MOLECULAR CLONING AND ANALYSIS OF TOMATO BLACKRING VIRUS

T H E S I S

submitted to

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by

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My special thanks go to my husband for proof-reading the manuscript and for his support during my course of study.

The author declares that the material contained in this thesis is her own work and does not appear in any other publication.

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SUMMARY

Tomato blackring virus RNA was reverse transcribed into single-strand complementary DNA. The RNA template was removed by alkaline hydrolysis, and the cDNA was transcribed into a double-stranded structure. The remaining single-stranded regions were removed by digestion with S1 nuclease and the recessed 3'-termini converted to a double-stranded structure. The cDNA was fractionated according to size.

Plasmid DNA (pUC9) was prepared and linearized by cleaving with a suitable restriction enzyme. The ends of the DNA were treated with calf intestine alkaline phosphatase to prevent re-annealing. Recombinant DNA molecules were formed by ligating TBRV cDNA and pUC9.

Competent *E. coli* cells were prepared and transformed with TBRV recombinant DNA. Amp^r, lac^- colonies were tested for the presence of TBRV cDNA sequences by the colony hybridisation method of Grunstein and Hogness. Colonies which gave a positive hybridisation signal were isolated and characterised in terms of length and which TBRV RNA molecules they hybridised to. Clones representing TBRV RNA-2 were successfully identified. Clones representing

either TBRV RNA-1 or TBRV RNA-3 which also hybridised to other RNA were found.

ABBREVIATIONS

EDTA	= ethylenediamine tetraacetic acid
Tris-HCl	= Tris hydroxymethyl methylamine
SDS	= sodium dodecyl sulphate
HEPES	= N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
DTT	= dithiothreitol
PVP	= polyvinyl pyrollidone
oligo (dT)	= oligodeoxythymidylic acid
cDNA	= complementary DNA
dNTPs	= 5mM solution of all four deoxynucleoside triphosphates
dCTP	= deoxycytidine triphosphate
poly U	= polyuridinylate
mol. wt.	= molecular weight
TBRV	= tomato blackring virus
BMV	= brome mosaic virus
Kb	= Kilobase
c.p.m.	= counts per minute
µCi	= curie x 10^{-6}
mA	= amperes x 10^{-3}

SUPPLIERS LIST

Reverse Transcriptase; Klenow Fragment of DNA Polymerase I - Anglian Biotechnology Limited, Unit 8, Hawkins Road, Colchester, Essex.

Deoxyribonuclease I; Oligo (dT) - PL Biochemicals Inc.

Hinc II endonuclease; DNA Polymerase I - Cambridge Biotechnology Lab.

Nuclease S1; T_4 DNA Ligase; Hind III, EcoR1 endonucleases; - Bethesda Research Laboratories (UK) Ltd., P.O. Box 145, Science Park, Cambridge CB4 4BE.

Human Placental Ribonuclease Inhibitor - Bolton Biologicals, 545 West 7th Street, St. Paul, Minnesota 55102.

Calf Intestine Alkaline Phosphatase; dNTPs; X-gal - B.C.L., Bell Lane, East Lewes, Sussex.

Sephadex G-100 - Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Biodyne A-Nylon 66 - Pall Process Filtration Ltd., Europa House, Havant Street, Portsmouth PD1 3PD.

^{32}P-dCTP (3×10^3 Ci mM^{-1}); ^{3}H-dCTP (19 Ci mM^{-1}) - Amersham, White Lion Road, Amersham, Bucks.

INTRODUCTION

Tomato blackring virus (TBRV) is a member of the nepovirus group of plant viruses and characteristically has a bipartite RNA genome and isometric particles. Viruses of this group are typically transmitted to host plants by ectoparasitic, free-living soil nematodes which feed on plant roots. Outbreaks of infection occur in patches in fields, which reflects the distribution of the vector. The nepoviruses are also disseminated in infected seed, pollen and planting material which allows the virus to spread over a wider area (Harrison & Murant, 1977).

TBRV has a wide host range and is an important pathogen of many crops. Ringspot diseases are caused by TBRV in bean, sugar-beet, lettuce, raspberry and strawberry. In addition black ring symptoms occur in tomato. Potato, peach, celery, cabbage and grapevine are also infected by this virus. TBRV invades all parts of infected plants including the seed, pollen and apical meristem (Murant, 1970).

Several strains of TBRV have been reported (Murant, 1970). These strains can be distinguished by their serological properties and this has enabled their separation into two groups. One group consists of the type, lettuce ringspot and potato bouquet strains, while the other group contains the beet ringspot and potato pseudoaucuba strains (Murant, 1970).

The different strains of TBRV are associated with separate nematode species: the type strain group are transmitted by Longidorus attenuatus, whereas the beet ringspot group are spread by L. elongatus. The specificity between vector species and virus strains is related to properties of the virus coat protein (Harrison & Murant, 1977). TBRV does not multiply in its vector, nor is it transmitted through the egg, and it is lost during moulting. Nematodes remain viruliferous for about eight weeks, then lose the ability to spread TBRV (Harrison & Murant, 1977). Virus-like particles have been detected on the stylet guiding sheath of nematodes which have fed on plants infected with TBRV. In experimental conditions, TBRV can be readily mechanically transmitted, although there is no evidence to suggest this occurs in nature (Murant, 1970).

Some strains of TBRV, including the beet ringspot and potato bouquet strains, are associated with satellite RNA (mol. wt. 4.8×10^5) (Murant et al, 1973). This RNA is not essential for infectivity of the "helper" RNA. Satellite RNA can be physically separated from genomic RNA on the basis of molecular weight. Strains cured of satellite RNA, do not contain the satellite RNA again (Murant et al, 1973). The satellite is dependent on the "helper" virus for replication and it is packaged in "helper" virus protein. The

satellite RNA may have originated either, by reduction of an ancestral TBRV genome, or from a host messenger RNA (Mayo et al, 1979). Satellite systems have been reported in other nepoviruses, namely tobacco ringspot, myrobalan latent ringspot and arabis mosaic virus (Mayo et al, 1982(a)).

TBRV has isometric particles which are about 30nm in diameter and have a 5- or 6-sided angular outline (Murant, 1970). After centrifugation, these particles sediment as three components; top component, T (55S), middle component, M (97S) and bottom component, B (121S). They contain respectively, 0, 28, and 38% single-stranded RNA (Murant, 1970). M component contains RNA-2 (mol. wt. 1.6×10^6), and B component contains RNA-1 (mol. wt. 2.8×10^6). TBRV particles have T=1 icosahedral structures with 60 molecules of coat protein (mol. wt. 5.5×10^4) (Harrison & Murant, 1977). The coat protein of nepoviruses are larger than those of other small isometric plant viruses. The comoviruses have two coat protein molecules (mol. wt. 2.5×10^4 and 4.4×10^4) (Harrison & Murant, 1977). Work with tobacco ringspot virus (a nepovirus) led Chu and Francki (1979) to propose that each protein is a stable tetramer of a smaller protein (mol. wt. 1.3×10^4) and that there are 240 in each particle.

Translation in wheat germ extracts and reticulocyte lysates showed that TBRV RNA-1 produces a maximum polypeptide product of 2.2×10^5 mol. wt. and a more prominant polypeptide of 1.9×10^5 mol. wt. (Fritsch et al, 1980). The smaller polypeptide may be the breakdown product of the larger polypeptide or may be a partial translation product. Both polypeptides failed to react with TBRV antiserum (Fritsch et al, 1980). TBRV RNA-2, similar ly translated in vitro, yielded a polypeptide of 1.6×10^5 mol. wt., part of which reacted with TBRV antiserum (Fritsch et al, 1980). Coat protein molecules were not produced directly from RNA-2 and Mayo and Barker (1983) suggest that the coat protein may be cleaved from the 1.6×10^5 mol. wt. polypeptide by a protease coded for by RNA-1. The smaller coat protein (mol. wt. 2.5×10^4) of cowpea mosaic virus (a comovirus) is produced by a similar method (Fritsch et al, 1980). In this respect the replication strategy of nepoviruses and comoviruses seems to differ from that of tobacco mosaic virus and a number of other RNA plant viruses including alfalfa mosaic virus and tobacco rattle virus whose coat protein is translated from a sub-genomic messenger RNA (Hunter et al, 1976). The large polypeptides produced by TBRV RNA-1 and RNA-2 represent 80% and 100% respectively of the coding capacity of each genome. TBRV satellite RNA was translated into a 4.8×10^4 mol. wt. polypeptide which did not react

with TBRV antiserum (Fritsch et al, 1980).

The 5'-termini of TBRV RNA-1, RNA-2 and satellite RNA have been shown to be covalently bound to a similar genome-linked protein (VPg) (Mayo et al, 1982(b)). Other nepoviruses also have a VPg, possession of which is a feature of the group. Treatment of nepovirus RNAs with pronase or proteinase K led to a loss of infectivity, whereas under similar conditions, the infectivity of cowpea mosaic virus (a comovirus) or tomato bushy stunt virus (a tombusvirus) was unaffected (Mayo et al, 1982(b)). The size of tobacco ringspot virus RNA (a nepovirus) was not changed by treatment with the proteolytic enzymes. Harrison and Barker (1978) showed that the VPg cannot be exchanged between the RNA molecules and concluded that it was firmly attached. The VPg must be attached to TBRV RNA-1 and RNA-2 in order to form local lesions (Mayo et al, 1982(b)). Picornavirus RNA eg. Polio virus and Bacillus subtilis phage Ø29 DNA also have covalently bound proteins at their 5'-termini. This protein is required for infectivity of phage Ø29, but not for the Picornavirus RNA (Harrison & Barker, 1978).

The nepovirus VPg differs between the members of this group, but is similar for the different strains of TBRV (Harrison & Barker, 1978). The role of VPg is not known but several suggestions have been proposed.

Harrison and Barker (1978) suggest that it is involved in replication and may be part of a replicase complex, or it may be involved in the association of coat protein with RNA. Other suggestions give the VPg a relatively non-specific role and compare it to the 5'-cap of tobacco mosaic virus (Mayo et al, 1982(b)).

The nepovirus VPg is probably virus-coded and Mayo et al (1982(b)) suggest that RNA-1 directs its synthesis since infection by isolated RNA-1 alone can induce its production in protoplasts. However, the possibility of RNA-2 containing a gene for VPg cannot be overlooked, as this protein is thought to be less than 50 amino acid residues long, and techniques used so far may have failed to detect it (Robinson et al, 1980).

Both TBRV RNA species and the satellite RNA have polyadenylate at their 3'-termini (Mayo et al, 1979). This is another feature characteristic of the nepovirus group. Mayo et al (1979) demonstrated that RNA from five nepoviruses, including TBRV, could be bound to oligo (dT) cellulose in high ionic strength buffers. Under these conditions the RNAs of tobacco mosaic virus and tobacco rattle virus did not bind. A polyadenylate tract is generally found at the 3'-end of eukaryotic messenger RNA molecules.

Indeed many vertebrate viral RNAs, which can act as a messenger, also have 3'-polyadenylate termini (Mayo et al, 1979). Cowpea mosaic virus has a 3'-polyadenylate tract which is probably characteristic of the comoviruses (Mayo et al, 1979).

TBRV RNA-1 and RNA-2 must be inoculated together in order to form lesions in whole plant experiments (Harrison & Murant, 1977). Further work, involving pseudo-recombinants of TBRV RNA-1 and RNA-2 from different strains, showed that each molecule carried separate genetic information. RNA-1 carries determinants for the host range, seed transmissibility and the kind of symptom produced, whereas RNA-2 determines serological specificity, nematode transmissibility and other kinds of symptom (Harrison & Murant, 1977).

The replication of isolated TBRV RNA-1 and RNA-2 molecules in tobacco mesophyll protoplasts have been investigated by Robinson et al (1980). Their results suggest that administration of RNA-2 alone does not allow replication of this molecule. Isolated RNA-1 was found to be capable of independant replication and the products were characterised to be RNA-1 by hybridisation with complementary DNA (cDNA) of RNA-1 and by electrophoresis on polyacrylamide-agarose gels. These authors also detected synthesis of the genome-linked protein in protoplasts inoculated

with isolated RNA-1 molecules and concluded that RNA-1 codes for this protein and that RNA-1 contains all the information required for its own synthesis. They further stated that if there was a virus-coded replicase involved in TBRV replication, then RNA-1 must code for it. So far there is no information about the enzymes involved in TBRV replication (Robinson et al, 1980).

Robinson et al (1980) attempted to hybridise TBRV RNA-1 with TBRV cDNA-2, and TBRV RNA-2 with TBRV cDNA-1. These authors detected no cross-hybridisation and concluded that there was no sequence homology, however, their techniques were limited, such that sequences of less than 250 nucleotides long, would not be detectable. This limitation also excludes hybridisation between the polyadenylate groups at the 3'-termini of both RNA molecules. These experiments were based on the untested assumption that the cDNA represented the entire genome (Robinson et al, 1980). There was no sequence homology detectable by hybridisation between the satellite RNA and either genomic RNA (Robinson, 1982). Direct nucleotide sequencing of the 3'-termini of RNA-1 and RNA-2 indicate a region of near homology extending approximately 100 bases from the polyadenylate tract (S. Dodd PhD Thesis, Dundee 1984.)

Objective of this Research Work.

Experiments involving isolated TBRV RNA-1 and RNA-2 have been hindered by the difficulty of obtaining pure preparations of either RNA molecule (Murant et al, 1973). Separation techniques have relied upon size differences between these molecules, however, degradation of TBRV RNA-1 could yield molecules which have a molecular weight nearer to that of TBRV RNA-2. It is also possible that RNA degradation products may aggregate and separation be incomplete. Results obtained from such experiments (Harrison & Murant, 1977; Fritsch et al, 1980; Robinson et al, 1980; Mayo et al. 1982(b); Robinson, 1982; Mayo & Barker, 1983), although providing strong evidence as to the roles of these molecules, cannot be considered absolutely conclusive because of background levels of replication ascribed to contamination of RNA-2 by RNA-1 and vice versa.

The objective of this research work is to develop a reliable method of separating the three TBRV RNA molecules. Separation by hybridisation selection was employed by Adams et al (1979).
Normally purification of about 50-fold can be achieved by a single cycle of hybridisation. Further purification may be achieved by repeating the cycle.

We propose to synthesise TBRV cDNA and to clone

these molecules into Escherichia coli (E. coli). Colonies containing recombinant DNA will be identified. These colonies will contain sequences derived from either TBRV RNA-1, -2 or -3, but not from more than one. The recombinant DNA can be denatured and coupled to a solid matrix such as epoxy-activated cellulose. Complementary RNA can then be hybridised to the bound DNA. RNA separated in this manner will be of one species only.

The recombinant DNA may also be used for sequencing studies.

Introduction to Techniques used in this Research Work.

Synthesis of Complementary DNA.

Single-stranded cDNA can be synthesised using reverse transcriptase (avian myeloblastosis virus). This reaction requires an RNA template base-paired to a primer which has a free 3'-OH group. TBRV RNA has a 3'-polyadenylate group which can be hybridised to a primer consisting of a oligo-deoxythymidine (dT) sequence. After first-strand synthesis has been completed, the RNA can be hydrolysed.

