A complex synthesizing the maize mitochondrial plasmid RNA *b*

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December 1993

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements of the degree of
Master of Science

Abstract

RNA *b* is the most abundant member of a family of autonomously replicating single- and double-stranded RNA plasmids found in mitochondria of several maize races. The extent to which this molecule associates *in vivo* with proteins was investigated by both rate zonal and CsCl equilibrium density gradient centrifugations of clarified lysate of mitochondria from maize plants with the S-type cytoplasm. A soluble endogenous complex of RNA *b*, responsible for synthesis of the more abundant (+) RNA *b* strand in *in vitro* conditions (in mitochondrial lysate), was identified. The complex had a density of 1.49 g/cm^3 but a surprisingly low sedimentation coefficient, only slightly larger than the naked RNA *b*. Only a minor fraction of RNA *b* molecules were bound in the complex; the majority of RNA *b* sedimented as naked RNA molecules. Complexes synthesizing other, less abundant, maize RNA plasmids were not identified. However, *in vitro* synthesis of all RNA plasmid species in mitochondrial lysate was resistant to heparin, suggesting that in all cases preformed RdRp-RNA template complexes, capable of elongating *in vivo* preinitiated RNA plasmid strands, were present.

Résumé

Le ARN *b* est le membre le plus abondant d'une famille de plasmides à ARN simple et double-brin à réplication autonome présents chez les mitochondries de plusieurs races de maïs. L'importance d'une association *in vivo* de cette molécule à des protéines a été examinée par des centrifugations de zone at en gradient de densité de chlorure de césium du lysat clair de mitochondries de plants de maïs porteurs du cytoplasme de type "S". Ceci a permi d'identifier un complexe soluble endogène de ARN *b*, responsable *in vitro* (en lysat mitochondrial) de la synthèse du brin le plus abondant (brin +) du ARN *b*. Ce complexe a une densité de 1.49g/cm^3 mais un coefficient de sédimentation étonnamment faible, n'étant que très légèrment supérieur à celui du ARN *b* nu. Seule une faible fraction des molécules de ARN *b* est fixée à ce complexe, la majorité d'entre elles sédimentant comme des molécules de ARN nues. Aucun complexe associé a la synthèse des autres plasmides a ARN (moins abondants) du maïs n'a été identifié. Cependant la synthèse *in vitro* de toutes les espèces de plasmides à ARN du lysat mitochondrial est résistante a l'héparine, suggérant dans tous les cas la présence de complexes de RdRp/matrice d'ARN prétormés, capables d'allonger *in vivo* des brins de plasmides à ARN préinitiés.

(Traduit par Martine Jean)

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Abbreviations

ATP 6 . . subunit 6 of F_O-ATPase
b . . . . . bases
BMV . . . brome mosaic virus
bp . . . . . base pair
CEV . . . citrus exocortis viroid
CMS . . . cytoplasmic male sterility
COX II . . subunit II of cytochrome *c* oxidase
cpm . . . . counts per minute
DEPC . . . diethylpyrocarbonate
ds . . . . . double-stranded
dsRNA . . double-stranded RNA
DTT . . . . dithiothreitol
EDTA . . ethylenediaminetetraacetic acid
EGTA . . ethylene glycol-bis-(β-aminoethyl ether) N,N,N',N' tetraacetic acid
EM . . . . electron microscopy
HEPES . . N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
ICR . . . . internal control region
kDa . . . . kilodalton(s)
kbp . . . . kilobase pair(s)
mRNA . . messenger RNA
mtDNA . mitochondrial DNA
ORF . . . open reading frame
PSTV . . . potato spindle tuber viroid
RdRp . . . RNA-dependent RNA polymerase
RF . . . . . replicative form
RI . . . . . replicative intermediate
rpm . . . . rotations per minute
rRNA . . . ribosomal RNA
SDS . . . . sodium dodecyl sulphate
ss . . . . . single-stranded
ssRNA . . single-stranded RNA
Tricine . . N-tris[Hydroxymethyl] methyl-2-aminoethane
Tris . . . . Tris[Hydroxymethyl] aminomethane
tRNA . . . transfer RNA
VLP . . . . virus-like particle
VPg . . . . viral genome-linked protein
VSV . . . . vesicular stomatitis virus

Acknowledgements

There are many who have helped me during the work on this project. I would like to first thank Dr. G.G. Brown, my thesis supervisor, for his help and support during all stages of the work. I would also like to thank members of my supervisory committee, Dr. G. Maclachlan and Dr. M. Zannis-Hadjopoulos, for their advice. I am indebted to Mingda Zhang for his help, patience with which he answered my numerous questions, and for providing me with a picture of the predicted secondary structure of RNA *b*. I would like to thank Rima Menassa for her encouragement, Martine Jean for translating the abstract of this thesis, and Richard Stahl for helping me master some of the techniques. I would also like to thank Antonio Monroy for valuable discussions, Dr. R. Chase for his help in difficult times, and Dr. W. Morgan for believing in my skills. I am grateful to McGill University for financial support in the form of a differential fee waiver. Last but not least, I take this opportunity to thank my family and Jean Pierre for their love and support throughout.

Dedicated to the memory of my grandmother

Introduction

Mitochondria of several maize races contain single- and double-stranded non infectious RNA replicons that have been commonly referred to as RNA plasmids. Although it has been shown that these molecules replicate in a DNA-independent fashion, neither the exact mechanism of the replication nor the enzymes involved are known. In the work presented below, I have investigated an activity, present in the mitochondria of maize S-type cytoplasm, that is able to synthesize new strands of RNA plasmids *in vitro*. I wished to establish whether plasmid RNA associates *in vivo* with proteins (and/or cellular structures), and if the active RNA polymerase-RNA template complex, responsible for the *in vitro* synthesis of plasmid RNA, could be identifed. In summary, the objectives of my study were:

- to determine whether the activity synthesizing new RNA plasmid molecules is soluble or membrane-bound;
- to characterize the activity by its resistance or sensitivity to different inhibitors;
- to establish whether RNA plasmids form complexes with proteins *in vivo*; if they do, are any of these active in the synthesis of new plasmid RNA ?

Most of the work presented here is concerned with the most abundant RNA plasmid molecule - single-stranded RNA *b*. Other plasmid species are present in far lower number and due to the limited amount of material obtainable (purified mitochondria), their investigation is difficult.

Literature review

Plant mitochondria

Mitochondria are organelles present in the cells of practically all eukaryotic organisms. They are capable of replicating and expressing their own genetic information, a process that is indispensable for several basic metabolic processes. The majority of mitochondrial proteins are, however, coded for by the nucleus and a complicated exchange of information and coordination of functions must therefore exist between the mitochondrion and the nucleus. In the following few paragraphs I would like to discuss some of the characteristics of mitochondrial genetic systems, restricting my attention to plant mitochondria.

One of the most distinct features of plant mitochondria is the size and structural heterogeneity of their genome. The length of plant mtDNAs, mapped to date, vary from 180 kbp (Lebacq and Vedel 1981) to about 2500 kbp (Ward *et al.* 1981), which is 10- to 200- fold more than animal mtDNA. The genomic DNA apparently exists as a mixture of molecules of different sizes (Synenki *et al.* 1978, Pring and Lonsdale 1985); although both linear and circular molecules have been detected, it remains unclear whether linear DNA molecules exist *in vivo* or result from cleavage of DNA circles during manipulations. It is now believed that the occurrence of subgenomic DNA molecules of different sizes is a result of frequent recombination between repeated sequences on mtDNA (for a review see Lonsdale 1989). Recombination events not only change the physical organization of the genome in the present population of the mitochondria but during the evolution they have also caused reorganization of coding sequences (recently reviewed by Bonen and Brown 1993). Rearrangements involving terminal regions of genes seem to be particularly common and an altered expression of such regions (and/or a creation of

new, expressed open reading frames, ORFs) are believed to be the cause of cytoplasmic male sterility (see below).

Another phenomenon that apparently resulted from the high frequency of recombination events in plant mitochondria is the occurrence of chloroplast DNA sequences in mtDNA. A fragment highly homologous to the chloroplast 16S rRNA and two tRNAs was first detected in maize (Stern and Lonsdale 1982). The evidence of integration of other sequences into mtDNA of other plants had followed (Lonsdale *et al.* 1983). Both chloroplast and mitochondrial DNA sequences have been also found in the nucleus (Kemble *et al.* 1983, Timmis and Scott 1983). These findings raise up a question: What is the molecular mechanism underlying transfer of genetic information between different cellular compartments? A possible clue was provided by Schuster and Brennicke (1987) They have described an ORF in *Oenothera* mtDNA with high homology to reverse transcriptase and showed that it is transcribed. That led them to the proposal that genetic information could be transferred from one organelle to another in the form of RNA, subsequently reverse transcribed into DNA and integrated into the genome. The hypothesis is supported by other lines of evidence; firstly, it was shown that mitochondria of several species of genus *Vigna* have lost the *coxII* gene and that its functional (i.e. edited) copy exists on the nuclear genome (Nugent and Palmer 1991), and secondly, Marechal-Drouard *et al.* (1988) have demonstrated that certain tRNA synthesized in the nucleus can be subsequently transported into the mitochondria.

Mitochondria of some plants contain small linear and circular extrachromosomal DNAs (reviewed by Brown and Zhang 1993). They replicate independently of the mitochondrial genome and thus are commonly referred to as DNA plasmids. Some of them can, however, occasionally integrate into the main mitochondrial chromosome and have been termed episomes (Schardl *et al.* 1984). The most extensively studied examples of mitochondrial episomes are linear S1 and S2 DNAs, found in mitochondria of the S-type male-sterile cytoplasm of maize (Pring *et al.* 1977). These plasmids possess covalently bound proteins at their

5' termini (Kemble and Thompson 1982), and, interestingly, S1 and S2 contain open reading frames coding for polypeptides homologous to DNA and RNA polymerases, respectively (Kuzmin and Levchenko 1987, Oeser 1988). Both ORFs are expressed (Zabala *et al* 1987, Zabala and Walbot 1988). In addition to extrachromosomal DNA elements, unusual RNA replicons have been described in maize mitochondria. As these are the subject of this thesis they will be discussed elsewhere in greater detail.

Despite its size, plant mtDNA does not appear to have a remarkably higher coding capacity than its animal or yeast counterparts. Mitochondrial genome of plants encodes transfer RNAs, all three ribosomal RNAs, some components of respiratory chain and ATP synthase enzymatic complex, and a few ribosomal proteins (summarized by Bonen and Brown 1993). In addition, several ORFs of unknown functions have been identified. The majority of "house-keeping" proteins, i.e. enzymes of DNA replication, transcription and most proteins taking part in translation, are probably imported from the cytoplasm.

Little information is available about the enzymes responsible for transcription of the plant mitochondrial genome. *In vitro* transcription is resistant to α-amanitin at a concentration that inhibits nuclear RNA polymerases II and III, and exhibits different rifampicin sensitivity than RNA synthesis in both chloroplast and bacteria (Finnegan and Brown 1987). Hence, the plant mitochondrial RNA polymerase is a distinct enzymatic complex with specific localization and function. The only organism in which mitochondrial RNA polymerase has been studied in greater detail is the yeast *Saccharomyces cerevisiae*. The core enzyme is a single polypeptide of about 145 kDa (Kelly and Lehman 1986) that shows high homology to the RNA polymerases of bacteriophages T3 and T7 (Masters *et al.* 1987). Two additional specificity factors interacting with the core polymerase have also been described (Winkley *et al.* 1985, Schinkel *et al.* 1987, Ticho and Getz 1988).

Transcription and post-transcriptional processing of RNAs in plant mitochondria have been investigated extensively during the last decade. Several

initiation sites of RNA transcription have been identified on mtDNAs of maize, wheat and soybean, and consensus sequences representing possible promoters have been proposed (reviewed by Gray *et al.* 1992). It should be noted, however, that the degree of conservation of these sequences is low. Newly transcribed RNA molecules are often processed further: polycistronic transcripts must be converted to monocistronic messages by specific endonucleotide cleavage and introns may be removed by splicing. In addition, many of the mRNAs are edited (C-to-U changes). Finnegan and Brown (1990) have observed that non-coding regions of mtDNA may be transcribed but that these transcripts are not always stable. Therefore, a mechanism of recognition and specific degradation of non-coding RNA sequences may exist in plant mitochondria.

An overview of plant mitochondria molecular biology would not be complete without mentioning one very common phenomenon - cytoplasmic male sterility (CMS). CMS can be defined as a maternally inherited inability of plants to produce pollen. It is thought to result from dysfunction of mitochondria under certain nuclear background. Rearrangements of mtDNA were shown to underlie the occurrence of CMS in *Petunia* (Young and Hanson 1987), *Brassica napus* (Singh and Brown 1991), and in one type of maize male sterile cytoplasm, *T-cms* (Dewey *et al.* 1986). An altered expression of normal mtDNA genes and/or expression of new chimeric genes that may result from such rearrangements could cause CMS. Sterile cytoplasms of maize have been divided into three groups: *T-cms*, *S-cms* and *C-cms*. The nature of CMS conferred by the C and S type cytoplasms is unclear.

RNA replicons in plants

A large variety of RNA replicons exist in plants; their types range from small parasitic RNAs (viroids) to complex genetic units that not only replicate but also express their genetic information (RNA viruses).

Majority of plant viruses are non enveloped RNA viruses carrying in their virion the plus (i.e. coding) RNA strand. Interestingly, replication of a few of them have been connected with a vesiculation of mitochondria suggesting a possible mitochondrial localization of the process (Francki 1987). The list of RNA viruses infecting plants also includes enveloped minus-strand RNA viruses (plant rhabdoviruses) and viruses with dsRNA genomes (reoviruses) (Matthews 1982). In addition, some plants support replication of dsRNA molecules enclosed in virus-like particles (VLPs) (Boccardo *et al.* 1986). These replicons were appropriately termed cryptic viruses.

Multiplication of an RNA virus in plant tissue is sometimes accompanied by a replication of a satellite virus or a satellite RNA (Murant and Mayo 1982). The former type of satellite replicon codes for its own capsid protein while the latter is encapsidated and transmitted in helper virus particles. Neither of them is able to replicate without a presence of the helper virus.

Lastly, plant cells accommodate replication of the smallest RNA replicons known - viroids - and of a group of replicating entities classified as RNA plasmids.

DsRNAs in plants

Cells of several plant species contain abundant dsRNAs that do not seem to be associated with VLPs (reviewed recently by Brown and Zhang 1993). Horizontal transfer has not so far been shown for any of these RNAs and they are therefore assumed to be non-infectious. Autonomous replication, i.e. replication independent of any DNA template, has been demonstrated for a few of them, namely ss and ds RNAs in maize (Finnegan and Brown 1986), dsRNA of *Vicia faba* (Lefebvre *et al.*

1990), dsRNA of *Brassica* (Monroy *et al.* 1990) and dsRNA found in tobacco leaves (Ikegami and Fraenkel-Conrat 1979). Molecules with these properties have been classified as RNA plasmids (Finnegan and Brown 1986).

