

ASPECTS OF THE MOLECULAR BIOLOGY OF TWO PLANT PATHOGENS

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by

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ABSTRACT

This thesis is in two parts. In the first part, the extrachromosomal DNA of *Fusarium oxysporum* f.sp. *lycopersici*, the tomato wilt fungus, was examined, following a report of a 46.7 kbp circular DNA molecule in the fungus which was correlated with both pathogenicity and resistance to the antibiotic nifuroxime. This work shows that no plasmid-like DNA can be extracted from the fungus. The DNA extracted from nuclease-treated mitochondria was shown, by electron microscopy and restriction mapping, to be a 46.5 kbp circular DNA molecule. This mitochondrial DNA showed no correlation with pathogenicity or resistance to nifuroxime. Indeed, the phenotypes reported for the isolates used in this work were shown to be not reproducible. The mitochondrial DNA was also shown by its characteristic *HaeIII* digestion pattern to occur in subcellular fractions in which the 46.7 kb circular DNA had previously been found, indicating that the previously reported DNA was mitochondrial DNA. Mitochondrial DNA was also detected in an isolate previously reported to lack the circular DNA molecule.

In the second part, the genome organisation and expression of the plant bipartite RNA virus, red clover necrotic mosaic virus, was studied. It was shown that the 5'-ends of the viral RNAs were blocked to end-labelling, but that there was no evidence for a genome-linked protein. A set of cDNA clones made from viral RNA were constructed in the *E. coli* plasmid pUC8, for use in analysis of RNA from virus particles and from infected plants. Northern blotting showed that the main species of RNA from both virus and infected plants were the two genomic RNAs. Three dsRNA species were

extracted from infected plants, of which the two larger species cross-hybridised with RNA-1, and the smallest hybridised to an RNA-2-specific clone. *In vitro* translation showed that RNA-1 gave rise to proteins of 38 K and 55 K, and RNA-2 (selected by a specific clone) to a protein of 34 K. The RNA-2 fraction extracted from a gel was translated to both the 34 K protein and the 40 K coat protein; the latter was probably translated from a subgenomic RNA derived from RNA-1, corresponding to the extra dsRNA band. This subgenomic RNA was encapsidated in virions to a small extent.

DEDICATION

This thesis is dedicated to my mother, Sarah, and to the memory of my father, Frank.

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ABBREVIATIONS

A ₂₆₀	absorbance at 260 nm
AfV	<i>Aspergillus foetidus</i> virus
APT	aminophenylthioether
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CIA	chloroform/isoamyl alcohol (see section 2.3.1)
CIP	calf intestinal phosphatase
c.p.m.	counts per minute
DEAE	diethylaminoethyl
DMSO	dimethylsulphoxide
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DPT	diazophenylthioether
ds	double-stranded (RNA etc.)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
Ggt	<i>Gaeumannomyces graminis</i> var. <i>tritici</i>
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
hr	hours
Iac	iodoacetate
IgG	immunoglobulin G
K	kilodalton

kb	kilobase (pairs)
m ⁷ GMP	7-methylguanosine 5'-monophosphate
min.	minutes
MOPS	3-[N-morpholinolpropanesulphonic acid
mtDNA	mitochondrial DNA
nDNA	nuclear DNA
Nfx	nifuroxime
NTP	nucleoside triphosphate
PAGE	polyacrylamide gel electrophoresis
PDA	potato dextrose agar
PEG	polyethylene glycol
PIPES	piperazine-N,N'-bis[2-ethanesulphonic acid]
<i>Psv</i>	<i>Penicillium stoloniferum</i> virus
RCNMV	red clover necrotic mosaic virus
RNase	ribonuclease
r.p.m.	revolutions per minute
rRNA	ribosomal RNA
SCH	sucrose casein hydrolysate (see section 2.3.3)
SDS	sodium dodecyl sulphate
ss	single-stranded (RNA etc.)
TAP	tobacco acid pyrophosphatase
TCA	trichloroacetic acid
TdT	terminal deoxynucleotidyl transferase
TEMED	N,N,N',N'-tetramethylethylenediamine
TMV	tobacco mosaic virus
tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
UV	ultraviolet

PART A. EXTRACHROMOSOMAL DNA IN *FUSARIUM OXYSPORUM*F.SP. *LYCOPERSICI*.CHAPTER 1. INTRODUCTION.1.1 *FUSARIUM OXYSPORUM*1.1.1 The fungus

Fusarium is a genus of the form-family Tuberculariaceae of the form-order Moniliales of the form-class Deuteromycetes, the imperfect fungi. These fungi reproduce only asexually, by means of conidial spores; any sexual stages are classified in other classes of the fungi. Deuteromycetes have septate branched hyphae with multinucleate compartments, with a cell wall composed of chitin and glucan (Bartnicki-Garcia, 1968), and are classified on the basis of the spore-bearing structures. The Tuberculariaceae are characterised by possession of a sporodochium, a conidiophore-bearing structure.

The *Fusaria* have a great variability in structure, usually with long crescent-shaped macroconidia borne on sporodochia, and small oval microconidia on single hyphae. The mycelium also produces chlamydospores, thick-walled resistant resting spores. The microconidia consist of a single cell with one nucleus, whereas the macroconidia have 3 to 5 cells with one nucleus each - the nuclei are genetically identical (Puhalla, 1981). The sexual stages of *Fusarium*, where known, are ascomycete fungi of the genera *Nectria* and *Gibberella*. Many *Fusaria* cause diseases of plants, including wilts and rots of tomato, pea, melon, potato, cotton,

flax, and ornamental plants (Snyder & Hansen, 1940). Some species are saprophagous.

The vascular wilt diseases form the species *F. oxysporum*, which is divided into 25 form-species on the basis of host specificity (Snyder & Hansen, 1940). *F. oxysporum* has one nucleus per hyphal cell, and four chromosomes per haploid genome (Puhalla, 1981). The form-species are morphologically and physiologically identical, differing only in their host-range. The form-species *melonis*, *niveum* and *cucumerium* are interconvertible by mutation, and some isolates can have two or three of the phenotypes simultaneously (Bouhot, 1981).

F. oxysporum f.sp. *lycopersici* causes the vascular wilt disease of tomato. It can be cultured in the laboratory on solid media such as potato dextrose agar, on which its optimum growth is at 24-28°C; the fungus dies at temperatures above 37°C or below 8°C (Haymaker, 1928). In liquid culture, in sucrose casein hydrolysate, the fungus produces masses of microconidia, which can be used to inoculate tomato plants. The spores enter through damaged roots, and the fungus spreads upwards through the plant. It sporulates in the xylem of the plant and the spores are carried upward by the transpiration stream until they are caught at the end walls of the vessel. Here the spores germinate and sporulate again. As this cycle repeats, the fungus ramifies stepwise up the plant stem (Aist, 1981). This mode of movement is important in enabling the fungus to outdistance the host resistance responses. *F. oxysporum* f.sp. *lycopersici* has optimal pathogenicity at 28°C (Jones & Woltz, 1981), causing stunting of the host, yellowing and wilting of the leaves, epinasty, and vascular browning which is visible in stem sections. At lower temperatures, the

fungus moves into the lower stem of the host but does not cause any external symptoms (Jones & Woltz, 1981).

Strains of *F. oxysporum* f.sp. *lycopersici* also cause root and crown rot of tomato (Leary & Endo, 1971). In this case the fungus enters the roots of the plant and progresses by means of intracellular hyphae through the cortex and xylem. The xylem is observed to be discoloured several cm in advance of the mycelium. There are two types of the crown rot pathogen, orange and purple (Leary, 1972), which differ in pigment production, morphology, and severity of symptoms in the host. Studying mutants of the purple type showed no correlation between purple colour and pathogenicity (Sanchez *et al.*, 1975).

1.1.2 Enzymes involved in pathogenicity

A range of plant cell wall degrading enzymes are produced by phytopathogenic fungi, including *F. oxysporum*. Plant cell walls consist mainly of fibrils of cellulose (a glucan) in a matrix of hemicellulose (a xylan containing some galacturonate) and pectin. The pectin consists of a backbone of polygalacturonate, with some rhamnose, with blocks of arabinogalactan (a galactose backbone with arabinose side-chains). The most important enzymes involved in degradation of the cell walls are the 'pectinases' (Byrde, 1982). This term covers polygalacturonases and pectin lyases which attack the rhamnogalacturonan chains, and arabinanases and galactanases which degrade the arabinogalactan blocks. Enzymes which cleave the polysaccharide chains in random locations (the 'endo' enzymes) are the principal causes of wall breakdown. One particular fungus usually has multiple forms of each type of enzyme (isoenzymes): each form may be adapted to a particular environmental situation (Byrde, 1982).

When *F. oxysporum* f.sp. *lycopersici* is cultured in a medium containing tomato cell walls, the first enzymes produced are the pectinases, and the activity of these enzymes is critical in the early stages of cell wall degradation *in vivo* (Cooper & Wood, 1975). When cultured on tomato cell walls, the enzymes are produced in the order: endopolygalacturonase (endoPG), endo-pectin transeliminase (endoPTE), arabinanase, β -galactosidase, xylanase, and cellulase. EndoPG and endoPTE are induced by D-galacturonate, arabinanase is induced by L-arabinose, β -galactosidase is induced by L-arabinose and D-galactose, and cellulase is induced by cellobiose. The synthesis of these enzymes is also under the control of catabolites - their synthesis is inhibited by the addition of readily metabolised carbon-sources (Cooper & Wood, 1975). Another important enzyme is pectin methylesterase (PME): the galacturonate subunits of the pectin are mostly esterified, as the methyl ester, and PME activity may be required to allow the PG to attack (MacHardy & Beckman, 1981). Polygalacturonate activity has been detected in infected tomato, as has PTE and hemicellulase activity. Cellulases are unimportant in pathogenesis, but may be active in the final stages of the disease (MacHardy & Beckman, 1981).

The wilt symptoms in the plant may result from impaired vascular transport. Possibly the vessels are blocked by pectic gels produced by the pectolytic enzymes (Jones *et al.*, 1982); the xylem may also become blocked by polysaccharides and glycoproteins secreted by the fungus (Dimond, 1972). It has been observed that inoculating tomato plants with pectinases induces wilt symptoms (Jones *et al.*, 1972). However, mutants of *F. oxysporum* f.sp. *lycopersici* made by ultraviolet irradiation of microconidia, which lacked PG activity, were still moderately pathogenic

(i.e. no stunting of the plants was observed) (McDonnell, 1958). These plants did show epinasty, yellowing and vascular occlusion, showing that pectolytic activity is not necessary for pathogenicity. PME activity was still associated with these mutants (McDonnell, 1958; Puhalla, 1981), and PME seems to have a major role in symptom development (MacHardy & Beckman, 1981).

1.2 EXTRACHROMOSOMAL DNA IN FUNGI

1.2.1 Introduction

Extranuclear inheritance in fungi was first noticed in *Saccharomyces cerevisiae* for the 'petite' mutants: the mutation segregates in a non-Mendelian manner following a cross with the wild-type yeast. This mutation has since been shown to be an alteration in mitochondrial DNA (see section 1.3.1). Other extranuclear phenotypes in fungi are also due to alterations in mitochondrial DNA, in several cases leading to the formation of autonomously replicating plasmids. As well as mitochondrial DNA and the plasmids derived from it, some fungi also contain other plasmids: these have no homology to either the mitochondrial genome or the chromosomes, and can be found either within the mitochondria or outside them, in some cases being associated with the nucleus.

1.2.2 Mitochondrial DNA in fungi

Mitochondria are cell organelles found in all eukaryotes, and are involved in respiration, oxidative phosphorylation and fatty acid biosynthesis. They possess their own DNA and protein synthesis machinery. The mitochondrial DNA of fungi ranges from 19 to 115 kb in size, being larger

Table 1.1 Fungal mitochondrial DNAs.

Species	Size (kb)	Structure	Reference
<i>Achlya ambisexualis</i>	49.8	c	
<i>Allomyces macrogynus</i>	49	?	
<i>Aspergillus amstelodami</i>	40.5	c	1
<i>Aspergillus nidulans</i>	31.5	c	
<i>Brettanomyces anomalus</i>	56.5	c	
<i>Brettanomyces custersii</i>	108	?	
<i>Candida parapsilosis</i>	35	c	
<i>Candida tropicalis</i>	52	c	
<i>Cephalosporium acremonium</i>	26.7	c	
<i>Claviceps purpurea</i>	45	c	
<i>Cochliobolus heterostrophus</i>	115	c	6
<i>Dekkera bruxellensis</i>	75	?	
<i>Dekkera intermedia</i>	63.5	?	
<i>Fusarium oxysporum</i>	46.5	c	2
<i>Gaeumannomyces graminis</i>	60	?	3
<i>Hansienaspora vinea</i>	26.7	?	
<i>Hansenula mrakii</i>	55	1	
<i>Hansenula wingei</i>	25.5	c	
<i>Kloeckera africana</i>	27.1	c	
<i>Kluyveromyces lactis</i>	36.4	c	
<i>Neurospora crassa</i>	60-73	c	
<i>Neurospora intermedia</i>	62-71	c	4
<i>Neurospora sitophila</i>	65	c	4
<i>Pachytrichospora transvaalensis</i>	41.4	?	

Table 1.1 continued

<i>Pichia lindneri</i>	52	c	
<i>Pichia pinus</i>	35.1; 76.9	c	
<i>Phytophthora infestans</i>	36.2	c	
<i>Podospora anserina</i>	95	c	
<i>Podospora curvicolla</i>	55	c	
<i>Saccharomyces carlsbergensis</i>	66.4	c	5
<i>Saccharomyces cerevisiae</i>	68-77.8	c	
<i>Saccharomyces exiguus</i>	23.7	c	
<i>Saccharomyces telluris</i>	34.8	?	
<i>Saccharomyces unisporum</i>	27.4	c	
<i>Saccharomyces uvarum</i>	61	c	
<i>Saccharomycopsis lipolytica</i>	48.5	c	
<i>Saprolegnia</i> sp.	42	c	
<i>Schizosaccharomyces pombe</i>	19.3	c	
<i>Torulopsis glabrata</i>	18.9; 20.3	c	
<i>Trichosporum cutaneum</i>	34	c	
<i>Ustilago cynodontis</i>	75	?	

Notes:

Structures: c, circular; l, linear; ?, not determined.

References: data was derived from Böckelmann *et al.* (1986), except for: 1, Lazarus *et al.*, 1980; 2, this thesis; 3, McFadden & Buck, 1983; 4, Collins & Lambowitz, 1983; 5, Sanders *et al.*, 1975; 6, Garber & Yoder, 1984.

than that of animals (14-18 kb) and smaller than that of plants (220-2400 kb) (Dawson *et al.*, 1984). The fungal mitochondrial DNAs (mtDNAs) which have been characterised are listed in table 1.1. In all cases where known, the mtDNA is circular, with the sole exception of the linear mtDNA of *Hansenula mrakii* (Wesolowski & Fukuhara, 1981); in several cases the DNA was isolated initially as linear fragments (Schäfer *et al.*, 1971; Hollenberg *et al.*, 1970) and was subsequently shown to be circular. Fungal mtDNAs encode ribosomal and transfer RNAs, plus 5-10 % of the mitochondrial proteins (the other proteins are nuclear products and are transported into the mitochondria) (Böckelmann *et al.*, 1986). MtDNA-encoded proteins include the three largest subunits of cytochrome c oxidase, a subunit of cytochrome b, subunits 6, 8 and 9 of ATPase (Tzagoloff *et al.*, 1979), and in the case of *Saccharomyces cerevisiae* RNA maturases. These are enzymes responsible for the correct splicing of mitochondrial transcripts, and are coded for by open reading frames within the introns of several genes on the mtDNA. Other fungi also contain long open reading frames in their mtDNA introns, which would encode proteins with homology to the RNA maturases; the second intron of the *cob* gene of *Neurospora crassa* could code for a maturase similar to those of the yeast *cob* gene, which are required for the correct splicing of transcripts of the *cob* and *oxi3* genes (Hellmer Citterich *et al.*, 1983). As shown in table 1.1, different species of the same genus can have mtDNAs greatly different in size, and there can also be differences between the mtDNAs of different strains of the same species. These differences are due in part to the large AT-rich regions which separate the genes, and the extensive untranslated regions at either end of the genes which are transcribed into mRNA (Böckelmann *et al.*, 1986). Also,

each fungus can have a different set of introns, and these can vary between strains of the same species. For example, the cytochrome c oxidase subunit I (COI) gene varies in size from 1.7 kb in *Neurospora crassa* (no introns) (Burger *et al.*, 1982) to 25 kb in the closely related *Podospora anserina* (at least 9 introns) (Böckelmann *et al.*, 1986). Usually the genes for cytochrome b, ATPase subunit 6, and large ribosomal RNA have introns as well as the COI gene; again this may vary between strains e.g. the COI gene of *N. crassa* has none, one, 3 or 4 introns depending on the strain (Collins & Lambowitz, 1983).

Mitochondrial DNA contains sequences which are recognised by the mitochondrial DNA polymerase as origins of replication (*ori* sequences). *Saccharomyces cerevisiae* mtDNA has at least 7 such sequences, characterised by a common stretch of 265 bp containing three short GC-rich clusters and an AT-rich palindrome of 23 bp (de Zamaroczy *et al.*, 1981).

Mitochondrial genes are transcribed by the nuclear RNA polymerases, and a nonanucleotide sequence which occurs at the 5'-ends of both rRNA genes in *S. cerevisiae* and *K. lactis* has been identified as a yeast mitochondrial promoter (Osinga *et al.*, 1982). The genetic code used in mitochondria differs slightly from the universal code, for example UGA, the universal 'stop' codon, codes for tryptophan in mitochondria (Heckman *et al.*, 1980). Also the number of tRNAs is very small in mitochondria, with only 24 required to read the full set of codons, as opposed to the usual 32. This is due to the ability of tRNAs with U as the first base of the anticodon to read all four codons of a four-codon family, and tRNAs with a modified U as first base of the anticodon to read codons with either purine at the 3'-end (Heckman *et al.*, 1980).

In some drug-resistant mutants of fungi, the mutation maps on the mtDNA. Oligomycin is an inhibitor of the mitochondrial ATPase, and mutants in the gene for subunit 9 of the ATPase confer resistance. This gene is located on the mtDNA in yeast, although it is a nuclear gene in *Aspergillus* and *Neurospora* (Tzagoloff *et al.*, 1979). Chloramphenicol is an inhibitor of mitochondrial protein synthesis, and both mitochondrial and nuclear resistant mutants have been described (Lazarus & Turner, 1977). In yeast, mitochondrial resistance to chloramphenicol is due to an alteration in the sequence of the large rRNA gene (Dujon, 1980). Mitochondrial mutants resistant to erythromycin also map in the large rRNA gene, mutants resistant to paromomycin map in the small rRNA gene, and mutants resistant to antimycin map in the cytochrome b gene (Tzagoloff *et al.*, 1979).

1.3 FUNGI WITH ALTERED MITOCHONDRIAL DNA

1.3.1 *Saccharomyces cerevisiae* 'petite' mutation

The 'petite' (or ρ^-) mutants of yeast are so called due to their slow growth rate, due to a deficiency in respiration. When crossed with a normal 'grande' strain, all four ascospores from the diploid cell are 'grande', showing the extranuclear character of the mutation. 'Petite' mutants have a complete lack of mitochondrially-encoded proteins, due to the deletion of a large region of the mtDNA, but can still grow since *S. cerevisiae* is a facultative anaerobe (Locker *et al.*, 1979). As much as 70 % or as little as 0.3 % of the wild-type mtDNA may be retained. The retained fragment can be from any part of the mtDNA so long as one of the seven or so *ori* sequences is retained, allowing the molecule to be replicated. The different efficiencies of the *ori* sequences may be responsible for different degrees of suppressiveness and unequal retention of markers from different map regions during mutation from ρ^+ to ρ^- . Suppressiveness is the ability of the 'petite' phenotype to be dominant over wild-type in crosses, and is due to the replicative advantage of the 'petite' mtDNA over wild-type mtDNA (de Zamaroczy *et al.*, 1981). All 'petite' mutants have the same phenotype since even the smallest deletion is sufficient to block the mitochondrial protein biosynthetic pathway. The retained fragment is amplified, and is found as long linear and circular multimers. The mechanism of generation of the amplified circles seems to involve excision of a mtDNA region at sites of partial DNA sequence homology, probably the AT-rich spacer regions. These smaller circles may be at a replicative advantage, or may reinsert into

full-length mtDNA, producing an instability leading to further breakdown of the mtDNA (Clark-Walker & Miklos, 1974).

1.3.2 *Neurospora crassa* [poky] mutation

The group I extranuclear mutants of *N. crassa* are characterised by slow growth, gross deficiencies of cytochromes aa₃ and b, excess of cytochrome c, and lack of the small rRNA of the mitochondria, leading to a deficiency of mitochondrial protein synthesis. The phenotype is cytoplasmically inherited (Mitchell & Mitchell, 1952). The primary defect in [poky] and other group I mutants is a deletion of 4 bp from the 5'-end of the 19S rRNA gene, which leads to a destabilised rRNA and probably impairs processing to the mature RNA (Akins & Lambowitz, 1984). This defect then results in a gross lack of small ribosomal subunits. Other alterations such as deletions or integrated amplifications of parts of the mtDNA can occur in some mutants (Collins & Lambowitz, 1981), for example [poky] and [exn1]548 retain one-third to one-half of the wild-type mtDNA, and have tandem repeats of a 2 kb sequence which may represent a major origin of replication. Not all group I mutants have deletions or amplifications, but the defect in mitochondrial ribosomes may lead to the generation or stabilisation of such altered mtDNAs (Collins & Lambowitz, 1981).

1.3.3 *Neurospora crassa* 'stopper' mutation

The 'stopper' or [stp] phenotype is characterised by a stop/start mode of mycelial growth, lack of cytochromes b and aa₃, excess of cytochrome c and non-Mendelian inheritance (a group III extranuclear mutation). This phenotype is due to an alteration in the mtDNA; the lesion is different to

that in the [*poky*] mutation, since group I and group III mutants can form normally-growing heteroplasmons (Bertrand & Pittenger, 1972). In wild-type *N. crassa*, most of the genes for mitochondrial protein synthesis are located in a 21 kb region bounded by direct repeats of the tRNA^{Met} genes (Gross *et al.*, 1984). In the 'stopped' phase of growth, the mitochondria contain a 21 kb circular molecule (cf. normal mtDNA is 60-65 kb; Bernard *et al.*, 1975), plus heteromorphic linear and nicked circular fragments, leaving about 20 % of the original mtDNA deleted. In some 'stopper' mutants, a region of 2 kb is tandemly amplified a variable number of times: this region may represent a major origin of replication (Collins & Lambowitz, 1981). When active growth recommences, two circular DNAs are found, the 21 kb circle and a 43 kb circle which is found in lower amounts. The two DNA circles are formed by intramolecular recombination at the site of the two tRNA^{Met} genes: the 21 kb molecule contains the *oxi2* and rRNA genes; the 43 kb molecule contains the remainder of the original mtDNA less a deleted region of variable size. This deletion leads to the genes *cob* and *oxi3* (which code for cytochrome b and a subunit of cytochrome c oxidase) being underrepresented (Gross *et al.*, 1984).

1.3.4 *Aspergillus amstelodami* 'ragged' mutation

The 'ragged' or *rgd* mutants of *A. amstelodami* have a similar phenotype to the mitochondrial mutants of *Neurospora* described above, namely a stop/start mode of mycelial growth, lack of cytochrome aa₃, excess of cytochrome c, and are cytoplasmically inherited. They are further characterised by the irregular growth of the colonies (Caten, 1972). The mitochondria of the *rgd* mutants contains the normal wild-type mtDNA of 40.5 kb, plus circular molecules composed of tandem repeats of a 0.9-2.7

kb monomer which is derived from the mtDNA (Lazarus *et al.*, 1980; Lazarus & Kuntzel, 1981). Of six *rgd* mutants analysed, in five cases the amplified "rgdDNA" was derived from one region of the mtDNA. Sequence analysis of three *rgd*DNAs revealed a common region of 215 bp in the amplified DNA, which has homology to an *ori* sequence of *S. cerevisiae* (Lazarus & Kuntzel, 1981). Loss of growth may be due to competition for the DNA replicative machinery by the amplified sequence, leading to lack of replication of the mtDNA.

1.3.5 Senescence in *Podospora anserina*

When *P. anserina* is grown mycelially, after a period of linear growth the growth rate declines and stops, ending in the death of the hyphae. The life span is specific for each different race (genotype) of the fungus. The senescence factor is maternally inherited, and is transmitted asexually by hyphal anastomoses, indicating its extranuclear location. A plasmid-like circular DNA (plDNA) was first detected in senescent mycelium of strain *s* as oligomeric supercoiled molecules by Stahl *et al.* (1978) - subsequently a range of similar DNAs have been detected in senescent mycelia, usually as circles composed of tandem repeats of a monomeric unit, and these are listed in table 1.2.

PlDNA was identified as the senescence factor by its ability to transform juvenile (i.e. non-senescent) cells to senescence (Tudzynski *et al.*, 1980). Plasmid-like DNA is found in all senescent mycelia, but not in juvenile cells. Hybridisation experiments showed that plDNA is derived from a specific region of the juvenile mtDNA (Kück *et al.*, 1981). In senescent cells, the juvenile mtDNA (a 95 kb molecule) is replaced by a 30 kb

Table 1.2 Plasmid-like DNAs of *Podospora anserina*.

Plasmid-like DNA	Monomer size	Conformation	Location on mtDNA	References
p1DNA	2593 bp	ccc	Intron 1 of COI	1,2,3
(= α senDNA)		tandem rep.	(= <i>oxi3</i>)	
β senDNA	9.8 kb	ccc	COIII (= <i>oxi2</i>)	4
		tandem rep.		
γ senDNA	?	?	Large rRNA	4
δ senDNA	?	?	Near COII (= <i>oxi1</i>)	4
θ senDNA	4-6.3 kb	ccc	COI ?	5,6
	(common 1.1 kb)	tandem rep.		

Notes: conformation: ccc, covalently closed circular; tandem rep., tandemly repeated monomer.

References: 1, Stahl *et al.*, 1980; 2, Kück *et al.*, 1981; 3, Stahl *et al.*, 1982; 4, Wright *et al.*, 1982; 5, Jamet-Vierny *et al.*, 1980; 6, Vierny *et al.*, 1982.

molecule which no longer hybridises to plDNA, showing that about two-thirds of the juvenile mtDNA has been deleted (Kück *et al.*, 1981).

The plDNA was linearised with *Sall* and cloned into the bacterial plasmid pBR322 (Stahl *et al.*, 1982). The hybrid plasmid is able to replicate in both *E. coli* and *P. anserina*, conferring senescence on the latter. This shows that the hybrid plasmid contains a eukaryotic *ori* sequence and is expressed in *P. anserina*; in fact, the prokaryotic portion is also expressed in *P. anserina* (the β -lactamase product was detected), although pBR322 alone is unable to replicate in the fungal host (Stahl *et al.*, 1982). When a restriction fragment of juvenile mtDNA, which included about 25 % of the plDNA sequence, was cloned in pBR322, this hybrid was also able to replicate in *P. anserina* and to induce senescence.

The mutant *mex1* grows slowly but does not become senescent.

Hybridisation studies showed that a 2.6 kb region of the mtDNA, corresponding to the plDNA sequence, has been deleted (Vierny *et al.*, 1982). The plDNA region, part of the *oxi3* gene, was shown to be integrated into the nuclear DNA of *mex1* as a single copy (Wright & Cummings, 1983a). These authors also showed that in senescent mycelia, plDNA was found integrated in the nuclear DNA as tandem repeats, and β senDNA (corresponding to part of the *oxi2* gene) was integrated in the nuclear DNA as a single copy. None of these sequences were detected in the nuclear DNA of juvenile cells. A strain of *P. anserina* which senesces very rapidly, race A⁺, was found to contain plDNA as free plasmid even in juvenile cells, leading to the hypothesis that the concentration of plDNA in the cells determines the life-span of the mycelium - possibly a high concentration of plDNA causes breakdown of the mtDNA by homologous recombination, leading to breakdown of the mitochondria and release of

the plDNA which is then free to integrate into the nuclear DNA (Wright & Cummings, 1983a). The plDNA is transcribed *in vivo* to an RNA of 2.4-2.5 kb, nearly the full length of the DNA (Wright & Cummings, 1983b). The complete nucleotide sequence of plDNA has been determined. It is 2539 bp long, and corresponds precisely to the first intron of the COI gene (Osiewacz & Esser, 1984). It contains an open reading frame which would be translated to a protein with some homology to the *Saccharomyces cerevisiae* RNA maturase enzymes. Both the plDNA, and another intron in the COI gene of the rapidly senescing race A, are classified as type II introns, and the proteins deduced from their DNA sequences have amino acid sequence homology to reverse transcriptases (Matsuura *et al.*, 1986). Since plDNA corresponds to an intron, it could arise by reverse transcription of the circular RNA released by intron excision, and reverse transcriptase activity has been detected in "middle-aged" race A cells (Matsuura *et al.*, 1986). The other amplified plasmids, which are never found without plDNA also (Wright *et al.*, 1982), seem to have more variable excision sites.

1.3.6 Other fungi with altered mitochondrial DNA

Podospora curvicolle also undergoes senescence, although unlike *P. anserina* it is able to resume growth after the stoppage. It does not have a region analogous to the plDNA of *P. anserina*, although circular tandem amplifications are found. The monomer units are 9.0-10.9 kb long, with variable excision sites, and all are derived from the large rRNA gene, as is the γ sendNA of *P. anserina* (Böckelmann & Esser, 1986). Isolate T40 of *Cochliobolus heterostrophus* ceases growth after 30-50 cm, unlike other isolates. Only T40 is observed to contain a tandemly

amplified plasmid, of monomer length 1.9 kb, in addition to the normal mtDNA (Garber & Yoder, 1984). The plasmid is derived from the mtDNA, and is transcribed to RNA *in vivo* (Yoder & Turgeon, 1985).

The [kal] mutants of *Neurospora intermedia* undergo irreversible senescence, correlated with deficiencies of cytochromes aa₃ and b, excess of cytochrome c, and altered mtDNA. These effects also correlate with the appearance of a unique fragment resembling a transposable element which does not hybridise to juvenile mtDNA (Griffiths & Bertrand, 1984).

1.4 FUNGAL PLASMIDS

1.4.1 The 2 µm plasmid of *Saccharomyces cerevisiae*

Most strains of *S. cerevisiae* contain a circular DNA plasmid which is 2 µm in length. It is present at 60-70 copies per cell, forming 2.4-3 % of the total cellular DNA, and has no homology with either the chromosomes or the mtDNA (Guérineau, 1979). It has a chromatin-like structure with the same 165 bp nucleosomal repeat length as the nuclear DNA (Nelson & Fangman, 1979), but is never found integrated into the genome (Cameron *et al.*, 1977). From restriction mapping (Cameron *et al.*, 1977) and more recently from the complete nucleotide sequence (Hartley & Donelson, 1980), the plasmid has been determined to exist in equal amounts of two isomers. The sequence of 6318 bp contains two non-tandem inverted repeats of 599 bp, and intramolecular recombination at these sites leads to two isomers differing in the orientation of the two unique regions, of 2346 and 2774 bp, with respect to each other. Such recombination could also lead to the formation of oligomers which have also been detected (Guérineau *et al.*, 1971). The sequence also shows three open reading frames, which would

code for proteins of 49,000, 43,000 and 33,000 molecular weight (Hartley & Donelson, 1980). Two of these reading frames correspond to the genes *REP1* and *REP2*, which are involved in the stable propagation and maintenance of a high copy number of the plasmid; a further locus, *REP3*, is also involved, and this region corresponds to direct repeats of a 62 bp sequence of the 2 μ m DNA. The origin of replication of the plasmid is in a 450 bp region which possesses much symmetry, and could be folded to produce large secondary structures (Hartley & Donelson, 1980). Replication of the 2 μ m plasmid requires the nuclear replication machinery, and is usually under control of the cell cycle (Jazwinski & Edelman, 1979).

Replication takes place early in the S-phase of the cell cycle, unless the copy number is too low, in which case the *REP* genes take over until the correct copy number is reached. The other gene of the plasmid, *FLP* (corresponding to the 49,000 molecular weight protein), is involved in the intramolecular recombination, and also promotes recombination of the bacterial transposon Tn5 (Jayaram & Broach, 1983).

The 2 μ m plasmid is probably located in the nucleus, at least during the S phase of the cell cycle, since it is associated with histones and it requires the chromosomal DNA replication machinery (Nelson & Fangman, 1979); furthermore, staining of cells with the fluorescent dye 4',6-diamidino-2-phenylindole indicates that the 2 μ m DNA is confined to the nucleus (Gunge *et al.*, 1982).

The function of the 2 μ m plasmid, other than maintenance of its own DNA, is obscure. One early report correlates loss of the plasmid with loss of resistance to oligomycin (Guérineau *et al.*, 1974) in spontaneous mutants; it has not been shown that regaining the plasmid restores resistance (Böckelmann *et al.*, 1986).

1.4.2 The plasmids of *Neurospora*

The first of these plasmids to be detected was in *N. crassa* strain Mauriceville-1c, and was noticed as an extra band visible after electrophoresis of an *Eco*RI digest of mtDNA (Collins *et al.*, 1981). Electron microscopy showed a monomer of 3.6 kb, plus a series of oligomers at least up to a hexamer. Restriction mapping confirmed the 3.6 kb monomer size, and revealed an unusual cluster of five *Pst*I sites within a 0.4 kb region (Collins *et al.*, 1981). Although the plasmid has no homology with the mtDNA, as demonstrated by Southern blotting, it was shown to be located within the mitochondria: the plasmid was isolated intact from nuclease-treated mitochondria, but this protection was lost in the presence of 0.1 % Triton X-100 which disrupts the mitochondrial membranes. The plasmid has no detectable effect on the phenotype of *N. crassa*, although Northern blotting experiments showed that the plasmid is transcribed *in vivo* (Collins *et al.*, 1981). A major transcript of 3.5 kb was detected, and a protein of 41-44,000 molecular weight was tentatively identified as not being present in mitochondria of strains lacking the plasmid. Subsequent sequence analysis has shown that the "Mauriceville" plasmid is 3581 bp long, with a single long open reading frame which would encode a protein of 82,000 molecular weight (Nargang *et al.*, 1984). The major transcript is equivalent to virtually the full length of the plasmid. The *Pst*-cluster consists of 8 *Pst*I sites arranged in five palindromic elements: the outermost of these elements are identical to an 18 bp GC-rich sequence found many times throughout the *N. crassa* mtDNA; the other three elements differ from this sequence by a few base-pairs. Analysis of the codon usage and the conserved sequences of the "Mauriceville" plasmid show that it is related to the group I mtDNA

introns (Nargang et al., 1984). Mitochondrial introns may have arisen by integration of such plasmids, since some introns encode proteins (RNA maturases); alternatively, the plasmid could have arisen from reverse transcription of an excised intron. The plDNA of *Podospora anserina* is also derived from a mitochondrial intron, in this case a group II intron in the COI gene (see section 1.3.5).

Similar plasmids have been detected in *N. intermedia* strains P405-Labelle and Fiji N6-6 (Stohl et al., 1982). These plasmids, with monomer sizes 4.1 kb and 5.2 kb respectively, are unrelated to either each other or to the "Mauriceville" plasmid. Like the "Mauriceville" plasmid they are located within the mitochondria while having little or no homology to the mtDNA, and have no apparent effect on the phenotype. Unlike the "Mauriceville" plasmid, the "Labelle" and "Fiji" plasmids do not have a cluster of *Pst*I sites, and no transcripts are detected *in vivo* (Stohl et al., 1982). A plasmid has also been detected in *N. intermedia* strain Varkud-1c: this plasmid is very similar to the "Mauriceville" plasmid (Nargang et al., 1984). It has a 3.8 kb monomer and is transcribed *in vivo*.

Further plasmids have been isolated from the mitochondria of four isolates of *N. tetrasperma*, namely "Hanalei" (5.0 kb monomer), "Lihue", "Surinam" and "Waimea Falls", which have similar but not identical restriction patterns (Natvig et al., 1984). Cross-hybridisation studies show three mutually hybridising groups of plasmids in *Neurospora* species: the first consists of the *N. crassa* "Mauriceville" and *N. intermedia* "Varkud" plasmids; the second consists of the four *N. tetrasperma* plasmids and the *N. intermedia* "Fiji" plasmid; and the third group contains the *N. intermedia* "Labelle" plasmid (Natvig et al., 1984).

1.4.3 The killer plasmids of *Kluyveromyces lactis*

Killer strains of *K. lactis* produce a toxin which kills a range of yeasts including non-killer strains of *K. lactis*, and to which the killer strains are immune. In contrast to the killer toxin of *Saccharomyces cerevisiae*, which is specified by a dsRNA mycovirus, the killer phenotype of *K. lactis* is correlated with two DNA plasmids (Gunge *et al.*, 1981). The plasmids, named pGK11 and 2, were shown by electron microscopy to be linear DNA molecules of 8.2 kb and 12.7 kb respectively. Both have a buoyant density in CsCl different to that of both the nuclear DNA and the mtDNA (Gunge *et al.*, 1981). Protoplasts of killer *K. lactis* were fused with protoplasts of *S. cerevisiae*, and in progeny *S. cerevisiae* cells which gained the pGK1 plasmids, the killer phenotype was also gained, showing that the two plasmids produce both killer toxin and immunity, and also that the plasmids are able to be replicated and expressed in *S. cerevisiae* (Gunge & Sakaguchi, 1982). The killer *S. cerevisiae* cells retained their 2 μ m plasmid in addition to the pGK1 plasmids, indicating that the linear plasmids do not use the same replication machinery as the 2 μ m plasmid. Further experiments showed that the plasmid DNAs alone could transform *S. cerevisiae* protoplasts to killer phenotype (Gunge *et al.*, 1982). These authors also showed that cells showing a weak killer phenotype possessed reduced levels of pGK11, indicating that the gene for killer toxin is located on pGK11. Subclones of these weak killers which had no pGK11, although retaining pGK12, were non-killers and were sensitive to the toxin, indicating that the gene for immunity is also on pGK11 (Gunge' *et al.*, 1982). The linear plasmids were shown to be present at 50-100 copies per cell in *K. lactis*, and staining of cells with 4',6-diamidino-2-phenylindole showed that the linear plasmids were present in

the cytosol, unlike the yeast 2 μ m plasmid which is confined to the nucleus (Gunge *et al.*, 1982).

Both pGK11 and pGK12 have their 5'-ends blocked, probably by a protein, and have terminal inverted repeats of 202 bp and 184 bp respectively, which are probably involved in replication (Sor *et al.*, 1983). The nucleotide sequence of pGK11 has been determined (Stark *et al.*, 1984): it is 8874 bp long, with four open reading frames, three of which produce detectable transcripts; the 3.5 kb "ORF 2" probably encodes the large subunit of the killer toxin (Stark *et al.*, 1984).

1.4.4 Other fungal plasmids

(i) Extrachromosomal DNA was reported in *Rhizoctonia solani* by Martini *et al.* (1978), who detected DNA bands of different sizes in cleared lysates of two different races of *R. solani* which differed in host specificity. More recently, a plasmid, named pRS64, was detected in three hypovirulent strains of *R. solani*. Electron microscopy showed that this plasmid was a linear molecule of 2.6 kb (Hashiba *et al.*, 1984). It did not appear to be associated with the mitochondria. The pathogenic parent strain lacked the plasmid, indicating some role in determination of pathogenicity for the plasmid. However, more recent results have established that virulence in *R. solani* is correlated with the presence of viral dsRNA, and that hypovirulent strains lack some or all of the dsRNA segments (Finkler *et al.*, 1985); hence the role of the DNA plasmid in hypovirulence is questionable.

(ii) A series of four linear DNA plasmids have been isolated from *Claviceps purpurea* strain K1, of sizes 6.6 kb (p11), 5.3 kb (p12), 10 kb (p13) and 1.1 kb (p14). Of these, p11 and p12 are the major bands. All

four were isolated from nuclease-treated mitochondria, and shown to be linear by electron microscopy. Hybridisation analysis indicates that pl1, 2 and 4 share common sequences, but are unrelated to the mtDNA. The ends of pl1 and 2 have inverted repeats of 300 bp. Related linear plasmids have been detected in other strains of *C. purpurea*, and in two of these strains parts of the plasmid sequences are found integrated into the mtDNA near the large rRNA gene (Tudzynski & Esser, 1986). The function of these plasmids is unknown, but their occurrence in wild-type strains suggests a possible function in the parasite-host relationship (Böckelmann *et al.*, 1986).

(iii) The morel *Morchella conica* has two linear plasmids of 6.09 and 8.0 kb; the smaller of these has 750 bp inverted repeats at each end (Meinhardt & Esser, 1984).

(iv) A plasmid-like DNA was detected in a total DNA preparation of *Cephalosporium acremonium*, having a buoyant density in CsCl different to that of the nuclear DNA and the mtDNA (Minuth *et al.*, 1982). The DNA was heterogeneous in size as judged by electron microscopy and restriction mapping, although a size of 21.0 kb has been reported (Esser *et al.*, 1983). The plasmid was not associated with the mitochondria.

(v) A linear plasmid of 6.4 kb has been reported in *Ascobolus immersus* (Francou, 1981). This plasmid is maternally inherited, and has 1.2 kb long inverted repeats at the ends. Two further linear plasmids of 5.5 and 7.9 kb have also been detected (Böckelmann *et al.*, 1986).

(vi) Two linear plasmids of 7.4 and 3.7 kb have been found in the mushroom *Agaricus bitorquis*. Both are located within the mitochondria, but have no homology to either mtDNA or nuclear DNA (Mohan *et al.*, 1984).

(vii) Circular DNAs 2 μ m in length (6.4 kb) have been detected in *Schizosaccharomyces pombe* (Del Giudice *et al.*, 1979) and *Saccharomyces italicus* (Jazwinski & Edelman, 1979), which in the latter case is very similar to the 2 μ m plasmid of *S. cerevisiae*.

(viii) A series of 3 μ m circular plasmids have been detected in the yeasts *Torulopsis glabrata*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*. The 3 μ m DNA of *S. cerevisiae* is a 9 kb molecule present at 4-5 copies /cell, and is apparently identical to the nuclear rDNA repeat unit (Clark-Walker & Azad, 1980). The 3 μ m DNAs of *T. glabrata* (Clark-Walker & Azad, 1980) and *S. pombe* (Fournier *et al.*, 1982) are both about 11 kb in length, and also contain complete copies of the nuclear ribosomal genes.

(ix) Finally, extrachromosomal DNA has been reported in *Fusarium oxysporum*: this is reviewed in section 1.6.

Apart from the killer plasmids of *Kluyveromyces lactis*, and a questionable role in pathogenicity for the linear plasmid of *Rhizoctonia solani*, none of the fungal plasmids described has any clear function. However, the wide distribution of such plasmids implies that they may provide some essential, although as yet undiscovered, role in their hosts (Böckelmann *et al.*, 1986).

1.5 GENETIC MANIPULATION OF FUNGI

1.5.1 Genetic manipulation of yeasts

Much of the work on genetic engineering of fungi has concentrated on yeasts, mostly *Saccharomyces cerevisiae*, partly due to the ease of handling of the single-celled organisms and partly due to the extensive characterisation of many genetic loci in yeast. Transformation systems have been developed for a range of yeasts, including the industrially important *Yarrowia* (*Saccharomycopsis*) *lipolytica*.

Transformation of yeasts involves formation of protoplasts, by treatment of the cells first with a reducing agent (2-mercaptoethanol or DTT) and then with a cell wall-degrading enzyme such as β -glucuronidase. The protoplasts are then transformed with exogenous DNA in the presence of calcium chloride and polyethylene glycol (Beggs, 1981). The transformed protoplasts are embedded in a solid matrix (3 % agar) and are allowed to regenerate on suitable selective media.

There are three basic types of yeast vectors, termed yeast integrating plasmid (YIp), yeast replicon plasmid (YRp) and yeast episomal plasmid (YEp). The first report of reproducible transformation of yeast used the YIp vector pYe*leu*10 (Hinnen *et al.*, 1978). The vector consisted of a yeast DNA fragment containing the *LEU2* gene cloned into a bacterial plasmid. The plasmid was isolated from *Escherichia coli* by its ability to complement the *leuB6* mutation of the bacterium. The plasmid transformed *leu2⁻* yeast to leucine prototrophy; transformants were selected by their ability to grow in the absence of leucine. As with other YIp vectors, the plasmid was not replicated in the yeast but became integrated into the nuclear DNA at the site of homology, in this case into chromosome III at

the *LEU2* locus. The phenotype is fairly stable, but about 1 % of cells have lost the phenotype 20 generations after transformation. YIp vectors typically transform 10^{-7} of the viable protoplasts, producing 1-10 transformants/ μ g DNA (Beggs, 1981). Other yeast genes have been cloned in *E. coli* by their ability to complement bacterial mutations. Most antibiotic marker genes are of little use in constructing yeast vectors, since yeast is resistant to most antibiotics: an exception is the antibiotic G418, for which the resistance gene is encoded by the bacterial transposon Tn601, which can be used to transform yeast to G418-resistance (Jimenez & Davies, 1980).

The transformation efficiency of a vector can be greatly increased by including a yeast autonomously replicating sequence (*ars*) in the plasmid. These *ars* sequences are the origins of DNA replication, and occur about 25 times per chromosome or once per 32-40 kb of DNA (Beggs, 1981). An example of these YRp's is YRp7 which consists of the yeast *TRP1* gene and *ars1* sequence inserted into the bacterial plasmid pBR322 (Struhl *et al.*, 1979). When an additional DNA fragment is inserted into the tetracycline-resistance gene of the pBR322 moiety, transformants can be selected by their ampicillin-resistance in *E. coli* and their ability to complement the *trp1*⁻ mutation in yeast. Transformants contain a few copies of free plasmid per cell. However, YRp vectors segregate very unstably at mitotic or meiotic division, even on selective media, and after sporulation of the diploid cells YRp7 is completely lost. When the cloned yeast DNA contains a centromere region, e.g. *CEN3* which is located close to the *LEU2* gene and an *ars* sequence, the plasmid behaves like a mini-chromosome and segregates with Mendelian characteristics (Clarke & Carbon, 1980). YRp

vectors typically transform 10^{-4} of the viable protoplasts, producing 100-10,000 transformants/ μg DNA (Beggs, 1981).

Similar vectors, containing a mitochondrial *ars* sequence, have been made by cloning a 5.4 kb 'petite' mtDNA sequence into YIp5 (Hyman *et al.*, 1982): the resulting YRmp vectors produced somewhat less transformants than YRp vectors (150-500 / μg DNA), but were more stable to mitosis. The YE_p vectors are based on the naturally occurring yeast 2 μm plasmid (section 1.4.1). Plasmid pJDB248, consisting of bacterial plasmid pMB9, the yeast *LEU2* gene, and the entire 2 μm plasmid sequence, transformed *leu2⁻* yeast with an efficiency of 10^3 - 10^5 transformants/ μg DNA (0.1 % of viable protoplasts transformed) (Beggs, 1978). The supercoiled plasmid could be reisolated from the transformants. Vectors need only the 350 bp *ori* region to give high frequency of transformation and multiple copies in yeast cells which already contain the 2 μm plasmid; such vectors are unstable in strains lacking the 2 μm DNA due to the absence of essential parts of the 2 μm sequence (Beggs, 1981).

The plasmid pJDB248 which could transform *leu2⁻* *S. cerevisiae* as described above, also transforms and is replicated in a *leu1⁻* mutant of *Schizosaccharomyces pombe* (Beach & Nurse, 1981).

The 3 μm plasmid (or PMO) of *S. pombe* has also been used as the basis of vectors (Fournier *et al.*, 1982). A plasmid consisting of pBR322, the yeast *URA3* gene and the PMO DNA was used to transform *ura4⁻* *S. pombe* to a *URA⁺* phenotype with an efficiency of about 60 transformants/ μg DNA. The transformants were fairly stable, and also expressed the prokaryotic β -lactamase gene (Fournier *et al.*, 1982).

Protoplasts of *Kluyveromyces lactis* are prepared and transformed in the same way as those of *S. cerevisiae* (Das & Hollenberg, 1982). A vector

based on the yeast 2 μ m replicon was able to replicate in *K. lactis*, but produced only 4-20 transformants/ μ g DNA. Efficiencies of 1000-30,000 / μ g DNA were obtained when certain *K. lactis* DNA fragments were cloned in the vector YRp7, to create the pKARS vectors. These fragments presumably contain *ars* sequences; plasmids containing the *S. cerevisiae ars* sequences are unable to replicate in *K. lactis*. Transformants made using the pKARS vectors were unstable, with 80-100 % losing the transformed phenotype in 10 generations (Das & Hollenberg, 1982).

Transformation systems based on plasmids containing genes from *S. cerevisiae* have recently been developed for other yeasts, for example the *S. cerevisiae ARG4* gene has been expressed in *Candida maltosa* and *Pichia guilliermondii* (Kunze *et al.*, 1985). A plasmid containing the *LEU2* gene of *Yarrowia lipolytica* has been expressed in *leu2⁻* mutants of both *Y. lipolytica* and *S. cerevisiae* (Davidow *et al.*, 1985).

1.5.2 Genetic manipulation of filamentous fungi

The genetic manipulation of filamentous fungi is less advanced than that of yeasts for several reasons. Protoplasts for use in transformation are less easy to prepare than are yeast protoplasts, mainly due to lack of suitable lytic enzymes (Hamlyn *et al.*, 1981), and the protoplasts produced can be heterogeneous with respect to number of nuclei, biochemical function and so on (Böckelmann *et al.*, 1986). Also, commercially important filamentous fungi are less well characterised genetically than are yeasts, and can be more difficult to culture. Despite these factors, transformation systems have been developed for several species of filamentous fungi.

Much work has been done on the transformation of *Aspergillus nidulans*. Integrating vectors, similar to the YIp vectors described above, have been constructed for *A. nidulans*; as in the case of yeasts, these vectors produce a relatively low number of transformants and become stably integrated into the genome. For example, a *trpC*⁻ strain of *A. nidulans* has been transformed to tryptophan prototrophy using the *trpC* gene cloned into the bacterial vector pBR329 (Yelton *et al.*, 1984). More than 20 stable transformants were produced per µg DNA. Similar results were obtained for a strain with defective acetamidase (*amdS* gene) using the wild-type *amdS* gene cloned into pBR322; the plasmid became integrated into the genome at the *amdS* locus (Tilburne *et al.*, 1983).

A. nidulans has also been transformed with a gene from *Neurospora crassa*: a *pyrG*⁻ mutant of *A. nidulans* was transformed to uracil prototrophy using the *pyr4* gene of *N. crassa* cloned into pBR322 (Ballance *et al.*, 1983). The *pyr4* gene has little homology to the *A. nidulans pyrG* gene, and so it became integrated at a variety of sites within the *A. nidulans* genome (Turner & Ballance, 1985). The *N. crassa pyr4* gene was also expressed in *S. cerevisiae*, although the corresponding yeast *URA3* gene was not expressed in *N. crassa* or *A. nidulans*. Since the pBR322 part of the vector was also integrated into the nuclear DNA, the transformants could then be further transformed with nonhomologous DNA cloned in pBR322, which would integrate into the *A. nidulans* DNA at the prokaryotic region (Ballance *et al.*, 1983).

