. Several of the partial digestion products were eluted and their nucleotide sequences investigated by total RNase T_1 digestion and paper electrophoretic fractionation, followed by pancreatic RNase digestion of the fractionated RNase T_1 products. The natures of the pancreatic RNase products were ascertained by paper electrophoresis, together with standards from complete MS2 RNA digested with RNase T_1 and pancreatic RNase.

The results of this work are summarised in Table 7. Dr. B. Johnson deduced from the possible permutations of the oligonucleotides that RNase T_1 product B14-35 was probably of the sequence:

C-A-C-G-G-G-U-C-C-G-C-A-A-A-G (sequence 17)

which occurs at positions 3444-3459 in the complete MS2 RNA sequence and that partial RNase T_1 product B14-40 was probably:

U-G-C-U-C-G-A-A-A-G-A-G-C-A-C-G (sequence 18)

TABLE 7

Partial RNase T_1 digestion of IF3 protected site ≠

Spot number	Products obtained with pancreatic RNase	Deduced structure*
B14-35a	C,G	C-G
-35b	A-A-A-G	A-A-A-G
-35c	C,A-C,G	C-A-C-G or A-C-C-G
-35d	U,C,C,G	U-C-C-G or C-C-U-G or C-U-C-G
-35e	G	G
B14-40a	A-A-A-G	A-A-A-G
-40b	U,C,C,G	U-C-C-G or C-C-U-G or C-U-C-G
-40c	C,A-C,G	C-A-C-G or A-C-C-G
-40d	A-G	A-G
-40e	U-G	U-G
B14-44a	A-A-C,G	A-A-C-G
-44b	C,C,G	C-C-G

* Where more than one possible structure is shown, those oligonucleotides found in RNase T_1 fingerprints of the IF3 protected site (Table 5) are underlined.

≠ This work was performed by Dr. B. Johnson.

which occurs at position 3432-3447. The possible sequence of partial digestion product B14-44 of A-A-C-G-C-C-G occurs at position 3344-3350, whereas the alternative permutation, C-C-G-A-A-C-G, does not occur in the MS2 RNA sequence. Additionally, this permutation would give the pancreatic RNase digestion product G-A-A-C, which does not occur in fingerprints of the protected site (Table 5).

4.2.5 Summary of sequence information for the IF3 protected site in MS2 RNA

The analysis by the 'wandering spot' sequencing technique of the RNA species protected by IF3 shows that they belong to three separate regions of the MS2 RNA molecule.

Region I (Fig. 40) corresponds to RNA species B60-1, B58-6 and B55-2 described above. B60-1 covers the region 3344-3360 of the complete MS2 RNA sequence at the 3'-terminus. The 5'-terminus of B55-2 could not be determined from the wandering spot, but the sequence could be read covering residues 3350-3360. The heterogeneity of the 3'-end of Region I could account for the observation that oligonucleotide T_1 from the RNase T_1 fingerprints of the protected site sometimes had a variable composition (B. Johnson pers. commun.).

Region I contains the partial RNase T_1 digestion product B14-44 (Table 7) covering the sequence 3344-3350. All the possible oligonucleotides that can be produced by pancreatic RNase and RNase T_1 digestion of Region I, with the exception of A-A-C, are present in the fingerprints of the protected site (Johnson and Szekely, 1977) (Fig. 40).

Region II corresponds to RNA species B60-2, B60-3, B58-2 and B58-3 and is shown in Fig. 41. All four RNA species start with a common 5'-terminus but there is some heterogeneity in the position of the 3'-terminus, both between different preparations of the protected site and also within the same preparation of the site. Two of the RNA

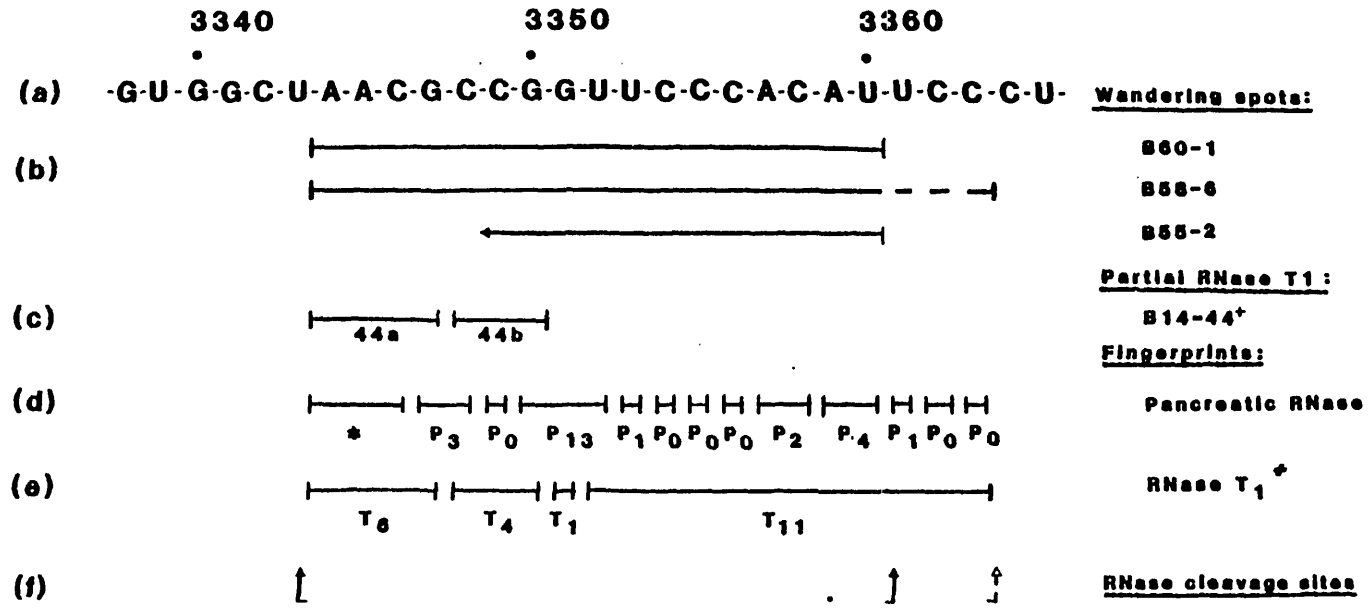


FIGURE 40 Summary of sequencing information for Region I.

(a) Nucleotide sequence of MS2 RNA (Iserentant et al., 1980).

(b) Wandering spot.

(c) Partial RNase T_1 digestion products. $^{+}$numbers refer to Table 7.

(d) and (e) Positions of digestion products found in pancreatic RNase and RNase T_1 fingerprint of IF3 protected site. $^{\neq}$numbers refer to Table 5. *oligonucleotide not observed in fingerprints.

(f) Position of pancreatic RNase cleavage points. ↑ certain, ⇧ probable.

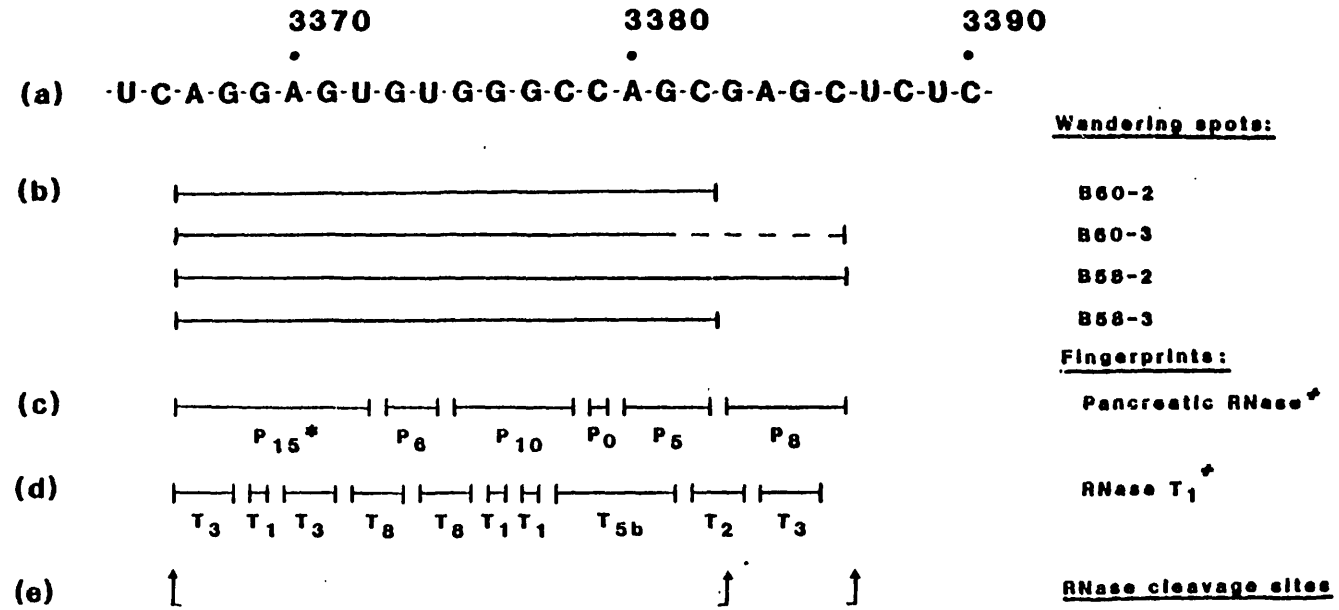


FIGURE 41 Summary of sequencing information for Region II.

(a) Nucleotide sequence of MS2 RNA (Iserentant et al., 1980).

(b) Wandering spots.

(c) and (d) Position of digestion products found in pancreatic RNase and RNase T_1 fingerprints of protected site. ≠ numbers refer to Table 5. * oligonucleotide unique in MS2 RNA sequence.

(e) Position of pancreatic RNase cleavage points.

species (B60-2, B58-3) cover the sequence 3367-3382, whereas B60-3 covers the sequence 3367 to possible 3386 and B58-2 the sequence 3367-3383. All of the possible products that can be produced by pancreatic RNase and RNase T_1 digestion of this region, including the unique pancreatic oligonucleotide A-G-G-A-G-U, can be accounted for in the fingerprints of the protected site (Fig. 41).

Region III corresponds to RNA species B60-4 and B58-1 and is shown in Fig. 42. Since the sequence of only the first 18 nucleotides of these RNAs could be determined from the wandering spots, the exact extent of this region is somewhat uncertain. However, as judged by its low mobility in the second dimension of the homochromatography fingerprints, it would be expected to be relatively large. The RNA species B60-4 and B58-1 both have a common 5'-terminus (residue 3427) and extend to residue 3444 and beyond. The partial T_1 digestion product B14-40 found by B. Johnson can be placed in the sequence covering part of this region from residues 3432-3447. Overlapping the 3'-portion of B14-40 is the partial RNase T_1 product B14-35, which covers the region 3444-3459. Since the protected fragment was prepared using pancreatic RNase, the region must extend up to, at the minimum, the next pyrimidine residue, after which the RNase could cut, i.e., residue 3460. The region 3427-3460 includes two pancreatic RNase digestion products which are found in the fingerprints of the protected site and which occur only once in the MS2 RNA sequence (P_{12}:G-A-A-A-G-A-G-C and P_9:G-A-A-A-G-C) (Fig. 42). All of the possible pancreatic RNase and RNase T_1 digestion products of this region can be found in the fingerprints of the protected site (Fig. 42). The exact 3'-terminus of this region cannot be ascertained from the sequence data presented in this work; however, it may extend a

FIGURE 42 Summary of sequence information for Region III.

(a) Nucleotide sequence of MS2 RNA (Iserentant et al., 1980).

(b) Wandering spots.

(c) Partial RNase T_1 digestion products. $^{+}$numbers refer to Table 7.

(d) and (e) Positions of digestion products found in pancreatic RNase and RNase T_1 fingerprints of protected site. $^{\not}$numbers refer to Table 5. *oligonucleotide unique in MS2 RNA.

(f) Position of pancreatic RNase cleavage points. ↑ certain, ⇧ probable.

further six residues to residue 3466 since the pancreatic RNase digestion product G-G-C (P_7) that is found in fingerprints of the protected site is absent from Regions I and II, which have been fully characterized but is present at position 3463-3466 (Table 8). The pancreatic RNase product G-G-U (P_{13}) which precedes P_7 in the MS2 RNA sequence also occurs in Region I.

With the exception of the RNase T_1, oligonucleotide T_{10} (U-C-U-C-G) and the two pancreatic RNase oligonucleotides P_{16} (G-A-A-A-G-G-U) and P_{12} (A-G-G-G-A-C), all of the digestion products found in the pancreatic RNase and RNase T_1 fingerprints of the IF3 protected site can be accounted for by Regions I, II and III (Table 8).

4.2.6 Base composition of protected regions I, II and III

The distribution of the different nucleotides present in the maximum extents of Regions I, II and III of MS2 RNA protected by IF3 (80 nucleotides in total) is given in Table 9. It is readily apparent from this table that the protected site is particularly rich in G residues (approximately 39% of the total residues), especially in Regions II and III, where the G content is 50% and 45% respectively.

The high cytidine content of Region I, together with its very close proximity to Region II in the primary sequence of MS2 RNA, would suggest that a high degree of base-pairing could occur between these two regions.

To determine whether the IF3 protected regions were located in an area of high G content, a histogram was plotted of the number of G residues present every 50 nucleotides in the sequence of Fiers et al. (1976) (Fig. 43a). The distribution of C residues is also shown (Fig. 43c) for comparison, since regions rich in both G and C residues would be expected to contain a large amount of secondary structure. From

TABLE 8

Occurrence of RNase T_1 and pancreatic RNase digestion products of IF3 protected site in Regions I, II and III

Digestion product*		Region I	Region II	Region III
		(No. of occurrences)		
T_1	G	1	3	7
T_2	C-G	-	1	2
T_3	A-G	-	3	1
T_4	C-C-G	1	-	-
T_{5a}	C-A-C-G	-	-	1
T_{5b}	C-C-A-G	-	1	-
T_6	A-A-C-G	1	-	-
T_7	A-A-A-G	-	-	2
T_8	U-G	-	2	3
T_{9a}	U-C-C-G	-	-	1
T_{9b}	C-U-C-G	-	-	1
T_{10}	U-C-U-C-G +	-	-	-
T_{11}	$(U_{3/4},C_{3/5}$,A-C,A-U)	1	-	-
P_O	C	6	1	3
P_1	U	2	-	1
P_2	A-C	1	-	1
P_3	G-C	1	-	2
P_4	A-U	1	-	-
P_5	A-G-C	-	1	-
P_6	G-U	-	1	1
P_7	G-G-C	-	-	1
P_8	(A-G,G)C	-	1	-
P_9	$(G,A\text{-}A\text{-}A\text{-}G)C^{\neq}$	-	-	1
P_{10}	G-G-G-C	-	1	-
P_{11}	$(G,G,A\text{-}G)C^{\neq}$ +	-	-	-
P_{12}	$(G,AG,A\text{-}A\text{-}A\text{-}G)C^{\neq}$	-	-	1
P_{13}	G-G-U	1	-	1
P_{14}	G-G-G-U	-	-	2
P_{15}	$(G,A\text{-}G,A\text{-}G)U^{\neq}$	-	1	-
P_{16}	$(G,G,A\text{-}A\text{-}A\text{-}G)^{\neq}$ +	-	-	-

* Prefix T = T_1, P = pancreatic RNase; $\neq$ sequences unique to MS2 RNA; + sequence not found in Regions I, II or III.

TABLE 9

Distribution of residues in the maximum extents of Protected Regions I, II and III

	No. of occurrences (%)			
	A	U	C	G
Region I (n = 20)	4 (20)	4 (20)	9 (45)	3 (15)
Region II (n = 20)	4 (20)	2 (10)	4 (20)	10 (50)
Region III (n = 40)	8 (20)	5 (12.5)	9 (22.5)	18 (45)
Total (I+II+III) (n = 80)	16 (20)	11 (13.75)	22 (27.5)	31 (38.75)

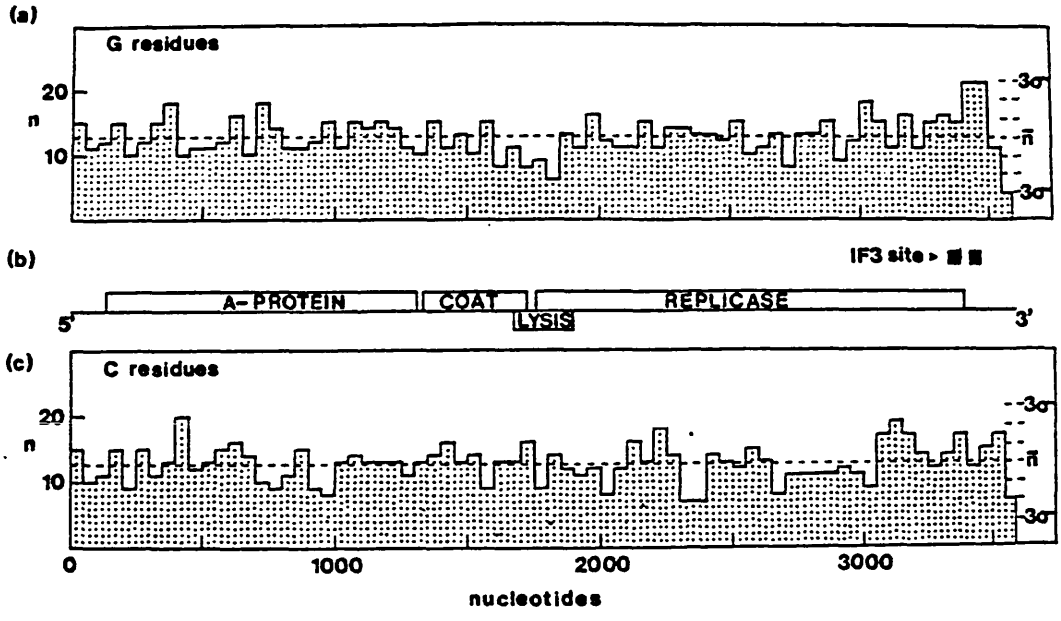


FIGURE 43 Distribution of G and C residues in MS2 RNA.
The number (n) of G and C residues present in 50 nucleotide segments of the sequence of MS2 RNA determined by Fiers et al. (1976) were plotted as histograms. (a) Distribution of G residues, $\bar{n}$ = 12.80, σ = 2.84. (b) Genetic map of MS2 RNA based on Fiers et al. (1976) and Beremand and Blumenthal (1979). (c) Distribution of C residues, $\bar{n}$ = 12.84, σ = 2.74.