The dsRNA of *Vicia faba* has unique features that distinguish it from other plant RNA replicons. Unlike RNA viruses, the RNA molecule is not enclosed in a nucleocapsid structure. It is, however, packaged together with its replicase in cytoplasmic membranous vesicles (Lefebvre *et al.* 1990) thus making the replicon most reminiscent of the vesicle-enclosed virus-like dsRNA conferring hypovirulence in *Cryphonectria parasitica* (chestnut blight fungus) (Hansen *et al.* 1985, Fahima *et al.* 1993). Surprisingly, the dsRNA of *Vicia faba* shows homology to the nuclear genome (Turpen *et al.* 1988). In order to explain this fact it has been suggested that its replication might involve reverse transcription into DNA (Turpen *et al.* 1988). Alternatively, the replicon might have integrated in its genome a nuclear transcript (Lefebvre *et al.* 1990). The presence of the dsRNA in cytoplasm of *Vicia faba* is believed to cause cytoplasmic male sterility although the exact mechanism of its interference is not known.

The precise cellular location of the majority of plant dsRNAs is unknown. Several of them have been reported to cosediment with mitochondria during cell fractionation procedures but conclusive evidence for a mitochondrial localization exists only for RNA plasmids of *Zea mays* (see below). These molecules thus represent the only RNA replicons known whose replication seems to be restricted to one particular cellular compartment - mitochondrion.

Mitochondrial RNA plasmids of maize

Single- and double-stranded RNA species that copurified with mitochondria during cell fractionation were originally discovered in maize strains containing the S-type cytoplasm (Schuster *et al.* 1983, Sisco *et al.* 1984), and in a male-fertile Latin American race of maize, Racimo de Uva (RU cytoplasm) (Schuster *et al.* 1983).

An investigation using more sensitive methods revealed their presence in a wide variety of maize cytoplasms, including T, C and also the fertile N (normal) cytoplasms (Zhang and Brown 1993, Zhang 1993), albeit at lower levels. However, teosintes that are considered to be the ancestors of modern maize races did not contain these molecules (Zhang 1993).

Sisco *et al.* (1984) investigated the possibility of a vertical transmission of the maize RNA plasmids. Attempts to transmit them by aphid or mechanical inoculation were unsuccessful. Moreover, no VLPs were observed in electron-microscope scans of leaf extracts (Sisco *et al.* 1984) and mitochondrial preparations (Finnegan 1989), suggesting that the RNAs were not components of a virus.

Several lines of evidence suggest that maize RNA plasmids reside in the mitochondrion:

- RNA plasmids are maternally inherited (Sisco *et al.* 1984);
- they cosediment with mitochondria in a linear sucrose gradient (Finnegan and Brown 1986) and in a discontinuous density Percoll gradient (Finnegan and Brown 1986, Monroy *et al.* 1990);
- synthesis of new RNA plasmid strands by intact mitochondria is resistant to RNase treatment but the activity is completely abolished in the presence of a non-ionic detergent (Finnegan and Brown 1986);
- the distribution of RNA plasmids within plant tissues, as elucidated by *in situ* hybridizations, is very similar to the distribution of mitochondrial *atp6* mRNA (Zhang 1993).

These data combined together represent a fairly conclusive evidence of the RNA plasmids' mitochondrial localization.

The maize plasmid family comprises of several RNA species that share sequence similarities among each other but not with the nuclear, mitochondrial, and chloroplast DNA (Schuster *et al.* 1983, Finnegan and Brown 1986, Zhang 1993). In plants with the S-type cytoplasm the most abundant member of the family, RNA *b*, is a 719b long ssRNA molecule (Schuster *et al.* 1983, Zhang and Brown

1993). A larger RNA species, RNA *a*, is about 2800b long (Finnegan and Brown 1986) and although majority is also single-stranded, part of it exists in a double-stranded form. In addition, double-stranded species of around 800b (RNA *b'*) and 2100b, together with an RNA larger than 4000b, have been described (Finnegan and Brown 1986, Zhang and Brown 1993).

The strand represented by single-stranded RNA *b* and RNA *a* molecules was arbitrarily designed the plus (+) strand and the complementary strand minus (-) strand (Zhang and Brown 1993). The availability of cDNA clones of RNA *b* sequence has allowed a direct detection of both strands by hybridization methods (Zhang and Brown 1993). Indeed, small amounts of (-) strands of both RNA *b* and RNA *b'* were detected. The exact ratio of (+) and (-) strands was not determined but it could be estimated that the (-) strand is about 50 to 100 times less abundant than the (+) strand (M.Zhang, personal communication).

Different maize cytoplasms do not contain exactly the same set of RNA plasmids (Zhang 1993). In some cytoplasms, new RNA species seem to be present. In addition, some variation in sizes of the individual RNAs is also observed. The LBN cytoplasm (a sub-group of S cytoplasm) is particularly interesting exception as it contains mostly double-stranded form of both RNA *a* and RNA *b* (Sisco *et al.* 1984).

Autonomous replication of maize RNA plasmids was first shown using mitochondria isolated from plants with S-type cytoplasm (Finnegan and Brown 1986). Intact mitochondria synthesized new strands of RNA plasmids at concentrations of actinomycin D that completely inhibited DNA-dependent RNA synthesis. The most prominent species observed was the single-stranded RNA *b*. Less abundant RNA *b'*, RNA *a* and a 2100b long RNA were also seen. Surprisingly, the radioactive labelled RNA *b'* and RNA *a* were shown to be double-stranded, suggesting that the newly made RNA strands remained base-paired with its template. Results of several experiments ascertained that the incorporation of radioactive label was due to a true elongation of RNA chain rather than to a terminal labelling.

Finnegan and Brown (1986) concluded that the maize RNA plasmids are replicated independently of any DNA template, i.e. in a way similar to RNA viruses. Indeed, this conclusion is now strongly supported by the direct evidence of (-) strands of both RNA *b'* and RNA *b* (see above).

The same method - *in organello* labelling - was used for investigating the replication of endogenous RNA plasmids in a wider selection of maize cytoplasms, and the results were compared with their distribution as determined by hybridizations (Zhang 1993). Similarly to S cytoplasm, replication of several species was seen. Synthesis of new RNA *b* strands seemed to coincide with the presence and/or replication of larger species in all cytoplasms examined.

It was not determined in any of the *in organello* labelling experiments discussed above whether the newly synthesized RNA plasmid molecules represented the (+) strand, more abundant in the steady-state population, or rather the complementary (-) strand.

RNA b and its structural characteristics

RNA *b* is the only member of the maize RNA plasmid family that has been completely sequenced and whose structure has been studied in a greater detail (Zhang and Brown 1993). It is a single-stranded linear molecule, 719b long, which does not seem to code for any proteins as it lacks any open reading frame of a substantial length. When database searches were conducted, the (-) strand of RNA *b* was found to share a 13 nucleotide long sequence with the cadang-cadang viroid (Haseloff *et al.* 1982). 11 of these 13 nucleotides fall within the viroid conserved region. Since central conserved region is thought to be important for viroid replication (Diener 1986) it was speculated that the analogous RNA *b* region might serve a similar function (Brown and Zhang 1993). It was, however, also noted that no circular forms of RNA *b* had been detected and thus the molecule was unlikely to replicate by a rolling-circle mechanism employed by viroids.

RNA *b* is characterized by an extensive secondary structure. Computer analysis predicted a high level of intra-molecular base-pairing that gives the molecule a partly double-stranded appearance (Zhang and Brown 1993). It seems probable that the secondary structure plays a role in RNA *b* replication, particularly in the recognition of the molecule by a replicating enzyme (Zhang and Brown 1993) as was suggested for RNA molecules replicated by Qß replicase (Priano *et al.* 1987, Munishkin *et al.* 1991). An unpublished picture of the RNA *b* predicted secondary structure was kindly provided by M.Zhang and G.Brown, and is enclosed, with the permission of the authors, in the appendix of this thesis.

The 5' and 3' termini of RNA *b* are inaccessible to modifying enzymes (Zhang and Brown 1993). This is thought to be due to the extensive secondary structure of the molecule. However, a possibility of unusual modifications of the termini was not excluded. Computer analysis have predicted a direct base-pairing between a GGGG sequence located at the extreme 5' terminus and the 3' terminal stretch of three cytosine residues that would result in a formation of a circular-like molecule. Interestingly, analogous sequences have been found at the termini of Qß RNA and other RNAs serving as templates for Qß replicase, and at least some of these RNAs are predicted to assume a secondary structure with a similar base-pair interaction between the termini (Priano *et al.* 1987, Munishkin *et al.* 1991).

RNA *b* is likely to represent an internally deleted form of RNA *a* (Zhang and Brown 1993). It was proposed that the relationship between RNA *a* and RNA *b* might be analogous to the relationship between the genomic and defective interfering (DI) RNAs of RNA viruses (Zhang and Brown 1993). It remains to be shown, however, whether RNA *a* or a larger member of the family code for proteins that are necessary for replication of these RNA molecules.

Origin of maize RNA plasmids

The origin of these peculiar self-replicating RNA molecules is completely unknown. It has been speculated by Finnegan (1989) and Zhang (1993) that they might have originated from a virus, which has lost some of its functional characteristics including infectivity. Alternatively, the RNAs may be derived from a nuclear or mitochondrial transcript that somehow acquired ability to self-replicate and whose sequence has been altered "beyond recognition" during the course of evolution. In the first scenario, replication of RNA plasmids would likely be mediated by a polymerase encoded by the replicons themselves. The second hypothesis implies an involvement of a host apparatus.

Replication complexes of RNA replicons

RNA replicons vary in size and complexity as do their replication strategies and enzymes involved. The purpose of this chapter is to give a brief overview of those features directly related to the formation of replicative complexes. Therefore, enzymes involved in the replication and signals that ensure their specific binding to the target RNA sequences will be discussed, together with the existing direct evidence of replication complexes. For the reason of clarity, replication strategies will be briefly mentioned as well.

Coliphage Qβ

Replication of Qß phage can serve as a typical example of general replication strategies utilized by plus-strand RNA viruses. Upon entering the host cell, the single-stranded RNA genome of phage Qß functions as a polycistronic mRNA, from which the virus-specific proteins are translated. Using the genomic RNA as a template, the phage-induced RNA-directed replicase than synthesizes a full length

(-) strand (Spiegelman *et al.* 1968, Weissmann *et al.* 1968), which later gives rise to single stranded (+) RNA progeny by an analogous mechanism (Feix *et al.* 1968). Effective replication of each strand is apparently dependent on the ability of the template and the nascent chain to assume an extensive secondary structure and in this manner prevent an inter-molecular base-pairing (Weissmann 1974). Double-stranded molecules are biologically inactive and are considered to be dead-end products (Weissmann *et al.* 1968).

Qß replicase (reviewed by Blumenthal and Carmichael 1979) is one of the very few viral RNA-directed RNA polymerases (RdRp) that has been well characterized. The enzyme consists of four subunits: a phage-coded polypeptide (Kamen 1970, Kondo *et al.* 1970) which represents the active polymerase subunit, 30S ribosomal protein S1 (Wahba *et al.* 1974), and protein synthesis elongation factors EF-Tu and EF-Ts (Blumenthal *et al.* 1972). The S1 protein seems to be involved in recognition of specific sites on Qß RNA and the Tu-Ts complex in initiation of replication, although the exact function of the latter is not completely clear (Blumenthal and Carmichael 1979). Since EF-Tu normally binds aminoacyl-tRNAs and since the 3' end of Qß RNA can resemble tRNA, including the presence of a 3' terminal -CCA_{OH} trinucleotide (Rensing and August 1969), it was suggested that EF-Tu might bind to the 3' end of the molecule and possibly supply the first GTP (Blumenthal *et al.* 1972, Landers *et al.* 1974). In addition to these polypeptides, a host factor required for initiation of Qß RNA replication has been identified (Franze de Fernandez *et al.* 1968).

Qß replicase can efficiently replicate a number of small RNA molecules ("variant" and "6S" RNAs), some of which do not show any homology to the Qß genome (Mills *et al.* 1973, Mills *et al.* 1975, Schaffner *et al.* 1977, Munishkin *et al.* 1991). Thus the replicase is likely to recognize a specific secondary and/or tertiary structure of a template RNA rather than any primary sequence signal at the 3' end (Priano *et al.* 1987). Indeed, it was shown that the Qß replicase binds strongly to internal regions of the Qß RNA (Meyer *et al.* 1981). This contact is thought to

result in a subsequent specific positioning of the 3' end in an active site of the enzyme and eventually in an initiation of RNA synthesis (Meyer *et al.* 1981). Although unlikely to be involved in the initial binding, the 3' terminal -CCC(A)$_{OH}$ sequence seems to be necessary for correct replication, as it is present in all RNA molecules replicated by Qß replicase.

As far as physiochemical properties of the Qß replicative complexes are concerned, a replicative intermediate (RI) of Qß phage (i.e. template with growing RNA strands and replicase molecules) was shown to sediment at 40-50S in a sucrose gradient, while a value of 30S was determined for a non-replicating phage RNA (Feix *et al.* 1967, Hori 1970).

Plus-strand RNA viruses

Replication strategies of eukaryotic plus-strand RNA viruses are similar to that of bacteriophage Qß (see above). Genomic (+) ssRNA is released from a nucleocapsid and translated into virus-specific proteins that trigger synthesis of the complementary (-) strand. The (-) strand is then copied into (+) strand viral RNAs, which would be later encapsidated. In some cases, the (-) strand also serves as a template for transcription of a subgenomic (+) RNA coding for structural proteins (Simmons and Straus 1972, Miller *et al.* 1985).

Replication of a plus-strand RNA virus in infected tissue is accompanied by an appearance of a heterogeneous group of partially double-stranded RNA molecules, the replication intermediates (RIs). Each RI is composed of a genome-length strand and varying number of complementary strands of length smaller or equal to genomic RNA (Baltimore and Girard 1966, Girard 1969, Savage *et al.* 1971, Simmons and Straus 1972). In addition, an accumulation of fully double-stranded RNA molecules - replicative forms (RFs) - is often observed. A long-standing question whether these molecules also represent intermediates in replication or dead-end products and/or possible artifacts of the experimental approach remains open (for

discussion on RFs and RIs, see Richards and Ehrenfeld 1990 and Marsh *et al.* 1990).

Genomic RNAs of plus-strand RNA viruses possess several specific features (Matthews 1982). The 5' end is often protected by either a cap structure (e.g. brome mosaic virus, Sindbis virus, and Semliki-forest virus) or by a covalently linked small protein, designed as VPg (poliovirus, cowpea mosaic virus). Interestingly, the linkage of VPg to poliovirus RNA occurs either at the time or immediately after initiation of new RNA strand synthesis and it was proposed that the protein may actually function as a primer in this process (reviewed in Richards and Ehrenfeld 1990). The 3' end of genomic RNA can contain a poly(A) tail, and in this respect resemble eukaryotic mRNAs, or it can assume a tRNA-like structure with the terminal trinucleotide -CCA_{OH}.