The *pyr4* gene has also been used in a cotransformation system: *A. nidulans* was transformed with a mixture of a *pyr4*-containing plasmid and the *oliC31* (oligomycin-resistance) gene cloned in a λ DNA vector, and 4 %

of the *pyr4*-containing transformants were also oligomycin-resistant (Turner & Ballance, 1985).

The *A. nidulans* sequence *ans1* produces a 100-fold increase in transformation efficiency when used in *pyr4*-based vectors, although it is apparently not an *ars* sequence since it probably causes integration into the genome. This sequence is the basis of the first self-cloning vector described for a filamentous fungus, pDJB3 (Turner & Ballance, 1985). This vector allows gene isolation by complementation of mutations in the homologous organism, rather than in *E. coli* as described above for yeast vectors. pDJB3 consists of the *ans1* sequence from *A. nidulans* and the *pyr4* gene from *N. crassa* cloned into pBR325, and produces 5000 transformants per μg DNA. For example, the *acuD* gene was isolated by cloning *A. nidulans* DNA into pDJB3, transforming a *pyrG⁻acuD⁻* mutant and selecting for a *Pyr⁺Acu⁺* phenotype. The plasmid, containing the *acuD* gene, was then rescued by subcloning in *E. coli* (Turner & Ballance, 1985). Another method for isolating *A. nidulans* genes uses a cosmid vector (containing the fungal *trpC* gene) to make a library of *A. nidulans* DNA. For example, the *yA⁺* gene (determining spore colour) was isolated by transforming a *trpC⁻yA⁻* strain with the library and selecting for *trpC⁺yA⁺* transformants (Yelton *et al.*, 1985).

An autonomously replicating plasmid has been reported recently in *A. nidulans*, based on the *pyr4* gene and a fragment of *A. nidulans* DNA which acts as an *ars* in yeast, although it appears that the ability to replicate freely does not increase the transformation efficiency of this plasmid (Barnes & MacDonald, 1986).

Autonomously replicating plasmids have also been constructed for other filamentous fungi. A plasmid containing a complete copy of the *N.*

intermedia "Labelle" plasmid and the *N. crassa* *qa-2*⁺ gene in pBR325 was used to transform *N. crassa* from *qa-2*⁻ to *qa-2*⁺ (Stohl *et al.*, 1984). Many of the plasmids recovered had the "Labelle" sequence deleted, implying that the *qa-2*⁺ gene has an *ars* sequence; a vector consisting of the *qa-2*⁺ gene in pBR322 showed free replication as well as integrating into the genome, and yielded 100 transformants/ μ g DNA (Hughes *et al.*, 1983).

The plDNA of *Podospora anserina* cloned into pBR322 was used to transform a long-lived strain of *P. anserina* to senescence, and the prokaryotic β -lactamase gene was also expressed in the eukaryotic host, as described in section 1.3.5 (Stahl *et al.*, 1982). A mitochondrial DNA fragment, containing part of the plDNA sequence, was also replicated in *P. anserina* after cloning into pBR322, and also induced senescence (Stahl *et al.*, 1982).

The phytopathogenic basidiomycete *Ustilago maydis* has been transformed with a vector based on the yeast 2 μ m plasmid (Banks, 1983): the vector contained the 2 μ m DNA and the gene for aminoglycoside phosphotransferase (derived from a bacterial transposon). Transformants arose at 10-15 / μ g DNA, and were resistant to the aminoglycoside neomycin. The vector was autonomously replicated at low levels in *U. maydis*, and in common with other autonomously replicating vectors it was mitotically unstable (Banks, 1983).

Another phytopathogenic fungus, *Cochliobolus heterostrophus*, has also been successfully transformed, and vectors have been developed using the *A. nidulans* *amdS* gene and the hygromycin B-resistance gene *hygB* as selectable markers (Yoder *et al.*, 1986).

As a final example, a vector pCP2 was constructed from pBR325, the *LEU2* gene of *Saccharomyces cerevisiae*, and a fragment of the mtDNA of *Cephalosporium acremonium*, which was able to replicate in *S. cerevisiae*, transforming it to leucine prototrophy (Tudzynski & Esser, 1982). This vector, presumably containing the major replicon of *C. acremonium* mtDNA, produced a transformation efficiency comparable to that of YEp vectors in yeast and was more stable than vectors based on the mtDNA replicons of yeast (Tudzynski & Esser, 1982); it has also been shown to replicate in *C. acremonium* and *P. anserina* (Esser *et al.*, 1983).

The ability to transform filamentous fungi, especially with vectors which can replicate in several hosts, is important in the strain improvement of industrially used species that are not accessible to sexual genetics, such as *C. acremonium* which is the main producer of the antibiotic cephalosporin; and transformation of non-pathogenic mutants of phytopathogenic fungi with gene libraries of the pathogenic variants may lead to identification and isolation of the genes involved in pathogenicity.

1.6 EXTRACHROMOSOMAL DNA IN *FUSARIUM OXYSPORUM*

1.6.1 Discovery of a plasmid-like DNA in *Fusarium oxysporum*

The first report of extrachromosomal DNA in *F. oxysporum* f.sp. *lycopersici* was by Martini *et al.* (1978) who reported a plasmid-like DNA present in a cleared lysate extract made from fragmented mycelium by the SDS-NaCl method (Hirt, 1967), visualised as a single band present after agarose gel electrophoresis. *E. coli* K-12 was transformed with the cleared lysate, and after testing with 24 antibiotics the transformants were found to be resistant to furadantin, unlike the parental bacterium. Furadantin is a member of the nitrofuran group of antibiotics, which inhibit synthesis of DNA, RNA and proteins in bacteria (McCalla, 1979); they cause single-stranded breaks in the DNA (Tu & McCalla, 1975). The transformed bacteria were also able to grow on agar as sole carbon-source, as is *F. oxysporum* f.sp. *lycopersici* due to its polygalacturonase activity. DNA extracted from the transformed *E. coli* comigrated with the DNA from *F. oxysporum* on an agarose gel (Martini *et al.*, 1978). The authors suggested that the polygalacturonase activity, which seemed to be associated with the plasmid-like DNA, may relate to the phytopathogenic mechanism of the fungus.

In a further report (Grimaldi *et al.*, 1978) the same group reported that the *F. oxysporum* DNA was a supercoiled molecule of apparent molecular weight 60×10^6 (equivalent to about 100 kb). They also noted that purple variants arose at a frequency of $0.5-1 \times 10^{-3}$ when the fungus was propagated from single spores following treatment with nitrosoguanidine or ethidium bromide, and that these purple variants no longer possessed the plasmid-like DNA (Grimaldi *et al.*, 1978).

The ability of the fungal plasmid-like DNA to replicate autonomously in a prokaryotic host is unusual in that all other fungal replicons tested require a bacterial replicon for their propagation in a bacterial host (see section 1.5).

1.6.2 Correlation of nifuroxime resistance with a plasmid-like DNA

The next publication from this group appeared four years later (Guardiola *et al.*, 1982). They observed that of a range of micro-organisms tested (including bacteria, yeasts, filamentous fungi, and plant and animal cell cultures), only *F. oxysporum* f.sp. *lycopersici* showed resistance to the nitrofurantoin antibiotic, nifuroxime (5-nitro-2-furaldehyde oxime). It was resistant at levels of up to 100 µg/ml nifuroxime. The wild-type pathogenic strain FC1, isolated from tomato, was resistant to the three nitrofurans nitrofurantoin, nifuroxime and furazolidone, when inoculated onto plates either as mycelium or as single spores: none inhibited FC1 at levels up to 100 µg/ml. A variant of FC1, isolated on the basis of its altered morphology, was unable to grow on 35 µg/ml nifuroxime, with both spore germination and mycelial growth being inhibited, and was also sensitive to furazolidone (Guardiola *et al.*, 1982).

Nifuroxime-sensitive mutants were made by heat-shocking FC1 in liquid culture at 42°C for 3-4 hr, and microconidia were selected by their inability to grow in the presence of 35 µg/ml nifuroxime. Sensitive spores formed more than 60 % of the total after the heat-shock treatment; no reversion to nifuroxime-resistance was detected. One such nifuroxime-sensitive mutant was denoted FC3 (Guardiola *et al.*, 1982). Nuclear mutants resistant to iodoacetate (FC4, derived from FC1) and cacodylate (FC5, derived from FC3) were selected by single-spore isolation from plates

containing 3 mg/ml iodoacetate or 2 mg/ml cacodylate, respectively. FC4 and FC5 were then allowed to anastomose, and the heterokaryons segregated to uninucleate spores which were then tested against nifuroxime, iodoacetate and cacodylate: out of 2256 spores tested, 41 % had the FC4 phenotype and 1.5 % had the FC5 phenotype; 56.5 % had the non-parental phenotype nifuroxime-resistant, cacodylate-resistant, and 1 % had the non-parental phenotype nifuroxime-sensitive, iodoacetate-resistant. These data were taken to show that nifuroxime-resistance segregated separately to the nuclear markers and hence was an extranuclear phenotype (Guardiola *et al.*, 1982).

DNA was extracted from the mycelium of FC1, and following electrophoresis on a 0.4 % agarose gel an extra band was seen migrating slower than the major nuclear DNA smear: this band was not seen in mitochondrial extracts of FC1, nor in total DNA extracted from FC3. When FC1 DNA was examined under the electron microscope, a circular molecule of 46.7 kb was seen. Treatment of a mitochondrial pellet with DNase showed that the circles were not protected, which was interpreted to indicate that they did not represent the mitochondrial DNA, which was extracted as linear fragments. The 46.7 kb circles were not detected in FC3 DNA. Further experiments showed that FC3 was not pathogenic on tomato plants, and also that FC3, unlike FC1, did not exhibit exo-polygalacturonase when grown on glucose as sole carbon-source (both FC1 and FC3 had endo-polygalacturonase activity when grown in the presence of D-galacturonate). The authors concluded that loss of the 46.7 kb plasmid-like DNA correlated with loss of nifuroxime-resistance, and that nifuroxime-sensitive mutants lacked exo-polygalacturonase activity (and hence were unable to grow on pectate as sole carbon-source) although retaining an inducible endo-

polygalacturonase. The lack of this enzyme was proposed to be a factor in the lack of phytopathogenicity of the sensitive isolates. Hence the plasmid-like DNA appeared to control both resistance to nifuroxime, and pathogenicity via the exo-polygalacturonase enzyme (Guardiola *et al.*, 1982). No further mention was made of the ability of the plasmid-like DNA to propagate in *E. coli*.

1.7 AIMS OF THE PRESENT WORK

The plasmid-like DNA of *Fusarium oxysporum* f.sp. *lycopersici* appeared to have two distinct affects on the phenotype of the fungus, namely resistance to nifuroxime and ability to cause wilt disease on tomatoes, unlike many fungal plasmids which have no clear effects on phenotype (section 1.4). Hence this plasmid was of interest for use in constructing cloning vectors for the genetic manipulation of *F. oxysporum* and possibly other filamentous fungi, and also for use in the investigation of the molecular basis of phytopathogenicity of fungi. It was intended first to extract the plasmid and characterise it by agarose gel electrophoresis, restriction mapping and electron microscopy, and then to analyse the gene products of the plasmid by hybridisation analysis and *in vitro* translation of fungal RNA. Following this characterisation, it was intended to analyse the ability of the plasmid to transform non-pathogenic isolates to pathogenicity, and to construct shuttle vectors using all or parts of the plasmid DNA.

CHAPTER 2. METHODS AND MATERIALS.

2.1 MISCELLANEOUS METHODS

2.1.1 Reagents and enzymes

All chemicals were of analytical reagent grade, unless stated otherwise. Most chemicals were obtained from BDH, Fisons or Sigma. Restriction enzymes were obtained from BRL or Amersham.

2.1.2 Sterilisation

All plasticware (reaction tubes and pipette tips) and all liquid reagents and buffers (except those containing heat-labile or volatile compounds) were sterilised by autoclaving at 121°C for 20 min. Glassware to be used for DNA or RNA solutions was sterilised by baking at 180°C overnight.

2.1.3 Water

All buffers and reagents were made up in glass-distilled water. "Sterile water" refers to autoclaved glass-distilled water

2.1.4 Dialysis tubing

Dialysis tubing was prepared by boiling it for 1 hr in 10 mM EDTA, pH 7.0, then for a further 1 hr in fresh 10 mM EDTA. It was then rinsed 10 times in distilled water, autoclaved in distilled water and stored at 4°C.

2.1.5 Phenol

Solid phenol (BDH Analar grade) was melted at 60°C and 8-hydroxy-quinoline was added to 0.1 % w/w. The phenol was water-saturated by

shaking with distilled water, allowing the phases to separate, collecting the phenol phase using a separating funnel, and then repeating the process.

Phenol extractions were performed by adding one volume of water-saturated phenol to the sample, vortexing or shaking until an emulsion was formed, and centrifuging at 10,000×g until the phases were completely separated. The upper aqueous phase was removed to a fresh tube, and the lower phenolic phase usually discarded. Traces of phenol were usually removed from the aqueous phase by a similar extraction with CIA (chloroform : isoamyl alcohol, 24:1 by volume).

2.1.6 Butanol extraction

Extraction with butanol was used to remove ethidium bromide from nucleic acid samples. 1-butanol was saturated with distilled water by shaking to an emulsion and allowing the phases to separate. Water-saturated butanol formed the upper phase. 1 volume of water-saturated butanol was added to the DNA sample, which was vortexed and the phases allowed to separate. The upper, butanol phase was discarded, and the process repeated until all traces of ethidium were removed from the aqueous phase. 2-propanol was sometimes substituted for 1-butanol in this process.

1-butanol, which had not been water-saturated, was used to reduce the volume of aqueous samples. At least one volume of butanol was added each time, extracting essentially as described above. When the aqueous phase reached the desired volume, residual butanol was removed by extracting twice with one volume of diethyl ether (discarding the upper phase each time), and finally the ether was removed by evaporation at 60°C.

2.1.7 Ethidium agarose plates

Plates were made by boiling 1 % w/v agarose in water, cooling to 60°C, adding ethidium bromide to 0.5 µg/ml, and pouring into Petri dishes.

The concentration of a DNA sample was estimated by spotting 1 to 2 µl onto a plate next to a dilution series of DNA of known concentrations, usually phage λ DNA (BRL), and comparing the fluorescence on a short wavelength UV transilluminator. For greater accuracy, a dilution series of the test DNA was compared to the standards.

2.1.8 Spectrophotometry

Concentrations of DNA and RNA solutions were determined by measuring the absorbance at 260 nm of a dilution of the sample in a Beckman model 35 spectrophotometer. It was assumed that 50 µg/ml DNA has an A_{260} of 1, and that 40 µg/ml RNA has an A_{260} of 1, in a cuvette of 1 cm pathlength.

2.1.9 Centrifugation

High speed centrifugation was performed in a MSE Hi-Spin 21 centrifuge, using the 8×50 ml or 6×300 ml fixed angle rotors as appropriate.

Ultracentrifugation was performed in a Beckman L8-55 ultracentrifuge; the rotors used are mentioned at the appropriate places in the text. Small volumes (i.e. less than 1.5 ml) were centrifuged in a Beckman Microfuge B, unless stated otherwise.

2.2 FUNGAL ISOLATES

Fusarium oxysporum f.sp. *lycopersici*, isolates FC1, FC3, and FC4, were kindly provided by Dr. F. Cervone, Università "La Sapienza", Rome. Wild-type race O isolates 4, 13 and BD were from the Imperial College Department of Pure & Applied Biology culture collection.

The phenotypes of the isolates were reported by Guardiola *et al.* (1982) to be:

FC1	Pathogenic	Nifuroxime-resistant
FC3	Non-pathogenic	Nifuroxime-sensitive
FC4	Pathogenic	Nifuroxime-resistant, Iodoacetate-resistant

The wild-type isolates are all pathogenic.

2.3 STANDARD BUFFERS AND MEDIA

2.3.1 Buffers

Tris-acetate (TAE):

40 mM tris, 5 mM acetic acid, 1 mM EDTA, pH 8.2. Stored as a 10× stock.

Tris-borate (TBE):

90 mM tris, 90 mM boric acid, 2 mM EDTA, pH 8.3. Stored as a 10× stock.

Chloroform/isoamyl alcohol (CIA):

24 parts chloroform : 1 part isoamyl alcohol by volume.

2.3.2 Solid Media

Potato Dextrose Agar (PDA):

39 g/l PDA (Difco) in distilled water.

Tryptone Agar:

10 g/l Bacto-tryptone (Difco), 5 g/l D-glucose, 15 g/l agar (Difco).

V8 Agar:

20% v/v 'V8' vegetable juice (Campbell Soup Company, Camden, New Jersey), adjusted to pH 6.0 with NaOH, 20 g/l agar.

Polypectate agar:

5.0 g/l NH_4Cl , 2.0 g/l KH_2PO_4 , 0.5 g/l MgSO_4 , 1/1000 volume of Trace elements, 15 g/l agar, 10 g/l sodium polypectate

2.3.3 Liquid Media

Complete Medium:

10 g/l Bacto-peptone, 5 g/l Yeast extract (Difco), 20 g/l D-glucose.

Trace Elements:

0.2 g/l FeSO_4 , 1.0 g/l ZnSO_4 , 0.02 g/l each of NaMoO_4 , CaSO_4 , MnCl_2 .

Sucrose Casein Hydrolysate (SCH):

4.6 g/l Casamino acids (Difco), 15 g/l Sucrose, 0.5 g/l D-glucose, 1.0 g/l KH_2PO_4 , 0.5 g/l MgSO_4 , 0.1 % v/v Trace elements.

All media were sterilised by autoclaving.

2.4 GROWTH AND MAINTENANCE OF FUNGUS

2.4.1 Growth and maintenance of fungal cultures

Fungal isolates were maintained on PDA plates at 4°C, and also on PDA slopes under sterile paraffin at 4°C.

Small pieces of mycelium from the growing edge were used as inocula onto plates or into liquid media. Mycelia on solid media were usually grown at 24°C. Liquid cultures were grown in conical flasks (100 to 200 ml per 500 ml flask) at 24°C, shaken in an orbital incubator at 150 r.p.m. When high yields of microconidia were required, flasks with baffles were used.

2.4.2 Response to antibiotics

(i) Mycelium

Fungus was inoculated onto tryptone agar plates supplemented with the appropriate concentration of antibiotic. Nifuroxime (Sigma) was added after autoclaving and cooling to 37°C, from a 14 mg/ml solution in absolute ethanol. Iodoacetic acid (Sigma) was added after autoclaving from a 600 mg/ml solution in distilled water, which was filter-sterilised. To construct growth curves, the diameter of the mycelium was measured daily over a period of 7 to 8 days after inoculation. Growth rate was defined as the gradient of the linear portion of a diameter vs. time plot.

(ii) Single microconidia

Microconidia were produced in liquid culture in SCH. The concentration of spores was measured using a Neubauer hæmocytometer. Spores were serially diluted in sterile water to ca. 100 cells/ml, and 1 ml samples spread on plates with or without nifuroxime. Plates were incubated at 24°C for a few days and then examined for the presence of mycelial colonies.

2.4.3 Spore germination assay

Mycelium was grown on V8 agar in the dark at 24°C for 6 to 7 days, then in a "Black-Light" UV incubator at 24°C for a further 3 to 7 days to induce spore formation. Spores were washed off the plates in 20 ml sterile water, concentrated by centrifugation at 2000 r.p.m. for 5 min., and resuspended to 30,000 macroconidia /ml. 50 µl of suspension was placed in the well of a glass cavity slide, and incubated overnight in a sealed container at 24°C. The spores were examined for germination using a light microscope. Germination was defined as the presence of a germ tube of greater length than the width of the macroconidium.

Nifuroxime was added by drying an aliquot of 0.5 mg/ml solution (in water) onto the cavity in the slide, such that adding 50 µl spore suspension gave the desired final concentration of nifuroxime. Each concentration was tested in triplicate.

2.4.4 Detection of exopolysaccharide activity

Mycelium was inoculated onto Polypectate agar plates, which contain sodium polypectate as sole carbon source (Hankin *et al.*, 1971). Control plates contained a medium identical except that the polypectate was replaced with 10 g/l D-glucose. The plates were incubated at 24°C.

2.5 PATHOGENICITY TESTING

2.5.1 Infection of plants

Plants, tomato variety Ailsa Craig or variety Money Maker, were grown in Levington or John Innes No. 2 compost in a glasshouse. Seedlings were infected about 3 weeks after planting the seeds. Fungal inoculum was prepared by growing liquid culture in SCH for 3 to 4 days, which was then collected by filtration, washed with distilled water, and resuspended in 200 ml sterile water.

The plants' roots were cut about 1 cm from the stem with a scalpel, and 20 to 30 ml fungal suspension was poured onto the soil. Plants were grown for a further 3 weeks before infection was assayed.

Infection was assayed by a variety of observations, which are described and compared in section 3.2.1.

2.5.2 Isolation of fungus from infected plants

A 2 to 3 cm length of stem was cut, from at least 1 cm above soil level. This was surface sterilised by soaking in 0.1 % mercury (II) chloride for 1 to 2 min., washed twice in sterile water, and sliced into 2 mm thick sections using a sterile scalpel blade. Each section was placed on a tryptone agar plate, with or without nifuroxime, and incubated at 24°C until the fungal mycelium had grown clear of the plant material.

2.6 GEL ELECTROPHORESIS OF NUCLEIC ACIDS

2.6.1 Agarose gel electrophoresis

The required amount of agarose (Sigma, type I) was dissolved in buffer by briefly boiling on a hot-plate. The solution was cooled to 60°C and poured into a horizontal template to a thickness of 3 to 5 mm, and allowed to set for at least 1 hr (gels of less than 0.6 % were set at 4°C).

Routine analysis was on "mini-gels", 10×6 cm. For restriction mapping, larger gels (11.5×15.5 cm) were used.

Initially tris-acetate buffer (TAE) was used; for restriction-mapping gels requiring long runs tris-borate buffer (TBE) was used as it is a better buffer.

DNA samples were loaded after the addition of 1/10 volume of 10×Reaction Stop (0.1 M EDTA, 20 % w/v Ficoll-400 (Sigma), 0.25 % w/v each of Xylene Cyanol FF and Bromophenol Blue (both from Sigma)). Gels were electrophoresed fully submerged at not more than 5 V/cm. At the end of the run gels were stained by soaking in 0.5 µg/ml ethidium bromide (Sigma) for at least 30 min., destained briefly in water, and DNA fragments were visualised using a short-wave UV transilluminator.

If desired, stained gels were photographed using an Exa I camera with yellow and UV filters, onto Kodak Pan F 35 mm film.

2.6.2 Polyacrylamide gel electrophoresis

To make 40 ml of gel, the following were mixed:

4 ml	40 % w/v acrylamide (BDH), 0.4 % NN'-methylene-bisacrylamide (BDH)
4 ml	10×TAE
0.3 ml	100 mg/ml ammonium persulphate (prepared freshly)
15 µl	TEMED
Distilled water to 40 ml	

The mixture was poured into a vertical mould of 20×16.5 cm, formed from two glass plates and 1 mm-thick perspex spacers. A perspex well-former (1 cm wide wells) was inserted at the top and the gel was allowed to polymerise at room temperature before removing the comb.

The electrode buffer was 1×TAE. The gel was pre-electrophoresed at 70 V for 30 to 60 min. before loading the samples, which contained 10 % v/v glycerol and 0.025 % w/v bromophenol blue. Gels were electrophoresed at 70 V for 18 hr.

Nucleic acid bands were stained with ethidium bromide, visualised and photographed, as described for agarose gels.

2.7 ANALYTICAL NUCLEASE DIGESTS

2.7.1 Ribonuclease:

A 25 µl sample was digested in a total volume of 30 µl, containing 10 µg/ml Ribonuclease A (Sigma), 10 mM sodium acetate pH 5.0, 5 mM magnesium sulphate. Digestion was at 37°C for 30 min.

2.7.2 Deoxyribonuclease:

Digestion conditions were as described for RNase, except that the RNase was replaced with 50 µg/ml DNase-I (Sigma).

2.7.3 S1 Nuclease:

Digestion conditions were as described for RNase, except that the RNase was replaced with 5 units S1 nuclease (Sigma), and that the buffer also contained 0.5 mM zinc sulphate.

2.7.4 Restriction endonucleases:

0.25 to 1 µg DNA was digested in a 20 µl reaction containing 1/10 volume of 10×Buffer and 2 to 5 units of enzyme. Buffers were as recommended by the manufacturers (see table 2.1). Digests were incubated at the optimal temperature for the particular enzyme, usually overnight.

Due to the high concentration of EDTA in the mtDNA samples, 1/20 volume of 0.2 M MgCl₂ was added. If found to be necessary, 1/20 volume of 100 mM spermidine trihydrochloride (Bouche, 1981) and of 2 mg/ml BSA (BRL, nuclease-free grade) were also added.

When using two enzymes, the buffer containing the lower salt concentration was usually used, except in the cases of *SmaI* (which requires KCl for activity) and *Sall* (which only works efficiently in high salt), for which the appropriate *SmaI*- or *Sall*-buffer was used.

Reactions were terminated by adding 1/10 volume of 10×Reaction Stop (see section 2.6.1), and analysed by agarose gel electrophoresis. Mapping gels were 0.6 % agarose in 1×TBE buffer, run for up to 18 hr at 30 V to resolve the largest bands.

Table 2.1 Buffers used for restriction endonuclease digestions.

Enzyme	Tris-HCl ^a	MgCl ₂ ^a	NaCl ^a	DTT ^a	pH
<i>AccI</i>	50	10	0	1	8.0
<i>BamHI</i>	20	7	100	1	8.0
<i>ClaI</i>	10	10	0	0	8.0
<i>EcoRI</i> ^b	50	10	50	1	7.5
<i>HindIII</i> ^c	50	10	50	0	8.0
<i>Sall</i>	8	6	150	0	7.6
<i>SmaI</i>	10	10	20 ^d	1	8.0
<i>XbaI</i>	6	6	100	0	7.6

^a concentrations expressed in mM

^b also used for *HindII*

^c also used for *AvaII*, *HaeII*, *HaeIII*, *PstI*, *XhoI*

^d NaCl was replaced by KCl

Restriction endonuclease fragments of phage λ DNA (BRL) were used as size markers, with values obtained from the DNA sequence (Sanger *et al.*, 1982).

2.8 PREPARATION OF PROTOPLASTS

2.8.1 Optimisation of conditions

A 200 ml liquid culture in SCH was incubated in a 500 ml flask with baffles at 24°C for 3 to 4 days. Spores were separated from mycelium by filtration through 77 μ m nylon mesh and collected by centrifugation at 10,000 r.p.m. for 10 min.

Protoplasting buffer contained 0.1 M citrate, 0.1 M KH_2PO_4 , pH 5.8 (Hamlyn *et al.*, 1981), plus various combinations of enzymes and osmotic stabilisers. The enzymes used were Novozym 234 (Novo), Cellulase-CP and Cellulase-CT (Sturge), each at 0.5 % w/v. Enzyme combinations were tested with 0.7 M NaCl as osmotic stabiliser, by incubating spores in a 1.5 ml volume at room temperature or 37°C for 5 hr. The yield of protoplasts was determined by examining a small aliquot using a light microscope.

Osmotic stabilisers tested were 0.7 to 1.2 M NaCl, $\pm$ 0.3 or 1.0 M sorbitol, using incubation periods of up to 19 hr.

The effect of pretreatment with a reducing agent was also tested by incubating the spores in 10 mM DTT for 1 hr at room temperature before the protoplasting reaction.

2.8.2 Small scale preparation of protoplasts

100 ml of culture in SCH was grown and the spores harvested as above. The spores were incubated in 10 mM DTT for 1 hr at room temperature and harvested by centrifugation, then incubated in 10 ml SSCP (0.7 M NaCl, 0.3 M sucrose, 0.1 M sodium citrate, 0.1 M KH_2PO_4 , pH 5.8) containing 50 mg each of Novozym 234 and Cellulase-CP at 37°C for 3 hr. Protoplasts were harvested by centrifugation (10 000 r.p.m. for 10 min.).

2.8.3 Large scale preparation of protoplasts

Fungus was grown in 200 ml Complete Medium for 7 days. The mycelium was collected by filtration on Whatman No 1 filter paper, and washed with distilled water. 50 g mycelium was resuspended in 500 ml 0.7 % v/v 2-mercaptoethanol and shaken at 24°C for 1 hr. It was then harvested and washed with water again, then resuspended in 1000 ml SSCP containing 0.5 % w/v each of Novozym 234 and Cellulase-CP, and incubated with shaking at 24°C for 16 hr. The protoplasts were harvested by centrifugation at 10,000 r.p.m. for 10 min.

2.9 ISOLATION OF MITOCHONDRIAL DNA

2.9.1 Large scale preparation of mtDNA

Protoplasts were prepared from 50 g mycelium as described in section 2.8.3. The protoplast pellet was resuspended in 50 ml 100 mM tris-HCl, 50

mM EDTA, pH 7.5, 15 % w/v sucrose. 50 g glass beads (0.45 to 0.50 mm diameter) were added and the slurry was shaken in a Braun model MSK cell homogeniser for 30 sec. at 2000 r.p.m. and 3 sec. at 4000 r.p.m. (Cummings *et al.*, 1979). The homogenate was then diluted with a further 50 ml 15 % sucrose, and debris and beads were removed by centrifuging at 4000 r.p.m. for 10 min. Mitochondria were pelleted from the supernatant by centrifuging at 13,000 r.p.m. for 30 min.

The pellet was resuspended in a total of 12 ml 20 % w/v sucrose in 100 mM tris-HCl, 50 mM EDTA, pH 7.5, and layered over 35 % + 60 % w/v sucrose step gradients (containing the same buffer) - each gradient consisted of 2 ml sample and 18 ml of each layer. The gradients were centrifuged in a Beckman SW28 rotor at 22,000 r.p.m. for 1.5 hr at 4°C. The dense pigmented band which forms at the 35-60 % sucrose interface was collected with a sterile pasteur pipette, diluted with 3 volumes of 10 mM tris-HCl, 1 mM EDTA, pH 8, and centrifuged at 12,000 r.p.m. for 30 min. at 4°C in a Beckman SW50.1 rotor.

The mitochondrial pellets were resuspended in 5 volumes 50 mM tris-HCl, 5 mM EDTA, 50 mM NaCl, pH 8.0, 2 % w/v SDS, and heated at 60°C for 2 hr. Proteinase-K (Sigma Protease Type XI) was added to 100 µg/ml and the lysates were incubated at 37°C for 2 hr. The samples were extracted twice with phenol and once with CIA. Solid CsCl was added to a density of 1.39 g/ml and ethidium bromide to 0.2 mg/ml, and this solution layered over an equal volume of 1.75 g/ml density CsCl, 0.2 mg/ml ethidium bromide in 50 mM tris-HCl, 5 mM EDTA, 50 mM NaCl, pH 8.0 (made by taking 25.6 g CsCl and 0.5 ml 10 mg/ml ethidium bromide and making up to 25 ml with buffer). These step gradients were centrifuged in a Beckman SW50.1 rotor at 35,000 r.p.m. for 17 hr at 20°C. The DNA band was located by

illumination with UV, collected with a sterile pasteur pipette, extracted 3 times with water-saturated 2-propanol, dialysed against 1 mM tris-HCl, 0.5 mM EDTA, 1 mM NaCl, pH 8.0, and finally concentrated by butanol-extraction.

2.9.2 DNase-treatment of mitochondria

The method used was based on those of Minuth *et al.* (1982) and Hahn *et al.* (1979).

A mitochondrial pellet was prepared from protoplasts as described in section 2.9.1. The packed mitochondria from 6 g mycelium were used for each digest. The mitochondria were resuspended in 1.0 ml 20 % w/v sucrose with the following additions:

5 µg pAT153 DNA;

5 µg pAT153, 20 µl 1 M tris-HCl pH 7.5, 10 µl 1 M MgCl₂, 0.1 ml DNase-I (1 mg/ml);

5 µg pAT153, 20 µl 1 M tris-HCl pH 7.5, 10 µl 1 M MgCl₂, 0.1 ml DNase-I (1 mg/ml), 10 µl Nonidet P-40 (BDH).

After 40 min. incubation at 4°C, 0.4 ml 0.5 M EDTA was added and the mitochondria were pelleted at 12,000 r.p.m. for 30 min. The pellets were resuspended in 2.5 ml 20 % sucrose, purified by sucrose step gradient centrifugation and the DNA was extracted as described in section 2.9.1. DNA was recovered from the digest supernatants by extracting with phenol and CIA, precipitating with 2 volumes of ethanol at -20°C overnight, and pelleting at 40,000 r.p.m. for 30 min. in a Beckman SW50.1 rotor. The pellets were dissolved in 1 ml sterile water, dialysed against distilled water for 6 hr, and precipitated with ethanol again. The final pellets

were dried, dissolved in 20 μ l sterile water and analysed by agarose gel electrophoresis.

2.9.3 Assay for Cytochrome c Oxidase activity

Intact mitochondria were prepared and centrifuged on a sucrose step-gradient as described in section 2.9.1. The gradient was fractionated into 1 ml aliquots from the top. Each fraction was then assayed for its ability to oxidise reduced cytochrome c, essentially as described by Schnarrenberg *et al.* (1971).

A stock of 20 mg/ml cytochrome c (Sigma, type III, molecular weight 12,384) was reduced by adding DTT to 0.1 mM. The stock was then scanned between 500 and 600 nm in a Beckman model 35 spectrophotometer to ensure it was at least 60 % reduced.

Assays were performed in glass cuvettes at room temperature: to 2 ml 20 % w/v sucrose, reduced cytochrome c was added to 17.8 μ M, followed by 1 μ l mitochondrial fraction. The absorbance at 550 nm was followed in the spectrophotometer for 5 min. Rate of change of A_{550} was then calculated, and converted to pmol cytochrome c oxidised per min. from:

$$A_{550}(\text{oxidised cytochrome c}) = 8100 \text{ /cm/M}$$

$$A_{550}(\text{reduced cytochrome c}) = 28400 \text{ /cm/M}$$

2.10 METHODS FOR ISOLATING A *FUSARIUM* PLASMID

2.10.1 Method 1 (based on Birnboim & Doly, 1979)

Protoplasts from a 100 ml liquid culture were resuspended in 3 ml 25 mM tris-HCl, 10 mM EDTA, pH 8.0 and 6 ml 0.2 M NaOH, 1 % w/v SDS was added. The sample was incubated on ice for 5 min., then 4.5 ml 3 M potassium acetate, pH 4.8, was added and the sample kept on ice for a further 30 min. The lysate was centrifuged at 14,000 r.p.m. for 30 min., and two volumes of ethanol were added to the supernatant. The precipitate was collected by centrifugation (section 2.9.2) and resuspended in 3 ml TAE. After addition of 1 ml 8 M LiCl, the sample was kept on ice for 30 min. and then centrifuged at 10,000 r.p.m. for 10 min. to remove the RNA precipitate. The supernatant was collected and after ethanol-precipitation as above the pellet was resuspended in 0.5 ml TAE. After addition of 30 μ l of 0.2 mg/ml RNase-A, the mixture was incubated at 37°C for 45 min. and phenol-extracted. After ethanol-precipitation and centrifugation the final pellet was dissolved in 50 μ l TAE.

As a positive control, 2 μ m-plasmid DNA was extracted by this method from protoplasts of *Saccharomyces cerevisiae* S288c (Guerineau *et al.*, 1971). Protoplasts were made from 200 ml liquid culture of S288c in Complete Medium: the cells were harvested by centrifugation (7000 r.p.m. for 10 min.), washed for 20 min. in 10 ml 1 M sorbitol, 25 mM EDTA pH 8.0 containing 4.5 % v/v 2-mercaptoethanol, and harvested as above. The cells were then resuspended in 3 ml 1.2 M sorbitol, 0.1 M sodium citrate pH 5.8, 10 mM EDTA, containing 5 % v/v β -glucuronidase (Sigma). After 1.5 hr at 37°C the resulting protoplasts were harvested as above, and plasmid DNA was extracted as described.

2.10.2 Method 2

A cleared lysate was prepared as in method 1. DNA was purified by PEG-precipitation, as described by Humphreys *et al.* (1975). To the supernatant, PEG-6000 was added to 10 % w/v, and the sample incubated at 4°C overnight. DNA was pelleted at 10,000 r.p.m. for 10 min., taken up in 1 ml TAE containing 10 µg/ml RNase-A and digested at 37°C for 1 hr. Proteinase-K was added to 50 µg/ml and SDS to 0.5 %, the sample was incubated at 37°C for 1 hr, extracted with phenol and CIA, and reprecipitated with PEG-6000.

As a variant of this method, the NaOH/SDS lysis step was performed at 60°C for 5 min.

2.10.3 Method 3 (based on Hirt, 1967)

Protoplasts from 200 ml liquid culture were resuspended in 20 ml 25 mM tris-HCl, 10 mM EDTA, pH 8.0, 13 ml 10 % w/v SDS were added and the mixture was incubated at 60°C for 1 hr. Solid NaCl was added to 1 M, and the sample was incubated at 4°C overnight. Debris was pelleted at 14,000 r.p.m. for 30 min. PEG-6000 was added to 10 % w/v and after 2 hr at 4°C the DNA was pelleted at 10,000 r.p.m. for 10 min. The pellet was redissolved in 4.5 ml TAE and 4.5 g CsCl was added. The sample was centrifuged at 17,000 r.p.m. for 1 hr in a Beckman SW50.1 rotor, the protein layer was removed from the top and ethidium bromide was added to 1 mg/ml. The sample was then centrifuged in the same rotor for 65 hr at 33,000 r.p.m., 15°C. The DNA band was located by UV illumination, taken off and extracted with water-saturated 2-propanol. It was dialysed for 4 hr against TAE, then NaCl was added to 0.5 M and PEG-6000 to 10 % w/v,

and the DNA precipitated at 4°C for 2 hr. After pelleting for 5 min. (Microfuge) the DNA was taken up in 100 µl TAE.

2.10.4 Method 4

DNA was extracted essentially according to Minuth & Esser (1982). 3 g mycelium was ground to a fine powder under liquid nitrogen. A NaCl/SDS cleared lysate was prepared and PEG-precipitated as in method 3, and then centrifuged on step gradients of 2.5 ml 47.6 % w/v CsCl and 2.5 ml 95.3 % w/v CsCl, in TAE containing 1 mg/ml ethidium bromide, for 20 hr at 35 000 r.p.m. in a SW50.1 rotor at 20°C. The DNA was collected and concentrated as in 2.10.3.

2.11 SUBCELLULAR FRACTIONATION OF *FUSARIUM*

Fusarium mycelium was fractionated into cytoplasmic, mitochondrial and nuclear extracts as described by Guardiola *et al.* (1982).

100 ml liquid culture (grown in SCH for 13 days) was harvested by filtration. The mycelium was ground to a fine powder under liquid nitrogen, allowed to thaw and resuspended in 20 ml 50 mM tris-HCl, 20 mM EDTA, pH 8.0, containing 0.6 M sorbitol. The nuclear fraction was collected by centrifuging at 6000 r.p.m. for 10 min.

Mitochondria were pelleted from the supernatant by centrifuging at 15,000 r.p.m. for 30 min. The high-speed supernatant was used as a "cytoplasmic" fraction. Nuclear and mitochondrial pellets were each suspended in 10 ml 50 mM tris-HCl, 20 mM EDTA, pH 8. SDS was added to all three fractions to 1 % w/v, and all were heated at 60°C for 10 min. Debris was removed by centrifuging at 14,000 r.p.m. for 30 min. NaCl was added to the

supernatants to 0.5 M and PEG-6000 to 10 % w/v. Nucleic acids were precipitated at 4°C overnight, and collected by centrifuging at 10,000 r.p.m. for 10 min. The DNA was purified by CsCl-ethidium step gradient centrifugation as described for mtDNA in section 2.9.1.

After dialysis the DNA was precipitated with ethanol and redissolved in 50 µl TAE.

2.12 PREPARATION OF NUCLEAR DNA

20 g mycelium was protoplasted and broken in the Braun homogeniser as described in sections 2.8.3 and 2.9.1. The 4000 r.p.m. pellet was made up to 40 ml with TAE and extracted repeatedly with phenol and CIA, precipitated with ethanol at -20°C overnight, and the pellet resuspended in a total of 3.5 ml TAE.

2.13 ISOLATION OF DOUBLE-STRANDED RNA

2.13.1 Method 1 (essentially as described by Morris & Dodds, 1979)

Fungus was grown as 200 ml liquid culture in SCH for 13 days. Mycelium was harvested by filtration and ground to a fine powder under liquid nitrogen. 10 ml GPS (0.2 M glycine, 0.1 M Na₂HPO₄, 0.6 M NaCl, pH 9.5), 1 ml 10 % SDS and 0.37 ml Bentonite (3 % w/v) were added. The mixture was extracted by shaking with 10 ml saturated phenol and 10 ml CIA, and centrifuged at 10,000 r.p.m. for 20 min. at 4°C. The aqueous phase was recovered and ethanol was added to 15 % v/v. A 5 ml cellulose column was prepared by equilibrating 1.25 g Cellex N-1 (Bio-Rad) in 25 ml STE (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.0) containing 15 % v/v ethanol,

and packing into a sterile syringe. The sample was applied, and then DNA and ssRNA were washed off with 25 ml STE, 15 % ethanol. DsRNA was eluted with two aliquots of 2.5 ml STE, and precipitated with two volumes of ethanol at -20°C overnight. The dsRNA was collected by centrifuging at 10,000 r.p.m. for 20 min., dried under vacuum, and redissolved in a total volume of 0.5 ml sterile water.

The yield was estimated by determining the absorbance at 260 nm. 25 µl samples were analysed by PAGE.

2.13.2 Method 2

As an alternative, a phenol-extracted sample prepared as in Method 1 was precipitated at -20°C for 1 hr with two volumes of ethanol. Nucleic acids were pelleted at 10,000 r.p.m. for 10 min. and redissolved in 7.5 ml TAE. 2.5 ml LiCl was added and the sample stored at 4°C overnight. The LiCl-insoluble fraction was removed by centrifuging at 10,000 r.p.m. for 10 min., and nucleic acids precipitated from the supernatant with two volumes of ethanol at -20°C for at least 3 hr. After centrifuging at 10,000 r.p.m. for 10 min., the pellets were dried under vacuum and redissolved in a total of 3 ml TAE. The sample was further purified by phenol- and CIA-extractions and ethanol precipitation, and the pellet washed with 70 % ethanol. The dried pellet was dissolved in a final volume of 1.5 ml TAE.

As a positive control, *Gaeumannomyces graminis* var. *tritici*, isolate 3b1a (Buck *et al.*, 1981), was grown in 200 ml Complete Medium at 15 to 17°C for 11 days, then harvested and the LiCl-soluble nucleic acids extracted as for *Fusarium*.

Samples were analysed by PAGE or agarose gel electrophoresis. Size markers were dsRNAs from the mycoviruses HmV, AfV and PsV (Bozarth *et al.*, 1981; Buck & Ratti, 1977; Bozarth *et al.*, 1971).

Some gels were stained with Acridine Orange: the gel was soaked in a 30 µg/ml solution of Acridine Orange (Sigma) for 30 min., then destained for 1½ hr in distilled water in an enamel pan.

2.14 DETERMINATION OF BUOYANT DENSITY OF DNA

The buoyant densities of mtDNA and nDNA in CsCl were determined by isopycnic centrifugation. Three tubes of 5 ml each were prepared for each DNA species. 5 µg DNA was dissolved in 5 ml 50 mM tris-HCl, 50 mM NaCl, 5 mM EDTA, pH 8.0, containing 90 % w/v CsCl (giving a starting density of 1.66 g/ml). These were then centrifuged to equilibrium in a Beckman SW50.1 rotor at 30,000 r.p.m., 20°C, for 65 hr. The gradients were fractionated by puncturing the bottom of the tube and allowing the liquid to drip out under gravity. Fractions of 12 drops were collected, and stored at -20°C until ready for analysis.

The location of the DNA band was determined by spotting 2 µl aliquots of each fraction onto ethidium-agarose plates.

The refractive index of each fraction was measured using an Abbé High Accuracy '60' refractometer (Bellingham & Stanley) with a sodium lamp (Gallenkamp). The corrected refractive index, n_{corr} , was calculated from the observed value, n_{obs} , and the values measured for buffer, n_{buff} , and distilled water, n_w , using:

$$n_{corr} = n_{obs} - (n_{buff} - n_w)$$

where n is the refractive index at 20°C at the wavelength of the sodium D₁ line (589.6 nm).

The density ($\rho^{20^\circ\text{C}}$) was then calculated from:

$$\rho^{20^\circ\text{C}} = 10.9276 \times n_{\text{corr}} - 13.593 \quad (\text{Bruner \& Vinograd, 1965}).$$

2.15 ELECTRON MICROSCOPY OF DNA

Polaron copper grids coated with Parlodion (from a 3.5 % solution in n-pentyl acetate) were provided by Ms. V. Lewis. The mtDNA examined was prepared from DNase-treated mitochondria (see section 2.9.2), and was spread using the Kleinschmidt cytochrome c technique (Kleinschmidt *et al.*, 1962).

Spreading mix contained:

10 μl DNA (50 ng mtDNA + 50 ng marker)

35 μl formamide (Rose, recrystallised)

0.5 - 1 μl cytochrome c (1 mg/ml in 2 M tris-HCl, 0.2 M EDTA, pH8.5)

double-distilled water to 105 μl .

Hypophase was 10 % v/v formamide, 10 mM PIPES pH7.7 in double-distilled water, and was made up freshly every 30 min.

Hypophase was poured into a glass Petri dish and its surface cleaned by two passes of a teflon bar. Spreading mix was introduced to the surface by running it slowly down a clean glass rod which was held at an acute angle to the hypophase surface. Each 105 μl of spreading mix was used to make two grids, by pressing the grids shiny-side-down onto the liquid

surface. The grids were then rinsed briefly in 90 % ethanol, stained for 15 sec. in uranyl acetate, rinsed again in 90 % ethanol, and allowed to dry shiny-side-up on filter paper. The stain was made by diluting a stock of 50 mM uranyl acetate in 50 mM HCl, to 50 μ M in 90 % ethanol just before use.

Grids were coated with platinum in an Edwards E306 Shadower, using 12 turns of platinum on tungsten wire, and a shadowing angle of 11°. They were then examined in a Hitachi H-600 electron microscope at 50 kV. Search magnification was 8000, photographs were taken at 2100 to 6300. Photographs were taken such that the field of view included at least one size marker molecule. Negatives were recorded on Kodak Technical Pan 2415 35 mm film, and then printed onto 5"x7" paper. Contour lengths of circular molecules were traced from the prints using a Hewlett-Packard 9864A digitiser coupled to a 9821A calculator.

The DNA size markers used were donated by Dr. P.W.J. Rigby, and were SV40 DNA (5243 bp; Van Heuverswyn & Fiers, 1979) and pTSV3 (8901 bp; it is SV40 DNA cloned into the EcoRI site of plasmid pAT153, and was constructed by Dr. C.E. Clayton and Dr. P.W.J. Rigby, Imperial College).

CHAPTER 3. RESULTS.

3.1 RESISTANCE OF *FUSARIUM OXYSPORUM* F. SP. *LYCOPERSICI* TO ANTIBIOTICS

3.1.1 Effect of nifuroxime on mycelial growth

The various *Fusarium* isolates were grown on Tryptone agar plates at 24°C, containing increasing concentrations of nifuroxime (Nfx). The isolates FC1, FC3 and FC4, described by Guardiola *et al.* (1982), were compared with three race 0 isolates, referred to as O-4, O-13 and O-BD, which had been isolated from tomato plants. Each concentration of Nfx was tested on between 2 and 8 separate plates. The growth rates in the presence of 0 to 100 µg/ml Nfx are summarised in table 3.1. To take into account the differences in growth rate between isolates in the absence of Nfx, these figures were converted to percentage of control growth rate for each isolate (see table 3.2).

These figures show little difference between the six isolates tested as regards their resistance to Nfx at 24°C. According to the phenotypes defined by Guardiola *et al.* (1982), all six isolates are intermediate between resistant (Nfx^r, not inhibited at 100 µg/ml Nfx) and sensitive (Nfx^s, unable to grow on 35 µg/ml Nfx).

To check if the Nfx^r phenotype was easily lost when the fungus was cultured in the absence of Nfx, isolate FC1 which had been maintained constantly in the presence of 35 µg/ml Nfx (named FC1*) was used. The response of FC1* to varying concentrations of Nfx was determined, as were the responses of the various isolates at 33°C (the incubation temperature used by Guardiola *et al.* (1982)).

Table 3.1 Growth rates of mycelium in the presence of Nfx. Concentrations of Nfx are in $\mu\text{g/ml}$; growth rates are in cm/day , and are mean values.

Concentration of Nfx	Isolates					
	FC1	FC3	FC4	O-4	O-13	O-BD
0	0.99	0.81	0.81	0.93	1.03	0.85
20	0.61	0.47	0.46	0.55	0.68	0.46
35	0.58	0.35	0.35	0.56	0.51	0.39
50	0.28	0.20	0.29	0.35	0.32	0.38
70	0.40	0	0	0.35	0.15	0.35
100	0	0.03	0.02	0	0.11	0

Table 3.2 Percentage growth rates of mycelium in the presence of Nfx. Concentrations of Nfx are in $\mu\text{g/ml}$.

Concentration of Nfx	Isolates					
	FC1	FC3	FC4	O-4	O-13	O-BD
0	100	100	100	100	100	100
20	61.0	58.1	57.5	59.2	66.2	54.3
35	58.3	42.9	43.1	60.6	50.0	45.9
50	28.5	24.0	36.2	38.1	30.7	44.8
70	40.4	0	0	37.7	14.6	41.4
100	0	3.8	2.1	0	10.4	0

Also, to check that the Nfx being used was inhibitory to other fungi, the mycelial fungus *Phialophora* sp. (lobed hyphopodia), formerly isolate 2.2 (Prr2.2; Jamil & Buck, 1984) was plated at increasing concentrations of Nfx and incubated at 24°C.

These growth rate data are summarised in tables 3.3 and 3.4.

Isolate FC1* is distinctly more resistant than the isolates listed in tables 3.1 and 3.2, when assayed at 24°C. However, when this isolate is compared to the others at 33°C, it is no more resistant than FC1 or FC4. FC3 is slightly more sensitive at this temperature, but is also the slowest growing isolate on control plates.

The fungus Prr2.2 is clearly more sensitive to Nfx than any of the *Fusarium* isolates.

3.1.2 Effect of Nfx on growth from single spores

In the preceding section, the inoculum used was a small piece of mycelium cut from a plate. To see if mycelial growth was more sensitive when initiated from single spores, preparations of microconidia were diluted and plated as described in section 2.4.2(ii). The number of mycelial colonies visible after 3 days at 24°C was counted.

In the first test, spores of FC1 and O-13 were plated on 0 or 50 µg/ml Nfx: on the control plates >1000 and 38 colonies were observed, respectively; no colonies grew on 50 µg/ml from either isolate.

FC1* was spread on 0 or 70 µg/ml Nfx, and the plates were incubated for 10 days: the control plate had ≈200 colonies, but none grew on 70 µg/ml even after 10 days. Since the FC1* spores used had been grown in liquid

Table 3.3 Growth of mycelium in the presence of Nfx. Growth rates are in cm/day; concentrations of Nfx are in $\mu\text{g/ml}$; - indicates not determined; incubation temperature used for each isolate is shown in brackets.