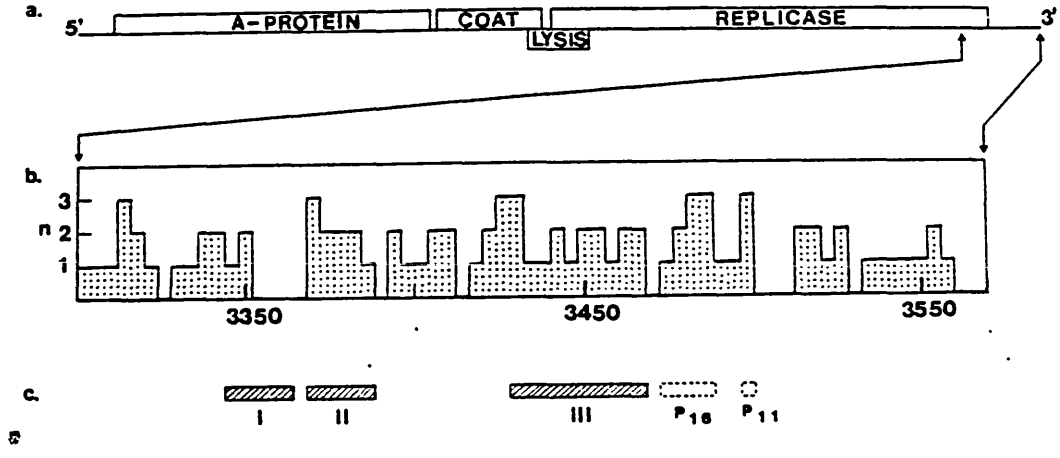


FIGURE 44 Distribution of G residues in the 3'-terminal portion of MS2 RNA

The number (n) of G residues present every 4 nucleotides in the 3'-terminal portion of MS2 RNA (residues 3300 - 3569) were plotted as a histogram. (a) Genetic map of MS2 RNA based on Fiers et al. (1976) and Beremand and Blumenthal (1979). (b) Distribution of G residues, $\bar{n} = 1.31, \sigma = 0.92$. (c) Location of IF3 protected Regions I, II and III, and pancreatic RNase oligonucleotides P_{16} and P_{11}.

Fig. 43a, it is clear that the 3'-terminal portion of MS2 RNA, where the IF3 protected site is partly located, is the most G rich part of the whole sequence, being some 3 standard deviations above the mean at 50 nucleotides resolution. This portion of the RNA is also, to some extent, rich in C residues (Fig. 43c).

However, the binding of IF3 to G-rich regions in MS2 RNA does not totally account for the location of the protected site since Region I of the protected site contains only 15% G residues and there are also nucleotide sequences near to the protected regions that are G-rich, yet are not protected by IF3 (Fig. 44).

4.2.7 Search for sequence homology between Regions I, II and III

To determine whether there was any sequence homology between the three protected regions, the RNA sequences were compared and aligned such that there was maximum common homology between the the three regions, with Region III being divided into a 5'- and 3'-portion (Fig. 45). There appears to be little homology between Region I and Regions II and III. In contrast, however, homology is seen between the 5'- and 3'-portions of Region III and this homology is to a large extent conserved in Region II. Additionally, the sequence G-A-A-A-G occurs twice in Region II and also occurs in oligonucleotide P_{16} found in fingerprints of the IF3 protected site (Johnson and Szekely, 1977).

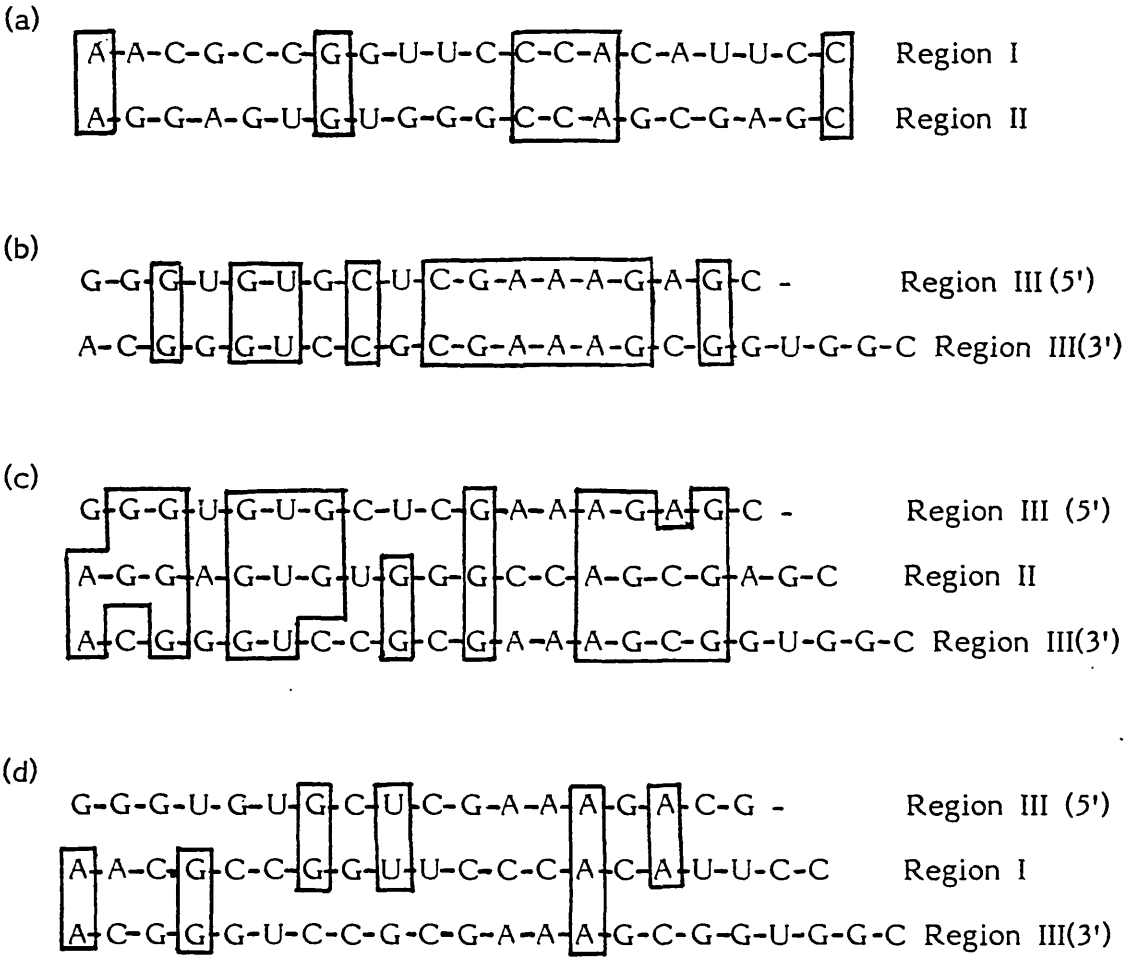


Fig. 45 Sequence homology between Regions I, II and III of the IF3 protected site in MS2 RNA.

Nucleotide sequences of Regions I, II and III were arranged so as to give maximum homology. Homologous nucleotides are shown boxed. (a) Region I compared with Region II; (b) Region III 5'-portion compared with the 3'-portion of the same region; (c) Region II compared with the 5'- and 3'-portions of Region III; (d) Region I compared with the 5'- and 3'-portions of Region III.

4.3 STUDIES ON THE SECONDARY AND TERTIARY INTERACTIONS OF MS2 RNA AT THE IF3 PROTECTED SITE

Since the regions of MS2 RNA protected by IF3 against pancreatic RNase digestion were found to migrate as a single band upon gel electrophoresis under non-denaturing conditions, and yet were separated into three regions of varying length in the strongly denaturing conditions of the homochromatography fractionation, it was clear that some secondary, and possibly tertiary, interactions were involved between the RNA species of the protected site. Studies were therefore undertaken to investigate whether modification of the secondary and tertiary structure of MS2 RNA influenced the binding and protection of the RNA by IF3. The IF3 protected site was also studied directly for evidence of secondary structure using two-dimensional gel electrophoresis under non-denaturing and denaturing conditions (Ross and Brimacombe, 1979).

4.3.1 Studies using mildly heat denatured MS2 RNA

Anderton and Szekely (pers. commun.) studied the effect of treatment of MS2 RNA with formaldehyde (Lodish, 1970) prior to binding to IF3 upon the protection afforded by the factor against pancreatic RNase digestion. Formaldehyde reacts with nucleotides to give methylol ($-CH_2OH$) adducts with the $-NH_2$ and =NH of nucleotide bases as the major products and thus prevents base pairing (McGhee and von Hippel, 1975a, 1975b). These adducts are unstable and most of the formaldehyde molecules can be expected to dissociate from the adduct when the RNA is purified by ethanol precipitation (McGhee and von Hippel, 1975a,b). An alternative reaction product could be the stable $-NH--CH_2-NH-$ cross-links (Lodish, 1975). Under the conditions described by Lodish (1970), modification results in the addition of only 10

formaldehyde molecules per f2 RNA molecule (Lodish, 1975) and denaturation is mild with the formaldehyde perhaps preventing tertiary interactions (van Dieijen et al., 1976).

Accordingly, mild formaldehyde denaturation, resulting in about 15% denaturation, as estimated by circular dichroism, was found to give very little difference in the amount of RNA protected against pancreatic RNase digestion relative to native RNA. Pancreatic RNase fingerprint analysis of the protected site, which migrated in a gel with a mobility similar to that of the native protected site, showed that the partially denatured protected site contained the same oligonucleotides to those found in the native protected site, together with a few additional spots which were not further characterised. Two-dimensional homochromatography fractionation revealed similar patterns for the native and partially denatured protected sites. . From these results it was concluded that partial denaturation had little effect on the structures present in the MS2 RNA at the IF3 protected site.

In view of these results, an alternative method of denaturation was chosen for further study of the binding and protection of partially denatured MS2 RNA by IF3. Heat has long been known to be capable of disrupting the pairing and stacking of bases of the secondary structure of RNA and DNA and the hyperchomicity, or increase in A_{260} upon heating a sample of DNA or RNA, can be used as a measure of the amount of secondary structure present in these molecules (e.g., Felsenfeld, 1971). The temperature at which the disruption of secondary structure occurs is termed the transition temperature or T_m. As a result of imperfect base pairing, a sharp transition like that found for DNA is not seen with RNA and a more gradual transition occurs with those regions held together more tightly by more hydrogen bonds

requiring a higher temperature for dissociation than those regions held together by fewer hydrogen bonds. Thus, if RNA is heated and then rapidly cooled so as to prevent the dissociated regions from re-forming their respective base pairs, different regions of the molecule can be denatured depending upon the extent of hydrogen bonding and the temperature to which the RNA was heated.

The effect of mild heat denaturation of MS2 RNA on the binding of IF3 and protection against pancreatic RNase digestion has therefore been investigated. Initial experiments used denaturation by heating for 3 minutes at 85°C in the presence of 10mM Tris buffer followed by rapid cooling. Binding of IF3 and washing of the IF3.MS2 RNA complexes were both at 0°C. This is in contrast to the binding and washing at 37°C and 4°C respectively used by others when binding to native and formaldehyde denatured MS2 RNA (Johnson and Szekely, 1977, 1979; T. Anderton, pers. commun.) and was chosen since at this temperature the probability of any renaturation of the RNA occurring would be reduced. The extent of denaturation caused by this process was assessed by comparison of the thermal denaturation curves of the native and heat denatured MS2 RNA (Fig. 46) and estimated to be in the order of 18%. The T_m of the both native and partially denatured RNA in the absence of Mg^{2+} ions, estimated from the mid-point of the transition, was approximately 60°C and compares well with the value of 54°C obtained by van Dieijen et al. (1976) which was determined under slightly different salt concentrations. Since, in the denaturation process, the temperature to which the RNA preparation had been heated was greater than the T_m, it would be expected that considerably more denaturation than the 18% determined would be found. However, temperature equilibration during the heating was probably sub-optimal as

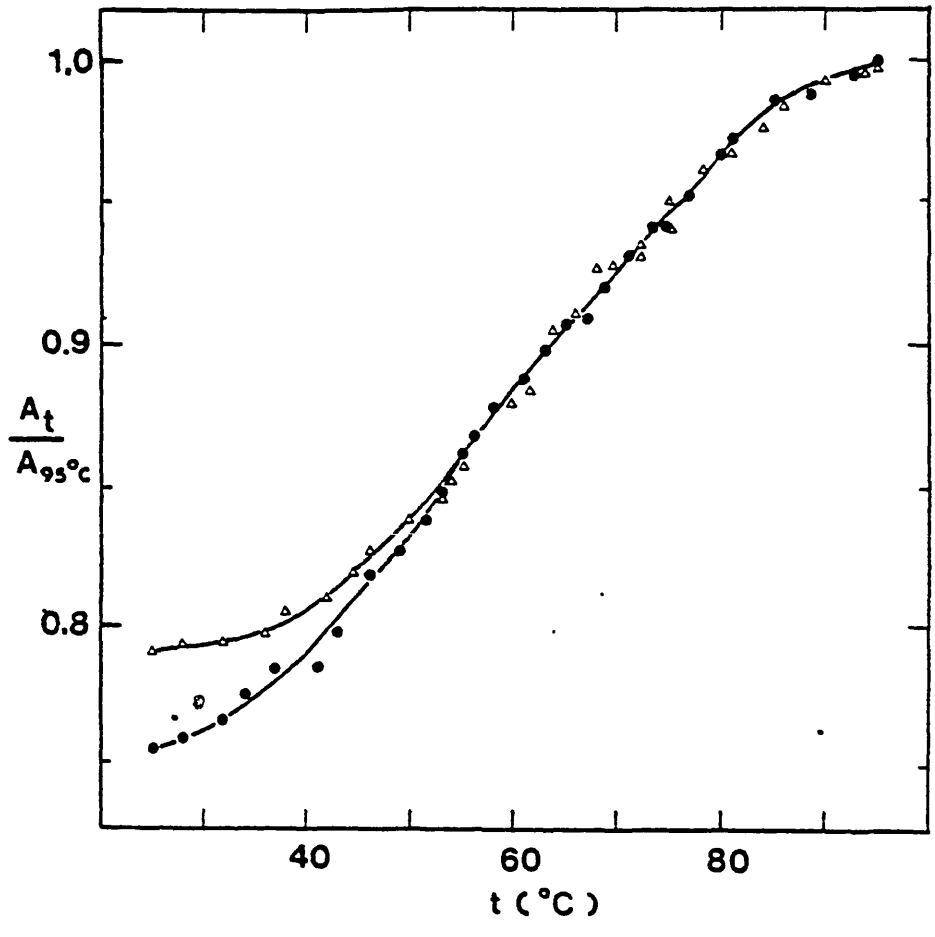


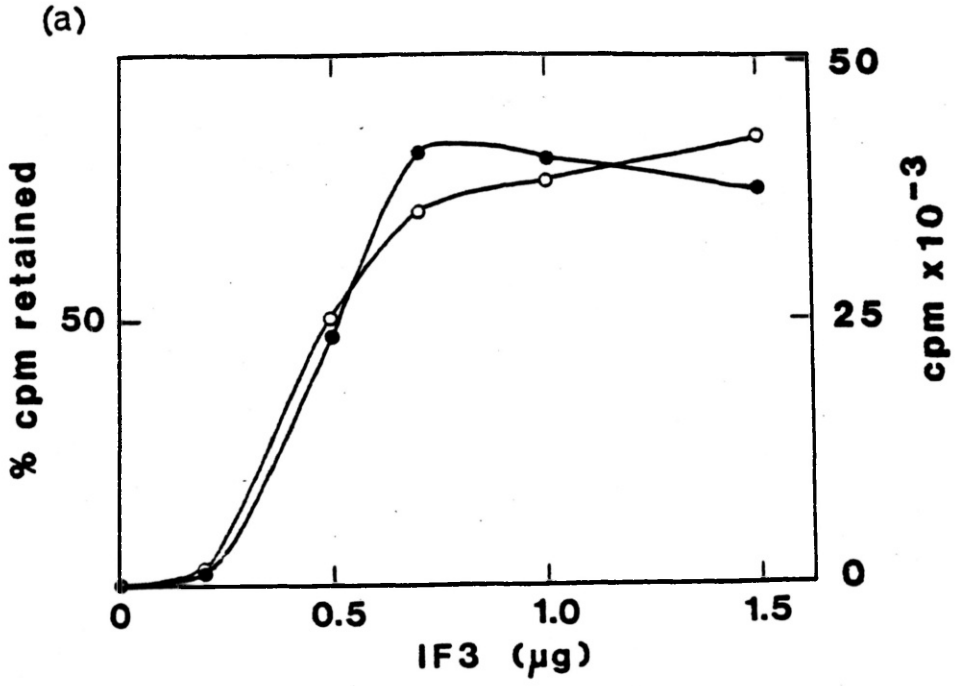
FIGURE 46 Thermal denaturation curves of native and partially heat denatured MS2 RNA.

MS2 RNA (32 µg) was partially denatured in 120µl of 10mM Tris HCl pH 8.0 as described in methods in section 3.9.1. Tm curves were determined as described in methods in section 3.9.2. Native MS2 RNA (●), partially denatured MS2 RNA (△).

was the rapidity of cooling, possibly allowing the more strongly base paired regions to re-form between heating and cooling.

The binding curves of IF3 to native and partially denatured MS2 RNA were determined, together with PAGE of the RNA preparations used. This latter determination was undertaken so as to exclude the possibility of any changes in the binding curves being due to fragmentation of the RNA during the denaturation process. The binding of IF3 to native MS2 RNA at 0°C (Fig. 47a) was similar to that described previously at 37°C with a 4°C wash (section 4.1.2, Fig. 19). The binding was saturable with maximum retention of MS2 RNA (approximately 80% of the total cpm) occurring at an input of 0.7μg of IF3. A slight reduction in the binding of MS2 RNA was seen at high concentrations of IF3. When heat denatured MS2 RNA was used, a curve similar to that obtained with native RNA was obtained, with the exception that at IF3 concentrations above the saturating maximum a slight increase in the retention of RNA was seen rather than the slight reduction in retention seen with native RNA (Fig. 47a). PAGE of the RNA preparations used in the binding curves shows that the heat denatured, as well as native, MS2 RNAs were, in the main, intact (Fig. 47b). This result would suggest that denaturation under these conditions had little effect on the binding of IF3 to the RNA and that, presumably, the conformation of the binding site was unaffected by the mild denaturation process.

Mg^{2+} ions have been shown to play a role in stabilising the secondary and tertiary structures of MS2 RNA (Jacobson, 1976), as well as those of other RNAs such as tobacco mosaic virus RNA (Boedtker, 1960) and the three-dimensional folding of tRNA (Quigley et al., 1978). Fukami and Imahori (1971), studying the control of protein synthesis by



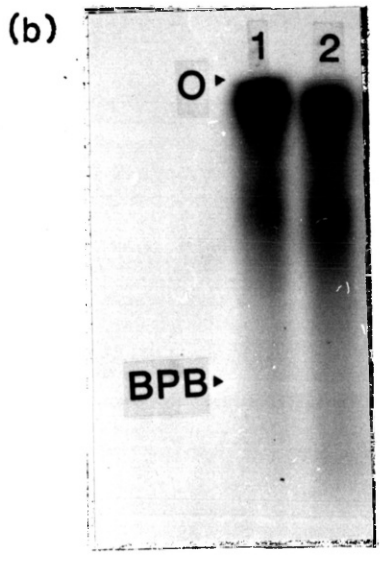


FIGURE 47 Binding of IF3 to native and partially denatured (^{32}P)-MS2 RNA.