Synthesis of a second cDNA strand, complementary to the first, can be carried out using DNA polymerase I (*E. coli*) which also requires a base-paired primer with a free 3'-OH group. cDNA is capable of forming, at its 3'-termini, hairpin structures by intramolecular base-pairing. This transient structure can act as a primer for the synthesis of a second-strand cDNA.

The hairpin loop and any single-stranded DNA at the other end can be removed by digestion with the single-strand-specific endonuclease, Nuclease S1 (*Aspergillus oryzae*). This yields a duplex DNA molecule (see figure 1). (For a general review, see Old & Primrose, 1982.)

Synthesis of a cDNA copy of a polyadenylated mRNA

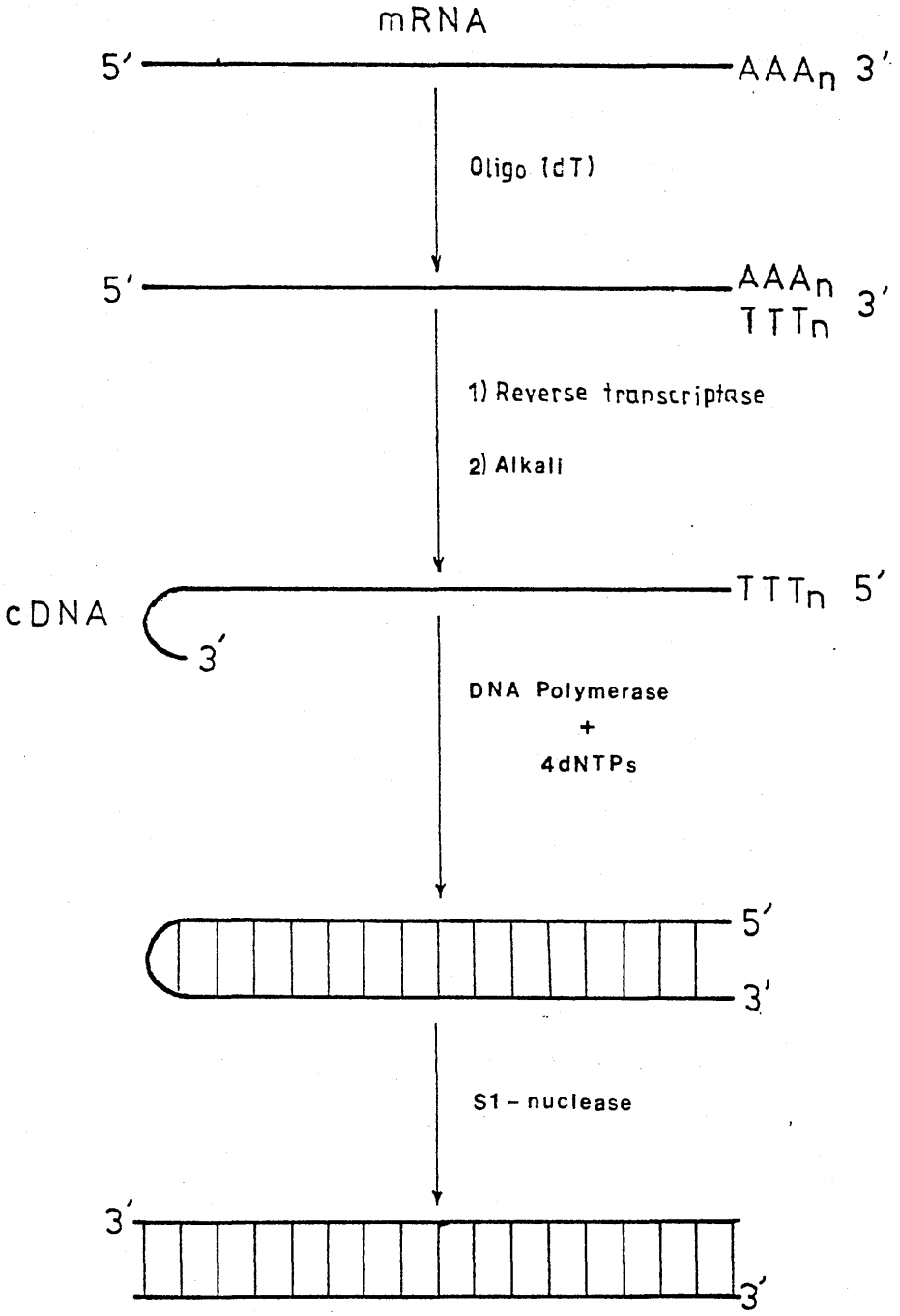

figure 1.

Cloning Recombinant DNA Molecules.

Cloning procedures involve joining foreign DNA (cDNA) to plasmid DNA to make a recombinant DNA molecule (Old & Primrose, 1982). Plasmids are covalently, closed circular molecules of double-stranded DNA, and are found in many bacterial species. The plasmid DNA can be linearized by cleaving it with a suitable restriction enzyme and the cDNA can be enzymatically joined into this site (see figure 2) (Maniatis et al, 1982).

In nature, many plasmids are self-transmissible to new hosts. This procedure can be achieved experimentally by treating host cells to make them temporarily receptive to the plasmid DNA (i.e. make the cells competent) (Cohen et al, 1973). This laboratory technique is termed transformation.

Many of the naturally occurring plasmids are unsuitable as cloning vehicles, but by applying the techniques of gene manipulation, plasmids have been constructed which possess properties which make them good vectors (Vieira & Messing, 1982). A plasmid vector should have the following properties (Maniatis et al, 1982) :

1. one or more marker functions to enable selection of bacteria carrying the plasmid.

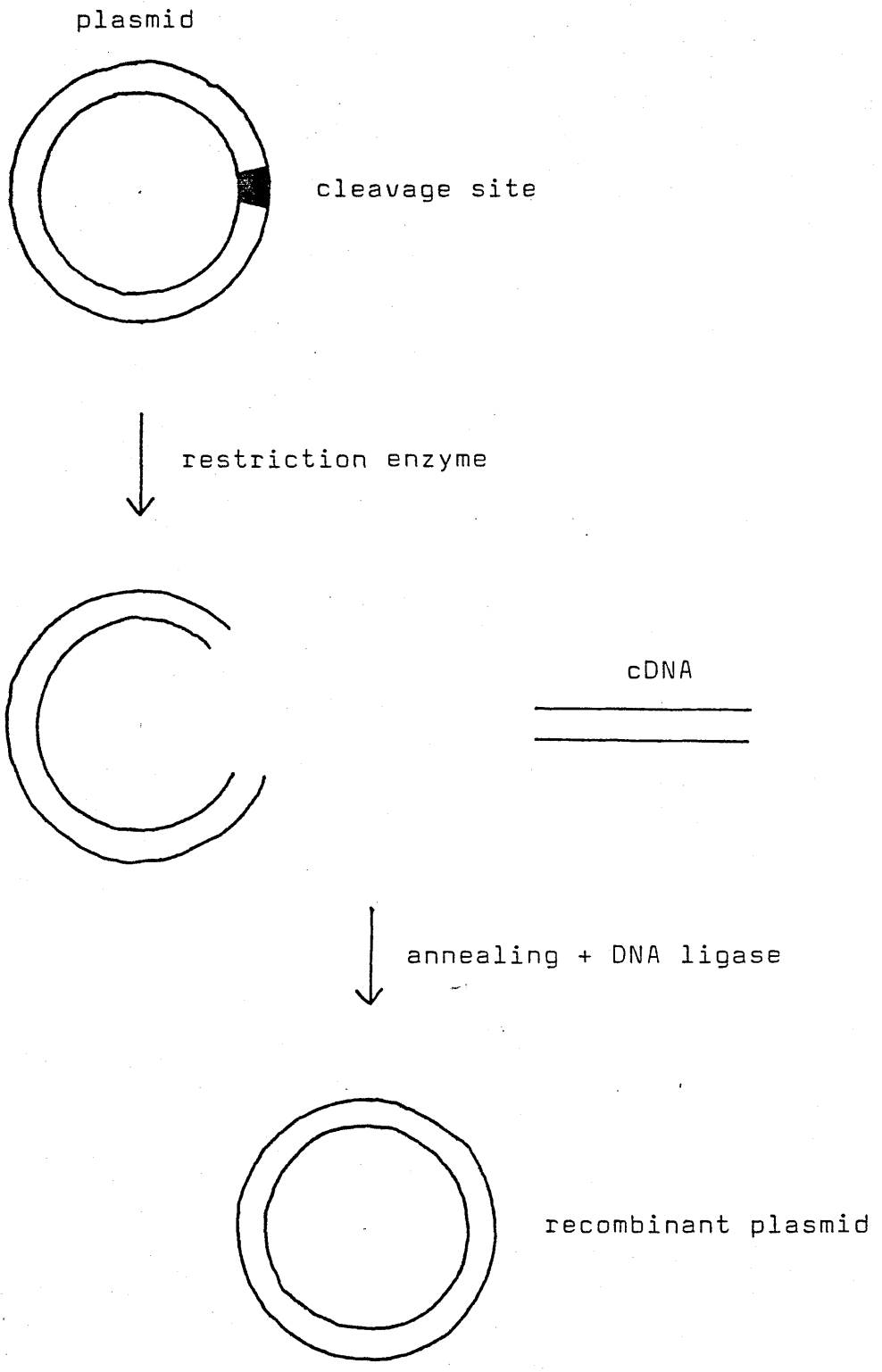

figure 2. formation of a recombinant DNA molecule

2. possess single restriction endonuclease cleavage sites in regions non-essential for DNA replication - ideally the restriction sites should be located within the genes coding for the marker function.
3. a plasmid should be as small as possible.
4. plasmid replication should be under "relaxed" control i.e. replicated independantly of the chromosomal DNA.

The plasmid vector which will be used in this work is pUC9 (Vieira & Messing, 1982). This plasmid has, as its selectable marker, a gene for ampicillin resistance; an origin of replication derived from pBR322 and a lac complementation gene derived from phage M13 mp9 (see figure 3). A multiple cloning site (restriction enzyme cleavage sites) is located within the lac complementation gene (see figure 4) (Vieira & Messing, 1982). The pUC9 plasmid is small (2.7Kb) and its replication is under relaxed control (Vieira & Messing,1982).

Insertion of pUC9 into a suitable host eg. JM83 (Vieira & Messing, 1982) renders it lac^+ by complementation with a defective β-galactosidase gene carried on the bacterial chromosome. Addition of the chromogenic substrate X-gal(5-bromo-4-chloro-3-indolyl-β-D-galactoside) to the medium allows identification

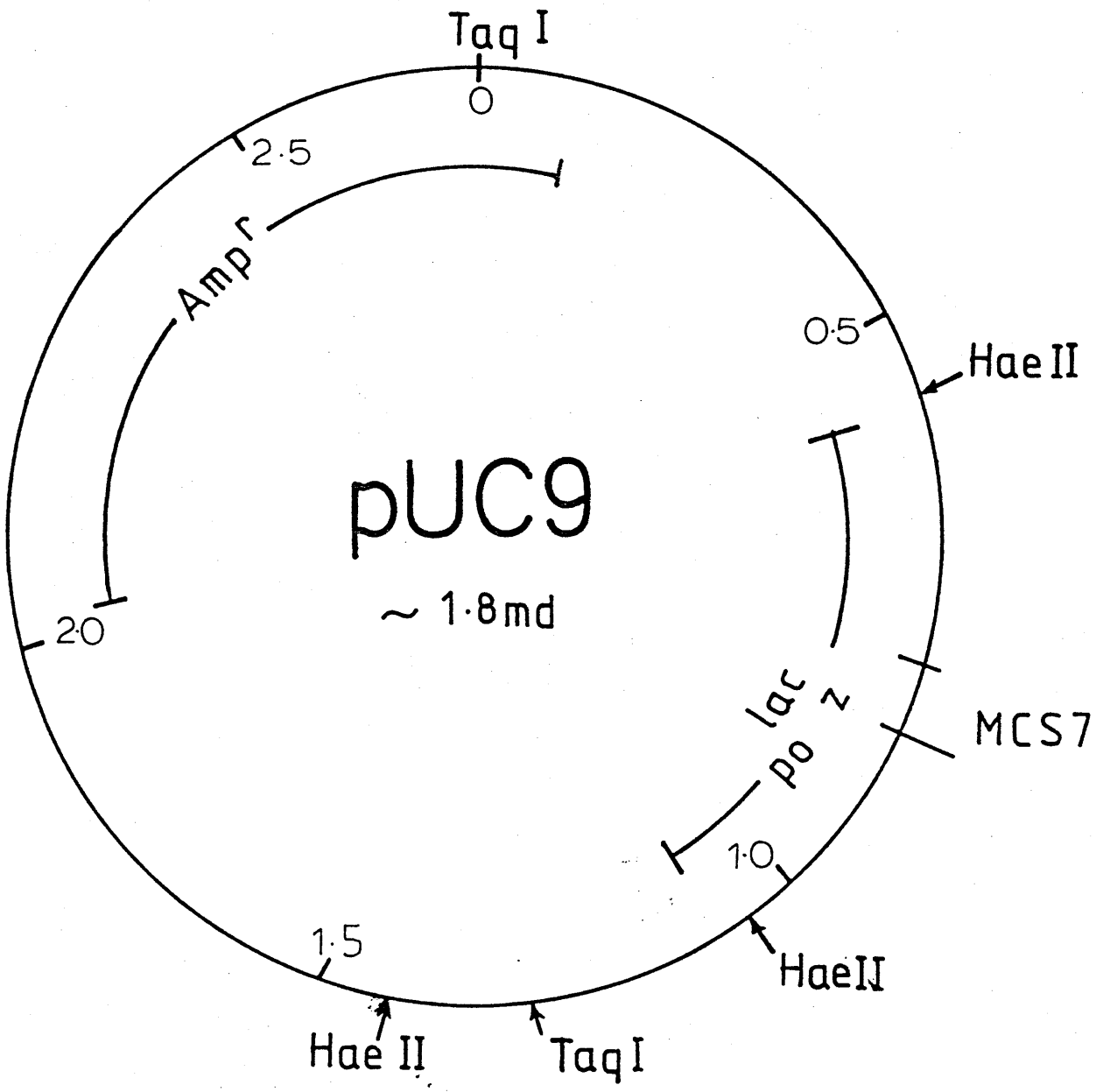
Taq I
0
2·5
Amp r
0·5
Hae II
pUC9
~ 1·8md
2·0
lac
po z
MCS 7
1·0
1·5
Hae II
Hae II
Taq I

figure 3.

pUC 9

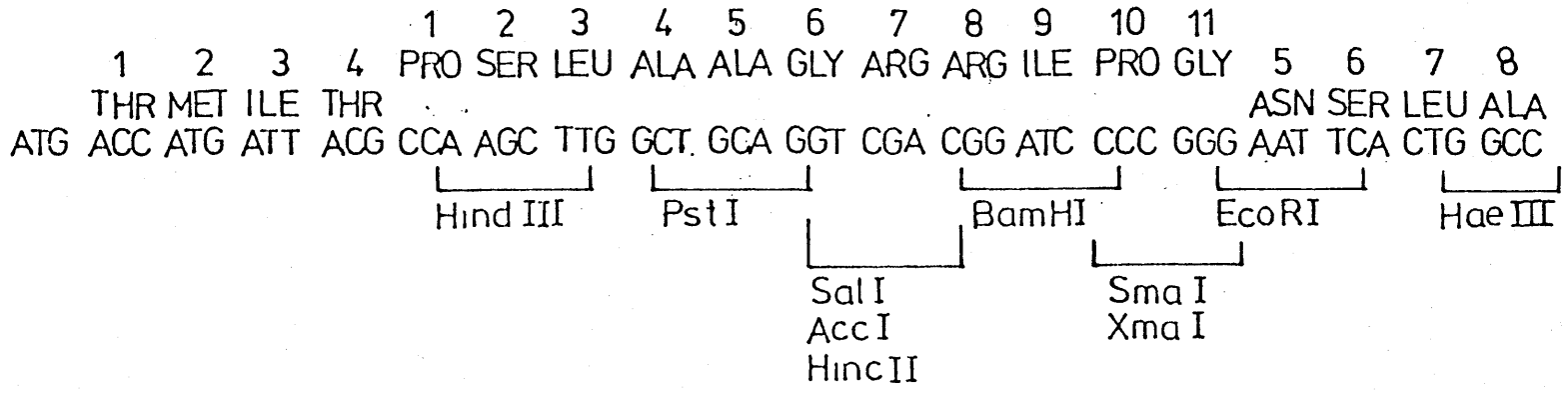

figure 4.

of lac^+ colonies which appear blue. Inserting foreign DNA into the multiple cloning site of pUC9 interrupts the coding sequence of the β-galactosidase fragment, therefore the X-gal substrate will not be metabolised and the bacterial colonies will remain white.