Concentration of Nfx	Isolates						
	FC1*	FC1*	FC1	FC3	FC4	O-13	Prr2.2
	(24°C)	(33°C)	(33°C)	(33°C)	(33°C)	(33°C)	(24°C)
0	0.80	0.24	0.28	0.20	0.27	0.35	1.34
20	0.65	0.15	0.17	0.11	0.19	-	0.25
35	0.50	0.12	0.11	0.04	0.15	0.08	-
50	0.42	0.09	0.10	0.02	0.09	-	0.09
70	0.32	-	-	-	-	-	-
100	0.19	0	0.01	0	0	-	0

Table 3.4 Data of table 3.3 expressed as percentage growth rates.

Concentration of Nfx	Isolates						
	FC1*	FC1*	FC1	FC3	FC4	O-13	Prr2.2
	(24°C)	(33°C)	(33°C)	(33°C)	(33°C)	(33°C)	(24°C)
0	100	100	100	100	100	100	100
20	81.2	60.7	61.4	55.5	68.8	-	18.7
35	62.8	47.1	40.7	20.5	55.8	23.7	-
50	52.1	35.2	35.7	11.5	31.6	-	6.4
70	39.8	-	-	-	-	-	-
100	24.4	0	3.6	0	0	-	0

culture without Nfx, the effect of growing the spores in SCH containing 30 µg/ml Nfx was tested: see table 3.5.

Table 3.5 Effect of growing spores in the presence of Nfx.

Nº colonies when spores of FC1* were produced in:

	SCH	SCH + 30 µg/ml Nfx
Control plates	78 to ~3000	41 to 120
Plates containing 70 µg/ml Nfx	0	0
Plates containing 100 µg/ml Nfx	0	0

In the next test, spores of FC1* and FC3 were compared when plated at a range of lower Nfx concentrations. The results are summarised in table 3.6.

Table 3.6 Comparison of FC1* and FC3 when grown from spores.

Nº colonies counted after 3 days:

Concentration of Nfx in plates	Spores from:		
	FC1* ^a	FC1* ^b	FC3 ^b
0	34-40	35	25-29
20	22-31	23	34-36
35	22-28	40	0-21
50	18-30	28	0-2

^a Spores produced in SCH + 30 µg/ml Nfx.

^b Spores produced in SCH with no antibiotic.

The above results can be summarised as follows:

None of the isolates tested could grow from single spores in the presence of ≥ 70 $\mu\text{g/ml}$ Nfx, even when the spores were produced in the presence of 30 $\mu\text{g/ml}$ Nfx. At concentrations of ≤ 50 $\mu\text{g/ml}$ Nfx, spores of FC1* were more resistant than spores of FC3, although in some experiments FC3 spores were able to develop on 50 $\mu\text{g/ml}$ Nfx, which is considerably higher tolerance than the Nfx^r phenotype described by Guardiola *et al.* (1982).

3.1.3 Effect of nifuroxime on germination of spores

Since the growth of mycelium from single spores seemed to differentiate between FC1* and FC3 to some extent, the ability of macroconidia to germinate in the presence of Nfx was tested since this should be as sensitive, or more sensitive than, the previous test.

Aliquots of ≈ 1500 macroconidia of isolates FC1 and FC3 were germinated with or without Nfx as described in section 2.4.3. The results are summarised in table 3.7.

Both FC1 and FC3 are weakly inhibited by 20 $\mu\text{g/ml}$ Nfx, and strongly inhibited by 50 $\mu\text{g/ml}$ Nfx. The concentrations required to cause inhibition of germination of 50% of spores were estimated by interpolation to be 41 $\mu\text{g/ml}$ for FC1 and 33 $\mu\text{g/ml}$ for FC3.

3.1.4 Effect of iodoacetate on mycelial growth

According to Guardiola *et al.* (1982), isolate FC4 is an iodoacetate-resistant (Iac^r) derivative of FC1. To check that this marker was reproducible, various isolates were grown at 24°C (or in one case at

Table 3.7 Effect of Nfx on spore germination. Figures are expressed as % of macroconidia which germinated; concentrations of Nfx are in $\mu\text{g/ml}$.

Concentration of Nfx	Isolates	
	FC1	FC3
0	96	91
20	80	70
35	84	37
50	8	1
70	0	0
100	0	0

33°C) on tryptone agar plates containing a range of concentrations of iodoacetate (Iac). In each case, the growth rates were recalculated as percentage of growth rate in the absence of Iac. These data are summarised in tables 3.8 and 3.9.

These figures show that all three isolates tested are inhibited by >2 mg/ml Iac, and are therefore Iac^r according to the definition of Guardiola *et al.* (1982). FC4 was also determined to be Iac^r at 33°C.

To attempt to select for a population of Iac-resistant cells in liquid culture, 1 ml aliquots of FC4, grown in SCH, were spread on plates containing 1.2 mg/ml Iac. No growth was observed after 6 days incubation at 24°C.

Table 3.8 Growth rates of mycelium in the presence of Iac. Concentrations of Iac are in mg/ml; growth rates are in cm/day; - indicates not determined.

Concentration of Iac	FC3 (24 °C)	FC4 (24 °C)	O-4 (24 °C)	FC4 (33 °C)
0	0.81	0.81	0.93	0.27
0.1	0.83	0.88	-	-
0.5	0.30	0.33	-	-
1	0.03	0.07	-	-
2	0	0	-	-
3	0	0	0	0

Table 3.9 Percentage growth rates in the presence of Iac.

Concentration of Iac	FC3 (24 °C)	FC4 (24 °C)	O-4 (24 °C)	FC4 (33 °C)
0	100	100	100	100
0.1	103	109	-	-
0.5	37.0	41.3	-	-
1	4.1	8.3	-	-
2 and 3	0	0	0	0

3.2 PATHOGENICITY OF *FUSARIUM* ISOLATES

3.2.1 Pathogenicity testing

The infection of a plant with *Fusarium* was assessed in various ways. The full symptoms, i.e. wilting and stunting of the plant, were rarely observed; more commonly seen were epinasty and yellowing of the leaves. Figure 3.1 shows symptoms in three Ailsa Craig tomato plants: the strong symptoms and weak symptoms were both produced by infection with FC1, which is clearly pathogenic. The failure to produce wilting and stunting in all plants was presumably due to growing the infected plants in a glasshouse, rather than under conditions of controlled temperature, and also the method of inoculation used, which was not very efficient. A more reproducible property was the browning of the vascular bundles, seen when the stem was cut a few cm above soil level. This is an indication that the fungus has spread from the point of infection (below soil level) up the stem of the plant. However, the best indication of infection of the plant was the ability to reisolate *Fusarium* from stem slices taken from above soil level.

Observations of the various criteria of pathogenicity are summarised below.

Experiment 1: infection of tomato var. Ailsa Craig.

Inoculum:	None	FC1	FC3	FC4
N ^o plants inoculated:	7	8	8	8
Plants showing strong symptoms:	0	1	0	0
Browning of vascular bundles:	0	5	6	0
<i>Fusarium</i> reisolated:	no	yes	yes	no

FC1 was reisolated from plants showing both strong and weak symptoms.



1

2

3

Figure 3.1 Symptoms in tomato var. Ailsa Craig. 1: healthy (mock-infected). 2: strong symptoms (infected with FC1). 3: weak symptoms (infected with FC1).

Experiment 2: infection of tomato var. Ailsa Craig.

Inoculum:	None	FC1*	O-4
Nº plants inoculated:	7	7	7
Strong symptoms:	0	1	1
Weak symptoms:	0	6	3
Browning of vascular bundles:	0	5	0
<i>Fusarium</i> reisolated:	no	yes	yes

Hence FC1* appears to be no more virulent under these growth conditions than FC1. To see if another variety of tomato would be more susceptible to *Fusarium* than Ailsa Craig, some of the above isolates were retested on var. Money Maker.

Experiment 3: infection of tomato var. Money Maker.

Inoculum:	None	FC1*	O-4	FC4
Nº plants inoculated:	5	5	5	5
Strong symptoms:	0	0	0	0
Weak symptoms:	0	2	2	0
Browning of vascular bundles:	0	0	4	0
<i>Fusarium</i> reisolated:	no	yes	yes	no

Hence using a different variety of tomato makes little difference to the symptoms observed. Since the lack of consistency of symptoms seen for each isolate was probably an artifact of the inoculation and growth conditions, the pathogenicity of each isolate was judged from the plant(s) showing the most marked symptoms. The pathogenicity of the isolates tested can be summarised as:

FC1, FC1*, FC3 and O-4: Pathogenic

FC4: Non-pathogenic.

3.2.2 Resistance of reisolated fungus to nifuroxime

The fungal isolates which had been reisolated from plants were compared to the original isolate used for the inoculum, to see if passage through the plant had selected for a more Nfx-resistant phenotype. The growth rates and percentage growth rates are shown in tables 3.10 and 3.11. Comparing these data to table 3.2 indicates that isolates FC1, FC3 and O-4 have become more resistant to Nfx after reisolation, and now have approximately the same response as FC1*. The resistance of FC1* is about the same after reisolation (see table 3.4). This indicates that the apparent increase in resistance to Nfx after passage through tomato plants is in fact due to reisolating and maintaining the fungus on plates containing 35 µg/ml Nfx (since FC1* is just FC1 which has constantly been cultured on Nfx-containing media).

3.2.3 Growth of *Fusarium* on Polypectate agar

According to Guardiola *et al.* (1982), isolates FC1 and FC3 differ in their growth on polypectate agar plates, in that FC3 has a lag period due to its lack of an exopolygalacturonase enzyme. Six isolates were grown on both polypectate agar and glucose agar plates, and the growth rates were compared (see table 3.12).

All six strains grew faster using polypectate as sole C-source than when using D-glucose; none showed a noticeable lag period.

Table 3.10 Growth rates of mycelium of fungus reisolated from plants.

Concentrations of Nfx are in $\mu\text{g/ml}$; growth rates are in cm/day . Plant: AC - reisolated from Ailsa Craig; MM - reisolated from Money Maker.

Isolate	Plant	Concentrations of Nfx			
		0	20	50	100
FC1	AC	0.76	0.68	0.43	0.26
FC3	AC	0.60	0.48	0.32	0.19
O-4	AC	0.82	0.63	0.35	0
FC1*	AC	0.83	0.58	0.37	0.13
FC1*	MM	0.80	0.65	0.41	0.27
O-4	MM	0.90	0.58	0.37	0.21

Table 3.11 Percentage growth rates of fungus reisolated from plants.

Units and abbreviations are as in legend to table 3.10.

Isolate	Plant	Concentrations of Nfx			
		0	20	50	100
FC1	AC	100	88.5	56.6	33.7
FC3	AC	100	79.2	53.2	31.3
O-4	AC	100	77.5	42.8	0
FC1*	AC	100	69.7	44.5	15.8
FC1*	MM	100	81.2	51.0	33.4
O-4	MM	100	64.8	40.8	23.1

Table 3.12 Growth rates of mycelium on polypectate agar. Growth rates are in cm/day.

Isolate	Growth rate on D-glucose;	on polypectate
FC1	0.34	1.27
FC3	0.26	1.27
FC4	0.28	0.90
O-4	0.28	1.22
O-13	0.32	1.23
O-BD	0.24	1.11

3.3 ATTEMPTS TO DETECT A PLASMID IN *FUSARIUM*

3.3.1 Detection of the 2 μ m plasmid in *Saccharomyces cerevisiae*

To show that the alkaline lysate method described in section 2.10.1 is suitable for isolating plasmid DNA from fungal, as well as bacterial, protoplasts, the 2 μ m plasmid was extracted from *Saccharomyces cerevisiae* S288c. Aliquots of the DNA prepared were digested with *Eco*RI and analysed by electrophoresis on an agarose gel (see figure 3.2). The DNA gave rise to two doublet bands after *Eco*RI digestion, with sizes calculated to be 4.18, 3.78, 2.39 and 2.25 kb. These values compare well with the expected sizes of *Eco*RI fragments deduced from the DNA sequence (Hartley & Donelson, 1980), i.e. 4.072, 3.911, 2.407 and 2.246 kb. This shows that reasonably pure plasmid DNA can be extracted from fungal protoplasts by this method.

3.3.2 Attempts to detect a plasmid in *Fusarium*

DNA was extracted from isolates FC1 and O-13 by the alkaline lysate method (2.10.1), and analysed by electrophoresis on an agarose gel. No discrete band of DNA was detected, only a low molecular weight smear, presumably degraded fragments of RNA.

Since precipitation with PEG is reported to be gentler than ethanol-precipitation for large DNA molecules (Humphreys *et al.*, 1975), DNA was extracted from isolates FC1, FC4 and O-13 by the modified alkaline lysate described in section 2.10.2. Again, no discrete band of DNA was detected after electrophoresis.

In case the alkaline lysis step was causing loss or degradation of a plasmid, a gentler lysis method was used, modified from the Hirt

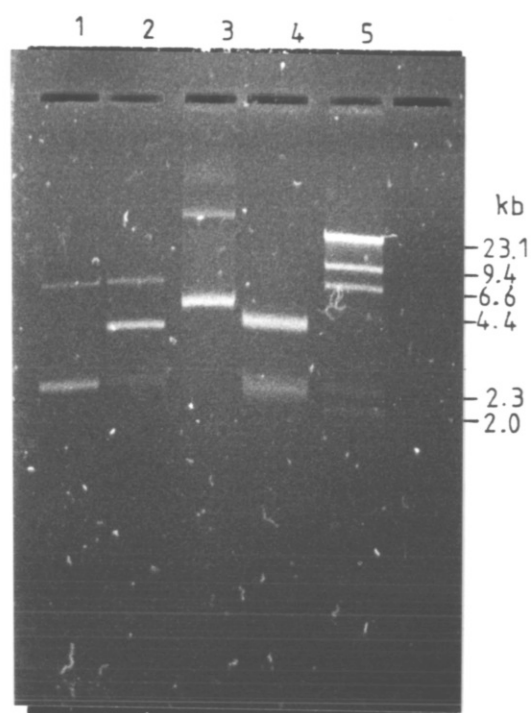


Figure 3.2 Restriction analysis of 2 μ m plasmid extracted from *S. cerevisiae*. Lane 1, pAT153 DNA; lane 2, pAT153 digested with *EcoRI*; lane 3, DNA from S288c; lane 4, DNA from S288c digested with *EcoRI*; lane 5, *HindIII* digest of λ DNA.

procedure for bacterial plasmids (Hirt, 1967). DNA was extracted from FC1 and FC3 as described in section 2.10.3. A single band of DNA was seen on the isopycnic CsCl gradients. When this band was extracted, a low yield of high molecular weight DNA was obtained, which comigrated with phage λ DNA when electrophoresed on a 0.4 % agarose gel. When digested with *HindIII*, this DNA produced a featureless smear on an agarose gel which indicates the preparation consisted of heterogeneous sheared fragments, probably of chromosomal DNA. No difference could be detected between the DNA extracted from FC1 and that from FC3.

Finally, DNA was extracted by the NaCl/SDS lysate method from FC1 mycelium ground under liquid nitrogen, as described in section 2.10.4. This method was originally used to isolate both plasmid and mtDNA from *Cephalosporium acremonium* (Minuth *et al.*, 1982). A single band of DNA was obtained which comigrated with λ DNA when electrophoresed on a 0.4 % agarose gel. When digested with *HindIII* this produced a featureless smear (figure 3.3, lane 2); when digested with *BamHI*, faint bands could be seen superimposed on the background smear (figure 3.3, lane 4). These bands are probably due to repeated DNA sequences in the chromosomal DNA. No evidence for a plasmid in *Fusarium* was obtained from any of these extraction procedures. DNA was also extracted from crude subcellular fractions (see section 3.7), and from purified mitochondria (sections 3.5 and 3.6).

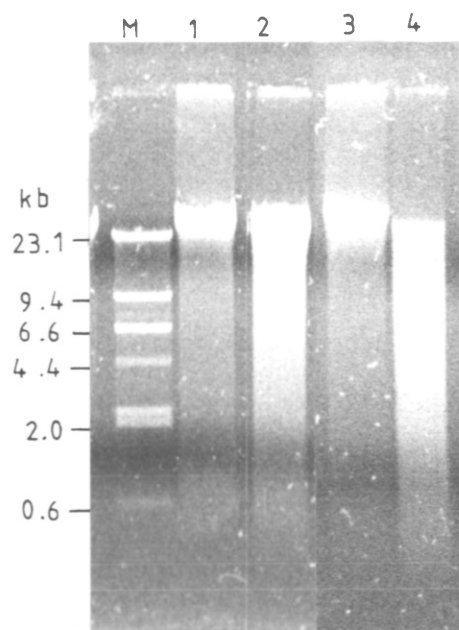


Figure 3.3 Restriction analysis of DNA extracted from *Fusarium*. Lanes 1 and 3, DNA from FC1, extracted by method 4; lane 2, FC1 DNA digested with *Hind*III; lane 4, FC1 DNA digested with *Bam*HI; lane M, *Hind*III digest of λ DNA.

3.4 DOUBLE-STRANDED RNA CONTENT OF *FUSARIUM OXYSPORUM* F. SP. *LYCOPERSICI*

3.4.1 Nucleic acids purified by cellulose column chromatography

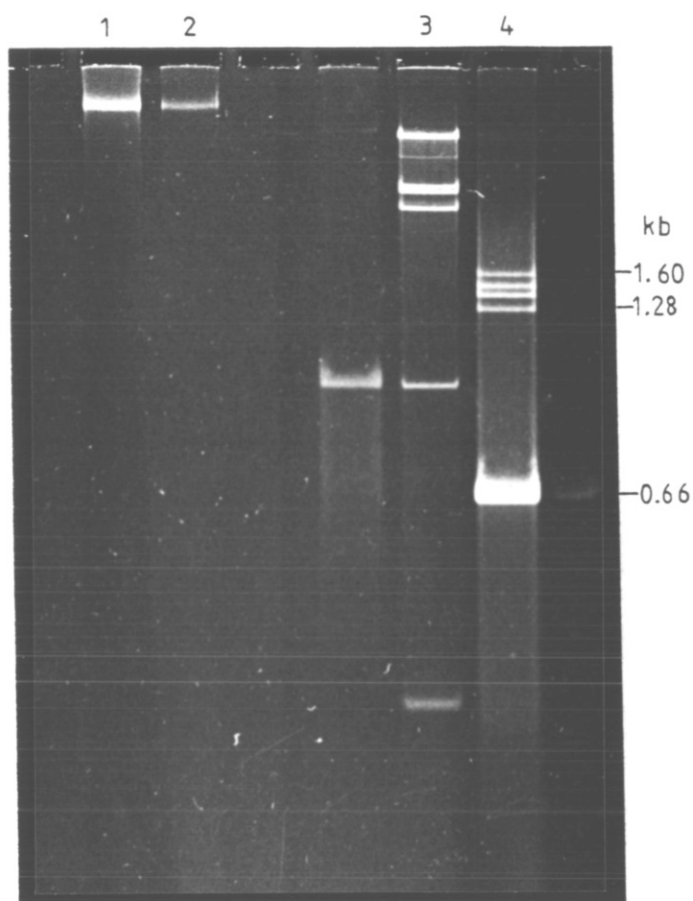
Nucleic acids were isolated from FC1* and O-13 by the method of Morris & Dodds (1979) (section 2.13.1). In this procedure, the nucleic acids are applied to a column of cellulose in 15 % v/v ethanol. Under these conditions, only dsRNA will bind to the column. Once the DNA and ssRNA has been removed by washing with 15 % ethanol, the dsRNA is eluted with buffer containing no ethanol.

Analysis by PAGE showed a single high molecular weight band from each isolate. To determine if these bands were DNA or RNA, digests of each with DNase, RNase and S1 nuclease were performed, as in section 2.7. The result is shown in figure 3.4. The *Fusarium* bands were digested by DNase but unaffected by RNase, indicating that the material was DNA. This DNA was apparently digested by S1; this may indicate that the DNA was substantially single-stranded, or more likely that the S1 preparation was contaminated with DNase.

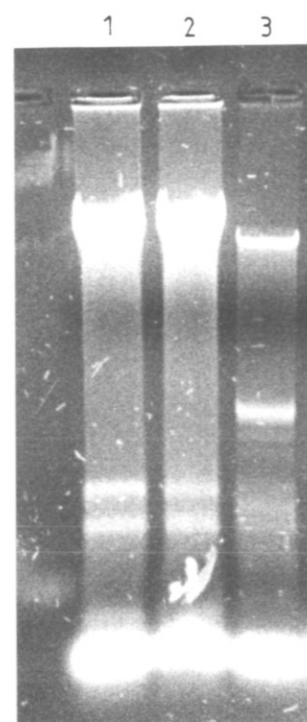
A similar single DNA band was also found in isolate FC3.

3.4.2 Total nucleic acid extracts

Fungal mycelium was ground under liquid nitrogen, and the LiCl-soluble nucleic acids purified as described in section 2.13.2. *F. oxysporum* isolates FC1* and FC3 were analysed, and also *Gaeumannomyces graminis* var. *tritici* (Ggt) isolate 3b1a, which is known to contain dsRNA (Buck et al., 1981). When analysed by agarose gel electrophoresis, FC1* and FC3 gave a single high molecular weight band, which was digested by DNase



3.4



3.5

Figure 3.4 PAGE of nucleic acids extracted by cellulose column chromatography. Lane 1, nucleic acids from O-13; lane 2, nucleic acids from FC1*; lane 3, dsRNA from *Ustilago maydis* P4 (Koltin & Day, 1976); lane 4, dsRNA from PsV (Appendix II).

Figure 3.5 Agarose gel electrophoresis of total nucleic acid extracts. Lane 1, total nucleic acids from FC1*; lane 2, total nucleic acids from FC3; lane 3, total nucleic acids from Ggt 3b1a.

but not by RNase. There were also two rather fuzzy low molecular weight bands which were digested by RNase. These smaller bands stained red with acridine orange, indicating they were ssRNA, whereas the DNA band stained green indicating it was dsDNA.

Ggt 3b1a gave rise to a series of additional bands seen on agarose gels (figure 3.5), which were resolved much better by PAGE and which migrated at the positions expected for the 3b1a viral dsRNAs. This indicates that the methods used are sufficiently sensitive to detect dsRNA when present. The *F. oxysporum* isolates tested had no dsRNA detectable by these methods, and certainly very much less than Ggt 3b1a.

3.5 PREPARATION OF MITOCHONDRIAL DNA

3.5.1 Preparation of protoplasts

In initial experiments, protoplasts were made from spores since these should have less cell wall material to digest away than mycelium. Using the citrate-phosphate buffer of Hamlyn *et al.* (1981), and enzymes at 5 mg/ml, the effect of using either Novozym 234 or a combination of Novozym 234 and Cellulase-CP, at either 24°C or 37°C, was tested using 0.7 M NaCl as osmotic stabiliser. The combination of both enzymes gave the highest yield of protoplasts, ≈50 % after 5 hr, with slightly more at 37°C, so these conditions were used to optimise osmotic strength.

A range of NaCl concentrations from 0.7 to 1.2 M was tested, giving the greatest yield of ≈50 % in 3 hr using 1 M NaCl.

Next the effect of pretreatment with a reducing agent (10 mM DTT) was tested, and also the effect of replacing some or all of the NaCl with

sorbitol. In all cases, treatment with DTT approximately doubled the yield of protoplasts, with 0.7 M NaCl + 0.3 M sorbitol giving >90 % protoplasts in a 3.5 hr incubation. The protoplasts were stable in this buffer for at least 18 hr.

For larger scale protoplast preparations (i.e. spores from 100 ml culture, see section 2.8.2) DTT was replaced with 0.7 % 2-mercaptoethanol, and 0.3 M sorbitol replaced with 0.3 M sucrose, for reasons of economy.

To maximise the amount of protoplasts for preparation of mitochondria, fungus was grown as mycelium to yield ≈ 50 g per 200 ml culture. To obtain a good yield of protoplasts (i.e. at least half the mycelium and spores converted to protoplasts), the incubation time was increased to 16 hr, and the temperature was reduced to 24°C to minimise chances of protoplast degradation.

3.5.2 Methods of extracting mtDNA

Since mtDNA was not resolved from nuclear DNA on isopycnic CsCl gradients during attempted plasmid preparations (section 3.3.2), it was decided to extract the mtDNA from purified mitochondria rather than crude cell extracts.

The sucrose step-gradient method of Tudzynski & Esser (1979) was used, following one cycle of differential centrifugation to remove nuclei and cellular debris (see section 2.9.1). Mitochondria were observed as a dense pigmented band at the interface of the 35 % and 60 % w/v sucrose layers after centrifugation (for proof of this identification see section 3.6.1). A gentle method of cell breakage, namely grinding the mycelium with sand in a mortar was used initially (Lambowitz & Luck, 1976; Collins *et al.*,

1981) but this gave a very low yield of mitochondria as judged by the band intensity, presumably due to insufficient breakage of the cells.

A much better yield was obtained by protoplasting the cells and then breaking the protoplasts in a Braun homogeniser as described in section 2.9.1.

A further problem was encountered when trying to extract the DNA from the purified mitochondria. A very low yield of DNA was obtained from any protocol involving precipitation of the DNA with ethanol; apparently the process of precipitation, pelleting and drying of the pellet caused the DNA to aggregate irreversibly, such that only a very small amount of the pelleted DNA was soluble, and the DNA which did dissolve was not cut by restriction enzymes. Hence, in the optimised protocol, the DNA was freed of CsCl and buffer components by dialysis against a very low concentration buffer and then concentrated by butanol extraction.

Using the optimised protocol of section 2.9.1, DNA was obtained which migrated as a single sharp band when electrophoresed on agarose gels, at the position of phage λ DNA.

The yield of this DNA was 60 μ g from 50 g mycelium, estimated using ethidium-agarose plates as in section 2.1.7.

3.6 CHARACTERISATION OF THE MITOCHONDRIAL DNA

3.6.1 Characterisation of the mitochondria

The putative mitochondrial preparation was fractionated on a sucrose step-gradient, and the fractions were assayed for Cytochrome c Oxidase activity by their ability to oxidise externally added reduced cytochrome

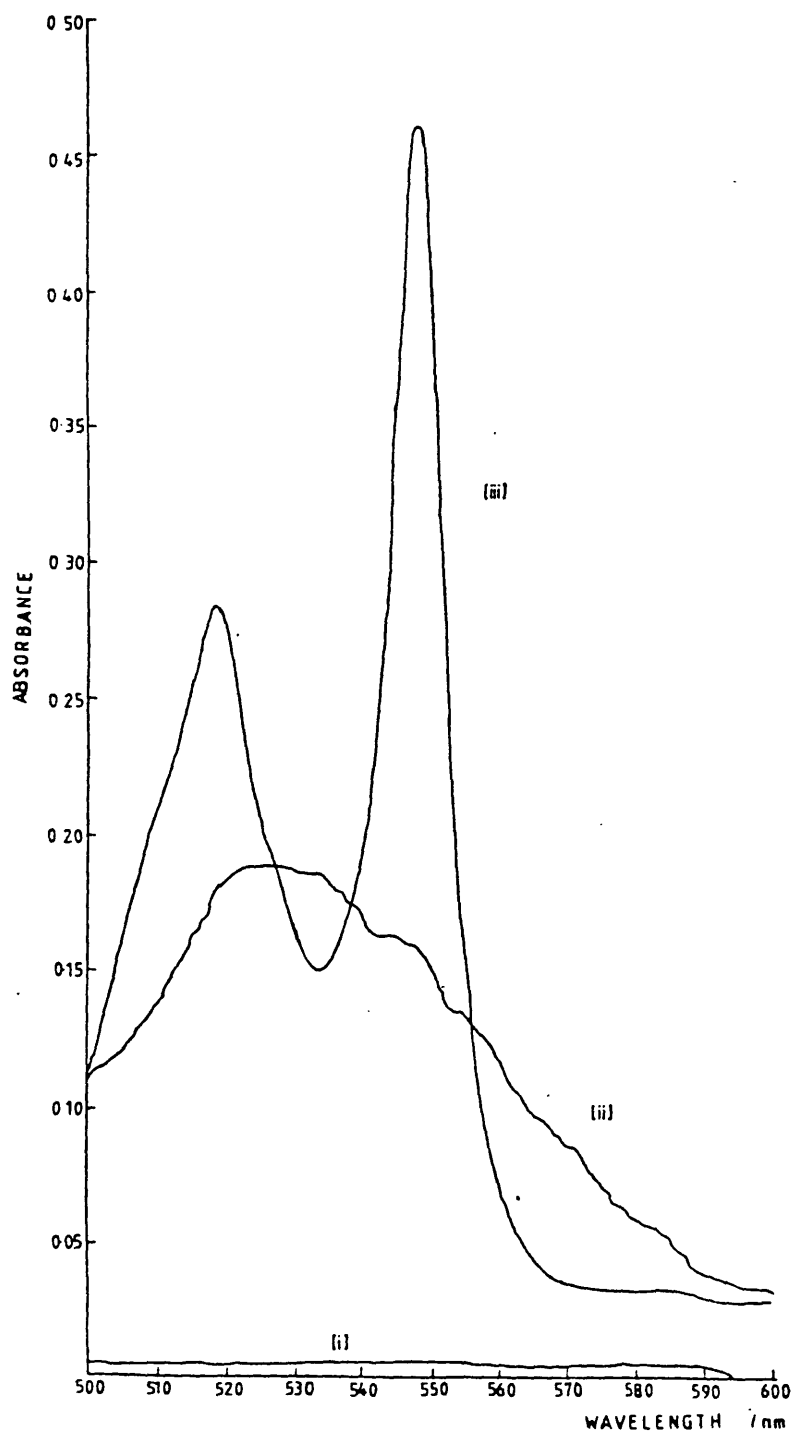


Figure 3.6 Absorbance spectra of oxidised and reduced cytochrome c. (i) no cytochrome c; (ii) oxidised cytochrome c, 17.8 μM ; (iii) reduced cytochrome c, 17.8 μM .

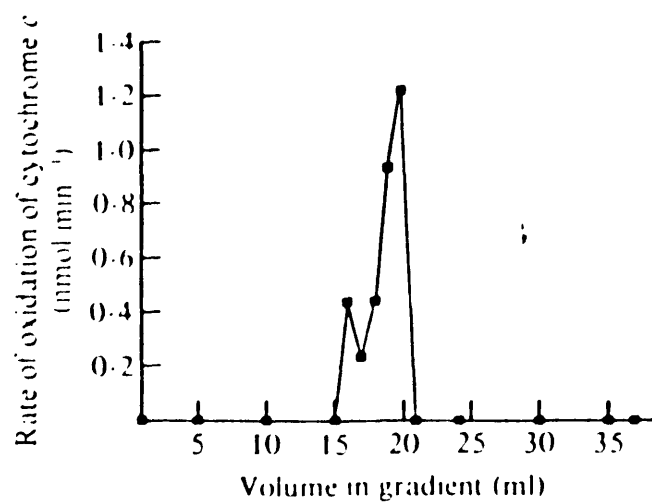


Figure 3.7 Cytochrome c Oxidase activity of fractions from a sucrose gradient.

c. Cytochrome c was reduced by adding an excess of DTT, and the oxidation state of the protein was determined by scanning the absorbance spectrum between 500 and 600 nm. Figure 3.6 shows that oxidised cytochrome c shows a broad peak with a maximum at ≈ 525 nm, and an absorbance of 8,100 /cm/M at 550 nm. When reduced, the spectrum is altered to two sharp peaks with maxima at 521 nm (β) and 550 nm (α), with the A_{550} increased to 28,400 /cm/M (Schnarrenberg *et al.*, 1971). Hence the proportion of a known concentration of cytochrome c which is reduced can be calculated from the measured A_{550} . The absorbance due to the mitochondria alone was negligible (A_{550} of 0.004 for 1 μ l in a 2 ml cuvette).

A stock which was ≈ 60 % reduced was then oxidised by adding 1 μ l aliquots from each fraction of the sucrose gradient, and A_{550} was monitored with time for about 5 min. using the chart recorder.

The A_{550} decreased linearly with time over this period. The slope of this line was measured for each fraction, and the rate of change of A_{550} was converted to nmol cytochrome c oxidised /min. using:

$d/dt (A_{550})$ of 1 /min. is equivalent to 4.93×10^4 nM/min.,

or 98.5 nmol/min. in each 2 ml reaction.

Activity was plotted against fraction number and the result is shown in figure 3.7. The peak of activity corresponds to the dense pigmented band which was collected, and from which DNA was extracted in other experiments. Since Cytochrome c Oxidase is an integral membrane enzyme located on the inner mitochondrial membrane, this shows that the subcellular fraction analysed did indeed contain the mitochondria.

3.6.2 Location of the mtDNA

In order to prove that the putative mtDNA was located within a membrane-enclosed compartment (i.e. the mitochondria), the following nuclease-protection experiment was performed.

Plasmid pAT153 DNA was added to 3 aliquots of mitochondria, and these were then treated with (i) no treatment; (ii) DNase; (iii) DNase + Nonidet P-40, as described in section 2.9.2.

The mitochondria were then purified and DNA extracted from them as usual, and also DNA was extracted from the extramitochondrial supernatant.

The pAT153 DNA recovered from the supernatant was analysed by agarose gel electrophoresis, shown in figure 3.8. The plasmid was detected only in the sample with no DNase, showing that the DNase was active in degrading extramitochondrial DNA even in the presence of 1 % Nonidet P-40.

The DNA from the mitochondrial fractions was analysed by digestion with EcoRI followed by agarose gel electrophoresis (see figure 3.9; note that the fraction of the total DNA recovered in lane 3 is twice that in lanes 1 and 2). The usual pattern of mtDNA bands was seen in all 3 fractions, but much less was obtained after treatment with both DNase and Nonidet P-40. The amounts recovered were estimated from agarose-ethidium fluorescence (section 2.1.7) to be: no treatment 12 μ g; treated with DNase 16 μ g; treated with DNase + Nonidet P-40 1.4 μ g.

This shows that the DNA extracted from mitochondria is protected from exogenous DNase, presumably by being located inside the mitochondria. After DNase treatment in the presence of 1 % Nonidet P-40, only 10 % of the control amount of mtDNA was recovered, implying that the nonionic

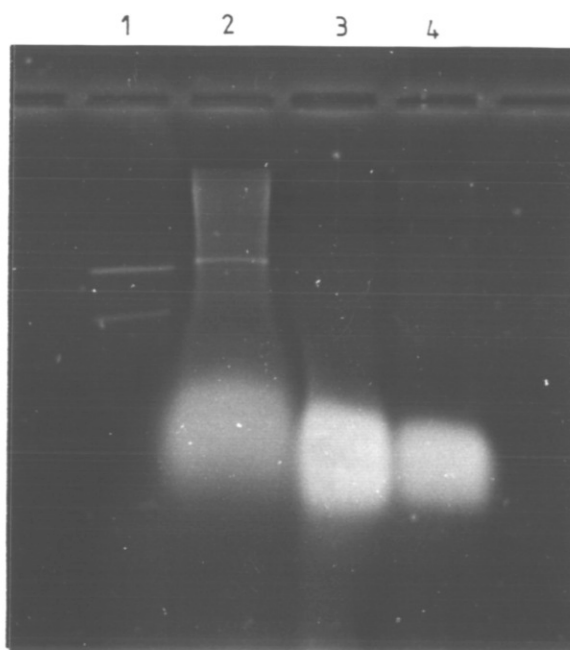


Figure 3.8 Recovery of extramitochondrial DNA. Lane 1, pAT153 DNA; lanes 2-4, DNA recovered after: 2, no treatment; 3, DNase treatment; 4, DNase + Nonidet P-40 treatment.

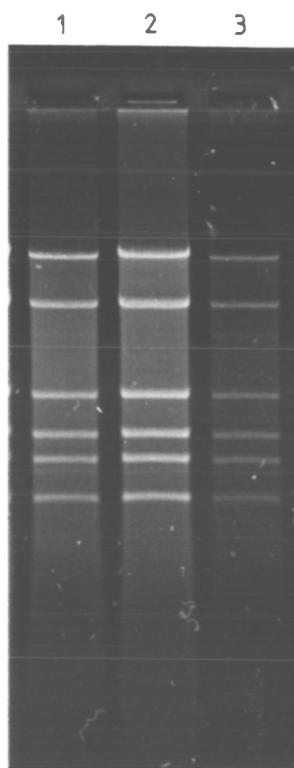


Figure 3.9 Recovery of intramitochondrial DNA. *Eco*RI digests of DNA recovered from mitochondria after: lane 1, no treatment; lane 2, DNase treatment; lane 3, DNase + Nonidet P-40 treatment.

detergent disrupted a lipid membrane, allowing DNase access to the mtDNA in ≈ 90 % of the mitochondria.

3.6.3 Restriction endonuclease analysis of mtDNA

In initial experiments in which either BSA or spermidine was omitted from restriction digests, the mtDNA was resistant to most enzymes, presumably due to inhibitory contaminants in the preparations. By adding both BSA and spermidine, digestion was observed with all the enzymes tested, either singly or in combination with other enzymes.

Examples of the patterns obtained are shown in figures 3.10 to 3.12.

The size of each band was determined using the phage λ DNA digests electrophoresed on the same gel as size standards. For each gel, a calibration curve of $\log(\text{size in bp})$ vs. migration was plotted, and the size of each mtDNA fragment read off this curve. Bands of >25 kb could not be sized accurately, even from overnight electrophoresis runs, and so their size was deduced from double digests.

A complete list of restriction fragment sizes is given in table 3.13.

The mean size, calculated from 22 single and double digests, is 46.5 kb.

The enzymes *Sall*, *SmaI* and *XhoI* produced no visible effect on the mtDNA band migration in single digests; however when used in combination with other enzymes each gave rise to one extra band, implying that each cuts the mtDNA once, converting it from a circular to linear form.

A faint extra band was sometimes seen in *BamHI* digests at 14.7 kb: it is presumably a partial digestion product, since its size is very close to that expected for the two smaller fragments combined.

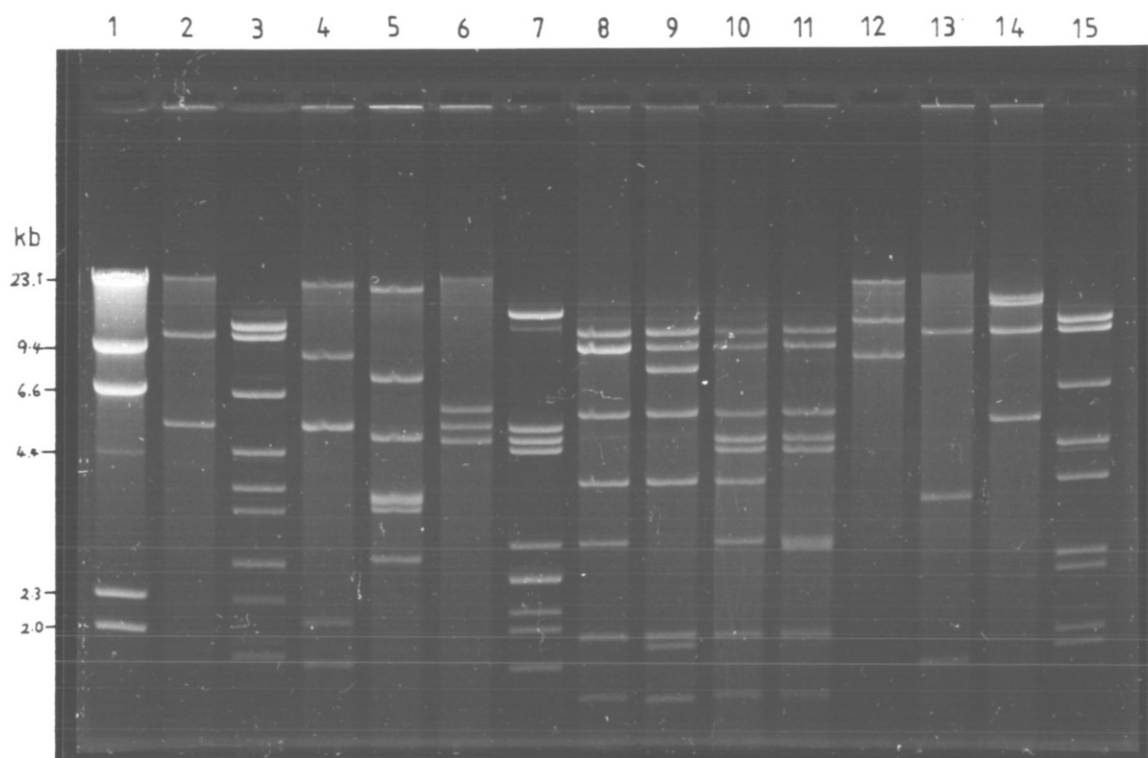


Figure 3.10 Restriction endonuclease analysis of mtDNA. Lane 1, λ DNA $\times$ *HindIII*; lane 2, mtDNA $\times$ *ClaI*; lane 3, λ DNA $\times$ *ClaI*; lane 4, mtDNA $\times$ *ClaI* + *BamHI*; lane 5, mtDNA $\times$ *ClaI* + *EcoRI*; lane 6, mtDNA $\times$ *ClaI* + *XhoI*; lane 7, λ DNA $\times$ *PstI*; lane 8, mtDNA $\times$ *PstI*; lane 9, mtDNA $\times$ *PstI* + *BamHI*; lane 10, mtDNA $\times$ *PstI* + *XhoI*; lane 11, mtDNA $\times$ *PstI* + *ClaI*; lane 12, λ DNA $\times$ *SmaI*; lane 13, mtDNA $\times$ *SmaI* + *ClaI*; lane 14, mtDNA $\times$ *SalI* + *ClaI*; lane 15, λ DNA $\times$ *SalI* + *ClaI*.

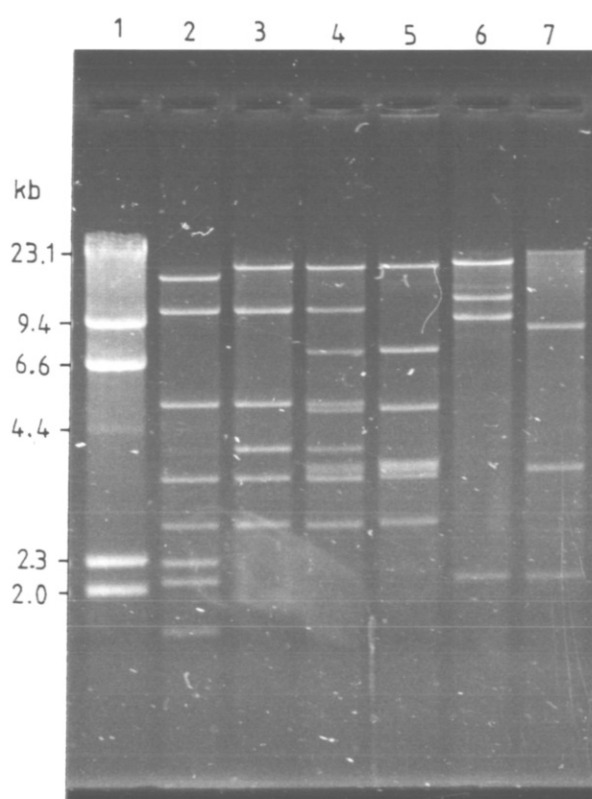


Figure 3.11 Restriction endonuclease analysis of mtDNA. Lane 1, λ DNA $\times$ *HindIII*; lane 2, mtDNA $\times$ *EcoRI* + *BamHI*; lane 3, mtDNA $\times$ *EcoRI*; lane 4, mixture of lanes 3 and 5; lane 5, mtDNA $\times$ *EcoRI* + *ClaI*; lane 6, mtDNA $\times$ *SalI* + *BamHI*; lane 7, mtDNA $\times$ *SmaI* + *BamHI*.

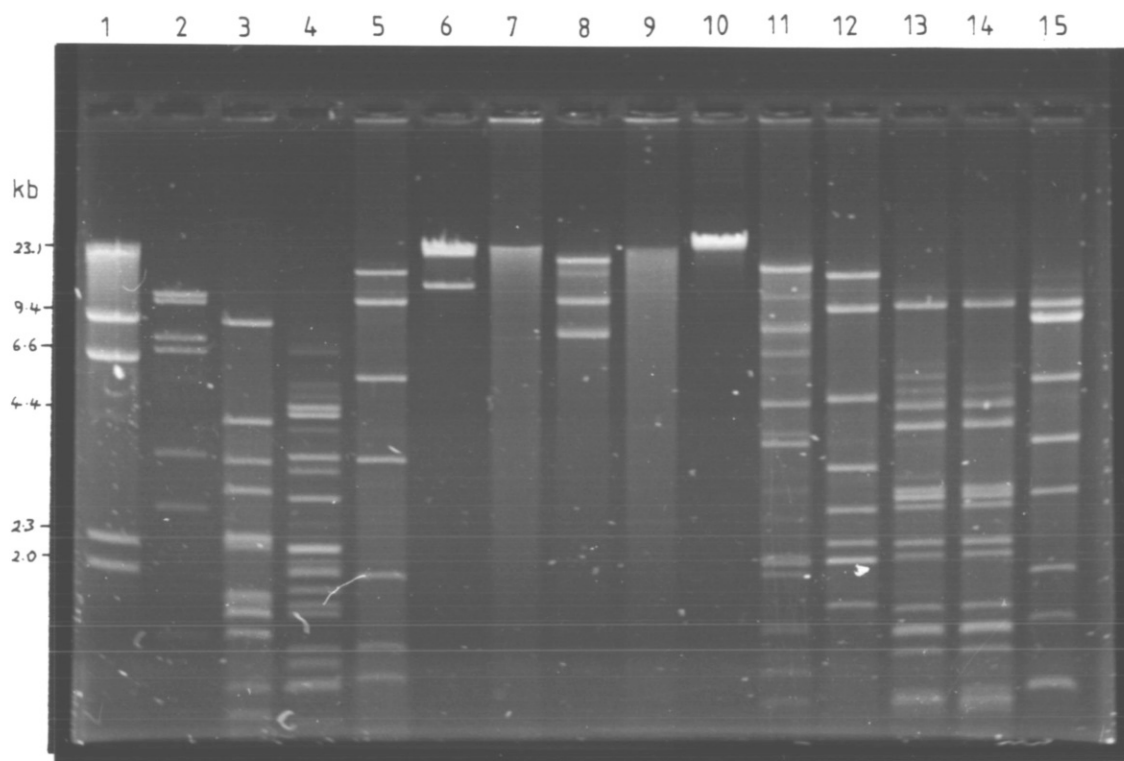


Figure 3.12 Restriction endonuclease analysis of mtDNA. Lane 1, λ DNA $\times$ *Hind*III; lane 2, λ DNA $\times$ *Acc*I; lane 3, mtDNA $\times$ *Acc*I; lane 4, λ DNA $\times$ *Hind*II; lane 5, mtDNA $\times$ *Hind*II; lane 6, λ DNA $\times$ *Sal*I; lane 7, mtDNA $\times$ *Sal*I; lane 8, λ DNA $\times$ *Sma*I; lane 9, mtDNA $\times$ *Sma*I; lane 10, λ DNA $\times$ *Xba*I; lane 11, mtDNA $\times$ *Xba*I; lane 12, mtDNA $\times$ *Eco*RI + *Bam*HI; lane 13, mtDNA $\times$ *Hind*III; lane 14, mtDNA $\times$ *Hind*III + *Xho*I; lane 15, mtDNA $\times$ *Pst*I.

Table 3.13 Restriction fragment sizes (in kb) of FC1 mtDNA. Σ refers to the sum of restriction fragment sizes for each combination of enzymes. The figure in brackets after the enzyme name(s) is the total number of fragments observed for that digest.

(i) Single enzymes

<i>Bam</i> HI (3)	31.6 ^a	12.3	2.11							Σ 46.0
<i>Cla</i> I (3)	30.7 ^a	11.2	5.26							Σ 47.2
<i>Eco</i> RI (6)	21.1	10.9	5.08	3.96	3.41	2.73				Σ 47.2
<i>Pst</i> I (9)	11.1	9.2	9.2	5.60	3.81	2.87	1.96	1.53	1.07	Σ 46.4
<i>Ava</i> II (10)	12.5	11.0	7.07	5.13	3.28	2.35	1.71	1.57	1.31	Σ 46.5
	0.50									Σ 46.7
<i>Hind</i> II (10)	19.1	11.9	5.80	3.53	1.95	1.33	1.11	0.75	0.64	Σ 46.7
	0.55									Σ 47.4
<i>Hae</i> II (12)	12.3	10.2	9.8	5.54	3.11	1.76	1.12	1.02	0.93	Σ 47.4
	0.77	0.49	0.33							Σ 47.2 ^c
<i>Xba</i> I ^b (>10)	19.5	8.65	5.03	4.00	2.16	2.16	2.03	1.52	1.18	Σ 47.2 ^c
	1.00									Σ 36.9 ^a
<i>Acc</i> I ^d (>15)	8.99	4.40	3.48	3.02	2.39	2.39	2.32	1.78	1.64	Σ 35.0 ^a
	1.64	1.47	1.09	0.92	0.76	0.57				Σ 43.8 ^a
<i>Hae</i> III ^d (>15)	5.26	4.25	3.58	2.98	2.85	2.63	2.33	2.00	1.91	Σ 43.8 ^a
	1.62	1.50	1.05	0.92	0.79	0.77				Σ 45.3
<i>Hind</i> III ^b (>17)	11.1	4.79	4.23	2.98	2.91	2.74	2.36	2.20	1.71	Σ 45.3
	1.52	1.52	1.35	1.05	0.99	0.91	0.79			

(ii) Double digests

<i>Bam</i> HI + <i>Xho</i> I (4)	28.8	11.6	2.78	2.07						Σ 45.3
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Table 3.13 (continued)

<i>Bam</i> HI + <i>Sma</i> I (4)	31.6*	9.16	3.62	2.21					£46.6
<i>Bam</i> HI + <i>Sal</i> I (4)	20.8	12.3	9.92	2.21					£45.2
<i>Cla</i> I + <i>Xho</i> I (4)	30.7*	5.93	5.35	4.92					£46.9
<i>Cla</i> I + <i>Sma</i> I (4)	30.7*	11.1	3.48	1.74					£47.0
<i>Cla</i> I + <i>Sal</i> I (4)	16.4	15.3	10.6	5.39					£47.8
<i>Cla</i> I + <i>Bam</i> HI (6)	22.8	8.74	5.26	5.26	2.11	1.77			£45.9
<i>Eco</i> RI + <i>Xho</i> I (7)	21.1	10.9	5.08	3.96	2.73	2.30	1.22		£47.3
<i>Eco</i> RI + <i>Bam</i> HI (9)	16.1	10.9	5.08	3.41	2.73	2.34	2.13	2.13	
		1.72							£46.5
<i>Eco</i> RI + <i>Cla</i> I (9)	21.1	7.3	4.89	3.57	3.53	3.41	2.73	0.35	
		0.17*							£47.0
<i>Pst</i> I + <i>Xho</i> I (10)	11.1	9.2	5.60	4.81	4.49	3.81	2.87	1.96	
		1.53	1.07						£46.4
<i>Pst</i> I + <i>Sma</i> I (10)	11.1	9.3	8.52	5.60	3.81	2.87	1.96	1.53	
		1.07	1.02						£46.8
<i>Pst</i> I + <i>Sal</i> I (10)	9.2	9.2	7.08	5.60	4.76	3.81	2.87	1.96	
		1.53	1.07						£47.1
<i>Pst</i> I + <i>Bam</i> HI (11*)	11.1	9.2	7.56	5.60	3.81	1.96	1.87	1.53	
		1.18	1.07	0.57					£45.5*
<i>Pst</i> I + <i>Cla</i> I (11*)	11.1	9.2	5.60	4.74	4.42	2.87	2.79	1.96	
		1.53	1.00	0.76					£46.0*
<i>Pst</i> I + <i>Eco</i> RI (15)	9.4	5.60	4.51	3.81	3.29	3.19	2.87	2.62	
		2.40	1.91	1.85	1.50	1.40	0.63	0.39	£45.2
<i>Hind</i> II + <i>Xho</i> I (10*)	19.1	11.9	5.80	3.31	1.95	1.33	1.11	0.75	
		0.64	0.55						£46.4*

Table 3.13 (continued)

<i>Hind</i> II + <i>Cla</i> I (12 ^a)	14.0	11.9	4.29	3.75	3.53	1.76	1.33	1.28
	1.11	0.75	0.64	0.55				Σ44.9 ^a
<i>Ava</i> II + <i>Cla</i> I (12 ^a)	12.5	7.07	5.13	4.98	4.59	3.20	2.35	1.71
	1.66	1.57	1.31	0.50				Σ46.6 ^a
<i>Hae</i> II + <i>Cla</i> I (15)	9.8	9.8	8.4	3.25	3.11	2.09	1.91	1.81
	1.76	1.12	1.02	0.93	0.77	0.49	0.33	Σ46.6

Notes

^a accurate value was deduced from double digests

^b partial digests only obtained; values given are those of the brightest bands

^c sum not included in calculation of mean size, since digest was not to completion

^d many smaller bands visible but not well resolved

^e sum not included in calculation of mean size, since there should be at least one extra band which was not detected in these experiments

^f fragment was not visualised; its size was calculated from the map

^g number of fragments too small, implying one extra fragment which was not detected.