(a) Binding curves. Varying amounts of IF3 were incubated with (^{32}P)-MS2 RNA (0.2µg, 50185 cpm), either native or partially heat denatured in 10mM Tris HCl pH 8.0 (methods section 3.9.1), as described in section 3.6 and filtered over nitrocellulose. A blank value (without IF3, 408 cpm) was subtracted. native (●), partially denatured (O) MS2 RNA. (b) Autoradiograph of 5% PAGE (methods 3.1.2) of native and partially denatured MS2 RNA used in part (a). Track 1 : native; track 2 : partially denatured.

the conformation of R17 RNA, found that the presence of Mg^{2+} ions was essential to attain the partially thermally denatured structures. Thus, the lack of a significant effect of denaturation upon the binding of IF3 may be due to a need for the presence of Mg^{2+} ions to stabilise a partially denatured conformation (Strauss and Sinsheimer, 1968). On the other hand, the presence of Mg^{2+} ions may also stabilise the IF3 binding site against heat denaturation.

Accordingly, MS2 RNA was partially thermally denatured in the presence of various concentrations of $Mg(OAc)_2$ and assayed for the binding of IF3 at 0°C and also for the degree of any fragmentation caused by the denaturation process by PAGE. The binding of IF3 to the variously denatured RNAs is shown in Table 10, where it can be seen that IF3 bound well to all of the RNAs. The binding of IF3 to MS2 RNA denatured in the absence of Mg^{2+} was similar to that observed previously (Fig. 47a), although the binding at 0.5µg IF3 was somewhat higher and lack of data points cannot rule out the possibility that this was merely an anomalous point. Denaturation in the presence of Mg^{2+} caused little difference in the binding of IF3 relative to native MS2 RNA although, at low IF3 concentration, denaturation in the presence of increasing Mg^{2+} concentration increased the retention of RNA on the filter. PAGE of the RNAs used in the binding assays (Fig. 48) showed that increasing the Mg^{2+} concentration in the denaturation mixes caused an increase in the degree of fragmentation of the RNA. Under similar conditions (10mM Mg^{2+}, pH 8.0), Dahlberg and Peacock (1971a, b) found that heating 16S and 23S rRNA at 70°C resulted in a similar fragmentation of these RNAs. The cause of such fragmentation is uncertain, but may be the result of stabilisation of a partially denatured structure in which 'hidden breaks' in the RNA (Gould, 1967)

TABLE 10

IF3 BINDING TO MS2 RNA PARTIALLY DENATURED IN THE PRESENCE OF Mg^{2+}

		MS2 RNA cpm retained on filter (%)		
Mg^{2+}*	IF3(µg):	0.2	0.5	1.5
Native:	-	361(2.0)	8442(47.2)	1322(73.9)
Denatured:				
	0	527(2.9)	11347(63.5)	13319(74.5)
	2 mM	1427(7.99)	9023(50.5)	11937(66.7)
	5 mM	2086(11.7)	7119(39.8)	12053(67.4)
	10mM	3471(19.4)	8444(47.2)	13100(73.3)

* Mg^{2+} concentration in denaturing buffer. Final Mg^{2+} concentration in the binding mix was always 1 mM. Varying amounts of IF3 were incubated with (^{32}P) MS2 RNA (0.2µg, 18773 cpm) which had been heat denatured as described in section 3.9.1 and filtered over nitrocellulose filters (section 3.6.1) followed by counting in a liquid scintillation counter. A blank value (without IF3, 890cpm) was subtracted.

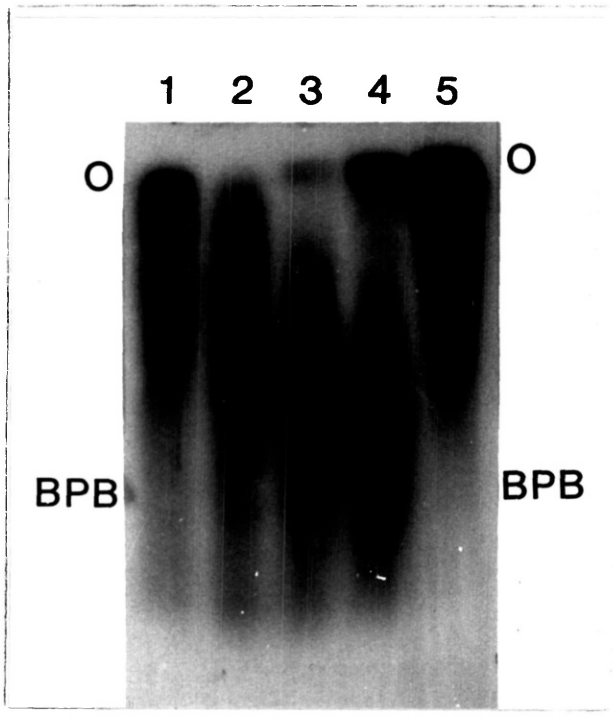


FIGURE 48 PAGE of (^{32}P)-MS2 RNA partially heat denatured in the presence of various concentrations of $Mg(OAc)_2$

(^{32}P)-MS2 RNA (2μg) was partially heat denatured in 10μl of 10mM Tris HCl pH 8.0 containing various concentrations of $Mg(OAc)_2$ as indicated, followed by electrophoresis through a 5% polyacrylamide gel and autoradiography. BPB marks the position of the bromophenol blue marker. Track 1 : MS2 RNA denatured in the absence of $Mg(OAc)_2$; Track 2 : denatured in 2mM $Mg(OAc)_2$; Track 3 : denatured in 5mM $Mg(OAc)_2$; Track 4 : denatured in 10mM $Mg(OAc)_2$; Track 5 : native MS2 RNA.

become apparent. Alternatively, the presence of Mg^{2+} ions in the denaturation mixes may be promoting the hydrolysis of the RNA at the mildly alkaline pH.

Since the sequencing results show that IF3 protects a single site near the 3'-end of the RNA, it is remarkable that whilst partial denaturation treatment in the presence of 10mM Mg^{2+} resulted in extensive fragmentation, some 72% of the total RNA was retained on the filter by 1.5µg of IF3. Lack of data points in Table 10 preclude the determination of the shape of the binding curve. However, this finding of extensive binding of the factor to fragmented denatured RNA suggests the possibilities that either native MS2 RNA contain additional binding sites for IF3, but which are not protected against pancreatic RNase and, therefore, do not appear in fingerprints of the protected site, or that partial denaturation treatment produces additional sites on the RNA to which IF3 can bind. These possibilities are further investigated in a later section of this thesis (section 4.3.2).

To further investigate the effect of partial heat denaturation treatment upon IF3 binding, the conditions which gave rise to the minimal amount of fragmentation in Fig. 48 were used; i.e., denaturation in the presence of 2mM $Mg(OAc)_2$. Optical melting curves determined in the presence of 2mM Mg^{2+} of native and denatured MS2 RNA are shown in Fig. 49. The effect of having Mg^{2+} in the buffer was to increase the T_m of the native RNA by some 12 degrees to 72°C, in agreement with the stabilising effect of Mg^{2+} on secondary structure and also with the observation of van Dieijen et al. (1976). MS2 RNA denatured in the presence of 2mM $Mg(OAc)_2$ was approximately 21% denatured and had a T_m of 68°C.

The binding of IF3 to MS2 RNA denatured in this way and also

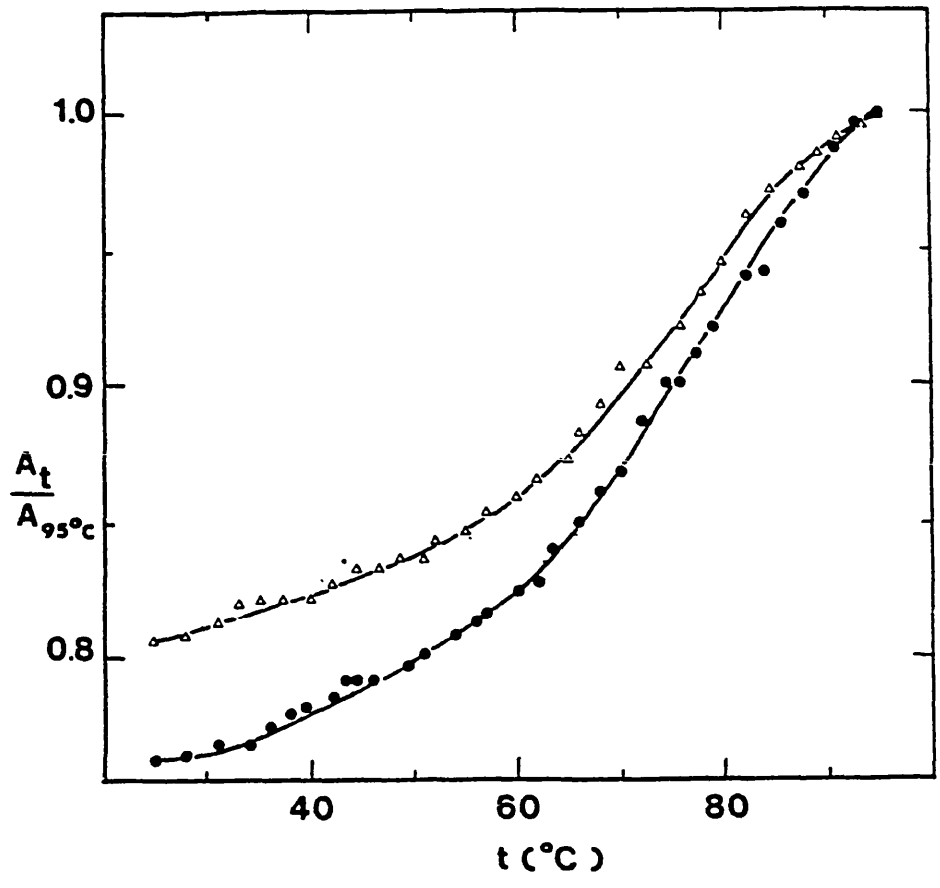


FIGURE 49 Thermal denaturation curves of native and partially heat denatured MS2 RNA.

MS2 RNA (32µg) was partially heat denatured in 120µl of 10mM Tris HCl pH 8.0, 2mM $Mg(OAc)_2$ as described in methods section 3.9.1. Tm curves were determined as described in section 3.9.2. Native MS2 RNA (●), partially denatured MS2 RNA (△).

to native MS2 RNA is shown in Fig. 50, together with PAGE analysis of the RNA preparation as a check of fragmentation incurred during denaturation. The greater amount of RNA being retained on the filters by IF3 relative to that shown in Fig. 47 is due to a more intact preparation of RNA being used (cf Fig. 50b and Fig. 47). It can be seen that the binding curve for the denatured RNA was shifted to the right relative to the native RNA curve and that the maximum amount of RNA bound was reduced, i.e., higher concentrations of IF3 were required to retain the same number of cpm of RNA on the filter than for native RNA. This would suggest that denaturation under these conditions decreases the affinity of the IF3 and MS2 RNA for each other. PAGE analysis shows that the denatured RNA used in the binding curve is, in the main, intact and therefore the change in binding characteristics was probably not due to fragmentation (Fig. 50b).

To investigate whether partial heat denaturation treatment under these conditions affected the protection of MS2 RNA by IF3 against pancreatic RNase digestion, IF3 was bound to native and partially denatured RNA at 0°C and digested with RNase at the same temperature with aliquots being assayed at various times to determine the amount of RNA retained on nitrocellulose filters. Fig. 51 shows that the partially denatured RNA was digested at a greater rate than the native RNA as would be expected, since the denaturation process would produce additional single-stranded pyrimidine residues which would be available for digestion by the pancreatic RNase. After 4 hours digestion at 0°C, 30 - 35% of the RNAs were still retained on the filters by IF3. Johnson and Szekely (1977), using digestion at 37°C with an RNase : RNA ratio of 1 : 100 (w/w), found that only 0.5 - 1.0% of the MS2 RNA was retained on the filters by IF3 after 3 hours digestion. To test whether under the conditions used in Fig. 51 the RNase

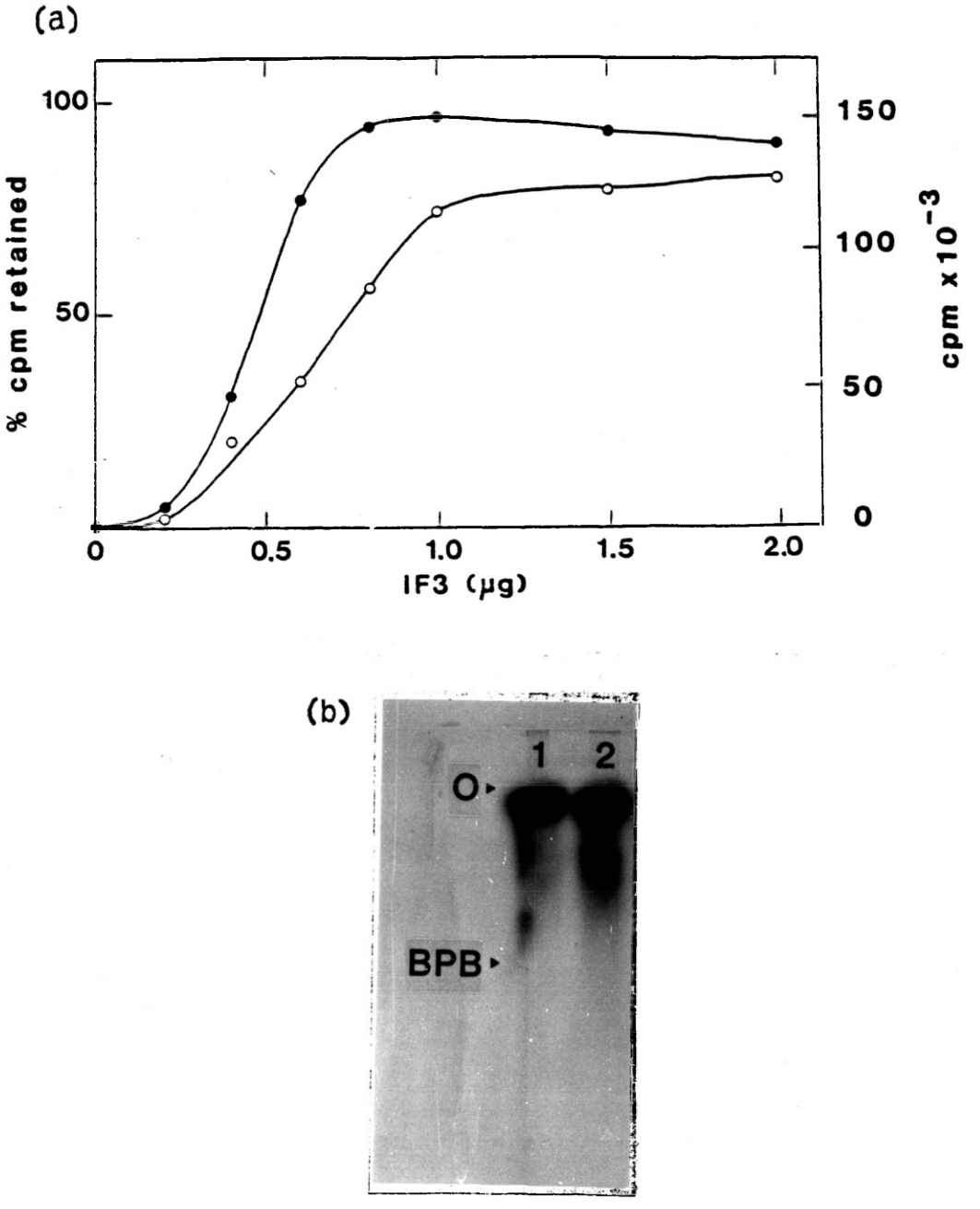


FIGURE 50 Binding of IF3 to native and partially denatured (^{32}P)-MS2 RNA.

(a) Binding curves. Varying amounts of IF3 were incubated with (^{32}P)-MS2 RNA (0.2µg, 144044 cpm) either native or heat denatured in 10mM Tris HCl pH 8.0, 2mM $Mg(OAc)_2$. A blank without IF3 (979 cpm) was subtracted. Native (●), partially denatured (O) MS2 RNA.
(b) Autoradiograph of 5% PAGE of native (1) and partially denatured (2) MS2 RNA used in part (a).

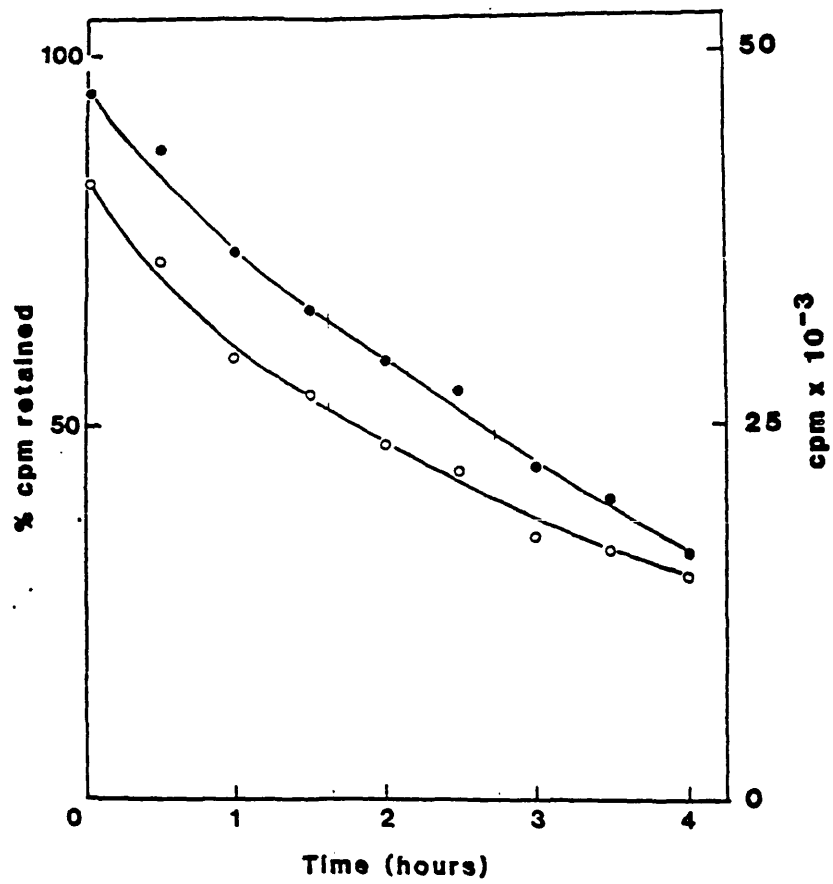


FIGURE 51 Protection of native and partially denatured MS2 RNA by IF3 at 0°C.

IF3 (17µg) was bound to (^{32}P)-MS2 RNA either native or partially denatured in the presence of 2mM $Mg(OAc)_2$ in 400µl of binding buffer at 0°C. Pancreatic RNase (0.2µg) was added and aliquots (40µl, 51 000 cpm total) were filtered over nitrocellulose at various times and the filters counted in a liquid scintillation counter. Native (●), partially denatured (O) MS2 RNA.

digestion had gone to completion, and that the high cpm retained on the filter was due to protection of larger amounts of RNA by IF3, native MS2 RNA was digested in the absence of IF3 at two different RNase : RNA ratios at 0°C and at one ratio at 30°C, with the extent of digestion being monitored by TCA precipitation (Fig. 52). It was found that under the conditions used for the protection experiment described previously, 54% of the MS2 RNA remained TCA precipitable after three hours digestion. Increasing the RNase : RNA ratio to 1 : 10 increased the rate of digestion, but some 36% of the total MS2 RNA remained precipitable after three hours digestion. Digestion at 30°C resulted in some 7% of the MS2 RNA remaining precipitable after three hours digestion.