The ampicillin resistant, lac^- colonies will be tested for the presence of TBRV cDNA sequences by the colony hybridisation method of Grunstein and Hogness (1975). Colonies containing recombinant DNA will be identified as TBRV RNA-1, -2, or -3 by the method of Thomas (1980).

MATERIALS AND METHODS

1. Analysis of TBRV RNA.

Purified TBRV RNA was supplied, as an ethanol precipitate, by Dr. M.A. Mayo (Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA). TBRV (strain G), belonging to the potato bouquet serotype, was propagated on Nicotiana clevelandii. The virus was purified from systemically infected leaves by the method of Mayo et al (1982(b)). The RNA was prepared from the virus particles by a two-phase phenol method (Harrison & Barker, 1978).

The RNA was dried under vacuum for 15 minutes and resuspended to a concentration of approximately 1mg ml^{-1} in 0.1x STE (1x STE is 180mM sodium chloride, 20mM Tris-HCl pH 7.4, 2mM EDTA). TBRV RNA was checked for purity and integrity by electrophoresis on a denaturing agarose gel (Rave et al, 1979). Brome mosaic virus RNA (mol. wt. 1.1, 1.0, 0.75, 0.3 x 10^6) (Lane, 1977) was electrophoresed as a standard. A 1.2% agarose gel was prepared by mixing 1.2g agarose, 10ml 10x running buffer (0.2M HEPES, 10mM EDTA, pH7.8) and 73ml water. After boiling for 15 minutes, the gel was cooled to 60^{o}c and 16.7ml 37% formaldehyde was added. The gel was poured into a horizontal gel former.

The RNA samples (4µg) were mixed with 23µl buffer X (deionised formamide, 10x running buffer,

37% formaldehyde, 5:1:2 by volume) and were denatured by incubation at 65°c for 10 minutes. The samples were quick-chilled on ice to prevent re-annealing and 3µl 0.02% bromophenol blue in 50% glycerol, 1x running buffer was added. The samples were loaded, the wells sealed with vaseline and electrophoresis was carried out at 25 - 35mA for 2 - 3 hours in 1x running buffer.

The bands were visualised by staining the gel in a 0.2% solution of toluidine blue in 0.4M sodium acetate and 0.4M acetic acid for 30 minutes, followed by washing in water.

2. Synthesis of Complementary DNA.

(a) First-Strand Synthesis.

A 20µl reaction mixture was prepared from sterile stock solutions and buffers by a modification (Maniatis et al, 1982) of the method of Efstratiadis and Villa-Komaroff (Efstratiadis & Villa-Komaroff, 1979).

buffer or solution	final concentration
1M Tris-HCl (pH 8.3 @ 42°c)	0.1M
1M KCl	0.15M
0.1M $MgCl_2$	0.01M
0.7M β-2-mercaptoethanol	0.04M
5mM dNTPs	0.5mM
oligo $(dT)_{12-18}$ (8 units ml^{-1})	0.016 units
Human Placental Ribonuclease Inhibitor	15 units
TBRV RNA	200µg ml^{-1}
^{32}P-dCTP (3×10^3 Ci mM^{-1})	10µCi
Reverse transcriptase	11 units
	20µl

Incubation was carried out at 42°c for 1 - 3 hours. The reaction was terminated by the addition of 2µl 0.25M EDTA. The products of five 20µl reactions were pooled.

The amount of first-strand cDNA made was estimated by gel filtration of a 5µl sample on a

Sephadex G-100 column (1cm x 15cm), equilibr ated in 0.1x STE. Twenty-five fractions (approximately 0.5ml) were collected and 200µl of each fraction, mixed with 5ml of liquid Scintillator Unisolve E (koch-Light), were counted in a Packard liquid scintillation spectrometer model 3380.

The reaction mixture was incubated at 65°c for 1 hour with 15µl 0.5M sodium hydroxide in order to hydrolyse the RNA template. The sodium hydroxide was then neutralised by the addition of 15µl 0.5M HCl and the cDNA was precipitated overnight at -20°c in the presence of 0.1 volume 4M potassium acetate pH 4.8 and 1.1 volumes 100% isopropanol. cDNA was pelleted by centrifugation at 10,000r.p.m. for 10 minutes in an MSE micro centaur centrifuge. The pellet was washed in 100% ethanol and dried under vacuum for 10 minutes.

(b) Second-Strand Synthesis.

The dried cDNA pellet from the first-strand synthesis experiment was resuspended in a 150µl reaction mixture using sterile stock solutions and buffers (Efstratiadis & Villa-Komaroff, 1979 as modified by Maniatis et al, 1982).

buffer or solution	final concentration
0.2M HEPES pH 6.9	0.1M
1M KCl	0.07M
0.1M $MgCl_2$	0.01M
20mM DTT	2.4mM
5mM dNTPs	0.5mM
Bovine serum albumin	1%
DNA polymerase I	20-50 units μg^{-1} DNA
^{3}H-dCTP (19 Ci mM^{-1})	50μCi
	150μl

The reaction was incubated overnight at 15^{o}c and was terminated by the addition of 15μl 0.25M EDTA. The amount of second-strand cDNA made was estimated by gel filtration of a 5μl sample on Sephadex G-100 (section 2(a)). A half volume each of chloroform and phenol/8-hydroxyquinoline was added to the reaction. The mixture was vortexed and the aqueous phase, containing the cDNA, was recovered by brief centrifugation. The organic phase was re-extracted with 0.1x STE and the two top phases were combined and precipitated overnight at -20^{o}c in the presence of 1.1 volume isopropanol and 0.1 volume 4M potassium acetate as described above (section 2(a)).

(c) Second-Strand Completion Reaction.

This reaction was carried out to allow the

extension of short-length second-strand cDNA. The dried pellet from the second-strand reaction was resuspended in a 25µl reaction mixture using sterile stock solutions and buffers (Efstratiadis & Villa-Komaroff, 1979 as modified by Maniatis et al, 1982).

buffer or solution	final concentration
0.5M Tris-HCl (pH 8.3 @ 42°c)	0.05M
1M KCl	0.14M
0.1M $MgCl_2$	0.01M
0.35M β-2-mercaptoethanol	0.04M
5mM dNTPs	0.5mM
Reverse transcriptase	11 units
^{3}H-dCTP (19 Ci mM^{-1})	25µCi
	25µl

The reaction was incubated at 42°c for 3 hours and was terminated by the addition of 3µl 0.25M EDTA. The reaction was monitored by separating a 2µl aliquot on Sephadex G-100 (section 2(a)). The reaction was extracted with chloroform and phenol as described in section 2(b) and the cDNA was precipitated overnight at -20°c in the presence of 1.1 volume isopropanol and 0.1 volume 4M potassium acetate. The cDNA was pelleted by centrifugation at 10,000r.p.m. for 10 minutes in a MSE micro centaur centrifuge. The pellet was vacuum dried for 10 minutes.

(d) S1 Nuclease Digest of cDNA.

The dried pellet from the second-strand completion reaction was resuspended in 88µl sterile water and 10µl 10x S1 buffer (0.3M sodium acetate pH 4.6, 2M sodium chloride, 10mM zinc sulphate) (Maniatis et al, 1982). S1 nuclease (2 units μg^{-1}DNA) was added and the reaction was incubated at 37°c for 1 hour. The reaction was terminated by the addition of 4µl 0.25M EDTA. The reaction was monitored by taking 1µl samples before the incubation and directly after termination. Both samples were separated by gel filtration on Sephadex G-100 (section 2(a)). Sixteen fractions (approximately 0.5ml) were collected and 200µl of each fraction were mixed with 5ml scintillant and were counted for radioactivity in a Packard liquid scintillation spectrometer model 3380. The amount of radioactivity associated with the cDNA in each sample was compared and the susceptibility to digestion by S1 nuclease was determined.

The S1 nuclease reaction mixture was extracted with chloroform and phenol (section 2(b)) and the cDNA was precipitated overnight at -20°c in the presence of 1.1 volume isopropanol and 0.1 volume 4M potassium acetate. The pellet was collected by centrifugation at 10,000r.p.m. for 10 minutes in a MSE micro centaur centrifuge.

(e) Production of Blunt-Ended Termini on cDNA.

S1 nuclease digestion of cDNA has been reported to leave short single-stranded regions at the end of a double-stranded cDNA (Seeburg et al, 1977). These recessed 3'-groups must be converted to a double-stranded structure before ligation into a vector molecule. The cDNA pellet was resuspended in a 50µl reaction mixture using sterile stock solutions and buffers (Seeburg et al, 1977).

buffer or solution	final concentration
0.1M $MgCl_2$	0.01M
1M KCl	0.07M
0.2M HEPES pH 6.9	0.1M
20mM DTT	2.4mM
5mM dNTPs	0.05mM
Klenow fragment of DNA polymerase I	20-50 units μg^{-1} DNA
	50µl

The reaction was incubated for 30 minutes at room temperature and was terminated by the addition of 5µl 0.25M EDTA. The cDNA was precipitated overnight at $-20^{o}c$ in the presence of isopropanol and potassium acetate (section 2(a)).

(f) Size Fractionation of Double-Stranded cDNA.

The experiments described above generate cDNA which vary in length. Usually the largest cDNA molecules are selected for cloning because they represent the largest proportion of the genome. Double-stranded cDNA was size fractionated by centrifugation on a 5-20% sucrose linear gradient (Gustafson et al, 1982). Polyallomer centrifuge tubes used for the gradient were soaked for 15 minutes in 0.5M sodium hydroxide and rinsed. Sterile sucrose solutions were prepared as shown below.

sucrose% (w/v)	volume of 60% sucrose (ml)	volume of 10x STE (ml)	volume of water (ml)	volume used for gradient (ml)
5	0.83	0.1	9.07	1.8
10	1.67	0.1	8.23	3.6
15	2.50	0.1	7.40	3.6
20	3.33	0.1	6.56	1.8

Sucrose gradients were prepared by carefully layering appropriate volumes of the above solutions with a pipette and then were stored overnight at 4°c to allow formation of a continuous gradient by diffusion. The double-stranded cDNA was dissolved in 200µl 0.1x STE and layered on top of the gradient. Centrifugation was carried out at 40,000r.p.m. for 7 hours in a Sorvall TST41.14 rotor.

Gradients were fractionated using a MSE model 185 density gradient fractionator. Twenty fractions (0.5ml volume) were collected and 10µl of each were mixed with 5ml liquid scintillator and were measured for ^{32}P and ^{3}H radioactivity in a Packard liquid scintillation spectrometer model 3380. Appropriate volumes (to give 1,000c.p.m. ^{32}P) were taken from suitable fractions and their sizes determined by gel electrophoresis.

A 1.2% agarose gel was prepared by mixing 1.2g agarose with 10ml 10x TBE buffer (0.98M tris-borate, 0.89M boric acid, 0.02M EDTA, pH 8.3) and 90ml water. The agarose was melted in a boiling water bath and poured into an appropriate gel former. The cDNA samples were made up to 20µl with water and 5µl 0.02% bromophenol blue in 50% glycerol, 5x TBE buffer was added. λ DNA digested with Hind III (23, 9.9, 6.6, 4.3, 2.2, 2.0 Kb) was used as a standard. Electrophoresis was carried out at 30mA for 3 hours. The lane containing λ DNA digested with Hind III was sliced off the gel, soaked for 20 minutes in a solution of ethidium bromide (3µg ml^{-1}) and the bands visualised under ultra violet illumination. The distances migrated by the standards were measured. The rest of the gel was dried at 80°c under vacuum for 2 hours and cDNA detected by autoradiography. The gel was exposed to Kodak XRP1 x-ray film for 1 - 4 days at -70°c using

Dupont Cronex intensifier screens. The size of the cDNA was determined by comparison to the standard.

The cDNA fractions were pooled into high mol. wt. (greater than 1Kb) and low mol. wt. (0.4Kb - 1Kb) fractions and precipitated overnight at -20^{o}c in the presence of 1.1 volume isopropanol and 0.1 volume 4M potassium acetate (section 2(a)). The cDNA was collected in 1.5ml eppendorf tubes by centrifugation at 10,000 r.p.m. for 10 minutes in a MSE micro centaur centrifuge. The pellet was washed twice in 100% ethanol and dried under vacuum for 10 minutes. The cDNA was resuspended in 20µl 0.1x STE and 1µl aliquots of each fraction were measured for ^{32}P radioactivity.

3. Cloning Recombinant DNA Molecules.

(a) Large Scale Preparation of Plasmid DNA.

Plasmid DNA was prepared by a modification of the alkaline method of Birnboim and Doly (1979). *E. coli* glycerol stock solution (20µl) was inoculated into 2ml sterile L-broth (5g sodium chloride, 5g yeast extract, 10g tryptone per litre water, pH 8.0) containing 50µg ml^{-1} ampicillin. The cells were grown by incubating at 37°c overnight with vigorous shaking. The 2ml culture was transferred to 50ml L-broth containing 50µg ml^{-1} ampicillin and was further incubated at 37°c until the culture reached the late log phase (OD_{600} of 0.6). An aliquot of 15ml cell suspension was transferred to 1 litre L-broth containing 50µg ml^{-1} ampicillin and was incubated at 37°c with vigorous shaking until the OD_{600} was approximately 0.4. Chloramphenicol, which inhibits chromosomal replication but not plasmid replication, was added to the 1 litre cell culture (75mg chloramphenicol and incubation at 37°c (vigorous shaking) was carried out for a further 12 - 16 hours (Clewell, 1972).