3' terminal structures that resemble tRNAs have been described for plant RNA viruses belonging to several different virus groups (for review see Mans *et al.* 1991). It is now generally accepted that these sequences function in the initiation of (-) strand synthesis (Ahlquist *et al.*1984, Miller *et al.* 1986). Litvak *et al.* (1973) and Hall and Wepprich (1976) speculated that eukaryotic elongation factors might be involved in replication of these viruses *via* recognition of their tRNA-like structures, in a manner analogous to the interaction of bacterial EF-Tu and EF-TS with Qß RNA. This concept seems to be supported by the fact that genomic RNAs of several viruses can be aminoacylated (Mans *et al.* 1991), and elongation factors bind preferentially to aminoacylated tRNAs. Some evidence of a direct interaction between elongation factors and RNA of plus-strand RNA viruses exists (Litvak *et al.* 1973, Bastin and Hall 1976, Joshi *et al.* 1986) but more information is clearly necessary.

Several plus-strand RNA viruses contain sequences closely resembling the internal control regions (ICRs) of tRNA genes (Marsh *et al.* 1987, Marsh *et al.* 1989, van der Vossen *et al.* 1993). In the case of brome mosaic virus (BMV), ICR sequences are located close to the 5' end (Marsh and Hall 1987) and affect primarily the (+) strand synthesis (Pogue e*t al.* 1990). Based on these results, the authors

proposed that host transcription factors normally interacting with the RNA polymerase III might in fact be responsible for a modification of the replicative complex that results in a switch from synthesis of the (-) strand to synthesis of the (+) strand (Marsh and Hall 1987, Marsh *et al.* 1990).

Replication complexes of plus-strand RNA viruses are membrane-bound (Friedman *et al.* 1972, Zabel *et al.* 1974, Froshauer *et al.* 1988, Martín and García 1991, Bienz *et al.* 1992, Lee *et al.* 1992); this has hindered purification and characterization of enzymes directly involved in replication. Consequently, substantial information about the components of replication complexes and their corresponding functions exists for only a few viruses, the best studied being that of poliovirus. Crude poliovirus replication complexes were shown to contain, in addition to ssRNAs, RIs, RFs and membranes, one major polypeptide representing the viral coded polymerase and several other viral and host-encoded proteins, including a precursor of the VPg and a "host factor" necessary for initiation of synthesis (reviewed by Ishihama and Nagata 1988 and Richards and Ehrenfeld 1990). Using EM immunochemistry, Bienz *et al.* (1992) have demonstrated that polioviruses replication complexes actually contained two membrane systems: rosette like virus-induced vesicles and compact membranes which enclosed the nascent chains.

Solubilized replication complexes of Sindbis virus and Semliki Forest virus, two closely related alphaviruses, have a density of 1.25g\cm^3 and sediment at 20-100S (Barton *et al.* 1991). Analysis of their protein components revealed presence of several virus encoded proteins as well as of one cellular protein. Two of the virus specific proteins detected in the complex, nsP1 and nsP2, are likely to possess capping functions and helicase activity, respectively (Cross and Gamotos 1981, Cross 1983, Gorbalenya and Koonin 1989). Interestingly, analogous functions have been assigned to two polypeptides encoded by brome mosaic virus (BMV) genome (see Marsh *et al.* 1990). Thus a helicase activity separating newly made (+) strands from quasi-double-stranded RIs might be one of the necessary components of viral RNA-dependent RNA polymerase complexes.

Replication of plus-strand RNA viruses requires cooperation between virus-encoded and cellular proteins, as suggested by presence of both in replication complexes and in template-dependent RdRp preparations (see above, also Dorssers *et al.* 1984, Mouches *et al.* 1984, Hayes and Buck 1990). One of the possible functions of cellular proteins might be to confer flexibility to the replication apparatus, that is, to enable regulation of synthesis at several specific initiation sites (minus- versus plus-strand synthesis and switch between transcription of subgenomic and genomic size RNA molecules). In this regard, their function would be similar to that of eukaryotic transcription factors. Elongation of nascent chains is then catalyzed by the virus-encoded RNA polymerase.

Minus-strand RNA viruses

Genomic RNA of minus-strand RNA viruses is not recognizable by the host cell translational machinery. Therefore, the first step in multiplication of these replicons inside a cell is the transcription of virus-specific mRNAs, mediated by a nucleocapsid-associated RdRp. After sufficient pool of new viral proteins is built up, the genomic (-) ssRNA serves as a template for synthesis of a full-length (+) strands that are later copied into the (-) strand progeny. By contrast to plus-strand RNA viruses, the template for any synthesis is not a naked RNA molecule but rather the RNA associated with nucleocapsid protein (Banerjee 1987, Ishihama and Nagata 1988). The RdRp-containing nucleocapsid of either strand can thus be regarded as a potential replication complex. Furthermore, it was reported that newly synthesized (+) and (-) RNA strands of the vesicular stomatitis virus (VSV), a member of family *Rhabdoviridae*, are found only in form of nucleocapsids suggesting that their encapsidation occurs immediately after synthesis (Soria *et al.* 1974). The VSV nucleocapsid structures are unusually stable. They sediment in CsCl gradient at a density of 1.3 g/cm^3 (Wertz *et al.* 1987) in comparison with density of 1.2 g/cm^3 observed for the enveloped VSV virion (Matthews 1982).

An interesting feature of minus-strand RNA viruses genomes is the presence of short inverted repeats at their termini (Lamb and Choppin 1983, Banerjee 1987). In other words, the 3' ends of (-) and (+) strands are alike and they can both be recognized by one replicating enzyme.

Double-stranded RNA viruses

Double-stranded RNA viruses contain a dsRNA molecule in their capsids. The first event in their multiplication is the transcription of full length (+) strands by a nucleocapsid-associated RdRp. In some double-stranded viruses, the replication is conservative, like that of the *Saccharomyces cerevisiae* dsRNA virus (Fujimura *et al.* 1986) and reoviruses (reviewed in Estes 1990). In others, the process is semiconservative, an example being that of bacteriophage ϕ6 (Usala *et al.* 1980). The only difference between these two types is whether the newly transcribed strand is released (conservative replication) or displaces the strand of the same polarity in dsRNA template (semiconservative replication). The (+) ssRNA is than extruded from the particle (Fujimura *et al.* 1986) and serves as a mRNA for translation of virus-specific proteins. In case of the *Saccharomyces cerevisiae* dsRNA virus, newly made structural and non-structural proteins assemble around the (+) ssRNA thus forming new virus particles that later complete the replication cycle by synthesizing the complementary (-) strand (reviewed by Wickner 1992). The (+) strand of bacteriophage ϕ6 is taken up by preformed procapsids (Gottlieb *et al.* 1990) suggesting that in at least some dsRNA viruses the assembly of nucleocapsids can precede the genome packaging.

It is clear from the previous discussion that replication complexes of double-stranded RNA viruses are represented by the whole nucleocapsids. Physiochemical properties of these particles will vary slightly among different groups of dsRNA viruses but the replicating VLP of *Saccharomyces cerevisiae* virus with its density of 1.35 g/cm^3 (Fujimura *et al.* 1986) serves as a good example.

Viroids

Viroids are circular single-stranded RNA pathogens of plants. As they represent the smallest and also the simplest RNA replicons known, considerable attention has been given to their investigation and numerous reviews concerning different aspects of viroid molecular biology have been published (recently by Owens and Hammond 1988, Diener 1991).

Viroids replicate by a rolling circle mechanism (Branch *et al.* 1981, Owens and Diener 1982, Branch and Robertson 1984). As they do not seem to code for any proteins themselves, their multiplication must be mediated solely by the host cell enzymes. It is now widely excepted that the host DNA-dependent RNA polymerase II is directly involved in the replication (Mühlbach and Sänger 1979, Rackwitz *et al.* 1981, Schindler and Mühlbach 1992). RNA polymerase I has also been suggested as a potential replicating enzyme (Semancik and Harper 1984, Spiesmacher *et al.* 1985) but its involvement is questionable.

Several viroid domains are thought to be important in their replication, among them the central conserved region (Diener 1986) and terminal domains that are capable of directly binding purified DNA-dependent RNA polymerase (Goodman *et al.* 1984). In addition, viroid RNA exhibits features, characteristic of group I and group II introns (reviewed by Hadidi 1986); they might be involved in a potential self-processing of the viroid RNA.

A replication complex of citrus exocortis viroid (CEV) has been extracted from nuclei-rich preparation of infected leaf tissue (Rivera-Bustamante and Semancik 1989). Solubilization of the complexes was achieved by treating the nuclei with sarkosyl. The complexes synthesized both linear and circular CEV RNA, suggesting that enzymes necessary for circularization of newly made linear CEV RNA molecules were present as well. Addition of exogenous RNAs did not affect the replication. Therefore, the complexes had been assembled *in vivo* and did not form after or during the extraction. When solubilized nuclear extracts were fractionated on a sucrose gradient, the viroid-replicating complexes sedimented slightly faster than

the majority of CEV RNA. Surprisingly, the radioactively labelled nascent CEV RNA cosedimented with the complexes, indicating that it remained in a close association with them rather than being released.

In vitro reconstitution studies revealed that potato spindle viroid (PSTV) form complexes with histones and a 41 kDa protein (Wolff *et al.* 1985). The presence of viroid complexes *in vivo* was assayed by sedimentation of nucleosomal fraction of nucleoli through linear sucrose gradient. The majority of viroid RNA was bound in complexes with sedimentation coefficients between 12 to 15S, a value characteristic for nucleosomes (Wolff *et al.* 1985). Given the basic character of histones, it is not clear whether their association with viroids has a physiological significance or results merely from non-specific interactions.

RNA plasmids

Information concerning replication of naked non-viroid RNA molecules is very scarce. DsRNA from tobacco leaves (Ikegami and Fraenkel-Conrat 1979) might be replicated a host enzyme, the RNA-dependent RNA polymerase, which was previously shown to be present in a variety of plants (Fraenkel-Conrat 1986). However, the conclusion is based only on a similar distribution of the dsRNA and the enzyme inside the cell. Single-stranded 20S RNA, found in some strains of yeast *Saccharomyces cerevisiae* (Wickner 1992), can be classified as a plasmid although it encodes a protein resembling the RNA-dependent RNA polymerase of (+) RNA viruses (Matsumoto and Wickner 1991). The 20S RNA is a naked (Widner *et al.* 1991), probably circular molecule (for a discussion see Wickner 1992). Interestingly, longer-than-one-unit molecules were observed (Matsumoto and Wickner 1991) and thus it is possible that the 20S RNA replicates by a rolling-circle mechanism. In addition, a linear dsRNA molecule (W RNA) has a virtually identical sequence and might thus represent the RF of the 20S RNA (Rodriguez-Cousino *et al.* 1991).

As mentioned previously, the dsRNA associated with CMS in *Vicia faba* is enclosed together with its replicase in membranous vesicles (Lefebvre *et al.* 1990). Upon treatment of the vesicles with a non-ionic detergent, the enzyme remains associated with its template; the solubilized replication complexes are capable of incorporating a radioactive nucleotide into dsRNA molecules. The composition and origin of the replicase are yet to be determined.

Konarska and Sharp (1989, 1990) have characterized two structurally related RNAs (X and Y) that are replicated *in vitro* by the DNA-dependent RNA polymerase of bacteriophage T7. Both RNAs may assume secondary structures resembling tRNAs, including the 3' terminal -CC_{OH} dinucleotide. In the case of X RNA, the 3'end could be adenylated by the *E. coli* nucleotidyl transferase (Konarska and Sharp 1990). Several conclusions can be made based on these results: not only can DNA-directed RNA polymerases efficiently replicate certain RNAs but they can recognize the same structure (tRNA element) as RNA-dependent RNA polymerases do, suggesting that both enzymes evolved from the same ancestor.

Materials and Methods

Materials

Inbred maize seeds (pedigree Mo17VgMsHt) were purchased from Mike Brayton Seeds (Ames,IA) or from Holden's Foundation Seeds (Williamsburg,IA). Maize seedlings were dark-grown at 28°C for 5 days. The plasmid clone containing a cDNA corresponding to the 5' terminal 620b of RNA *b* sequence was described previously (Zhang and Brown 1993). [α-^{32}P]-UTP was obtained from ICN Biomedicals Canada. RNA Transcription Kit was from Stratagene. Ultrapure rNTPs and RNA guard™ (placental RNase inhibitor) were from Pharmacia. DNase and RNase free BSA, actinomycin D, and cordycepin-5'-triphosphate were from Boehringer Mannheim, Hybond™-N nylon transfer membrane from Amersham, and Percoll from Sigma. Restriction enzymes, DNA ligase, and RNA ladder were purchased from Gibco BRL.

Methods

Isolation of mitochondria

Crude mitochondria were obtained by a modification of the method originally described by Kemble *et al.* (1980). Briefly, the etiolated maize shoots were homogenized in a grinding buffer (0.4M mannitol, 10mM HEPES pH 7.2, 1mM EGTA, 5mM sodium bisulfate, 0.25% BSA). The resulting homogenate was filtered and then clarified by centrifugation at 1000xg for 10 min. Mitochondria were collected by centrifugation at 12,000xg for 10 min. For the majority of purposes, mitochondria were further purified essentially as described by Jackson *et al.* (1979). The crude mitochondrial pellet was resuspended in the grinding buffer

(20 ml of buffer/100 g of tissue), and the suspension was cleared for a second time by centrifugation at 1000xg for 10 min. followed by final pelleting at 12,000xg for 15 min. The mitochondria were resuspended in grinding buffer (around 2 ml/100 g of tissue) and carefully layered on top of a discontinuous Percoll gradient. Each gradient comprised 3 ml of 45%, 3 ml of 21%, and 3 ml of 13.5% Percoll mixtures, each containing 0.25M sucrose, 5mM HEPES (pH 7.2), 10mM EGTA, and 0.1% BSA. Between 2 and 3 ml of mitochondria suspension was loaded on each gradient. Gradients were centrifuged in a SW 41 rotor at 15,000 rpm for 35 min. at 4°C. The mitochondria were collected from the lowest interface, diluted at least 10 times with wash buffer (0.3M mannitol, 10mM HEPES pH 7.2, 10mM EDTA) and collected by centrifugation at 12,000xg for 15 min. The mitochondrial pellets were frozen in liquid nitrogen and stored at -70°C.

In experiments, where quantity rather than quality of mitochondria was required (isopycnic sedimentation of mitochondrial lysate in CsCl gradients), the crude mitochondrial pellet (see above) was resuspended in a grinding buffer and recentrifuged (10,000xg for 10 min.) through a sucrose cushion (0.6M sucrose, 20mM EDTA, 10mM Tris pH 7.6), essentially as described by Kemble *et al.* (1980). The mitochondrial pellets were frozen in liquid nitrogen and stored at -70°C.

Preparation of mitochondrial lysate

Frozen mitochondrial pellet was quickly thawed by an addition of ice-cold 1xR/L (replication/lysis) buffer (40mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 25mM $(NH_4)_2SO_4$, 3mM DTT). For most experiments, 50-100 µl of buffer was added to mitochondria isolated from 10 g of tissue. The suspension was subjected to two cycles of quick freezing (in liquid nitrogen) and thawing (in 37°C) and the resulting lysate was clarified by centrifugation at 12,000xg for 15 min.