Since it was clear that digestion at 0°C with an increased concentration of RNase would still be very slow, the protection experiment was repeated, using binding of IF3 to native and heat denatured MS2 RNA at 0°C, followed by digestion at 30°C with an RNase : RNA ratio of 1 : 40 (w/w). The protection curves obtained from this experiment are shown in Fig. 53. After 3 hours digestion, some 5% of the RNA was retained on the filters, in the case of both native and partially denatured MS2 RNA and, although digestion did not appear to have gone to completion, it is clear that there was no significant difference in the protection afforded by IF3 to native and partially denatured MS2 RNA. It is, of course, possible that during the incubation for 3 hours at 30°C, some renaturation of the secondary structure in the region of the binding site may have occurred.

4.3.2 Studies using S_1 nuclease fragmented MS2 RNA

In view of the observation that IF3 bound to highly fragmented partially denatured MS2 RNA (Table 10 above), the effect of fragment-

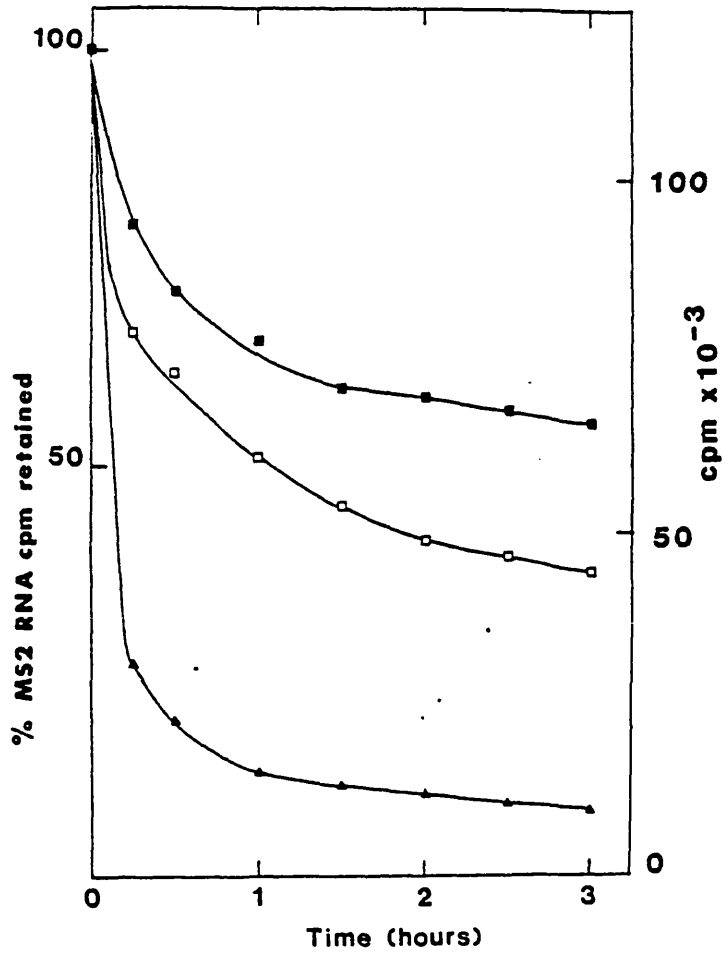


FIGURE 52 Digestion of (^{32}P)-MS2 RNA in the absence of IF3 at 0°C and 30°C.

(^{32}P)-MS2 RNA (4.2µg, 1229770 cpm) was digested with the indicated ratio of pancreatic RNase in 800µl of binding buffer at either 0°C or 30°C. Aliquots (80µl) were removed at various times and RNA precipitated with TCA as described in methods section 3.1.6, followed by counting in a liquid scintilliaton counter. Digestion at 0°C: (■) 1 : 50 RNase : RNA; (□) 1 : 10 RNase : RNA. Digestion at 30°C : (▲) 1 : 50 RNase : RNA (w/w).

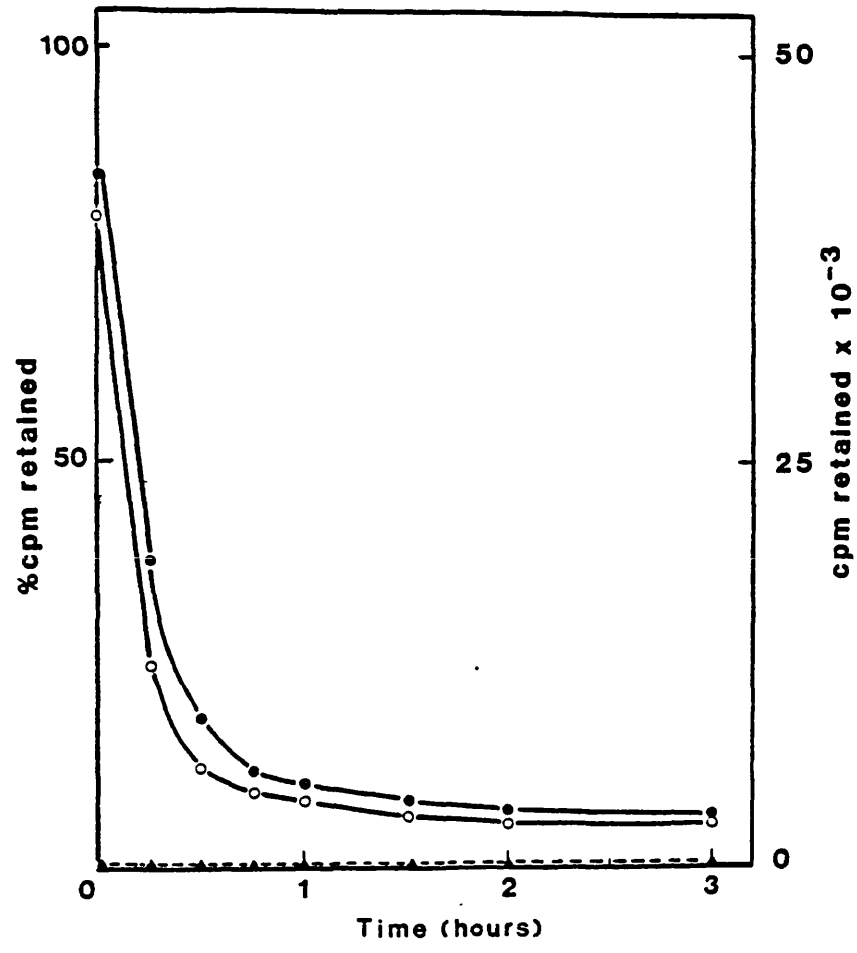


FIGURE 53 Protection of (^{32}P)-MS2 RNA by IF3: binding at 0°C and digestion at 30°C.

IF3 (17µg) was bound to (^{32}P)-MS2 RNA (2µg), either native or partially denatured in the presence of 2mM $Mg(OAc)_2$, in 400µl of binding buffer at 0°C. Pancreatic RNase (0.05µg) was added and the mixtures incubated at 30°C. Aliquots containing 0.2µg RNA (150 000 cpm) were filtered over nitrocellulose at various times and followed by liquid scintillation counting. A parallel mixture lacking IF3 was used as a blank. Native (●), partially denatured (○) MS2 RNA, (▲) blank.

ation of MS2 RNA with and without partial denaturation was investigated. Nuclease S_1 (EC 3.1.30.1) from Aspergillus oryzae hydrolyses single-stranded nucleic acids (Ando, 1966) or single-stranded regions within nucleic acids (Rushizky et al., 1975). At suitable ionic strength and temperature, it cleaves tRNA specifically at the anticodon loop and the 3'-end (Harada and Dahlberg, 1975). This enzyme can therefore be used to specifically fragment MS2 RNA by hydrolysing at the single-stranded regions present in the secondary structure (Rushizky et al., 1975). (^{32}P)MS2 RNA was digested with various amounts of nuclease S_1 and the extent of digestion determined by PAGE (Fig. 54a). As expected from the work of Rushizky et al. (1975), increasing the amount of nuclease S_1 decreases the size of the digestion products. The digestion conditions of 0.05U/μg RNA was chosen for a preparative digestion for use in binding studies, since this ratio resulted in a reasonable size range of digestion products. If the digestion products were too small, the binding of IF3 to the specific site near the 3'-terminus of the RNA would be barely detectable using the filtration binding assay. A preparative digest of (^{32}P)MS2 RNA was made for use in the following experiments and an aliquot assayed by PAGE (Fig. 54b).

The binding of IF3 to S_1 nuclease fragmented MS2 RNA (S_1F-MS2 RNA) at 0°C is shown in Table 11, together with the binding to native MS2 RNA. It can be seen that, whereas native MS2 RNA was bound well by IF3, S_1F-MS2 RNA was bound only to a very small extent. This may be due either to the S_1 nuclease cutting around the binding site to such an extent that the amount of RNA retained by IF3 on the filter was very small compared with the total RNA present, or that the 3'-terminal binding site contains some single-stranded regions essential for IF3 binding and that S_1 nuclease digestion has therefore destroyed the site. These two possibilities could have been tested by PAGE of the RNA that may have been bound to IF3, although this was not attempted.

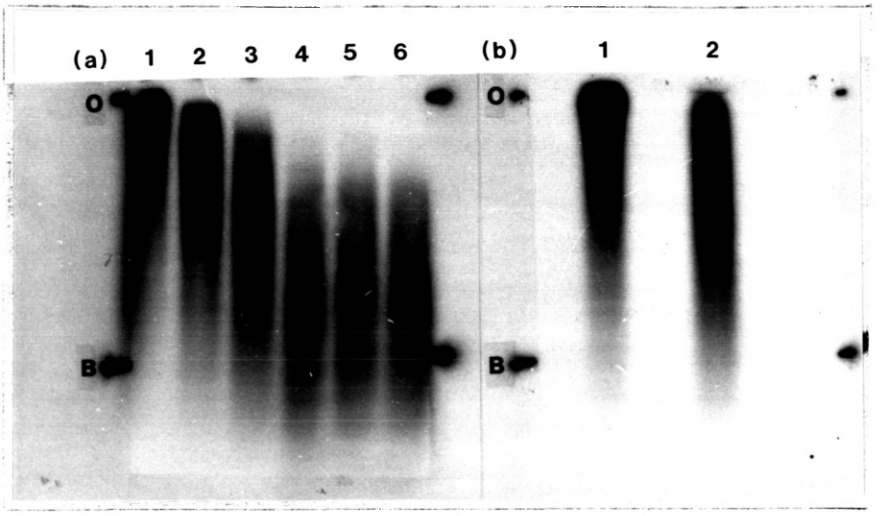


FIGURE 54 Digestion of (^{32}P)-MS2 RNA with S_1 nuclease.

(a) (^{32}P)-MS2 RNA (2.5µg) was digested with various amounts of S_1 nuclease as described in methods section 3.10 and an aliquot (0.5µg) analysed by 5% PAGE. Track 1, native MS2 RNA, Track 2, 0.04U/µg S_1; Track 3, 0.08U/µg; Track 4, 0.16U/µg; Track 5, 0.25U/µg; Track 6, 0.33U/µg.

(b) Autoradiograph of preparative S_1 nuclease digest of (^{32}P)-MS2 RNA. 22.5µg of MS2 RNA was digested with 2.25 units of S_1 nuclease and an aliquot (0.5µg) fractionated on 5% PAGE. O and B show the positions of the origin and BPB marker respectively. Track 1 : native, Track 2 : digested.

TABLE 11

BINDING OF IF3 TO PARTIALLY DENATURED AND NON-DENATURED S_1 NUCLEASE FRAGMENTED MS2 RNA

(a) Non-denatured

	cpm bound (%)	
IF3 (μg)	Intact MS2 RNA	S1-F MS2 RNA
0	194 (0.2)	856 (1.4)
0.6	80899 (83.5)	1469 (2.4)
1.0	71697 (74.0)	1897 (3.1)
1.5	72179 (74.5)	2142 (3.5)

(b) Partially denatured

	cpm bound (%)	
IF3 (μg)	Intact MS2 RNA	S1-F MS2 RNA
0	290 (0.3)	734 (1.2)
0.6	48442 (50.0)	2693 (4.4)
1.0	60456 (62.4)	20809 (34.0)
1.5	-	28766 (47.0)

Various amounts of IF3 were bound to denatured or undenatured intact MS2 RNA (0.15μg, 96 885 cpm) or S1-F MS2 RNA (0.15μg), 61205 cpm) at 0°C and followed by filtration over nitrocellulose filters and scintillation counting.

The binding of IF3 to partially heat denatured S_1F-MS2 RNA was also investigated. Table 11b shows that IF3 at high concentration bound partially heat denatured S_1F-MS2 RNA to a far greater extent than that found for undenatured S_1F-MS2 RNA (cf Table 11a). This observation, together with those noted previously that IF3 bound to partially denatured MS2 RNA to a greater extent than was expected from the amount of fragmentation, can be explained by proposing that upon heat denaturation, additional sites are produced on the MS2 RNA molecule to which IF3 can bind. That this binding is weak is shown by the observation that the protection of partially denatured MS2 RNA by IF3 against RNase digestion is not significantly different than for native MS2 RNA and also that high concentrations of the factor were required to retain the RNA in the nitrocellulose filtration assay.

4.3.3 Studies on the effect of ribosomal protein S1

E. coli ribosomal protein S1 has been shown to bind to and disrupt the secondary structure of synthetic and natural single-stranded polynucleotides, including MS2 RNA (Szer et al., 1976, 1977; Bear et al., 1976; Thomas et al., 1978, 1979). The effect of S1 protein upon the protection of MS2 RNA by IF3 was therefore investigated to determine whether there was any effect of the protein through its disruption of the MS2 RNA secondary structure on the binding of IF3.

Ribosomal protein S1, isolated from E. coli using poly-A cellulose column chromatography, as described by Carmichael (1975), was the generous gift of Dr. R. Foreman, who also provided some technical assistance in the following experiments. The preparation was approximately 95% homogeneous as judged by SDS-PAGE. S1 protein was bound to (^{32}P)MS2 RNA in conditions similar to those used for IF3 binding (Fig. 55). Maximum retention, 55% of the total MS2 RNA cpm,

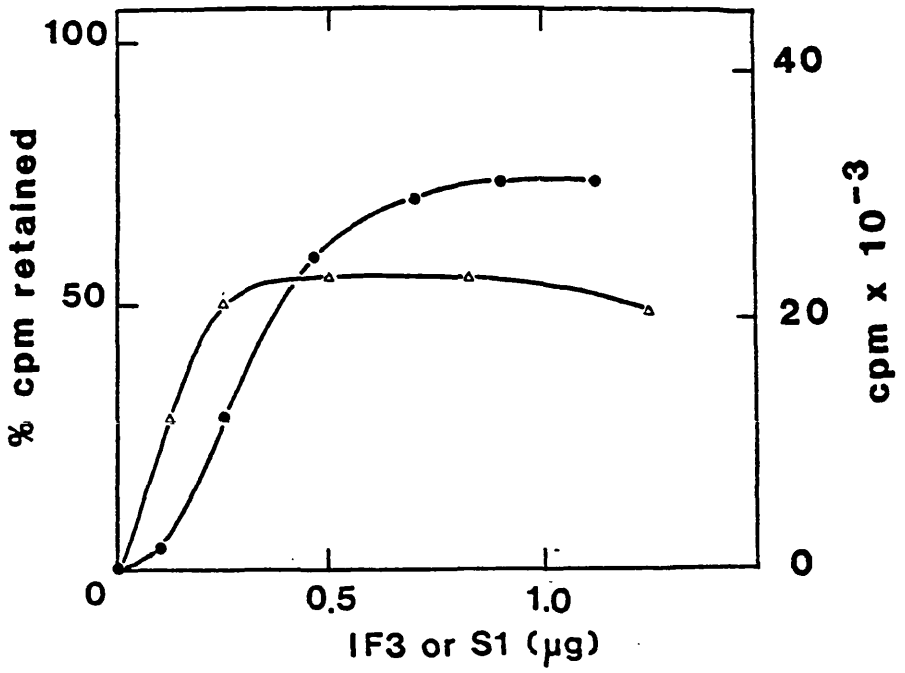


FIGURE 55 Binding of IF3 and ribosomal protein S1 to (^{32}P)-MS2 RNA Varying amounts of IF3 or S1 were incubated with (^{32}P)-MS2 RNA (0.2µg, 42453 cpm) as described in section 3.6 and filtered over nitrocellulose filters. A blank value (without either IF3 or S1, 415 cpm) was substracted. IF3 (●), S1 (△).

occurred at an input of 0.5μg of S1 protein into the reaction and, therefore, bound with an affinity similar to that observed by Thomas et al. (1979) and also by Senear and Steitz (1976), using the closely related R17 RNA. IF3 bound well the same preparation of MS2 RNA with approximately 1μg of factor being required to maximally retain over 70% of the RNA on the filter (Fig. 55).

To determine whether S1 protein influenced the protection of MS2 RNA by IF3, complexes of MS2 RNA with IF3, S1 and S1 + IF3 were digested with pancreatic RNase and the amount of RNA retained on nitrocellulose filters determined at various times (Table 12). It was found that under the conditions used for digestion, S1 protein did not protect MS2 RNA from RNase digestion to any significant extent. IF3 alone protected some 1.31% of the MS2 RNA and, in the presence of S1 protein, the protection was reduced by approximately 50% to 0.68%.

To determine whether the decrease in protection by IF3 in the presence of S1 protein was due to the modification of the protected site, the digestion of MS2 RNA in the presence of IF3 and IF3 + S1 protein was repeated and the protected sites fractionated by PAGE (Fig. 56). In the presence of IF3 alone, the characteristic band of the protected RNAs was seen together with an additional, fainter band of slower mobility. The pattern in the case of IF3 + S1 protein was similar, but with the bands being slightly less intense. This experiment would suggest that S1 protein does not alter the regions of MS2 RNA protectd by IF3 since, if additional or lesser regions of the RNA were protected, then the mobility of the protected fragments in the gel would be expected to be different from the native protected site. S1 protein does, however, reduce the total amount of RNA protected by IF3 and may do so by either unwinding some secondary structure

TABLE 12

INFLUENCE OF RIBOSOMAL PROTEIN S1 ON THE PROTECTION OF (^{32}P)-MS2 RNA BY IF3

	cpm retained on filter (% cpm)		
Hrs.	IF3	IF3 + S1	S1
0	122000 (69.7)	170000 (97)	85000 (48.6)
0.5	6500 (3.7)	5200 (2.9)	1200 (0.68)
1.0	3200 (1.82)	2200 (1.25)	300 (0.17)
1.5	3800 (2.17)	1750 (1.0)	100 (0.06)
2.0	2300 (1.31)	1200 (0.68)	112 (0.06)

(^{32}P)-MS2 RNA (2μg) was bound to IF3 (15μg), S1 protein (7μg) and IF3 + S1 protein (15μg and 7μg respectively) in 400μl of binding buffer. Pancreatic RNase (0.2μg) was added and the mixture incubated at 37°C. Aliquots containing 0.4μg of RNA (175 290 cpm) were filtered over nitrocellulose at various time intervals and followed by liquid scintillation counting.