The cells were harvested by centrifugation at 7,000r.p.m. for 10 minutes (4°c) in a MSE scientific instruments 18 centrifuge. The pellets were combined and resuspended in 10ml lysis solution (50mM glucose,

25mM tris-HCl pH 8.0, 10mM EDTA, 5mg ml^{-1} lysozyme) and left at room temperature for 5 minutes. Twenty ml alkaline solution (0.2M sodium hydroxide, 1% SDS) was added and the cells were left on ice for 10 minutes. Ice cold 5M potassium acetate pH 4.8 (15ml) was added and the cells were left on ice for a further 10 minutes. Bacterial debris and cell DNA was removed by centrifugation at 20,000r.p.m. for 20 minutes ($4^{o}c$) in an MSE 18 850 rotor. Isopropanol (0.6 volumes) was added and the cells were incubated at $-20^{o}c$ for 1 hour. The DNA was recovered by centrifugation at 15,000r.p.m. for 5 minutes in a MSE 18 850 rotor. The supernatant was discarded and the tubes inverted and left to drain.

Further purification of the plasmid was carried out by isopycnic centrifugation in caesium chloride-ethidium bromide gradients. The pelleted DNA was resuspended in 8 ml TE buffer (10mM tris-HCl, 1mM EDTA, pH 8.0) and 1g solid caesium chloride was added per 1ml DNA solution. Ethidium bromide (0.8ml) (10mg ml^{-1}) was added per 10ml caesium chloride solution. The DNA was centrifuged in a Sorvall 865.1 rotor for 40 hours at 40,000r.p.m. ($20^{o}c$). The DNA was visualised by its fluorescence under a long-wavelength UV light. The DNA separated into two fractions, the upper fraction consisting of chromosomal and nicked circular plasmid DNA, the lower of closed circular pUC9 DNA.

The lower fraction was removed by syringe and the ethidium bromide removed by shaking the plasmid solution with six changes of an equal volume of isopropanol saturated with water. The lower phase (containing the plasmid) was dialysed against several changes of TE buffer pH 8.0 and 5mg ml^{-1} proteinase K was added. The reaction was incubated at $20^{o}c$ for 20 minutes, followed by centrifugation at 5,000r.p.m. for 5 minutes in a MSE 18 850 rotor. The solution was extracted with phenol and chloroform (secton 2(b)) and the plasmid was precipitated overnight at $-20^{o}c$ in the presence of 1.1 volume isopropanol and 0.1 volume 4M potassium acetate. After centrifugation at 15,000r.p.m. for 15 minutes in an MSE 18 850 rotor, the plasmid pellet was resuspended in 0.5ml 0.1 x STE and re-precipitated in the presence of isopropanol and potassium acetate. The plasmid DNA was finally resuspended in 0.5ml 0.1 x STE.

(b) Construction of Recombinant Plasmid.

Covalently, closed circular plasmid DNA must be linearized prior to the formation of a recombinant DNA molecule (Maniatis et al, 1982). pUC9 was linearized by cleaving with the restriction enzyme, Hinc II which generates blunt-ended termini in the multiple cloning site. A 50µl reaction was prepared from

sterile stock solutions and buffers as detailed on Bethesda Research Laboratories information sheet. The final solution contained 1 x Hinc II buffer(0.01M Tris-HCl, 0.06M sodium chloride, 6.6mM magnesium chloride), 10µg pUC9, 0.04% bovine serum albumin, 1mM DTT, 0.05M spermidine, 20 units of Hinc II endonuclease. The reaction was incubated at 37°c for 3 hours and terminated by the addition of 2µl 0.5M EDTA. The reaction mixture was extracted with phenol and chloroform and the DNA was precipitated overnight in the presence of 1.1 volume isopropanol and 0.1 volume 4M potassium acetate (section 2(a)). The DNA was pelleted by centrifugation at 10,000r.p.m. for 10 minutes in a MSE micro centaur centrifuge and was resuspended in 20µl 0.1 x STE. A 0.5µg aliquot of DNA was electrophoresed on a TBE gel (section 2 (f)) along with a standard (λ DNA digested with HindIII) and uncut pUC9 to check that linearization had taken place.

In order to prevent the re-circularisation of pUC9, the plasmid was incubated in the presence of calf intestine alkaline phosphatase, which removes terminal 5'-phosphate groups from both DNA strands (Seeburg et al, 1977). Ten µg pUC9 linearized with Hinc II was incubated with 50mM Tris-HCl pH 8.0, 1mM EDTA and 3.9 units of alkaline phosphatase for 1 hour at 37°c. The enzyme was inactivated by the addition of 3µl 0.2M sodium nitriloacetic acid pH 7.5 followed

by incubation at 65°c for 1 hour (Boehringer instruction sheet). Fifty µl 0.1 x STE was added and the reaction was extracted with three changes of phenol and chloroform. The DNA was precipitated overnight in the presence of 1.1 volume isopropanol and 0.1 volume 4M potassium acetate. The DNA was pelleted by centrifugation at 10,000r.p.m. as described above and resuspended in 0.1 x STE.

Ligation reactions were prepared by mixing 600ng linearized, phosphatased pUC9 and 280ng TBRV cDNA high mol. wt. fraction. TBRV cDNA (200ng) low mol. wt. fraction and linearized, phosphatased pUC9 (400ng) were similarily prepared. Both mixtures were dried under vacuum for 15 minutes and were resuspended in 4µl 20mM Tris-HCl pH 7.6, 10mM magnesium chloride, 10mM DTT, 0.6mM ATP, 0.04% bovine serum albumin and 1µl T_4 DNA ligase (BRL information sheet). The reactions were incubated overnight at 20°c and terminated by the addition of 2µl 0.25M EDTA. Before phenol/chloroform extraction, 8µl (3mg ml^{-1}) tRNA was added as a carrier. The recombinant DNA was precipitated overnight in the presence of 1.1 volume isopropanol and 0.1 volume 4M potassium acetate, pelleted by centrifugation at 10,000r.p.m. for 10 minutes as described above, and resuspended in 10µl 0.1 x STE.

(c) Transformation of *E. coli*.

Transformation of *E. coli* involves introducing the recombinant DNA molecules into host cells which can be made "competent" to the uptake of foreign DNA by calcium chloride treatment (Cohen *et al*, 1973). The modified procedure of Dagert and Ehrlich (1979), which was used in this work, yields four to six times as many transformed cells as Cohens original procedure. One drop of a glycerol stock of *E. coli* cells (strain JM83) was inoculated into 5ml sterile L-broth and the cells were grown by incubating overnight at 37°c (shaking incubator). A 0.5ml aliquot of cell suspension was withdrawn under sterile conditions, inoculated into 50ml sterile L-broth and further multiplied by incubating at 37°c for 2 - 3 hours in a shaking incubator to an absorbance value (A_{650nm}) of 0.2 - 0.3. The cell culture was immersed in ice for at least 20 minutes to stop cell growth and the cells were harvested by centrifugation in a MSE scientific instruments 18 centrifuge 850 rotor at 7,000r.p.m. for 5 minutes (4°c) using sterile centrifuge tubes. The supernatant was removed and the cells were gently resuspended in 20ml ice-cold sterile 0.1M calcium chloride. The cell suspension was incubated on ice for 20 minutes and collected by centrifugation at 7,000r.p.m. for 5 minutes (4°c). At this stage, rather than a normal pellet, the cells form a pellet with the

shape of a halo. The cells were resuspended in 0.5ml 0.1M calcium chloride and stored on ice until required (approximately 24 hours later).

In order to ensure that the cells had been rendered competent, 50µl of cells were withdrawn after 1 hour incubation with calcium chloride and transformed with 50ng pUC9 DNA. Cells which yielded less than 2×10^6 amp^r colonies per µg of DNA were discarded. Transformation was carried out by mixing 50µl cells and 5µl suitably diluted plasmid in a sterile tube which was incubated on ice for 10 minutes. The cells were heat-shocked at 37^oc for 5 minutes. One ml sterile L-broth was added and the cells were incubated at 37^oc for 1 hour (shaking incubator).

Agar plates were prepared by mixing 100ml L-broth, 1g agar, 100mg l^{-1} ampicillin (added after autoclaving) and 50 µg ml^{-1} X-gal. Ten µl of transformed cells were inoculated onto 82mm diameter plates. The inoculated agar plates were incubated overnight at 37^oc.

4. Selection of Recombinant Clones.

Amp^r, lac^- colonies were tested for the presence of TBRV cDNA sequences by the colony hybridisation method of Grunstein and Hogness (1975) with some modifications (D. Hughes, personal communication).

(a) Transfer of Colonies to Nitrocellulose.

Colonies were transferred to nitrocellulose by gently pressing a nitrocellulose membrane (Schleider & Schnell BA83, 82mm diameter) onto the agar surface until the filters became wet. The filters, with the colonies attached, were removed and air dried for 30 minutes and then placed for another 30 minutes on two thicknesses of 3MM filter paper (soaked in 0.5M sodium hydroxide and 0.8M sodium chloride) in order to lyse the cells and denature the DNA. The alkali was neutralised by placing the filters for 30 minutes on two thicknesses of 3MM filter paper soaked in 1M Tris-HCl, pH 7.5 and 1.5M sodium chloride. Lipids were removed from the nitrocellulose filters by placing them for 20 seconds on two thicknesses of 3MM filter paper soaked in chloroform. After washing in 2x SSC (0.3M sodium chloride, 0.03M sodium citrate, pH 7.0), the filters were air-dried for 30 minutes and the DNA fixed permanently by baking for 2 - 3 hours under vacuum at $80^{o}c$. The filters were placed back to back in a sealable polythene bag.

(b) Hybridisation to Nitrocellulose Filters.

Recombinants were identified by hybridisation using ^{32}P-labelled TBRV cDNA as probe according to Grunstein and Hogness (1975) with minor modifications. Hybridisation buffer was prepared from sterile stock solutions.

stock solution	volume (ml)
20x SSC	3.0
2% (w/v) PVP/ficol	0.1
2% (w/v) BSA	0.5
sheared denatured herring sperm DNA (5mg ml^{-1})	0.25
10% SDS	0.5
water	5.65

The nitrocellulose filters were sealed in polythene bags with 3ml of hybridisation buffer and prehybridisation was carried out at 65^{o}c for 3 hours in a shaking water bath. The bags were opened and the buffer replaced with a fresh 3ml of hybridisation buffer containing approximately 100,000 c.p.m. of TBRV cDNA (see below) and hybridisation was carried out overnight at 65^{o}c in a shaking water bath. the nitrocellulose filters were washed twice by shaking in 2x SSC containing 0.1% SDS, pH 7.0 for 15 minutes at room temperature followed by two washes for 15 minutes at 65^{o}c. Hybridisation was detected by autoradiography to Kodak XRP1 x-ray film for 1 - 3 days at -70^{o}c using Dupont Cronex intensifier screens.

(c) Synthesis of High Specific Activity cDNA.

High specific activity cDNA was prepared by the same method detailed in section 2(a), except that the concentration of dCTP was 0.025mM and 20μCi ^{32}P-dCTP was used.

5. Isolation and Characterisation of Recombinant DNA.

(a) Isolation of Recombinant DNA.

Colonies which gave a positive hybridisation signal were inoculated into 2ml sterile L-broth containing 100mg ml^{-1} ampicillin and were multiplied by shaking overnight at $37^{o}c$. Recombinant plasmid DNA was prepared by the method of Holmes and Quigley (1981). *E. coli* cells were harvested by centrifugation at 10,000 r.p.m. for 2 minutes in a 1.5ml centrifuge tube in a MSE micro centaur centrifuge. The cells were resuspended in 35µl STET buffer (8% sucrose, 5% triton X-100, 50mM EDTA, 50mM Tris-HCl pH 8.0), vortexed hard and left at room temperature for 10 minutes. Five µl of freshly prepared lysozyme solution (10µg ml^{-1} in water) was added, the mixture vortexed hard and left at room temperature for 5 minutes. The mixture was boiled for 2 minutes, immediately chilled on ice, and centrifuged at 10,000 r.p.m. for 10 minutes. The gelatinous pellet was removed by a sterile toothpick and the crude plasmid DNA was precipitated from the supernatant in the presence of 1.1 volume isopropanol and 0.1 volume 4M potassium acetate at $-20^{o}c$ for 5 minutes. The DNA was pelleted by centrifugation at 10,000 r.p.m. for 10 minutes, washed twice with 100% ethanol, dried under vacuum for 15 minutes and resuspended in 20µl 0.1x STE.

In order to remove contaminating RNA, .1µl ribonuclease A (40µg ml^{-1}) and 1µl ribonuclease T_1 (2 units μl^{-1}) were added and the mixture was incubated at 37°c for 30 minutes. To remove the ribonucleases and other contaminating proteins, 2µl proteinase K (5mg ml^{-1}) was added, the mixture incubated for 30 minutes at 37°c and extracted with phenol/chloroform as described in section 2(b). Plasmid DNA was precipitated from the aqueous phase with isopropanol as described above and finally resuspended in 20µl 0.1x STE.

(b) Size Determination of Recombinant DNA.

The recombinant DNA molecules were digested with restriction enzymes, chosen to cleave out the inserted cDNA, and the length of the inserts determined by agarose gel electrophoresis (Maniatis *et al*, 1982). The inserted cDNA was cleaved from the plasmid vector by digestion with a mixture of *Hind III* and *EcoR1* (*Hind III* restriction enzyme information sheet). The reaction mixture contained in a total volume of 20µl; 50mM Tris-HCl pH 8.0, 10mM magnesium chloride, 50mM sodium chloride, 0.05% (w/v) BSA, 2.5mM spermidine, 1mM DTT, approximately 0.5µg plasmid DNA, 5 units *HindIII* and 5 units *EcoR1*. The reaction was incubated at 37°c for 3 hours and terminated by the addition of 2µl 0.25M EDTA. Five µl of 0.02% bromophenol blue in 50%

glycerol, 5x TBE buffer was added and the DNA electrophoresed on a 1.2% agarose TBE gel (section 2(f)). DNA (λ DNA digested with HindIII and EcoR1; 21226, 5148, 4973, 3530, 2027, 1904, 1584, 1330, 983, 831, 564, 125 base pairs and pHP34 digested with Taq1;1444, 1307, 616, 378, 315/312 base pairs) (Prentki & Krisch, 1981) was used as standards. The gels were stained with ethidium bromide and the DNA visualised under UV light.

(c) Blot Transfer Hybridisation of Plasmid DNA to TBRV RNA

TBRV RNA from a satellite-containing strain was electrophoresed on a 1.2% agarose formaldehyde gel (section 1) in thirteen parallel lanes, each containing 2µg RNA. The RNA in one lane was visualised by staining and the rest was transferred to Biodyne A-Nylon 66 membrane according to Thomas (1980) as modified by Pall Corporation (Biodyne instruction manual). The gel was placed on top of 3MM filter paper (soaked in 20x SSC) and supported by a glass plate over a reservoir of 20x SSC. Strips of parafilm were placed closely around the gel to ensure that capillary movement of buffer did not by-pass the gel. A strip of Biodyne A membrane (cut to the same size as the gel) was placed on top and was covered with two layers of 3MM filter paper (soaked in 20x SSC) and a stack of paper towels, approximately 5cm thick. A flat weight of 1kg was placed on top and the

apparatus left overnight.