In vitro synthesis of maize RNA plasmids molecules and analysis of the products

The constituents of the RNA plasmid labelling reactions that were constant unless specified otherwise were: 40 mM Tris-HCl (pH 8.0), 10mM $MgCl_2$, 25mM $(NH_4)_2SO_4$, 3mM DTT, 400μM ATP, CTP, and GTP, 40μM UTP, 10 μCi of [α-^{32}P]-UTP (3000 Ci/mmol), 50 μg/ml of actinomycin D, and 1000U/ml of RNA guard™. Experiments investigating the RNA plasmid synthesis activity in mitochondrial lysate were typically carried out in a volume of 20 μl, and contained lysate derived from Percoll purified mitochondria obtained from 1-2 g of tissue. For detection of the activity in fractions from sucrose and CsCl gradients, up to 50 μl of each fraction was used and the reaction volume was kept as low as possible while increasing the concentration of [α-^{32}P]-UTP up to 20 μCi per reaction. The reaction mixtures were incubated at 27°C for 60 min. An equal volume of stop buffer (0.5% SDS, 50 μg/ml of proteinase K, and 200 μg/ml of *E. coli* tRNA) was added to the reactions, and the incubation continued at 37°C for another 15 min. Products of the reaction were then phenol/chloroform-extracted, precipitated by ethanol, and resolved on a 1% agarose/5M urea gel, as described previously (Finnegan and Brown 1986), or on a 4% polyacrylamide/urea gel (Maniatis *et al.* 1982).

Rate zonal centrifugation

Mitochondrial lysate derived from Percoll purified mitochondria from 20 g of tissue was divided into two equal parts: one was left untreated while the other was supplemented with 0.2% SDS and 100 μg/ml of proteinase K and incubated for 30 min. at 37°C. The samples were loaded on linear 10-40% sucrose gradients in 1xR/L buffer, and centrifuged in a SW 50.1 rotor at 39,800 rpm for 14 hrs. and 20 min. at 4°C. Fractions were collected by displacement. Part of each fraction was extracted twice by phenol/chloroform and nucleic acids were precipitated by ethanol, resolved on 1% agarose/urea gel, and stained by ethidium bromide. For specific detection of RNA plasmids, RNAs were transferred from the gel to a nylon

membrane and detected by Northern hybridization (see below). An aliquot of each fraction of untreated gradient was assayed for the RNA plasmids labelling activity.

Density gradient centrifugation

Mitochondria from 400 g of tissue were purified on sucrose cushion (see above) and lysed in 1xR/L buffer. The clarified lysate was supplied with saturated solution of CsCl to a final density ρ_o^{25} = 1.46 g/cm^3 (refraction index η_D^{25} = 1.3773) while keeping the concentration of components of R/L buffer constant, and centrifuged in a SW 50.1 rotor at 39,800 rpm for 40 hrs. at 4°C. The gradient was fractionated by collecting drops from the bottom, and the refraction index η_D^{25} of each fraction was measured. The fractions were then dialyzed against 500 ml of 1xR/L for 3 x 2 hrs. at 4°C. The dialyzation tubing had been treated in 1xR/L containing 0.5 mg/ml of DNase and RNase free BSA (1 hr. at 50°C) prior to the dialysis. This treatment was necessary for the subsequent recovery of RNA plasmid labelling activity. Dialyzed fractions were analyzed similarly to the sucrose gradient fractions, with the exception that nucleic acids were not stained by ethidium bromide but directly transferred to a nylon membrane and hybridized to RNA *b* sequences. The refraction index η_D^{25} of each fraction was converted to a density ρ^{25} value according to a table in the manual "Techniques of Preparative, Zonal, and Continuous Flow Ultracentrifugation" (by O.M.Griffith, Spinco Division, Beckman Instruments, Inc.).

Preparation of RNA b probes

Radioactive RNA *b* probes were prepared by run-off *in vitro* transcription of RNA *b* cDNA clones. The (+) RNA *b* strand was transcribed from the original plasmid (Zhang and Brown 1993) that had been linearized by *Eco*RI. For synthesis of the (-) strand, a 44bp long *Not*I-*Not*I fragment, located between the promoter for synthesis of the (-) strand and the first 5' nucleotide of the (-) RNA *b* cloned

sequence was removed. The resulting plasmid was linearized by *Hind*III. *In vitro* transcriptions were performed using RNA Transcription Kit (Stratagene); the conditions of the reactions were as recommended by the manufacturer for the production of probes with very high specific activity.

Northern hybridization

After separations of the mitochondrial RNAs on agarose/urea gels, the gels were washed with distilled water to remove urea, and the RNAs were transferred to a Hybond^{TM}-N membrane by electroblotting for 1 hr. in 0.5xTAE (20mM Tris pH 7.8, 10mM sodium acetate, and 0.5mM EDTA) under constant current of 1 A (Biorad electroblotting apparatus). The blots were either baked for 2 hrs. at 80°C or UV crosslinked. Prehybridization of the blots was carried out for 1-2 hrs. at 55°C in a solution containing 50% formamide, 5xDenhardt's reagent, 5xSSPE, 0.1% SDS, 100 µg/ml of denatured, fragmented salmon sperm DNA, and 50 µg/ml of denatured yeast RNA. Heat-denatured radioactive RNA probe at a concentration of 1-5x10^5 cpm/ml was added to the hybridization buffer and the incubation continued at 55°C overnight. The membranes were washed three times in 1xSSPE/0.5% SDS at 65°C for 15 min. each, and then once in 0.1xSSPE/0.5% SDS for 15 min. at 60°C Blots were stored wet; if required, the probe was stripped off the membrane by a boiling solution of 0.1% SDS which was poured on the membrane and allowed to cool to room temperature.

RNase A protection analysis of the in vitro labelled RNA plasmids

Plasmid RNA was labelled by a standard *in vitro* labelling reaction using mitochondrial lysate equivalent to 15 g of tissue. The RNAs were phenol/chloroform extracted, precipitated by ethanol and resuspended in DEPC-treated water. Aliquots of the labelled RNAs were added to 40 µl of hybridization buffer (0.7M NaCl, 0.1M Tricine pH 7.8, 2mM EDTA, and 50% formamide), together with 400 ng of

in vitro transcripts of RNA *b* cDNA clones or without an addition of exogenous RNA ("mock-hybridized" control). The samples were heated at 85°C for 10 min., and hybridized overnight at 57°C. After cooling to room temperature, the samples were diluted by an addition of 240 µl of dilution buffer to a final concentration of 300µM NaCl/10mM Tricine pH 7.8/5mM EDTA. 5 µg of RNase A together with 10 µg of *E.coli* tRNA were added and the samples were incubated at 37°C for 1 hr. The digestion was stopped by the addition of 0.5% SDS, 200 µg/ml of proteinase K, and 10 µg of *E.coli* tRNA per reaction, and the reaction was continued at the same temperature for another 30 min. Protected RNA fragments were extracted twice with phenol/chloroform, ethanol precipitated, and resolved on a 4% polyacrylamide/urea gel.

Results

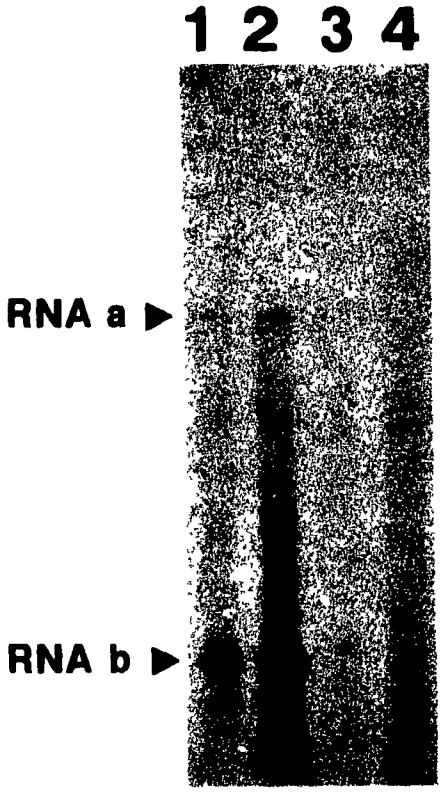
In vitro synthesis of plasmid RNA in maize mitochondrial lysate

In previous studies, *in vitro* synthesis of new strands of maize mitochondrial RNA plasmids took place inside intact mitochondria (Finnegan and Brown 1986). I attempted to develop an assay for this activity in which a mitochondrial lysate rather than the intact organelles could be used. Mitochondria from maize plants with the S-type cytoplasm were purified on a Percoll gradient, disrupted by freeze/thaw cycles, and the lysate was assayed for synthesis of endogenous plasmid RNA as described in Materials and Methods. Several plasmid RNAs were synthesized under these conditions (Fig. 1). As in *in organello* labelling (Finnegan and Brown 1986), the most highly labelled product was the 719b long RNA, RNA *b*. Labelling of RNA *a* (around 2800b) was also observed. Removal of membranes from the crude mitochondrial lysate by centrifugation did not result in a significant decrease in the number of labelled products (Fig. 1, lane 1, as compared to Fig. 1, lane 2) indicating that all components of the activity were soluble.

We had previously observed that maize mitochondria can, under certain conditions, cosediment with an unspecified soluble nucleotidyl transferase activity (unpublished results). In order to exclude the possibility that this activity might have contributed to the labelling of RNA plasmids molecules in mitochondrial lysate, the nucleotide requirements of the RNA plasmid synthesis activity was examined. As shown in Fig. 2A, efficient incorporation of radioactive UTP into plasmid RNAs required the presence of all four nucleotides When the reaction contained the UTP alone (Fig. 2A, lane 5, also Fig.1, lanes 3 and 4), or when one nucleotide was omitted (Fig. 2A, lanes 2, 3, and 4), little or no labelling of RNA *b* was observed. In addition, the activity was inhibited by the 3'deoxy- analog of ATP, cordycepin-5'-triphosphate (Fig. 2C, lanes 3, 4, and 5). These results confirmed that the observed

Figure 1. *In vitro* labelling of maize RNA plasmids in a mitochondrial lysate

Maize mitochondria, isolated by centrifugation through a Percoll gradient, were lysed by two consecutive freeze/thaw cycles. A crude, membranes-containing lysate (lanes 2 and 4), and a lysate that had been clarified by centrifugation at 12,000xg for 15 min. (lanes 1 and 3) were assayed for their ability to support synthesis of endogenous RNA plasmid strands *in vitro*. Products of the reaction were extracted, resolved on a 1% agarose/urea denaturing gel and visualized by autoradiography. Lanes 1 and 2 show results of reactions, in which all four nucleotides were present, while lanes 3 and 4 represent controls, to which only the labelled UTP was added. Positions of RNA *b* and RNA *a* are indicated.


1 2 3 4
RNA a
RNA b

incorporation of labelled nucleotides into plasmid RNAs was due to a synthesis of new RNA molecules.

Further investigation of the RNA plasmid synthesis activity was carried out by treating the mitochondrial lysate with proteinase K prior to the reaction. The activity was completely abolished by such a treatment (Fig. 2B, lane 2) and has, therefore, a protein component. Addition of DNase I to the lysate did not have any effect while RNase A treatment resulted, not surprisingly, in a disappearance of the signal (not shown). The activity was resistant to rifampicin (Fig. 2C, lane 2) but inhibited by ethidium bromide (not shown). It was also sensitive to pyrophosphate; however, concentrations higher than 1mM were necessary for a complete inhibition (not shown). It is possible that an active pyrophosphatase might have been present in the mitochondrial lysate.

Heparin is a polyanion which disrupts weak binding between nucleic acids and proteins. It inhibits the initial, non-specific, interaction of RNA polymerase with its template; once an initiation complex is formed (and/or elongation started), the heparin sensitivity is lost. It has been reported that heparin concentrations as low as 40nM completely inhibited initiation of transcription by the yeast mitochondrial RNA polymerase (Biswas 1992). Interestingly, the synthesis of new maize RNA plasmid strands was not inhibited by heparin, even at concentrations as high as 200μM (Fig. 2D). This suggested that the observed labelling of plasmid RNAs was due to an elongation of plasmid RNA molecules preinitiated *in vivo*. Thus, preformed complexes, comprising an RNA template, RdRp, and nascent chains, must have been present in the lysate. This conclusion was further supported by the fact that neither dilution of the lysate 12.5 fold nor an addition of exogenous RNA *b* to the reaction had any effect on the total amount of RNA *b* synthesized (not shown). If the components necessary for plasmid RNA synthesis assembled in the lysate, it is expected that these manipulations would alter their concentration and hence that the total amount of RNA *b* synthesized would be altered. The results presented in Fig. 2D also show that higher concentrations of heparin seemed to result in

Figure 2. Characterization of the activity that synthesizes new strands of maize RNA plasmids

(A) Nucleotide requirements of the activity. Lane 1, standard reaction; lanes 2,3, and 4, reactions from which ATP, CTP, or GTP were omitted, respectively; lane 5, only the labelled UTP present.

(B) Sensitivity to proteinase K. Mitochondrial lysate was pretreated for 20 min. at 27°C in absence (lane 3) or presence (lane 2) of proteinase K at a concentration of 300 μg/ml, and then assayed for the activity. Lane 1, standard (unpretreated) reaction.

(C) Effect of cordycepin-5'-triphosphate and rifampicin. Lane 1, standard reaction; lane 2, reaction in the presence of 50 μg/ml of rifampicin; lanes 3,4, and 5, reactions supplemented with cordycepin-5'-triphosphate at a concentration of 4μM, 40μM, and 400μM, respectively.

(D) Effect of heparin. Lane 1, standard reaction; lanes 2, 3, 4, and 5, reactions supplemented with heparin at a concentration of 1μM, 10μM, 100μM, and 1mM, respectively.

In all experiments, a lysate of Percoll gradient purified mitochondria was used. Products of the reactions were extracted, resolved on 1% agarose/urea gels, or 4% polyacrylamide/urea gels, and visualized by autoradiography.