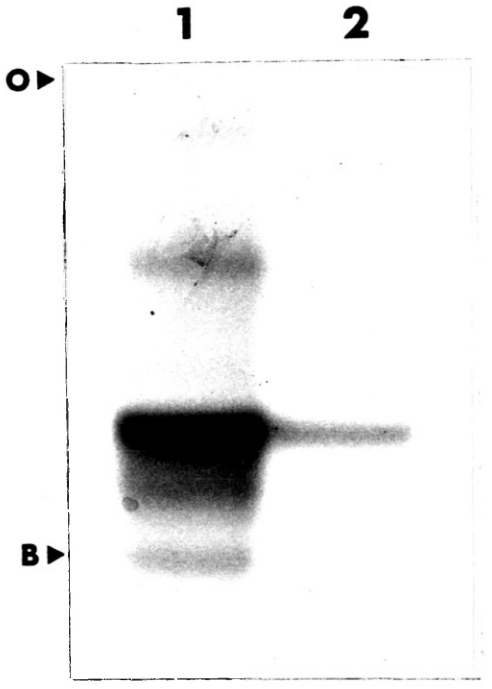


FIGURE 56 PAGE of (^{32}P)-MS2 RNA protected by IF3 in the presence and absence of ribosomal protein S1.
IF3 (150μg) was bound to (^{32}P)-MS2 RNA (20μg) in the presence or absence or S1 protein (70μg) and digested with pancreatic RNase for 1 hour as described in Table 12. IF3 protected MS2 RNA was isolated as described in methods section 3.6.2 and electrophoresed through a 15% polyacrylamide gel followed by autoradiography. Track 1 : IF3 alone; Track 2 : IF3 + S1 protein. B marks the position of the BPB dye marker.

present in the 3'-terminal site and thus prevent IF3 from binding, or by competing with the factor for the specific site, but not protecting the site from RNase digestion.

The nature of the bands of slower mobility observed in Fig. 56 is uncertain. Although such bands were occasionally observed by Dr. B. Johnson (pers. commun.), apart from Fig. 56, they were never observed in other preparations of the IF3 protected fragment made during the course of the work reported in this thesis. It is possible that they may represent IF3 protected fragments originating from some other region of the RNA or, alternatively, they may correspond to a larger section of the specific binding site described in this work. Such a species may have arisen from slightly different digestion conditions used to isolate the protected fragments (e.g., use of an old RNase preparation of low specific activity) with the additional sequences arising from a strong secondary structure, reducing digestion by the old RNase and also holding the RNA species in a distinct secondary structure. These possibilities could have been distinguished by fingerprint analysis of the bands, however, as noted above since these bands were not seen again in this work this was not possible.

4.3.4 Studies on the secondary strcuture of the IF3 protected site

The experimental determination of RNA secondary structure using the two-dimensional gel electrophoresis technique of Ross and Brimacombe (1979) has been applied to the determination of the secondary structure of E. coli 16S ribosomal RNA (Glotz and Brimacombe, 1980). The method involves a two-dimensional gel electrophoresis procedure in which mixtures of interacting RNA fragments are separated under non-dissociating conditions in one dimension and then are dissociated in the second dimension. The interacting fragments thus appear as pairs or

families of fragments in the second dimension.

Studies were made on 5'-end labelled IF3 protected MS2 RNA fragments using this technique. The protected fragment was run on a non-denaturing 15% polyacrylamide gel, autoradiographed (Fig. 57a) and followed by electrophoresis of the track in the second dimension, in the presence of 8M urea. The autoradiograph (Fig. 57b) showed several spots forming families, both vertically and horizontally; however, the main IF3 protected material lay below the diagonal smear as an intense spot. This was located on the diagonal formed by the origin of the first dimension, the XCFF and BPB dyes and the (γ-^{32}P)-ATP. Since the buffer systems are similar in both dimensions, with the exception that the second dimension contains 8M urea, the marker dye-ATP diagonal would be expected to be the diagonal upon which an RNA molecule that is unaffected by the second dimension would be expected to run. On the other hand, the RNA species forming the visible diagonal smear had different mobilities in the second dimension, perhaps by their having some intramolecular secondary structure. Thus, the observation that the IF3 protected fragment runs below the 'RNA diagonal' suggests that either the fragment has no secondary structure at all, or that it possesses a very stable secondary structure that is unaffected by the denaturing conditions of the second dimension.

Glyoxal (ethanedial) reacts with nucleic acids, nucleotides and their component bases (Nakaya et al., 1968). At high concentrations, it has been reported to react with all bases of both RNA and DNA (Nakaya et al., 1968); however, the guanosine-glyoxal adduct is by far the most stable (Nakaya et al., 1968; Broude and Budowsky, 1971; Shapiro et al., 1970). Glyoxalation introduces an additional ring into guanosine residues (Shapiro and Hackmann, 1966), thus sterically hinder-

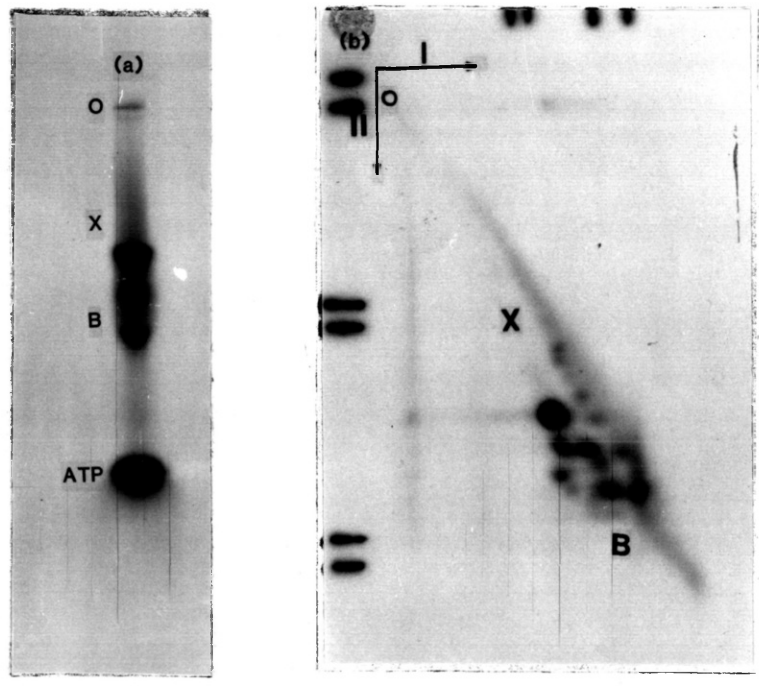


FIGURE 57 Two-dimensional PAGE of IF3 protected MS2 RNA.
(a) Autoradiograph of first dimension. IF3 protected MS2 RNA was 5'-end labelled and electrophoresed through a 15% non-denaturing PA gel.
(b) Autoradiograph of second dimension. The strip of gel from (a) was electrophoresed in the second dimension as described in section 3.1.2 using TAE as running buffer. O, X and B refer to the positions of the origin, XCFF and BPB markers respectively. I and II show the directions of the first and second dimensions.

ing the formation of G-C base pairs and consequently the renaturation of the native structure.

McMaster and Carmichael (1977) found that under their conditions for sample preparation, the glyoxal-guanosine adduct was stable and therefore the running buffers for gel electrophoresis of the denatured RNA did not need to contain denaturing agents. Thus, it is possible using this technique to run both native and denatured molecules on the same gel.

5'-end labelled IF3 protected MS2 RNA was denatured using glyoxal and electrophoresed on a 20% polyacylamide gel, together with native IF3 protected MS2 RNA and, as a control, protected RNA that had been treated in the absence of glyoxal. Autoradiography (Fig. 58a), showed that both the native material and the control treated in the absence of glyoxal had the same characteristic main band and faster running band, as seen before, together with several bands of slower mobility. These bands were similar to those seen with the IF3 protected site in the presence and absence of S1 protein (section 4.3.3; Fig. 56). The glyoxal treated material consisted of a wide band, possibly comprising of two bands, larger than the main band in the native track, together with two bands of slower mobility. As noted by McMaster and Carmichael (1977), denaturation and glyoxalation cause a reduction in the electrophoretic mobility of RNA. This probably reflects a change in molecular weight of the RNA upon addition of the glyoxal adduct and, in the case of the IF3 protected fragments which are particularly guanosine-rich (section 4.2.6), this change may be large.

When the RNA species from the first dimension were run in the second dimension (in this case a 20% gel, using the buffer system of Maxam and Gilbert (1977), as used by Glotz and Brimacombe, 1980),

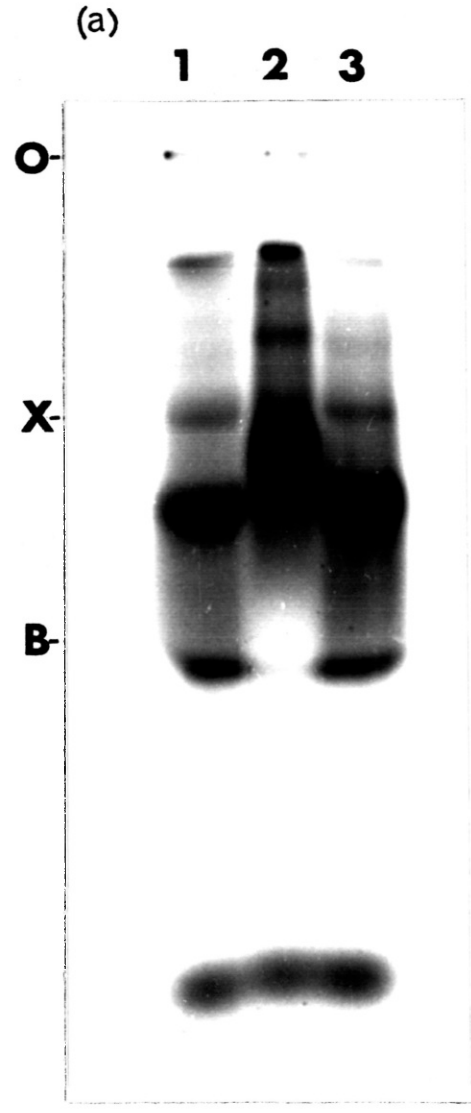


FIGURE 58 Two-dimensional PAGE of IF3 protected MS2 RNA.
(a) Autoradiograph of first dimension of 15% PAGE of 5'-end labelled IF3 protected MS2 RNA. Track 1, native protected fragment; Track 2, protected fragment denatured with glyoxal as described in section 3.1.2; Track 3, protected fragment treated as for Track 2 but in the absence of glyoxal.

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FIGURE 58 continued:

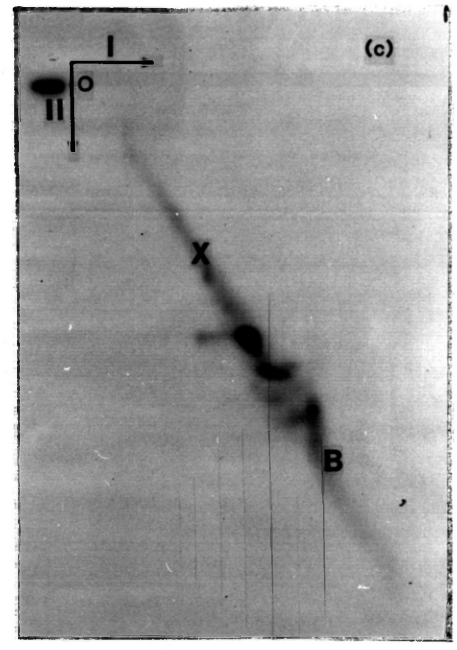


FIGURE 58 continued:

(b) Autoradiograph of second dimension of native protected fragment. The strip from the first dimension was electrophoresed in a denaturing gel containing the running buffer of Maxam and Gilbert (1977) as described in section 3.1.2.

(c) Autoradiograph of Track 3 material from part (a) (protected fragment treated in the absence of glyoxal) electrophoresed as described in part (b) of this figure.

(d) Autoradiograph of Track 2 material from part (a) (glyoxal denatured protected fragment) electrophoresed as described in part (b) of this figure. O, X and B show the positions of the origin, XCFF and BPB markers. I and II show the directions of the first and second dimensions respectively.

the autoradiographs of the untreated and the treated in the absence of glyoxal RNA species showed a similar pattern (Figures 58b, c) with an intense spot running on the RNA diagonal, rather than below as found before (Fig. 57). This result can be explained in that, in the case of the 20% gel / the diagonal formed by the marker dyes coincides with the diagonal corresponding to RNA species having no inter-RNA secondary structure, but possibly having intra-RNA secondary structure. When the glyoxal treated RNA was fractionated in this manner (Fig. 58d), the RNA species lay on the diagonal and the few interacting species below the intense spot were not seen.

These observations suggest that the Band I protected fragment may consist of RNA lacking in any secondary structure or, alternatively, that the fragment consists of several RNA species that are strongly held together by interactions that are not disrupted by the denaturing second dimension.

4.4 POSSIBLE HOMOLOGY BETWEEN THE IF3 PROTECTED SITE ON MS2 RNA AND 16S rRNA

Finally, in view of the observation that IF3 appears to bind to the 30S subunit through the 16S rRNA moiety (see section 1.1.5), a search was made for nucleotide sequence homology between the IF3 protected site on MS2 RNA and the nucleotide sequence of 16S rRNA (Carbon et al., 1978; Brosius et al., 1978; Noller and Woese, 1981) with a view to attempting to localise the IF3 binding site on that molecule.

Firstly, since the IF3 protected site was found to be relatively G-rich (overall some 39% - section 4.2.6), a search was made for G-rich

regions in the 16S rRNA sequence (Fig. 59). This molecule is, to start with, overall G-rich and comprises some 31.6% of the total residues, as compared with MS2 RNA, where the overall G content is 25.6% (cf Fig. 43). At 50 nucleotides resolution (Fig. 59a), several regions of the 16S rRNA sequence stand out as being relatively G-rich. Notable are the regions covering residues 100 - 150 (46% G residues) and the region 650 - 750 (40% G residues). At 100 nucleotides resolution (Fig. 59b), the region 700 - 800 is particularly G-rich and contains approximately 37% G residues.

Secondly, a search was made using a simple computer program for the presence of the oligonucleotides found in fingerprints of the IF3 protected site (Table 5) of the size 5 residues and larger (namely, T_{10}, P_9, P_{11}, P_{12} and P_{16}), but excluding residue T_{11} because of its uncertain composition. Of these oligonucleotides, only two, namely T_{10} (U-C-U-C-G) and P_9 (G-A-A-A-G-C), occur in the 16S rRNA sequence at residues 656 - 660 and 764 - 769 respectively (numbering system of Noller and Woese, 1981). As we have seen in section 4.2.5, oligonucleotide T_{10} does not appear in the protected Regions I, II and III and is probably not a true component of the IF3 protected site on MS2 RNA (see section 5.2.1). Oligonucleotide P_9 is of interest, since it is located in one of the previously determined regions of the molecule that is G-rich (Fig. 59), namely, residues 700 - 800.

Finally, a search was made for homology of the nucleotide sequences of protected Regions I, II and III with the 16S rRNA sequence (Fig. 60). For Region I, one region of homology showing some 60% similarity over 20 residues was found at positions 1455 - 1474 (Fig. 60a). Likewise, for Region II, three regions of homology were found (Fig. 60b): residues 353 - 372 (55% homology over 20 nucleotides),

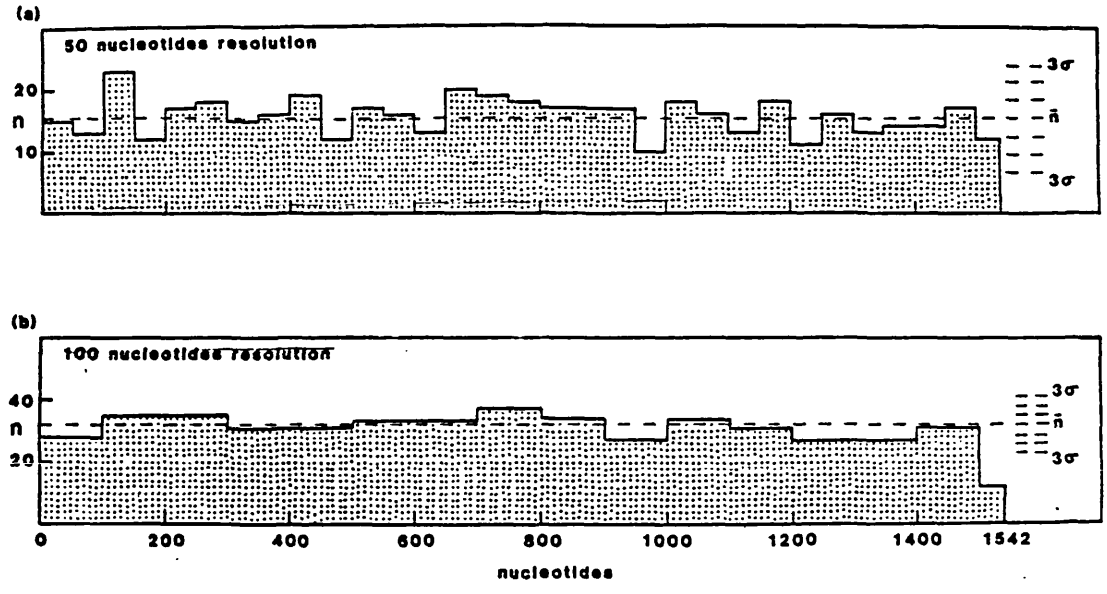


FIGURE 59 Distribution of G residues in 16S rRNA.
The number (n) of G residues present in either 50 or 100 nucleotide segments of 16S rRNA (Noller and Woese, 1981) were plotted as histograms. (a) Distribution of G residues at 50 nucleotides resolution ($n = 15.67$, $\sigma = 2.88$). (b) Distribution of G residues at 100 nucleotides resolution ($n = 31.6$, $\sigma = 3.11$).