Following transfer, the Biodyne A membrane was removed and the RNA fixed by drying at 80°c for 1 hour. The Biodyne A membrane was cut into individual lanes which were separately sealed in polythene bags. The immobilised TBRV RNA was hybridised to selected plasmid DNA which was labelled with ^{32}P-dCTP by a modification of the nick translation method of Rigby et al (1977). Deoxyribonuclease was stored as a 1mg ml^{-1} stock in 0.1M HCl at -20°c. Two µl of this stock was diluted 1:9 in activation buffer (10mM tris-HCl pH 7.5, 5mM magnesium chloride, 100µg ml^{-1} BSA) and left on ice for 2 hours. Immediately before use, a 1:49 dilution, followed by a 1:39 dilution of deoxyribonuclease with activation buffer was made to give a final enzyme concentration of 50ng ml^{-1}. The nick translation reaction mixture was prepared from sterile stock solutions.

buffer or solution	volumes (µl)
0.1mM dCTP	0.8
0.4mM dNTP (-dCTP)	1.0
2x nick translation buffer	1.5
activated deoxyribonuclease	0.4
^{32}P-dCTP (dried)(3000 Ci mM^{-1})	10µCi
plasmid DNA	50ng

(2x nick translation buffer is 66.7mM potassium phosphate, 6.7mM magnesium chloride, pH 7.5)

The reaction was incubated at room temperature for 60 seconds, immediatly chilled on ice, and approximately 0.5µl of DNA polymerase I (representing 1 - 4 units) was added. The reaction was incubated overnight at 15°c and was terminated by the addition of 50µl 0.1x STE and 5µl 0.25M EDTA pH 8.0. Eight µl $tRNA$ (3mg ml^{-1}) was added as a carrier and the deoxyribonuclease was denatured by incubation at 70°c for 10 minutes. The ^{32}P-labelled plasmid DNA was separated from unreacted nucleotides by precipitation overnight at -20°c in the presence of 1.1 volume isopropanol and 0.1 volume 4M potassium acetate as previously described (section 2(a)).

Immediatly prior to hybridisation, the ^{32}P-labelled plasmid DNA was denatured by incubating an aliquot at 65°c for 10 minutes with 0.1 volume 1M sodium hydroxide. The reaction was chilled on ice to prevent re-annealing and the alkali was neutralised by the addition of 0.1 volume 1M HCl.

The strips of Biodyne A membrane were sealed in polythene bags with 1.5ml hybridisation buffer (0.75M sodium chloride, 0.04M sodium phosphate pH 6.8, 0.01M EDTA, 50% deionized formamide, 0.1% (w/v) of

ficoll, PVP and BSA, 0.3% (w/v) SDS, 0.25mg ml^{-1} sheared denatured herring sperm DNA and 0.04mg ml^{-1} poly U) (Thomas, 1980) and prehybridisation was carried out by shaking overnight at 42°c.

The buffer was replaced with a fresh solution containing 0.5 x 10^6 c.p.m. ^{32}P-labelled plasmid DNA and hybridisation was carried out by shaking overnight at 42°c. The Biodyne strips were washed twice (each 5 minutes) in 100ml 1x SSC/0.1% SDS at room temperature, followed by two washes (each 15 minutes) at 50°c in the same buffer. Hybridisation was detected by autoradiography to Kodak XRP1 x-ray film for 1 - 3 days at -70°c using Dupont Cronex intensifier screens.

1. Analysis of TBRV RNA.

TBRV RNA samples were analysed by agarose gel electrophoresis. All batches could be resolved into three species as expected (see photograph of gel, figure 5). The molecular weights of the RNA bands, calculated by comparison to BMV RNAs, were 2.2×10^6, 1.5×10^6 and 4.8×10^5 close to the expected values of 2.8×10^6, 1.6×10^6 and 4.8×10^5 (Murant et al, 1973; Harrison & Murant, 1977). (see figure 6).

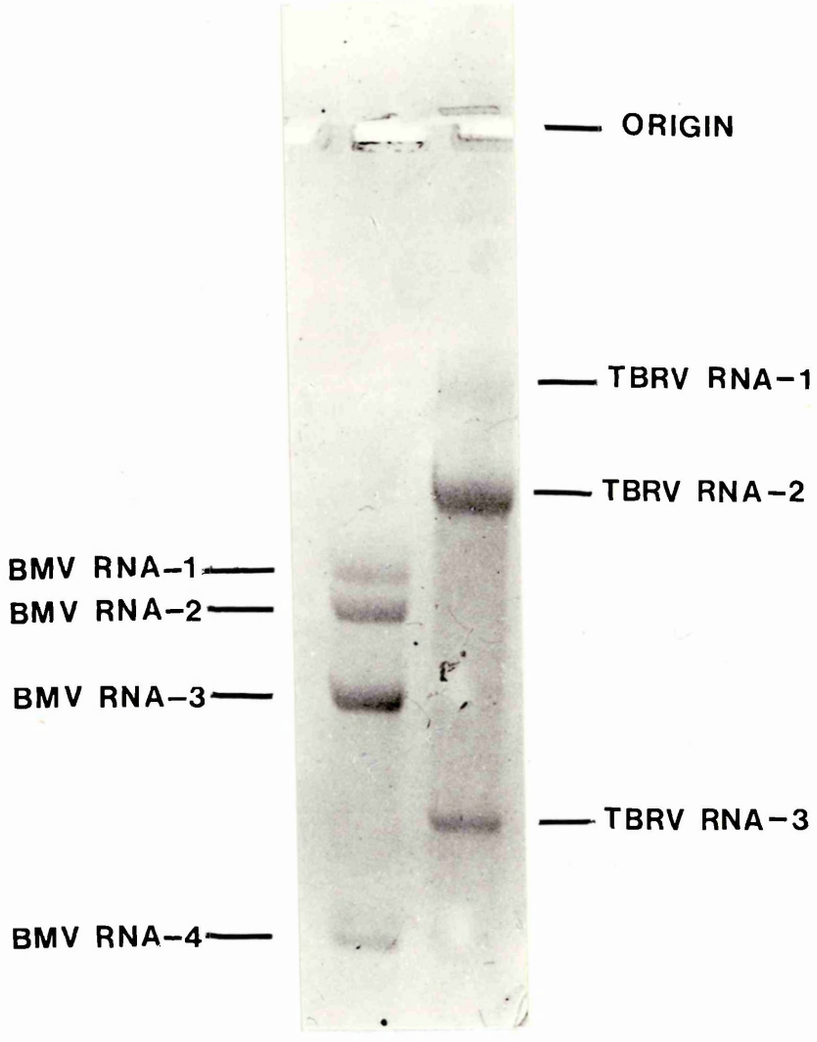
ORIGIN
TBRV RNA-1
TBRV RNA-2
BMV RNA-1
BMV RNA-2
BMV RNA-3
TBRV RNA-3
BMV RNA-4

figure 5.

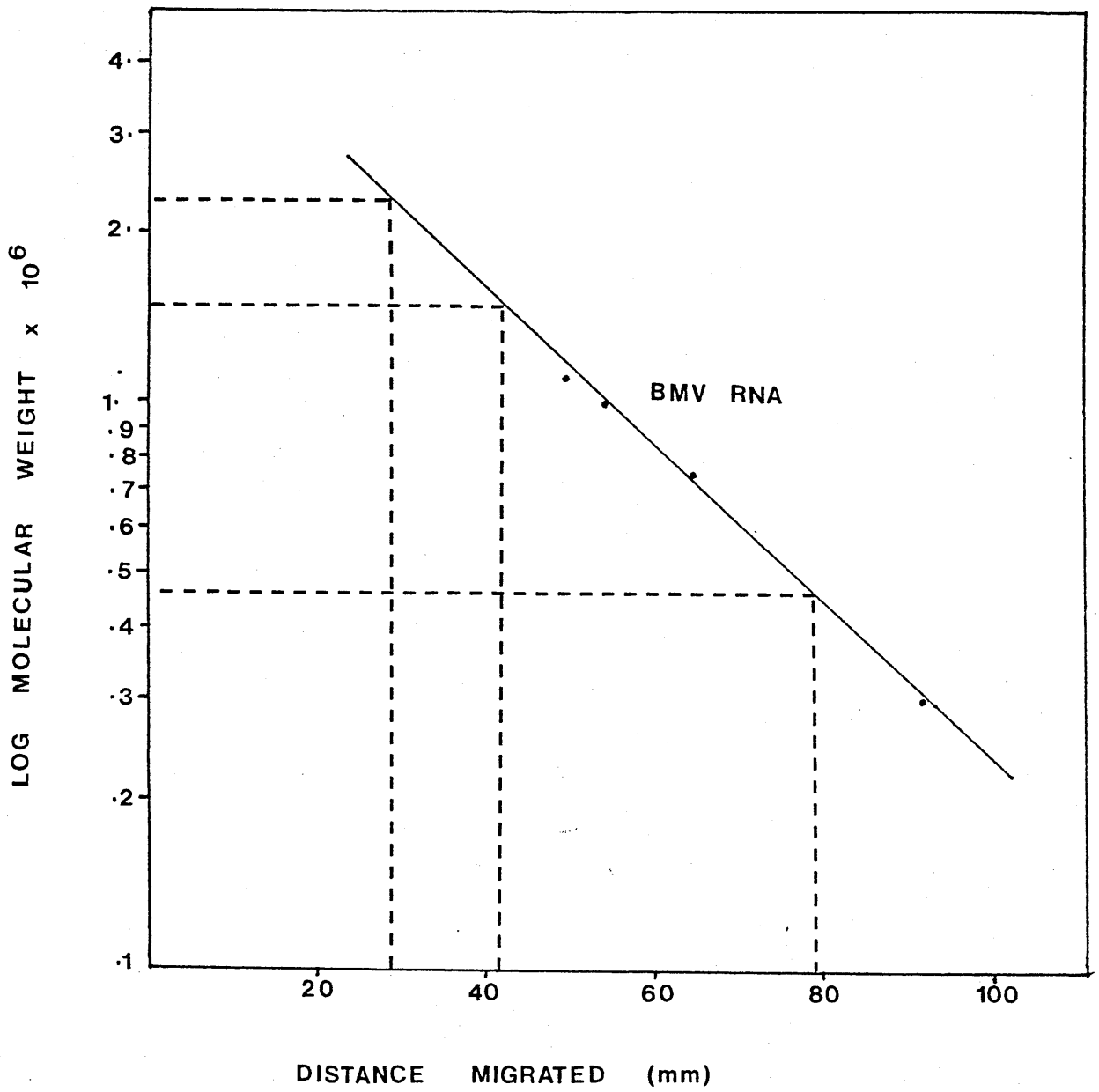
LOG MOLECULAR WEIGHT x 10^6
4
3
2
1
.9
.8
.7
.6
.5
.4
.3
.2
.1
BMV RNA
20
40
60
80
100
DISTANCE MIGRATED (mm)

figure 6.

2. Synthesis of Complementary DNA.

(a) First-Strand Synthesis.

TBRV cDNA was synthesised as described in Materials and Methods section 2(a). An aliquot (5µl) was analysed by gel filtration on Sephadex G-100. Twenty-five fractions (0.5ml) were collected and an aliquot (200µl) of each counted for ^{32}P-radioactivity. A typical profile is shown in figure 7. The cDNA was eluted as a distinct peak in fractions 4 - 6; unincorporated ^{32}P-dCTP eluted in fractions 8 - 15. The percentage of total ^{32}P-dCTP incorporated into cDNA was calculated. Since the total amount of dCTP in the reaction mixture is known, this allows calculation of the quantity of cDNA synthesised (assuming that the cytosine represents 25% of the nucleotides in the cDNA). The product to template ratio, defined as mass of cDNA synthesised/mass of RNA template, was 20%.

In early experiments, product to template ratios were variable, ranging from 0 - 20%. Attempts were made to optimise experimental conditions in order to maximise the percentage of cDNA. Several factors were found to be critical in obtaining maximal yields and reducing variability in product to template ratio:

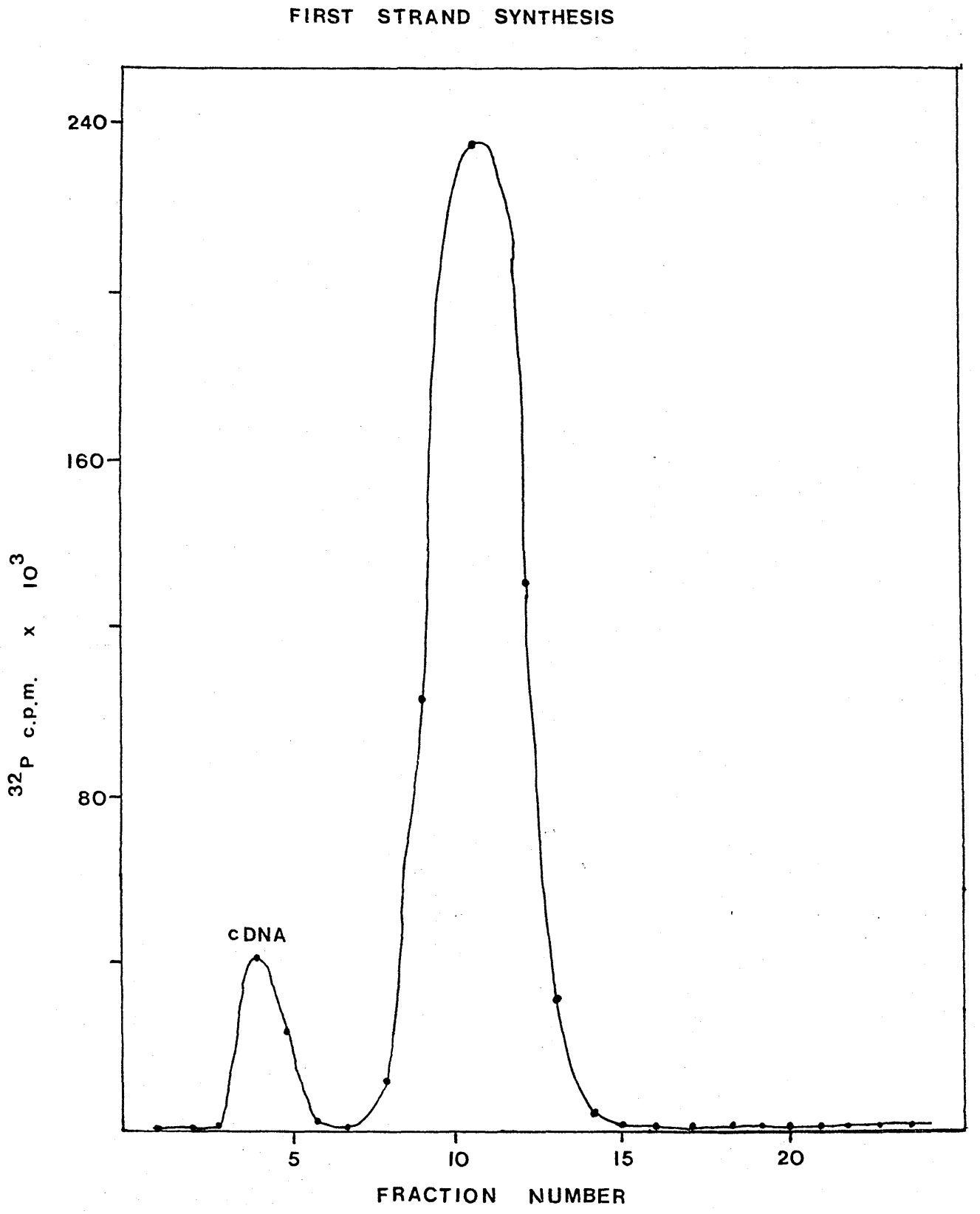
FIRST STRAND SYNTHESIS
240
160
80
^{32}P c.p.m. x 10^3
cDNA
5
10
15
20
FRACTION NUMBER

figure 7.