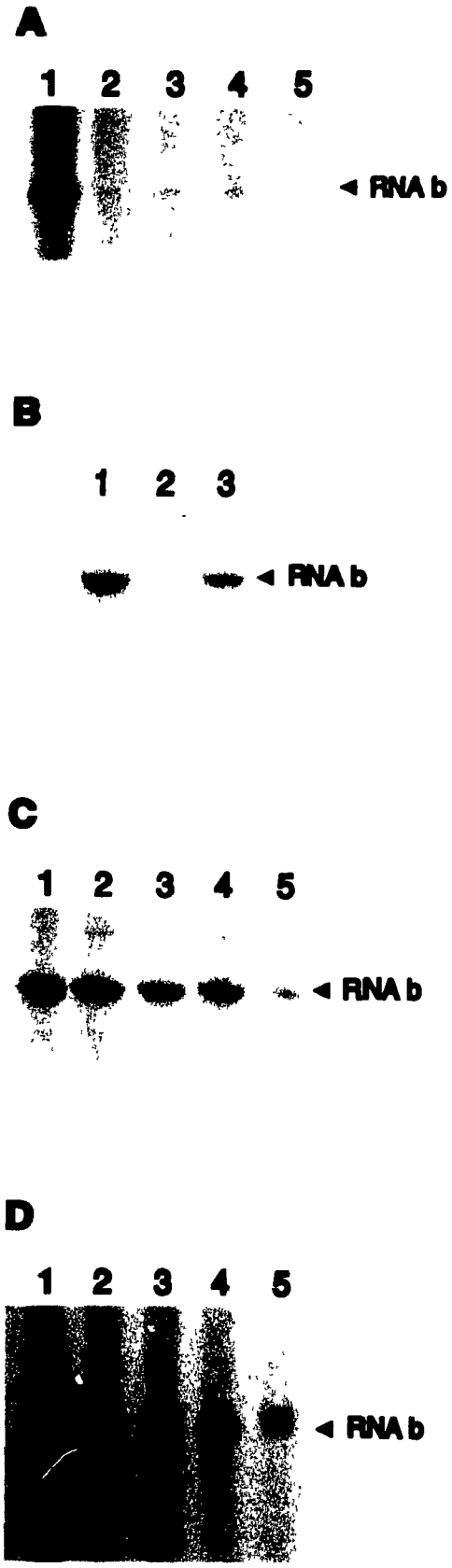
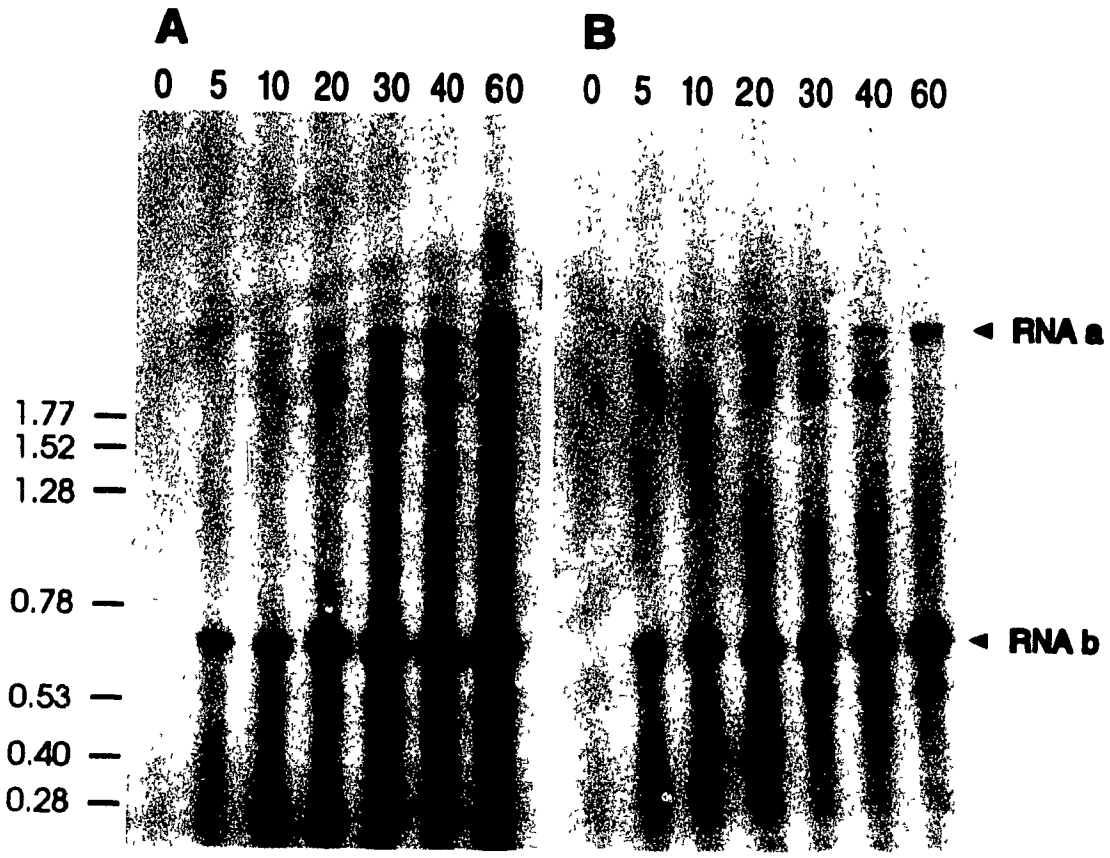

A
1 2 3 4 5
◄ RNA b
B
1 2 3
◄ RNA b
C
1 2 3 4 5
◄ RNA b
D
1 2 3 4 5
◄ RNA b

Figure 3. Time course of the synthesis of new RNA plasmid strands

Time course of the RNA plasmid labelling activity was examined both in standard conditions (panel A) and in the presence of 10 μM heparin (panel B). At the times indicated (in min.), aliquots were taken from each reaction and the RNAs were extracted, resolved on a 1% agarose/urea gel and analyzed by autoradiography. Positions of marker RNA molecules are indicated (sizes in kilobases).


A
0 5 10 20 30 40 60
B
0 5 10 20 30 40 60
1.77
1.52
1.28
0.78
0.53
0.40
0.28
RNA a
RNA b

an increased labelling of RNA *b'*, a plasmid species of around 800b. The significance of this is, however, unclear.

Time course of the synthesis of new RNA plasmid strands has been investigated with heparin both absent (Fig. 3A) and present (concentration of 10μM, Fig. 3B). In both experiments, the reaction rate was constant for at least 20 min.; then it slowly leveled off. Although heparin caused an overall slight decrease in the number of products, the kinetics of the reaction seemed to be very similar to that of the standard reaction. This finding is in agreement with the hypothesis that predicts an existence of preformed RdRp-RNA template complexes (see above).

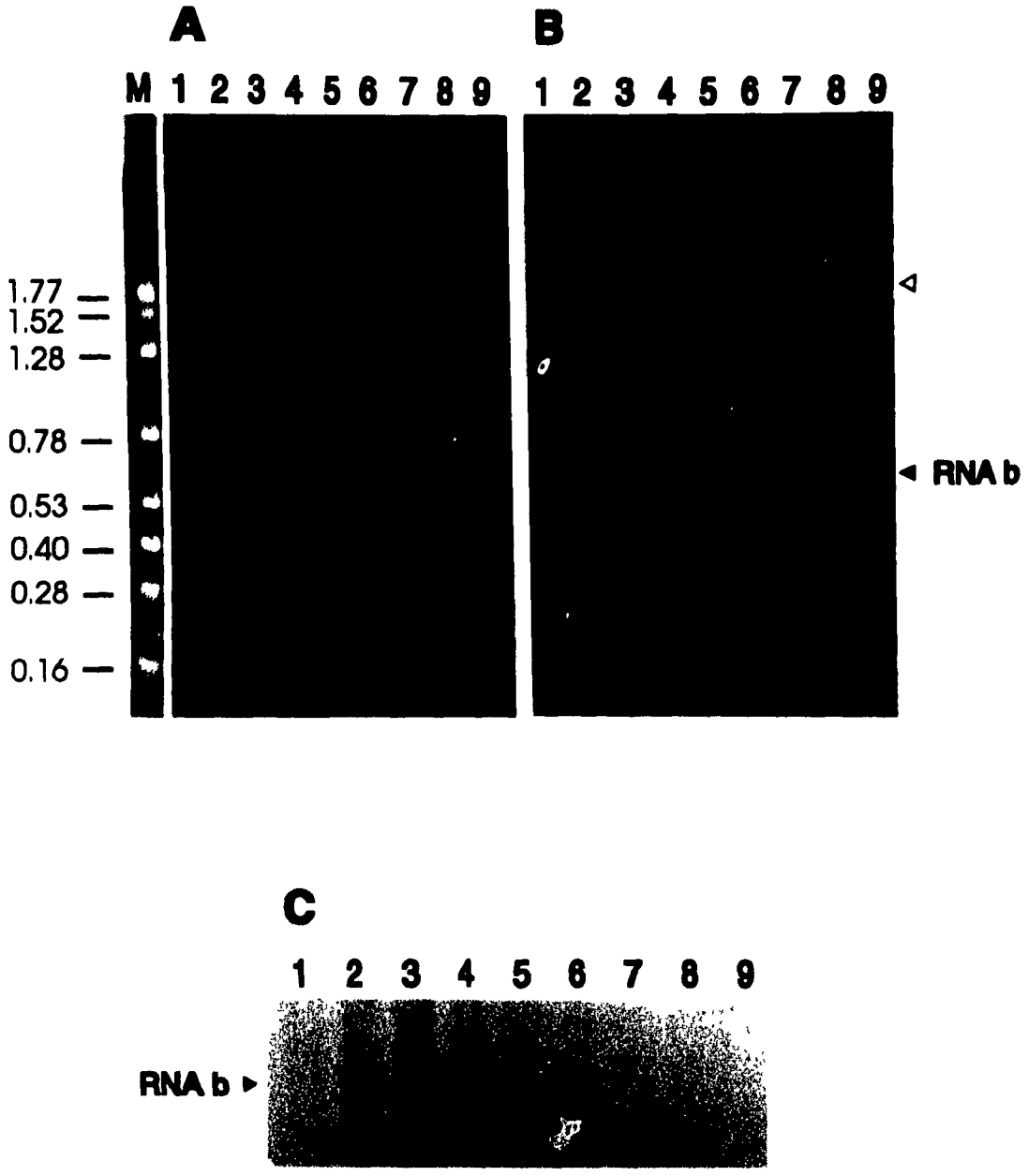
Rate zonal centrifugation of RNA *b* and the synthesis activity

In order to determine whether RNA *b*, the most abundant maize plasmid species is associated *in vivo* with proteins, a clarified lysate from Percoll purified maize mitochondria was fractionated on a 10-40% sucrose gradient, and the sedimentation position of RNA *b* (Fig. 4A) was compared with a sedimentation of the same molecule in a parallel gradient, on which proteinase K pretreated lysate was loaded (Fig. 4B). In both gradients, majority of RNA *b* was found in fraction 4. This indicated that the most prevalent form of RNA *b in vivo* was a naked molecule. The same experiment was repeated with freshly prepared sonicated mitochondria in order to exclude the possibility that any major complexes were dissociated by the freeze/thaw cycles, as had been reported for some ds RNA viruses (Nash *et al.* 1973). The results obtained (not shown) were virtually the same as those shown in Fig. 4A and 4B.

Distribution of the less abundant (-) strands in the fractions of the sucrose gradient was examined by Northern hybridizations. RNAs, resolved on the gel (Fig. 4A and 4B), were transferred on a membrane, and hybridized with (+) or (-) *in vitro* transcript of RNA *b* cDNA clones (for detection of (-) or (+) strands, respectively). Most of the RNA *b* (-) strand was detected in the same fraction where

Figure 4. Rate zonal centrifugation of RNA *b* and the synthesis activity

Clarified mitochondrial lysate, both untreated and pretreated with proteinase K, was fractionated by centrifugation through a 10-40% sucrose gradient (SW 50.1 rotor, 39,800 rpm for 14 h 20 min.). Fractions taken from each gradient were analyzed for their RNA content: nucleic acids were extracted, resolved on a 1% agarose/urea denaturing gel, and stained by ethidium bromide (panel A, untreated lysate; panel B, proteinase K treated lysate). Aliquots taken from each fraction of the untreated gradient were assayed for the ability to synthesize new strands of endogenous RNA plasmids; products of the reaction were extracted, resolved on a 1% agarose/urea gel and visualized by autoradiography (panel C). Sedimentation was from the right to the left. Position of RNA *b* and mitochondrial small rRNA (Δ) are indicated. Lane M, size marker (sizes in kilobases).


A
B
M 1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
1.77
1.52
1.28
0.78
0.53
0.40
0.28
0.16
RNA b
C
1 2 3 4 5 6 7 8 9
RNA b

the majority of (+) strands was present - in fraction 4, although as expected the amount of (-) strand was much lower (data not shown). Northern hybridization also allowed for the detection of RNA *a* (+) strand in fractions 1 and 2 of both gradients (not shown).

Each fraction of the untreated gradient was assayed for its ability to synthesize new molecules of RNA plasmids (Fig. 4C). Unexpectedly, the peak of the activity synthesizing RNA *b* was found in the same fraction as the majority of naked RNA *b*, that is in fraction 4. However, the activity sedimented slightly faster than the RNA *b* alone; it was present in fractions 3 and 2, and absent in fraction 5, while the amount of RNA *b* strands in fraction 5 was higher than that observed in fraction 3 (Fig. 4A). Similar observations were made when conditions of sedimentation - speed and time of centrifugation - were changed (not shown); the activity that synthesized new RNA *b* molecules seemed to sediment slightly faster than the majority of RNA *b* under all conditions. On the basis of the degree of sedimentation observed for the endogenous 18S and 26S rRNAs and other RNA markers run in separate gradients, it was estimated that RNA *b* alone sediments at around 12-13S, while the activity sediments between 14S and 20S. It seemed likely that the sedimentation of "activity" represented in fact the sedimentation of an active RdRp-RNA *b* template complexes, which would then be fairly small, only slightly larger than naked RNA *b* molecules.

The activity, capable of synthesis of the larger RNA *a*, was seen in fractions 2 and 3. These results are not included in Fig. 4 because the bands were too faint to be clearly visible on photographic prints.

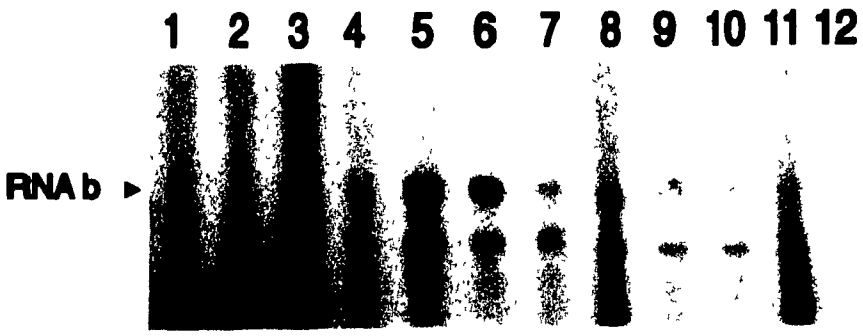
Isopycnic centrifugation of the complex active in RNA *b* synthesis

The activity synthesizing new RNA *b* strands was further characterized by isopycnic sedimentation in CsCl gradients. Mitochondria were sedimented through a sucrose cushion (see Materials and methods), lysed by two freeze/thaw cycles, and

Figure 5. Density gradient centrifugation of the complex active in RNA *b* synthesis

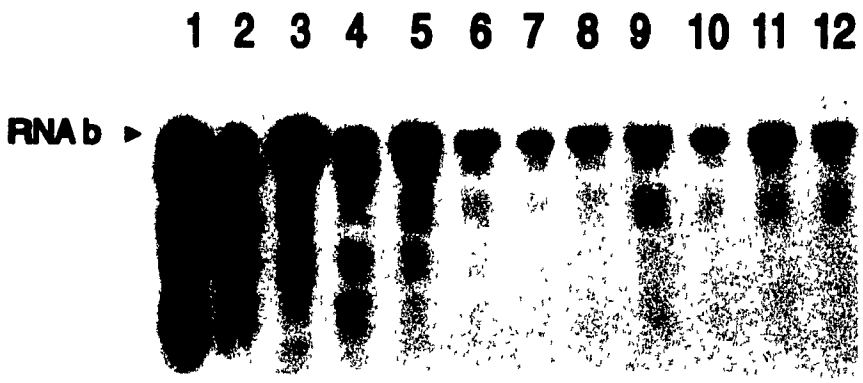
Maize mitochondria, purified by centrifugation through a sucrose cushion, were lysed by two freeze/thaw cycles; the lysate was clarified and fractionated by centrifugation on a CsCl gradient (SW 50.1 rotor, 39,800 rpm for 40 h). Dialyzed fractions were assayed for their ability to synthesize new strands of RNA plasmid and products of the reaction were analyzed as described in Fig. 4 (panel A). Nucleic acids were extracted from the remaining portion of the fractions, resolved on a 1% agarose/urea gels, transferred onto a membrane, and hybridized to (-) or (+) *in vitro* transcripts of RNA *b* cDNA clones for detection of (+) or (-) strands, respectively (panel B; the bottom blot was exposed 2.5-fold longer than the upper). Sedimentation is from the right to the left.