3.6.4 Construction of a restriction map of mtDNA

The fragments of FC1 mtDNA produced by digestion with *XhoI*, *Sall*, *SmaI*, *BamHI*, *ClaI* and *EcoRI* fit unambiguously onto a circular map of 46.5 kb, as shown in figure 3.13. Numbering is in kb from the unique *XhoI* site. The precise relative locations of the two sets of nearby *ClaI* and *EcoRI* sites (at ≈ 5 kb and ≈ 10 kb on the map) were worked out by mixing digests of mtDNA with *EcoRI*, and with *EcoRI* + *ClaI*, and electrophoresing them in the same lane of a gel to resolve bands which are very close in size (see figure 3.11, lane 4).

3.6.5 Electron microscopy of mtDNA

MtDNA (from DNase-treated mitochondria) was mixed with SV40 or pTSV3 DNAs and spread on Parlodion-coated grids. Six grids were searched for intact circular molecules. When found, large circular molecules were photographed such that at least one size-marker molecule was in the picture.

The photographs were printed and the contour lengths of each molecule determined relative to the marker, and hence converted to size in kb. Each circle was measured at least twice, and the mean value taken, to reduce error in measurements.

The great majority of DNA observed on the grids was long linear molecules. These were not measured since there was no way of judging if these molecules had suffered two or more breakages. All these linear molecules were presumably derived from the circular form by breakage, either during extraction or (more likely) during spreading.

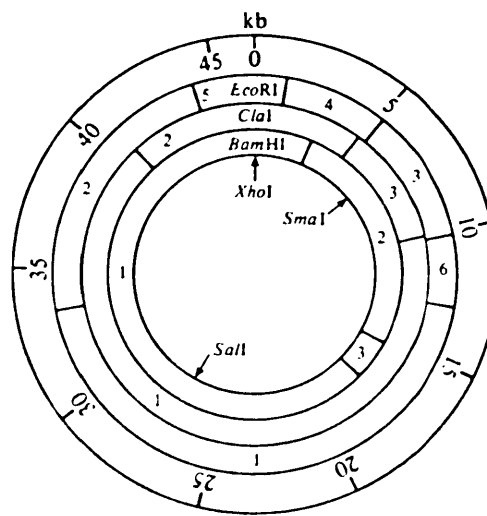


Fig 3.13 Circular restriction map of *F. oxysporum* strain FC1 mtDNA.

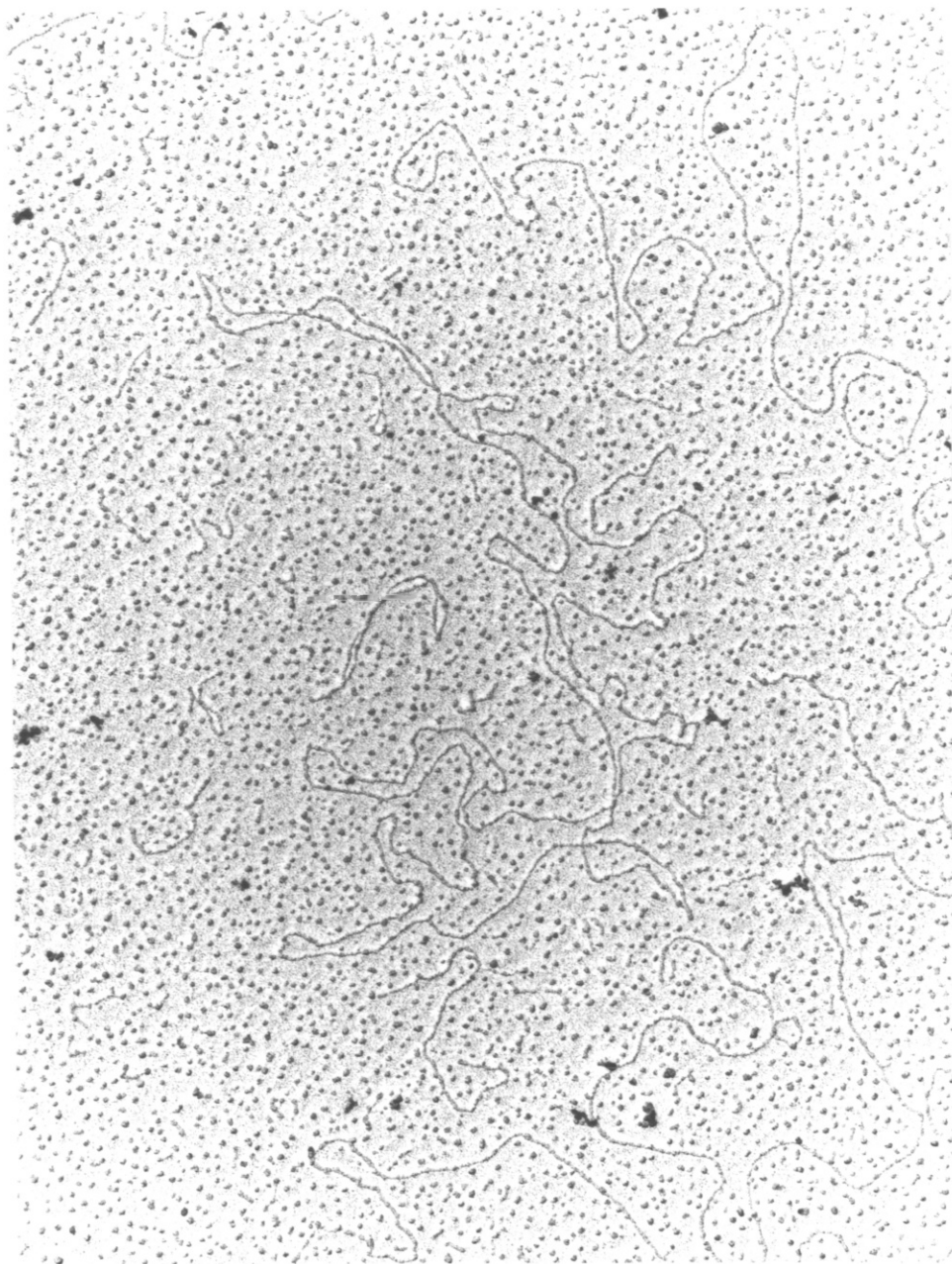


Figure 3.14 Electron micrograph of FC1 mtDNA circular molecule.

No difference was observed between grids spread with 0.5 μ l or 1 μ l of cytochrome c stock.

18 separate mtDNA molecules were measured, and the mean size was calculated to be 46.8 ± 0.7 kb, in good agreement with the size obtained from the restriction map.

One such circular mtDNA molecule is shown in figure 3.14, with an SV40 marker molecule.

3.6.6 Buoyant density of *Fusarium* DNA in CsCl

MtDNA and nDNA (prepared as described in section 2.12) were centrifuged to equilibrium in CsCl gradients, in the absence of ethidium bromide. Each fraction was assayed for DNA by spotting on agarose-ethidium plates, and its refractive index was measured and converted to density.

Three gradients were fractionated for each DNA species; the densities of fractions at the centres of the peaks were:

mtDNA 1.685, 1.685, 1.686; mean 1.685 g/ml

nDNA 1.690, 1.693, 1.693; mean 1.692 g/ml

3.7 DETECTION OF MITOCHONDRIAL DNA IN FUNGAL EXTRACTS

3.7.1 Screening subcellular fractions for mtDNA

DNA was extracted from "nuclear", "mitochondrial" and "cytoplasmic" fractions of FC1 by the method of Guardiola *et al.* (1982), as described in section 2.11. These DNA samples were then digested with various restriction enzymes and analysed by agarose gel electrophoresis (figure 3.15).

Digestion with *EcoRI* gave a smear down the gel with a pattern of bands of various intensities superimposed. The "mitochondrial" fraction showed a typical mtDNA pattern, plus extra bands which may mean it was contaminated with nuclear DNA (lane 3). "Nuclear" and "cytoplasmic" fractions showed a pattern with the major bands not corresponding to mtDNA bands - these could be due to repeated DNA sequences in the nDNA (lanes 4 and 1, respectively). Some of the fainter bands may correspond to mtDNA bands, but this is not clear.

Digestion with *ClaI* gave smears with no clear bands superimposed. However, since *HaeIII* has a 4 bp recognition site and would be expected to produce a background smear of much lower mean molecular weight, this enzyme was used (see figure 3.15, lanes 6 and 7). Using *HaeIII*, the 11 largest mtDNA fragments can also be detected in "cytoplasmic" DNA. Since Guardiola *et al.* (1982) isolated their 46.7 kb circular DNA from "cytoplasmic" DNA, this makes it likely that their molecule was mtDNA, released from mitochondria by grinding under liquid nitrogen.

3.7.2 Screening other isolates for mtDNA

Since the above digestion with *HaeIII* clearly showed the presence of mtDNA in a sample containing much nuclear DNA, a preliminary experiment was conducted to detect mtDNA in FC3. A total DNA fraction was used (prepared as in section 2.13.2) and when digested with *HaeIII* gave three largest bands of 5.75, 4.66 and 3.67 kb, which compare well with the three largest *HaeIII* fragments of FC1.

These data indicate that certain regions of FC1 and FC3 mtDNAs are similar, but does not rule out the possibility of major rearrangements or deletions.

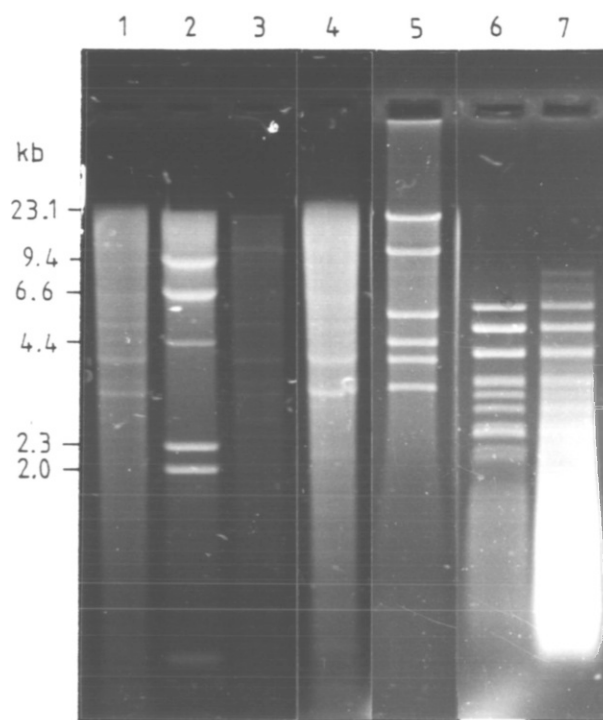


Figure 3.15 Restriction digests of DNA from various subcellular fractions. Lane 1, "Cytoplasmic" fraction DNA digested with *EcoRI*; lane 2, λ DNA digested with *HindIII*; lane 3, "Mitochondrial" fraction DNA digested with *EcoRI*; lane 4, "Nuclear" fraction DNA digested with *EcoRI*; lane 5, purified mtDNA digested with *EcoRI*; lane 6, mtDNA digested with *HaeIII*; lane 7, "Cytoplasmic" fraction DNA digested with *HaeIII*.

CHAPTER 4. DISCUSSION.

4.1 THE PHENOTYPE OF *FUSARIUM OXYSPORUM* F.SP. *LYCOPERSICI*

4.1.1 Resistance to antibiotics

Of the six isolates tested, namely FC1, FC3 and FC4 of Guardiola *et al.* (1982), and three race O isolates, all had roughly the same resistance to nifuroxime. Several showed slow growth on 100 µg/ml nifuroxime, and all grew at 43-61 % of the control rate on 35 µg/ml nifuroxime. This phenotype is intermediate between those described by Guardiola *et al.* (1982) for FC1 (nifuroxime-resistant) and FC3 (nifuroxime-sensitive). The strains which were inhibited most by nifuroxime (FC3, FC4 and O-BD) also had the slowest growth rates on control plates. The reason for this discrepancy from earlier work is not clear. Fresh samples of FC1 and FC3 were obtained from F. Cervone, and were found to have the same phenotype as those received previously. Maintaining the mycelium constantly in the presence of nifuroxime did lead to increased resistance to the higher concentrations of nifuroxime (strain FC1*), but the nifuroxime-resistant phenotype of Guardiola *et al.* (1982) was not reproduced. Similar results were obtained when nifuroxime was used to inhibit growth from single spores, or germination of spores. The filamentous fungus *Phialophora* sp. (lobed hyphopodia) was strongly inhibited at low concentrations of the antibiotic, showing that the preparation of nifuroxime used in this work was not inactive.

In addition to the above data, the isolate FC4 was found to be sensitive to iodoacetate, rather than resistant as claimed by Guardiola *et al.* (1982).

4.1.2 Pathogenicity on tomato plants

The failure to observe an equal severity of symptoms on all plants infected with a particular isolate of *F. oxysporum* could be due in part to the rather inefficient method of inoculation used, and also to growing the infected plants at a sub-optimal temperature (Jones & Woltz, 1981). Hence the pathogenicity of a particular isolate was deduced from the plant(s) showing the most marked symptoms. The strongest symptoms for each isolate were seen on tomato var. Ailsa Craig, and can be briefly summarised as: O-4 and FC1 (whether maintained in the presence of nifuroxime or not) were strongly pathogenic; FC3 was weakly pathogenic; and FC4 was non-pathogenic and could not be reisolated from inoculated plants. Once again, these results contrast to some extent with those of Guardiola *et al.* (1982). Isolates which were reisolated from plants showed a slight increase in resistance to nifuroxime: this could be attributed to the fact that they were reisolated in the presence of nifuroxime. All six isolates were able to grow on polypectate-agar without a lag period, possibly due to constitutive polygalacturonase activity; however, the possibility was not ruled out that the polypectate contained some free galacturonate which could induce an inducible polygalacturonase activity.

A comparison of the phenotypes determined in this work with those previously reported is shown in table 4.1.

4.2 ATTEMPTS TO DETECT EXTRACHROMOSOMAL NUCLEIC ACIDS

4.2.1 Attempts to detect a plasmid-like DNA

Preparations of DNA from *F. oxysporum* isolates FC1, FC4 and O-13, made

Table 4.1 Phenotypes of isolates FC1, FC3 and FC4.

Isolate	This work	Previous work ⁽¹⁾
FC1	Intermediate resistance to Nfx	Resistant to Nfx
	Sensitive to Iac	Sensitive to Iac
	Pathogenic	Pathogenic
FC3	Intermediate resistance to Nfx	Sensitive to Nfx
	Sensitive to Iac	Sensitive to Iac
	Pathogenic	Non-pathogenic
FC4	Intermediate resistance to Nfx	Resistant to Nfx
	Sensitive to Iac	Resistant to Iac
	Non-pathogenic	Pathogenic

Notes: Nfx, nifuroxime; Iac, iodoacetate; ⁽¹⁾ Guardiola *et al.* (1982).

by an alkaline lysate method which was successfully used to prepare the yeast 2 μ m plasmid, showed no signs of a plasmid-like DNA. Lysates made from FC1 and FC3 by a gentler SDS-NaCl method also failed to detect a plasmid-like DNA. Since the plasmid-like DNA reported in isolate FC1 by Guardiola *et al.* (1982) was within the size-range of fungal mtDNA molecules, mtDNA was prepared from FC1 to compare with the plasmid-like DNA. The characterisation of the mtDNA is discussed in section 4.3.

4.2.2 Attempts to detect dsRNA

Since the presence of various dsRNA molecules has been correlated with increases or decreases in pathogenicity in some fungi (Van Alfen, 1986; Rogers *et al.*, 1986; Stanway, 1985), nucleic acid extracts were made from isolates FC1, FC3 and O-13 by methods which were able to detect the dsRNA content of *Ggt* 3b1a. No dsRNA was detected in the *F. oxysporum* isolates.

4.3 PREPARATION AND CHARACTERISATION OF THE MITOCHONDRIAL DNA

4.3.1 Identification of the mtDNA

A method is described which produces a high yield of protoplasts from both spores and mycelium of *F. oxysporum* FC1 (section 2.8). Mitochondria were prepared by gentle breakage of the protoplasts, which was observed to give a higher yield of mitochondria than grinding the frozen mycelium. A fraction containing intact mitochondria was prepared by banding a partially purified preparation on sucrose density gradients; the integrity of the mitochondria was established by detection of cytochrome c oxidase activity, which is due to an integral protein of the inner mitochondrial

membrane. The DNA from this mitochondrial preparation was protected against nuclease digestion in the absence of detergent, but not in the presence of a nonionic detergent, implying that the DNA was enclosed within the mitochondrial membrane and was in fact the mtDNA. The results establish the presence of a single DNA species in mitochondrial preparations.

4.3.2 Characterisation of the mtDNA

Restriction endonuclease mapping of the mtDNA produced a unique circular restriction map on which the sites for six restriction enzymes can be placed unambiguously. The length of the mtDNA was calculated to be 46.5 kb, which lies roughly in the middle of the range of reported sizes for fungal mitochondrial DNAs (see section 1.2.2). Electron microscopy of DNA from nuclease-treated mitochondria also revealed the presence of circular molecules, with a contour length calculated to be 46.8 kb. The majority of DNA molecules seen by this method were linear, presumably resulting from breakage of the DNA during preparation of the grids. The size of the mtDNA of strain FC1 is remarkably similar to that reported for the plasmid-like DNA of *Guardiola et al.* (1982) (46.7 kb).

The buoyant density of the mtDNA in CsCl was 1.685 g/ml, compared to 1.692 g/ml for the nuclear DNA; the small difference could explain the difficulty in resolving the total DNA into two bands on CsCl-ethidium bromide gradients. The density is also within the range reported for other fungal mtDNAs (table 4.2).

Table 4.2 Buoyant densities of fungal mitochondrial DNAs.

Species	Density (g/ml)	Reference
<i>Saccharomyces cerevisiae</i>	1.684	Guérineau, 1979
<i>Saprolegnia</i> sp.(47-15a)	1.685	Clark-Walker & Gleason, 1973
<i>Fusarium oxysporum</i>	1.685	This work
<i>Hansenula wingei</i>	1.686	O'Connor <i>et al.</i> , 1975
<i>Torulopsis glabrata</i>	1.686	O'Connor <i>et al.</i> , 1976
<i>Saccharomycopsis lipolytica</i>	1.687	Kück <i>et al.</i> , 1980
<i>Cephalosporium acremonium</i>	1.689	Minuth <i>et al.</i> , 1982
<i>Schizosaccharomyces pombe</i>	1.689	Del Giudice <i>et al.</i> , 1979
<i>Aspergillus nidulans</i>	1.690	Lopez Perez & Turner, 1975
<i>Kluyveromyces lactis</i>	1.692	O'Connor <i>et al.</i> , 1975
<i>Podospora anserina</i>	1.693	Stahl <i>et al.</i> , 1980
<i>Candida parapsilosis</i>	1.698	O'Connor <i>et al.</i> , 1975
<i>Neurospora crassa</i>	1.698	Schäfer <i>et al.</i> , 1971

4.4 COMPARISON OF MITOCHONDRIAL DNA WITH THE PLASMID-LIKE DNA

The mtDNA of FC1 was the only circular DNA molecule detected in the fungus, and had a contour length of 46.5 kb. This makes it highly likely that the 46.7 kb plasmid-like DNA detected by Guardiola *et al.* (1982) was in fact the mtDNA. When subcellular fractions were prepared by the method of Guardiola *et al.* (1982), which involves grinding the mycelium under liquid nitrogen, mtDNA was detected by its characteristic *HaeIII* restriction pattern in both the mitochondrial and cytoplasmic fractions, the latter presumably due to breakage of some mitochondria. Guardiola *et al.* (1982) were unable to isolate the mtDNA intact, and reported that the circular DNA was not protected from nuclease digestion; again this result can be explained in terms of disruption of the mitochondria. Guardiola *et al.* (1982) also reported that strain FC3 lacked the 46.7 kb DNA; however my preliminary results suggest that a similar (if not identical) mtDNA can be detected in a *HaeIII* digest of total DNA from FC3. Indeed, it would be difficult to see how FC3 could grow as it does if it lacked all, or a major part, of its mtDNA. The various phenotypes correlated with the presence of the 46.7 kb DNA by Guardiola *et al.* (1982) were not reproducible in my hands, and it is unlikely that these phenotypes could correlate with the presence or absence of the mtDNA. Since no genetic markers appeared to correlate with the mtDNA, and production of strains lacking the mtDNA would be very difficult, its use as a cloning vector seemed of limited value and it was decided to discontinue work on this topic.

4.5 PROSPECTS FOR FUTURE WORK

4.5.1 Recent publications on this topic

After completion of this work, the results on characterisation of the mtDNA of strain FC1 were published (Marriott *et al.*, 1984). One further report on *Fusarium oxysporum* DNA has appeared: De Lorenzo *et al.* (1984) reported that the buoyant densities of nuclear and mitochondrial DNAs were 1.712 g/ml and 1.685 g/ml respectively (cf. my values of 1.692 and 1.685 g/ml). The 46.7 kb circular DNA was identified with the mtDNA, in agreement with my work.

4.5.2 Future work

It remains to be demonstrated whether the genes controlling nifuroxime-resistance and production of exo-polygalacturonase are located on the mtDNA or in the nuclear DNA. In this context, it may be interesting to characterise the mtDNAs of other strains of *F. oxysporum* f.sp.

lycopersici, notably strains which can be shown to have markedly different phenotypes to FC1.

The mtDNA of FC1 appears to have little use in the construction of vectors for use with *F. oxysporum*, since the marker genes reported by Guardiola *et al.* (1982) did not have reproducible phenotypes. However, the method reported here for the preparation of protoplasts from *F. oxysporum* may be of more general use in both isolation of DNA from the fungus, and development of a transformation system for use in the genetic engineering of the fungus.

PART B. GENOME ORGANISATION AND EXPRESSION
OF RED CLOVER NECROTIC MOSAIC VIRUS.

CHAPTER 5. INTRODUCTION.

5.1 RED CLOVER NECROTIC MOSAIC VIRUS

5.1.1 Discovery and physical characterisation of the virus

Red clover necrotic mosaic virus (RCNMV) was first reported as a pathogen of red clover (*Trifolium pratense*) in Czechoslovakia (Musil, 1969). RCNMV infects mainly red clover growing as field crops or on meadows and pastures (Musil & Gallo, 1982). The virus produces mosaic, malformation and veinal necrosis on the leaves of infected plants, the symptoms being most marked in the summer (Gerhardsen & Lindsten, 1973), and it has also been isolated from white clover (*T. repens*). RCNMV is widely distributed, with isolates reported from Czechoslovakia, Poland, Sweden, Britain (Hollings & Stone, 1977), Australia, Canada (Hollings & Stone, 1980), and New Zealand (Morris-Krsinich *et al.*, 1983). It was first detected in Britain in 1971, but was unimportant in British clovers until 1975 when some serious outbreaks occurred (Hollings *et al.*, 1977). Red clover is an important crop, accounting for 45 % of herbage legume seed sold in Britain, and RCNMV is a serious pathogen causing losses of greater than 50 % (Bowen & Plumb, 1979). RCNMV is present in the leaves, stems and roots of infected clovers, and the occurrence of necrotic flecks on the leaves distinguishes RCNMV from other viruses found in clovers, such as alfalfa mosaic virus and red clover mottle virus (Hollings & Stone, 1977).

Various strains of RCNMV infected 47 of 106 plant species tested, and infected members of 15 out of 30 plant families tested. Diagnostic species include *Chenopodium quinoa*, on which the virus produces necrotic local lesions; *Phaseolus vulgaris*, on which the virus produces necrotic lesions followed by systemic invasion; and *Vigna sinensis* on which different strains of the virus produce different patterns of infection, such as necrotic local lesions (English H and Scottish strains), necrotic lesions with systemic mottle (Swedish strain), faint chlorotic lesions followed by systemic infection (Czech strain TpM-48), or chlorotic lesions with necrotic margins which expand to produce systemic infection (Czech strain TpM-34) (Hollings & Stone, 1977). The virus can be propagated in *P. vulgaris*: high yields are obtained when the plants are grown in growth cabinets at 20°C, although yields from plants grown in a glasshouse during summer are low (Hollings *et al.*, 1978).

RCNMV is transmitted through soil, and is exuded into the soil by infected *T. pratense* and *Nicotiana clevelandii* (Hollings & Stone, 1977). The fungus *Olpidium* may be the biological vector, although RCNMV is also spread mechanically by machinery or grazing (Bowen & Plumb, 1979); RCNMV has been transmitted to clover by zoospores of *Olpidium radicale* (Macfarlane, 1982).

Viral particles are seen in the electron microscope to be isometric, with a diameter of 27 to 34 nm (Hollings & Stone, 1977; Gould *et al.*, 1981). A single component is detected in the ultracentrifuge with an $s_{20,w}$ value of 126S (Gerhadsen & Lindsten, 1973) to 135S (Gould *et al.*, 1981). The virus forms a single major band upon isopycnic centrifugation in CsCl, with a buoyant density of 1.36 to 1.37 g/ml (Hollings & Stone, 1977).

The virus is a good immunogen, and the various strains cross-react serologically to different degrees. These serological cross-reactions have led to the classification of isolates of RCNMV into four serotypes (Musil & Gallo, 1982; Rao *et al.*, 1985):

serotype A includes Czech strain TpM-34;

serotype B includes Czech strain TpM-48, English strain H and Canadian strain C (formerly clover primary leaf necrosis virus);

serotype C includes Swedish isolate 63/70;

serotype D includes English strain E and the Australian strain.

RCNMV reacts weakly with antisera to sweet clover necrotic mosaic virus, and is more distantly related to carnation ringspot virus (Hiruki *et al.*, 1984).

5.1.2 The Dianthovirus group of plant viruses

RCNMV has a number of similarities to sweet clover necrotic mosaic virus (SCNMV) and carnation ringspot virus (CRSV), and the three viruses have been classified together in the Dianthovirus group, with CRSV as the type member (Matthews, 1982). The group properties are as follows: the genome is composed of two molecules of (+)-sense RNA, of molecular weights 1.5 and 0.5×10^6 ; the virus particles contain a single species of polypeptide with a molecular weight of about 40,000, with no detectable lipid or carbohydrate; the particles are isometric with a diameter of 31-34 nm, a density in CsCl of about 1.37 g/ml, an $s_{20,w}$ of about 135S, and a particle molecular weight of 7.1×10^6 (Matthews, 1982).

SCNMV is similar but serologically distinct to RCNMV (Okuno *et al.*, 1983);

CRSV is only distantly related to the other two Dianthoviruses, and

differs in that it is transmitted through soil by nematodes (Kleinhempel *et al.*, 1980).

5.1.3 Molecular biology of the Dianthoviruses

The first Dianthovirus to be well characterised was CRSV. The genome of single-stranded RNA was shown to be bipartite: it consists of two molecules (molecular weights 1.5 and 0.5×10^6) both of which are necessary to initiate an infection (Dodds *et al.*, 1977). Two strains of CRSV, A and N, differ by their ability to aggregate in 0.5 % SDS at pH 5. When pseudorecombinants were made from separated RNAs from the two strains (i.e. RNA-1 from N plus RNA-2 from A, or *vice versa*) and assayed for their behaviour in 0.5 % SDS at pH 5, the property of aggregation was correlated with RNA-1. Since the protein responsible for viral aggregation is the 38,000 molecular weight (38 K) coat protein, the result showed that the gene for coat protein is located on RNA-1 (Dodds *et al.*, 1977). Viral-specific double-stranded RNA (dsRNA) was isolated from plants infected with CRSV, and this was composed of three species. The major bands of 3 and 1×10^6 molecular weight have twice the molecular weight of the genomic RNAs (Dodds *et al.*, 1977), and the minor band of 1.2×10^6 has twice the molecular weight of a minor single-stranded RNA detected in the viral RNA (Henriques & Morris, 1979). The significance of dsRNA in virus-infected plant tissue is discussed in section 5.5.

The bipartite nature of the RCNMV genome was demonstrated by Gould *et al.* (1981) for the Australian strain; it has also been demonstrated for the Swedish and Canadian strains of RCNMV, and for the other Dianthovirus SCNMV (Okuno *et al.*, 1983). Gould *et al.* (1981) hybridised cDNA made from RNA-1 with RNA-2 and *vice versa*, which showed that there

was no detectable sequence homology between the two genomic RNAs. The kinetics of the cDNA/RNA reassociation reactions gave values of the complexities of the two RNAs as 4.41 and 1.47 kb.

An attempt was made to map gene functions onto one or other of the genomic RNAs using pseudorecombinants derived from the Swedish and Canadian strains of RCNMV and SCNMV by Okuno *et al.* (1983). The coat proteins of the different strains could be distinguished both by molecular weight (varying between 37.5 K and 38.5 K) and serologically. In every case, the coat protein of the pseudorecombinant was identical to that of the parental strain which donated RNA-1, indicating that the gene for coat protein is on RNA-1 (as for CRSV). The other properties for which the pseudorecombinants were assayed were the phenotypes on various host plants (Okuno *et al.*, 1983). There were no simple correlations between symptoms and RNAs, other than the observation that systemic infection of sweet clover at 26°C requires RNA-1 derived from SCNMV; the other symptoms produced apparently depended on an interaction between RNA-1 and RNA-2.

A pseudorecombinant was made from RNA-1 of RCNMV and RNA-2 of CRSV, which could replicate in *Nicotiana clevelandii*, indicating that the RNA-dependent RNA replicase of one Dianthovirus can support the replication of RNA from another Dianthovirus, even though the two are only distantly related serologically (Lommel & Morris, 1982). A preliminary report suggests that the pattern of dsRNA bands extracted from RCNMV-infected plants is similar to that from CRSV-infected plants, i.e. three bands with the two smaller bands being similar in size (Morris, 1983); no corresponding minor single-stranded viral RNA species has been reported for RCNMV.

The translation of RCNMV in the rabbit reticulocyte lysate system was studied by Morris-Krsinich *et al.* (1983), for the New Zealand strain RCNMV-D. Total viral RNA was translated to give three major proteins of molecular weights 39 K, 36 K and 34 K. The 39 K protein comigrated with the viral coat protein and was precipitated by anti-RCNMV immunoglobulins, indicating that it was indeed the coat protein. This was further confirmed by comparing partial proteolytic digests of the major *in vitro* translation products with a digest of the coat protein; the 36 K and 34 K molecular weight proteins were unrelated either to the coat protein or to each other.

The viral RNA species were partially separated by sedimentation through sucrose gradients containing 50 % formamide, and each fraction was translated (Morris-Krsinich *et al.*, 1983). The 36 K protein was translated mainly from fractions enriched for RNA-1. The 34 K protein was translated mainly from fractions enriched for RNA-2. The coat protein was translated from most fractions of the gradient. Further information was obtained from hybrid-arrested translation of total viral RNA: when the RNA was hybridised with cDNA made to gel-purified RNA-1 before translation, the formation of both the 36 K and 39 K proteins was blocked. Hence it appears that RNA-1 encodes both the 36 K protein and the coat protein; RNA-2 encodes the 34 K protein; and although the gene for coat protein is located on RNA-1, the coat protein is apparently not translated from the same RNA species as the 36 K protein (Morris-Krsinich *et al.*, 1983). Further work would be required to ascertain precisely which RNA species are translated to which protein products.

The strategies of gene expression employed by other plant RNA viruses, and their implications for the gene expression of RCNMV and the other Dianthoviruses, will be discussed in the following sections.

5.2 GENOME STRUCTURE OF PLANT RNA VIRUSES

5.2.1 Introduction

In this section the structures of the genomes of the plant single-stranded RNA viruses will be reviewed, and in the following sections the strategies of gene expression, mechanisms of genome replication, and functions of viral-encoded proteins will be discussed.

There are 24 groups of viruses with single-stranded RNA genomes which infect plants, along with several ungrouped viruses (Matthews, 1982). Of these, two groups (the Rhabdoviruses and the tomato spotted wilt virus group) are enveloped and have genomes of negative-sense RNA (or of unconfirmed polarity in the latter case), and these will not be discussed further. The remaining groups, including the Dianthoviruses, have genomes of positive- (i.e. messenger-) sense RNA and have no envelope of lipid. In this, they resemble three families of animal viruses, the Picornaviridae, the Caliciviridae and the Nodaviridae, and comparisons will be made with these families where appropriate.

The main features of the genome structures of the non-enveloped single-stranded RNA viruses are summarised in table 5.1.

Table 5.1 Genome structures of the non-enveloped single-stranded RNA viruses.

Virus group	No. of segments ¹	5'-ends ²	3'-ends ³
<u>(i) Plant viruses</u>			
Tobamovirus	1	cap	t
Tymovirus	1	cap	t
Luteovirus	1	VPg	OH
Tombusvirus	1	?	OH?
Sobemovirus	1	VPg	OH
Closterovirus	1	?	?
Carlavirus	1	?	?
Potyvirus	1	VPg	polyA
Potexvirus	1	cap	polyA ⁴
Tobacco necrosis virus	1	P	OH?
Maize chlorotic dwarf virus	1	?	?
Carnation mottle virus	1	cap	OH
Turnip crinkle virus	1	cap	OH?
Dianthovirus	2	?	?
Comovirus	2	VPg	polyA
Nepovirus	2	VPg	polyA
Tobravirus	2	cap	OH
Furovirus	2	P? ⁵	OH
Pea enation mosaic virus	2	VPg	OH
Cucumovirus	3	cap	t
Bromovirus	3	cap	t
Ilarvirus	3	cap	OH
Hordeivirus	3	cap	t ⁶

Table 5.1 (continued)

Alfalfa mosaic virus	3	cap	OH
Beet necrotic yellow vein v.	4 ⁷	cap	polyA
<u>(ii) Animal viruses</u>			
Picornaviridae	1	VPg	polyA
Caliciviridae	1	VPg	polyA
Nodaviridae	2	cap	protein

Notes:

- ¹ Number of RNA segments comprising the genome
- ² VPg refers to genome-linked protein; P refers to free phosphate
- ³ t refers to tRNA-like structure; polyA refers to polyadenylate tract; OH refers to free hydroxyl when neither of the previous two structures are present
- ⁴ Not all Potexviruses have a polyadenylate tract (Guilford & Forster, 1986)
- ⁵ Soilborne wheat mosaic virus RNA-2 apparently has a heterogeneous uncapped 5'-end (Hsu & Brakke, 1985)
- ⁶ Hordeivirus genomic RNAs have an internal polyadenylate tract 210 bases from the 3'-end (Stanley *et al.*, 1984)
- ⁷ One or both of the two smaller RNAs of BNYVV are required for transmission by fungus, but not for replication in mechanically inoculated plants (Koenig *et al.*, 1986), and so are considered as part of the genome for the purposes of this discussion. Although BNYVV is a possible Furovirus, it differs from other Furoviruses in having a poly(A) tail (Bouzoubaa *et al.*, 1986).

Most of the data in this table were from Matthews (1982); other references are mentioned in the text.

5.2.2 Segmentation of the genome

The genome of an RNA virus consists of the total unique sequence required to initiate infection and viral replication. Plant virus genomes can be monopartite, or consist of two, three or possibly four segments (see table 5.1): each segment is packaged separately in a shell of viral coat protein. The advantage of a monopartite genome is that a single particle (or type of particle) is required to initiate an infection, whereas two or more different particles must infect the same cell to initiate a successful infection in the case of a multipartite virus. However, various advantages arise from a multipartite genome: reassortment of RNA components from similar but different clones gives rise to an expanded pool of variation for natural selection (although variation may be high simply due to replication errors: Pressing & Reanney, 1984); smaller RNA molecules are less prone to developing adverse mutations due to the lack of proofreading and error repair in RNA replication (Pressing & Reanney, 1984); expression of genes on different segments can be regulated separately (see section 5.3; Goldbach, 1986).

5.2.3 5'-terminal structures

Most RNA viruses have a 5'-terminal blocking structure, either a cap or a genome-linked protein (VPg) (see table 5.1). Of the plant RNA viruses, only tobacco necrosis virus and its satellite, and RNA-2 of soilborne wheat mosaic virus have been shown to lack a blocking group and have a free 5'-phosphate as do RNA bacteriophages (Lesnaw & Reichmann, 1979; Van Emmelo *et al.*, 1984; Hsu & Brakke, 1985).

The cap consists of a 7-methylguanosine residue linked through its 5'-triphosphate to the 5'-terminal nucleotide. Such structures are common to

virtually all eukaryotic cellular messages (Shatkin, 1976). The cap is added to nascent RNA polymerase II transcripts by guanylyl- and methyl-transferases in the nucleus (Konarska *et al.*, 1984), and is involved in both processing of mRNA precursors, and translation of the messages. Uncapped cellular transcripts are spliced much less efficiently than capped ones *in vitro* (Konarska *et al.*, 1984), although splicing is not known to be involved in plant RNA virus transcription. The cap has a role in the formation of the initiation complex of translation: it interacts with a cap-binding protein, which then associates with initiation factors eIF-4A and eIF-4B to form the complex between RNA, ribosomal 40S subunit and Met-tRNA_i (Shatkin, 1985). However, the presence of a cap is not necessary for efficient translation of all RNAs, as shown by the many uncapped RNA viruses, which do not produce capped transcripts. The cap has another role for some viruses: its presence is necessary for the infectivity of RNA from tobacco mosaic virus (Dawson *et al.*, 1986), brome mosaic virus (Ahlquist *et al.*, 1984b), and black beetle virus (Dasmahapatra *et al.*, 1986); and RNA-4 of alfalfa mosaic virus and tobacco streak virus requires a cap in order to activate infectivity of the three genomic RNAs (Langereis *et al.*, 1986). One function of the cap in promoting infectivity of RNA may be protection from exonucleases (Konarska *et al.*, 1984).

Viral RNAs tend to have a long leader sequence (70-260 bases) between the cap and the first translated codon, although subgenomic transcripts have shorter leaders (10-50 bases) (Cornelissen *et al.*, 1983). The 5'-end of the RNA presumably contains signals for the capping enzyme(s) and the RNA replicase, as well as the ribosome binding site (Koper-Zwarthoff *et al.*, 1980). The sequence cap-G-[U+A-rich region]-AUG is common to tobacco

mosaic virus and its subgenomics, and RNAs-3 and -4 of brome mosaic virus and alfalfa mosaic virus; it is known as the "Butler box" and is uncommon in cellular messages (Goelet *et al.*, 1982).

Six groups of plant viruses (and two groups of animal viruses) have a small protein, the VPg, covalently attached to the 5'-end of the RNAs (see table 5.1). These proteins are encoded by the viral RNA and range from 2400 to 12,000 in molecular weight (Wimmer, 1982). In the case of the Nepoviruses, the VPg is necessary for the infectivity of the RNA (Mayo *et al.*, 1982); however, the RNAs of Picornaviruses, Comoviruses and pea enation mosaic virus are still infective after prolonged protease digestion (Goldbach, 1986). The VPg is not required for translation of the RNA *in vitro* (Davies & Hull, 1982); in the case of poliovirus, the *in vivo* translated RNA lacks the VPg, and it is possible that the presence of a VPg on a viral RNA destines that RNA for encapsidation rather than translation (Wimmer, 1982). The VPg is found on both strands of the poliovirus replicative form, and the intracellular form VPg-pUpU may act as the primer of picornaviral RNA replication (Flanegan & Baltimore, 1977; Vartapetian *et al.*, 1984), although more recent results indicate that VPg may be added after initiation of replication (Young *et al.*, 1985). The involvement of VPg in the replication of a plant virus has not yet been demonstrated, although the (-)-strands of the cowpea mosaic virus replicative form do have the VPg attached (Goldbach, 1986).

5.2.4 3'-terminal structures

Several groups of RNA viruses have polyadenylate tails at the 3'-ends of the viral RNAs, as do many eukaryotic cellular RNAs. Unlike the cellular mRNAs, which have the poly(A)-tail added post-transcriptionally, the

poly(A) of viral RNAs is replicated along with the rest of the genome and is present as poly(U) at the 5'-end of the (-)-strand, at least for Picornaviruses (Vartapetian *et al.*, 1984) and Comoviruses (Goldbach, 1986). Picornaviruses and Comoviruses lack the polyadenylation signal AAUAAA which is common to cellular polyadenylated RNAs, although the signal is present near the 3'-end of the polyadenylated tomato black ring virus RNA-3 (Meyer *et al.*, 1984).

Many non-polyadenylated viral RNAs have a high degree of secondary structure at the 3'-end. For several groups, the 3'-end can fold into a tRNA-like structure, which can be aminoacetylated in a manner analogous to tRNAs. For example, Bromoviruses and Cucumoviruses can accept tyrosine, Tobamoviruses can accept histidine or valine, and Tymoviruses can accept valine (Hall, 1979). In the case of turnip yellow mosaic virus, the RNA-valine adduct binds to the *E. coli* elongation factor EF-Tu.GTP, which normally binds to aminoacetylated tRNA during translation (Joshi *et al.*, 1984). The 3'-ends of Tobamoviruses have an identical "pseudoknotted" secondary structure, which has "arms" analogous to the aminoacyl-acceptor arm and anticodon arm of tRNAs (Rietveld *et al.*, 1984). The role of these structures *in vivo* is not clear, although the conserved tRNA-like structure of the Bromoviruses and Cucumoviruses presumably contains recognition site for the RNA replicase and the site of initiation of encapsidation (Ahlquist *et al.*, 1984a). The RNAs of barley stripe mosaic virus have a tRNA-like structure which can accept tyrosine at the 3'-end, and also a poly(A) tract about 210 bases upstream of the 3'-end (Stanley *et al.*, 1984). Brome mosaic virus RNA-3 has a similar internal poly(A), 16-22 residues long, in the region between the two coding regions (Ahlquist *et al.*, 1981).

The RNAs of alfalfa mosaic virus and the Ilarviruses have a series of hairpin loops near the 3'-end, which form the putative recognition sites for the RNA replicase, and for coat protein which binds in the 3'-most 88 bases to activate the RNA for infectivity: it may be that the coat protein is necessary to initiate viral synthesis (Koper-Zwarthoff & Bol, 1980).

The only RNA virus known to have a blocking group at the 3'-end is the Nodavirus, black beetle virus, which apparently has a covalently-bound protein at the 3'-ends of the RNAs (Dasmahapatra *et al.*, 1986).

5.3 STRATEGIES OF GENE EXPRESSION IN PLANT RNA VIRUSES

5.3.1 Introduction

In general, eukaryotic ribosomes translate only the 5'-most gene on a given RNA molecule, and cellular mRNAs are usually monocistronic. Since many viral RNAs are polycistronic, a variety of strategies have evolved to allow ribosomes to translate internal or "closed" cistrons. The strategies which have been postulated can be briefly summarised as follows:

- (i) dividing the genome into two or more components, thus "opening" more cistrons;
- (ii) transcription of internal genes into subgenomic messages;
- (iii) partial readthrough of weak "stop" codons to allow expression of downstream genes;
- (iv) translation of the entire RNA into a polyprotein which is subsequently proteolytically cleaved;
- (v) alternate initiation sites at the 5'-end of the RNA;

(vi) polycistronic RNAs / internal initiation.

As may be expected, a given virus may employ two or more of these strategies to express its genes.

The division of the genome into two or three components, and the advantages and disadvantages of this, have been discussed in section 5.2.2. The advantage with respect to gene expression is that the genome has more than one gene that can be translated directly, without the need for protein cleavage or synthesis of a subgenomic RNA; and also that expression of genes on different segments can be regulated independently, which is important for viruses which translate the whole RNA segment into a single polyprotein.

A summary of the strategies employed by the various groups of viruses is shown in table 5.2.

5.3:2 Subgenomic RNAs

Subgenomic RNAs are viral-specific messages transcribed from the viral genome, and allow translation of genes which do not lie at the 5'-end of the genomic RNAs. In almost all cases analysed in detail, these subgenomic RNAs form a 3'-coterminial set with the appropriate genomic RNA.

An example of such a 3'-coterminial set of RNAs, which has been studied in detail, is provided by tobacco mosaic virus (TMV). The TMV genome comprises a single RNA molecule of 6395 bases (Goelet *et al.*, 1982). In TMV-infected plants, three other major virus-specific RNAs of 692, 1500 and 2990 bases are detected, and each of these is translated into a different protein product *in vitro* (Goelet & Karn, 1982; Sulzinski *et al.*, 1985). The genomic RNA is translated to two proteins (126 K and 183 K),

Table 5.2 Strategies of gene expression among the non-enveloped single-stranded RNA viruses.

(i) Plant viruses

Tobamovirus	2, 3
Tymovirus	2, 3, 4?
Tombusvirus	2
Sobemovirus	2, 3
Potyvirus	4
Potexvirus	2, 6?
Tobacco necrosis virus	2, 6?
Carnation mottle virus	2, 3
Turnip crinkle virus	2
Dianthovirus	1, 2?
Comovirus	1, 4, 5
Nepovirus	1, 4
Tobravirus	1, 2, 3
Furovirus	1, 2?, 3
Pea enation mosaic virus	1, 6?
Cucumovirus	1, 2
Bromovirus	1, 2
Ilarvirus	1, 2
Hordeivirus	1, 2
Alfalfa mosaic virus	1, 2, 3
Beet necrotic yellow vein virus	1, 2, 3

(ii) Animal viruses

Picornaviridae	4, 5
Nodaviridae	1, 2

Key:	1	Multicomponent genome	4	Polyprotein
	2	Subgenomic RNAs	5	Alternate initiation sites
	3	Readthrough	6	Internal initiation

Most of the data in this table is from Matthews (1982) and Davies & Hull (1982): for further references see the text.

initiated from the same AUG codon; the 1500-base RNA is translated to a 30 K protein, and the 692-base RNA is translated to the 17 K coat protein (Goelet *et al.*, 1982). The 2990 base RNA is translated to a 54 K protein which corresponds to the carboxyl-terminal 54 K of the 183 K protein (Sulzinski *et al.*, 1985). Sequence analysis shows that the three subgenomic RNAs are 3'-coterminial with the genome, and the gene order along the genome is 5'-126 K-54 K-30 K-17 K-3'. The coat protein mRNA is capped like the genome, although the 30 K mRNA may be uncapped (Joshi *et al.*, 1983). The origin of assembly of TMV lies within the 30 K gene, and so the genome and 1.5 kb RNA, which both contain this sequence, are encapsidated into viral rods; the coat protein message is not (Takamatsu *et al.*, 1983). In the case of a similar Tobamovirus, TMV-Cc (the cowpea strain of TMV), the origin of assembly lies only about 400 bases from the 3'-end, and so for this virus the coat protein message does become encapsidated into short rods (Takamatsu *et al.*, 1983). In general, a subgenomic RNA will become encapsidated into virus particles if it contains the origin of assembly.

Another well-studied example is RNA-3 of brome mosaic virus (BMV). This is an RNA of 2117 bases encoding two genes, one at the 5'-end for a 35 K protein, and the other at the 3'-end encoding the 20 K coat protein (Ahlquist *et al.*, 1981). BMV RNA-3 is translated only to the 35 K protein *in vitro*; the message for the coat protein is the subgenomic RNA-4, which is found both in infected plants and in virus particles. RNA-4 comprises the 3'-terminal 876 bases of RNA-3 (Ahlquist *et al.*, 1981). The synthesis of this subgenomic RNA has been studied using a BMV-specific RNA replicase extract *in vitro* (Miller *et al.*, 1985). (+)-strands of RNAs 3 and 4 are transcribed to full-length (-)-strands by the replicase; but

whereas the (-)-strand of RNA-3 is transcribed to the (+)-strand of RNA-4, the (+)-strand of RNA-3 does not produce (-)-strands of RNA-4. This indicates that the replicase is capable of initiating internally on the (-)-strand of RNA-3 to produce the coat protein message, and a hairpin structure occurring just upstream of the RNA-4 initiation site may form the recognition signal for the replicase (Miller *et al.*, 1985). RNA-4 is not replicated autonomously *in vivo*, since its sequence does not contain a replicase initiation signal on the (-)-strand (Miller *et al.*, 1985). A similar process for the synthesis of the subgenomic RNA-4 by internal initiation on the (-)-strand of the genomic RNA-3 has been proposed for cucumber mosaic virus (Collmer & Kaper, 1985).

One case where a subgenomic RNA does not have the same 3'-end as the genomic RNA is barley stripe mosaic virus (BSMV). In this case also, the subgenomic coat protein message, RNA-4, is derived from the genomic RNA-3 (Gustafson *et al.*, 1982). However, BSMV RNA-3 has an internal poly(A) sequence about 210 bases from the 3'-end, and RNA-4 is composed of the 800 bases 5' to this sequence, terminating with the poly(A) tail (Stanley *et al.*, 1984).

There is no direct evidence for a subgenomic RNA in a Dianthovirus, although CRSV and possibly RCNMV have three dsRNA bands present in infected tissue which implies the existence of a third viral single-stranded species (Dodds *et al.*, 1977; see section 5.1.3). Amongst the other bipartite RNA viruses, subgenomics have been detected as derived from both genomic RNAs of the Tobraviruses (Bisaro & Siegel, 1980; Bocarra *et al.*, 1986; Hughes *et al.*, 1986), and from RNA-1 of the Nodavirus, black beetle virus (Guarino *et al.*, 1984).

5.3.3 Readthrough of termination codons

Two functionally different polypeptides may be translated from the same initiation codon at the 5'-end of an RNA molecule by partial readthrough of a termination codon. An example of this is the translation of the genomic RNA of TMV, which is translated to two proteins of 126 K and 183 K *in vitro*. Readthrough of the UAG codon (i.e. substituting an amino acid at this point) at the 3'-end of the 126 K gene allows formation of the 183 K protein, which contains the whole of the 126 K polypeptide sequence at its amino-terminus (Goelet *et al.*, 1982). The two proteins may have different functions *in vivo* (Van Telgen *et al.*, 1985). This strategy can even allow expression of three overlapping genes: a 30 K protein is translated from the 5'-end of the genomic RNA of carnation mottle virus (CarMV) *in vitro*. Addition of amber suppressor tRNA to the *in vitro* translation promotes formation of proteins of 77 K and 100 K at the expense of the 30 K protein, by increasing the amount of readthrough at two amber (UAG) termination codons (Harbison *et al.*, 1985). The overlapping nature of the three genes, with a common start codon and two UAG codons, has been confirmed from the complete sequence of the CarMV genome (Guilley *et al.*, 1985).

