Two *Ustilago maydis* viral dsRNAs of different size code for the same product

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ABSTRACT

UmV is a double-stranded RNA (dsRNA) virus of the corn fungus *Ustilago maydis*. There are three viral subtypes, P1, P4 and P6, which differ in the specificity of their secreted killer toxins. Each has three size classes of dsRNAs: H (heavy), M (medium), and L (light). We find that, unique among dsRNA viruses, two segments of different size code for the same product - the toxin resistance factor. The smaller dsRNA (L) is homologous to one end of the larger (M), and may have arisen by replication and packaging of a sub-genomic mRNA. We have also compared all the UmV dsRNAs with each other and with the dsRNAs of the similar yeast virus (ScV) by Northern gel and by 3' sequence analysis. Like those of ScV, many of the UmV dsRNAs have one 3' terminus with the sequence UUUUCA_{OH} or UUUUUCG_{OH}. The H and L dsRNAs of similar size in different viral subtypes are generally related in sequence. The UmV H dsRNAs of different size are not detectably related in sequence.

INTRODUCTION

Most fungal viruses are segmented double-stranded RNA (dsRNA) viruses without infectious cycles that persist indefinitely in their host cells (1). There are two organisms in which such viruses are known to be responsible for the synthesis of extracellular toxins that kill sensitive cells of the same species: *Ustilago maydis* and *Saccharomyces cerevisiae*. In the latter, one viral dsRNA (L) encodes a capsid polypeptide, while a second dsRNA (M) encodes the toxin (2,3). There are a number of yeast viruses with different toxin specificities, of which two (k_1 and k_2) have been characterized in detail (4,5). The k_1 viruses generally displace the k_2 viruses when both are introduced into the same diploid, a phenomenon entitled exclusion (5). The determination of the 3' end sequences of these viral dsRNAs has enabled us to predict the site of initiation of the viral transcriptase (6,7), to compare k_1 and k_2

viral dsRNAs (8), and to understand viral exclusion (9,10).

In the Ustilago system, there are also a number of viruses with different toxin specificities: P1, P4, and P6 (11). All have three groups of viral dsRNAs: H (heavy), M (medium), and L (light). The major species of viral dsRNAs present in each subtype are constant. Strain P1 has H1, H2, M1, M2, M3, and L. Strain P4 has H1, H2, H3, H4, M2, M3, and L. Strain P6 has H1, M2, and L. The dsRNAs of the same apparent size in the three different strains have been designated with the same numbers. Each strain also has a number of other dsRNA species present in much lower molar quantities. Until the present work, there have been no data on the relatedness of the viral dsRNAs within a strain or among strains.

The ability to synthesize the viral polypeptide toxin and the specificity of that toxin segregates with the M dsRNA species in inter-strain crosses and in curing experiments (12-16). One of the products of in vitro translation of the denatured M species is antigenically related to toxin (15). All the UmV viruses have one major viral capsid polypeptide of about 75,000 daltons (14). In vitro translation of the H species results in polypeptides with antigenic similarity to this capsid polypeptide (15), and there are strains of Ustilago with only one of the H segments (H1) encapsidated in particles with this polypeptide (14). Strains with only H2, H3, and H4, or only H3 and H4 also have viral particles of the same characteristics as those of the wild-type (14). Consequently, all the H segments may encode similar capsid polypeptides (16). Genetic experiments also indicate that the L segments encode a factor necessary for immunity to toxin (17). Just as with the yeast viruses, there are distinct exclusion relationships among the Ustilago viruses (18, 19).

We have made a survey of the UmV dsRNAs for sequence homologies and determined the 3' sequences of most of the dsRNAs. This physical evidence supports the functional differentiation between H, M, and L segments based on genetic evidence and shows that the Ustilago and yeast viruses are evolutionarily related. Surprisingly, two of the viral dsRNAs, M and L, both encode the toxin resistance factor: the sequence of L is derived entirely from one end of M.

MATERIALS AND METHODS

Preparation of dsRNA. The viral dsRNAs were isolated from agarose or polyacrylamide gels after extraction and purification on CF11 columns as previously described (6, 19). The strains used have been described (16). Each viral dsRNA used for sequence analysis, Northern analysis, or heteroduplex analysis was purified by extended gel electrophoresis and contained only one species (by size) except for M2 and M3 and H3 and H4, which were inadequately resolved and used as mixtures (e.g. M2+3).

Sequence Analysis. The 3' end labeling, isolation of 3' termini, and sequencing were performed as previously described (6, 7). Sequencing was performed using the chemical method of Peattie (20). We refer to the sequences of the 3' terminal T₁ oligonucleotides without the added pCp and with the deduced 5' Gp.

Northern gel analysis. Northern gels of undenatured dsRNAs were performed as described (9). Individual 3' end labeled, denatured dsRNAs or cDNAs made using individual viral dsRNAs as templates and calf thymus DNA random primer (21) were used as probes.

Electron microscopy. Electron microscopy of dsRNA with pBR322 open circular DNA internal standard was performed as previously described (8). Measurements were made of 50 to 200 molecules and standard deviations were less than 10%. Calculations assumed 3.4 angstroms/bp for dsDNA and 3.0 angstroms/bp for dsRNA.

Agarose gel electrophoresis. The DNA restriction fragments used for sizing dsRNAs were the AluI fragments of pBR322, the RsaI fragments of pLL-26 and the EcoRI fragment of pLL-26 (9, 22). Correction was made for the difference in weight per bp: 687 for dsRNA, 660 for dsDNA.

RESULTS

Sizes of viral dsRNAs. A summary of the UmV dsRNAs easily resolved on agarose gels is given in Table 1. There is no definitive method of sizing dsRNAs (23), but we have applied several of the more rapid techniques to the UmV dsRNAs, obtaining values generally in agreement (within 10%) with previously published values. Note that UmV L is the smallest known viral dsRNA.