A

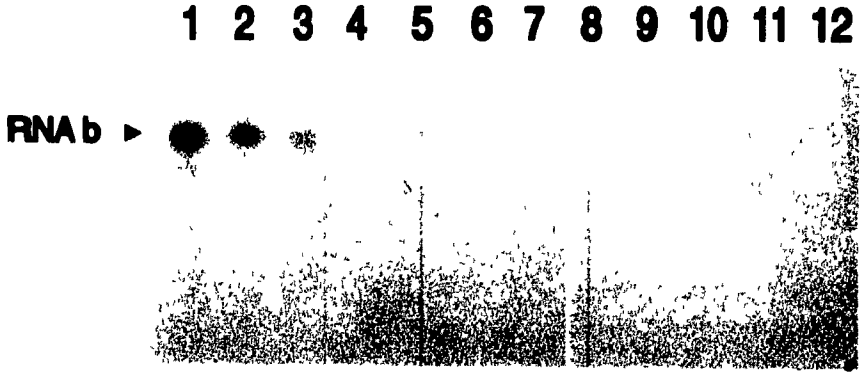


B

(+) strand



(-) strand



the clarified lysate was fractionated on a CsCl gradient. Results from a gradient that had a starting density $\rho_0^{25} = 1.46$ g/cm^3 are shown in Fig. 5. The complex active in synthesis of new RNA *b* molecules sedimented in the middle of the gradient; the peak of the activity was found in fraction 5 (Fig. 5A), at a density $\rho^{25} = 1.49$ g/cm^3 although some activity was found in all lighter fractions. By contrast, the majority of both (+) and (-) RNA *b* strands (as detected by Northern hybridizations) sedimented at the bottom of the gradient (Fig. 5B, lanes 1 and 2); this finding was not unexpected since naked RNA molecules have a density of 1.8 g/cm^3 or higher (Brakke 1960, Brenner *et al.* 1961). It can be concluded from these results that new RNA *b* molecules are indeed transcribed from preformed complexes, and that these complexes can be distinguished from the bulk of naked RNA *b* by their buoyant density.

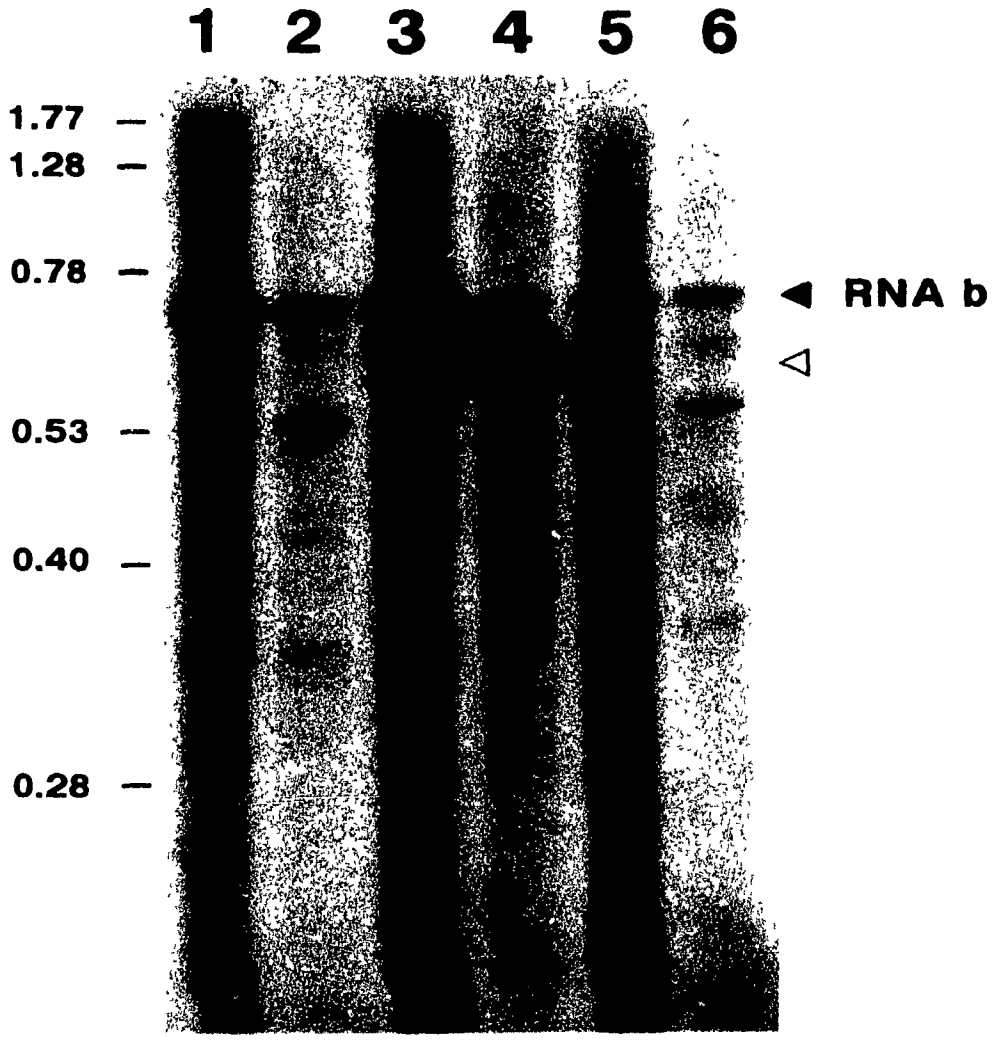
Attempts to identify protein components of the RNA *b* synthesis complex were unsuccessful. Also, the activity synthesizing new RNA *a* strands has not been detected in the CsCl gradient fractions.

In vitro synthesized RNA *b* molecules represent the (+) strands

In order to determine whether the more common (+) or rather the complementary (-) strands of RNA *b* were synthesized in the mitochondrial lysate, products of a labelling reaction were extracted, hybridized to (+) or (-) *in vitro* transcripts of RNA *b* cDNA clones, and the resulting hybrids treated with RNase A in a high salt concentration (results shown in Fig. 6). While the (-) transcript protected a 600b long fragment (lane 4), which contained most of the radioactivity originally present in the RNA *b* (lane 3), no band at a corresponding position was seen when (+) transcript was used (lane 6). The length of the rescued fragment is as expected, since available cDNA clones contain only 620 out of 719b of the RNA *b* sequence (Zhang and Brown 1993). Hence, the newly produced RNA *b* strands are (+) strands.

Figure 6. RNase protection analysis of the *in vitro* synthesized RNA *b* molecules

RNA that had been labelled in an *in vitro* labelling reaction was extracted, denatured, and hybridized to (-) (lanes 3 and 4) or (+) (lanes 5 and 6) *in vitro* transcripts of RNA *b* cDNA clones; alternatively, they were "mock-hybridized" (lanes 1 and 2). Each reaction was either left untreated (lanes 1, 3, and 5), or subjected to a treatment by RNase A at a concentration of 18 g/ml (lanes 2,4, and 6). RNAs from each sample were extracted, resolved on a 4% polyacrylamide/urea gel, and visualized by autoradiography. The positions of RNA *b* and the protected fragment (Δ), are indicated. Sizes of marker RNA molecules are in kilobases.


1 2 3 4 5 6
1.77
1.28
0.78
0.53
0.40
0.28
RNA b

Interestingly, in a control where no exogenous RNA was added two protected fragments could be seen, one at a position of RNA *b* and the other about 500b in size (Fig. 6, lane 2). This finding confirmed that endogenous (-) strands of these plasmid species were present. When the same experiment was repeated with a low salt concentration in the RNase A digestion step, no bands were visible on the autoradiograph (not shown).

Discussion

The replication complex of an RNA replicon can be defined as a structure that contains the template RNA, nascent chains, and the replicating enzymes, and that produces full-length (genomic) RNA molecules. In the work described here, a complex responsible for synthesis of the most abundant maize RNA plasmid molecule - single-stranded (+) RNA *b* - in a mitochondrial lysate was identified. Since RNA *b* sequence does not code for any protein, and therefore there is no evidence indicating that RNA *b* might represent a transcript of a larger RNA plasmid, the identified complex is likely to be involved in one step of replication of this molecule - the synthesis of the more abundant (+) strand.

This complex had been formed *in vivo*; I found no evidence that assembly of the complex and, consequently, initiation of synthesis, occurred in the mitochondrial lysate. Perhaps additional protein factors absent from the clarified mitochondrial lysate are required for the initiation. Alternatively, conditions of the reaction might have been unfavorable for such a reaction to occur (e.g. dilution of the mitochondrial protein, composition of the reaction buffer).

The complex had a buoyant density of 1.49 g/cm^3 and a sedimentation coefficient between 14 and 20S, only slightly higher than that of naked RNA *b* molecule (12-13S). If we assume that the complex is composed of RNAs and proteins only, and there is currently no evidence suggesting otherwise, the density of 1.49 g/cm^3 would indicate presence of a significant amount of protein. More specifically, the percentage of protein content, calculated according to a formula that describes a correlation between density and protein content of viral nucleocapsids (Sehgal *et al.* 1970), is around 60%. If this was so, the value would surely be reflected in the value of a sedimentation coefficient. That is, however, not the case. At present, I do not know how to satisfactorily explain this apparent discrepancy. It is possible that the increased mass that results from the association of RNA *b* with

protein is offset by effects on the conformation of the RNA, which have the effect of lowering the sedimentation coefficient of the complex. Alternatively, the complex may have an unusual composition: perhaps it contains only a small number of proteins that are able to significantly "loosen up" its conformation and thus decrease its density. Possibly, a third, lighter component, perhaps lipid, is present in the complex. The possibility that the complex had lost some of its components during sedimentation through sucrose gradient while the density gradient purified complex remained relatively intact seems highly improbable; considering the harsh conditions of CsCl gradient, the opposite would be expected to occur.

The RNA *b* synthesis complex is not abundant. The lysate of mitochondria purified from 1g of maize seedlings was able to synthesize between 10^7 and 10^8 new molecules of RNA *b* in 60 min. (data not shown). Assuming that initiation of synthesis did not occur and that each of these molecules represented one active complex, the same number of complexes was originally present in the lysate. The number of RNA *b* molecules in the same sample can be estimated to be roughly somewhere around 10^{11} molecules. Thus the complex is far from being abundant, and this may explain why attempts to identify protein components associated specifically with the CsCl gradient purified complex have thus far failed.

New (+) RNA *b* strands, produced by the complex, must be copied from a (-) strand template. Interestingly, the majority of (-) strands sedimented in the CsCl gradient as naked RNA molecules (Fig. 5B, bottom panel), and only a minute fraction (lane 5) was found in the same fraction as the RNA *b* synthesis activity, suggesting that only a minority of (-) strand was associated with the complexes. It was not determined whether the RNA template present in the complex is single- or double-stranded. It also remains to be seen, whether the naked (-) RNA *b* is free (single-stranded), or exists in its majority in a double-stranded form and might thus potentially represent a replicative intermediate, analogous to dsRI-RNAs of plus-stranded RNA viruses (Marsh *et al.* 1990). The majority of (+) strands of RNA *b* is single-stranded (Zhang and Brown 1993) and naked (this report).

However, some of these molecules banded in CsCl gradient at densities that indicated their association with proteins (Fig. 5B, upper panel). Since no function can be assigned to these complexes, their significance remains unclear.

Plasmid species other than RNA *b* were synthesized by the maize mitochondrial lysate (see Fig. 1 and 3). Their synthesis was, similarly to the synthesis of RNA *b*, resistant to heparin, and therefore represented also an elongation of *in vivo* preinitiated molecules. Since the amount of *in vitro* synthesized RNA is likely to reflect the amount of the corresponding complex, these molecules must have been synthesized by complexes that are much lower in abundance than the RNA *b* complex. Alternatively, some of these RNA molecules might have been by-products of the replication of other plasmid species, or replicative intermediates (as speculated by Zhang (1993) for *in organello* labelled plasmids); in that case, one complex would be responsible for the "appearance" of more than one plasmid species.

RNA *b* is most probably an internally deleted form of RNA *a* (Zhang and Brown 1993); the RNA *b* and RNA *a* synthesis activities were, however, separated by rate zonal centrifugation (data not shown), indicating that both molecules were synthesized by distinct complexes. Further investigation would be needed to clarify the relationship between other newly synthesized plasmid RNAs, and also to determine strandedness and identity of these molecules. It is interesting to mention in this connection that previously reported results (Finnegan and Brown 1986) suggest an *in organello* synthesis of (-) strands of some RNA plasmid species, in particular of the RNA *b'*, a species slightly larger than RNA *b*.

In summary, results presented here indicate that the majority of the most abundant maize mitochondrial RNA plasmid - RNA *b* - is naked and only a small fraction of it is associated with a complex that is able to synthesize new molecules of (+) RNA *b*, and is therefore likely to represent an active replication complex. What RNA polymerase is present in the complex ? Is it encoded by the host genomic DNA or by one of the RNA plasmids ? Answers to these question might

provide insight into the origin as well as replication of these peculiar RNAs. The possibility of purification of the RNA *b* synthesis complexes should allow for a more detailed investigation in this direction.

References

Ahlquist, P., Bujarski, J., Kaesberg, P. and Hall, T.C. (1984). Localization of the replicase recognization site within brome mosaic virus RNA by hybrid-arrested RNA synthesis. *Plant. Mol. Biol.* **3**, 37-44.

Baltimore, D. and Girard, M. (1966). An intermediate in the synthesis of poliovirus RNA. *Proc. Natl. Acad. Sci. USA.* **56**, 741-748.

Banerjee, A.K. (1987). Transcription and replication of rhabdoviruses. *Microbiol. Rev.* **51**, 66-87.

Barton, D.J., Sawicki, S.G. and Sawicki, D.L. (1991). Solubilization and immunoprecipitation of alphavirus replication complexes. *J. Virol.* **65**, 1496-1506.

Bastin, M. and Hall, T.C. (1976). Interaction of elongation factor 1 with aminoacylated brome mosaic virus and tRNAs. *J. Virol.* **20**, 117-122.

Bienz, K., Egger, D., Pfister, T. and Troxler, M. (1992). Structural and functional characterization of the poliovirus replication complex. *J. Virol.* **66**, 2740-2747.

Biswas, T.K. (1992). Transcriptional commitment of mitochondrial RNA polymerase from *Saccharomyces cerevisiae*. *J. Mol. Biol.* **226**, 335-347.

Blumenthal, T. and Carmichael, G.G. (1979). RNA replication: function and structure of Qβ-replicase. *Annu. Rev. Biochem.* **48**, 525-548.