Two tRNA^{tyr} species have been isolated from tobacco which read the UAG codon as tyrosine, and it may be that tobacco does not use UAG as a terminator (Beier *et al.*, 1984).

5.3.4 Polyproteins

An RNA molecule can contain a single long open reading frame which is translated into a "polyprotein", which is subsequently cleaved into a number of functionally different proteins by viral-specific proteases. The

classic example of this strategy is poliovirus: the genome is translated into a polyprotein of 247 K, which is cleaved, while still nascent, into at least ten proteins via a series of intermediates (Argos *et al.*, 1984). This strategy is also used by both the genomic RNAs of cowpea mosaic virus (CPMV). The 200 K protein from RNA-1 and the 105 K protein from RNA-2 are cleaved into the various final products by two proteases, one specific for glutamine/methionine dipeptides, the other for glutamine/glycine and glutamine/serine dipeptides. Both proteases are derived from the RNA-1 polyprotein, and the initial cleavages are autocatalytic on the nascent 200 K polypeptide (Davies & Hull, 1982; Goldbach, 1986). Similarly, each RNA of the bipartite Nepoviruses is translated to a polyprotein which is then cleaved proteolytically (Fritsch *et al.*, 1980; Jobling & Wood, 1985).

The monopartite genome of the Potyviruses is translated into a single polyprotein, and as for the Picornaviruses only a single virus-specific, genome-length RNA is found associated with polysomes *in vivo* (Dougherty, 1983; Vance & Beachy, 1984).

This strategy usually involves the translation of entire genomic RNA(s) into polyproteins, but in the case of turnip yellow mosaic virus (TYMV) a polyprotein is produced from the 5'-end of the genome, whereas the coat protein gene at the 3'-end of the genome is translated from a subgenomic RNA (Davies & Hull, 1982). The 195 K protein of TYMV, which is produced by readthrough at the 3'-end of the gene for a 150 K protein, undergoes autocatalytic proteolysis *in vivo* to yield products of 82 K, 78 K and 35 K (Morch *et al.*, 1982).

5.3.5 Other strategies of gene expression

Although eukaryotic ribosomes usually initiate translation at the 5'-most AUG codon on a message, in certain cases a "weak" initiation signal can allow translation to start at an internal AUG codon. The best example of this among the plant viruses is the case of cowpea mosaic virus (CPMV) RNA-2. *In vitro* translation of RNA-2 gives rise to two proteins of 105 K and 95 K; sequence analysis of the RNA reveals a single open reading frame corresponding to the 105 K protein (van Wezenbeek *et al.*, 1983). Apparently some of the ribosomes fail to initiate at the first AUG at base 161, since it does not correspond to the optimum sequence A/GNNAUGG (Kozak, 1984), and instead initiate at the AUG codon at base 512 (or possibly 524) to produce the 95 K protein (van Wezenbeek *et al.*, 1983). The identity of the initiating codon for the 105 K protein has been confirmed using frameshift deletions introduced into CPMV RNA-2 via the cloned cDNA, followed by *in vitro* transcription and translation (Vos *et al.*, 1984). Cleavage of the 95 K or 105 K proteins should give rise to proteins of 48 K or 58 K respectively; as yet only the 48 K protein has been detected *in vivo* (Goldbach, 1986). A similar case of polyproteins having different amino-termini has been described for the foot and mouth disease Picornavirus (Clarke *et al.*, 1985).

RNA splicing has not been reported for any plant viruses, but it does occur in the enveloped animal Coronaviruses. The genomic RNA of murine hepatitis virus gives rise to a set of 3'-coterminial subgenomic RNAs, however each subgenomic RNA contains a leader sequence derived from the 5'-end of the genome (Spaan *et al.*, 1983). The "splicing" sites between the leader and coding regions have no consensus sequence, and the cellular splicing mechanism is not involved; instead a model of RNA

polymerase "jumping" has been proposed in which the polymerase starts at the 3'-end of the viral (-)-strand, synthesises a short leader sequence and then translocates to a specific internal site to commence synthesis of the coding region of the subgenomic RNA (Spaan *et al.*, 1983).

Polycistronic messages, i.e. RNAs on which ribosomes initiate at internal sites, have been tentatively reported for the plant viruses pea enation mosaic virus (PEMV; Gabriel & de Zoeten, 1984), papaya mosaic virus (PMV; Short & Davies, 1983), tobacco necrosis virus (TNV; Davies & Hull, 1982) and carnation mottle virus (CarMV; Salomon *et al.*, 1978). However, the gene products of CarMV have recently been explained entirely in terms of readthrough and subgenomic RNAs (Guilley *et al.*, 1985); the results with PMV have been attributed to cleavage of the viral RNA during *in vitro* translation (Bendena *et al.*, 1985); the results with PEMV could be attributed either to cleavage of the RNA *in vitro* or to rapid proteolysis of a precursor protein (Gabriel & de Zoeten, 1984); and dsRNAs have been detected in TNV-infected tissue which imply the existence of at least two subgenomic RNAs (Condit & Fraenkel-Conrat, 1979). Hence there is no firm evidence that plant viral RNAs can act as polycistronic messages.

5.4 FUNCTIONS OF VIRAL PROTEINS

5.4.1 Structural proteins

The best characterised viral-specific proteins are the structural proteins, i.e. the coat protein which forms the viral capsid. Nearly all plant viruses have a single coat protein; the Comoviruses have two coat proteins, occurring in nearly equimolar quantities (Goldbach, 1986). Capsids of plant ssRNA viruses fall into four morphological groups, roughly spherical or isometric, bacilliform, rigid rods, or long flexuous rods (Matthews, 1982). More than half of the groups of plant RNA viruses (including the Dianthoviruses) have isometric capsids, and those which have been studied in detail have a very similar structure, namely 180 coat protein subunits arranged icosahedrally with T=3 surface lattice symmetry: these include Tymoviruses, Tombusviruses, Sobemoviruses, Cucumoviruses, Bromoviruses and tobacco necrosis virus (Matthews, 1982). The genes specifying coat proteins can in many cases be identified by *in vitro* translation of the appropriate viral-specific RNA, followed by immunoprecipitation with antiserum raised to purified virus. The position of the coat protein gene on the viral genome tends to be similar for many groups of plant viruses.

Among the monopartite viruses, the coat protein gene is at the 3'-end of the genome and is translated from a 3'-coterminial subgenomic RNA in the case of Tobamoviruses, Tymoviruses, Sobemoviruses, Potexviruses, turnip crinkle virus and carnation mottle virus (Davies & Hull, 1982).

Potyviruses express their genome as a polyprotein (section 5.3.4), but again the coat protein gene forms the 3'-most portion of the polyprotein gene (Allison *et al.*, 1985). This contrasts with the animal

Picornaviridae: in this case the four viral proteins are derived from the 5'-most portion of the genome (Argos *et al.*, 1984).

Among the multipartite viruses, the coat protein gene is often, but not always, located on the smallest genome component. Considering the bipartite viruses, the coat protein gene is at the 3'-end of RNA-2 in the case of the Tobraviruses (expressed from a subgenomic RNA), Comoviruses and Nepoviruses (cleaved from a polyprotein); it is translated from the 5'-end of RNA-2 of the Furoviruses, beet necrotic yellow vein virus and the animal Nodaviridae; and it is translated from RNA-1 of pea enation mosaic virus as a larger precursor (Gabriel & de Zoeten, 1984). The Dianthoviruses resemble the latter virus in that the coat protein gene is on RNA-1 (see section 5.1.3).

The Bromoviruses, Cucumoviruses, Ilarviruses and alfalfa mosaic virus all express coat protein from the 3'-end of RNA-3 via a subgenomic RNA (Davies & Hull, 1982). Certain viruses do not fit the general scheme however: the tripartite barley stripe mosaic virus expresses coat protein from the 5'-end of RNA-2 (Davies & Hull, 1982), as do the bipartite Furoviruses (Mayo & Reddy, 1985) and the tetrapartite beet necrotic yellow vein virus (Bouzoubaa *et al.*, 1986).

5.4.2 RNA replicase subunits

Uninfected plants contain RNA-dependent RNA polymerases, with molecular weights of 130-140 K (Dorssers *et al.*, 1984), and the amounts of these are amplified upon viral infection. However, they are not template-specific (Miller & Hall, 1984) and synthesise short heterogeneous (-)-strands using both viral and host RNAs as templates *in vitro* (Goldbach, 1986). Their function *in vivo* remains unclear.

For several plant viruses, an RNA replicase which is specific for the viral RNA (and which is absent from healthy plants) has been isolated, at least in a partially pure form, and it seems likely that all RNA viruses encode at least one subunit of the viral replicase complex. The replicase isolated from barley infected with brome mosaic virus (BMV) shows specificity for BMV RNA over RNA from a related Bromovirus (cowpea chlorotic mottle virus, CCMV), or RNA from another virus which infects barley (barley stripe mosaic virus), implying that the template-specificity of the replicase is determined by a BMV-encoded product (Miller & Hall, 1983). Similarly, the replicase isolated from cowpeas infected with CCMV will not use RNA from cowpea mosaic virus (CPMV) as template, although it is less specific than the BMV replicase in that it will also use BMV RNA as template (Miller & Hall, 1984). The BMV-specific replicase complex contains a 110 K protein which has a similar tryptic fingerprint to the 110 K product of BMV RNA-1 (Bujarski *et al.*, 1982). The CPMV-specific replicase has been characterised at the polypeptide level, and consists of a 110 K protein (encoded by CPMV RNA-1) plus two host proteins (Dorssers *et al.*, 1984). The host proteins are isolated in variable amounts, and the RNA polymerase activity correlates with the amount of the 110 K protein, indicating that the 110 K protein is indeed the core polymerase (Goldbach, 1986). The CPMV 110 K protein also has a region of homology with the poliovirus protein 3D, the RNA polymerase core enzyme (Argos *et al.*, 1984).

Another well-studied replicase is that of cucumber mosaic virus (CMV). The enzyme is composed of two polypeptides of 110 K and 100 K, but neither of these components gives the same peptide fingerprint as the

CMV-specified 100 K protein, so the presence of a viral protein in this enzyme has yet to be established (Khan *et al.*, 1986).

There are several other lines of evidence implicating the involvement of virally encoded proteins in RNA replication. In the case of multicomponent viruses, if it can be shown that one component can replicate in the absence of others in plant protoplasts, this indicates that the former encodes the viral component(s) of the replicase. For example, although none of the RNAs of alfalfa mosaic virus or brome mosaic virus can replicate alone, RNAs-1 and 2 can replicate in the absence of RNA-3 in protoplasts; hence the gene products of RNAs-1 and 2 are both involved in RNA replication (Nassuth & Bol, 1983; Kiberstis *et al.*, 1981). Similarly RNA-1 of cowpea mosaic virus and tomato black ring virus replicate in protoplasts in the absence of RNA-2, and therefore encode the replicase (Goldbach *et al.*, 1980; Robinson *et al.*, 1980). RNA-1 of tobacco rattle virus also encodes the viral replicase: RNA-1 can initiate an infection in whole plants, although RNA-2 is required for the production of viral coat protein (Harrison & Robinson, 1982, and references cited therein).

Studies using temperature-sensitive mutants of tobacco mosaic virus (TMV) deficient in RNA replication indicate that two different viral-encoded functions are required for TMV synthesis: both are required for the synthesis of viral RNA, but only one is required for synthesis of the double-stranded replicative form (Dawson & White, 1979).

Another line of evidence is homology between the amino acid sequences of various viral proteins, usually inferred from the known sequence of the RNA in question. The core polymerase 3D of poliovirus has homology both with the putative replicases of other Picornaviruses and with the 110 K

and 87 K proteins of CPMV (Argos *et al.*, 1984). Three blocks of homology have been detected in the proteins of TMV, alfalfa mosaic virus (AlMV), BMV, CMV and the animal Alphavirus sindbis (SBV), and these are described in table 5.3. Homology block 2 contains a highly conserved region which is also present in the Picornaviral and CPMV replicases, the reverse transcriptases of animal Retroviruses and Hepadnaviruses and cauliflower mosaic virus, the bacteriophage MS2 replicase, and the putative RNA polymerase of influenza virus (Kamer & Argos, 1984), as well as the putative polymerases of black beetle virus and carnation mottle virus (Guilley *et al.*, 1985). This sequence may represent the active site and/or recognition site of the enzymes (Kamer & Argos, 1984). The similarities are thought to arise from a conserved function (RNA synthesis) among divergent viruses (Ahlquist *et al.*, 1985).

Other proteins may be involved in the regulation of the synthesis of (+) and (-)-strands of RNA, and the coat protein of AlMV appears to reduce the amount of (-)-strands synthesised relative to the amount of (+)-strands (Nassuth & Bol, 1984; see section 5.5.2).

The role of the VPg in viruses which encode one is unclear (see section 5.5.3).

5.4.3 Other non-structural proteins

A function which is presumably encoded by all plant RNA viruses, other than replicase components, is a protein enabling cell-to-cell transfer of the virus (or RNA) as it spreads through the plant. Two such proteins have been tentatively identified. Mutants of the tomato strain of tobacco mosaic virus (TMV-L) have been isolated which can replicate normally in

Table 5.3 Blocks of homology among putative RNA replicases.

Homology block	Protein of:		
	AlMV, CMV, BMV	TMV	SBV
1N	N-terminal portion of RNA-1 product	N-terminal portion of 126 K product	Protein nsP1
1C	C-terminal portion of RNA-1 product	C-terminal portion of 126 K product	Protein nsP2
2	RNA-2 product	C-terminal portion of 183 K product	Protein nsP4

Abbreviations: AlMV alfalfa mosaic; CMV cucumber mosaic; BMV brome mosaic; TMV tobacco mosaic; SBV sindbis viruses.

References: Cornelissen & Bol, 1984; Ahlquist *et al.*, 1985; Rezaian *et al.*, 1985.

protoplasts, but which are deficient in cell-to-cell transfer (Leonard & Zaitlin, 1982; Ohno *et al.*, 1983). The lesion was identified as a single amino acid substitution in the 30 K protein, which has no other known function. Similarly, a temperature-sensitive mutant of TMV, in which lesions do not expand at the restrictive temperature, was shown to have an A→G mutation in the 30 K gene which causes a non-conservative arginine→glycine substitution (Zimmern & Hunter, 1983). This region of the TMV RNA also contains the origin of viral assembly, and the mutant is also defective in viral assembly at the restrictive temperature (Zimmern & Hunter, 1983). Many plant viruses encode a protein of around 30 K, and possibly these perform similar functions. The 32 K protein (or P3) of alfalfa mosaic virus is found attached to plant cell walls, implying a role in cell-to-cell spread (Godefroy-Colburn *et al.*, 1986); P3 is encoded by RNA-3, which component is not required for RNA replication, but is required to initiate infection of a plant (Nassuth & Bol, 1983).

Apart from the proteases which are required to cleave the polyproteins of some plant viruses (section 5.3.4), the functions of other viral proteins remain obscure. Some may have a function in establishing infection of a plant, or in vector transmission; for example, RNAs-3 and 4 of beet necrotic yellow vein virus can become deleted or lost altogether when the virus is transferred by mechanical inoculation, but not when it is transmitted by the fungal vector (Koenig *et al.*, 1986): this implies a role for the gene products of RNAs-3 and 4 in soil transmission and root infection. The 126 K protein of TMV has been detected as associated with host chromatin, leading to the suggestion that it is involved in modifying host gene expression, and hence altering the growth of infected leaves (Van Telgen *et al.*, 1985).

5.5 REPLICATION OF RNA VIRUSES

5.5.1 Replicative forms of plant viruses

The replication of RNA viruses is independent of the host DNA-dependent RNA synthesis. Unlike DNA-dependent RNA polymerases, RNA replication is insensitive to actinomycin D, at least for viruses such as tobacco mosaic virus, cowpea chlorotic mottle virus and cucumber mosaic virus, although the replication of potato virus X is sensitive to actinomycin D in the early stages of infection (Motoyoshi & Hull, 1974; Otsuki *et al.*, 1974). Virus-specific double-stranded RNA molecules have been found in plant tissue infected with all RNA viruses investigated (Morris, 1983; Morris *et al.*, 1983). These replicative forms (RFs) reflect the gene expression of the virus in that they correspond in size to double-stranded forms of both the genomic RNA(s) and any subgenomic RNAs (Henriques & Morris, 1979). The presence of these RFs is presumably a consequence of RNA replication, since viral (+)-strands must be synthesised on a (-)-strand template (and vice versa). Three such RFs have been detected in Dianthoviruses, as described in section 5.1.3. Two are presumably the RFs of the genomic RNAs; the third implies the existence of a subgenomic RNA in infected plants.

However, the fully double-stranded RFs detected are probably not directly involved in the replicative pathway, but are either side-products or artifacts of the extraction procedure. In the case of RNA bacteriophages such as R17, no dsRNA can be detected in cell extracts before deproteinisation; however replicative intermediates (RIs) are detected which show one or more nascent strands connected by short regions of base-pairing to a mostly single-stranded template strand (Thach & Thach,

1973). The base-paired region is presumably at the active centre of the replication complex. Since RNA replicases appear to recognise initiation sequences by their secondary structure, a replicase could not recognise a fully double-stranded RF, and in the case of R17 the RFs are produced from the RIs by deproteinisation (Thach & Thach, 1973). It has also been shown that the RI of poliovirus is mostly single-stranded, with up to nine nascent chains per template strand (Richards *et al.*, 1984). Similarly, *in vivo* labelling of the RI of turnip yellow mosaic virus shows that the RI is mostly single-stranded, and no fully dsRNA was detected in infected leaves (Garnier *et al.*, 1980): the dsRNA of plant viruses may represent deproteinised RIs, or old non-replicating RIs. The dsRNA RFs of subgenomic RNAs do not necessarily imply autonomous replication of the subgenomic RNAs, but may simply represent side-products due to the synthesis of (-)-strands on subgenomic RNAs which are not associated with polysomes, since 3'-coterminial subgenomic RNAs must still possess the replicase binding site at the 3'-end of the (+)-strand. Hence the basic model of replication for single-stranded RNA viruses (whether in bacterial, animal or plant hosts) is synthesis of nascent (+)-strands on a mostly single-stranded (-)-strand template, and *vice versa*.

5.5.2 Replication of capped plant viruses

A model of the replicative cycle of cucumber mosaic virus (CMV) has been proposed, based on the observations that the RFs of CMV are uncapped, lack an A residue from the 3'-end of the (+)-strand, and have an extra unpaired G residue at the 3'-end of the (-)-strand (Collmer & Kaper, 1985). The extra G on the (-)-strand and the A usually at the 3'-end of

the (+)-strand must be added post-transcriptionally. The extra G is also detected on the (-)-strands of the RFs of animal Togaviruses, and the RNA phage Q β , but its function is unclear. The extra A on the (+)-strand generates a sequence CCA which can be aminoacetylated; in the aminoacetylated form the RNA can bind to elongation factor EF-I, which may prevent the replicase from binding and thus keep that particular RNA free for translation. The proposed replication cycle is as follows: on entering a cell the viral (+)-strands are translated to produce the viral proteins necessary for replication. The replicase initiates (-)-strand synthesis on some (+)-strands of RNAs-1 to 3, starting at the penultimate C residue, and ending with a post-transcriptionally-added G at the 3'-end. (+)-strands are then synthesised on free (-)-strands, with (+)-RNA-4 being internally initiated on (-)-RNA-3. (+)-strands which become adenylated at the 3'-end may be destined for translation or encapsidation (Collmer & Kaper, 1985). This model also fits the replication of Bromoviruses, and possibly of alfalfa mosaic virus (AlMV); in the latter case, the balance between synthesis of (+) and (-)-strands appears to be regulated by the coat protein (Nassuth & Bol, 1983). When protoplasts are infected with RNAs-1 and 2 alone (plus some coat protein to activate the infection), (+) and (-)-strands are synthesised in roughly equal amounts; when RNA-3 is also present, the synthesis of (-)-strands is greatly reduced relative to that of (+)-strands. Since it is known that coat protein binds near the 3'-ends of AlMV RNAs, it has been proposed that a bound coat protein molecule is necessary for the replicase to initiate (-)-strand synthesis, and when the viral RNA has been expressed and formed a high concentration of coat protein, the coat protein binds to the replicase and prevents it from initiating any

further (-)-strands (Nassuth & Bol, 1983). Temperature-sensitive mutants in both RNAs-1 and 2 of AlMV have been isolated which are defective in the synthesis of (-)-strands and of coat protein, indicating that the products of RNAs-1 and 2 are involved in the synthesis of (-)-strands, and in the production of the coat protein via the subgenomic RNA-4 (Sarachu *et al.*, 1985).

5.5.3 Replication of uncapped plant viruses

The replication cycle of plant viruses possessing a VPg is presumably similar to that of the animal Picornaviruses. The replication cycle of poliovirus is still not completely understood. It is known that protein 3D is the core replicase, but purified protein 3D synthesises RNA only in a primer-dependent manner (Young *et al.*, 1985). The VPg is involved in replication, since antibodies directed to the VPg inhibit replication (Goldbach, 1986); however the VPg is not necessary for priming. The replicase requires a "host factor" to initiate (-)-strand synthesis, and this host factor has been shown to be a terminal uridylyltransferase which adds about five uridylate residues to the 3'-end of the (+)-strand (Andrews & Baltimore, 1986). The oligo(U) tract can then form a hairpin loop by base-pairing with the poly(A) tail, possibly stabilised by the host factor, which acts as the primer for (-)-strand synthesis (Andrews & Baltimore, 1986); dimeric poliovirus RNA molecules have been isolated which consist of (+) and (-)-strands covalently linked at the 3'-end of the (+)-strand (Young *et al.*, 1985). VPg may be added to the 5'-end of the (-)-strand after its cleavage from the (+)-strand. Since the replicase is synthesised in equimolar amounts with the VPg (from the polyprotein), and one VPg molecule is required for each viral

RNA molecule, it has been suggested that each replicase molecule can only synthesise one RNA molecule, and cannot reinitiate (Goldbach, 1986). This has led to a highly speculative model for the replication of cowpea mosaic virus (CPMV): a membrane-bound complex is formed consisting of the viral 60 K and 110 K proteins, host factors, and a viral RF. The 60 K protein binds to the membrane, and the VPg, which forms the carboxyl-terminus of the 60 K protein, is cleaved from it by the 24 K protease (which forms the amino-terminus of the 110 K protein). The VPg, which may become uridylylated, then acts as primer for the synthesis of a new (+)-strand catalysed by the 110 K protein. As the new (+)-strand is elongated, the old (+)-strand is displaced from the RF, and is finally released as a free (+)-strand. The replication complex cannot support another cycle of replication since it no longer contains VPg (Goldbach, 1986). Obviously further work is required to determine the role of VPg in RNA synthesis, and to determine whether terminal uridylyltransferase activity is also involved in the replication of Comoviruses and other plant viruses, as described above for an animal Picornavirus.

5.6 AIMS OF THE RCNMV PROJECT

An understanding of the molecular basis of RNA virus replicative processes and pathogenicity would be of great value in the control of plant virus infections. As has been described in this chapter, little is known of the functions of non-structural virus proteins and of the interaction between the virus and its host at the molecular level. RCNMV was chosen since it is important as a pathogen of red clover, and its bipartite nature should allow independent analysis of the gene functions

of each segment. Little was previously known of the molecular biology of any Dianthovirus, and the group differs in several respects from other groups of bipartite plant RNA viruses.

The long-term aim of the project is to fully characterise the viral genome and its gene products, and to establish the relationships of these products to pathogenicity. Several approaches are being taken towards this objective. The strategy of gene expression used by RCNMV is to be investigated by the study of intracellular virus-specific RNA species and their translation *in vitro*, and the functions of these RNA species can then be further studied by analysis of separate RNA species in a plant protoplast system. Individual genes can be studied using mutants with specific lesions within a gene, and there are two approaches to obtaining such mutants. One is the isolation of spontaneous or chemically-induced mutants of the RNA. The other approach is to construct full-length cloned copies of the RNA, which can be modified in a site-specific manner, transcribed to RNA *in vitro* and analysed by infecting plants or plant protoplasts. To construct such cloned copies, the end sequences of the RNAs must be known, and also the 5'-end structure and its effect on infectivity should be determined. To accurately map the viral genes and to aid in construction of mutations, the complete sequence of the RNA component in question should be determined, via sequencing of DNA clones. Such sequence data and clones would also allow insertion of cloned copies of individual genes into the plant genome, using a Ti plasmid-based vector, so that the effect of individual viral genes on the plant could be examined in the absence of viral RNA replication.

The work described in this thesis was started with the following objectives:

to determine the structures at the 5'-ends of the viral RNAs and their effects on infectivity of the RNA;

to construct a set of cDNA clones to use as probes in the analysis of intracellular virus-specific single- and double-stranded RNAs;

translation *in vitro* of the separated viral RNAs, and of any further intracellular virus-specific RNAs which could be purified by hybrid-selection using the cDNA clones.

CHAPTER 6. METHODS AND MATERIALS.

6.1 MISCELLANEOUS METHODS

6.1.1 Miscellaneous methods

The following were exactly as described in section 2.1:

reagents, sterilisation, water, dialysis tubing, phenol extraction, butanol extraction, spectrophotometry, centrifugation.

6.1.2 Standard buffers

CIA, TAE and TBE were as listed in section 2.3.1.

TE:

10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

SSC:

0.15 M NaCl, 15 mM trisodium citrate, pH 7.0. This was made as a 20×SSC stock, using 175.2 g/l NaCl and 88.2 g/l trisodium citrate.

6.1.3 Restriction endonuclease digests

These were performed essentially as described in section 2.7.4. 1/20 volume of 0.1 M spermidine was usually included. The buffers used are listed in table 2.1; *TaqI* digests used the *HindIII*-buffer and were incubated at 65°C under sterile paraffin oil; *Sau3AI* digests used the *EcoRI* buffer, *HincII* was used rather than *HindII*; the same reaction conditions were used.

6.1.4 Deionisation

Formamide and glyoxal were deionised by stirring with Amberlite MB-3 mixed-bed resin (BDH), about 5 g per 100 ml, for 30 min., then the resin was removed by filtration through a 0.2 μ m pore-size nitrocellulose filter (Whatman) and the formamide or glyoxal stored in small aliquots at -70°C .

6.1.5 Determination of incorporation of radioactivity into DNA

Incorporation of radioactivity was determined by spotting 1 μ l aliquots of the sample onto 2.3 cm DE-81 discs (Whatman). One disc was untreated (to show the input of radioactive label); the other disc was washed in six changes of 5 min. each of 0.5 M Na_2HPO_4 (20 ml per disc), then briefly in two changes of distilled water and two changes of ethanol, and then dried.

Both discs were then added to plastic scintillation vials containing 10 ml scintillant, and the radioactivity determined using a Packard Tri-carb Liquid Scintillation Counter.

The scintillant was made by dissolving 4g 2,5-diphenyloxazole and 0.1 g 1,4-bis[5-phenyl-2-oxazolyl]-benzene in 666 ml toluene and 333 ml absolute ethanol.

6.1.6 Radioactively labelled compounds used

$[\alpha\text{-}^{32}\text{P}]\text{dCTP}$, >400 Ci/mmol, was from Amersham.

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 7000 Ci/mmol, was from New England Nuclear.

L- $[\text{S}\text{-}^{35}\text{S}]\text{methionine}$, >800 Ci/mmol, and L-[4,5- $^3\text{H}]\text{leucine}$, 120-190 Ci/mmol, were from Amersham.

6.1.7 End-labelling of restriction fragments

Restriction fragments of DNA were labelled at the 3'-end either for use as radioactive size-markers, or to visualise fragments too small to detect by staining with ethidium bromide.

For size-markers, 0.5 µg λ DNA was digested with *Hind*III in a 20 µl reaction, then 10 µl of this was added to 1.5 µl 1 mM dATP, 1.5 µl 1 mM dGTP, and 2 µl water containing 5 µCi [α -³²P]dCTP and 1 unit DNA Polymerase I (Klenow fragment) (Amersham). The mixture was incubated at room temperature for 10 min., and the reaction stopped by freezing. 1 to 2 µl of this were used for electrophoresis in one track of a gel.

*Taq*I fragments were labelled essentially as above, except that the unlabelled dNTPs were replaced by 2 µl of 0.5 M Tris-HCl, pH 7.8, 50 mM MgCl₂, 0.1 M 2-mercaptoethanol, and only 0.5 µCi [α -³²P]dCTP was used. After incubation, the mixture was analysed by PAGE.

6.2 GEL ELECTROPHORESIS OF NUCLEIC ACIDS

6.2.1 Non-denaturing agarose gel electrophoresis

Agarose gels for DNA were essentially as described in section 2.6.1., using the TBE buffer system. Occasionally, ethidium bromide was added to the gel after cooling to 60°C, to 0.5 µg/ml; DNA bands could then be visualised without prior staining.

Single-stranded RNA was also analysed on this type of gel. To prevent degradation of the RNA by contaminants in the agarose, 0.1 % SDS was added to the agarose after boiling. The SDS was removed by washing the gel in water 15 to 30 min. before staining with ethidium bromide. RNA

samples were heated to 60°C in 50 % v/v formamide before loading to prevent the formation of aggregates.

6.2.2 Polyacrylamide gel electrophoresis

PAGE of DNA and dsRNA was as described in section 2.6.2, except that TBE was used rather than TAE.

6.2.3 Hydroxide-agarose gel electrophoresis

To make 100 ml gel, 1.2g agarose was dissolved in 90 ml water by boiling and cooled to about 40°C. Then 10 ml 0.3 M NaOH, 10 mM EDTA (freshly prepared) were added and the gel was poured immediately. Electrode buffer was 30 mM NaOH, 1 mM EDTA; the gel was either connected to the buffer with Whatman 3MM paper wicks and electrophoresed at 50 V overnight; or electrophoresed partly submerged at 20 V overnight.

To a DNA sample, first 1/10 volume 0.2 M EDTA was added, then 1/10 volume 1 M NaOH and 1/10 volume glycerol containing Bromophenol Blue, before loading onto the gel.

6.2.4 Glyoxal-agarose gel electrophoresis

Gels contained 1 % agarose in 10 mM sodium phosphate, pH 7.0, which was also the electrode buffer. Samples were denatured before electrophoresis

by mixing:

- 5 µl RNA
- 2 µl 0.1 M sodium phosphate, pH 7.0
- 3 µl 40 % glyoxal (deionised)
- 10 µl DMSO

The sample was incubated at 50°C for 1 hr before adding a little Bromophenol Blue and loading on the gel.

If required, gels were stained by soaking in 0.05 % w/v Toluidine Blue (Hopkin & Williams) in 60 mM sodium phosphate pH 7.0, for 30-60 min., and destained by soaking in several changes of 10 mM sodium phosphate pH 7.0. Bands were observed using a white light transilluminator.

6.2.5 Formaldehyde-agarose gel electrophoresis

0.3 g agarose was dissolved in 21.9 ml water and 3 ml 10×MOPS buffer by briefly boiling. The mixture was cooled to 60°C, then 4.9 ml 40 % w/v formaldehyde (BDH Analar grade) was added and the gel was poured immediately. Gels were poured and allowed to set in a fume-cupboard, and electrophoresed fully submerged at 50 V, using 1×MOPS buffer as the electrode buffer.

10×MOPS buffer is 0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA, adjusted to pH 7.0 with NaOH.

Samples were denatured before electrophoresis by mixing:

- 4.8 µl RNA
- 3.2 µl 40 % w/v formaldehyde
- 2 µl 10×MOPS buffer
- 10 µl formamide (deionised)

The sample was incubated at 65°C for 5 min. before adding a little Bromophenol Blue and loading onto the gel.

If required, gels could be stained in 100 ml water containing 10 µl ethidium bromide (10 mg/ml) and 70 µl 2-mercaptoethanol.

6.2.6 Drying and autoradiography of gels

To detect ³²P-labelled bands on a gel, the gel was dried onto 3MM paper (Whatman) under vacuum using a Bio-Rad gel dryer. The dried gel was then

exposed to X-ray film (Kodak X-Omat S or Fuji RX) in a cassette with intensifying screens at -70°C . After the appropriate time, the film was developed in a Kodak X-Omat industrial processor.

6.3 GEL ELECTROPHORESIS AND FLUOROGRAPHY OF PROTEINS

6.3.1 Gel electrophoresis of proteins

Proteins were analysed under denaturing conditions on SDS-containing discontinuous buffer polyacrylamide gels (SDS-PAGE). Gels were cast in a vertical mould of $20 \times 16.5 \times 0.1$ cm, as described in section 2.6.2.

The acrylamide stock was 40 % w/v acrylamide, 1 % NN'-methylene-bisacrylamide, and was filtered before use.

30 ml of 10 % resolving gel was made by mixing:

7.5 ml Acrylamide stock

3.75 ml 3 M Tris-HCl pH 8.8

0.3 ml 10 % w/v SDS

18.2 ml distilled water

250 μl 10 % w/v ammonium persulphate (prepared freshly)

15 μl TEMED

This was poured into the mould until it was about three-quarters full, and then gently overlaid with water. After the resolving gel had set, the water was poured off and a 3 % stacking gel was set on top of this so that there was ≈ 2.5 cm stacking gel between the sample wells and the resolving gel. 10 ml of 3 % stacking gel was made by mixing:

0.75 ml Acrylamide stock

2.5 ml 0.5 M Tris-HCl pH 6.8

0.1 ml 10 % w/v SDS

6.6 ml distilled water

75 μ l 10 % w/v ammonium persulphate

5 μ l TEMED

Electrode buffer (pH 8.3) contained 6 g Tris, 28.8 g glycine, and 1 g SDS, per litre. Sample buffer was made by mixing:

250 μ l 0.5 M Tris-HCl pH 6.8

150 μ l glycerol

200 μ l 10 % w/v SDS

50 μ l 2-mercaptoethanol

350 μ l 0.25 % Bromophenol Blue

At least one volume of this was added to the sample, and samples were heated at 100°C for 2-3 min. and cooled on ice before loading onto the gel. Gels were electrophoresed at 35 V for 18 hr.

After electrophoresis, protein bands were visualised by staining in 0.25 % w/v Coomassie Brilliant Blue R (Sigma), 45 % v/v methanol, 9 % v/v acetic acid, for 1 hr, and then destaining in 45 % methanol, 9 % acetic acid for 2-4 hr. Bands were observed using a white light transilluminator.

6.3.2 Fluorography

To visualise proteins labelled with ^{35}S or ^3H , gels were fluorographed. After staining and destaining, the gel was washed for at least 30 min. in distilled water, then soaked for 15-30 min. in 1 M sodium salicylate (BDH). The gel was then dried onto 3MM paper at 80°C under vacuum using an LKB gel drier. The dried gel was exposed to X-ray film at -70°C (without intensifying screens) as described in section 6.2.6.

6.4 PROPAGATION AND ISOLATION OF VIRUS

6.4.1 Propagation of virus

The isolate used, isolate 890 of the Czech strain TpM-34 of RCNMV, was originally obtained from Glasshouse Crops Research Institute, Littlehampton, and had been purified by three cycles of single lesion isolation (Osman *et al.*, 1986). Frozen sap containing the virus was stored at -20°C and was used as inoculum.

RCNMV was propagated in *Phaseolus vulgaris* cv. The Prince. Primary leaves were inoculated by first sprinkling on carborundum powder, and then rubbing on infected sap. Excess sap and carborundum were rinsed off by spraying with water, and the infected plants were incubated at 17°C in a Fitotron 600H growth cabinet, with 18 hr per day of illumination at an intensity of 5 klux (Hollings & Stone, 1977). Leaves were harvested 4-7 days after inoculation, and were picked at the end of a dark period to give a lower starch content. The leaves were sometimes stored at -70°C before use.

6.4.2 Extraction of virus

Typically 150-200 g leaves were processed. The amounts given below are for 100 g leaves. 10×SE buffer is 0.4 M Na₂HPO₄, 0.267 M KH₂PO₄, 0.1 M EDTA, pH 7.0.

100 g leaves were homogenised in a Kenwood blender with 200 ml 1×SE and 0.2 ml 2-mercaptoethanol. The homogenate was squeezed through two layers of muslin. To the liquid, 0.5 volume chloroform and 0.5 volume butanol were added and mixed thoroughly. The mixture was separated by centrifugation at 10,000 r.p.m. for 10 min. (6×300 ml rotor). The aqueous

phase was collected and PEG-6000 and NaCl were added to 10 % w/v and 1 % w/v respectively. This mixture was then stirred at 4°C for 1.5 hr. Precipitated virus was collected by centrifugation at 10,000 r.p.m. for 20 min. (6x300 ml rotor) to give two pellets per 100 g starting material. Each pellet was resuspended in 8 ml 1xSE by shaking at 0°C for 1 hr. After centrifugation at 10,000 r.p.m. for 10 min. (8x50 ml rotor) to remove insoluble debris, the supernatants were layered over 1 ml sucrose cushions (10 % w/v sucrose in 1xSE) and centrifuged at 50,000 r.p.m. for 1½ hr at 4°C in a Beckman type 65 rotor. Each pellet was resuspended in 0.5 ml 1xSE, and after centrifuging for 10 min. (Microfuge) the liquid was layered onto a 10-50 % w/v sucrose gradient, which was made by freezing 38 ml 25 % w/v sucrose, 1xSE in a Beckman SW28 tube at -70°C, and thawing at room temperature (Davis & Pearson, 1978). The gradients were centrifuged in a Beckman SW28 rotor at 27,000 r.p.m., 10°C for 1½ hr. The gradients were illuminated from above with white light, and the bluish-white opalescent virus band was collected with a sterile pasteur pipette. The virus-containing sucrose was diluted 3- to 8-fold with 1xSE, and the virus was pelleted in a Beckman type 65 rotor at 50,000 r.p.m. for 1 hr at 4°C.

6.4.3 Extraction of viral RNA

Virus pellets were resuspended in 500 µl 25 mM Tris-HCl, pH 7.7, 25 mM NaCl, 2.5 mM EDTA, and after the addition of 50 µl 10 % w/v SDS, the suspension was incubated at 60°C for 10 min. The samples were then extracted three times with phenol and twice with CIA. 1/10 volume 3 M sodium acetate, pH 5.2, and 2.5 volumes of ethanol were added. After

storing at -70°C overnight, the RNA was pelleted (Microfuge, 10 min.). Pellets were washed twice with 70 % v/v ethanol, dried under vacuum and redissolved in sterile water. The yield was determined spectrophotometrically.

6.4.4 Purification of virus by isopycnic gradient centrifugation

Virus prepared as in section 6.4.2 was taken up in 5 ml 1×SE containing 49 % w/v CsCl (density 1.36 g/ml), and centrifuged at 38,000 r.p.m. for 17 hr at 10°C in a Beckman SW50.1 rotor. The gradient was illuminated from above with white light, and the bluish viral band was collected with a Gilson pipette. The virus was dialysed against several changes of distilled water at 4°C, and then diluted to 500 µl with TE. 50 µl 10 % w/v SDS was added, and RNA was extracted as described in the previous section.

6.5 EXTRACTION OF RNA FROM PLANT TISSUE

6.5.1 Extraction of total single-stranded RNA

Infected *P. vulgaris* leaves were prepared as described in section 6.4.1. 1 g tissue was ground to a fine powder under liquid nitrogen using a mortar and pestle. To this was added 2 ml 0.1 M Tris-HCl pH 7.8, 4 % v/v 2-mercaptoethanol, 0.8 % w/v SDS, 1 ml water-saturated phenol and 1 ml CIA. After thawing the mixture was vortexed, and centrifuged at 10,000 r.p.m. for 10 min. The aqueous phase was collected, and the organic phase was extracted with 2 ml 0.1 M Tris-HCl pH 9.0, 5 % v/v 2-mercaptoethanol, 2 % w/v SDS and centrifuged as above. The aqueous phases were pooled and extracted with a further 2 ml phenol + 2 ml CIA. After centrifugation as

above the aqueous phase was collected, and the organic phase was extracted with 0.5 ml 0.1 M Tris-HCl pH 7.8 and centrifuged again. The aqueous phases were pooled and made up to 8 ml by the addition of 1.35 g CsCl and the appropriate amount of sterile water. This was then underlayered with 2 ml 96 % w/v CsCl, 10 µg/ml ethidium bromide. Samples were centrifuged in a Beckman type 65 rotor at 40,000 r.p.m. for 17 hr at 4°C. After discarding the liquid, the RNA pellet was dissolved in 400 µl TE + 4 µl 2-mercaptoethanol. After extracting several times with water-saturated butanol to remove the ethidium bromide, 40 µl 3 M sodium acetate pH 5.2 and 1 ml ethanol were added. After storing at -70°C for 1 hr, the RNA was pelleted (Microfuge, 10 min.), dried under vacuum and dissolved in 0.1 ml sterile water.

6.5.2 Extraction of polysomal RNA

Polyribosomes were extracted according to Hari (1980). 2 g infected *P. vulgaris* tissue was ground in a sterile glass mortar with 12 ml 0.2 M Tris-HCl pH 9.0, 0.4 M KCl, 0.2 M sucrose, 35 mM MgCl₂, 25 mM EGTA, 10 mM 2-mercaptoethanol, 25 mM sodium diethyldithiocarbamate (Sigma), 0.5 % v/v Nonidet P-40. After filtration through sterile muslin, debris was removed by centrifugation at 15,500 r.p.m. for 15 min. The supernatant was layered over a 2 ml cushion of 60 % w/v sucrose, 40 mM Tris-HCl pH 9.0, 0.2 M KCl, 30 mM MgCl₂, 5 mM EGTA, and centrifuged at 40,000 r.p.m. for 3 hr in a Beckman type 65 rotor at 4°C. After draining, the pellets were resuspended in 1 ml 0.1 M Tris-HCl pH 9.0, 5 mM EGTA, 1 % SDS, and extracted with 0.5 volume phenol + 0.5 volume CIA. The organic phase was back-extracted once at room temperature and once at 60°C with 1 volume 0.1 M Tris-HCl pH 9.0, 5 mM EGTA, 1 % SDS, and the aqueous phases were

pooled and extracted once with phenol:CIA (1:1) and once with CIA. 1.5 volume propan-2-ol was added and after storing at -70°C overnight the RNA was collected by centrifugation at 20,000 r.p.m. for 30 min. in a Beckman SW50.1 rotor at 4°C. The pellets were redissolved in 250 µl 0.3 M sodium acetate pH 5.2, 500 µl ethanol was added and after 2 hr at -70°C the RNA was pelleted (Microfuge, 10 min.), dried under vacuum and dissolved in 0.1 ml sterile water.

6.5.3 Extraction of double-stranded RNA

50 g infected *P. vulgaris* leaves were ground to a fine powder under liquid nitrogen. 4 ml 1 M Tris-HCl pH 7.8, 1.6 ml 2-mercaptoethanol, 3.2 ml 10 % w/v SDS, 31.8 ml sterile water, 20 ml phenol and 20 ml CIA were added, and after vortexing, the phases were separated by centrifugation at 10,000 r.p.m. for 15 min. The aqueous phase was collected, 20 ml propan-2-ol were added, and after 45 min. at -70°C the sample was centrifuged at 10,000 r.p.m. for 15 min. The pellet was taken up in 8 ml 10 mM Tris-HCl pH 7.6, 1 % v/v 2-mercaptoethanol, 1 % w/v SDS, and extracted three times with 0.5 volume phenol + 0.5 volume CIA. The aqueous phase was collected and 1/3 volume 8 M LiCl was added. After 30 min. at 0°C the sample was centrifuged at 10,000 r.p.m. for 10 min., and the supernatant was added to 2 volumes of ethanol. After 1 hr at -70°C followed by centrifugation at 10,000 r.p.m. for 10 min., the pellet was dissolved in 16 ml TSE containing 15 % v/v ethanol. TSE is 50 mM Tris-HCl, pH 7.0, 100 mM NaCl, 1 mM EDTA.

A 10 ml cellulose column was prepared by equilibrating 3 g CF-11 (Whatman) in 50 ml TSE containing 15 % v/v ethanol. The sample was applied to the column and then washed with 50 ml TSE containing 15 % v/v

ethanol. DsRNA was eluted with two aliquots of 10 ml TSE and collected directly into 40 ml ethanol. The sample was stored at -70°C overnight, then centrifuged at 10,000 r.p.m. for 15 min. The pellet was dissolved in 1.2 ml sterile water and 0.4 ml 8 M LiCl was added. After 30 min. at 0°C followed by centrifugation (Microfuge, 10 min.), the supernatant was collected and added to 2 volumes ethanol. After 6 hr at -70°C , followed by centrifugation (Microfuge, 10 min.), the pellet was dried under vacuum and dissolved in 100 μl sterile water.

6.6 ISOLATION AND PURIFICATION OF DNA AND RNA SPECIES

6.6.1 DEAE-cellulose chromatography

Plasmids were purified by DEAE-cellulose column chromatography. A 0.3 ml column of DEAE-Sephacel (Sigma) was poured in a sterile 1 ml syringe. The column was washed with three aliquots of 1.5 ml 10 mM Tris-HCl, 1 mM EDTA, pH 7.6, containing 0.6 M NaCl, zero salt, and 0.1 M NaCl respectively. The DNA sample ($< 10\ \mu\text{g}$) was then loaded, and the column was washed with $2 \times 0.75\ \text{ml}$ 10 mM Tris-HCl, 1 mM EDTA, pH 7.6, 0.3 M NaCl. The DNA was eluted with $3 \times 0.25\ \text{ml}$ 10 mM Tris-HCl, 1 mM EDTA, pH 7.6, 0.6 M NaCl, one volume propan-2-ol was added, and after 1 hr at -70°C , the DNA was recovered by centrifugation (Microfuge, 10 min.).

6.6.2 Recovery of DNA from agarose gels

The method of Dretzen *et al.* (1981) was used. DEAE-cellulose paper (Whatman DE-81) was prepared by soaking it in 2.5 M NaCl for 3 hr and washing several times with distilled water. It was stored in 1 mM EDTA, pH 8.0, at 4°C .

DNA was electrophoresed in a 1 % agarose gel in the presence of ethidium bromide, and the bands of interest were located by UV illumination. Small strips of the DE-81 paper were inserted into slits in the gel just ahead of the band, and also just behind. The DNA was then electrophoresed onto the first strip of DE-81 at 90 V. The paper was removed from the gel, and shredded and vortexed in 0.5 ml 20 mM Tris-HCl, 1 mM EDTA, 1.5 M NaCl, pH 7.5. After incubating at 37°C for 2 hr, the paper fragments were pelleted by brief centrifugation, and the liquid was removed to a fresh tube. Liquid was recovered from the pellet by brief centrifugation through siliconised glass wool. The pooled liquid was extracted with 1 ml water-saturated butanol, 1 ml ethanol was added, and after 1 hr at -70°C the DNA was recovered by centrifugation (Microfuge, 10 min.).

6.6.3 Recovery of DNA from low melting point agarose

Gels were cast and electrophoresed as usual, except that low melting point agarose (BRL "Ultra-Pure" grade) was used. After staining with ethidium bromide, the bands of interest were excised with a sterile scalpel blade, and soaked in 10 volumes of sterile water for 1-2 hr at 4°C. The water was removed and 5 volumes TE were added. The gel was melted at 65°C and vortexed, and then extracted once with phenol, once with equal volumes of phenol and CIA, and once with CIA. 1/10 volume 3 M sodium acetate, pH 5.2, and 1 volume propan-2-ol were added, and after storage overnight at -70°C the DNA was recovered by centrifugation (Microfuge, 10 min.).

6.6.4 Recovery of RNA from low melting point agarose

Non-denaturing gels of 1 % low melting point agarose were used. The buffer was 1×TBE containing 0.1 % SDS. After electrophoresis the gel was washed briefly in distilled water and stained briefly with ethidium bromide. The bands were located by UV illumination and excised with a sterile scalpel blade. 4 volumes 0.5 M ammonium acetate and 1/100 volume 10 % w/v SDS were added, and the gel was melted at 65°C and vortexed. After phenol and CIA extractions as for DNA above, 2 volumes ethanol were added and the sample was stored at -70°C overnight. The RNA was collected by centrifugation (Microfuge, 10 min.), washed with 70 % v/v ethanol, dried under vacuum, and dissolved in 5 to 20 µl of sterile water.

6.7 HYBRIDISATION ANALYSIS OF NUCLEIC ACIDS

6.7.1 Preparation of random oligodeoxynucleotide primers

Random primers were prepared according to Taylor *et al.* (1976). Salmon sperm DNA (Sigma) was dissolved to 5 mg/ml in 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, containing 70 µg/ml DNase-I (Sigma). After 2 hr incubation at 37°C the DNA was autoclaved for 20 min. The resultant solution was used as a 5 mg/ml stock of random primers.

6.7.2 Random cDNA probes

Reverse transcriptase was used to synthesise randomly primed cDNA on RNA templates. The 50 µl reaction contained 50 mM Tris-HCl, pH 8.3, 70 mM KCl, 8 mM MgCl₂, 8 mM DTT, ≤1 µg RNA, 25 µl random primer stock solution, 0.5 mM each dATP, dGTP and dTTP, 5-15 µCi [α -³²P]dCTP, and 10 units AMV reverse transcriptase (Life Sciences). The mixture was incubated at 42°C

for 1 hr. The reaction was terminated by adding 1 μ l 0.5 M EDTA, pH 8.0. The RNA was hydrolysed by adding 5 μ l 1 M NaOH and incubating at 65°C for 1 hr. Excess alkali was neutralised with 5 μ l 1 M HCl, 90 μ l sterile water was added, and the incorporation of ^{32} P was determined by spotting 1 μ l onto DE-81 discs and scintillation counting (section 6.1.5).

6.7.3 Nick-translated probes

Probes were made from double-stranded DNA essentially as described by Rigby *et al.* (1977). A 50 μ l reaction contained 0.5–1 μ g DNA, 50 mM Tris-HCl, pH 7.8, 5 mM MgCl₂, 10 mM 2-mercaptoethanol, 20 μ M each dATP, dGTP, and dTTP, 2 μ M dCTP, 5 μ Ci [α - ^{32} P]dCTP, and 3 μ l 0.1 μ g/ml DNase-I (freshly diluted from a 5 mg/ml stock in 50 % v/v glycerol). After 10 min. at 14°C, 1 μ l (3 units) DNA Polymerase I (Amersham) were added, and the reaction was incubated at 14°C for a further 1.5 hr. The reaction was terminated by the addition of 2 μ l 0.5 M EDTA, pH 8.0.

6.7.4 Randomly-primed DNA probes

As an alternative to nick-translation, random primers were used with DNA templates. 5 μ l DNA (<1 μ g) was added to 25 μ l random primers, and heated to 100°C for 10 min. The DNA was cooled on ice and then made up to 50 μ l containing 50 mM Tris-HCl, pH 7.8, 5 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.5 mM each dATP, dGTP, and dTTP, 5 μ Ci [α - ^{32} P]dCTP, and 4 units DNA Polymerase I (Klenow fragment). The reaction was incubated at room temperature for 1.5 hr, and terminated by the addition of 2 μ l 0.5 M EDTA, pH 8.0,

This method was also used to make probes from DNA fragments in the presence of low melting point agarose. The band of interest was excised

from a gel and washed as described in section 6.6.3, and 30 μ l of DNA-containing gel was used to make a probe as described above (except that the incubation time was increased to 2.5 hr).