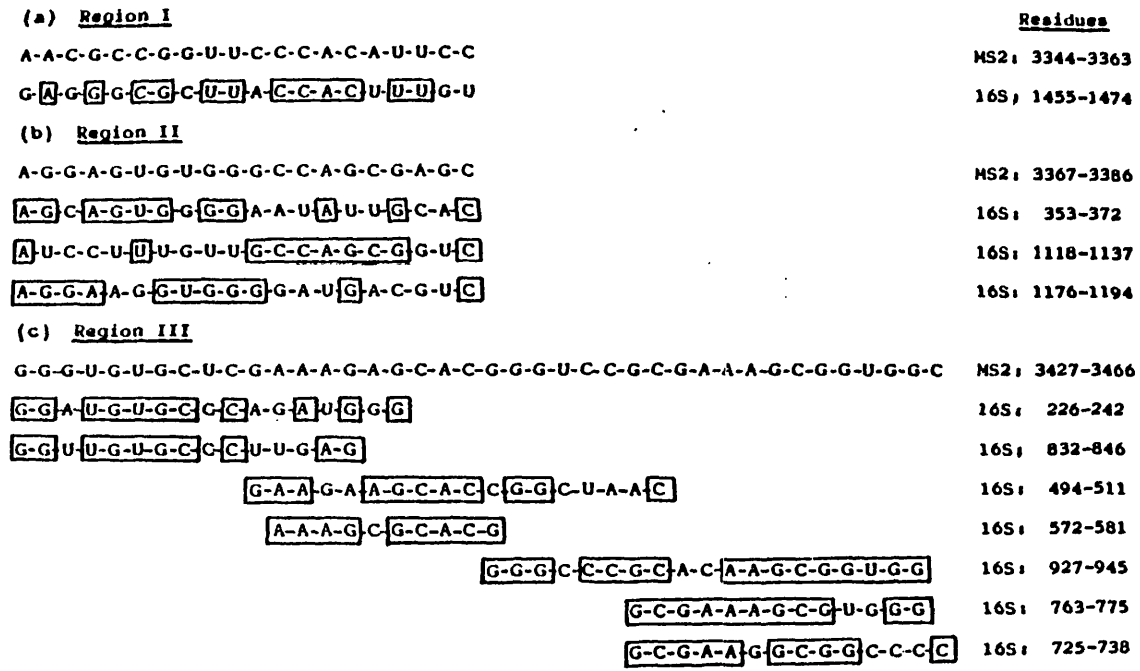


FIGURE 60 Search for homology between Regions I, II and III and 16S rRNA

The nucleotide sequences of protected Regions I, II and III and 16S rRNA (Noller and Woese, 1981) were compared and regions of homology are shown boxed. (a) Comparison of 16S rRNA with Region I; (b) Comparison with Region II; (c) Comparison with Region III

residues 1118 - 1137 (50% homology over 20 nucleotides) and residues 1176 - 1194 (55% homology over 20 nucleotides). Finally, for Region III, several regions of homology were found (Fig. 60c), showing homology of up to 84% in the case of residues 763 - 775 and 927 - 945. The significance of such homology is discussed later in section 5.5.3.

DISCUSSION

5.1 PREPARATION OF IF3 PROTECTED MS2 RNA

5.1.1 Isolation of functionally active homogeneous IF3

Homogeneous IF3, as judged by SDS-PAGE, was isolated from E. coli and migrated in an SDS-polyacrylamide gel with a mobility corresponding to an M_r of 23000 ± 10%. This determination agrees well with other estimates of the factor's M_r, being in the region of 21000 - 23500 (section 1.1.1, Table 1), but is somewhat higher than the values determined by chemical analysis of the two forms of IF3 (Table 1) (Brauer and Wittmann-Liebold, 1977). In general, however, M_r values of basic protein determined by SDS-PAGE are somewhat higher than those determined by chemical analysis (Hames, 1981).

The purified IF3 preparations were functionally active as assayed by their ability to stimulate in vitro initiation complex formation using MS2 RNA in messenger. An estimate of the approximate specific activity of a typical preparation showed that some 90 moles of IF3 were required to stimulate the binding of 1 mole of f(^{35}S)Met tRNA$_f^{Met}$ to the ribosome. In contrast to this, Vermeer et al. (1973a) found that approximately 3 moles of IF3 were required to bind 1 mole of the amino-acyl tRNA in a similar assay. As noted in section 4.1.1, this difference in specific activities may have been due to the irreversible denaturation of a large amount of the IF3 preparation during chromatography on Sephadex G75 in the presence of urea. Alternatively, the use in this work of a sub-optimal assay system or a combination of both of these factors may have been responsible for the apparent low specific activity.

Whilst after treatment with urea a renatured state of many

ribosomal proteins can be demonstrated after removal of the denaturing agent (see Wittmann, 1982), this renaturation is not always quantitative and is sensitive to minor variations in the renaturation process and, also, the particular protein in question: i.e., with some proteins,renaturation is greater than with other proteins under identical conditions. It is possible that under the conditions used in this study, renaturation of the IF3 preparations was poor. Alternatively, since the factor is, to a large extent, readily labile during purification (C. Gualerzi, pers. commun.) a considerable amount of inactivation could have already occurred before the chromatography step in the presence of urea.

The IF3 preparations used in this study were also active in binding to MS2 RNA with, in a typical case, approximately 220 moles of IF3 being required to maximally retain one mole of MS2 RNA in the filtration assay. This ratio compared well with those determined previously by Johnson and Szekely (1977, 1979; B. Johnson, pers. commun.) which ranged from 100 - 250 moles of IF3 per mole of MS2 RNA, but was somewhat higher than the 50 : 1 (IF3 : MS2 RNA) molar ratio reported by Jay et al. (1974a) for R17 RNA. It is unlikely that this difference arises from a greater affinity of the factor for R17 RNA since, in addition to having closely related nucleotide sequences (Robertson and Jeppesen, 1972), the 3' terminal regions of the Group I coliphages, the region where IF3 binds to MS2 RNA (Johnson and Szekely, 1977; this work, section 5.2), are highly conserved (Inokuchi et al., 1982). It is more likely that this reflects differences in the specific activities of the IF3 preparations used in these studies.

The low specific activities of the IF3 preparations used in this study, together with the intrinsic limitations of the binding assay,

preclude a detailed analysis of the binding parameters of this interaction (e.g., Woodbury and von Hippel, 1983). However, it is clear that the binding is saturable and, based on the estimate of the specific activity of the factor preparation used in the binding experiment, has an approximately equimolar stoichiometry.

Since the conformation of a protein has been shown to be important for binding to RNA (Littlechild et al., 1977), it is possible that treatment of IF3 with urea may have altered the factor's RNA binding characteristics. However, since Johnson and Szekely (1977) found no significant differences in the fingerprint patterns of IF3 protected MS2 RNA with IF3 purified either in the presence or absence of urea, it would appear that the binding of the purified factor used in this study to MS2 RNA is representative of the binding of native IF3 to this molecule. It is, of course, possible that in view of the low specific activity of the factor preparation, those IF3 molecules binding to MS2 RNA are not the same as those molecules that are functionally active in the initiation complex formation assay. However, the possibility that the binding of IF3 to the RNA is an artefact resulting from the use of a high concentration of functionally inactive protein is unlikely in view of the high degree of specificity of the interaction (Johnson and Szekely, 1977; see later section 5.2).

In view of these observations, it would be perhaps better for any future experiment to use an IF3 purification procedure that is rapid and does not involve chromatography in the presence of urea or other denaturing agent. In this respect, use could be made of the nucleic acid binding properties of the factor (section 1.1.3) by devising a procedure involving chromatography of a crude NH_4Cl ribosomal wash through, for example, poly-A cellulose. A similar procedure is in fact used in the

purification of other nucleic acid binding proteins, such as ribosomal protein S1, RNA polymerase and the Qβ-phage host factor (Carmichael, 1975). A further advantage of such a method would be that the purified IF3 would be active in binding to nucleic acid and therefore potentially functionally active, whereas already denatured IF3 (which would probably not bind to the poly-A) would not be purified with the active IF3.

5.1.2 Isolation and 5'-end labelling of IF3 protected MS2 RNA

The isolated IF3 protected MS2 RNA site migrated in a non-denaturing polyacrylamide gel, mainly as a single band (Band I) with a mobility relative to the bromophenol blue marker dye, similar to that found for the in vivo labelled protected fragment (Johnson and Szekely, 1977, 1979). A minor band (Band II), migrating ahead of Band I, was also found in some preparations of the protected fragment with an intensity varying from one preparation to another. Homochromatography fractionation of this material showed that it consisted of small oligonucleotides. It is possible that this material originated from the Band I material, since a change in the relative proportions of Bands I and II was seen in the same preparation of protected fragment labelled with the same preparation of polynucleotide kinase after storage for one month. As noted in section 4.1.5, an origin of this band from the PcV-ds-RNAs used as carrier can be excluded by the observation that a similar band pattern (Bands I and II) was also seen with some preparations of the protected site derived from in vivo labelled MS2 RNA.

In all of the preparations of the protected fragments, the incorporation of the ^{32}P label into the protected material by the polynucleotide kinase was never found to be greater than 4 - 5% of that expected, based on estimates of the size of the protected fragments, and the

number of 5'-termini present. This low incorporation of label was not due to an inability of the enzyme to label oligonucleotides of this size, since the preparations used were shown to label well with a partial RNase T_1 digest of MS2 RNA. Nor could the low incorporation have been due to the presence of 5'-phosphate groups on the protected RNAs since the preparations had been prepared using pancreatic RNase A which cleaves RNA to give 3'-phosphate and 5'-hydroxyl RNase A (e.g., Barnard, 1969). Mild HCHO denaturation of the protected site had no effect on the incorporation of ^{32}P into the protected fragment, suggesting that weak secondary and tertiary interactions were probably not responsible for preventing the labelling. It is possible, however, that the protected fragment possessed secondary and tertiary interactions that were resistant to the HCHO denaturation and, in this respect, it has been reported that the HCHO adducts are unstable and can dissociate when the RNA is purified by ethanol precipitation (McGhee and von Hippel, 1975a,b). Thus, the extent of denaturation could have been very mild indeed. As noted in section 4.1.5, stronger denaturating conditions were not used to improve uptake of the label since HCHO has been reported to cross-link RNA strands (Lodish, 1975) and this may have caused problems upon subsequent homochromatography fingerprinting and sequence analysis of the protected site.

Homochromatography fingerprinting of Band I protected material showed that it consisted of several species of RNA of different lengths. Whilst considerable heterogeneity in the patterns of spots was observed, some similarities between preparations was apparent. A similar situation was found by Dr. B. Johnson for in vivo labelled protected material (Johnson and Szekely, 1979; B. Johnson, pers. commun.). The possibility that the various RNA species observed upon

homochromatography originated from the degradation of a single linear RNA species during elution from the gel is unlikely since homochromatography fingerprinting of the protected site prior to gel electrophoresis gave a similar pattern to that of the sum of the patterns of the Band I and Band II material. Thus, it would appear that the material migrating as a discrete band upon non-denaturing PAGE consists of several RNA species of various lengths and which are held together by secondary, and possible tertiary, interactions which are destroyed in the strongly denaturing conditions of the homochromatography fingerprinting system.

5.2 THE NUCLEOTIDE SEQUENCES OF THE IF3 PROTECTED MS2 RNA FRAGMENTS

5.2.1 Determination of the nucleotide sequences

Band I RNA was shown to consist of three separate regions of the complete MS2 RNA sequence. Whilst the sequences of several of the oligonucleotides from preparation B58 could not be determined since the 'wandering spot' patterns were either very faint or were contaminated by more than one pattern, all four large oligonucleotides from preparation B60 were sequenced and no additional sequences corresponding to additional protected regions were found. It is possible, however, that the small oligonucleotides that were present in preparation B60 and which were not sequenced may have comprised additional species of RNA present in the protected site. These oligonucleotides may have consisted of: (1) additional sequences of RNA that were originally of short length; (2) products resulting from 'hidden breaks' in the three characterized regions; or (3) additional protected regions that may have been preferentially degraded during isolation. In view of the

heterogeneity in the lengths of the RNA species corresponding to the three protected regions determined from the wandering spots, it is probable that many of the spots seen in the case of complex homochromatography patterns represent the same basic regions of the RNA, but of differing lengths.

Region I covered the residues 3344 - 3363 of the MS2 RNA sequence and showed some heterogeneity at the 3'-terminus with two of the sequenced fragments, terminating at position 3360. This region is located in the 3'-terminal portion of the replicase gene of MS2.

Region II covered residues 3367 - 3386 and, although the 5'-terminus was the same, some heterogeneity in length of the fragments was observed with two fragments terminating at residue 3382 and one, possibly two, at residue 3386. This region is located four residues downstream of the maximum extent of Region I and, like that region, is located in the 3'-terminal portion of the replicase gene.

Region III covered the residues 3427 - 3460, although the exact 3'-terminus of this region could not be determined from the sequencing data, but most probably extended to residue 3466. This region is located some 40 nucleotides downstream of the maximum extent of Region II within the 3'-untranslated region of the MS2 RNA molecule.

With the exception of the pancreatic RNase A oligonucleotide A-A-C originating from Region I, all of the possible RNase T_1 and pancreatic RNase oligonucleotides that would originate from Regions I, II and III occurred in the fingerprints of the IF3 protected site obtained by Johnson and Szekely (1977) (Table 8). Additionally, with the exception of the RNase T_1 oligonucleotide T_{10} (U-C-U-C-G) and the two pancreatic RNase oligonucleotides P_{16} (G-A-A-A-G-G-U) and P_{12} (A-G-G-G-A-C), all of the digestion products found by Johnson and

Szekely (1977) could be accounted for by regions I, II and III (Table 8).

The reason for Johnson and Szekely (1977) not finding the trinucleotide A-A-C in the pancreatic RNase fingerprints of the protected site is uncertain. Since the RNase T_1 product A-A-C-G is present, then the trinucleotide A-A-C would also be expected to be present. It is unlikely that this trinucleotide was not transferred from the first dimension of the paper fingerprint, as appears to be the case for the mononucleotide Cp (see Table 5), since it would be expected to migrate, based on the mobilities of oligonucleotides described by Sanger et al. (1965), between spots P_2 (A-C) and P_3 (G-C), both of which are present in fingerprints of the protected site.

The oligonucleotide T_{10}, U-C-U-C-G, occurs twice in the MS2 RNA sequence: at residues 1253-1257 in the A-protein gene and at residues 3136-3140 in the replicase gene. In the latter case, the oligonucleotide is some 200 residues distant from the start of protected Region I. In both cases, however, very few of the pancreatic RNase and RNase T_1 digestion products identified in fingerprints of the protected site simultaneously fit the flanking sequences to this oligonucleotide (Fig. 61). B. Johnson (pers. commun. and Fig. 3 in Johnson and Szekely, 1979) observed that on several fingerprints of the protected site, the spot corresponding to T_{10} was faint, suggesting that this oligonucleotide may be a contaminant rather than a true component of the IF3 protected site.

Oligonucleotide P_{16}, G-A-A-A-G-G-U, occurs once in the complete MS2 RNA sequence at residues 3473 - 3479, and is some seven nucleotides downstream of the maximum extent of Region III (Fig. 62). The large RNase T_1 oligonucleotide, C-U-C-C-A-C-C-G, covering the residues between the end of Region III and P_{16}, was not found in

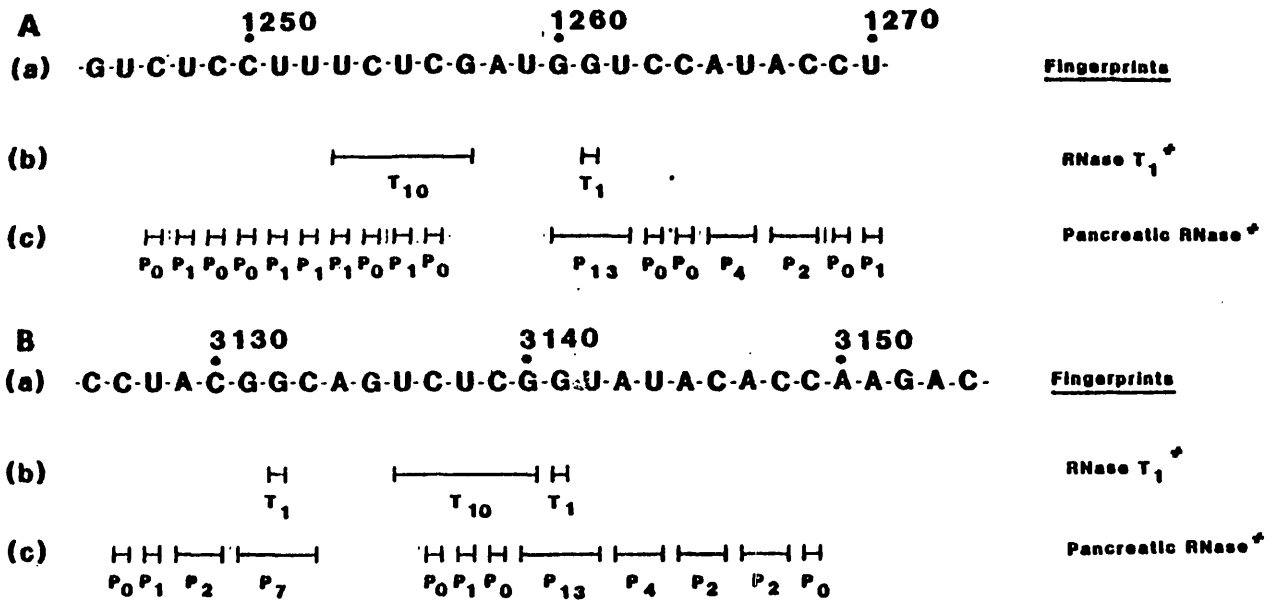


FIGURE 61 Locations of oligonucleotide T_{10} in the MS2 RNA sequence.

A. Location in the A-protein gene (residues 1253 - 1257).

(a) Nucleotide sequence (Fiers et al., 1975).

(b) and (c) Location of RNase T_1 and pancreatic RNase digestion products found in fingerprints of the IF3 protected site. # numbers refer to Table 5.

B. Location in the Replicase gene (residues 3136 - 3140).

(a) Nucleotide sequence (Fiers et al., 1976).

(b) As for Part A above.

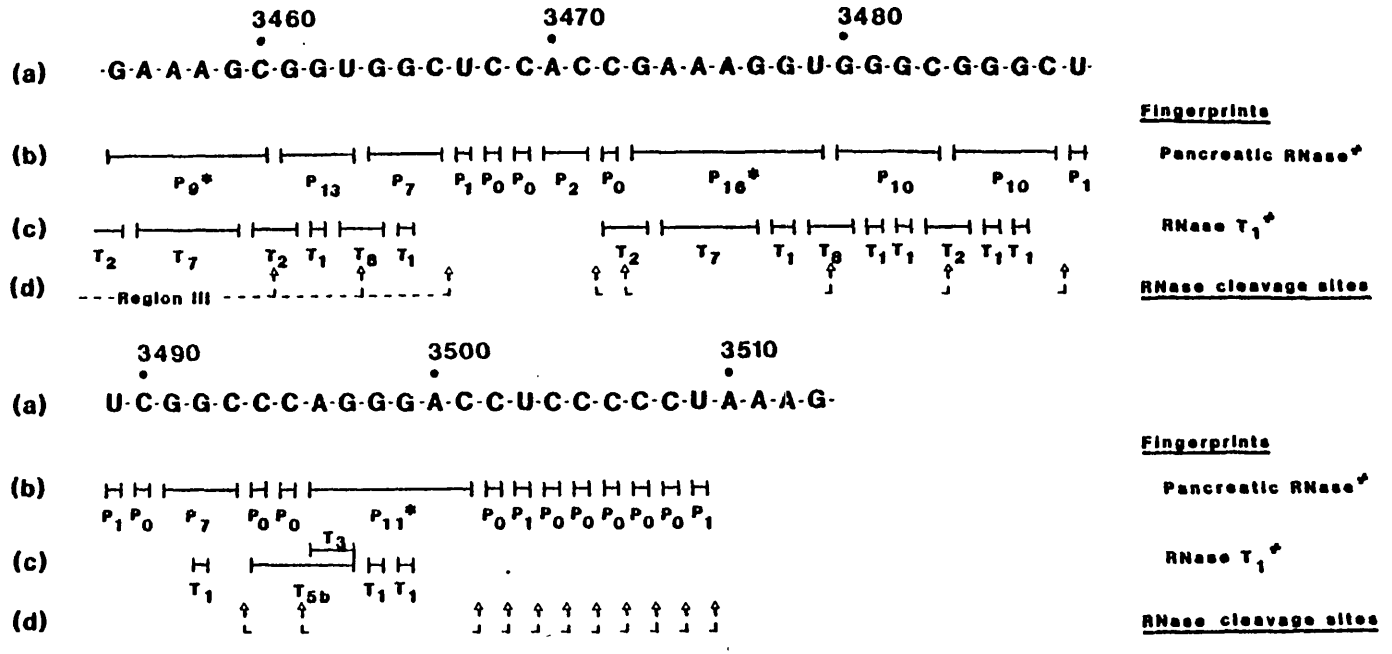


FIGURE 62 Locations of oligonucleotides P_{11} and P_{16} in the MS2 RNA sequence.