Blumenthal, T., Landers, T.A. and Weber, K. (1972). Bacteriophage Qβ replicase contains the protein biosynthesis elongation factors EF Tu and EF Ts. *Proc. Natl. Acad. Sci. USA.* **69**, 1313-1317.

Boccardo, G., Lisa, V., Luisoni, E. and Milne, R.G. (1986). Cryptic plant viruses. *Adv. Virus Res.* **32**, 171-213.

Bonen, L. and Brown, G.G. (1993). Genetic plasticity and its consequences: Perspectives on gene organization and expression in plant mitochondria. *Can. J. Bot.* **71**, 645-660.

Brakke, M.K. (1960). Density gradient centrifugation and its application to plant viruses. *Adv. Virus Res.* **7**, 193-224.

Branch, A.D. and Robertson, H.D. (1984). A replication cycle for viroids and other small infectious RNA's. *Science* **223**, 450-455.

Branch, A.D., Robertson, H.D. and Dickson, E. (1981). Longer-than-unit-length viroid minus strands are present in RNA from infected plants. *Proc. Natl. Acad. Sci. USA.* **78**, 6381-6385.

Brenner, S., Jacob, F. and Meselson, M. (1961). An unstable intermediate carrying information from genes to ribosomes for protein synthesis. *Nature* **190**, 576-581.

Brown, G.G. and Zhang, M. (1993). Mitochondrial plasmids: DNA and RNA. In: *Molecular Biology of the Mitochondria*, Levings III, C.S. and Vasil, I.K., eds. Kluwer, in press.

Cross, R.K. (1983). Identification of a unique guanine-7-metyltransferase in Semliki Forest virus (SFV) infected cell extracts. *Virology* **130**, 452-463.

Cross, R.K. and Gamotos, P.J. (1981). Concominant methylation and synthesis *in vitro* of Semliki Forest virus (SFV) ss RNAs by a fraction from infected cells. *Virology* **114**, 542-554.

Dewey, R.E., Levings III, C.S. and Timothy, D.H. (1986). Novel recombinations in the maize mitochondrial genome produce a unique transcriptional unit in the Texas male-sterile cytoplasm. *Cell* **44**, 439-449.

Diener, T.O. (1986). Viroid processing: A model involving the central conserved region and hairpin I. *Proc. Natl. Acad. Sci. USA.* **83**, 58-62.

Diener, T.O. (1991). Subviral pathogens of plants: Viroids and viroidlike satellite RNAs. *Faseb J.* **5**, 2808-2813.

Dorssers, L., Van der Krol, S., Van der Meer, J., Van Kammen, A. and Zabel, P. (1984). Purification of cowpea mosaic virus RNA replication complex: Identification of a virus encoded 110,000-dalton polypeptide responsible for RNA chain elongation. *Proc. Natl. Acad. Sci. USA.* **81**, 1951-1955.

Estes, M.K. (1990). Rotaviruses and their replication. In: *Fields virology*, 2nd edn., pp. 1329-1352, Fields, B.N. and Knipe, D.M., eds. Raven Press, New York, NY.

Fahima, T., Kazmierczak, P., Handsen, D.R., Pfeiffer, P. and Van Alfen, N.K. (1993). Membrane-associated replication of an unencapsidated double-stranded RNA of the fungus, *Cryphonectria parasitica*. *Virology* **195**, 81-89.

Feix, G., Slor, H. and Weissmann, C. (1967). Replication of viral RNA. XIII. The early product of phage RNA synthesis *in vitro*. *Proc. Natl. Acad. Sci. USA.* **57**, 1401-1408.

Feix, G., Pollet, R. and Weissmann, C. (1968). Replication of viral RNA. XVI. Enzymatic synthesis of infectious viral RNA with noninfectious Qβ minus strand as template. *Proc. Natl. Acad. Sci. USA.* **59**, 145-152.

Finnegan, P.M. (1989) RNA synthesis in maize mitochondria: The identification of autonomously replicating RNA species and a kinetic analysis of transcripts accumulation, Ph.D. *Thesis*. McGill University, Montreal.

Finnegan, P.M. and Brown, G.G. (1986). Autonomously replicating RNA in mitochondria of maize plants with S-type cytoplasm. *Proc. Nat. Acad. Sci. U.S.A.* **83**, 5175-5179.

Finnegan, P.M. and Brown, G.G. (1987). *In organello* transcription in maize mitochondria and its sensitivity to inhibitors of RNA synthesis. *Plant Physiol.* **85**, 304-309.

Finnegan, P.M. and Brown, G.G. (1990). Transcriptional and post-transcriptional regulation of RNA levels in maize mitochondria. *Plant Cell* **2**, 71-83.

Fraenkel-Conrat, H. (1986). RNA-directed RNA polymerases of plants. *CRC Crit. Rev. Plant Sci.* **4**, 213-226.

Francki, R.I.B. (1987). Responses of plant cells to virus infection with special reference to the sites of RNA replication. In: *Positive strand RNA viruses*, pp. 423-436, Brinton, M.A. and Rueckert, R.R., eds. Alan R. Liss, New York, NY.

Franze de Fernandez, M.T., Eoyang, L. and August, J.T. (1968). Factor fraction required for the synthesis of bacteriophage Qβ-RNA. *Nature* **219**, 588-590.

Friedman, R.M., Levin, J.G., Grimley, P.M. and Berezesky, I.K. (1972). Membrane-associated replication complex in arbovirus infection. *J. Virol.* **10**, 504-515.

Froshauer, S., Kartenbeck, J. and Helenius, A. (1988). Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes. *J. Cell Biol.* **107**, 2075-2086.

Fujimura, T., Esteban, R. and Wicker, R.B. (1986). *In vitro* L-A double-stranded RNA synthesis in virus-like particles from *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA.* **83**, 4433-4437.

Girard, M. (1969). *In vitro* synthesis of poliovirus ribonucleic acid: role of the replicative intermediate. *J. Virol.* **3**, 376-384.

Goodman, T.C., Nagel, L., Rappold, W., Klotz, G. and Riesner, D. (1984). Viroid replication: equilibrium association constant and comparative activity measurements for the viroid-polymerase interaction. *Nucleic Acids Res.* **12**, 6231-6246.

Gorbalenya, A.E. and Koonin, E. (1989). Viral proteins containing the purine NTP-binding sequence pattern. *Nucleic Acids Res.* **17**, 8413-8440.

Gottlieb, P., Strassman, J., Qiao, X., Frucht, A. and Mindich, L. (1990). *In vitro* replication, packaging, and transcription of the segmented double-stranded RNA genome of bacteriophage ϕ6: Studies with procapsids assembled from plasmid-encoded proteins. *J. Bacteriol.* **172**, 5774-5782.

Gray, M.W., Hanic-Joyce, P.J. and Covello, P.S. (1992). Transcription, processing and editing in plant mitochondria. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 145-175.

Hadidi, A. (1986). Relationship of viroids and certain other plant pathogenic nucleic acids to group I and II introns. *Plant. Mol. Biol.* **7**, 129-142.

Hall, T.C. and Wepprich, R.K. (1976). Functional possibilities for aminoacylation of viral RNA in transcription and translation.

Hansen, D.R., Van Alfen, N.K., Gillies, K. and Powell, W.A. (1985). Naked dsRNA associated with hypovirulence of *Endothia parasitica* is packaged in fungal vesicles. *J. Gen. Virol.* **66**, 2605-2614.

Haseloff, J., Mohammed, N.A. and Symons, R.H. (1982). Viroid RNAs of cadang-cadang disease of coconuts. *Nature* **299**, 316-321.

Hayes, R.J. and Buck, K.W. (1990). Complete replication of a eukaryotic virus RNA *in vitro* by a purified RNA-dependent RNA polymerase. *Cell* **63**, 363-368.

Hori, K. (1970). Replication of viral nucleic acids. I. Enzyme-RNA complexes involved in the synthesis of Qβ RNA. *Biochim. Biophys. Acta.* **217**, 394-407.

Ikegami, M. and Fraenkel-Conrat, H. (1979). Characterization of double-stranded ribonucleic acid in tobacco leaves. *Proc. Nat. Acad. Sci. U.S.A.* **76**, 3637-3640.

Ishihama, A. and Nagata, K. (1988). Viral RNA polymerases. *CRC Critical Rev. Biochem.* **23**, 27-76.

Jackson, C., Dench, J.E., Hall, D.O. and Moore, A.L. (1979). Separation of mitochondria from contaminating subcellular structures utilizing silica sol gradient centrifugation. *Plant Physiol.* **64**, 150-153.

Joshi, R.L., Ravel, J.M. and Haenni, A.-L. (1986). Interaction of turnip yellow mosaic virus val-RNA with eukaryotic elongation factor EF-1a. Search for a function. *EMBO J.* **5**, 1143-1148.

Kamen, R. (1970). Characterization of the subunits of Qβ replicase. *Nature* **228**, 527-533.

Kelly, J.L. and Lehman, I.R. (1986). Yeast mitochondrial RNA polymerase. Purification and properties of the catalytic subunit. *J. Biol. Chem.* **261**, 10340-10347.

Kemble, R.J. and Thompson, R.D. (1982). S1 and S2, the linear mitochondrial DNAs present in a male-sterile line of maize, possess terminally attached proteins. *Nucleic Acids Res.* **10**, 8181-8190.

Kemble, R.J., Gunn, R.E. and Flavell, R.B. (1980). Classification of normal and male sterile cytoplasms in maize. II. Electrophoretic analysis of DNA species in mitochondria. *Genetics* **95**, 451-458.

Kemble, R.J., Mans, R.J., Gabay-Laughnan, S. and Laughnan, J. (1983). Sequences homologous to episomal mitochondrial DNAs in the maize nuclear genome. *Nature* **304**, 744-747.

Konarska, M.M. and Sharp, P.A. (1989). Replication of RNA by the DNA-dependent RNA polymerase of phage T7. *Cell* **57**, 423-431.

Konarska, M.M. and Sharp, P.A. (1990). Structure of RNAs replicated by the DNA-dependent T7 RNA polymerase. *Cell* **63**, 609-618.

Kondo, M., Gallerani, R. and Weissmann, C. (1970). Subunit structure of Qβ replicase. *Nature* **228**, 525-527.

Kuzmin, E.V. and Levchenko, I.V. (1987). S1 plasmid from cms-S maize mitochondria encodes a viral-type DNA polymerase. *Nucleic Acids Res.* **15**, 6758.

Lamb, R.A. and Choppin, P.W. (1983). The gene structure and replication of influenza virus. *Annu. Rev. Biochem.* **52**, 467-506.

Landers, T.A., Blumenthal, T. and Weber, K. (1974). Function and structure in ribonucleic acid phage Qβ ribonucleic acid replicase. *J. Biol. Chem.* **249**, 5801-5808.

Lebacq, P. and Vedel, F. (1981). *Sal*I restriction enzyme analysis of chloroplast and mitochondrial DNAs in the genus *Brassica*. *Plant Sci. Lett.* **23**, 1-9.

Lee, J.-Y., Marshall, J.A. and Bowden, D.S. (1992). Replication complex associated with the morphogenesis of rubella virus. *Arch. Virol.* **122**, 95-106.

Lefebvre, A., Scalla, R. and Pfeiffer, P. (1990). The double-stranded RNA associated with the '447' cytoplasmic male sterility in *Vicia faba* is packaged together with its replicase in cytoplasmic membranous vesicles. *Plant. Mol. Biol.* **14**, 477-490.

Litvak, S., Tarrago, A., Tarrago-Litvak, L. and Allende, J.E. (1973). Elongation factor-viral genome interaction dependent on the aminoacylation of TYMV and TMV RNAs. *Nature New Biology* **241**, 88-90.

Lonsdale, D.M. (1989). The plant mitochondrial genome. In: *The biochemistry of plants*, vol. 15, pp. 229-295, Marcus, A., ed. Academic Press, New York, NY.

Lonsdale, D.M., Hodge, T.P., Howe, C.J. and Stern, D.B. (1983). Maize mitochondrial DNA contains a sequence homologous to the ribulose-1,5-biphosphate carboxylase large subunit gene of chloroplast DNA. *Cell* **34**, 1007-1014.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). *Molecular Cloning: A laboratory manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, NY.

Mans, R.M.W., Pleij, C.W.A. and Bosch, L. (1991). tRNA-like structures. Structure, function and evolutionary significance. *Eur. J. Biochem.* **201**, 303-324.

Marechal-Drouard, L., Weil, J.-H. and Guillemaut, P. (1988). Import of several tRNAs from the cytoplasm into the mitochondrion in bean *Phaseolus vulgaris*. *Nucleic Acids Res.* **16**, 4777-4788.

Marsh, L.E. and Hall, T.C. (1987). Evidence implicating a tRNA heritage for the promoters of (+) strand RNA synthesis in brome mosaic virus and related viruses. *Cold Spring Harbor Symp. Quant. Biol.* **52**, 331-341.

Marsh, L.E., Pogue, G.P. and Hall, T.C. (1989). Similarities among plant virus (+) and (-) RNA termini imply a common ancestry with promoters of eukaryotic tRNAs. *Virology* **172**, 415-427.

Marsh, L.E., Pogue, G.P., Huntley, C.C. and Hall, T.C. (1990). Insight to replication strategies and evolution of (+) strand RNA viruses provided by brome mosaic virus. *Oxford Surv. Plant Mol. Cell Biol.* **7**, 297-334.

Martín, M.T. and García, J.A. (1991). Plum potyvirus RNA replication in a crude membrane fraction from infected *Nicotiana clevelandii* leaves. *J. Gen. Virol.* **72**, 785-790.

Masters, B.S., Stohl, L.L. and Clayton, D.A. (1987). Yeast mitochondrial RNA polymerase is homologous to those encoded by bacteriophages T3 and T7. *Cell* **51**, 89-99.

Matsumoto, Y. and Wickner, R.B. (1991). Yeast 20 S RNA replicon. Replication intermediates and encoded putative RNA polymerase. *J. Biol. Chem.* **266**, 12779-12783.

Matthews, R.E.F. (1982). Classification and nomenclature of viruses. *Intervirology* **17**, 1-199.

Meyer, F., Weber, H. and Weissmann, C. (1981). Interactions of Qβ replicase with Qβ RNA. *J. Mol. Biol.* **153**, 631-660.

Miller, W.A., Dreher, T.W. and Hall, T.C. (1985). Synthesis of brome mosaic virus subgenomic RNA *in vitro* by internal initiation on (-) sense genomic RNA. *Nature* **313**, 68-70.

Miller, W.A., Bujarski, J.J., Dreher, T.W. and Hall, T.C. (1986). Minus-strand initiation by brome mosaic virus replicase within the 3' tRNA-like structure of native and modified RNA templates. *J. Mol. Biol.* **187**, 537-546.

Mills, D.R., Kramer, F.R. and Spiegelman, S. (1973). Complete nucleotide sequence of a replicating RNA molecule. *Science* **180**, 916-927.

Mills, D.R., Kramer, F.R., Dobkin, C., Nishihara, T. and Spiegelman, S. (1975). Nucleotide sequence of microvariant RNA: Another small replicating molecule. *Proc. Natl. Acad. Sci. USA.* **72**, 4252-4256.