6.7.5 DNA "dot-blots"

Large numbers of plasmids were screened by spotting onto a nylon-based membrane. 2 μ l of each plasmid was spotted onto GeneScreen (New England Nuclear) in a grid pattern and the spots were air-dried. The membrane was laid on a pad of filter paper soaked in 0.5 M NaOH, 2.5 M NaCl for 30 seconds, and then on a second pad soaked in 0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl for 30 seconds. The membrane was air-dried and then baked at 80°C for 2 hr.

6.7.6 Colony lifts

Large numbers of clones were screened by *in situ* lysis on GeneScreen membrane. Each colony was inoculated onto an ampicillin-containing nutrient plate (see section 6.8.1) in a grid pattern, and the plate was incubated at 37°C until the colonies were 1-2 mm in diameter. GeneScreen was laid onto the plate and marked in the corners with a needle (to help with alignment later). After a few seconds the membrane was carefully peeled off, and laid colony-side-up on pads of filter paper soaked in : 10 % w/v SDS for 3 min.; 0.5 M NaOH, 1.5 M NaCl for 5 min.; 0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl for 2 $\times$ 5 min. The membrane was air-dried at room temperature for 30-60 min., then baked at 80°C for 2 hr.

6.7.7 "Southern" blotting

DNA was transferred to GeneScreen membrane according to the manufacturer (New England Nuclear). After electrophoresis, the gel was soaked in 0.2 M NaOH, 0.6 M NaCl for 30 min., then in three washes of 20 min. each in 1×SSC. The gel was laid on Whatman 3MM paper wicks connected to a reservoir of 1×SSC. GeneScreen membrane, cut to the size of the gel and prewetted in 1×SSC, was laid on the gel and covered with 5 layers of 3MM paper, all cut to the size of the gel. Finally a stack of paper towels was placed on top, and held down with a small weight. Transfer was allowed to proceed at room temperature overnight. The membrane was removed from the gel, dried at room temperature, and baked at 80°C for 2 hr.

6.7.8 "Northern" blotting

RNA was transferred from formaldehyde-agarose gels to GeneScreen Plus membrane according to the manufacturer (New England Nuclear). After electrophoresis as described in section 6.2.5, the gel was soaked in distilled water (at 60°C) for 5 min., 50 mM NaOH for 20 min., and 25 mM sodium phosphate pH 6.5 for 30 min. GeneScreen Plus membrane was wetted in distilled water, soaked in 10×SSC for 15 min., and then placed on the gel. Capillary transfer was set up as described for DNA gels, except that the transfer buffer was 10×SSC. After transfer the membrane was air-dried, then baked at 80°C for 2 hr.

6.7.9 Transfer of dsRNA from nondenaturing gels

DsRNA was electrophoresed on 1 % agarose, 1×TBE gels as for DNA. The gel was soaked in 0.2 M NaOH, 0.6 M NaCl for 30 min., then in 25 mM sodium phosphate, pH 6.5 for three changes of 20 min. each. DsRNA was then

transferred to GeneScreen as described for DNA, except that the transfer buffer was 25 mM sodium phosphate, pH 6.5. After transfer the membrane was air-dried, then baked at 80°C for 2 hr.

6.7.10 Hybridisation of membrane-bound nucleic acids

GeneScreen with bound DNA or RNA was prehybridised in 5-10 ml of 50 % v/v formamide, 10 % w/v dextran sulphate (Sigma, molecular weight 500,000), 1 M NaCl, 0.1 mg/ml heat-denatured salmon sperm DNA, and 1×P buffer. 1×P buffer contains 0.2 % each of BSA, polyvinylpyrrolidone (PVP-40, Sigma), and Ficoll-400 (Sigma), 50 mM Tris-HCl pH 7.5, 0.1 % sodium pyrophosphate, 0.1 % SDS. The prehybridisation fluid was sealed in a plastic bag with the membrane (after excluding any air bubbles) and incubated at 42°C with shaking for 4-6 hr. The radioactive probe was denatured by heating to 100°C for 10 min., cooled on ice, and added to the mixture in the bag. Hybridisation was allowed to proceed at 42°C with shaking for 16-24 hr. The probe was then discarded and the blot was washed. DNA blots were washed twice at room temperature in 1×NTE for 5 min., twice at 60°C in 1×NTE, 1 % SDS for 30 min., and twice at room temperature in 0.1×NTE for 30 min. 1×NTE is 60 mM Tris-HCl pH 8.0, 2 mM EDTA, 300 mM NaCl. RNA blots were washed twice at room temperature in 2×SSC for 5 min., twice at 60°C in 2×SSC, 1 % SDS for 30 min., and twice at room temperature in 0.1×SSC for 30 min. The membrane was then dried, covered with Saranrap, and autoradiographed at -70°C using intensifying screens.

GeneScreen Plus was treated similarly, except that: prehybridisation fluid contained only 50 % v/v formamide, 10 % w/v dextran sulphate, 1 M NaCl, and 1 % SDS; the probe was denatured and added along with 0.5 ml 5 mg/ml

salmon sperm DNA; blots were washed and autoradiographed as for RNA blots on GeneScreen.

6.8 CONSTRUCTION OF COMPLEMENTARY DNA CLONES

6.8.1 Media and antibiotics

2×TY broth is 1.6 % w/v Bacto-tryptone (Difco), 1 % Yeast extract (Difco), 0.5 % NaCl. 2×TY agar contains 1.5 % w/v agar (Difco) in addition.

Ampicillin (Sigma) was made up as a 25 mg/ml stock, filter-sterilised and stored at -20°C. It was added to broth or agar after autoclaving and cooling to 40°C, to a final concentration of 100 µg/ml.

BCIG (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) (Anglian) was stored as a 20 mg/ml stock in dimethylformamide, at 4°C. IPTG (isopropyl β-D-thiogalactopyranoside) (Anglian) was stored at 4°C as a 24 mg/ml stock in sterile water.

Recombinant bacteria were maintained on 2×TY agar containing ampicillin at 4°C, restreaked every two weeks, and also in 2×TY broth containing ampicillin and 15 % v/v glycerol at -70°C.

The *Escherichia coli* host used was:

JM101: Δ*lacpro*, *thi*, *supE*, F' *traD36*, *proAB*, *lacI^q*, ZΔ*M15* (Messing, 1983).

6.8.2 Preparation of competent cells

An exponential culture of JM101 was prepared by inoculating 30 µl saturated culture into 15 ml 2×TY broth, and incubating with shaking at 37°C for 4-4.5 hr. The exponential cells were cooled on ice for 20 min., then harvested in a bench centrifuge at 4°C (5 min. at 80 % speed in a Hettich EBA 3S bench centrifuge). The bacterial pellets were resuspended

in 10 ml 50 mM CaCl_2 , kept on ice for 20 min., and harvested as above. The bacterial pellets were resuspended in 2 ml 50 mM CaCl_2 , and stored at 4°C for 24 hr before use.

6.8.3 Transformation of competent cells

DNA in 20-100 μl buffer was added to 200 μl competent JM101. The cells were incubated at 0°C for 30 min., heat-shocked at 42°C for 3 min., and inoculated into 2.5 ml 2xTY broth. This was incubated at 37°C with shaking for 30 min., 10 μl of ampicillin was added, and 0.2 ml aliquots were taken out for spreading. To each 0.2 ml aliquot, 50 μl BCIG and 10 μl IPTG were added, and the mixture was spread on a 2xTY agar plate containing ampicillin. Plates were incubated at 37°C, inverted, overnight.

6.8.4 Small-scale preparation of plasmids

"Mini-preps" were performed by the rapid boiling method of Holmes & Quigley (1981). A bacterial colony from a plate was inoculated into 1.5 ml 2xTY broth containing ampicillin using a sterile toothpick. The broth was incubated with shaking at 37°C overnight. The bacteria were pelleted in the Microfuge for 3 min., and resuspended in 0.35 ml 10 mM Tris-HCl, 50 mM EDTA, pH 8.0, 0.5 % v/v Triton X-100, 8 % w/v sucrose. 25 μl 10 mg/ml lysozyme (Sigma) were added and the tube was vortexed. The sample was then heated at 100°C for 15-40 seconds, and centrifuged at room temperature for 10 min. (Microfuge). The pellet was discarded, and 40 μl 3 M sodium acetate, pH 5.2 and 0.42 ml propan-2-ol were added to the supernatant. After at least 15 min. at -70°C, the DNA was pelleted (Microfuge, 10 min.), drained, and redissolved in 80 μl TE. The DNA was now suitable for analysis by electrophoresis or dot-blotting; if required

for restriction endonuclease analysis or other enzymic reactions, the sample was further purified. It was extracted with 40 μ l phenol and 40 μ l CIA, then 20 μ l 10 M ammonium acetate and 200 μ l ethanol were added. After at least 1 hr at -70°C the DNA was pelleted (Microfuge, 10 min.), dried under vacuum, and redissolved in 50 μ l TE.

6.8.5 Large-scale preparation of pUC8

Plasmid pUC8 (Vieria & Messing, 1982) was prepared for use as a cloning vector. Competent JM101 cells were transformed with pUC8 as described above, and the 2.5 ml culture was inoculated into 20 ml 2 $\times$ TY containing ampicillin, and incubated at 37°C with shaking overnight. This culture was then inoculated into 400 ml 2 $\times$ TY containing ampicillin, and shaken at 37°C for a few hours. The bacteria were chilled on ice for 30 min., then harvested by centrifugation, 7000 r.p.m. for 10 min. at 4°C in the 6 $\times$ 300 ml rotor. The pellets were resuspended in a total of 20 ml 25 mM Tris-HCl, 10 mM EDTA, pH 8.0, and pelleted at 7000 r.p.m. for 10 min. at 4°C in the 8 $\times$ 50 ml rotor. The pellets were resuspended in a total of 6 ml 25 mM Tris-HCl, 10 mM EDTA, pH 8.0, 50 mM D-glucose, 12 mg/ml lysozyme, and incubated on ice for 30 min. 12 ml 0.2 M NaOH, 0.5 % SDS were added, and the sample was mixed gently and kept on ice for a further 5 min. Then 9 ml 3 M potassium acetate pH 4.8 were added, and after a further 30 min. on ice, the lysate was cleared by centrifuging at 13,000 r.p.m. for 10 min. at 4°C (8 $\times$ 50 ml rotor). The supernatant was collected, 2 volumes of ethanol were added, and after 45 min. at -20°C the sample was centrifuged at 10,000 r.p.m. for 10 min. at 4°C . The pellet was redissolved in 4.5 ml 50 mM Tris-HCl, pH 7.8, 100 mM NaCl, and 1.5 ml 8 M LiCl were added. After 30 min. at 4°C , the RNA was pelleted (10,000 r.p.m., 10 min., 4°C)

and the supernatant was collected. Two volumes of ethanol were added and after 15 min. at -20°C the DNA was pelleted (10,000 r.p.m., 10 min., 4°C). The pellet was dried and redissolved in 1 ml 50 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, pH 7.8. 5 μl RNase-A (10 mg/ml) were added, and after 1 hr incubation at 37°C , the sample was extracted once with phenol and once with CIA. More 50 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, pH 7.8, was added to give a total of 2×20 ml. To each 20 ml, 20 g CsCl and 2 ml ethidium bromide (10 mg/ml) were added, the samples were overlaid with liquid paraffin in Beckman "Quickseal" tubes, and centrifuged in a Beckman VTi50 rotor at 40,000 r.p.m. for 20 hr at 20°C . The DNA bands were visualised by UV illumination and collected with a syringe by side-puncturing the tubes. Ethidium bromide was removed by extractions with water-saturated propan-2-ol, and 2 volumes sterile water and 6 volumes of ethanol were added. After storage at -20°C overnight, the DNA was pelleted in a Beckman SW50.1 rotor (20,000 r.p.m. for 20 min.), washed with 70 % v/v ethanol, dried under vacuum, and redissolved in a total of 200 μl sterile water. The concentration was determined by spectrophotometry.

6.8.6 Synthesis of first-strand cDNA

Complementary DNA was made on RCNMV RNA template using AMV reverse transcriptase in the presence of random primers and Actinomycin D. The standard 100 μl reaction contained 10 μg RCNMV RNA and 50 μl random primer stock solution in 50 mM Tris-HCl pH 8.3, 70 mM KCl, 8 mM MgCl_2 , 20 $\mu\text{g/ml}$ Actinomycin D (Sigma), 8 mM DTT, 0.5 mM each dATP, dGTP and dTTP, 0.1 mM dCTP, 10-20 μCi [α - ^{32}P]dCTP, and 20 units reverse

transcriptase (Life Sciences). The reaction was incubated at 42°C for 1 hr and terminated by the addition of 2 µl 0.5 M EDTA, pH 8.0.

The optimal concentration of random primers was determined in 10 µl reactions which were analysed by alkaline agarose electrophoresis. The effects of varying the KCl concentration between 70-140 mM, and of including placental ribonuclease inhibitor (RNasin, P&S), were also examined.

For protocols not requiring DNA/RNA hybrid, the RNA was hydrolysed by adding 6 µl 1 M NaOH per 100 µl reaction and incubating at 65°C for 1 hr. The alkali was neutralised by the addition of 6 µl 1 M Tris-HCl pH 8.0 and 6 µl 1 M HCl. The sample was extracted with equal volumes of phenol and CIA. The organic phase was back-extracted with 60 µl sterile water, and the pooled aqueous phase was passed down a 1 ml column of Sephadex G-100 in sterile water. 2-drop fractions were collected and analysed by Cerenkov counting, and the first peak of radioactivity was pooled and precipitated with 2 volumes of ethanol at -70°C overnight. The cDNA was collected by centrifugation (Microfuge, 10 min.).

6.8.7 Priming with oligo(dT)₁₀

First-strand cDNA reactions were performed in 20 µl volumes essentially as described above, except that the random primers were replaced by 2 µg oligo(dT)₁₀.

6.8.8 Synthesis of second strand by self-priming

Second strand of cDNA was synthesised using reverse transcriptase followed by DNA polymerase I (Klenow fragment), and the resultant ssDNA regions were cleaved with S1 nuclease (Maniatis *et al.*, 1982).

The first-strand cDNA from a 100 μ l reaction was redissolved in 50 μ l containing 50 mM Tris-HCl pH 8.3, 70 mM KCl, 8 mM $MgCl_2$, 8 mM DTT, 1 mM each dATP, dCTP, dGTP, and dTTP, and 300 units/ml reverse transcriptase. The reaction was incubated at 42°C for 90 min., then 2 μ l 0.5 M EDTA pH 8.0 were added. The sample was extracted with phenol and CIA, and precipitated with 150 μ l ethanol at -70°C for 3 hr. The DNA was pelleted (Microfuge, 10 min.), dried, and redissolved in 100 μ l containing 0.1 M HEPES-KOH pH 6.9, 10 mM $MgCl_2$, 10 mM DTT, 70 mM KCl, 0.5 mM each dATP, dCTP, dGTP, and dTTP, and 150 units/ml Klenow enzyme. The reaction was incubated at 12°C for 17 hr and stopped by the addition of 2 μ l 0.5 M EDTA, pH 8.0. After extracting with phenol and CIA, the DNA was passed down a column of Sephadex G-100, and the peak of radioactivity was pooled and precipitated, as described in section 6.8.6. The DNA pellet was redissolved in 50 μ l sterile water.

The optimum S1 concentration was determined by digesting 0.4 μ l aliquots of the DNA in 20 μ l reactions containing 10 mM sodium acetate, pH 4.5, 2 mM $MgCl_2$, 1 mM $ZnSO_4$, 0.2 M NaCl and 0-6 units S1 nuclease (Sigma). After 30 min. at 37°C, the samples were analysed by alkaline-agarose gel electrophoresis.

For cloning, 1.2 μ g ds-cDNA was treated with 120 units S1 in a 100 μ l reaction, in the same buffer as above. After 30 min. at 37°C, 2 μ l 0.5 M EDTA, pH 8.0, and 2.5 μ l 2 M Tris base were added. The sample was extracted with phenol and passed down a column of Sephadex G-100 as described above. The final pellet was taken up in 20 μ l sterile water.

6.8.9 Homopolymer tailing of DNA

Homopolymer tracts were added at the 3'-ends of DNA using terminal deoxynucleotidyl transferase (TdT).

Initial experiments used 25 μ l reactions containing 0.1 M potassium cacodylate, 25 mM Tris pH 6.9, 1 mM CoCl_2 , 0.2 mM DTT and 500 units/ml TdT (Anglian). The vector, pUC8 which had been cleaved with *Pst*I, was tailed with dGTP: 0.225 μ g pUC8 in a 25 μ l reaction containing 0.1 mM dGTP was incubated at 37°C for 20 min. After adding 2 μ l 0.5 M EDTA pH 8.0 to stop the reaction, the sample was extracted with phenol and CIA. The S1-treated cDNA was tailed with dCTP: 90 ng cDNA in 25 μ l with 0.1 mM dCTP was incubated at 37°C for 5-60 min.

In the second method, 20 μ l reactions containing 0.1 M potassium cacodylate pH 7.2, 2 mM CoCl_2 , and 0.2 mM DTT were used. 0.1 μ g *Pst*I-cleaved pUC8 was tailed using 10 μ M dGTP and 4.5 units TdT (BRL), by incubating at room temperature for 20 min. 30-40 ng cDNA was tailed with 10 μ M dCTP and 7.5 units TdT using the same incubation conditions. Reactions were terminated by the addition of 1 μ l 0.5 M EDTA, pH 8.0.

6.8.10 Annealing of homopolymer-tailed DNA

5 μ l dC-tailed DNA made by the first method above, or 20 μ l made by the second method, were mixed with 25 ng dG-tailed pUC8, and DNA was precipitated by the addition of 2.5 volumes of ethanol. After 1 hr at -70°C, the DNA was pelleted (Microfuge, 10 min.), dried, and dissolved in 50 μ l 10 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA. The sample was incubated at 65°C for 5 min., 57°C for 1 hr, and then cooled slowly to room temperature. The annealed DNA was used to transform 0.2 ml competent JM101 cells as described in section 6.8.3.

6.8.11 Preparation of vectors cleaved with restriction enzymes

Plasmid pUC8 was cleaved and treated with alkaline phosphatase for use in cloning restriction fragments of cDNA, or in cloning cDNA to which synthetic linkers had been added. pUC8 was cleaved with *Sall*, *Bam*HI, *Hinc*II, *Pst*I, or *Acc*I, as described previously, and precipitated with ethanol. The dried pellet was dissolved up in 15 μ l 50 mM Tris-HCl pH 8.3, and 0.1 unit calf intestinal phosphatase (CIP; from BCL) was added. After a 1 hr incubation at 37°C, the DNA was electrophoresed on a 1 % agarose gel, and the linear band was extracted from the gel using DE-81 paper (section 6.6.2). The DNA was redissolved in TE to a concentration of 50 ng/ μ l.

6.8.12 Addition of synthetic linkers to cDNA

Linkers were added essentially as described by Goodman & MacDonald (1979). The linker used (New England Biolabs) was the octamer d(GGTCGACC) which contains a site for *Sall*. Linkers were phosphorylated and labelled as follows. 0.5 μ l linkers (0.5 μ g) was added to 2.5 μ l [γ - ^{32} P]ATP (25 μ Ci), 1 μ l 10 $\times$ Kinase buffer (0.5 M Tris-HCl pH 7.6, 100 mM MgCl₂, 10 mM spermidine, 50 mM DTT), 5 μ l sterile water, and 1 μ l (6 units) T4 polynucleotide kinase (Amersham). After 30 min. at 37°C, a further 1 μ l kinase, 1 μ l 10 $\times$ Kinase buffer, 1 μ l 10 mM ATP, and 7 μ l water were added, and the incubation was continued for a further 30 min. at 37°C. The reaction was stopped by extracting once with phenol and twice with CIA. At this stage the linkers were checked by self-ligating 1 μ l labelled linkers in a 10 μ l reaction containing 66 mM Tris-HCl pH 7.6, 6.6 mM MgCl₂, 10 mM DTT, 1 mM ATP and 2.5 units T4 DNA ligase (Anglian). After incubating at 13°C for 1 hr, the ligated linkers were compared with

unligated, labelled linkers by electrophoresis on a 7 % polyacrylamide gel.

S1-treated cDNA (section 6.8.8) was made blunt-ended in a 40 μ l reaction containing 0.18 μ g cDNA, 1 mM ATP, 60 mM Tris-HCl pH 7.5, 8 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.2 mM each dATP, dCTP, dGTP and dTTP, and 4 units DNA polymerase I. The reaction was incubated at 10°C for 10 min., then linkers were added by mixing 9.2 μ l labelled linkers, 1.5 μ l 0.6 M Tris-HCl, 80 mM $MgCl_2$, pH 7.5, 1.5 μ l 10 mM ATP, 0.8 μ l 0.2 M 2-mercaptoethanol, and 2 μ l (2.5 units) T4 DNA ligase. After 24 hr at 14°C, the reaction was heated to 65°C for 5 min. to kill the enzymes. The sample was diluted to 100 μ l including the addition of 25 units *Sal*I and 10 μ l 10 $\times$ *Sal*I buffer (section 2.7.4). The reaction was incubated at 37°C for 18 hr, then excess linkers were removed by passing the sample down a column of Sephadex G-200 in sterile water. The first peak eluted from the column was pooled and precipitated with 2 volumes of ethanol. The final cDNA pellet was dissolved in 16 μ l sterile water.

The linker-cDNA was cloned into pUC8 in a ligation reaction containing 8 μ l linker-cDNA ($\approx$ 50 ng), 150 ng *Sal*I-cut, CIP-treated pUC8, 1 mM ATP, 66 mM Tris-HCl pH 7.6, 6.6 mM $MgCl_2$, 10 mM DTT and 5 units T4 DNA ligase in a final volume of 15 μ l. After 16 hr incubation at 14°C, the mixture was used to transform 0.2 ml competent JM101 cells.

6.8.13 Treatment of cDNA with Mung Bean Nuclease

Double-stranded cDNA was made blunt-ended by treatment with the single-strand-specific mung bean nuclease. 90 ng cDNA was treated in a 20 μ l reaction containing 50 mM sodium acetate, pH 4.5, 1 mM $ZnSO_4$, 50 mM NaCl, and 1 unit mung bean nuclease (P-L). After 10 min. at 37°C, the reaction

was stopped and the DNA was precipitated by the addition of 2 volumes of ethanol.

6.8.14 Cloning of cDNA restriction fragments

60 ng aliquots of double-stranded cDNA were cleaved in 20 μ l reactions with *TaqI*, *Sau3AI*, *AccI*, *PstI*, or *BamHI* (see sections 2.7.4 and 6.1.3 for reaction conditions). After the digestion, the DNA was recovered by precipitation with 2 volumes of ethanol. The dried pellets were redissolved in 20 μ l 66 mM Tris-HCl pH 7.6, 6.6 mM MgCl₂, 10 mM DTT, 1 mM ATP, containing 100 ng CIP-treated vector (section 6.8.11) and 5 units T4 DNA ligase. The samples were incubated at 14°C overnight, then 10 μ l aliquots of the ligated DNA were used to transform 0.2 ml aliquots of competent JM101 cells. The vectors used were:

AccI-cut pUC8 for cDNA cleaved with *TaqI* or *AccI*;

BamHI-cut pUC8 for cDNA cleaved with *Sau3AI* or *BamHI*;

PstI-cut pUC8 for cDNA cleaved with *PstI*.

Blunt-ended cDNA was ligated into *HincII*-cut pUC8 in a slightly different manner. 5 ng cDNA and 50 ng vector were incubated at 15°C overnight in 20 μ l 66 mM Tris-HCl pH 7.6, 6.6 mM MgCl₂, 10 mM DTT, 1 mM ATP, containing 5 units T4 DNA ligase and 12 % w/v PEG-6000. The reaction mixture was then used to transform 0.2 ml competent JM101 cells.

6.8.15 Synthesis of second strand of cDNA using RNase-H

As an alternative to the procedure described in section 6.8.8, cDNA was made double-stranded using the method of Gubler & Hoffman (1983).

Randomly-primed cDNA was made with reverse transcriptase in a 100 μ l reaction as described, and after adding 4 μ l 0.5 M EDTA pH 8.0 to stop

the reaction, the RNA/DNA hybrid was passed down a column of Sephadex G-200 in sterile water. The first peak eluted from the column was pooled and precipitated by adding 2 volumes of ethanol. After 1 hr at -70°C , the hybrid was pelleted (Microfuge, 10 min.), dried under vacuum, and dissolved in sterile water to a concentration of 0.2 mg/ml. 1 μg hybrid was then treated with DNA polymerase I and RNase-H in a 100 μl reaction containing 20 mM Tris-HCl pH 7.5, 5 mM MgCl_2 , 100 mM KCl, 10 mM ammonium sulphate, 50 $\mu\text{g/ml}$ BSA (BRL nuclease-free grade), 40 μM each dATP, dCTP, dGTP and dTTP, 25 units DNA polymerase I (Amersham) and 0.8 unit *E. coli* RNase-H (P-L). The reaction was incubated at 12°C for 1 hr and 22°C for 1 hr, then terminated by the addition of 4 μl 0.5 M EDTA pH 8.0, 25 μl 10 M ammonium acetate, and 250 μl ethanol. After storage at -70°C overnight, the cDNA was pelleted (Microfuge, 10 min.), dried under vacuum, and dissolved in a small amount of sterile water.

Before cDNA made by this method was tailed with TdT, it was made blunt-ended using the Klenow enzyme. A 20 μl reaction contained 50-60 ng cDNA and 1 unit DNA polymerase I (Klenow fragment) in 50 mM Tris-HCl pH 7.8, 5 mM MgCl_2 , 10 mM 2-mercaptoethanol, and 0.1 mM each of the four dNTPs. After 30 min. at room temperature, the cDNA was recovered by precipitation with ethanol as described above.

6.9 *IN VITRO* TRANSLATION

6.9.1 *In vitro* translation of RNA

RNA was translated using messenger-dependent, nuclease-treated rabbit reticulocyte lysate (Amersham N.90), essentially as described by Pelham & Jackson (1976). A standard 10 μ l translation reaction contained 8 μ l reticulocyte lysate, 10 μ Ci L-[^{35} S]methionine or 10 μ Ci L-[4,5- ^{3} H]leucine, 0.1-0.5 μ g RNA, and 1 μ g yeast tRNA (Sigma). The lysate was incubated at 30°C for 60 min., and then 5-10 μ l of the mixture was analysed by SDS-PAGE and fluorography (section 6.3).

In some translations, the cap analogue 7-methylguanosine 5'-monophosphate (m^7 GMP; Sigma) was included at 4-8 mM final concentration (Joshi *et al.*, 1983).

Incorporation of label into proteins was determined by precipitation with TCA. 1 μ l translation mix was added to 0.5 ml 1 M NaOH, 5 % v/v H_2O_2 ('100 volume'), and incubated at 37°C for 10 min. The sample was cooled on ice and 2 ml cold 25 % w/v TCA, 2 % w/v casein hydrolysate, were added. After a further 15-30 min. on ice, the precipitated protein was collected by filtration on a Whatman GF/C filter, and washed with 2 $\times$ 3 ml ice-cold 8 % w/v TCA, 2 $\times$ 5 ml ice-cold ethanol, and 5 ml ether. The filter was dried, and the radioactive protein content was determined by scintillation counting (section 6.1.5).

6.9.2 Preparation of RCNMV coat protein

RCNMV coat protein was used as an unlabelled size marker on protein gels. The virus from one sucrose gradient was pelleted as described (section 6.4.2), and resuspended in 500 μ l 25 mM Tris-HCl pH 7.7, 2.5 mM EDTA, 25

mM NaCl, 0.5 % SDS. This stock was stored at -20°C, and used without removal of the RNA.

Other unlabelled protein size markers were purchased from BCL, and stored as 10 mg/ml stocks at -20°C.

6.9.3 Immunoprecipitation

Labelled coat protein was precipitated from a mixture of translation products using a protocol based on that of Harbison *et al.* (1985). The basic method was as follows: 10 µl translation products were preincubated with 1 µl rabbit preimmune serum, 150 µl 1 M NaCl, 1 % v/v Triton X-100, 40 µl sterile water, and 60 µl formalin-fixed Staph A cells (BRL Immunoprecipitin, 375 µg resuspended in 25 mM Tris-HCl pH 7.5). This mixture was incubated at 4°C for 2 hr, then the Staph A cells were pelleted (Microfuge). The supernatant was collected, and the pellet was washed three times with 60 µl 0.75 M NaCl, 1 % Triton X-100. The washings were pooled with the supernatant, and 20 µl anti-RCNMV IgG (1 mg/ml) and 60 µl Staph A cells were added. The mixture was incubated at 4°C overnight. The Staph A cells were pelleted and the supernatant was discarded. The pellet was washed five times with 60 µl 0.75 M NaCl, 1 % Triton X-100, and finally resuspended in protein sample buffer for analysis by SDS-PAGE.

The anti-RCNMV IgG was prepared by the method of Clark & Adams (1977), from the serum of a rabbit immunised with highly purified RCNMV virions, and was a gift from T.A. Osman.

Variations to the above method were: the preimmune serum was omitted from the preincubation step; the preincubation step was omitted; the amount of IgG used was reduced to 4 µg or 1 µg.

6.10 HYBRID-SELECTION OF RNA SPECIES

6.10.1 Hybrid-selection using nitrocellulose

The method was essentially as described by Miller *et al.* (1983). 4 μ l DNA (2 μ g) was diluted to 10 μ l with TE, and heated to 100°C for 10 min. The DNA was quickly cooled on ice and spotted onto a small square of nitrocellulose (Amersham Hybond-C) which had been soaked in 20 $\times$ SSC. The nitrocellulose was dried, washed in 6 $\times$ SSC for 30 min., and baked at 80°C for 2 hr. The nitrocellulose was then hybridised in 100 μ l containing 65 % v/v deionised formamide, 0.4 M NaCl, 40 mM PIPES pH 6.4, 1mM EDTA, 0.2 % SDS, 30 μ g/ml yeast tRNA, and 10 μ l RCNMV-infected plant ssRNA (section 6.5.1), at 48°C for 2 hr. The hybridisation fluid was discarded and the nitrocellulose was washed 10 times at 60°C in 1 $\times$ SSC, 0.5 % SDS, and three times at room temperature in 2 mM EDTA. 300 μ l 1 mM EDTA containing 10 μ g tRNA were added, and bound RNA was eluted by heating at 100°C for 1 min., quickly cooling on ice, and transferring the liquid to a fresh tube containing 20 μ l 3 M sodium acetate, pH 5.2 and 900 μ l ethanol. After storage at -70°C overnight, the eluted RNA was pelleted (Microfuge, 10 min.), dried under vacuum and redissolved in sterile water for analysis by Northern blotting or *in vitro* translation.

6.10.2 Preparation of DPT-paper

As an alternative to using nitrocellulose as above, RNA was hybrid-selected using DNA covalently bound to diazophenylthioether (DPT) paper. Aminophenylthioether (APT) paper was made as described by Maniatis *et al.* (1982). 2 g Whatman 50 paper was cut into strips and sealed in a plastic tube with 21 ml 0.5 M NaOH and 9 ml 1,4-butanediol diglycidyl ether

(Aldrich). This was agitated by rotating end-over-end at 30 r.p.m. for 20 hr at room temperature. The liquid was discarded, and 25 ml 0.5 M NaOH and 25 ml 2-aminothiophenol (2 % v/v in ethanol) were added. After incubating at room temperature for a further 2 hr, the paper was washed in ethanol (15 min.) and then in 0.1 M HCl (15 min.). These washes were repeated another two times, then the paper was washed in distilled water for 1-2 hr, washed in ethanol, and dried. The APT-paper was stored at -20°C, and was activated just before use. It was diazotised by soaking in 1.2 M HCl containing 0.27 mg/ml NaNO₂ at 0°C for 30 min. The DPT-paper was washed twice in cold water and twice in cold 20 mM sodium acetate, pH 4.0, cut into small squares, and the DNA was applied. 10 µg DNA in 100 µl TE was denatured by adding 50 µl 1 M NaOH and heating at 100°C for 5 min. The DNA was cooled on ice, and 40 µl 1 M HCl, 20 µl 3 M sodium acetate pH 5.2, and 500 µl ethanol were added. After 1 hr at -70°C, the DNA was pelleted (Microfuge, 10 min.), dried, and redissolved in 25 µl sterile water. The DNA was heated at 100°C for 1 min., cooled, spotted onto the DPT-paper, and allowed to dry at room temperature overnight. Finally the paper was washed three times in water, 4 times in 0.4 M NaOH, and 4 times in water.

6.10.3 Hybridisation of DPT-paper

The procedure used was essentially that of Maniatis *et al.* (1982). The hybridisation fluid contained 50 % v/v deionised formamide, 0.1 % SDS, 0.6 M NaCl, 4 mM EDTA, 80 mM Tris-HCl pH 7.8, and 20 µg RCNMV-infected plant ssRNA in a total volume of 200 µl. The mixture was preheated at 65°C for 10 min., then the DPT-paper with bound DNA was added. Hybridisation was at 48°C for 2.5 hr, then the paper was washed 10

times at 60°C in 1×SSC, 0.5 % SDS. RNA was eluted by adding 0.2 ml 98 % formamide, 10 mM Tris-HCl pH 7.8, 10 µg/ml tRNA, and heating at 65°C for 15 min. The paper was removed (and recycled for further use by washing in 0.1 M NaOH), and 0.2 ml sterile water, 40 µl 3 M sodium acetate pH 5.2, and 1.1 ml ethanol were added. The RNA was recovered as described above.

6.11 ANALYSIS OF THE 5'-ENDS OF RCNMV RNA

6.11.1 Treatment with phosphatase and polynucleotide kinase

10 µg RNA was incubated in a 50 µl reaction containing 50 mM Tris-HCl pH 8.3 and 0.05 units of CIP, at 37°C for 30 min. The sample was extracted twice with phenol and once with CIA, precipitated by adding 2 volumes of ethanol, and redissolved to 1 mg/ml in sterile water.

3 µg CIP-treated RNA was end-labelled in a 20 µl reaction containing 50 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 1 mM spermidine, 5 mM DTT, 0.5 µCi [γ -³²P]ATP, 10 pmol unlabelled ATP, and 5 units T4 polynucleotide kinase (Amersham). After a 30 min. incubation at 37°C, the reaction was stopped by extracting with CIA; the RNA was recovered by precipitation with 2 volumes of ethanol, and analysed by electrophoresis on a glyoxal agarose gel.

6.11.2 Chemical cleavage of the 5'-cap structure

The periodate/aniline cleavage method of Fraenkel-Conrat & Steinschneider (1968) was used. 10 µg RNA was incubated at 25°C for 1 hr in the dark, in 10 µl 40 mM sodium acetate, pH 5.2, 0.2 mM NaIO₄. 10 µl 4 M ammonium acetate and 60 µl ethanol were added, and after 2 hr at -70°C the RNA was recovered by centrifugation (Microfuge, 10 min.). The RNA was then

dissolved in 20 μ l 10 mM sodium acetate, 0.3 M aniline-HCl, pH 5.0, and incubated for 3 hr at 25°C in the dark. The RNA was recovered by another precipitation with ethanol.

6.11.3 Enzymic cleavage of the 5'-cap structure

10 μ g RNA was treated in a 20 μ l reaction containing 50 mM sodium acetate, pH 6.0, 5 mM DTT, 1 mM EDTA, and 0.1 unit tobacco acid pyrophosphatase (TAP; from BRL), for 30 min. at 37°C (Efstratiadis *et al.*, 1977). The RNA was recovered by precipitation with ethanol as usual.

6.11.4 Assay for acid pyrophosphatase activity

Acid pyrophosphatase activity in the TAP preparations was assayed in a 10 μ l reaction containing 50 mM sodium acetate, pH 6.0, 5 mM DTT, 1 mM EDTA, 0.5 μ Ci [γ - ^{32} P]ATP, and 0.05 units TAP. After 30 min. at 37°C, the reaction was analysed by chromatography on polyethyleneimine (PEI) cellulose. PEI-cellulose thin-layer plates (Polygram CEL300 PEI/UV₂₅₄, Machery-Nagel) were predeveloped in distilled water and dried. The TAP reaction was spotted on 1 cm from the bottom of the plate, and then the plate was developed by ascending chromatography using 0.75 M KH₂PO₄, pH 3.5, for 2 hr. The plate was dried, and marker spots of unlabelled ATP were located by UV illumination. The plate was then wrapped in cellophane and autoradiographed at room temperature.

6.11.5 Iodination of RCNMV RNA

RNA was iodinated using Iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycoluril; Mayo *et al.*, 1982). Tubes were prepared by pipetting 100 μ l Iodogen (Pierce Chemical Company), 20 μ g/ml in chloroform, into

27x6 mm glass 'Durham' tubes. The chloroform was removed under vacuum. 10 μ l RNA (50 μ g), 20 μ l 0.5 M sodium phosphate pH 7.5, and 2 μ l [^{125} I]NaI (100 μ Ci) were added to the tube and incubated at 0°C for 5 min. The sample was then passed down a column of Sephadex G-50 in 10 mM Tris-HCl pH 8.0, 0.2 % SDS. 3-drop fractions were collected into 600 μ l ethanol and counted in a γ -ray counter (LKB Wallac 80000 Gamma Sample Counter). The first peak of activity was pooled, stored at -70°C for 1 hr, and pelleted (Microfuge, 10 min.). The pellet was dissolved in 50 μ l sterile water and reprecipitated with 2 volumes ethanol. The final pellet was dissolved in 50 μ l sterile water.

If necessary, the [^{125} I]RNA was further purified by pelleting through CsCl. The RNA was taken up in 3 ml 1.0 M CsCl, 50 mM Tris-HCl pH 7.6, 50 mM NaCl, and layered over 2 ml 5.7 M CsCl in the same buffer. The sample was centrifuged in a Beckman SW50.1 rotor for 17 hr at 30,000 r.p.m., 20°C. The pellet was dissolved in 100 μ l 10 mM Tris-HCl pH 7.6, 1 % v/v 2-mercaptoethanol, then 25 μ l 10 M ammonium acetate and 375 μ l ethanol were added. After 3 hr at -70°C, the RNA was pelleted (Microfuge, 10 min.) and redissolved in 20 μ l sterile water.

6.11.6 Analysis of iodinated RNA

[^{125} I]RNA was digested with proteases or nucleases and analysed by gel electrophoresis. Protease digestion: 1-3 μ l [^{125} I]RNA was treated with 10 μ g pronase (Sigma, Protease type V) and 10 μ g proteinase-K (Sigma, Protease type XI) in 10 μ l TE containing 0.5 % SDS, at 37°C for 1 hr. Nuclease digestion: 3-5 μ l [^{125} I]RNA was treated with 2 μ g RNase-A and 0.4 units RNase-T2 (Sigma) in 10 μ l 125 mM Tris-HCl pH 6.8, at 37°C for 1 hr. Samples were electrophoresed on SDS-containing agarose gels, or 20

% polyacrylamide protein gels. The gels were dried down onto 3MM paper, and autoradiographed at -70°C using intensifying screens.

6.11.7 Effect of protease digestion on infectivity of RNA

RCNMV RNA was treated with proteases essentially as described by Mayo *et al.* (1982). The protease stocks had been predigested at 37°C for 1 hr to remove any contaminating RNase. 2 µg RNA was treated in 80 µl 1×SSC, 0.5 % SDS, with 0.1 mg/ml each proteinase-K and pronase, at 37°C overnight. After extracting with phenol and CIA, the RNA was precipitated with 2 volumes of ethanol, and redissolved in 0.5 ml 50 mM sodium phosphate pH 7.0, 200 µg/ml Bentonite, 10 mg/ml Celite (Dodds *et al.*, 1977). 100 µl aliquots of this were inoculated onto cowpea (*Vigna sinensis* cv. Blackeye No. 5) leaves by gentle abrasion. The number of lesions which appeared was compared to that on similar leaves inoculated with untreated RNA.

CHAPTER 7. RESULTS.

7.1 EXTRACTION OF VIRUS AND VIRAL RNA

7.1.1 Extraction of virus and viral RNA

Initial extractions of virus using the phosphate buffer system of Gould *et al.* (1981) repeatedly resulted in RNA in an extensively degraded state as judged by glyoxal-agarose gel electrophoresis. Inclusion of 10 mM EDTA in all steps of the extraction procedure (section 6.4.2) resulted in a single intense virus band on the sucrose-gradients, and a good yield of intact RNA, typically in excess of 600 μ g RNA from 100 g leaf material. Yields were higher (600 μ g from 100 g) when the plants were grown under controlled conditions rather than in a glasshouse (100-200 μ g from 100 g), and could be further increased (to $\approx$ 1.4 mg from 100 g) by processing only the inoculated leaves, which have a higher concentration of virus.

7.1.2 Electrophoresis of viral RNA under denaturing conditions

The sizes of the RCNMV viral RNAs had not been determined before under fully denaturing conditions. Both glyoxal- and formaldehyde-agarose gels were used with a range of ssRNA markers of known sizes (tobacco mosaic virus RNA, 6395 bases (Goelet *et al.*, 1982); *E. coli* ribosomal RNAs, 2904 and 1542 bases (Brosius *et al.*, 1980; Brosius *et al.*, 1978); tomato black ring virus RNAs, 7760, 4790, and 1375 bases (Murant *et al.*, 1981; Meyer *et al.*, 1984)). The sizes of the RCNMV RNAs were calculated from graphs of log (molecular weight) against mobility. The mean estimates (nine determinations) of the RCNMV RNA sizes were:

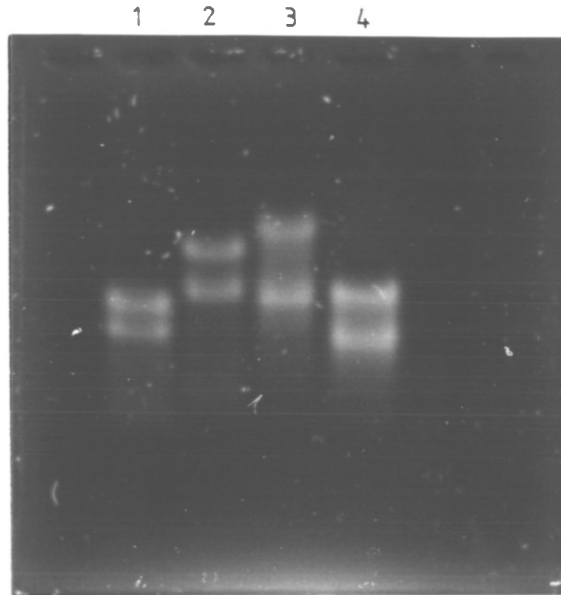


Figure 7.1 Electrophoresis of RCNMV RNA under denaturing and nondenaturing conditions. Lane 1, *E. coli* rRNA untreated; lane 2, *E. coli* rRNA glyoxalated; lane 3, RCNMV RNA glyoxalated; lane 4, RCNMV RNA untreated. Samples were electrophoresed as in section 6.2.4, and stained with ethidium bromide.

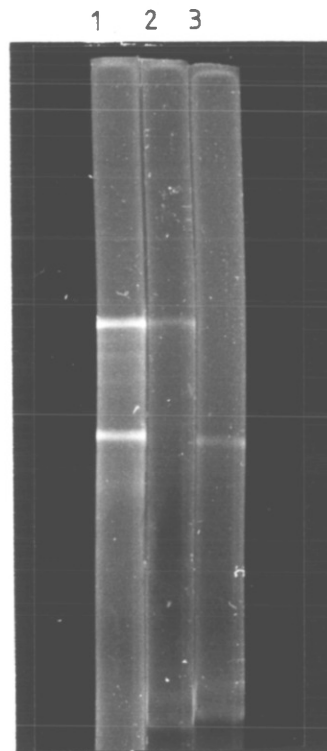


Figure 7.2 Separation of viral RNAs. The RNA was extracted from low melting point agarose, electrophoresed under nondenaturing conditions, and stained with ethidium bromide. Lane 1, total viral RNA; lane 2, RNA-1; lane 3, RNA-2.

RNA-1 3990 bases

RNA-2 1390 bases

Figure 7.1 shows both native and glyoxalated RCNMV RNA after agarose gel electrophoresis. It can be seen that the native RNA migrates more rapidly through the gel than does glyoxalated RNA. Glyoxalation leads to complete denaturation of the RNA (McMaster & Carmichael, 1977), and this removal of secondary structure leads to a more extended conformation of the molecule resulting in slower migration. Hence the result shown implies that the native conformation of RCNMV RNA contains much secondary structure.

7.1.3 Separation of viral RNAs

RNAs-1 and -2 were extracted from low melting point agarose as described in section 6.6.4. The inclusion of 0.1 % SDS in the gel was found to be necessary to prevent degradation of the RNA during extraction. A typical batch of separated RNAs is shown in figure 7.2. The separated bands were free from cross-contamination as judged by agarose gel electrophoresis. When a cDNA probe, made from gel-purified RNA-1, was used to probe a Northern blot of viral RNA, the only band which hybridised was RNA-1 (see figure 7.3). This also indicates that the RNA-1 was free of contaminating RNA-2, and confirms the absence of any substantial homology between RNA-1 and RNA-2, previously deduced for an Australian isolate of RCNMV by Gould *et al.* (1981). No minor species hybridising to RNA-1 were detected.

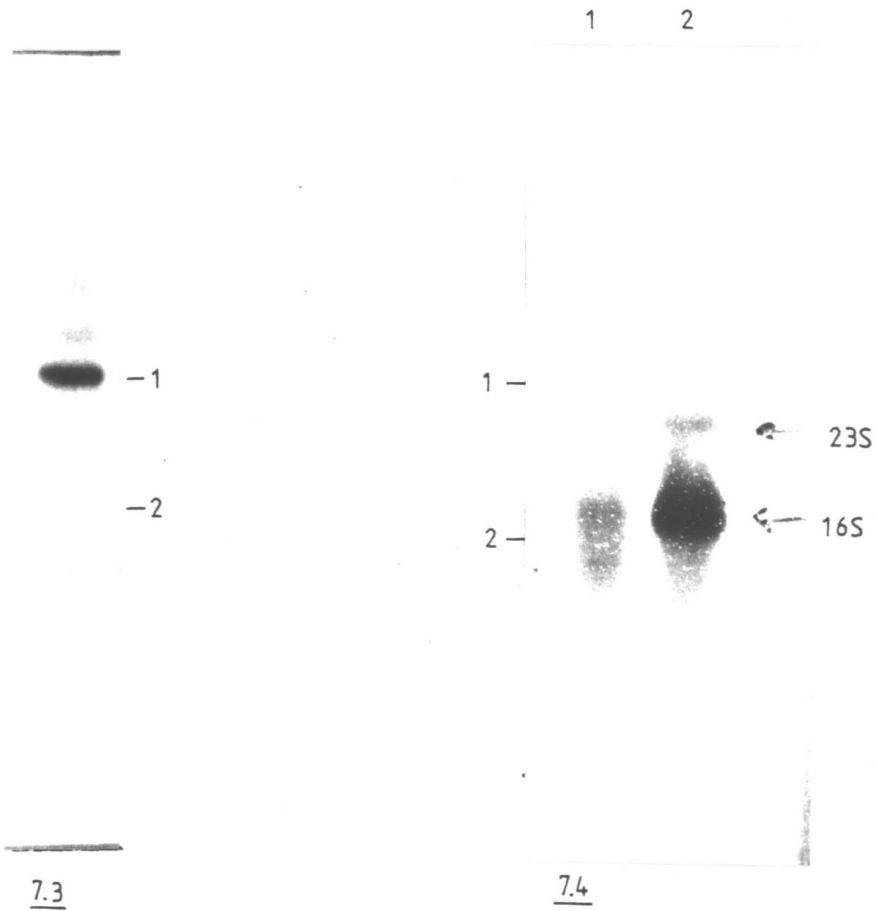


Figure 7.3 Hybridisation of cDNA-1 to viral RNA. 0.1 μ g viral RNA was electrophoresed on a formaldehyde-agarose gel, transferred to GeneScreen Plus, and probed with cDNA to RNA-1.

Figure 7.4 Autoradiogram of 5'-end-labelled RNA. See text for details. Lane 1, RCNMV RNA; lane 2, *E. coli* ribosomal RNA.

7.2 ANALYSIS OF THE 5'-ENDS OF VIRAL RNA

7.2.1 Treatment with phosphatase and polynucleotide kinase

In parallel reactions, RCNMV RNA and *E. coli* ribosomal RNA were first treated with calf intestinal phosphatase (CIP) to remove any 5'-phosphate, then phosphorylated by T4 polynucleotide kinase in the presence of [γ - ^{32} P]ATP, and analysed by glyoxal-agarose gel electrophoresis. An autoradiogram of the resultant gel is shown in figure 7.4. Both the 23S and 16S RNAs are visible as labelled bands; these RNAs are known to have a phosphate group at the 5'-end (Brosius *et al.*, 1978; 1980), which is removed by CIP leaving a 5'-hydroxyl which is a substrate for kinase. By contrast, no bands corresponding to the RCNMV RNAs could be detected, indicating that the 5'-ends of these RNAs are blocked. The featureless smear which is produced is probably the result of end-labelling random degradation products of RCNMV RNAs 1 and 2.

7.2.2 Treatment with periodate and aniline

If the 5'-ends of the RCNMV RNAs were blocked with the 7-methylguanosine cap structure seen in many eukaryotic RNAs, removal of the cap followed by treatment with CIP would result in 5'-hydroxyl RNA which would then be a substrate for kinase. One way to remove the cap is oxidation of the ribose moiety of the capping nucleotide with periodate, followed by β -elimination using aniline (Fraenkel-Conrat & Steinschneider, 1968). When RCNMV RNA was treated in this manner, followed by CIP and kinase reactions, the resulting labelled RNA showed only a featureless smear upon electrophoresis and autoradiography. This may have been due to

degradation of the RNA during the chemical reactions. A similar result was obtained using TMV RNA, which is known to be capped at the 5'-end.

7.2.3 Treatment with tobacco acid pyrophosphatase

Since the chemical method used above seemed to be too harsh a treatment, it was attempted to remove a cap structure enzymatically, using TAP. Following treatment with TAP, the RNA was treated with CIP and then kinase/[γ - ^{32} P]ATP as before. Again, only a featureless smear was seen following electrophoresis and autoradiography. However, the TMV capped control was not end-labelled using this protocol, so the TAP was assayed to check if it was in fact active. The enzyme was assayed by its ability to cleave the γ -phosphate group from ATP, thus releasing the label from [γ - ^{32} P]ATP as inorganic phosphate, which can be separated from the ATP by chromatography on PEI-cellulose (section 6.11.4). Figure 7.5 shows the result of such an assay: the ATP is clearly not being cleaved by the TAP. Two separate batches of TAP purchased from BRL both showed a complete lack of activity in this assay system.

Since an active preparation of TAP did not appear to be available commercially, it was then attempted to prepare TAP from leaves of *Nicotiana tabacum* cv. Xanthi, according to the method of Efstratiadis *et al.* (1977). Preparations of TAP made in this way were active in cleaving ATP, but caused severe degradation of RNA samples, presumably due to contaminating ribonuclease. Inclusion of the RNase inhibitor, RNasin, at up to 750 units/ml in the TAP reaction was not sufficient to prevent degradation of the RNA.