(a) Nucleotide sequence (Iserentant et al., 1980).

(b) and (c) Locations of Pancreatic RNase and RNase T_1 digestion products found in fingerprints of the IF3 protected site. # numbers refer to Table 5. * denotes the unique oligonucleotides P_{11} and P_{16}.

(d) Locations of possible pancreatic RNase cleavage sites during isolation of the protected fragments.

fingerprints of the protected site (Johnson and Szekely, 1977). This suggests that P_{16} is not a continuation of Region III. Immediately following P_{16} in the MS2 RNA sequence is the sequence, G-G-G-C-G-G-G-C, the pancreatic RNase digestion products of which (2 x G-G-G-C) are found in fingerprints of the protected site, but also occur in Region II (Fig. 62 and Table 8).

Like oligonucleotide P_{16}, oligonucleotide P_{11}, A-G-G-G-A-C, also occurs only once in the complete MS2 RNA sequence at a position some 16 residues downstream of P_{16} at position 3496 - 3501 (Fig. 62). It is flanked on its 5'-side by the sequence, G-C-C-C and, if the pancreatic RNase used in preparing the protected site were to cleave after the first or third C residue, then the RNase T_1 oligonucleotides C-C-A-G or A-G respectively would be produced, both of which are found in fingerprints of the protected site (Fig. 62). However, C-C-A-G also occurs in Region II and A-G in Regions II and III (Table 8). The 3'-flanking region of P_{11} contains a polypyrimidine cluster, followed by A-A-A-G. If this whole sequence were present in the protected site, then the unique RNase T_1 digestion product:

A-C-C-U-C-C-C-C-C-U-A-A-A-G

would be expected to be found in fingerprints of the protected fragment. Since this was not the case, the pancreatic RNase must cut at a position somewhere before the sequence A-A-A-G to give the possible RNase T_1 products of either A-C_{1-2}, or A-C-C-U, or A-C-C-U-C_{1-5}, or A-C-C-U-C-C-C-C-C-U. If the site of cleavage was close to the A-A-A-G sequence, then an oligonucleotide with a mobility similar to that of T_{11} ($U_{3/4}$,$C_{3/5}$,A-C,A-U) which occurs in the protected site may be expected, based on the mobilities determined by Sanger et al. (1965) using a similar fingerprinting system. Two spots were sometimes

seen in fingerprints of the protected site near the position of T_{11} (B. Johnson, pers. commun.; Johnson and Szekely, 1977, 1979). However, they may alternatively be due to some heterogeneity in the length of the 3'-terminal portion of Region I which comprises T_{11}.

The relationship of oligonucleotides P_{11} and P_{16} to the protected site, as defined by Regions I, II and III, is uncertain. It is possible that they may represent additional RNA species of the protected site that were not labelled by the polynucleotide kinase through having their 5'-termini inaccessible to the enzyme, possibly through the fragment possessing a strong secondary structure. Alternatively, they may represent very short regions of the protected fragment that migrated near to the top of the homochromatograph and which were not sequenced. In this latter respect, it should be noted that homochromatography fingerprints of in vivo labelled protected sites gave rise only to fragments of large size (Johnson and Szekely, 1979).

A third possibility relates to the observation that the ability of an RNA to bind protein is dependent on the method of initiation of the RNA. In the case of 16S rRNA, preparation by an acetic acid-urea technique permits the binding of an additional six or seven 30S proteins than preparation by a phenol extraction procedure (Hochkeppel et al., 1976; Hochkeppel and Gordon, 1978). Whilst in the above case such differences in isolation procedure may involve major changes to the tertiary structure of the RNA, the use in this study of unlabelled MS2 RNA isolated by a slightly different procedure to that used by Johnson and Szekely (1977) may have resulted in some minor alteration of the RNA tertiary structure in the region of the IF3 binding site involving oligonucleotides P_{11} and P_{16}. If this was the case, then the region(s) of RNA including P_{11} and P_{16} must not be essential for the binding of

the factor to the specific site.

5.2.2 Analysis of the nucleotide sequences of Regions I, II and III

The IF3 protected site is particularly rich in G residues (overall approximately 39%), especially in Regions II and III, where the G content is 50% and 45% respectively. This is in agreement with the proposal that IF3 contains the potential information in its primary structure to recognise G residues in both single- and double-stranded configurations (Bruhns and Gualerzi, 1980). Although the binding site is located in a region of MS2 RNA that is particularly rich in G residues, a preference for these residues does not totally account for the location of the protected site, since Region I contains only 15% G residues and, furthermore, there are several sequences close to the protected regions that are G-rich and yet were not found to be protected by IF3.

Little sequence homology was seen between Regions I and II. However, in contrast, some homology was seen between the 5'- and 3'-portions of Region III, and this homology was to a large extent conserved in Region II. Such sequence homology may reflect the presence of a possible recognition sequence for IF3 binding. If this is the case, the lack of such homology in Region I may be due to Region I and II being associated by secondary structure interaction and that IF3 may be recognising the homologous sequence in Region II. Alternatively, such putative homology could be merely a fortuitous similarity between the nucleotide sequences which, in the case of the two portions of Region III, may have arisen by a duplication process during the evolution of the phage.

5.3 STUDIES ON THE SECONDARY STRUCTURE INTERACTIONS AT THE IF3 BINDING SITE ON MS2 RNA

5.3.1 Studies on the effect of mild heat denaturation

Mild heat denaturation treatment of MS2 RNA in the absence of Mg^{2+} resulting in approximately 18% denaturation, was found to have little effect on the binding of IF3 relative to native RNA. At high concentrations of the factor, a reduction in binding seen in native RNA was not observed with the denatured RNA; however, in contrast, a slight increase in binding was observed. Since up to the saturating amount of IF3, the shapes of the native and heat denatured IF3 binding curves were similar, this would suggest that partial heat denaturation has little effect on the conformation of the RNA at the specific IF3 binding site. However, at IF3 concentrations above the saturating concentration, the increase in binding of the denatured RNA would suggest that either the binding of the factor to the specific site was stabilised by the mild denaturation process or that mild denaturation caused additional binding sites on the RNA to become available to the factor. Further evidence for the latter possibility comes from the observation that, whereas heat denaturation in the presence of Mg^{2+} was found to cause considerable fragmentation of the RNA, binding of the factor to this fragmented, denatured material was approximately 70% of the total cpm. Since the sequencing studies presented earlier in this work have shown that IF3 binds to and protects a single site in MS2 RNA and, assuming that once binding has occurred the RNA is retained on the nitrocellulose filter, the effect of fragmentation would be expected to be to reduce the total amount of RNA bound in proportion to the location of the fragmentation sites to the left and right of the binding site. Since the denatured RNA was observed to be fragmented to a considerable extent, the amount of RNA retained by IF3 would be

expected to be small and not the observed 72%.

Analysis by thermal denaturation curves showed that heat denaturation treatment in the presence of 2mM Mg^{2+}, at which fragmentation was minimal, resulted in approximately 21% denaturation. The binding curve of the factor to RNA treated in this way showed that higher concentrations of IF3 were required to retain the same amount of RNA on the filter and gel electrophoresis of the denatured RNA showed that fragmentation was very limited. Thus, the change in binding characteristics was probably not due to fragmentation, but due to the effect of partial heat denaturation in the presence of Mg^{2+}, possibly by altering the secondary structure of the specific protected site.

A time course of the protection of partially denatured MS2 RNA by IF3 against pancreatic RNase showed that there was no significant difference in the amount of RNA protected in the cases of both native and partially denatured MS2 RNA. Whilst the digestion did not go to completion, this observation would suggest that any binding of the factor to new sites on the RNA formed by denaturation was not sufficient to be protected against RNase digestion. Gel electrophoresis of the protected material was not undertaken and thus it is not possible to say whether the denatured RNA protected against digestion originated from the 3'-specific site, although this is perhaps most likely.

5.3.2 Studies using S_1 nuclease fragmented MS2 RNA

The binding of IF3 to S1 nuclease fragmented MS2 RNA (S_1F-MS2 RNA) giving digestion products in the size range similar to that observed upon heat denaturation in the presence of Mg^{2+}, showed that relative to native MS2 RNA, S_1F-MS2 RNA was bound by the factor

to a very small extent. This may be due to either the nuclease digesting around the specific binding site to such an extent that the amount of RNA retained by the IF3 on the filter (and hence the number of cpm) was very small, or that the specific binding site contains some single-stranded RNA regions that are essential for IF3 binding and which were digested by the nuclease. Without gel electrophoresis of the protected material, it is not possible to differentiate between these two possibilities.

IF3 was found to bind to heat denatured S_1F-MS2 RNA to a greater extent than to native S_1F-MS2 RNA. These observations suggest that upon heat denaturation of MS2 RNA, additional sites are made available on the molecule to which the factor can bind. That this binding is weak was shown by the earlier observation that protection of heat denatured MS2 RNA by IF3 against RNase digestion was not higher than that for native MS2 RNA. In this respect, IF3 has been shown by other workers to bind to a variety of nucleic acid molecules of both single- and double-stranded conformations (cf section 1.1.3). It is possible that the binding of the factor to heat denatured MS2 RNA is non-specific as appears to be the case with its binding to homopolynucleotides (Wickstrom et al., 1980).

5.3.3 Studies on the influence of ribosomal protein S1

Both IF3 and S1 bound to MS2 RNA in agreement with reports of the binding of S1 protein to MS2 and R17 RNAs (Thomas et al., 1979; Senear and Steitz, 1976). Under the conditions used, S1 did not significantly protect MS2 RNA from digestion by pancreatic RNase. This is in agreement with the finding of Senear and Steitz (1976), who found that, whereas S1 could protect R17 RNA against digestion with RNase T_1, the protein did not protect the RNA against pancreatic

RNase digestion.

In the presence of S1 protein, the protection of MS2 RNA by IF3 was reduced by a factor of approximately 50% from 1.3 to 0.68% of the total. Gel electrophoresis of the IF3 protected site in the presence and absence of S1 protein showed that the band pattern observed was the same in both cases, but that in the presence of S1 the total amount of RNA protected was less, in agreement with the observed reduction in protection. These studies suggest that S1 protein reduces the total amount of RNA protected by the factor, but not the number of regions of MS2 RNA, since such an action would be expected to produce a protected fragment with an altered mobility. It is possible that S1 protein may cause the reduction in binding by either unwinding some aspect of the secondary structure of the IF3 binding site essential for binding, but distal from the site of interaction of the S1 protein and thus prevent the factor from binding. Alternatively, S1 protein may compete with IF3 for binding to the same region of the RNA molecule, thereby preventing the factor's binding, but not protecting such a region from RNase digestion, as in the case for IF3.

5.3.4 Studies on the secondary structure of the IF3 protected site

Studies using the two-dimensional gel electrophoresis system of Ross and Brimacombe (1979) showed that Band I IF3 protected MS2 RNA was unaffected by the denaturing conditions of the second dimension. This observation would suggest that the protected site either consists of RNA species lacking in secondary structure or that the secondary structure was sufficiently strong to be unaffected by the denaturing conditions. Since the sequencing results and homochromatography fractionation of the protected site have shown that this material corresponds to several RNA species of different length and

nucleotide sequence, they would therefore have been expected to have been separated in the second dimension of the gel fractionation procedure. Clearly, the denaturing conditions were not sufficiently strong enough to disrupt the secondary structure of the protected fragment. At neutral pH, 8M urea has been reported to be not sufficient to destroy the secondary structure of RNA, especially of regions of very high G+C content (Reijnders et al., 1973), whereas at low pH, as is the case in the first dimension of the homochromatography fractionation procedure, and at high temperature, urea is an efficient denaturing agent (Reijnders et al., 1973; Gould and Matthews, 1976).

The stability of the secondary structure interactions at the IF3 protected site would thus appear to be greater than those observed in the case of E. coli 16S rRNA (Ross and Brimacombe, 1979; Glotz and Brimacombe, 1980). In this respect, the site resembles some of the regions of 5S RNAs of E. coli and B. stearothermophilus which remain interacting in the presence of high concentrations of urea (Zimmermann and Erdmann, 1978; Pieler and Erdmann, 1982).

5.4 SECONDARY STRUCTURE MODELS OF THE IF3 PROTECTED SITE

As we have seen in section 1.2.4, several secondary structure models have been proposed for the 3'-terminal region of MS2 RNA. However, as noted in that section, the validity of these models is somewhat open to criticism and they must be treated with caution until models are available built directly on experimental evidence, as is the case, for example, for E. coli 16S ribosomal RNA (Ross and Brimacombe, 1979; Glotz and Brimacombe, 1980; Noller and Woese, 1981)

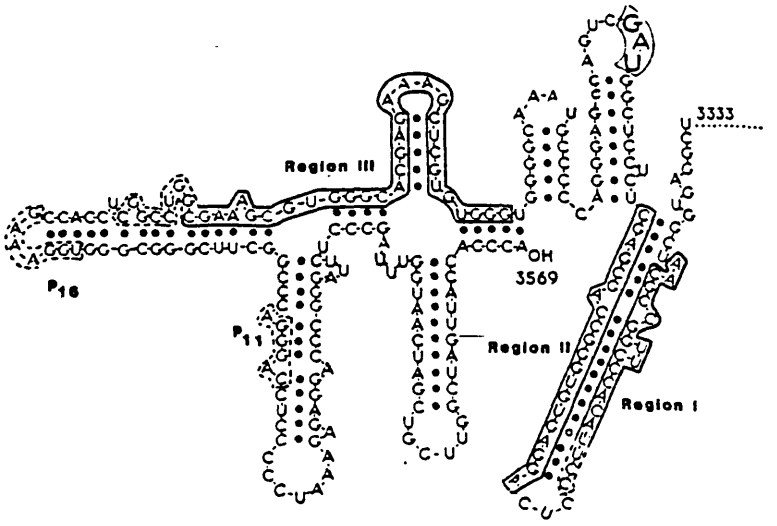
and the 5' end of MS2 RNA (Iserentant and Fiers, 1979) (section 1-2-4).

The location of the maximum extents of the IF3 protected Regions I, II and III in two of these models is shown in Fig. 63. In the model of Fiers et al. (1976) (Fig. 63a), the IF3 protected regions consist of two distinct portions of RNA, separated by two 'hairpin loop' structures and are not linked by secondary structure (although tertiary interactions may, however, occur). Protected Regions I and II form a strongly base paired structure.

In the model of Iserentant et al. (1980) (Fig. 63b), the protected Regions I, II and III are all linked by secondary structure interactions. This model can account for the observation that whereas Regions I and II are close together in the primary sequence and Region III is some 40 nucleotides distant from Region II, all three migrate together as a single band upon gel electrophoresis, as though they were linked. However, the two-dimensional gel electrophoresis studies and heat denaturation studies reported in the work suggest that the protected regions are held together by strong secondary and/or tertiary interactions and it is unlikely that the 5 base-pair region linking II and III would remain stable in the denaturing conditions of the second dimension of the two-dimensional fractionation procedure. However, tertiary interactions may further stabilise this interaction.

Alternative models for the secondary structure of the IF3 binding site can be drawn, incorporating the findings of this work, i.e., (i) that the 5' and 3' ends of the protected regions should be accessible to digestion by pancreatic RNase (i.e., not strongly base paired); (ii) that the fragments produced by partial RNase T_1 digestion of the protected site should have cleavage points that are either weakly or not base paired; (iii) that the protected site should be strongly base

(a)



(b)

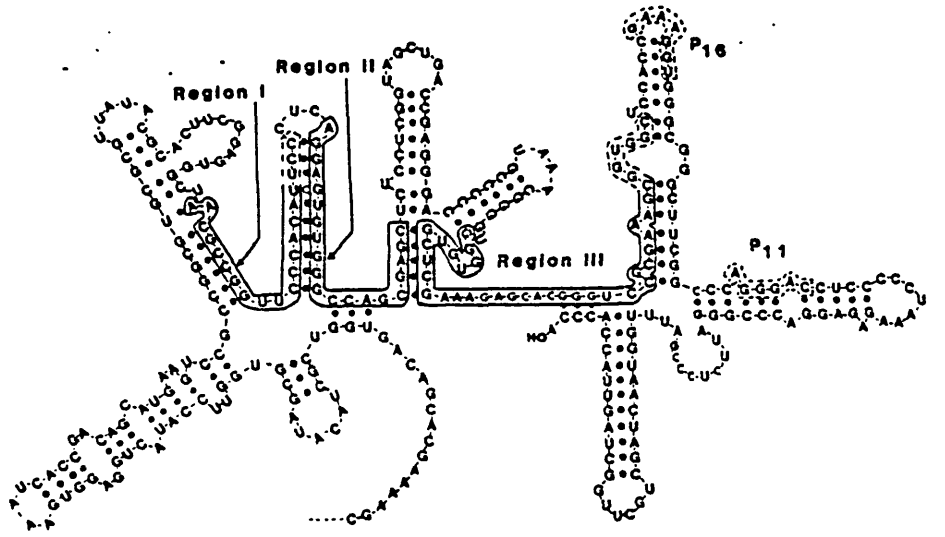


FIGURE 63 Locations of IF3 protected Regions I, II and III, and oligonucleotides P_{11} and P_{16} in secondary structure models of the 3'-terminal segment of MS2 RNA.

(a) Secondary structure model of Fiers et al. (1976).

(b) Secondary structure model of Iserentant et al. (1980).