(1) The use of analar quality water when preparing solutions and buffers was found to be critical. Solutions prepared from distilled tapwater gave product to template ratios of between 4 to 7%.

(2) Rigorous control of the reaction conditions was necessary, in particular maintaining the reaction buffer at pH 8.3. A deviation of $\pm$ 0.5pH units was found to result in a 5-fold decrease in product to template ratio (Maniatis et al, 1982).

(3) Increasing the RNA concentration from 100µg ml^{-1} (Maniatis et al, 1982) to 200µg ml^{-1} gave an average increase in product to template ratio of 10% to 20%.

(4) Reactions were carried out in 20µl volumes because yields were reduced in larger volumes.

(b) Second-Strand Synthesis.

TBRV cDNA second-strand was synthesised as described in Materials and Methods section 2(b). An aliquot (5µl) was analysed by gel filtration on Sephadex G-100. Twenty-five fractions (0.5ml) were collected and 200µl of each counted for ^{32}P- and ^{3}H-radioactivity. A typical profile of ^{3}H-dCTP incorporation is shown in figure 8. The cDNA was eluted in fractions 4 - 6 and unincorporated ^{3}H-dCTP in fractions 8 - 16. The percentage of total ^{3}H-dCTP incorporated into cDNA was calculated and the amount of cDNA synthesised

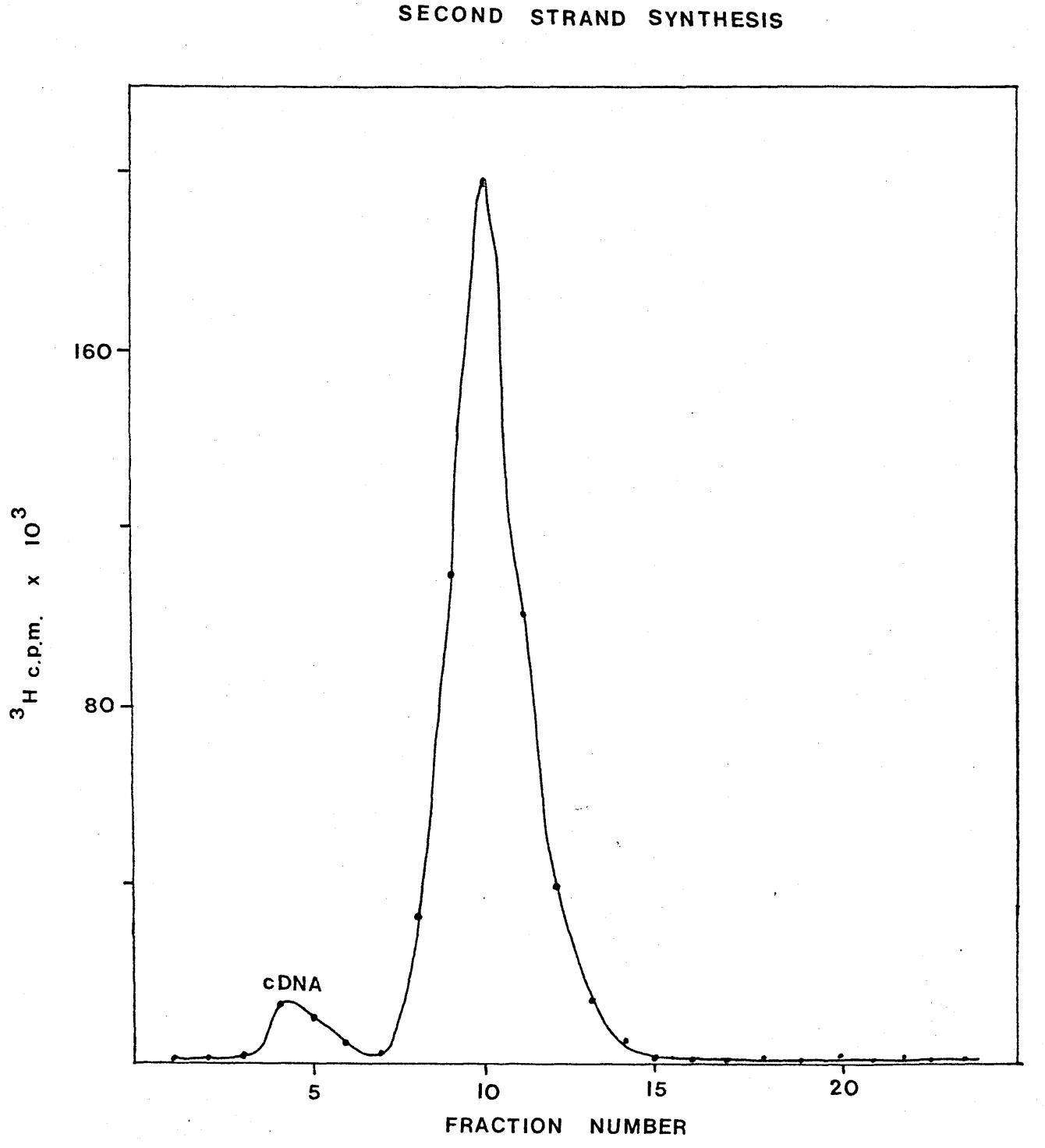
SECOND STRAND SYNTHESIS
160
80
^{3}H c.p.m. x 10^3
cDNA
5
10
15
20
FRACTION NUMBER

figure 8.

determined as a percentage of the first-strand. In the experiments shown in figure 7 and 8, 63% DNA was transcribed into double-stranded cDNA. Typically, 60 - 100% of the first-strand was transcribed by the DNA polymerase I.

As with first-strand synthesis, there were several important criteria which determined whether maximum yields of DNA were obtained:

(1) All solutions and buffers were prepared using analar quality water.

(2) Maintaining the reaction buffer at pH 6.9 was necessary.

(3) In some cases the Klenow fragment of DNA polymerase I was used as it lacks the exonucleotide activity.

(c) Second-Strand Completion Reaction.

The second-strand completion reaction was carried out as described in Materials and Methods section 2(c). The reaction was monitored by separating a 2µl aliquot on Sephadex G-100. Twenty-five fractions (0.5ml) were collected and 200µl of each counted for

^{3}H- and ^{32}P-radioactivity (figure 9 shows a typical ^{3}H profile). The cDNA was eluted in fractions 4 - 6; unincorporated ^{3}H-dCTP eluted in fractions 8 - 15. The percentage of ^{3}H-dCTP incorporated into cDNA was calculated and the second-strand to first-strand ratio determined. For the data represented in figures 7 and 8, 63% of first-strand DNA was transcribed into double-stranded cDNA. After the second-strand completion reaction, 70% of the first-strand had been transcribed.

In this experiment strict control of reaction conditions, such as pH and water purity, were important (see section 2(a) and (b)).

(d) S1 Nuclease Digest of cDNA.

S1 nuclease digest of cDNA was carried out according to Materials and Methods section 2(d). The reaction was monitored by taking aliquots (1µl), before the incubation and directly after termination, and separating these by gel filtration on Sephadex G-100. Sixteen fractions (0.5ml) were collected and 200µl of each counted for ^{32}P- and ^{3}H-radioactivity (see figure 10). The cDNA was eluted in fractions 4 - 5 and the co-precipitating nucleotides in fractions 8 - 13. The amount of radioactivity associated with the cDNA

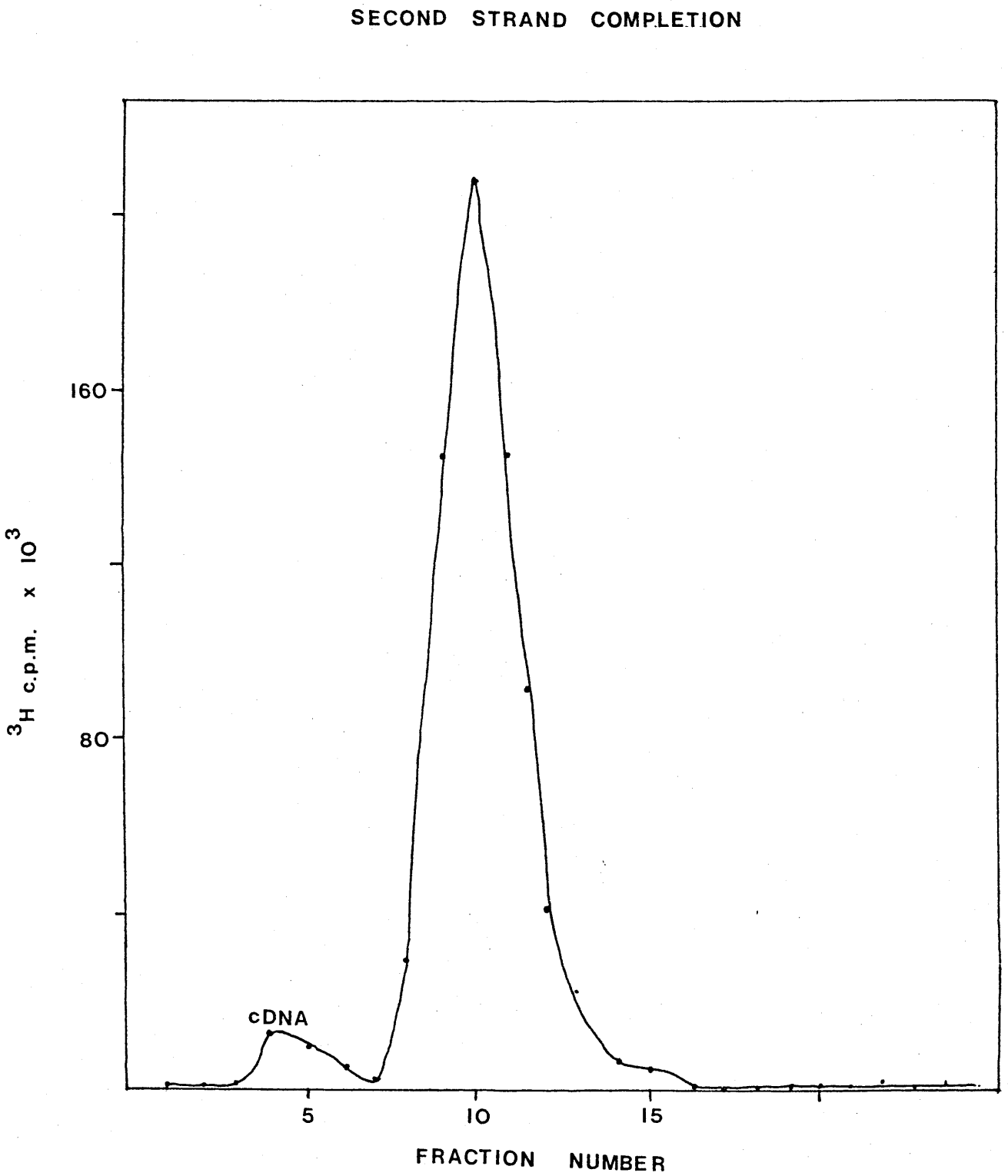
SECOND STRAND COMPLETION
160
80
^{3}H c.p.m. x 10^3
cDNA
5
10
15
FRACTION NUMBER

figure 9.

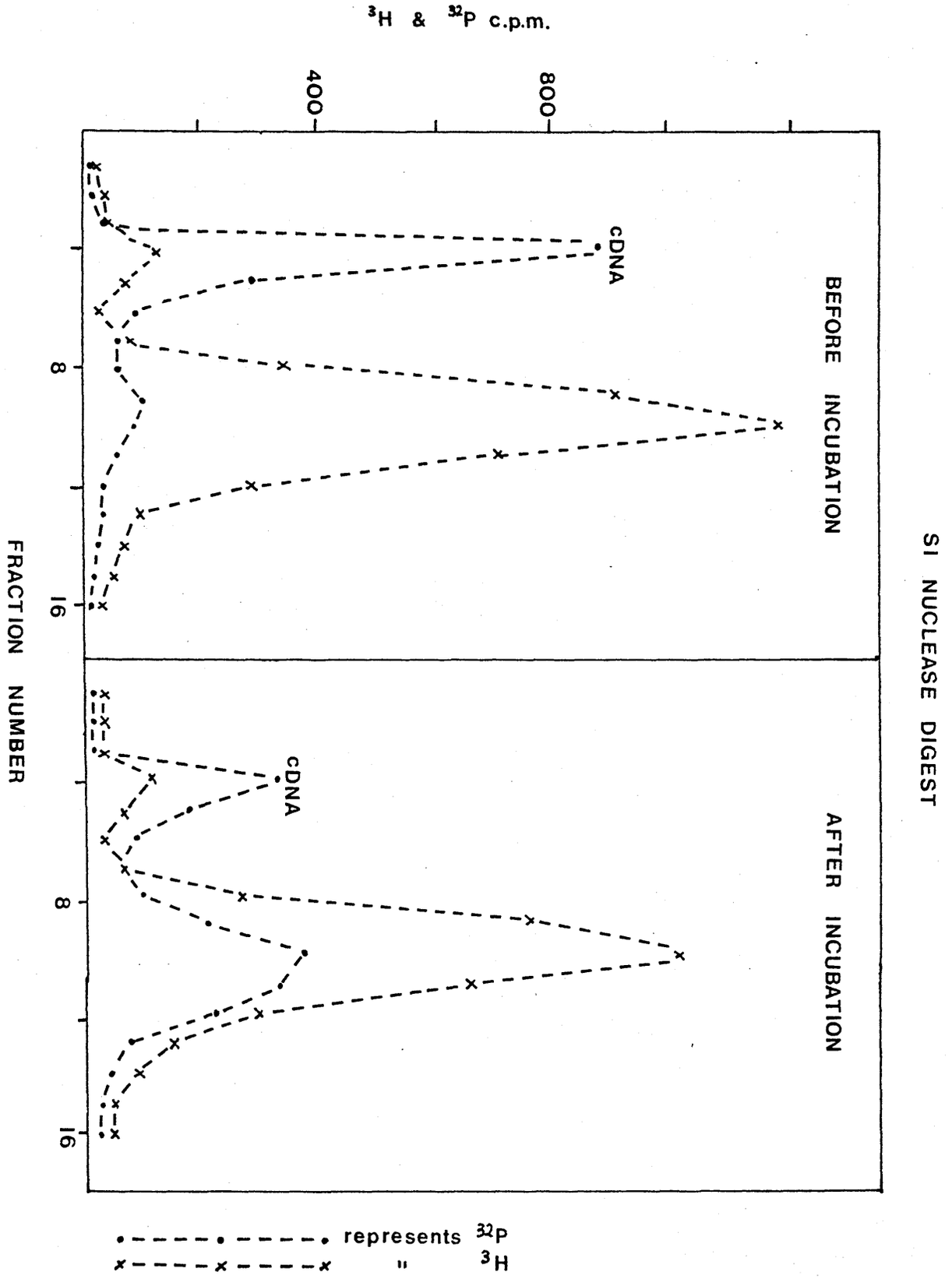
^{3}H & ^{32}P c.p.m.
400
800
BEFORE INCUBATION
AFTER INCUBATION
cDNA
cDNA
8
16
8
16
FRACTION NUMBER
S1 NUCLEASE DIGEST
represents ^{32}P
" ^{3}H

figure 10.

fractions was compared and the susceptibility to digestion by S1 nuclease determined.

before digestion with S1:

% of ^{3}H c.p.m. in cDNA = 3%

% of ^{32}P c.p.m. in cDNA = 67%

after digestion with S1:

% of ^{3}H c.p.m. in cDNA = 3%

% of ^{32}P c.p.m. in cDNA = 24%

These results show that the second-strand cDNA was resistant to digestion by S1 nuclease. Most of the first-strand cDNA was also found to be resistant to the enzyme.