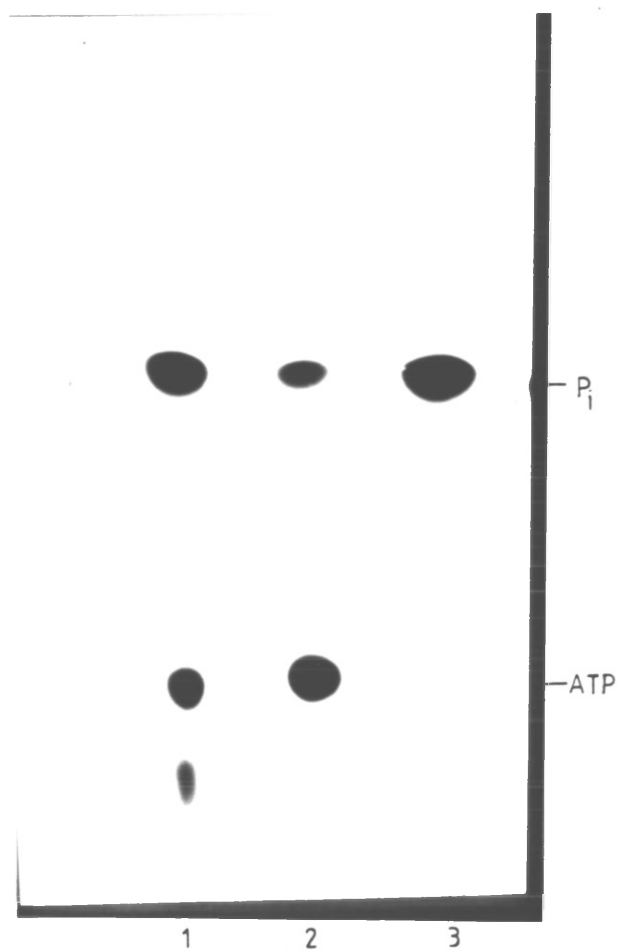


Figure 7.5 Assay for TAP activity. Autoradiogram of a PEI-cellulose chromatogram loaded with: lane 1, 0.5 μCi [γ - ^{32}P]ATP; lane 2, as lane 1 but digested with 0.05 unit TAP (BRL); lane 3, as lane 1 but digested with 0.02 unit CIP. The ATP spot was located by comparison with a spot of unlabelled ATP. Direction of chromatography is from bottom to top.

7.2.4 Iodination of viral RNA

Since the above experiments did not rule out the possibility of a genome-linked protein blocking the 5'-ends of the RCNMV RNAs, it was attempted to label such a protein with ^{125}I as described in section 6.11.5. These methods were described for the labelling of the genome-linked proteins of six nepoviruses (Mayo *et al.*, 1982). 50 μg RCNMV RNA was treated with 100 μCi ^{125}I using the Iodo-gen catalyst, and after gel filtration the peak of excluded material contained 2.4×10^5 c.p.m. of ^{125}I . When analysed by SDS-PAGE, an autoradiogram of this material showed two bands of labelled material. One band failed to enter the 20 % acrylamide resolving gel (as for intact RCNMV RNAs); the other band had a migration corresponding to a protein of 68,000 molecular weight. To ensure that any labelled proteins present were covalently attached to the RNA, half of the RNA from above was further purified by centrifugation through a cushion of 5.7 M CsCl. 1.2×10^4 c.p.m. of ^{125}I was recovered after this step. The resultant material was analysed by protease and nuclease digestions as described in section 6.11.6. These reactions were sufficient to digest to completion 5 μg cytochrome c, or 2 μg RCNMV RNA, respectively. When electrophoresed on an SDS-containing agarose gel (figure 7.6), two labelled bands which comigrated with the RCNMV viral RNAs were seen. This indicates that the ^{125}I was not removed from the RNA by pelleting through CsCl, and was presumably covalently attached to the RNA. Digestion with RNases removed all the labelled material from the gel (lane 3), confirming that the digestion conditions were sufficient to completely degrade the RNA. Digestion with proteases had no detectable effect on either the amount of ^{125}I in the bands, or on their migration. This shows that the ^{125}I was not attached to the RNA in a protease-

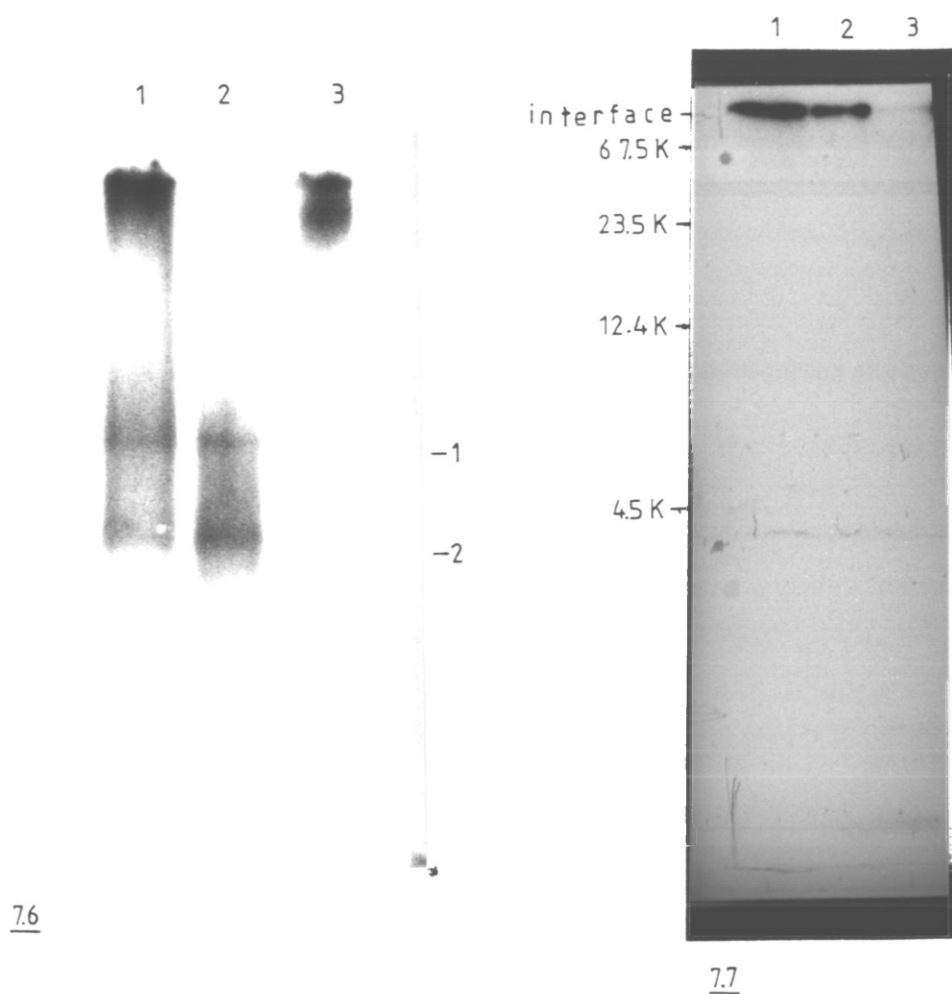


Figure 7.6 Electrophoresis of ^{125}I -labelled RNA on a 1 % agarose gel. Lane 1, untreated; lane 2, digested with proteases; lane 3, digested with RNases.

Figure 7.7 SDS-PAGE of ^{125}I -labelled RNA. Lane 1, untreated; lane 2, digested with proteases; lane 3, digested with RNases.

sensitive manner. When similar samples were analysed by SDS-PAGE (figure 7.7), the undigested material formed a single band of labelled material at the interface of the 3 % and 20 % polyacrylamide regions, which is where intact unlabelled RCNMV RNAs migrate. The 68,000 molecular weight band had been removed by centrifugation through CsCl, and so was not firmly attached to the RNA. The origin of this band is not clear, and it was not analysed further. Again, digestion with proteases had no effect on the intensity or mobility of the RNA band. Digestion with RNases completely removed the RNA band, and no smaller labelled bands were detected.

Using this gel system, proteins of apparent molecular weights of more than about 1000 should be resolved; the smallest unlabelled marker used (glucagon, molecular weight 4500) migrated about half-way down the gel. There are two possible explanations for these results. One is that RCNMV RNA lacks a genome-linked protein, and the incorporation of ^{125}I is due to a background labelling of the RNA itself; this effect has been observed for the RNAs of tobacco rattle virus and tomato bushy stunt virus when labelled with ^{125}I (Mayo *et al.*, 1982). The other explanation is that RCNMV RNA does possess a genome-linked protein, but the only residue (or residues) which can be labelled with ^{125}I remains covalently bound to the RNA after protease digestion (cf. strawberry latent ringspot virus RNA releases only half the covalently bound ^{125}I when treated with pronase (Mayo *et al.*, 1982)). However, this explanation also requires that the genome-linked protein migrates as a band of molecular weight much less than 1000 on a 20 % polyacrylamide gel, following RNase digestion, and thus escapes detection by the method described. This would contrast with the apparent molecular weights observed for the genome-linked proteins of nepoviruses (about 4000) and of picornaviruses and

comoviruses (4000-6000) (Mayo *et al.*, 1979; 1982). Also, it seems unlikely that a protein could migrate faster than the bromophenol blue/ion-front on an SDS-polyacrylamide gel, and no substantial amount of ^{125}I was detected comigrating with the bromophenol blue (figure 7.7). Hence the likeliest explanation is that RCNMV RNAs do not possess genome-linked proteins, at least not ones similar to those of nepoviruses and comoviruses.

7.2.5 Effect of protease digestion on the infectivity of RCNMV RNA

RCNMV RNA was inoculated onto cowpea leaves, with or without prior digestion with proteases (section 6.11.7). The total number of lesions which appeared on five leaves after inoculation with untreated RNA was 190; the total number of lesions which appeared on five leaves after inoculation with protease-treated RNA was 178. Hence infectivity of RCNMV RNA does not require any protease-sensitive structure.

7.3 CONSTRUCTION OF COMPLEMENTARY DNA CLONES

7.3.1 Synthesis of first strand of cDNA

DNA was synthesized on a viral RNA template using reverse transcriptase and random primers as described in section 6.8.6. The ratio of primers to template was optimised with respect to yield and length of cDNA by electrophoresis of the reaction products from a 10 μ l reaction on an alkaline agarose gel. 1 μ g RCNMV RNA was primed with 0.01, 0.1, 1, 10 and 20 μ g random primers. Autoradiography of the dried gel showed that 0.01-1 μ g primers produced no detectable cDNA, 10 μ g produced some low molecular weight cDNA, and 20 μ g produced a smear of cDNA up to $\approx$ 3 kb in length. When the amount of primers was tested at 10, 25, or 50 μ g per μ g template, cDNA was seen of roughly equal sizes for all three amounts of primers, with 25 μ g primers producing maximum yield of cDNA (figure 7.8). The smear of cDNA was not featureless, but showed two broad bands of approximately 1.0 and 2.5 kb, and a maximum length of 3.6 kb. Increasing the KCl concentration to 100 or 140 mM, and including RNasin at 600 units/ml, had a negligible effect on the length and yield of cDNA. The optimum conditions used are described in section 6.8.6. The yield of cDNA was calculated from the incorporation of [α - ^{32} P]dCTP into DNA (determined using DE-81 discs), assuming that the cDNA consisted of 25 % dC residues. Typically the yield of cDNA from a 100 μ l reaction (10 μ g template) was 1.0 μ g after the gel filtration step; some batches of reverse transcriptase yielded up to 3.4 μ g, with a maximum length up to 4 kb.

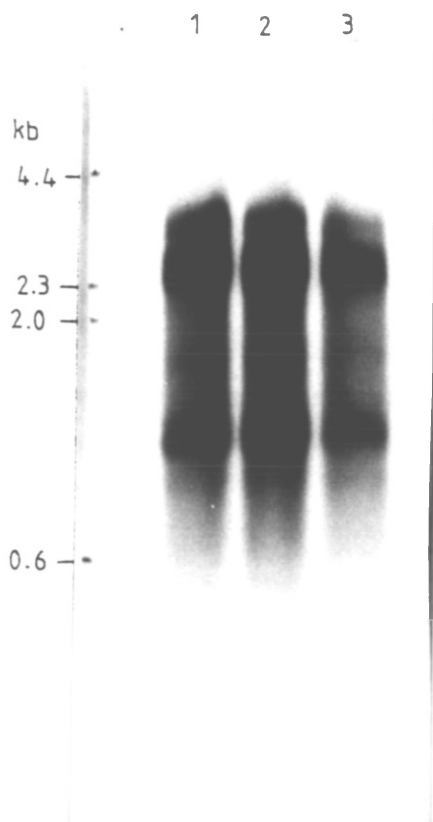


Figure 7.8 First strand cDNA synthesised from RCNMV RNA using random primers. Autoradiogram of the dried alkaline agarose gel. Lane 1, 10 μ g primers; lane 2, 25 μ g primers; lane 3, 50 μ g primers.

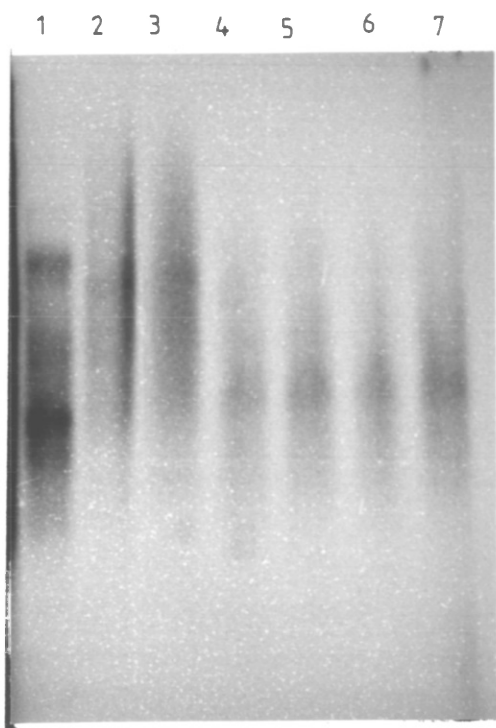


Figure 7.9 Optimisation of S1 digestion of double-stranded cDNA. Lane 1, first strand of cDNA; lane 2, cDNA after second strand reaction; lanes 3-7, 10 ng double-stranded cDNA digested with 0, 1, 2, 4 and 6 units S1 nuclease respectively.

7.3.2 Priming of cDNA synthesis by oligodeoxythymidylate

Three 20 μ l cDNA reactions were performed, with (i) 2 μ g RCNMV RNA, no primer; (ii) 2 μ g RCNMV RNA + 2 μ g oligo(dT)₁₀; (iii) 2 μ g tomato black ring virus (TBRV) RNA + 2 μ g oligo(dT)₁₀. The labelled cDNA products were analysed by alkaline agarose gel electrophoresis and autoradiography, and the incorporation of label into cDNA was determined for each.

(i) Incorporation was 0.9 %; a faint low molecular weight smear was seen on the autoradiogram.

(ii) Incorporation was 1.1 %; a faint smear was seen as for (i).

(iii) Incorporation was 24.7 %; three bands of approximately 1.4, 4.8 and 7.8 kb were seen on the autoradiogram. These presumably correspond to the three viral RNAs, which are polyadenylated at the 3'-ends (Mayo *et al.*, 1979) and hence primed by oligo(dT).

This experiment shows that RCNMV RNA does not possess an extensive poly(A) tract, and that no priming above background can be achieved using oligo(dT).

7.3.3 Synthesis of second strand of cDNA

Single-stranded cDNA was made double-stranded by self-priming as described in section 6.8.8. Both reverse transcriptase and the Klenow enzyme were used to ensure maximum length of the second strand. This procedure results in a hairpin loop at one end of the cDNA (corresponding to the 5'-end of the RNA), which must be cleaved by S1 nuclease before homopolymer tails or synthetic linkers can be added. The double-stranded cDNA migrates on a denaturing gel as a molecule of twice the length of the single-stranded cDNA from which it was made, and the optimum S1

concentration is defined as the minimum amount required to reduce the apparent size of the double-stranded cDNA to that of the single-stranded cDNA. Figure 7.9 shows the autoradiogram of an alkaline agarose gel of double-stranded cDNA digested with increasing concentrations of S1 nuclease: in this case 1 unit of S1 was optimal in cleaving the cDNA (10 ng), so in larger scale reactions 100 units S1 were used per μg of cDNA. In a typical experiment, 1 μg first strand cDNA input yielded 1.2 μg double-stranded cDNA; after digestion with 120 units S1 and further gel filtration, the final yield was 0.9 μg .

As an alternative method, double-stranded cDNA was made by the method of Gubler & Hoffman (1983; section 6.8.15), which is reported to give a higher yield of second strand, and to remove the need for treatment with S1 nuclease. In this method, the RNA/DNA hybrid from the first strand reaction is treated with RNase-H, which partially digests the RNA strand of the hybrid. The resultant RNA fragments, still attached to the DNA strand, can then act as primers for the synthesis of the second strand by DNA polymerase I. Typically, 1 μg hybrid (0.5 μg first strand cDNA) yielded 0.6 μg double-stranded cDNA.

7.3.4 Cloning of cDNA by homopolymer tailing

S1-treated double-stranded cDNA was tailed with dCTP, and *Pst*I-cut pUC8 was tailed with dGTP, using the first method in section 6.8.9. Aliquots of cDNA tailed for between 5 and 60 min. were annealed to dG-tailed pUC8 and used to transform JM101. All the time-points of cDNA tailing gave a similar yield of colonies: $\approx 55\%$ of colonies were white, and the yield was approximately 1.4×10^5 white colonies per μg cDNA input. 100 of the white colonies were picked for further analysis, clones TM1-100. Colony lifts of

these clones were probed with cDNA made to RCNMV RNA: none hybridised. Plasmid "mini-preps" were made from 11 of these clones and analysed by agarose gel electrophoresis. All 11 were identical in size, approximately 5.5 kb, cf. pUC8 is 2.66 kb. It is unclear how these plasmids arose, but none contained an RCNMV-specific insert.

Attempts were made to quantify the tailing reaction by measuring the incorporation of [α - ^{32} P]dCTP when *Pst*I-cut pUC8 was used as primer for the TdT reaction. When dCTP was used at a concentration of 100 μ M, a tail of 25 dC residues (i.e. the length suitable for cloning) was added in less than 2 minutes, so the precise tail length was very difficult to control. Hence in the second method of tailing described in section 6.8.9, the dCTP concentration was reduced to 10 μ M; this limits the tail length to about 20 dC residues. Double-stranded cDNA, made by the RNase-H/polymerase I method, was electrophoresed on a 1 % gel of low melting point agarose, and fractions of 0.4-1 kb and 1-4 kb were extracted. Aliquots of each size class were tailed in the presence of 10 μ M dCTP, annealed to dG-tailed pUC8, and used to transform JM101. 60 ng of the 0.4-1 kb cDNA produced a total of 18 white colonies (clones GC1-18); 40 ng of the 1-4 kb cDNA produced a total of 10 white colonies (clones GC19-28). Plasmids were isolated from all the white colonies, and dot-blots of the plasmids were probed with cDNA made from RCNMV RNA. None of the plasmids made from the 1-4 kb fraction hybridised, but 8 of those made from the 0.4-1 kb fraction did, indicating RCNMV-specific inserts (see figure 7.10). The inserts could be cut out of the plasmids using *Pst*I, and had sizes between 270 and 840 bp (figure 7.11). Note that clone GC4 was cut only once with *Pst*I, but the insert could be cut out using *Eco*RI and *Hind*III.



Figure 7.10 Dot-blot analysis of clones GC1-28. The dot-blot was probed with cDNA to RCNMV RNA. Top row GC19-23, second row GC24-28, third row GC1-5, fourth row GC6-9 and pUC8 control.

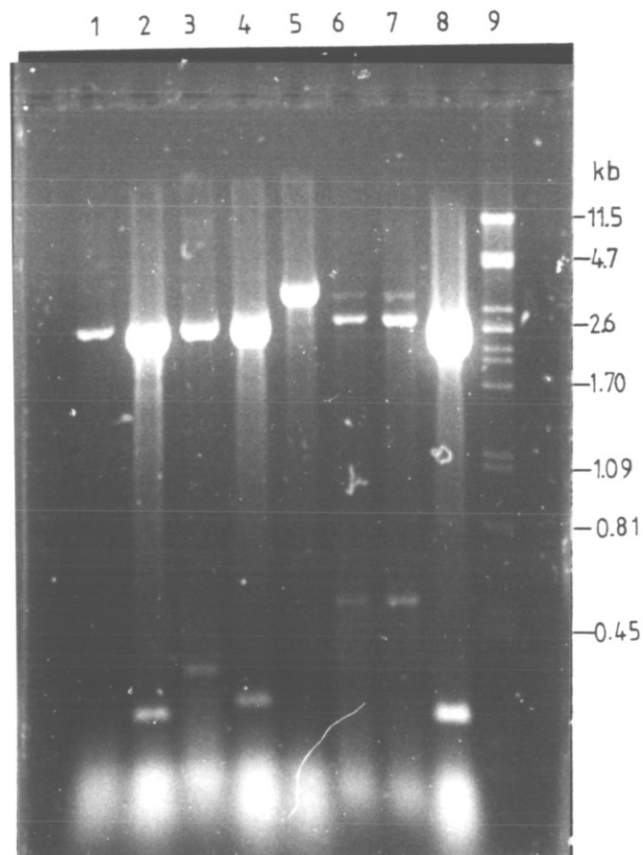


Figure 7.11 Analysis of clones GC1-7. Each plasmid was cut with *Pst*I and electrophoresed on a 1 % agarose gel. The higher molecular weight bands are overexposed in order to show the smaller insert bands. Lane 1, pUC8 cut with *Pst*I; lanes 2-8, clones GC1-7 cut with *Pst*I; lane 9, λ DNA cut with *Pst*I.

7.3.5 Cloning of cDNA by addition of synthetic linkers

Octameric linkers were added to S1-treated cDNA as described in section 6.8.12. When the phosphorylated linkers were self-ligated and analysed by PAGE and autoradiography, a ladder of linker oligomers was seen, indicating that the linkers were correctly phosphorylated and ligatable. The phosphorylated linkers were ligated onto cDNA, the excess linkers were digested off with *Sall*, and the resultant cDNA was ligated into *Sall*-cut pUC8. When this was used to transform JM101, 180 white colonies were produced from 50 ng of cDNA starting material. These clones, TM201-380, were analysed initially by probing colony lifts with cDNA to RCNMV RNA. Several of the colonies gave faint positive signals. The plasmids were extracted from 30 of these colonies and further analysed by dot-blotting and Southern blotting: none hybridised even slightly to RCNMV cDNA under these conditions, indicating a lack of RCNMV-specific inserts. When analysed by digestion with *TaqI*, 6 of these plasmids were shown to have deletions of between 20 and 500 bp, relative to the pUC8 vector.

7.3.6 Cloning of restriction fragments of cDNA

Double-stranded cDNA, made by self-priming but not treated with S1 nuclease, was cut with a range of restriction enzymes, and the resulting fragments were ligated into pUC8 which had been cut with the appropriate enzyme, and treated with CIP to prevent recircularisation (6.8.11 and 6.8.14). Note that *Sau3AI* fragments will ligate into the *BamHI* site of the vector to regenerate *Sau3AI* sites; similarly *TaqI* fragments will ligate into the *AccI* site of the vector to regenerate *TaqI* sites; only one in four of the possible *AccI* cleavage sites generates ends which are

compatible with the *AccI* site of the vector. The number of white colonies generated from cDNA cut with the various enzymes was:

AccI: 44 from 40 ng cDNA: clones AC1, AC2 etc.

BamHI: 5 from 20 ng cDNA: clones BM1-5.

PstI: none.

Sall: none.

Sau3AI: 71 from 40 ng cDNA: clones SU1, SU2 etc.

TaqI: 170 from 40 ng cDNA: clones TQ1, TQ2 etc.

Colony lifts, probed with RCNMV cDNA, showed that more than half of the white colonies hybridised; dot-blot of the plasmid DNAs gave the same result, but were in general clearer and with less nonspecific background than the colony lifts. When analysed by agarose gel electrophoresis, the plasmids migrated somewhat slower than the vector, indicating small inserts in the range 40 to 550 bp (see figure 7.12). The size of the smaller inserts was estimated more accurately by digesting the plasmid with *TaqI*, which has 3 sites in the vector DNA, end-labelling the fragments with [α - ^{32} P]dCTP as described in section 6.1.7, electrophoresing them on a 5 % polyacrylamide gel, and detecting the labelled bands by autoradiography. Examples of such autoradiograms are shown in figure 7.13. The insert sizes which were determined are listed in table 7.1.

7.3.7 Cloning of blunt-ended cDNA

In an attempt to generate larger inserts than obtained from cloning restriction fragments of cDNA, double-stranded cDNA made by the RNase-H/polymerase I method was made blunt-ended by digestion with the single-strand-specific mung bean nuclease. The resulting DNA was ligated into *HincII*-cut pUC8 (which also has blunt ends) and used to transform JM101.

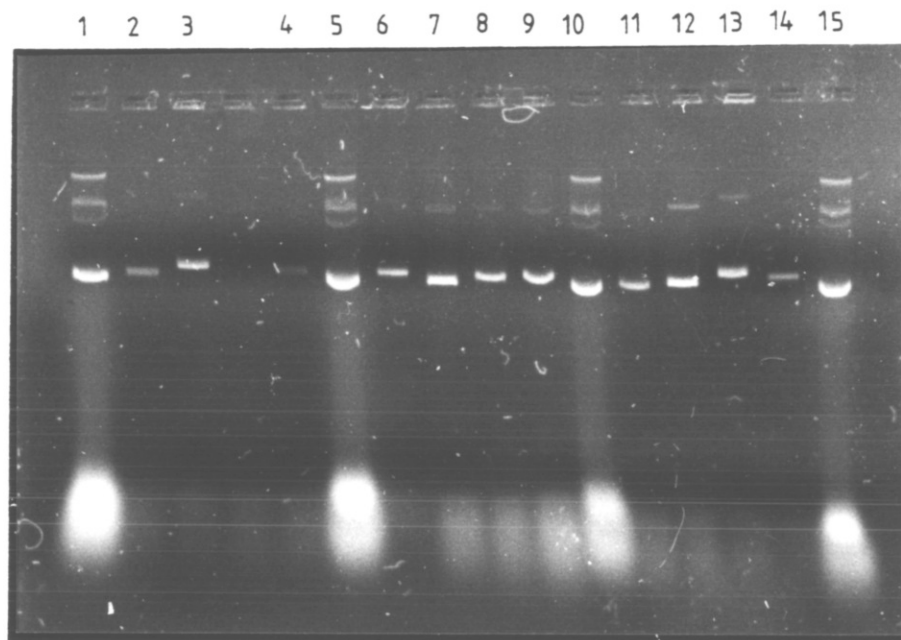


Figure 7.12 Agarose gel electrophoresis of cloned plasmids. Lanes 1, 5, 10, and 15, pUC8; lane 2, TQ5; lane 3, TQ7; lane 4, TQ15; lane 6, AC7; lane 7, AC10; lane 8, AC15; lane 9, SU11; lane 11, BM1; lane 12, BM2; lane 13, BM3; lane 14, BM4.

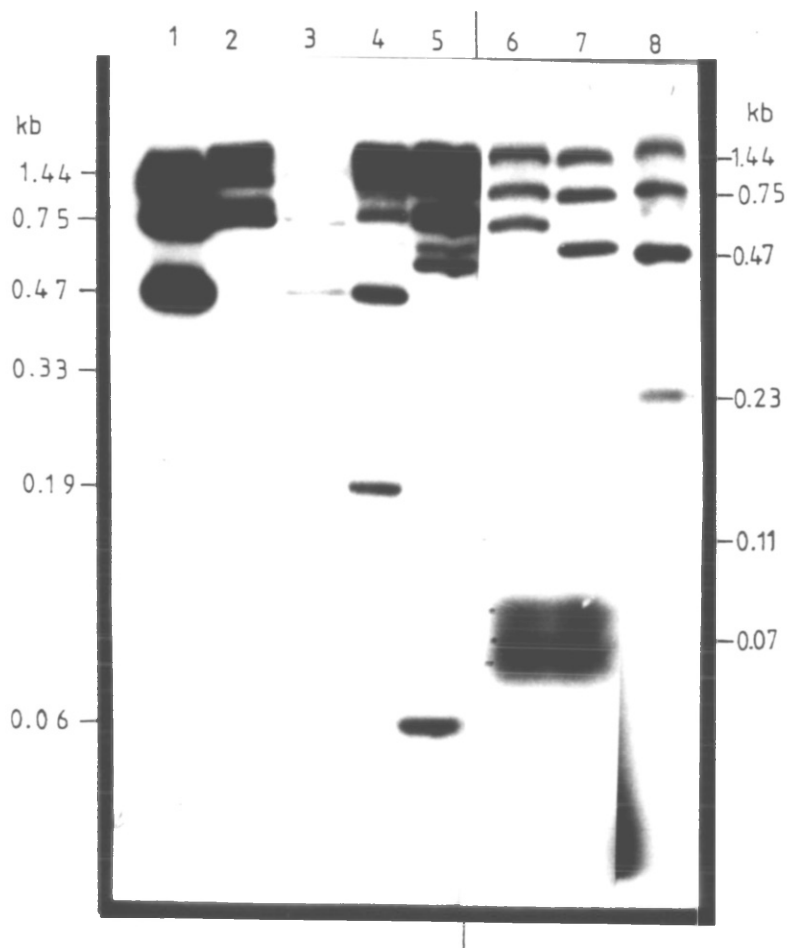


Figure 7.13 PAGE of end-labelled restriction fragments. *TaqI* digests of: lane 1, pUC8; lane 2, TQ7; lane 3, TQ15; lane 4, AC7; lane 5, AC15; lane 6, BM3; lane 7, BM4; lane 8, TQ5.

Only 5 white colonies were obtained from 10 ng cDNA; of these only two, clones BL1 and BL3, hybridised to RCNMV cDNA in a dot-blot analysis. Both had inserts of less than 300 bp, as judged from agarose gel electrophoresis of the plasmids.

7.3.8 Further analysis of cDNA clones

Seven aliquots of 0.5 µg RCNMV viral RNA were electrophoresed on a formaldehyde-agarose gel and transferred to GeneScreen Plus. The membrane was cut into seven strips, and six of these were hybridised with probes made from six cloned plasmids; the seventh was probed with RCNMV cDNA to locate the bands of RNA-1 and RNA-2. An autoradiogram of the resulting blots is shown in figure 7.14. Clearly clones BM2, BM3, TQ5 and TQ7 are specific for RNA-1; GC4 and GC9 are specific for RNA-2. The smear of material seen below RNA-2 in the GC9-probed track presumably consists of degradation products of RNA-2. A faint band at about 1.5 kb (i.e. approximately the position of RNA-2) is seen in the tracks probed with BM2 and TQ5: the significance of this band is discussed later. Larger numbers of clones were screened by probing dot-blot of plasmid DNAs with cDNA made to gel-purified RNA-1 or RNA-2 (figure 7.15). This method was not as clear-cut as probing a Northern blot with a cloned probe, but it was possible to class most of the clones as RNA-1-specific or RNA-2-specific by this method. In a similar procedure, clones were cross-hybridised with each other by probing a dot-blot of plasmid DNAs with a probe made from the gel-purified insert of one of the clones (the whole plasmid could not be used, as all the clones contain the pUC8 sequences). The properties of the best characterised clones are listed in table 7.1.

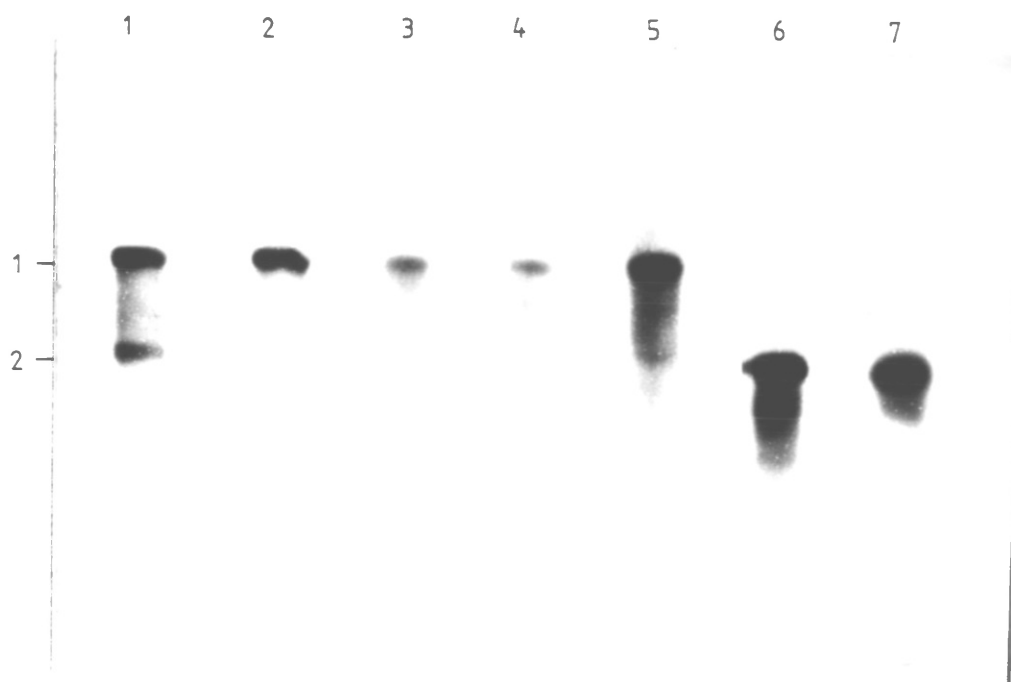


Figure 7.14 RCNMV viral RNA hybridised with cloned probes. Each track represents 0.5 μ g RNA and after agarose gel electrophoresis and blotting was probed with: lane 1, cDNA to RCNMV; lane 2, BM3; lane 3, BM2; lane 4, TQ7; lane 5, TQ5; lane 6, GC9; lane 7, GC4.

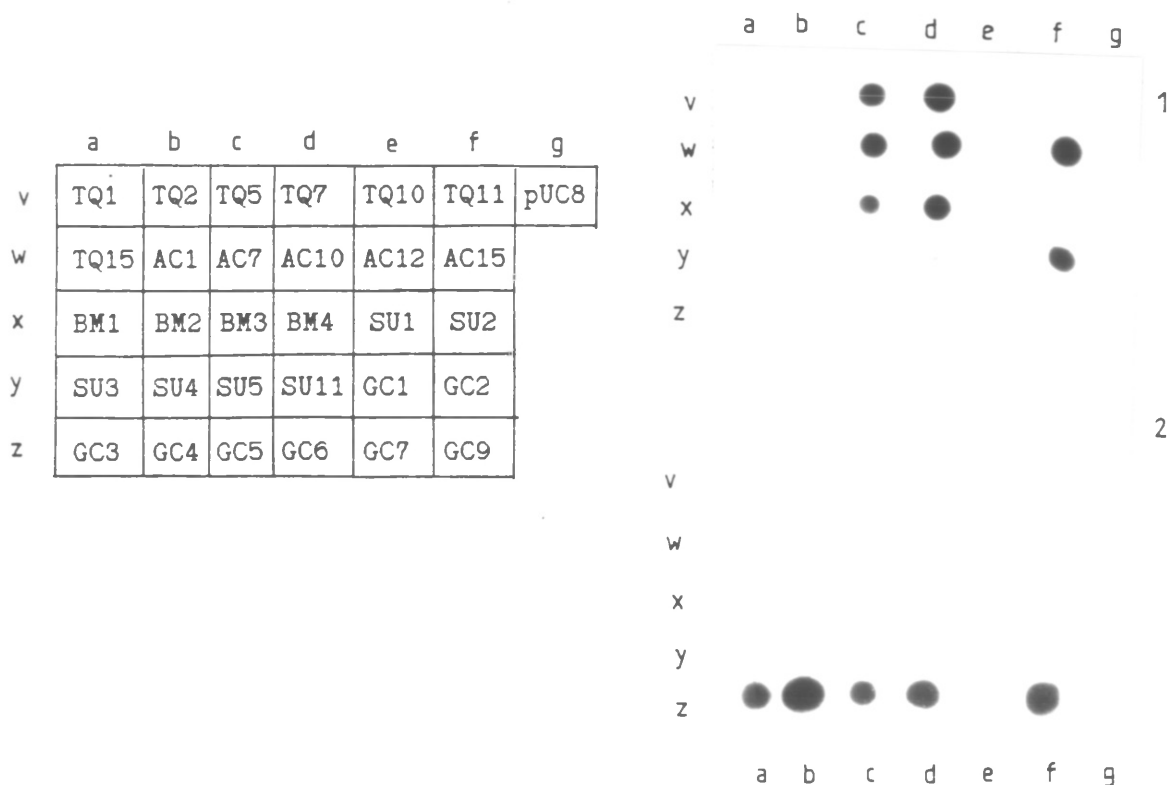


Figure 7.15 Dot-blot of cloned plasmids probed with cDNA to separated RNAs. Blot 1, cDNA to RNA-1; blot 2, cDNA to RNA-2.

Table 7.1 Properties of some cDNA clones.

Sizes of RCNMV-specific inserts are listed for each clone.

RNA-1 clones:

TQ5	235 bp (a, f)	AC10	115 bp (b, c, f)
TQ7	520 bp (a)	AC15	160 bp (b, c, f)
TQ10	40 bp (b, f)	BM1	135 bp (b)
TQ11	75 bp (b, f)	BM2	330 bp (a, d)
TQ23	≈100 bp (b, c)	BM3	520 bp (a, c, f)
TQ30	≈150 bp (b, c)	BM4	250 bp (b, c, f)
TQ34	≈200 bp (b, c)	GC1	280 bp (a)
TQ35	≈100 bp (b, c)	GC2	370 bp (b)
SU3	190 bp (b)	GC7	260 bp (b)
SU4	≈200 bp (b)	BL1	≈290 bp (b, d)
SU11	200 bp (b)	BL3	≈290 bp (b)
AC7	245 bp (b, c, f)		

RNA-2 clones:

SU5	260 bp (a)	GC5	575 bp (b, e)
GC3	310 bp (b, e)	GC6	565 bp (b, e)
GC4	840 bp (a, e)	GC9	355 bp (a, e)

Notes:

- (a) Northern blot of RCNMV RNA was probed with this clone
- (b) Clone hybridised to cDNA made from separated RNA
- (c) Clone hybridised to insert of BM3
- (d) Clone hybridised to insert of BM2
- (e) Clone hybridised to insert of GC9
- (f) Insert size determined by digestion with *TaqI* and end-labelling

7.4 ANALYSIS OF RNA FROM PLANT TISSUE INFECTED WITH RCNMV

7.4.1 Total single-stranded RNA from plant tissue

Single-stranded RNA was extracted from *P. vulgaris* tissue as described in section 6.5.1. The yield was typically 550 µg per g of tissue, estimated spectrophotometrically. When analysed by electrophoresis on formaldehyde-agarose gels followed by staining with ethidium bromide, a range of bands was seen, presumably mostly host rRNA (Leaver, 1979). No difference was detected between the ssRNA from healthy and RCNMV-infected tissue: in particular no band of RNA-1 was seen in the infected track (figure 7.16). When a similar gel was transferred to GeneScreen and hybridised to cDNA from viral RNA, two strongly hybridising bands corresponding in size to RCNMV viral RNAs were detected in the infected tissue extract (figure 7.17). There was no hybridisation detected to the healthy tissue extract, even on prolonged exposure to X-ray film, indicating that (i) the healthy tissue contained no RCNMV-specific RNA and (ii) the viral RNA from which the probe was made contained no host-specific RNA, i.e. RCNMV does not encapsidate any host RNA. The band of RNA-2 seen in the RNA from infected tissue was somewhat less intense relative to RNA-1 than its counterpart in the viral RNA; this effect was also produced by mixing viral RNA with RNA from healthy tissue before electrophoresis and transfer, and was presumably an effect of the large amount of host rRNA present at that position on the gel (figure 7.17). Faint bands could be seen below RNA-1, at about 3 kb, in both viral RNA and infected tissue RNA: these bands were not always detected in similar experiments, for example figure 7.17 lanes 5 and 6. No major ssRNA species were detected in infected tissue other than the two genomic RNAs.

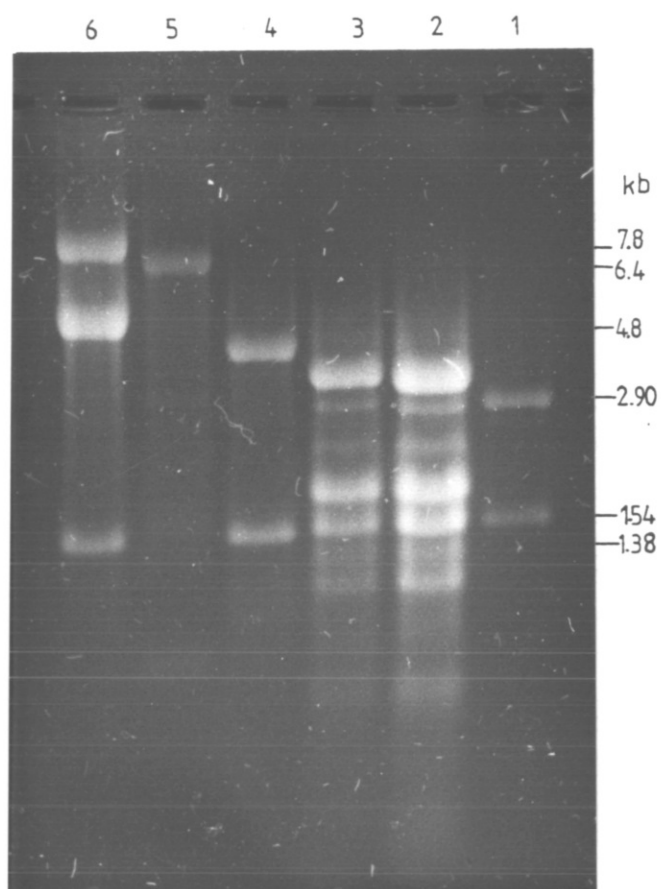


Figure 7.16 Gel electrophoresis of plant ssRNA extracts. The RNA was electrophoresed on a formaldehyde-agarose gel and stained with ethidium bromide. Lane 1, *E. coli* RNA; lane 2, 4 µg ssRNA from healthy tissue; lane 3, 4 µg ssRNA from infected tissue; lane 4, RCNMV viral RNA; lane 5, TMV RNA; lane 6, TBRV RNA.

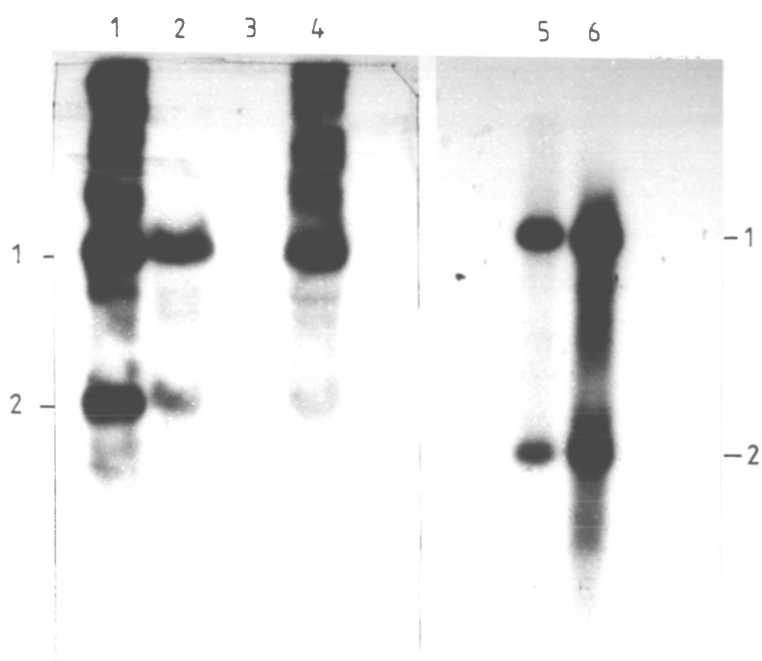


Figure 7.17 Northern blot analysis of plant ssRNA extracts. The RNA was electrophoresed on a formaldehyde-agarose gel and transferred to GeneScreen, which was probed with RCNMV cDNA and autoradiographed. Lane 1, 1 μ g RCNMV RNA; lane 2, 4 μ g RNA from infected tissue; lane 3, 4 μ g RNA from healthy tissue; lane 4, 4 μ g RNA from healthy tissue + 1 μ g RCNMV RNA; lanes 5 and 6 are from a separate experiment: lane 5, 0.1 μ g RCNMV RNA; lane 6, 4 μ g RNA from infected tissue.

The plant ssRNA extracts were also probed with cloned probes. Clone GC9 hybridised to RNA-2, plus a smear of smaller material; clones BM3 and BM2 hybridised to RNA-1, again plus a smear of smaller material (figure 7.18). The banding pattern seen in the BM3-probed track is probably an electrophoretic artifact: the areas which hybridise less to the probe appear to correspond to the strong non-hybridising host RNAs. Infected plant RNA was also probed with cDNA made to gel-purified RNA-1: again only RNA-1 was detected (figure 7.18, lane 4).

The RNA extracted from tissue at 2, 3, 4, and 7 days after infection was compared by electrophoresis followed by staining or by Northern blotting and probing with cDNA to RNA-1: no differences were detected in any of the samples from the results described above.

7.4.2 Polysomal RNA from plant tissue

RNA was extracted from intact polyribosomes as described in section 6.5.2. No attempt was made to exclude virus particles from the preparation, so the RNA produced resembled the total RNA fraction described above except that the polysomal fraction should be enriched for RNA species being translated. The yield of polysomal RNA was 100-150 μ g per g tissue. When electrophoresed on a formaldehyde-agarose gel and stained with ethidium bromide, the pattern of bands was the same as that shown in figure 7.16. When a gel of this material was Northern blotted and probed with cDNA to RNA-1, only the RNA-1 band plus a smear of smaller material was detected, as described for the ssRNA extract above. No additional bands were detected to those described above.

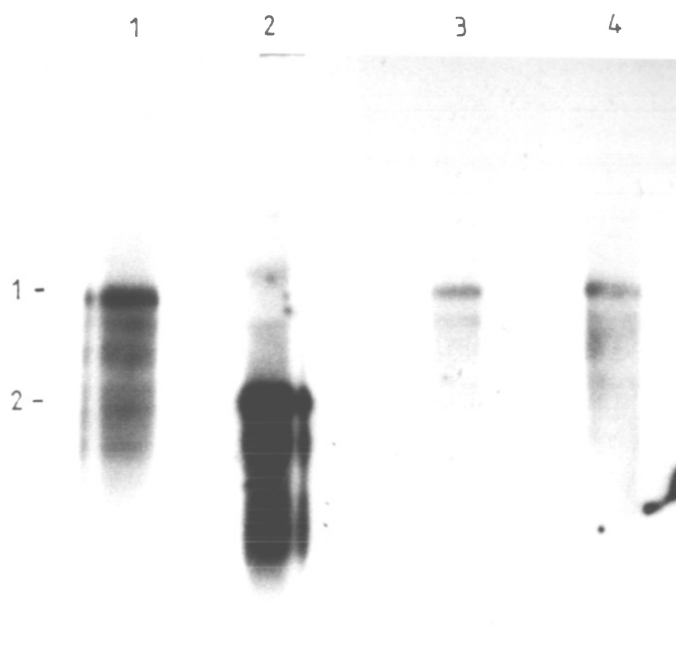


Figure 7.18 Total plant RNA hybridised with cloned probes. Each track contained 4 μ g RNA from infected tissue; after electrophoresis on a formaldehyde-agarose gel, the RNA was transferred to GeneScreen Plus and hybridised to various probes. Lane 1, BM3 probe; lane 2, GC9 probe; lane 3, BM2 probe; lane 4, cDNA probe made to RNA-1.

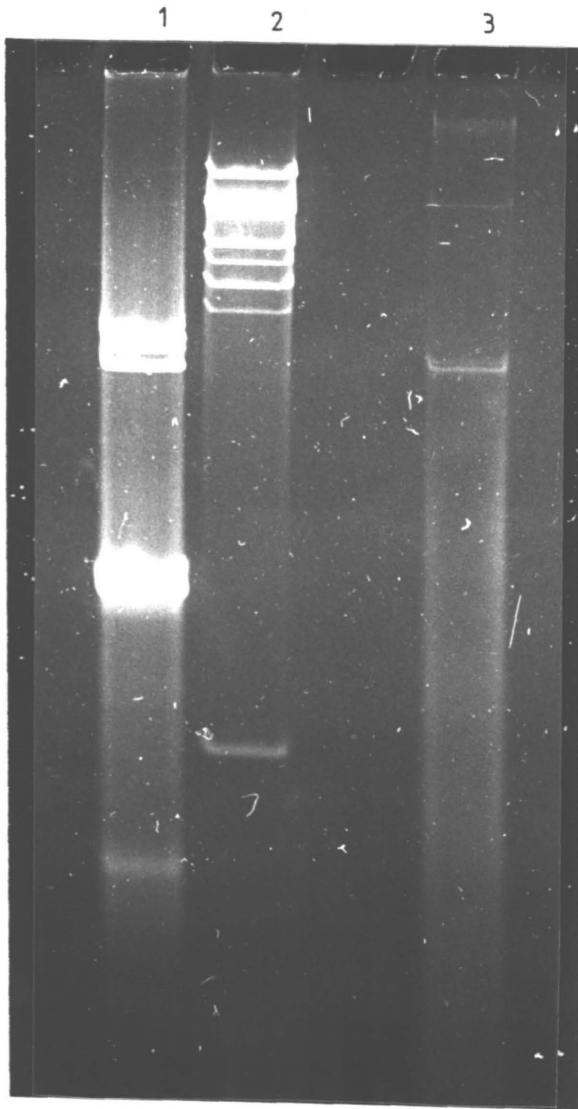
7.5 DOUBLE-STRANDED RNA FROM TISSUE INFECTED WITH RCNMV

7.5.1 Gel electrophoresis of dsRNA

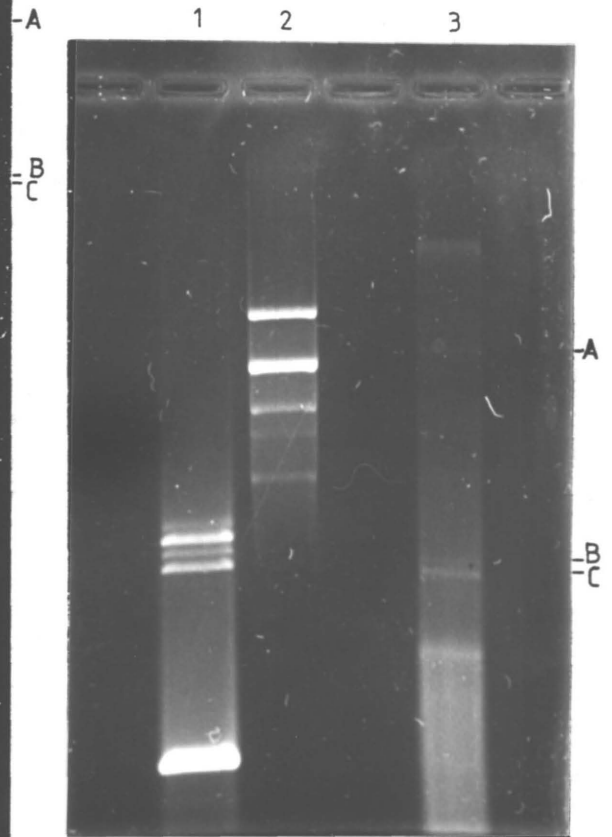
Double-stranded RNA was isolated from *P. vulgaris* tissue infected with RCNMV by chromatography of the LiCl-soluble nucleic acids on CF-11, as described in section 6.5.3. When the nucleic acids eluted from the CF-11 column were examined by PAGE, a large amount of single-stranded RNA was seen, presumably mostly host rRNA. Most of this ssRNA was removed by a further precipitation from 2 M LiCl; the LiCl-soluble fraction was electrophoresed on a 4 % polyacrylamide gel (figure 7.19). Lane 3 represents the total dsRNA fraction from 3.6 g plant material. The uppermost, smeary band was judged to be DNA from its migration on the gel, and the low molecular weight smear probably consisted of partially degraded ssRNA. The three sharp bands of dsRNA, here termed ds-A, ds-B and ds-C, were estimated to have sizes of 4.35, 1.32 and 1.30 kb respectively, from plots of log (molecular weight) against mobility, relative to the mycoviral dsRNA size markers (sizes listed in Appendix II). Ds-A and ds-B were of approximately equal intensities; ds-C was the most intense of the three bands.