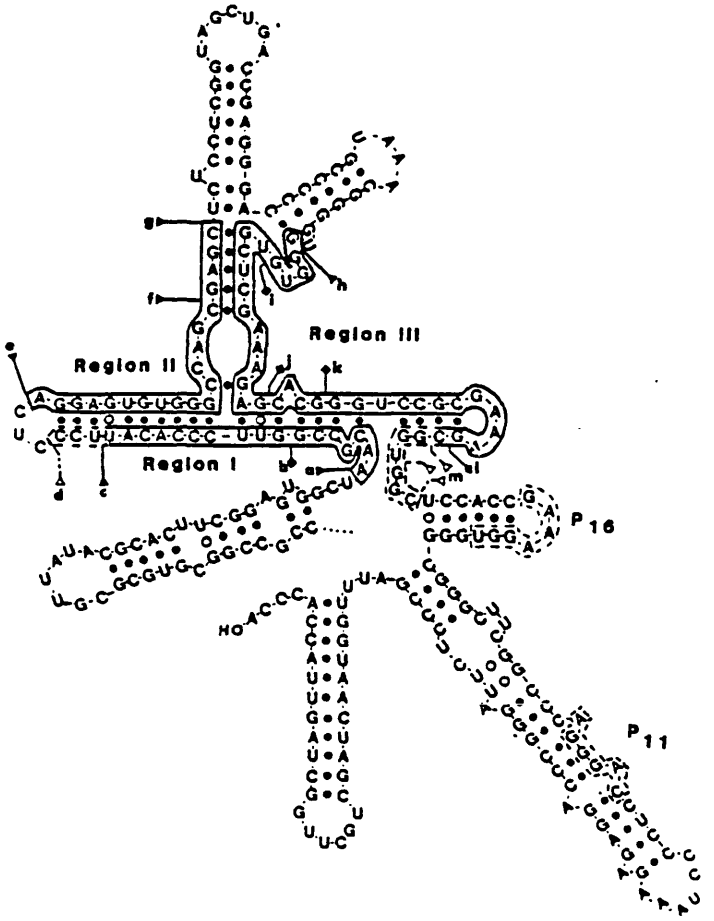
paired as suggested by the two-dimensional gel electrophoresis studies; (iv) that the protected site may possibly contain some single-stranded regions, as may be suggested by the S_1 nuclease digestion studies; and (v) that, based on the heat denaturation studies, the model for the isolated fragment may be most probably the same as that for the intact MS2 RNA molecule.

One such model is shown in Fig. 64. This model is highly structured, with all three protected regions being held together by secondary structure interactions. Some 70% of the bases present in this structure are in a base-paired conformation, thereby being in agreement with estimates of the overall degree of base-pairing in the Group I phage RNAs (section 1.2.1).

Furthermore, in agreement with the observed specificity of pancreatic RNase being in the main towards pyrimidine residues in a single-stranded conformation (Barnard, 1969; Edy et al., 1976), the 5' and 3' termini of the maximum extents of Regions I, II and III are located in single-stranded portions of the proposed structure (Fig. 64). Likewise, other termini representing the heterogeneity in lengths of the three protected regions are, in the main, located at discontinuities of the proposed secondary structure. Whilst the termini of the partial RNase T_1 digestion products of the protected fragment are not located in single-stranded regions or discontinuities of the proposed secondary structure (Fig. 64), it is possible that this may reflect slight differences in the secondary structure of the isolated protected fragment and the secondary structure of this region in the complete MS2 RNA molecule.

In order to further validate this model, the partial RNase T_1 and CM-RNase digestion data reported by Fiers et al. (1976) for this portion of the MS2 RNA molecule can be applied to the proposed structure

(a)



Key :

Pancreatic RNase cleavage points:

certain

probable

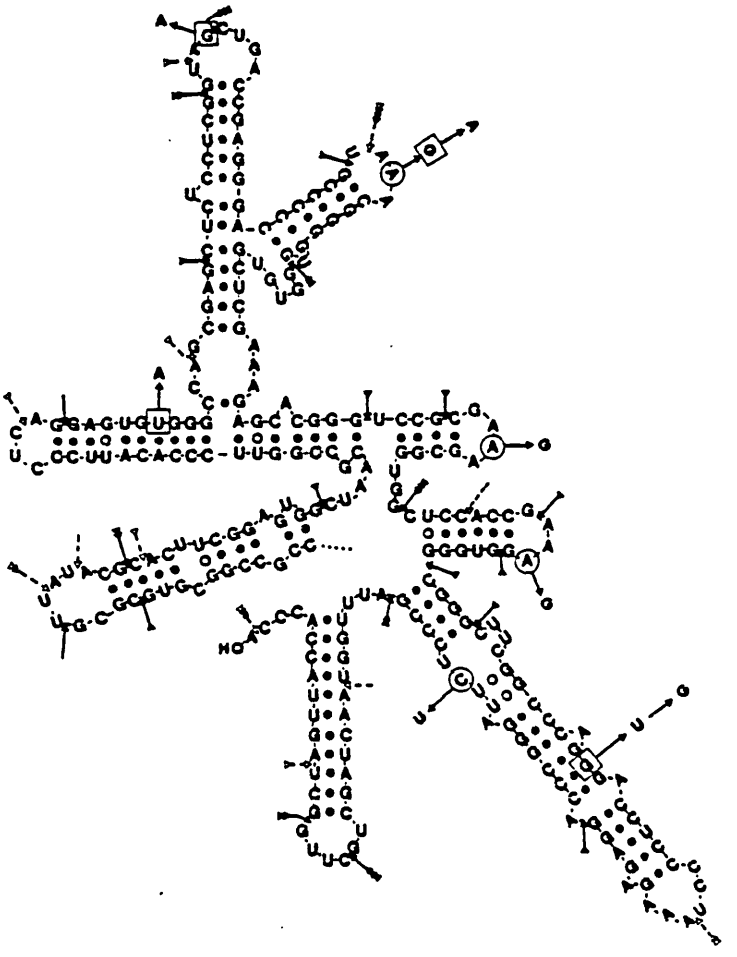
Partial RNase T_1 cleavage points:

FIGURE 64 Proposed secondary structure model of the 3'-terminal segment of MS2 RNA and locations of IF3 protected regions I, II and III , and oligonucleotides P_{11} and P_{16}. (a) Proposed secondary structure. (b) Table of locations of RNase cleavage points shown in part (a).

FIGURE 64 continued:

(b)

Letter	Residue	Wandering spot	Partial RNase T_1
a	3344	B60-1,B58-6,?B55-2	B14-44
b	3350		B14-44
c	3360	B60-1,B55-2	
d	3363	B58-6	
e	3367	B60-2,B60-3,B58-2,B58-3	
f	3382	B60-2,B53-3	
g	3386	B58-2,?B60-3	
h	3427	B60-4,B58-1	
i	3432		B14-40
j	3444		B14-35
k	3447		B14-40
l	3459		B14-35
m	3460	?B60-4,?B58-1	
	3463		
	3466		



Key:

Partial RNase T_1: ——→

Partial CM RNase: - - - →

Sensitivity of bonds:

——→ split very seldom

——→ seldom

——→ rather often

——→ very often

——→ always

FIGURE 65 Partial RNase T_1 and CM-RNase data of Fiers et al. (1976) as applied to the proposed secondary structure model shown in Fig. 64. The locations of mutations (circled) and reversions (boxed) obtained by Iserentant et al. (1980) are also shown.

and are shown in Fig. 65. In general, the sites of cleavage relate to the location of single-stranded regions and discontinuities in the proposed secondary structure. Additionally, mutations obtained by nitrous acid treatment of MS2 RNA (Iserentant et al., 1980) are found in single-stranded regions of the proposed structure, whereas reversions are located in both single-stranded and double-stranded regions, as was the case for an alternative model of this region (Iserentant et al., 1980).

Finally, based on estimates of the values of the thermodynamic quantities (ΔH, ΔS and ΔG) for the addition of a base pair to a double helix (Borer et al., 1974) and also loop formation (Gralla and Crothers, 1973), the average T_m (at approximately 1M Na^+ concentration) for the proposed secondary structure can be estimated to be approximately 107°C and the total free energy of formation of this structure ($\Delta G_{(25°C)}$) approximately -148 kcal. Since, in the case of DNA, a reduction of the Na^+ concentration by a factor of 10 reduces the T_m by about 17°C (Felsenfeld, 1971), the average T_m of the proposed structure at the more physiological Na^+ concentration of 0.1m would be expected to be approximately 90°C. Bearing in mind that such a calculated T_m is somewhat approximate, because of limitations in the determination of the thermodynamic parameters (ΔH, ΔS and ΔG) (Borer et al., 1974), the resulting estimate agrees with the observation that the protected fragment appears to possess a strong secondary structure and that partial heat denaturation treatment had little effect on the binding of IF3 to the specific binding site.

It should be noted, however, that such a proposed secondary structure is highly speculative and is no substitute for a model built directly on experimental data.

5.5 NATURE OF THE IF3 BINDING SITE ON MS2 RNA AND COMPARISON WITH OTHER PROTEIN : RNA INTERACTIONS

5.5.1 Nature of the IF3 binding site on MS2 RNA

In this work, as in other studies of protein : RNA protected regions, the extents of the MS2 RNA sequences that have been characterised must be regarded as the extents of the IF3 protected site, rather than the extents of the binding site, where the intimate interaction(s) of the factor with the RNA occurs. Whilst the factor may interact with some sequences present in the protected site, the overall protected site will most probably be larger than the binding site for several reasons. Firstly, IF3 bound to the RNA may sterically prevent the pancreatic RNase from hydrolysing pyrimidine residues immediately adjacent to the binding site during isolation of the protected fragment. Secondly, since pancreatic RNase was used to isolate the protected fragment, the observation that this portion of the RNA molecule is relatively purine-rich may mean that the nearest accessible pyrimidine residue to the binding site is some residues distant. Thirdly, since the protected site appears to be strongly structured, pyrimidine residues close to the binding site may be protected from RNase digestion by the secondary structure of the RNA. Finally, it is possible that the factor may be binding to only one of the protected regions but, because of the strong secondary structure, other regions appear in the protected site. Thus, from this work, it is not possible to localise exactly the site of intimate interaction (or binding site) of the factor within the chracterised protected regions. However, in view of the apparent high degree of secondary structure possessed by the protected site, it is possible that the factor may be recognising some aspect of a three-dimensional structure in this region.

Such an influence of secondary structure of the RNA in the vicinity of the IF3 binding site could account for the observed heterogeneity in lengths of the protected regions. If the extremities of the protected Regions I and II were not truly protected by the factor, but were present because of the strong secondary structure, then any 'breathing' of such a structure may result in additional sites for the pancreatic RNase to cleave to become available during isolation of the protected fragment.

In order to further investigate the location of the binding site within the protected fragment, the factor could be cross-linked with the RNA and the points of cross-link determined. Zweib and Brimacombe (1979) have used this approach to localise the site of ultraviolet light induced cross-linking of Met^{112} of ribosomal protein S7 to U^{1240} of E. coli 16S rRNA (based on the numbering system of Noller and Woese, 1981). However, care must be exercised in using this approach, since Ehresmann et al.(1980a), using a similar system, found that S7 was cross-linked to C^{1262}. Further studies of the nature of the IF3 binding site on MS2 RNA could be made by investigating the effect of chemical modification of the RNA residue (e.g., kethoxal modification of G residues - Staehelin, 1959) in the presence and absence of IF3. Such studies would identify those residues involved at the binding site and this approach has been used to investigate the binding of ribosomal protein S15 to 16S rRNA (Müller et al., 1979) and also the interaction of E. coli RNA polymerase and the lac UVS and T7 A3 DNA promoters (Siebenlist et al., 1980).

5.5.2 Comparison of the IF3-MS2 RNA specific interaction with other protein-RNA interactions

In section 1.3 of this thesis, the characteristics of several proteins, or groups of proteins, interacting with MS2 RNA, were described.

Of these interactions, the interaction of the viral coat protein with RNA formed into a specific secondary structure, covering the replicase cistron initiation site (section 1.3.3; Fig 8) has the closest resemblance to the interaction of IF3 with the RNA. In both cases, the interaction is specific and, at least in the case of the coat protein and most probably in the case of IF3, this interaction involves the recognition of some aspect of a specific secondary structure. Furthermore, the overall lengths of the protected sites are similar to the slightly smaller coat protein (M_r 14000 - section 1.2.3), protecting either 59 or 103 residues (depending on the amount of protein bound) and IF3 protecting maximally some 80 residues. One difference, however, is that whereas in the case of the coat protein protected site, the protected region is linear, in the case of IF3, the protected RNA sequences are non-contiguous and probably held together by secondary structure interactions into a three-dimensional structure. One further possible difference relates to the stoichiometry of the binding between the two proteins and the RNA. Whilst the stoichiometry of binding of IF3 is uncertain, but is probably approximately equimolar (section 5.1.1), the binding of the viral coat protein may involve the co-operative binding of dimers forming a hexameric protein cluster on the RNA (see section 1.3.3).

The IF3 protected site, furthermore, resembles the interaction of several E. coli ribosomal proteins with 16S and 23S rRNA in which the respective protein protected regions consist of several non-contiguous stretches of RNA (for recent reviews see: Brimacombe et al., 1976; Brimacombe, 1978; Noller and Woese, 1981; Wittmann, 1982). Of these interactions, that of S4 protein with 16S rRNA has, perhaps, been the most extensively characterised, both by determin-

ation of the nature of the protected region (Ungewickell et al., 1975a, 1975b, 1977; Ehresmann et al., 1977, 1980) and also by direct visualisation of the interaction by electron microscopy (Vasiliev et al., 1977; Cole et al., 1978). Two protected fragments (namely, S4 RNA-I and S4 RNA-II), encompassing several hundred nucleotides were obtained after mildly digesting a complex of S4 and 16S rRNA with RNase T_1. The S4 RNA-II complex contained all of the RNA sequence from residue 6 to residue 557, with excision at 248 - 278, 298 - 301, 325 - 362 and 513 - 530 and five additional 'hidden breaks' (numbering system of Noller and Woese, 1981). The S4 RNA-I complex contained S4 RNA-II plus the fragments 558 - 575, 819 - 858 and 870 - 887. All these regions form secondary structure interactions with each other in the model of Noller and Woese (1981) and it is of interest that a fragment almost identical to S4 RNA-II can be obtained by digestion of 16S rRNA with carrier-bound pancreatic RNase in the absence of S4 (Ehresmann et al., 1977, 1980). Results from the electron microscopic examination of the S4-RNA complex are in agreement with the non-contiguous nature of the protected site (Cole et al., 1978) and also show that the complex has some structural similarity to the 30S subparticle (Vasiliev et al., 1977).

Whilst IF3 is only slightly smaller than S4 protein, which has an M_r of 22550 (Reinbolt and Schiltz, 1973) and a radius of gyration (r_g) of 19.5 $\pm$ 1Å (Serdyuk et al., 1981) (compared with IF3 r_g = 15Å - Gualerzi and Pon, 1981), it is perhaps strange that the IF3 protected site on MS2 RNA is some six times smaller than the S4 protected site on 16S rRNA. However, as noted above (section 5.5.1), there are several factors that can make a protein's protected site larger than the actual binding site (or site of intimate interaction). In the case

of S4, it is possible that under the mild conditions used to prepare the protected site (digestion at 0°C), the protein directly interacts with only a portion of the protected regions, possibly partly buried in the RNA structure, and additional regions, to which the protein does not directly interact, appear in the protected fragment because of secondary structure interaction. Indeed, Noller and Woese (1981) propose that S4 binds to two long-range interactions between residues 27 to 37 and 547 to 556 and between residues 564 to 570 and 880 to 886 and thereby stabilises the RNA structure.

5.5.3 Nature of the IF3 binding site on 16S RNA

In view of the similarity between the IF3 protected site on MS2 RNA and ribosomal protein protected sites on rRNA, it is possible that IF3 may be recognising in MS2 RNA some aspect of a specific structure similar to that found at its binding site on 16S rRNA, through which it binds to the 30S subunit (see section 1.1.5). In order to attempt to localise the factor's binding site on 16S rRNA, a comparison was made between the 16S rRNA nucleotide sequence and the IF3 protected site on MS2 RNA.

A direct sequence comparison between 16S rRNA and the IF3 protected Regions I, II and III demonstrated that there was some degree of similarity between the sequences. However, in view of the high G content of the sequences compared (approximately 32% for 16S rRNA and overall 39% for the protected regions), a reasonably high degree of chance similarity can be expected. Of the 16S rRNA sequence, those regions covering residues 763 - 775 and 572 - 581 are of interest since, as well as containing the sequence G-A-A-A-G (which occurs twice in Region III and once in oligonucleotide P_{16} of the protected site) at residues 763 - 765, these regions are located close together in

the secondary structure models of the rRNA (Noller and Woese, 1981; Zwieb et al., 1981) and the region 763 - 775 is, furthermore, located in a particularly G-rich portion of the rRNA. Such homology, together with the observation that these regions lie in the 16S rRNA fragment to which IF3 was found to be mainly cross-linked (Pon et al., 1977; section 1.1.5), as well as the finding that this region of the RNA secondary structure maps close to the location of the IF3 binding site on the 30S subunit (Noller and Woese, 1981) suggests that this area of the RNA secondary strucutures may form at least one of the sites of interaction of the factor with the rRNA. However, confirmation of this proposal will have to await the results of future studies on the direct interaction of IF3 with the rRNA.

5.6 BIOLOGICAL SIGNIFICANCE OF THE INTERACTION OF IF3 WITH MS2 RNA

In this thesis, evidence has been presented for two types of interaction of IF3 with MS2 RNA: (1) a specific interaction at a site located near the 3'-end of the molecule; and (2), an apparently non-specific interaction between the factor and partially heat denatured MS2 RNA. Excluding, of course, the possibility that these interactions may be merely an artefact resulting from a large amount of denatured IF3 (although, as has been discussed in section 5.1.1., this is perhaps unlikely at least for the first type of interaction), some comment should be made on the possible biological significance of these interactions.

Dealing with the second type of interaction first, this apparently non-specific interaction with partially heat denatured MS2 RNA may be similar to the observation by Wickstrom et al. (1980) that IF3 binds

non-specifically to single-stranded homopolynucleotides. It is unlikely that such binding to partially denatured RNA has any biological significance and is most probably a reflection of the nucleic acid binding property of IF3 (see section 1.1.3).

As to the first type of interaction, the location of the protected site such a long way from the cistron initiation sites would rule out a function of IF3 in the direct recognition of these sites and is in agreement with a mechanism of action involving the 30S subunit (section 1.1.7). It is of interest, however, that the sequence A-C-C-U-C-C, which is homologous to the 16S rRNA sequence involved in the Shine-Dalgardo interaction during the initiation of protein synthesis, is present close to the IF3 protected site at residues 3500 - 3505 (Fig. 62), although this may be merely coincidental.

One possible function of the specific binding of the factor to the RNA may be that it is involved in the 'take-over' of the cell's protein synthesizing machinery by the phage. By binding to the RNA, the factor could be kept away from host-specific mRNAs, thus resulting in some degree of inhibition of host-specific macromolecule synthesis (Weissmann, 1974), together with a switchover to phage-specific protein synthesis.

Furthermore, it has been proposed that when 70S ribosomes translating the f2 coat protein gene reach the termination signal for the gene, the 50S subunit is released first, resulting in a transient 30S mRNA complex (Martin and Webster, 1975). IF3, by binding to the 3' end of the molecule, would be in relatively close proximity to the released 30S subunit to which, since the affinity of the subunit for IF3 is greater than MS2 RNA (Vermeer et al., 1973a), the factor may transfer, thereby preventing reassociation with the 50S subunit and also

facilitating reinitiation at the coat protein initiation site. IF3 released upon 30S initiation complex formation could be 'picked-up' by the 3' end of the mRNA and thus be ready to bind to the next 30S subunit resulting from termination of the polysome at the termination codon.

Thus, in this model, IF3 by binding to MS2 RNA may be involved in the 'take-over' of the host cell by the phage and also, at least during early stages of infection, when the amount of IF3 outweighs that of the RNA, may be involved in the efficient translation of the phage mRNA.

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