(e) Production of Blunt-Ended Termini on cDNA.

The recessed 3'-termini of the cDNA were converted to a double-stranded structure by Klenow fragment of DNA polymerase I as described in Materials and Methods section 2(e). The products of this reaction were not monitored.

(f) Size Fractionation of Double-Stranded cDNA.

Double-stranded cDNA was size fractionated by centrifugation on a 5 - 20% sucrose gradient as described in Materials and Methods section 2(f). Twenty fractions (0.5ml) were collected and an aliquot (10µl) of each counted for ^{3}H- and ^{32}P-radioactivity. A typical profile for ^{32}P is shown in figure 11. The cDNA was eluted in fractions 2 - 4. The size of the cDNA in these fractions was estimated by agar electrophoresis of aliquots containing about 1,000 c.p.m. of ^{32}P. λ DNA digested with HindIII was used as a standard. After electrophoresis the standard was visualised and the bands measured. The rest of the gel was dried and the cDNA bands were detected by autoradiography (see photograph of exposed X-ray, figure 12). A plot of distance migrated against molecular weight was made for the λ DNA standard and the sizes of the cDNA determined (figure 13). The cDNA fractions were pooled into a high molecular weight fraction (greater than 1Kb) and a low molecular weight fraction (0.4 - 1Kb).

After precipitation, the cDNA was resuspended and 1µl aliquots of each fraction were counted for ^{32}P-radioactivity.

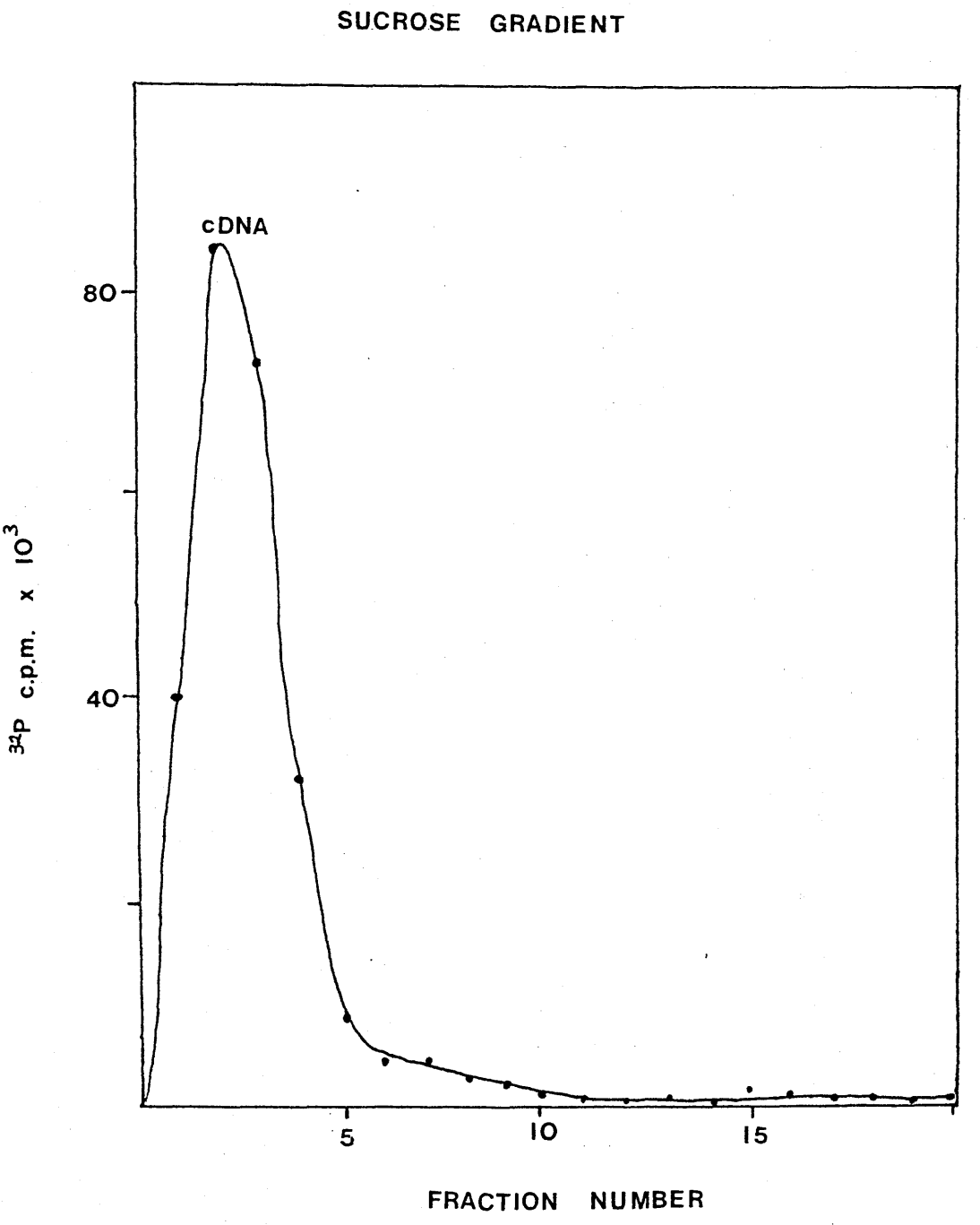
SUCROSE GRADIENT
cDNA
80
40
^{32}P c.p.m. x 10^3
5
10
15
FRACTION NUMBER

figure 11.

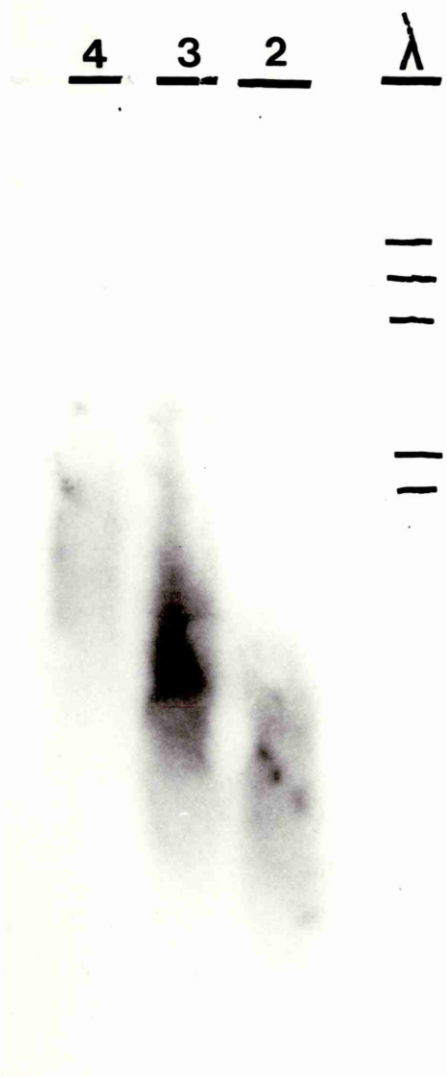

figure 12.

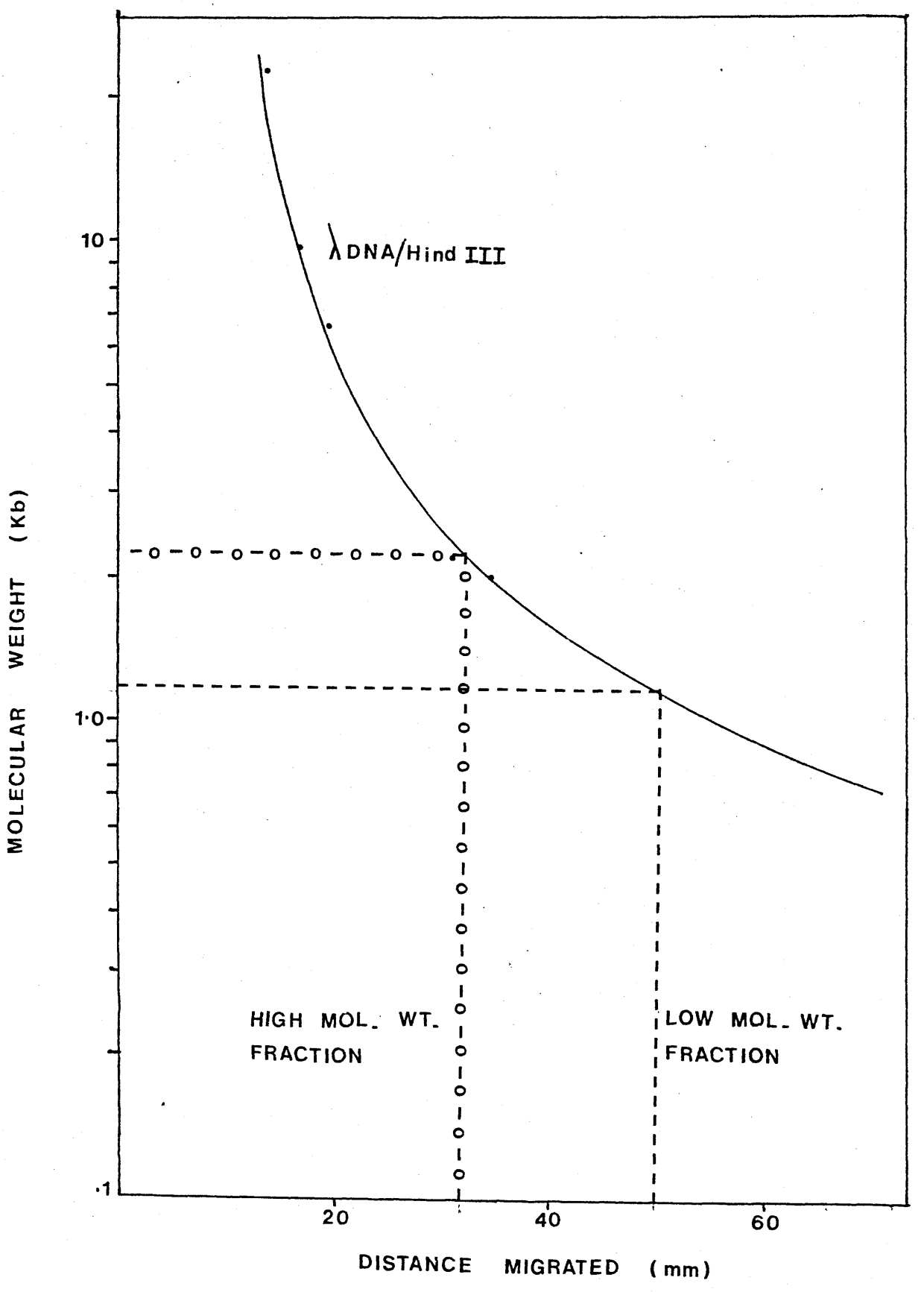
λ DNA/Hind III
10
1·0
·1
MOLECULAR WEIGHT (Kb)
HIGH MOL. WT. FRACTION
LOW MOL. WT. FRACTION
20
40
60
DISTANCE MIGRATED (mm)

figure 13.

	High mol. wt. fraction	Low mol. wt. fraction
^{32}P c.p.m.	1398	1021

The recovery of cDNA was approximately 560ng for the high molecular weight and 400ng for the low molecular weight fractions. Approximately 840ng cDNA was loaded on the gradient; therefore recovery was quantitative.

3. Cloning Recombinant DNA Molecules.

(a) Large Scale Preparation of Plasmid DNA.

Plasmid DNA (pUC9) was prepared as described in Materials and Methods section 3(a). This method typically yielded about 2mg of plasmid per litre of culture.

(b) Construction of Recombinant Plasmid.

Linearization of pUC9 with HincII was carried out as described in section 3(b) of Materials and Methods. An aliquot (0.5µg) of pUC9 was electrophoresed on an agarose gel to check that the enzyme had cleaved all the plasmid.

The pUC9 was treated with calf intestine alkaline phosphatase to prevent linearized plasmid from re-circularising (see Materials and Methods section 3(b)).

The plasmid DNA and TBRV cDNA were ligated as described in Materials and Methods section 3(b). Ligation occurred most efficiently when the reaction volume was kept as small as possible. It was not possible to check by gel electrophoresis whether ligation had occurred due to the small amount of DNA ligated.

(c) Transformation of *E. coli*.

Preparation of competent *E. coli* cells was carried out as described in Materials and Methods section 3(c). After one hour incubation on ice, an aliquot (50µl) of cells was withdrawn and transformed with pUC9 DNA (50ng). The following day the number of colonies per plate were counted and the transformation efficiency calculated. Only cells which yielded transformation efficiencies of more than 2×10^6 colonies per µg DNA were used.

Transformation of competent *E. coli* cells by recombinant DNA was carried out after 24 hours incubation on ice. The number of blue and white colonies per plate was counted after 24 hours incubation at 37^{o}c and the efficiency of transformation calculated. A typical set of results is shown below.

	E. coli transformed with high mol. wt. TBRV recombinant DNA	*E. coli* transformed with low mol. wt. TBRV recombinant DNA
average no. of blue colonies per plate	63	55
average no. of white colonies per plate	25	34
% of white colonies	28	38
transformation efficiency per µg DNA	4×10^4	6×10^4

The higher percentage of white colonies obtained for the low molecular weight TBRV recombinant DNA probably reflects the greater efficiency of ligation of short DNA (Maniatis *et al*, 1982).

In total 168 white colonies from the high molecular weight TBRV recombinant DNA, and 273 from the low molecular weight TBRV recombinant DNA were replica plated onto L-amp agar plates.