Monroy, A.F., Gao, C., Zhang, M. and Brown, G.G. (1990). Double-stranded RNA molecules in *Brassica* are inherited biparentally and appear not to be associated with mitochondria. *Curr. Genet.* **17**, 427-431.

Mouches, C., Candresse, T. and Bove, J.M. (1984). Turnip yellow mosaic virus RNA-replicase and virus-encoded subunits. *Virology* **134**, 78-90.

Mühlbach, H.-P. and Sänger, H.L. (1979). Viroid replication is inhibited by α-amanitin. *Nature* **278**, 185-187.

Munishkin, A.V., Voronin, L.A., Ugarov, V.I., Bondareva, L.A., Chetverina, H.V. and Chetverin, A.B. (1991). Efficient templates for Qβ replicase are formed by recombination from heterologous sequences. *J. Mol. Biol.* **221**, 463-472.

Murant, A.F. and Mayo, M.A. (1982). Satellites of plant viruses. *Annu. Rev. Phytopathol.* **20**, 49-70.

Nash, S.H., Douthart, R.J., Ellis, L.F., Van Frank, R.M., Burnett, J.P. and Lemke, P.A. (1973). On the mycophage of *Penicillium chrysogenum*. *Can. J. Microbiol.* **19**, 97-103.

Nugent, J.M. and Palmer, J.D. (1991). RNA-mediated transfer of the gene *coxII* from the mitochondrion to the nucleus during flowering plant evolution. *Cell* **66**, 473-481.

Oeser, B. (1988). S2 plasmid from *Zea mays* encodes a specific RNA polymerase: an alternative alignment. *Nucleic Acids Res.* **16**, 8729.

Owens, R.A. and Diener, T.O. (1982). RNA intermediates in potato spindle tuber viroid replication. *Proc. Natl. Acad. Sci. USA.* **79**, 113-117.

Owens, R.A. and Hammond, R.W. (1988). Structure and function relationships in plant viroid RNAs. In: *RNA Genetics*, vol. 2, pp. 107-125, Domingo, E., Holland, J.J. and Ahlquist, P., eds. CRC Press, Boca Raton, Fla.

Pogue, G.P., Marsh, L.E. and Hall, T.C. (1990). Point mutations in the ICR2 motif of brome mosaic virus RNAs debilitate (+) strand replication. *Virology* **178**, 152-160.

Priano, C., Kramer, F.R. and Mills, D.R. (1987). Evolution of the RNA coliphages: the role of secondary structures during RNA replication. *Cold Spring Harbor Symp. Quant. Biol.* **52**, 321-339.

Pring, D.R. and Lonsdale, D.M. (1985). Molecular biology of higher plant mitochondrial DNA. *Int. Rev. Cytol.* **97**, 1-46.

Pring, D.R., Levings III, C.S., Hu, W.W.L. and Timothy, D.H. (1977). Unique DNA associated with mitochondria in the "S"-type cytoplasm of male-sterile maize. *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2904-2908.

Rackwitz, H.-R., Rohde, W. and Sänger, H.L. (1981). DNA-dependent RNA polymerase II of plant origin transcribes viroid RNA into full-length copies. *Nature* **291**, 297-301.

Rensing, U. and August, J.T. (1969). The 3'-terminus and the replication of phage RNA. *Nature* **224**, 853-856.

Richards, O.C. and Ehrenfeld, E. (1990). Poliovirus RNA replication. *Curr. Top. Microbiol. Immunol.* **161**, 89-119.

Riviera-Bustamante, R.F. and Semancik, J.S. (1989). Properties of a viroid-replicating complex solubilized from nuclei. *J. Gen. Virol.* **70**, 2707-2716.

Rodriguez-Cousino, N., Esteban, L.M. and Esteban, R. (1991). Molecular cloning and characterization of W double-stranded RNA, a linear molecule present in *Saccharomyces cerevisiae*: Identification of its single-stranded RNA form as 20 S RNA. *J. Biol. Chem.* **266**, 12772-12778.

Savage, T., Granboulan, N. and Girard, M. (1971). Architecture of the poliovirus replicative intermediate RNA. *Biochimie* **53**, 533-543.

Schaffner, W., Rüegg, K.J. and Weissmann, C. (1977). Nanovariant RNAs: Nucleotide sequence and interaction with bacteriophage Qβ replicase. *J. Mol. Biol.* **117**, 877-907.

Schardl, C.L., Lonsdale, D.M., Pring, D.R. and Rose, K.R. (1984). Linearization of maize mitochondrial chromosomes by recombination with linear episomes. *Nature* **310**, 292-296.

Schindler, I.-M. and Mühlbach, H.-P. (1992). Involvement of nuclear DNA-dependent RNA polymerases in potato spindle tuber viroid replication: A reevaluation. *Plant Sci.* **84**, 221-229.

Schinkel, A.H., Groot Koerkamp, M.J.A., Touw, E.P.W. and Tabak, H.F. (1987). Specificity factor of yeast mitochondrial RNA polymerase. Purification and interaction with core polymerase. *J. Biol. Chem.* **262**, 12785-12791.

Schuster, A.M., Sisco, P.H. and Levings III, C.S. (1983). Two unique RNAs in CMS-S and RU maize mitochondria. In: UCLA Sym. on Molecular and Cellular Biology, New series, Vol. 12: *Plant Molecular Biology*, pp. 437-444, Goldberg, R., ed. Alan R Liss, Inc., New York, NY.

Schuster, W. and Brennicke, A. (1987). Plastid, nuclear and reverse transcriptase sequences in the mitochondrial genome of *Oenothera*: is genetic information transferred between organelles via RNA? *EMBO J.* **6**, 2857-2863.

Sehgal, O.P., Jean, J.-H., Bhalla, R.B., Soong, M.M. and Krause, G.F. (1970). Correlation between buoyant density and ribonucleic acid content in viruses. *Phytopathol.* **60**, 1778-1784.

Semancik, J.S. and Harper, K.L. (1984). Optimal conditions for cell-free synthesis of citrus exocortis viroid and question of specificity of RNA polymerase activity. *Proc. Nat. Acad. Sci. U.S.A.* **81**, 4429-4433.

Simmons, D.T. and Strauss, J.H. (1972). Replication of Sindbis virus. II. Multiple forms of double-stranded RNA isolated from infected cells. *J. Mol. Biol.* **71**, 615-631.

Singh, M. and Brown, G.G. (1991). Supression of cytoplasmic male sterility by nuclear genes alters expression of a novel mitochondrial gene region. *Plant Cell* **3**, 1349-1362.

Sisco, P.H., García-Arénal, F., Zaitlin, M., Earle, E.D. and Gracen, V.E. (1984). LBN, A male-sterile cytoplasm of maize, contains two double-stranded RNAs. *Plant Sci. Lett.* **34**, 127-134.

Soria, M., Little, M.S. and Huang, A. (1974). Characterization of vesicular stomatitis virus nucleocapsids. I. Complementary 40S RNA molecules in nucleocapsids. *Virology* **61**, 270-280.

Spiegelman, S., Pace, N.R., Mills, D.R., Levisohn, R., Eikhom, T.S., Taylor, M.M., Peterson, R.L. and Bishop, D.H.L. (1968). The mechanism of RNA replication. *Cold Spring Harbor Symp. Quant. Biol.* **33**, 101-124.

Spiesmacher, E., Mühlbach, H.-P., Tabler, M. and Sänger, H.L. (1985). Synthesis of (+) and (-) RNA molecules of potato spindle tuber viroid (PSTV) in isolated nuclei and its impairment by transcription inhibitors. *Biosci. Rep.* **5**, 251-265.

Stern, D.B. and Lonsdale, D.M. (1982). Mitochondrial and chloroplast genomes of maize have a 12-kilobase DNA sequence in common. *Nature* **299**, 698-702.

Synenki, R.M., Levings III, C.S. and Shah, D.M. (1978). Physicochemical characterization of mitochondrial DNA from soybean. *Plant Physiol.* **61**, 460-464.

Ticho, B. and Getz, G.S. (1988). The characterization of yeast mitochondrial RNA polymerase: a monomer of 150,000 daltons with a transcription factor of 70,000 daltons. *J. Biol. Chem.* **263**, 10096-10103.

Timmis, J.N. and Scott, N.S. (1983). Sequence homology between spinach nuclear and chloroplast genomes. *Nature* **305**, 65-67.

Turpen, T., Garger, S.J. and Grill, L.K. (1988). On the mechanism of cytoplasmic male sterility in the 447 line of *Vicia faba. Plant. Mol. Biol.* **10**, 489-497.

Usala, S.J., Brownstein, B.H. and Haselkorn, R. (1980). Displacement of parental RNA strand during *in vitro* transcription by bacteriophage ϕ6 nucleocapsids. *Cell* **19**, 855-862.

Van der Vossen, E.A.G., Neeleman, L. and Bol, J.F. (1993). Role of the 5' leader sequence of alfalfa mosaic virus RNA 3 in replication and translation of the viral RNA. *Nucleic Acids Res.* **21**, 1361-1367.

Wahba, A.J., Miller, M.J., Niveleau, A., Landers, T.A., Carmichael, G.G., Weber, K., Hawley, D.A. and Slobin, L.I. (1974). Subunit I of Qβ replicase and 30 S ribosomal protein S1 of *Escherichia coli. J. Biol. Chem.* **249**, 3314-3316.

Ward, B.L., Anderson, R.S. and Bendich, A.J. (1981). The mitochondrial genome is large and variable in a family of plants (*Cucurbitaceae*). *Cell* **25**, 793-803.

Weissmann, C. (1974). The making of a phage. *FEBS Lett.* **40**, S10-S18.

Weissmann, C., Feix, G. and Slor, H. (1968). *In vitro* synthesis of phage RNA: The nature of the intermediates. *Cold Spring Harbor Symp. Quant. Biol.* **33**, 83-100.

Wertz, G.W., Howard, M.B., Davis, N. and Patton, J. (1987). The switch from transcription to replication of a negative-strand RNA virus. *Cold Spring Harbor Symp. Quant. Biol.* **52**, 367-371.

Wickner, R.B. (1992). Double-stranded and single-stranded RNA viruses of *Saccharomyces cerevisiae*. *Annu. Rev. Microbiol.* **46**, 347-375.

Widner, W.R., Matsumoto, Y. and Wickner, R.B. (1991). Is 20S RNA naked? *Mol. Cell. Biol.* **11**, 2905-2908.

Winkley, C.S., Keller, M.J. and Jaehning, J.A. (1985). A multi-component mitochondrial RNA polymerase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **260**, 14214-14223.

Wolff, P., Gilz, R., Schumacher, J. and Riesner, D. (1985). Complexes of viroids with histones and other proteins. *Nucleic Acids Res.* **13**, 355-367.

Young, E.G. and Hanson, M.R. (1987). A fused mitochondrial gene associated with cytoplasmic male sterility is developmentally regulated. *Cell* **50**, 41-49.

Zabala, G. and Walbot, V. (1988). An S1 episomal gene of maize mitochondria is expressed in male sterile and fertile plants. *Mol. Gen. Genet.* **211**, 386-392.

Zabala, G., O'Brien-Vedder, C. and Walbot, V. (1987). S2 episome of maize mitochondria encodes a 130-kilodalton protein found in male-sterile and fertile plants. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7861-7865.

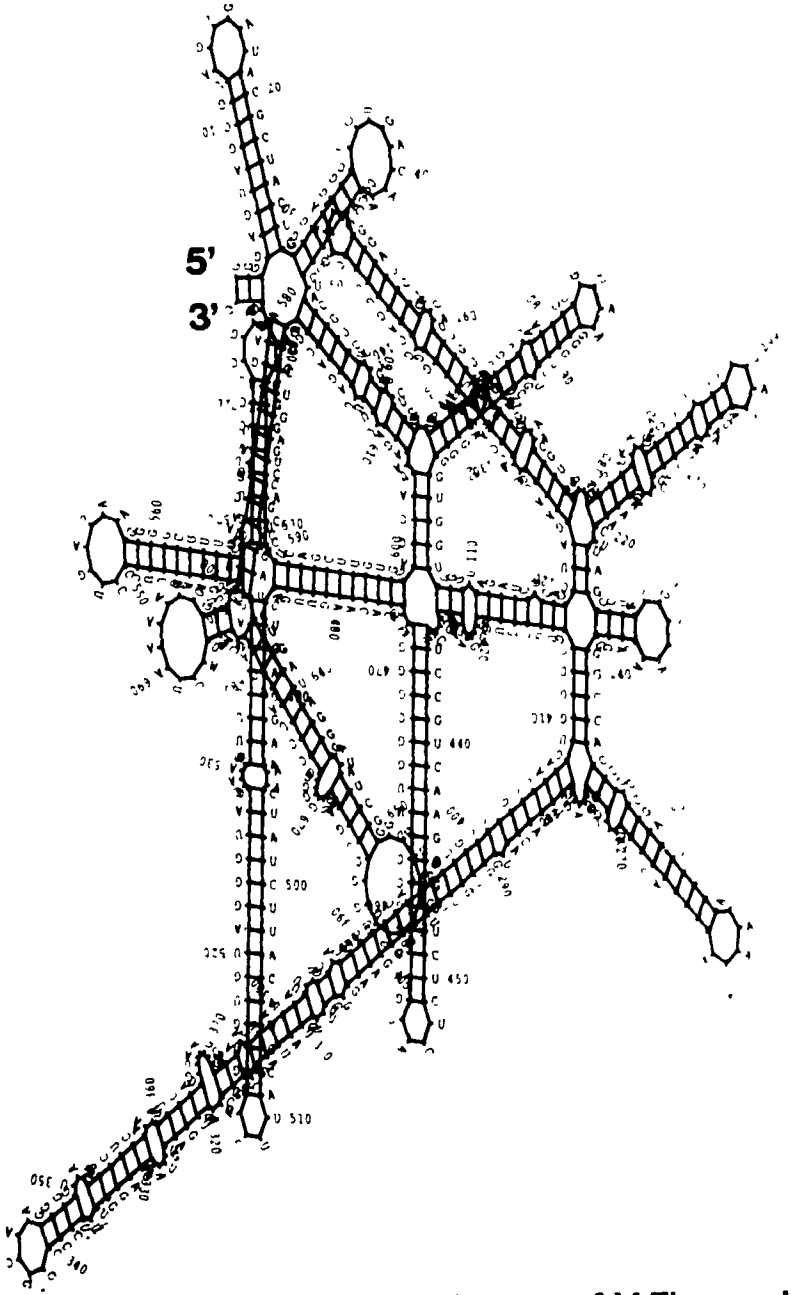
Zabel, P., Weenen-Swaans, H. and Van Kammen, A. (1974). *In vitro* replication of cowpea mosaic virus RNA. I. Isolation and properties of the membrane-bound replicase. *J. Virol.* **14**, 1049-1055.

Zhang, M. (1993) Plant mitochondrial RNA: replicon characterization and developmentally regulated distribution, Ph.D. *Thesis*. McGill University, Montreal.

Zhang, M. and Brown, G.G. (1993). Structure of the maize mitochondrial replicon RNA *b* and its relationship with other autonomously replicating RNA species. *J. Mol. Biol.* **230**, 757-765.

Appendix

Predicted secondary structure of RNA *b*



(courtesy of M.Zhang and G.G.Brown)