The dsRNA was also electrophoresed on a 1 % agarose gel (figure 7.20). The pattern seen here was similar to that produced by PAGE; the blurry band seen superimposed on the smear of ssRNA corresponded to a ssRNA of ≈ 1.5 kb. The dsRNA bands were estimated to have sizes of 4.53, 1.32 and 1.26 kb from this gel.

No similar sharp dsRNA bands were detected in extracts from healthy plant tissue.



7.19



7.20

Figure 7.19 PAGE of dsRNA from RCNMV-infected tissue. Lane 1, dsRNA from *Penicillium stoloniferum* viruses (PsV); lane 2, dsRNA from *Aspergillus foetidus* viruses (AfV); lane 3, dsRNA from 3.6 g RCNMV-infected tissue.

Figure 7.20 Agarose gel electrophoresis of dsRNA. Lane 1, dsRNA from PsV; lane 2, dsRNA from AfV; lane 3, dsRNA from 9 g RCNMV-infected tissue.

7.5.2 Hybridisation analysis of dsRNA

The dsRNA fraction from infected and healthy tissue was electrophoresed on a 1 % agarose gel and transferred to GeneScreen, then hybridised with cDNA to RCNMV viral RNA. The resultant autoradiogram (figure 7.21) shows a large band of ≈ 4.2 kb and a stronger band of ≈ 1.4 kb, corresponding to the bands ds-A and ds-B/C seen by staining with ethidium bromide. The bands were not sensitive to DNase-I. No hybridisation was detected with material from uninfected plants, confirming the viral origin of the dsRNAs. Bands ds-B and ds-C were not resolved by this system.

The dsRNA was also analysed by electrophoresis under denaturing conditions on a formaldehyde-agarose gel, followed by transfer to GeneScreen Plus and hybridisation with cDNA to RCNMV (figure 7.22). Bands comigrating with viral RNA-1 and -2 were seen, plus a smear of lower molecular weight material. Digestion of the dsRNA with 25 ng RNase-A in 2 $\times$ SSC prior to electrophoresis had no effect on the bands, whereas a similar digestion of 10 ng viral RNA greatly reduced the intensity of the bands seen on the autoradiogram, further indicating the double-stranded nature of the dsRNA. From this it appears that ds-A is denatured to a band which comigrates with viral RNA-1, and ds-B and -C are denatured to bands which comigrate with viral RNA-2 (the two were not resolved).

7.5.3 Hybridisation analysis of separated dsRNA bands

Since the dsRNA bands ds-B and ds-C were so close in size, it was not possible to resolve them using the blotting systems described above. To overcome this problem, RCNMV dsRNAs were first electrophoresed on a non-denaturing 1 % agarose gel. After brief staining with ethidium bromide, the three dsRNA bands were excised from the gel, the agarose slices were

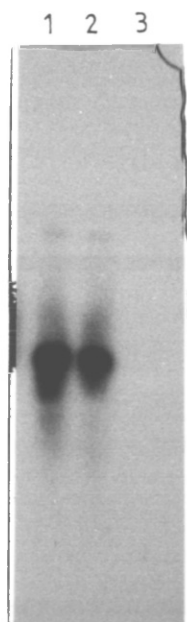


Figure 7.21 Hybridisation analysis of dsRNA electrophoresed under nondenaturing conditions. Lane 1, dsRNA from infected tissue; lane 2, dsRNA from infected tissue, treated with DNase-I before electrophoresis; lane 3, dsRNA from healthy tissue.

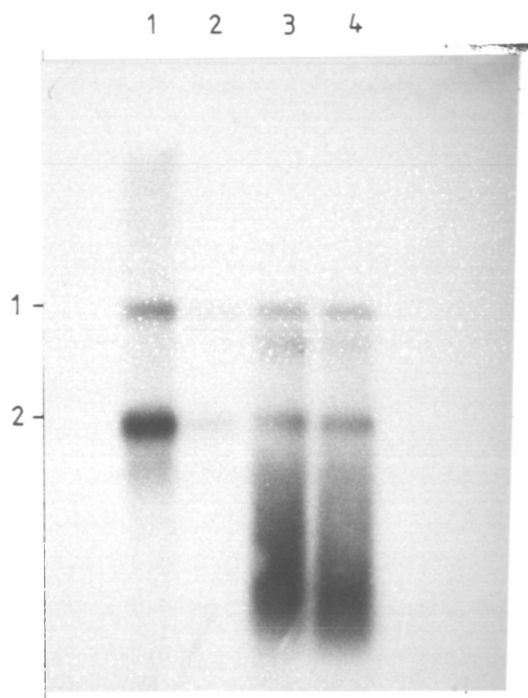


Figure 7.22 Northern blot analysis of dsRNA. RNA was electrophoresed under denaturing conditions. Lane 1, viral RNA; lane 2, viral RNA treated with RNase-A; lane 3, dsRNA from infected tissue; lane 4, dsRNA from infected tissue treated with RNase-A.

positioned 1-2 cm apart, and a new gel of 1 % agarose was cast around them. This new gel was then treated with alkali and the RNA transferred to GeneScreen as for other nondenaturing dsRNA gels (section 6.7.9). The blot was then hybridised to probes specific for one or other of the viral RNAs (figure 7.23). When probed with either the RNA-1-specific clone BM3, or with cDNA to gel-purified RNA-1, all three dsRNA bands hybridised. However, when probed with the RNA-2-specific clone GC4, only ds-C hybridised. Since viral RNA-1 migrates between ds-A and ds-B on an agarose gel, it is possible that both ds-B and ds-C could be contaminated with fragments of RNA-1 which being single-stranded would transfer and hybridise more efficiently than the dsRNA bands. Viral RNA-2 migrates faster than ds-C on an agarose gel, so fragments of RNA-2 could not contaminate the dsRNA bands. These observations suggest that ds-A is the double-stranded form of RNA-1, ds-C is the double-stranded form of RNA-2, and ds-B is derived from RNA-1.

An attempt was made to discriminate against viral-sense ssRNA by making a probe specific for the (-)strand of the dsRNA. Unlabelled cDNA was made from RCNMV RNA, and after removal of the RNA with alkali labelled cDNA was made using the unlabelled cDNA as template. However, this probe still hybridised to the viral (+)strand RNAs, so no further information could be gained in this manner.

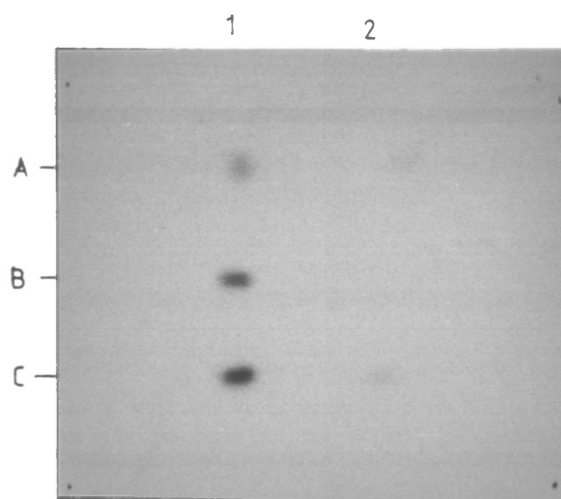


Figure 7.23 Hybridisation analysis of separated dsRNAs. Autoradiograms of the blot after probing with: lane 1, cDNA to RNA-1; lane 2, clone GC4.

7.6 *IN VITRO* TRANSLATION OF RCNMV RNA.

7.6.1 *In vitro* translation of RCNMV viral RNA

RCNMV viral RNA was translated in the rabbit reticulocyte lysate system, at a concentration of 50 µg/ml RNA (section 6.9.1). The translation products were separated by SDS-PAGE and visualised by fluorography. Figure 7.24 shows a comparison of using [³⁵S]methionine or [³H]leucine as the labelled amino acid. In each case, tobacco mosaic virus RNA was also translated as a control to ensure the activity of the reticulocyte lysate. Both radioisotopes gave a similar pattern of three major bands, two fainter higher molecular weight bands, and a range of less well defined lower molecular weight bands. The molecular weights of the bands seen on the autoradiogram were determined by comparison of the X-ray film with the dried gel, on which the unlabelled molecular weight markers (stained with Coomassie Blue) could be seen. Molecular weights were then calculated from a plot of log (molecular weight) against mobility. The molecular weights of the three major bands were calculated to be 40,000, 38,000 and 34,000 (here named p40, p38 and p34); the higher molecular weight bands were 56,000 and 54,000 (p56/54). The band of 51,000 molecular weight, seen only in the ³⁵S-labelled samples, is an artifact caused by messenger-independent labelling, and could also be seen in translations which had no RNA input (e.g. figure 7.26, lane 8). The lower molecular weight bands varied in amounts and sizes from one translation to the next, and are presumed to result from premature termination or nonspecific initiation of translation. Unlabelled RCNMV coat protein was visualised by staining with Coomassie Blue, and had a calculated molecular weight of 40,000 (figure 7.25): it comigrated with p40.

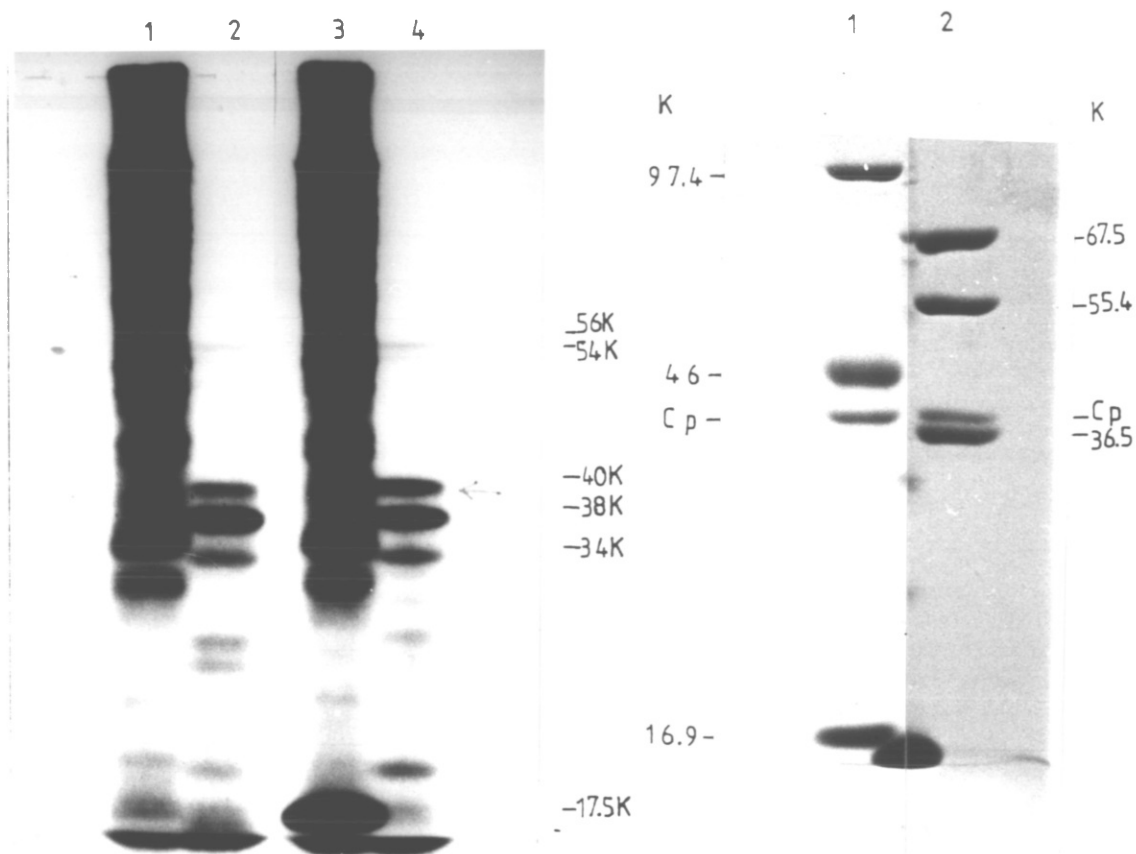


Figure 7.24 SDS-PAGE of *in vitro* translation products of RCNMV viral RNA. Lanes 1 and 2, labelled with ^{35}S ; lanes 3 and 4, labelled with ^3H . Lanes 1 and 3, 50 $\mu\text{g/ml}$ TMV RNA; lanes 2 and 4, 50 $\mu\text{g/ml}$ RCNMV RNA.

Figure 7.25 SDS-PAGE of RCNMV coat protein. Molecular weight values are given in brackets for each size marker. Lane 1, phosphorylase b (97,400), ovalbumin (46,000), RCNMV coat protein, and myoglobin (16,900); lane 2, bovine serum albumin (67,500), glutamate dehydrogenase (55,400), RCNMV coat protein, lactate dehydrogenase (36,500).

The RCNMV translation products labelled with ^{35}S or ^3H are of approximately equal intensities as seen in figure 7.24; however the ^{35}S -labelled tracks were exposed to X-ray film for 1 day, and the ^3H -labelled tracks for 8 days, to produce these images, so in all future experiments [^{35}S]methionine was used as the labelled amino acid.

The amount of incorporation of label into protein was determined by precipitation with TCA: depending on the freshness of the batch of lysate used, 50 $\mu\text{g/ml}$ RCNMV RNA produced between 18,000 and 80,000 TCA-precipitable c.p.m. / μl in a 1 hr translation (background 500 - 1000 c.p.m. / μl).

Treatment of the translation mix with RNase-A (1 μg RNase-A, incubated at 37°C for 15 min.) after translation had no effect on any of the protein bands, showing that none were due to aminoacyl-tRNA complexes. A time-course of translation was performed by taking 5 μl aliquots from a translation mix containing 50 $\mu\text{g/ml}$ RCNMV RNA at various times and analysing each aliquot by SDS-PAGE and fluorography (figure 7.26, lanes 3 to 6). After 4 min. at 30°C, no labelled products could be detected; after 8 min. p40, p38 and p34 could all be detected; and after 15 min. all the products seen in figure 7.24 could be detected. Hence there was no evidence for high molecular weight precursor proteins being formed; p54/56 (not resolved from each other on this fluorogram) was not detectable until 15 min. after starting translation, and its intensity increased slightly after that.

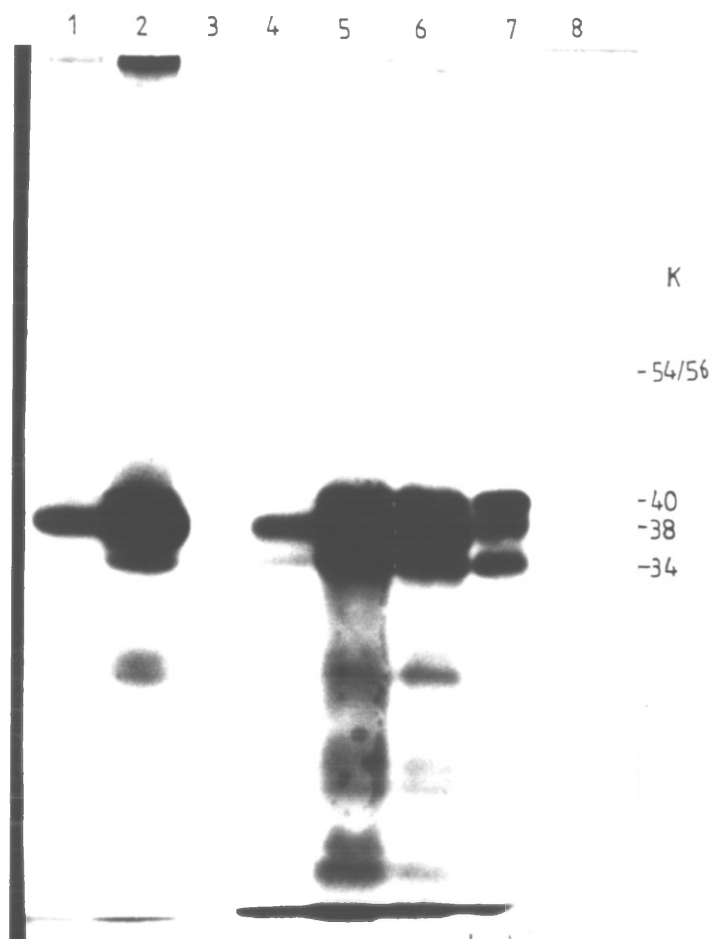


Figure 7.26 Effect of various parameters on translation of RCNMV RNA, analysed by SDS-PAGE. Lanes 1 and 2, translation of RNA from CsCl-⁻ purified virus at 5 μ g/ml and 30 μ g/ml respectively; lanes 3 to 6, translation of 50 μ g/ml RNA stopped after 4, 8, 15, and 60 min. respectively; lane 7, translation of RNA at 1 mg/ml; lane 8, no added RNA.

7.6.2 Effect of RNA concentration on translation of RCNMV RNA

Increasing the concentration of RCNMV RNA in the translation reaction 20-fold, i.e. to 1 mg/ml, altered the relative intensities of the major translation products (figure 7.26, lane 7). The intensities of the bands were determined by excising them from the dried gel after alignment with the fluorogram, adding each to scintillant and counting in the scintillation counter. The intensity of each band was expressed as a fraction of the total c.p.m. for the four bands, to allow for differences in the total amounts loaded onto the gel:

Protein	50 µg/ml RNA	1 mg/ml RNA
p54/56	0.02	0.03
p40	0.23	0.38
p38	0.57	0.29
p34	0.18	0.30

Hence increasing the concentration of RNA 20-fold enhanced the formation of p54/56, p40 and p34 by 60-70 %, and reduced formation of p38 to about 50 % of its previous level. This shows that under certain conditions p40 can be the most abundant translation product, and implies that the relative amounts of the translation products observed *in vitro* do not necessarily reflect the relative amounts produced *in vivo*.

To check that this p40 message was genuinely encapsidated, and not just loosely attached to the virus, a sample of virus was further purified by banding it on a CsCl gradient (section 6.4.4). When the RNA extracted from this virus was translated, protein p40 was still produced (figure 7.26, lanes 1 and 2) indicating that the message was indeed encapsidated.

7.6.3 Effect of cap analogue on the translation of RCNMV RNA

The cap analogue m⁷GMP has been reported to inhibit the translation of capped viral RNA (Joshi *et al.*, 1983). When TMV RNA was translated in the presence of 4 mM m⁷GMP, translation of the full-length 126,000 molecular weight protein was inhibited relative to the smaller partial-length products (figure 7.27, lanes 1 to 3). The 126,000 molecular weight protein is known to be translated from the capped TMV viral RNA. When RCNMV RNA at 10-50 µg/ml was translated in the presence of 4 mM m⁷GMP, no inhibition was observed, similarly with 10 µg/ml RNA and 8 mM m⁷GMP. However, when RCNMV RNA at 2 µg/ml was translated in the presence of 8 mM m⁷GMP, all the translation products were reduced in amount (figure 7.27, lanes 4 to 7). Hence the translation of RCNMV RNA is inhibited by the cap analogue to a much smaller extent than is the translation of TMV RNA.

7.6.4 In vitro translation of separated RNA species

The bands of RCNMV viral RNA were extracted from gels of low melting point agarose as described in section 6.6.4, and translated. The fluorogram in figure 7.28 shows that RNA-1 is translated mainly to p38, with a smaller amount of p54/56 (the two were not resolved). No p34 or p40 were produced. RNA-2 was translated to p40 and p34 in roughly equal amounts, as seen when total viral RNA was translated, plus a smaller amount of p38. No p54/56 was produced. Lane 3 shows the translation products of the RNA extracted from the region of gel below RNA-2, where a blurred band of RNA could sometimes be detected; no major proteins were produced, implying that this band probably consisted of breakdown products of the larger RNA bands.

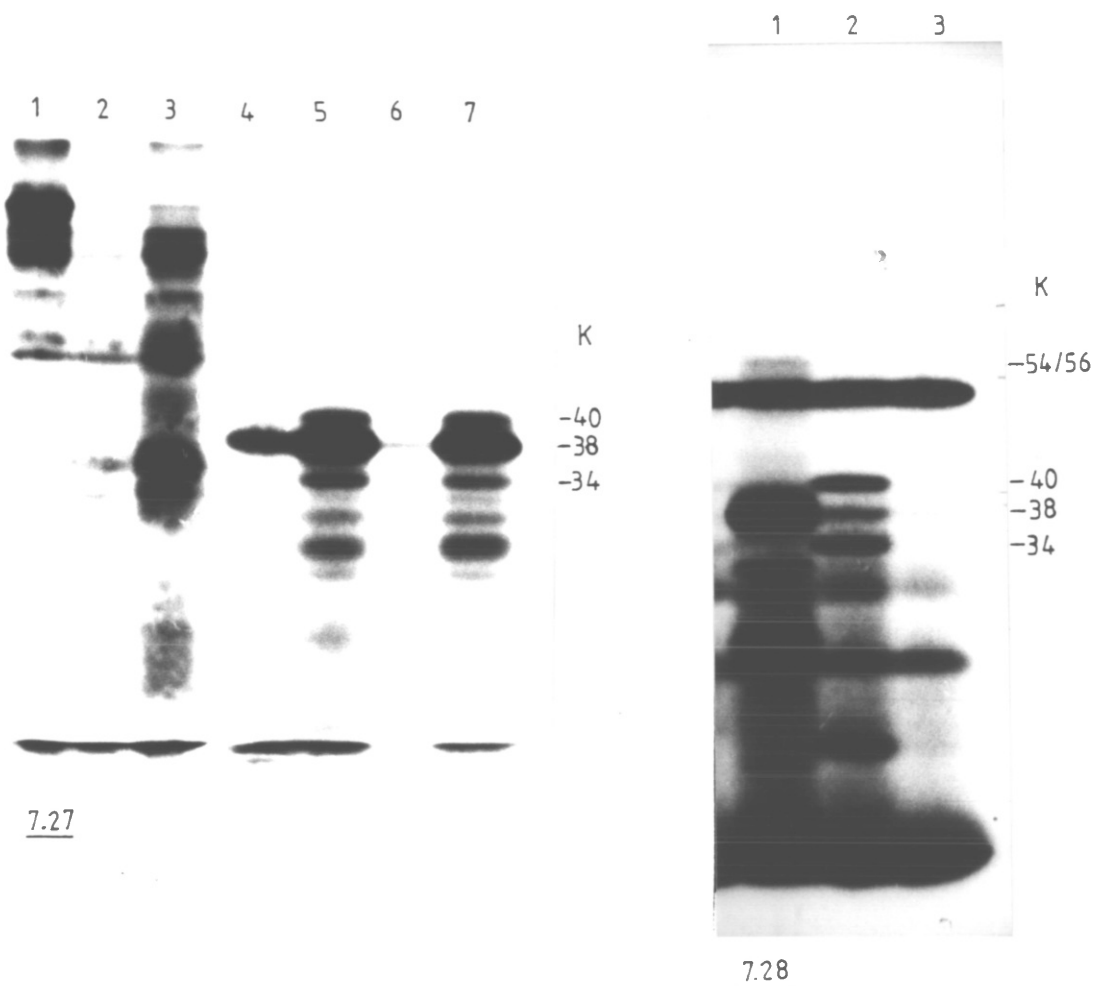


Figure 7.27 Effect of cap analogue on translation, analysed by SDS-PAGE. Lane 1, 10 $\mu\text{g/ml}$ TMV RNA; lane 2, 10 $\mu\text{g/ml}$ TMV RNA + 4 mM $m^7\text{GMP}$; lane 3, 50 $\mu\text{g/ml}$ TMV RNA + 4 mM $m^7\text{GMP}$; lane 4, 2 $\mu\text{g/ml}$ RCNMV RNA; lane 5, 10 $\mu\text{g/ml}$ RCNMV RNA; lane 6, 2 $\mu\text{g/ml}$ RCNMV RNA + 8 mM $m^7\text{GMP}$; lane 7, 10 $\mu\text{g/ml}$ RCNMV RNA + 8 mM $m^7\text{GMP}$.

Figure 7.28 SDS-PAGE of *in vitro* translation products of separated RNAs. Lane 1, RNA-1; lane 2, RNA-2; lane 3, RNA migrating faster than RNA-2 on an agarose gel.

7.6.5 *In vitro* translation of hybrid-selected RNA

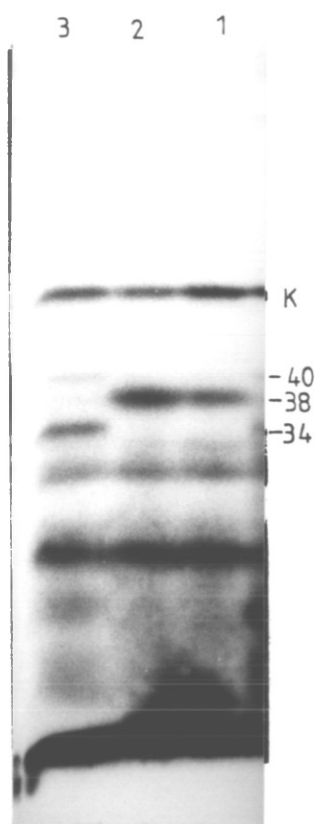
Cloned plasmids specific for one or other of the viral RNAs were bound to nitrocellulose (section 6.10.1) and used to select RNA from the total ssRNA of infected plant tissue. This RNA was then translated in the reticulocyte lysate (figure 7.29). RNA selected with the RNA-1-specific clones BM3 and TQ7 gave rise to p38, plus a trace amount of p54/56. RNA selected with the RNA-2-specific clone GC9 gave rise mainly to p34, plus a smaller amount of p40.

Similar hybrid-selections were performed using plasmid DNA bound to DPT-paper (sections 6.10.2 and 6.10.3); the resulting protein bands were extremely faint, but p38 could just be detected in the translation products derived from clones BM2 and TQ5. Attempts were made to use cloned DNA to select specific RNA species from gel-purified RNA-2, and to use cDNA made from gel-purified RNA-1 to select RNA from gel-purified RNA-2; no translation products were obtained, presumably due to degradation of the small amounts of RNA-2 being used in the hybridisation reactions.

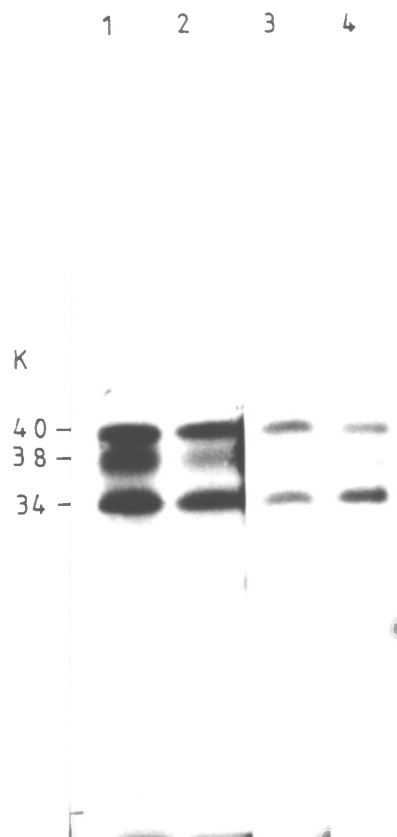
7.6.6 Immunoprecipitation of translation products

In order to establish which of the labelled translation products corresponded to the viral coat protein, the translation products were immunoprecipitated with antiserum to intact virus. In initial experiments, translation products were incubated with antiserum and the antigen-antibody complex was precipitated with Staph A cells in the presence of Nonidet P-40. Using this method, all the major translation products were precipitated equally, regardless of the extent to which the antiserum was diluted; indeed, the products were also precipitated by preimmune serum.

To make the reaction more specific, the method of Harbison *et al.* (1985) was used, with modifications as described in section 6.9.3. Figure 7.30 shows the fluorogram of the precipitated translation products. Lanes 1 and 2 were precipitated using 20 μ g IgG, with and without preincubation with preimmune serum. Without preincubation the major products were p40 and p34, with a smaller amount of p38; the amount of p38 was further reduced relative to the other two bands by including the preincubation step. It was then attempted to make the reaction more specific by reducing the amount of IgG used to 4 μ g (lane 3) or 1 μ g (lane 4): the relative amount of p38 was further reduced. Hence p38 clearly did not react with anti-RCNMV antiserum, but both p40 and p34 did. It was noted that p40 comigrated with purified RCNMV coat protein upon SDS-PAGE.



7.29



7.30

Figure 7.29 SDS-PAGE of *in vitro* translation products of hybrid-selected RNA. The RNA to be translated was selected by nitrocellulose filters with bound: lane 1, clone BM3; lane 2, clone TQ7; lane 3, clone GC9.

Figure 7.30 SDS-PAGE of immunoprecipitated RCNMV RNA *in vitro* translation products. Lane 1, using 20 μ g IgG, preincubation omitted; lane 2, using 20 μ g IgG; lane 3 using 4 μ g IgG; lane 4, using 1 μ g IgG.

CHAPTER 8. DISCUSSION.

8.1 STRUCTURE OF THE RCNMV GENOME

8.1.1 RCNMV viral RNA

As with other strains of RCNMV and other Dianthoviruses, the viral RNA of RCNMV isolate TpM-34 consists of two species, with sizes determined by electrophoresis to be 3.99 and 1.39 kb. These figures may be compared with the data obtained by Okuno *et al.* (1983) for three other Dianthoviruses:

Virus	RNA-1	RNA-2
SCNMV	3.91 kb	1.59 kb
RCNMV-Sw	4.20 kb	1.71 kb
RCNMV-C	4.49 kb	1.77 kb

The figures of Okuno *et al.* (1983) were derived from partially denaturing PAGE, whereas the sizes for TpM-34 were calculated from fully denaturing agarose gel electrophoresis. A comparison of the migration of RCNMV RNA under denaturing and nondenaturing conditions (section 7.1.2) showed that native RNA migrates faster, indicating a more compact structure presumably due to a high degree of secondary structure.

There is no homology between RNA-1 and RNA-2 of RCNMV-TpM-34 detectable by filter hybridisation (see section 7.1.3); a similar conclusion was obtained for RCNMV-Aus using solution hybridisation (Gould *et al.*, 1981).

8.1.2 The 5'-terminal structure of RCNMV RNA

In common with most eukaryotic cellular and viral RNAs, the 5'-ends of RCNMV RNAs are blocked to phosphatase and kinase reactions. The presence

of a 7-methylguanosine cap was not demonstrated due to practical problems encountered with both the chemical and enzymic methods of cap removal (sections 7.2.2 and 7.2.3), and the translation of RCNMV RNA was much less strongly inhibited by a cap analogue than that of the capped TMV RNA (section 7.6.3). However, there was no evidence for a genome-linked protein on the RCNMV RNAs either: RCNMV RNA does not require a protease-sensitive structure to infect plants (section 7.2.5), and no labelled protein could be detected when RCNMV RNA was treated with ^{125}I under conditions which labelled the VPg's of several Nepoviruses (section 7.2.4). Hence it seems most likely that the RCNMV RNAs are capped, in common with the majority of other plant RNA viruses (section 5.2.3).

8.1.3 The 3'-terminal structure of RCNMV RNA

Since it was not known whether the Dianthovirus RNAs were polyadenylated or not, it was attempted to prime cDNA synthesis on the RCNMV RNA using oligo(dT) as primer: although full-length cDNA could be made from the polyadenylated RNAs of tomato black ring virus (TBRV) in this way, no cDNA was made from RCNMV RNA, indicating the lack of a substantial polyadenylate tract (section 7.3.2). This result was further confirmed by the observation that RCNMV RNA did not bind to oligo(dT)-cellulose in 0.5 M NaCl, whereas polyadenylated TBRV RNA did and required zero salt buffer to elute it (S.M. Dodd, personal communication).

Although RCNMV RNA is not polyadenylated, the 3'-end is not blocked to labelling using [^{32}P]-5',3'-cytidine bisphosphate and RNA ligase (S.M. Dodd & V.E. Prince, personal communications), and so the RNAs presumably terminate in a 3'-hydroxyl group. Digestion of RCNMV RNA-2 labelled this way with RNase-T2, followed by chromatography on PEI-cellulose, indicates

that the 3'-terminal nucleotide of RNA-2 is cytidine (S.M. Dodd, personal communication).

8.2 CLONING OF COMPLEMENTARY DNA OF RCNMV

Since cDNA synthesis could not be primed on RCNMV RNA using oligo(dT), the first strand of cDNA was primed with random oligodeoxynucleotides. This produced a good yield of cDNA, but the cDNA synthesised was not full-length. After synthesis of the second strand of cDNA by standard protocols, the double-stranded DNA was cloned by a variety of methods. Addition of synthetic oligonucleotide linkers containing a *Sall* restriction site was unsuccessful, possibly due to failure to produce cDNA with both ends blunt, or possibly due to incomplete cleavage of added linkers with *Sall*. A more successful method was homopolymeric tailing of the cDNA followed by cloning in a vector with complementary homopolymer tails (section 7.3.4 and table 7.1).

However, the best yield of clones was obtained by ligating restriction fragments of cDNA into a vector with compatible ends. Although enzymes which cut DNA infrequently would be expected to produce larger fragments for cloning, a much larger number of clones was produced using the enzymes *TaqI* and *Sau3AI* which cut DNA frequently (section 7.3.6 and table 7.1). Of the clones tested, the majority were derived from RNA-1, presumably due to its greater size and hence number of restriction fragments produced.

When cloned DNA was used as a probe against blotted viral RNA, each clone was specific for one or other of the RNAs, confirming the lack of detectable homology between the two segments of the RCNMV genome.

8.3 ANALYSIS OF RNA FROM INFECTED PLANTS

8.3.1 Single-stranded RNA from infected plants

Northern blot analysis of single-stranded RNA from infected *Phaseolus vulgaris* tissue showed only the two genomic RNAs when probed with cDNA to RCNMV viral RNA (section 7.4.1). Although various faint bands or smears were sometimes also detected, these were not reproducible and are not considered to represent RCNMV-specific subgenomic RNAs, since experiments of this type are prone to artifactual bands (Dougherty, 1983; Palukaitis *et al.*, 1983; Hayes *et al.*, 1984). This contrasts with a virus such as tobacco mosaic virus (TMV), where the non-encapsidated subgenomic coat protein message is abundant and easily detected in infected tissue (Goelet & Karn, 1982).

No further bands were detected in polysomal RNA, which would be expected to be enriched in species which were being translated, i.e. viral-specific messages. The use of cloned probes did not detect any major non-genomic RNA species either.

8.3.2 Double-stranded RNA from infected plants

When dsRNA from infected tissue was analysed by electrophoresis on polyacrylamide or agarose gels, three species were detected, ds-A, B and C (section 7.5.1). Ds-B and ds-C were only about 20 bp apart in size. Since both genomic and subgenomic RNAs produce corresponding double-stranded RFs in plants (as described in section 5.5.1), this implies the existence of a subgenomic RNA in plants which is very close in size to RNA-2. Blotting of the dsRNA from both denaturing and non-denaturing gels showed that ds-A was the RF of RNA-1; however ds-B and C were not

resolved by these methods. To overcome this the bands were excised from an agarose gel, physically separated and then blotted. Only ds-C hybridised to an RNA-2-specific clone, showing that ds-C was the RF of RNA-2 (section 7.5.3). Hence ds-B must be the RF of a subgenomic RNA, presumably derived from RNA-1, which is apparently 20 bases larger than RNA-2 and which cannot be resolved from RNA-2 by agarose gel electrophoresis.

8.4 *IN VITRO* TRANSLATION OF RCNMV RNA

8.4.1 Translation of viral RNA

Translation of total RNA extracted from virions of RCNMV strain TpM-34 in the rabbit reticulocyte lysate cell free system produced three major products of 40 K, 38 K and 34 K, plus two fainter products of 54 K and 56 K which were sometimes not resolved by SDS-PAGE (section 7.6.1). This may be compared to the translation of RCNMV strain D in the same system, which produced major products of 39 K, 36 K and 34 K, plus a faint band of 56 K (Morris-Krsinich *et al.*, 1983). An identical pattern of proteins was produced from RNA extracted from virus which had been further purified by banding in a gradient of CsCl.

The 40 K product (p40) comigrated with purified RCNMV coat protein, although immunoprecipitation of the translation products with anti-RCNMV IgG precipitated both p40 and the 34 K product (p34). It seems highly likely that p40 is indeed the coat protein, and the reason for the co-precipitation of p34 (which is derived from a different genome segment, as described below) is unclear. Hence the coat protein mRNA is encapsidated within virus particles.

A time-course of translation (section 7.6.1) showed that all the products appeared at roughly the same rate, and there was no indication of any larger precursor proteins: this is evidence against RCNMV employing a polyprotein strategy of gene expression.

8.4.2 Translation of individual RNA species

Full-length RNA-1 was extracted from an agarose gel following electrophoresis of virion RNA, and upon translation produced p38 along with a small amount of p54/56 (section 7.6.4). It is possible that the p38 gene is located at the 5'-end of RNA-1, and p54/56 is produced by readthrough at the end of the p38 gene. RNA hybrid-selected from RCNMV-infected plant RNA by the RNA-1-specific clones BM3 and TQ7 was also translated to p38 and a smaller amount of p54/56, indicating that these clones were hybridising to full-length RNA-1 (section 7.6.5). If any smaller RNA-1-derived RNAs were also selected by these clones, their translation products were below the limit of detection.

When RNA-2 was extracted from an agarose gel of virion RNA and translated it produced both p34 and p40 in roughly equal amounts (section 7.6.4). Since pseudorecombinant experiments have shown that RNA-1 of Dianthoviruses encodes the coat protein (see section 5.1.3), it is possible that the band of RNA-2 seen on an agarose gel contains both the genomic RNA-2 and an RNA-1-specific subgenomic RNA of very similar size. When RNA selected from RCNMV-infected plant RNA by the RNA-2-specific clone GC9 was translated the major product was p34, showing that RNA-2 encodes p34, and therefore the coat protein (p40) is probably translated from a 1.4 kb subgenomic RNA. These results extend the work done on the translation of partially separated RNAs of RCNMV-D (Morris-Krsinich et

al., 1983), and this work is the first report of the translation of fully-purified RNA species from a Dianthovirus.

8.5 STRATEGY OF GENE EXPRESSION OF RCNMV

8.5.1 Evidence for a subgenomic RNA

There are several lines of evidence for the existence of a 1.4 kb subgenomic RNA derived from RNA-1 of RCNMV, which serves as the coat protein message.

(i) Three dsRNA bands were detected in infected tissue. Each genomic RNA would be expected to produce a double-stranded RF *in vivo*, and the third band is presumably the RF of a subgenomic RNA. The second-largest band, ds-B, has an apparent size about 20 bp larger than ds-C, but only ds-C hybridises to an RNA-2-specific clone, thus implying that ds-B represents the RF of a subgenomic RNA derived from RNA-1. A third dsRNA band has also been reported for CRSV (Dodds *et al.*, 1977), and tentatively for (presumably a different strain of) RCNMV (Morris, 1983).

(ii) Translation of the 1.4 kb fraction of viral RNA extracted from an agarose gel produces both p34 and p40, even though p40 (the coat protein) is known to be specified by RNA-1 of other Dianthoviruses. Hybrid-selection for RNA-2-specific sequences showed that only p34 is translated from purified RNA-2: hence encapsidated viral RNA contains a 1.4 kb subgenomic coat protein message, which is not resolved from RNA-2 upon electrophoresis. In the case of CRSV, a minor encapsidated RNA was detected which migrated somewhat slower than RNA-2 (Dodds *et al.*, 1977); this may represent the coat protein message of CRSV.

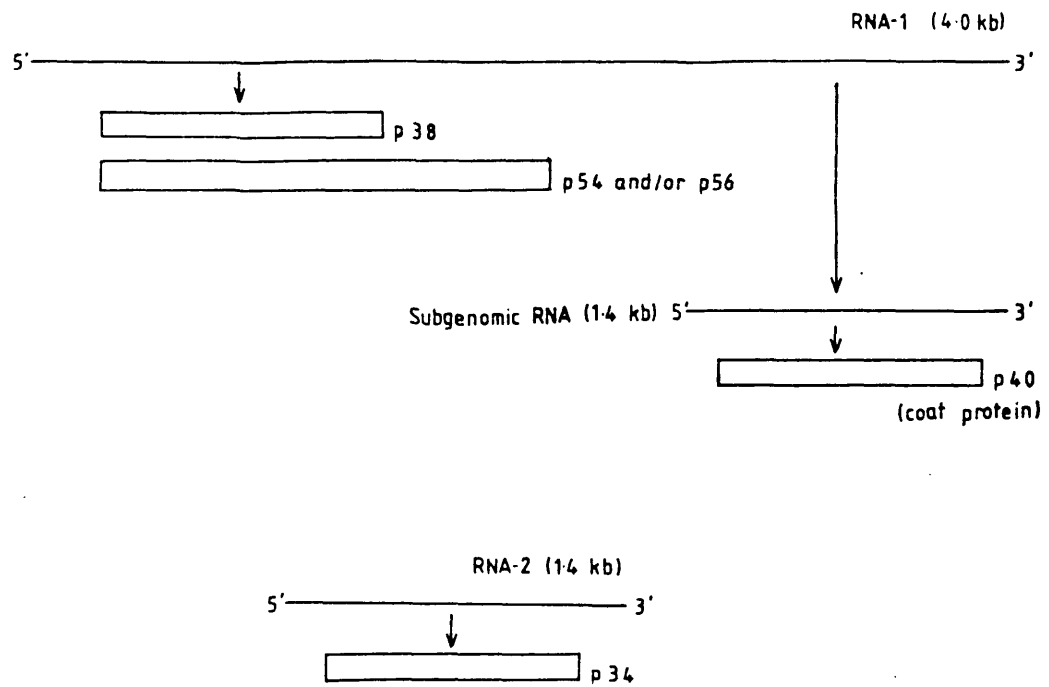


Figure 8.1 Proposed model for the gene expression of RCNMV. Horizontal lines represent RNA molecules, open bars represent proteins. The model is described in section 8.5.2.

(iii) A very faint band of 1.4 kb was sometimes detected in Northern blots of virion RNA when probed with RNA-1-specific clones, notably BM2 and TQ5 (see section 7.3.8). This may represent the subgenomic RNA, since there is apparently no homology between RNA-1 and RNA-2. The reason why this subgenomic RNA was so difficult to detect by blotting may be a combination of its low concentration, even in infected plants, and its comigration with RNA-2 which may interfere with its transfer to the GeneScreen membrane. The low concentration of the coat protein message is unusual, since coat protein must be produced in large amounts during the infection cycle and hence its mRNA would be expected to be present in large amounts also: perhaps the RCNMV coat protein message is unstable, and is either produced only transiently in plants, or is readily degraded during extraction of RNA. The subgenomic RNA must also be present in low concentration in virus particles, since it is not easily detectable by hybridisation; however, substantial amounts of coat protein are produced upon translation of virion RNA *in vitro*, indicating that the subgenomic RNA is very efficiently translated.

8.5.2 Proposed strategy of gene expression

Taking into account the above observations, the following pattern of gene expression can be proposed. RNA-2 encodes a single protein, p34. RNA-1 produces p38 by initiation at the 5'-most AUG codon, and readthrough at a weak termination codon at the end of the p38 gene allows formation of p54 and p56 (either two alternative stop codons, or p54 is produced from p56 by post-translational processing). The 3'-most 1.4 kb of RNA-1 is transcribed into a subgenomic RNA, which is then translated to produce the coat protein p40. This proposed model is illustrated in figure 8.1.

The coding capacity of RNA-1 is approximately equivalent to 145 K of protein, so it is possible to fit the p38, p56 and p40 genes onto RNA-1 without overlap; however, this would require internal initiation to form the second gene product from full-length RNA, and this has not been reliably reported for any other plant RNA virus.

Assuming that p56 is a readthrough product of p38, this leaves RNA with a coding capacity of 50 K unaccounted for; similarly, RNA-2 has a coding capacity of 50 K of which 16 K is unaccounted for. Further work would be required to determine if any further subgenomic RNAs and/or gene products remain to be detected.

The genome organisation of RCNMV and the other Dianthoviruses is unique in that no other group of segmented plant RNA viruses has the coat protein gene on the largest RNA segment, except the ungrouped pea enation mosaic virus (PEMV). PEMV differs from RCNMV in that the coat protein appears to be expressed via protein cleavage in the former, rather than via formation of a subgenomic RNA (see section 5.4.1).

8.6 CURRENT AND FUTURE WORK ON RCNMV

8.6.1 Other results from the RCNMV project

While the work described in this thesis was being undertaken, another line of investigation was also underway in the same laboratory.

Pseudorecombinants were constructed between strain TpM-34 and the English strain H, and their effects on plants were studied (Osman *et al.*, 1986). Strains TpM-34 and H differ in their symptoms on *Vigna sinensis*: TpM-34 produces ring-like necrotic lesions which expand to become confluent as the virus spreads through the plant systemically; strain H

produces small necrotic lesions which do not expand, and the infection does not become systemic. Pseudorecombinant T1:H2 (i.e. RNA-1 from TpM-34 and RNA-2 from H) reacted with antiserum to TpM-34 but not to H, and produced symptoms like H on cowpea; pseudorecombinant H1:T2 reacted with antiserum to H but produced symptoms like TpM-34. This confirms that RNA-1 specifies the coat protein (and hence immunological specificity), and shows that RNA-2 specifies lesion morphology and ability to systemically invade cowpea (Osman *et al.*, 1986).

It was then shown that TpM-34 RNA is able to replicate in cowpea protoplasts to produce intact virus (Osman & Buck, 1987). RNA-2 was unable to replicate in the absence of RNA-1, but RNA-1 was able to replicate in the absence of RNA-2. Replication of RNA-1 produced virus particles indistinguishable from genuine virus by morphology or serology, and an RNA was detected in the protoplasts which comigrated with RNA-2 but which hybridised to clone BM3 (RNA-1-specific) but not to GC9 (RNA-2-specific), thus confirming my evidence for a 1.4 kb subgenomic RNA derived from RNA-1 (Osman & Buck, 1987).

Since RNA-1 can replicate by itself, it must encode the virus-specific portion of the RNA replicase: perhaps p38 or p54/56 are involved in this function. Since RNA-1 can replicate and form intact virus particles, but cannot infect plants, RNA-2 must be involved in the cell-to-cell spread of RCNMV through the plant, as deduced from the pseudorecombinant experiment: p34 may be the cell-to-cell transport protein, analogous to the 30 K protein of TMV and the 32 K protein of alfalfa mosaic virus. RCNMV contrasts with other bipartite viruses of which RNA-1 can replicate alone in protoplasts (Comoviruses, Nepoviruses and

Tobraviruses) in that only RCNMV has the coat protein gene on RNA-1 and can therefore produce virus particles in the protoplasts.

8.6.2 Prospects for further work on RCNMV

The sequence analysis of RCNMV RNA-2 is currently underway, using the six RNA-2-specific clones listed in table 7.1. Determination of the complete sequence will allow identification of open reading frames on RNA-2, and allow sequence comparisons between the RNA-2 product p34 and other putative cell-to-cell transfer proteins. A similar approach could employ other clones described in this thesis to determine the sequence of RNA-1. Knowledge of the sequence of RNA-2 will allow the construction of a full-length clone in a transcription vector, which could then be used to introduce site-specific mutations into both the coding and non-coding regions of RNA-2 via *in vitro* transcription. Such specifically altered RNA-2 could then be used in studies of viral replication and pathogenesis in whole plants or in protoplasts. Full-length clones of RNA-2 could also be used in other ways: introduction of the cloned DNA into the host genome via a Ti plasmid vector could allow the expression of viral gene products in a plant in the absence of virus replication; or specific parts of the cloned DNA introduced into the host genome could be used to express antisense RNA for creating resistant plants. The availability of clones of RNA-2 of other strains of RCNMV would allow the construction of recombinant RNAs *in vitro*, which could be used to localise sequences important for the cell-to-cell transfer function. Such approaches could be extended to RNA-1 as well, eventually allowing elucidation of both replication and pathogenicity pathways of the virus.

APPENDIX I

NAMES AND ADDRESSES OF MAJOR SUPPLIERS

Aldrich: Aldrich Chemical Co. Ltd., The Old Brickyard, New Road,
Gillingham, Dorset, SP8 4JL.

Amersham: Amersham International plc, UK Sales Office, Lincoln
Green, Green End, Aylesbury, Bucks., HP20 2TP.

Anglian: Anglian Biotechnology Ltd., Unit 8, Hawkins Road,
Colchester, Essex, CO2 8JX.

BCL: Boehringer Corporation (London) Ltd., Bell Lane, Lewes,
East Sussex, BN7 1LG.

BDH: BDH Chemicals Ltd., Broom Road,
Poole, Dorset, BH12 4NN.

Beckman: Beckman-RIIC Ltd., Progress Road, Sands Industrial
Estate, High Wycombe, Bucks., HP12 4JL.

Bio-Rad: Bio-Rad Laboratories Ltd., Greenhill Crescent, Holywell
Industrial Estate, Watford, Herts., WD1 8RP.

BRL: BRL-Gibco Ltd., PO Box 35, Washington Road, Abbotsinch
Industrial Estate, Paisley, Scotland, PA3 4EP.

Difco: Difco Laboratories Ltd., PO Box 14B, Central Avenue,
East Molesy, Surrey, KT8 0SE.

Fisons: Fisons Scientific Apparatus, Loughborough, Leics.

Life Sciences: Life Sciences Inc., 2900 72nd Street North,
St. Petersburg, Florida 33710, USA.

New England Biolabs: New England Biolabs Inc., CP Laboratories, PO Box 22,
Bishops Stortford, Herts., CM23 3DH.

New England Nuclear: DuPont (UK) Ltd., NEW Products Division, Wedgwood
Way, Stevenage, Herts., SG1 4QN.

Novo: Novo Enzyme Products Ltd., 2B Thames Avenue,
Windsor, Berkshire, SL4 1QP.

P-L: Pharmacia Ltd., Pharmacia House, Midsummer Boulevard,
Milton Keynes, Bucks., MK9 3HP.

Rose: Rose Chemicals Ltd., 83 Darent Road, London N16 6EB.

Sigma: Sigma Chemical Company Ltd., Fancy Rd.,
Poole, Dorset, BH17 7NH.

Sturge: John & E. Sturge, Denison Road, Selby, North Yorks.,
YO8 8EF.

Whatman: Whatman Ltd., Springfield Mill, Maidstone, Kent, ME14 2LE.

APPENDIX IIDouble-stranded RNA size markers.*Aspergillus foetidus* viruses (AfV)

5.92 kb

3.90 kb

3.75 kb

2.70 kb

2.45 kb

2.08 kb

1.79 kb

0.39 kb

Penicillium stoloniferum viruses (PsV)

1.60 kb

1.43 kb

1.36 kb

1.28 kb

0.66 kb

Helminthosporium maydis virus (HmV)

8.23 kb

References: Ratti & Buck (1972); Buck & Ratti (1977); Bozarth et al.
(1971); Bozarth & Harley (1976); Bozarth (1977).

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