4. Selection of Recombinant Clones.

The replica plated white colonies (i.e. amp^r, lac^- colonies) were tested for the presence of TBRV cDNA sequences by the colony hybridisation method (Grunstein & Hogness, 1975) as described in Materials and Methods section 4. The bacterial colonies were transferred to nitrocellulose filters, the cells lysed, the DNA denatured and fixed permanently by baking. Colonies containing recombinant DNA were identified by hybridisation using ^{32}P-labelled TBRV cDNA as probe. The nitrocellulose filters were prehybridised to prevent any non-specific binding of probe and hybridisation was carried out using approximately 100,000 c.p.m. of TBRV cDNA. Autoradiography showed that of the 168 high molecular weight TBRV recombinant DNA colonies, 101 gave a positive hybridisation signal. For the low molecular weight TBRV recombinant DNA colonies, 273 were inoculated and 177 gave a positive hybridisation signal.

5. Isolation and Characterisation of Recombinant DNA.

(a) Isolation of Recombinant DNA.

Colonies which gave a positive hybridisation signal were multiplied and the plasmid DNA prepared as described in Materials and Methods section 5(a). The colonies which contained recombinant DNA from the high molecular weight TBRV fraction were selected first because they represent more of the genome. This method commonly yielded approximately 2ug DNA from a 2ml culture.

(b) Size Determination of Recombinant DNA.

The inserted cDNA was cleaved from selected plasmids as detailed in Materials and Methods section 5(b). In total 62 plasmids were examined, of which 41 were from the high molecular weight TBRV recombinant DNA and 21 from the low molecular weight TBRV recombinant DNA. Under UV light, inserts were observed in 38 of the plasmids. Ten plasmids, which were selected for further characterisation, are shown in figure 14. In lanes 1 - 3 and 10 - 12 the insert bands were very faint and do not show clearly in the photograph. The lengths of the inserts were found to be approximately 200 base pairs for lane 1; 320 base pairs for lanes 2 - 5; 870 base pairs for lane 8; 380 for lane 9; and 320 base pairs for lanes 10 - 12.

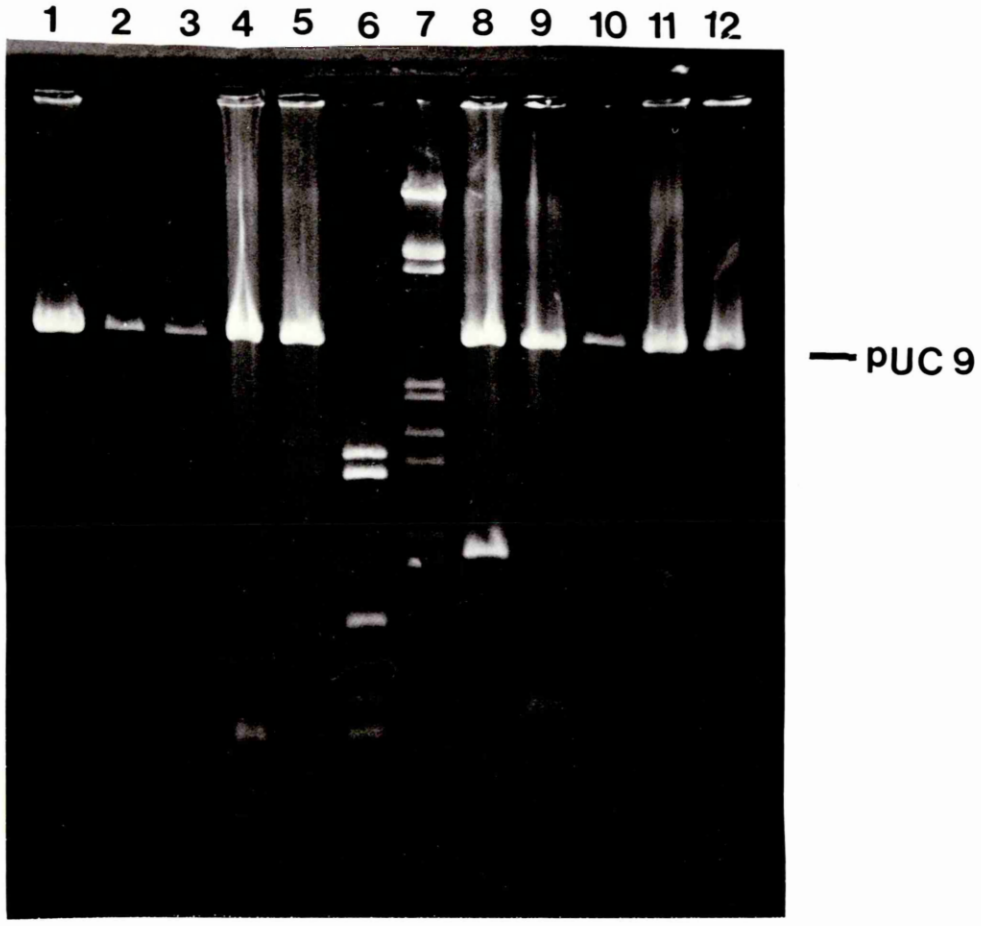

figure 14.

(c) Blot Transfer Hybridisation of Plasmid DNA to TBRV RNA.

During the synthesis of cDNA, a mixed population of TBRV RNA was reversed transcribed therefore it was necessary to identify whether TBRV RNA-1, 2 or 3 was represented by a particular recombinant DNA molecule. TBRV RNA (from a satellite-containing strain) was electrophoresed on an agarose gel and transferred to Biodyne A-Nylon 66 membrane as described in Materials and Methods section 5(c). The membrane was cut into individual lanes which were separately sealed in polythene bags and hybridisation was carried out using ^{32}P-dCTP labelled plasmid DNA. Hybridisation was detected by autoradiography. The positions of the visualised TBRV RNA bands were marked on the x-ray film (lane 11) to show which species was represented by each clone (see photograph of x-ray, figure 15).

lane number	clone	insert length (base pairs)
1	PTBR2-A	200
2	PTBR1/2-B	320
3	PTBR2-C	320
4	PTBR2-D	320
5	PTBR3-E	320
6	PTBR3-F	870
7	PTBR2-G	380
8	PTBR1-H	320
9	PTBR2-I	320
10	PTBR3-J	320

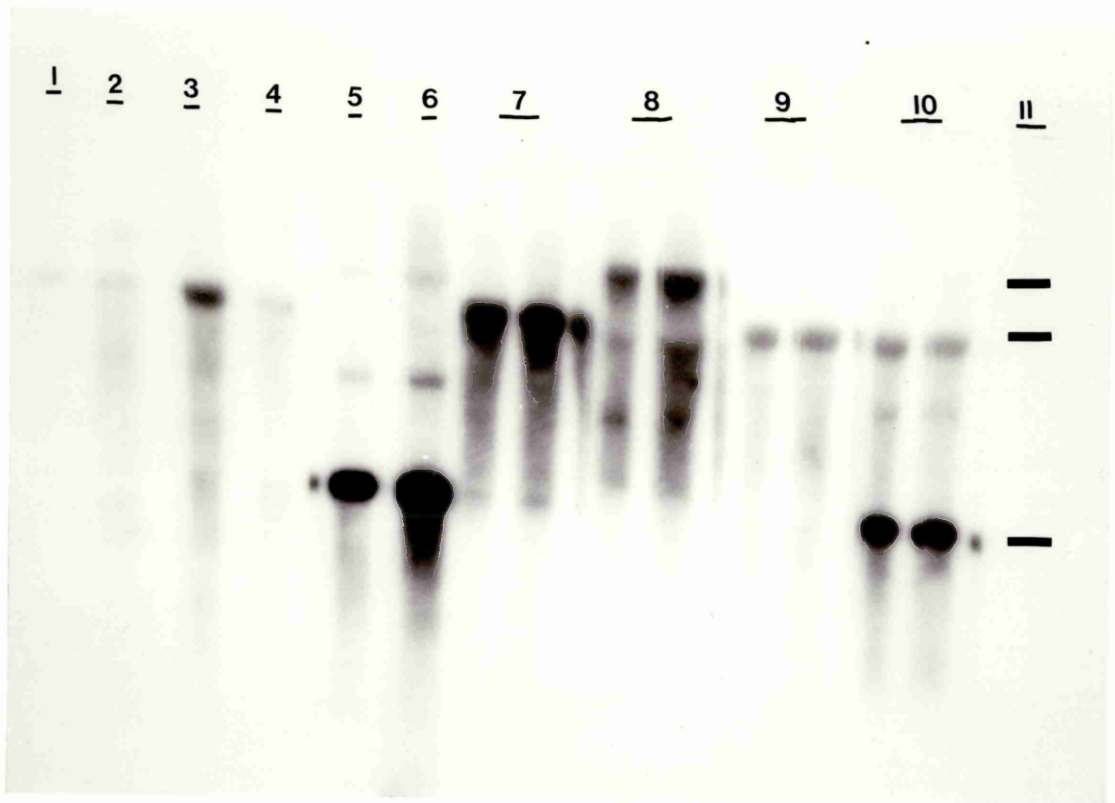

figure 15.

where PTBR1 = clone predominantly to TBRV RNA-1

" PTBR2 = clone exclusively to TBRV RNA-2

" PTBR3 = clone predominantly to TBRV RNA-3

" PTBR1/2 = clone representing TBRV RNA-1 and 2

DISCUSSION

TBRV RNA samples were checked for purity and integrity by electrophoresis and the molecular weights were calculated by comparison to BMV RNA which has values less than that of TBRV RNA-1 and RNA-2. The molecular weight of TBRV RNA-1 was measured to be lower than its reported value, possibly due to errors incurred by extrapolation with the standard. Alternatively it is possible that RNA-1 had partially re-annealed and therefore electrophoresed at an apparently lower molecular weight.

Several problems were encountered in the first step of the synthesis of complementary DNA. Initially, the cDNA yields from the first-strand synthesis reaction were inconsistent and a range of values was obtained. Optimisation of the reaction conditions improved the efficiency of this process. Significantly, it was discovered that doubling the RNA concentration improved yields. The reason for this effect is not known but perhaps providing more template per unit volume allows a greater chance for the reverse transcriptase to associate with the RNA. In addition reliable yields of cDNA were obtained by rigorous control of the reaction conditions. The most efficient reaction volume was found to be 20µl. It was believed that in larger volumes the increased amount of impurities was inhibitory to the reaction.

During all stages of cDNA synthesis, the solutions and buffers were prepared using Analar quality water, since it was suspected that residual trace quantities of ions present in the laboratory distilled water were inhibiting the enzyme activities.

During second-strand synthesis, typically 60 - 100% of the first-strand cDNA was transcribed by DNA polymerase I. As with first-strand synthesis, it was necessary to exercise strict control of the reaction conditions. The Klenow fragment of DNA polymerase I was used occassionally because it lacks the exonucleotide activity and therefore overcomes the possibility of the first-strand template being digested during the reaction.

The second-strand to first-strand cDNA ratio was increased by 5 - 13% by carrying out the second-strand completion reaction. Maniatis et al (1982) report that incubating the first-strand cDNA separately with reverse transcriptase and DNA polymerase I produces longer second-strand cDNA.

The hairpin loop and untranscribed cDNA were removed by digestion with S1 nuclease. As expected the second-strand cDNA was resistant to digestion. This was demonstrated because the amount of

incorporated ^{3}H remained the same. In contrast the amount of incorporated ^{32}P decreased after digestion which corresponded to the enzyme sensitive single-stranded regions. The recessed 3'-termini left by S1 nuclease digestion were converted to a double-stranded structure by Klenow fragment of DNA polymerase I. This step is necessary prior to blunt-end ligation into a vector molecule.

A mixed population of lengths was generated during the synthesis of cDNA and all samples were fractionated according to size. λDNA digested with Hind III was used as a standard. The preparation of λDNA used lacked the 4.3Kb fragment (see figure 12), which may have been caused by annealing of 3'-recessed ends. The longest cDNA was selected for cloning. Normally there was a larger proportion of short-length cDNA compared to longer-length cDNA, which resulted in usually half being discarded.

At each stage of the synthesis, there was an uncontrollable loss of cDNA during the phenol/chloroform extractions. A small amount of cDNA was lost at each isopropanol/potassium acetate precipitation. Ideally, in order to get 250ng sized DNA, it was necessary to start with 5μg first-strand cDNA.

Plasmid DNA (pUC9), linearized with Hinc II, was treated with calf intestine alkaline phosphatase to prevent the plasmid re-circularising during the ligation reaction. Recombinant DNA molecules were formed by ligating TBRV cDNA and linearized, phosphatased pUC9 DNA. Ligation occurred most efficiently when the reaction volume was kept as small as possible. Ligating using the blunt-end method results in a preferential ligation of the smallest DNA in the population (Maniatis et al, 1982). Since the large cDNA fraction (1Kb and larger) also contained some shorter DNA it would possibly have been more efficient to ligate using linkers.

Competent E. coli cells, which yielded a transformation efficiency greater than 2×10^6 colonies per µg DNA, were transformed with recombinant DNA. After incubation the plates were found to contain blue and white colonies. The blue colonies (ie lac^+ colonies) contained pUC9 which did not have inserted cDNA. These plasmids may have arisen from incomplete phosphatase treatment following linearization. Such plasmids would re-circularise during the ligation reaction. The percentage of blue colonies was always much greater than the percentage of whites.

The amp^r, lac^- colonies (ie white colonies) were tested for the presence of TBRV cDNA sequences by the colony hybridisation method of Grunstein and Hogness (1975). Autoradiography showed that 63% of the colonies contained recombinant DNA. Those colonies which did not react in colony hybridisation probably contained plasmids with very short inserts.

Colonies which gave a positive hybridisation signal were grown and the plasmid DNA prepared. The inserted cDNA was cleaved from selected plasmids and the lengths of the inserts determined. The largest insert was found to be 870 base pairs, and although this is shorter than the TBRV genome (8 and 5Kb) it is sufficient for separating RNAs by hybridisation selection. The inserts obtained were shorter than expected. This is probably because blunt-end ligation preferentially selects short cDNA. Longer inserts may have been ligated by using either synthetic linkers or homopolymeric tailing (Maniatis et al, 1982).

Inserts were not detected in all the lac^-, $hybridisation^+$ colonies. These colonies probably contained plasmids with very short inserts which could not be detected by the UV/ethidium bromide

method. Inserts of less than 300 base pairs are not easily visible by this method (P.T. Richardson, unpublished data).

Blot transfer hybridisation was carried out to identify which TBRV RNA was represented by each colony. Some clones reacted with more than one RNA species. In lanes 5 and 8 (see figure 15) the additional bands are not of the size of TBRV RNA-1, -2 or -3. This may be due to breakdown products of genomic RNAs. In this work 2µg RNA per lane was transferred to Biodyne A membrane, although it is possible to use as little as 10ng RNA (J.J. Milner, unpublished data). This will accentuate bands due to low levels of breakdown products. These non-standard bands are of one or two similar sizes in all lanes. The additional bands of genomic size, found in lanes 2, 6 and 10 (see figure 15) may have arisen by slight leakage of samples during electrophoresis.

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