Homologies among the UmV dsRNAs. The most striking result of our series of 13 Northern blots is that the L and M species within a subtype

Table 1. Sizes of UmV dsRNAs (base pairs)

UmV dsRNA ¹	2.8% PAGE ²	1% agarose ³	1% agarose ⁴	em
H1	6100	6200	-	6700
H2	4500	5000	4600	-
H3	3000	3800	3500	-
H4	2600	3600	3200	-
M1	1400	1700	1500	1500
M2-6	1000	1200	1100	-
M2-1,4	960	1100	960	1000
M3	920	1100	960	1000
L	360	-	350	360

¹M2-6 is the M2 segment of P6; M2-1,4 is the M2 segment of P1 and P4.

²Calculated from the values given by Bozarth et al (24).

³Size standards were the ScV dsRNAs.

⁴Size standards were dsDNA restriction fragments.

have extensive sequence homology (Fig. 1). The homologies among the L and M segments are unique among dsRNA viruses. Each L segment has extensive homology to one M segment of its own viral subtype as well as to one or more of the L dsRNAs of other viral subtypes. Unless either L or M is defective, this implies that the immunity function is encoded by both L and M. Specifically, P4 L is closely related to P4 M2+3 (M2 and M3 were not resolved on these gels) and to P1 L. It shows homology to P1 M1, but not to P1 M2+3. As expected from these results, the P1 M1 probe has homology to P4 M2+3 but not to P1 M2+3 (Fig. 1, Table 2). The P1 L probe appears to hybridize more strongly to P4 L than to P1 L because of the greater amount of P4 L present in the Northern transfer (Fig. 1). The hybridization results also demonstrate that P1 and P4 are much more closely related to each other than either is to P6.

Some dsRNAs of the same size are homologous among the subtypes. The H1 segments of all three subtypes are homologous, although the H1 segments of P1 and P4 are most similar. The homology of P6 H1 to P4 H1 and P1 H1 was more easily detectable with the P6 H1 probe (Fig. 1) than with the P4 or P1 H1 probes (Table 2). The P1 H2 and the P4 H2 segments are also closely related. Even though H1 alone, or H3 and H4 in combination are sufficient for the

Figure 1. Northern blots of UmV dsRNAs. These three Northern blots were performed by transfers onto nitrocellulose of dsRNAs separated on 1.4% agarose gels such as that shown stained with ethidium bromide (a). Probes were pCp labeled P6 H1 (b), reverse transcribed P1 M1 (c) and reverse transcribed P1 L1 (d). The lanes in each gel had P1 dsRNAs (P1), P4 dsRNAs (P4), P6 dsRNAs (P6), and ScV dsRNAs (ScV).

formation of viral particles of similar properties, and may therefore code for similar major capsid polypeptides, the H segments within a viral subtype have no detectable sequence homology. On the other hand, it is not surprising that several H segments might

Table 2. Hybridization analysis of UmV dsRNAs

dsRNA on nitrocellulose													
Probe	P1					P4					P6		
	H1	H2	M1	M2+3	L	H1	H2	H3+4	M2+3	L	H1	M2	L
P6 H1	+	-	-	-	-	+	-	-	-	-	+	-	-
P6 M2	-	-	-	-	-	-	-	-	-	-	-	+	+
P6 L	-	-	-	-	-	-	-	-	-	+	-	+	+
P4 H1	+	-	-	-	-	+	-	-	-	-	+	-	-
P4 H2	-	+	-	-	-	-	+	-	-	-	-	-	-
P4 H3+4	-	-	-	-	-	-	-	+	-	-	-	-	-
P4 M2+3	-	-	+	-	+	-	-	-	+	+	-	-	-
P4 L	-	-	+	-	+	-	-	-	+	+	-	-	-
P1 H1	+	-	-	-	-	+	-	-	-	-	-	-	-
P1 H2	-	+	-	-	-	-	+	-	-	-	-	-	-
P1 M1	-	-	+	-	+	-	-	-	+	+	-	-	-
P1 M2+3	-	-	-	+	-	-	-	-	-	-	-	-	-
P1 L	-	-	+	-	+	-	-	-	+	+	-	-	-

code for a major viral capsid polypeptide of similar amino acid sequence and yet have no detectable sequence homology, since this is exactly the case with ScV (9, 10, 25).

No homology was detected by Northern hybridization between any of the UmV dsRNAs and the ScV dsRNAs (e.g. Fig. 1). An ScV-L₁ cDNA probe also failed to react with any of the UmV dsRNAs.

Heteroduplex mapping of L and M.

Since the homologies between the L dsRNAs and one M dsRNA in each viral subtype are easily detected by Northern gels, we expected that there would be extensive regions of homology between these dsRNAs. This expectation is confirmed by heteroduplex analysis. Pl L is entirely derived from one end of Pl M1 (Fig. 2). The heteroduplexes take the form of "rabbit ears," in which the ears, of equal length, represent one terminus of M1 duplexed with the entire length of L.

These heteroduplex regions (b and c of Table 3) have a mean length of 371 bp. This is the length of L, independently determined on native L not subjected to denaturation and renaturation (Table 1). The remaining portion of M1, not hybridized to L, constitutes 1097 bp. The total length of M1 in the heteroduplexes is thus 1468 bp. Again, this is in agreement with the length of M1, which was

Figure 2. Heteroduplex of Pl M1 with Pl L. A "rabbit ears" heteroduplex is shown with a pBR322 open circle and molecules of L.

Table 3. Heteroduplex analysis of L and M

Hetero- duplex	a (bp)		(b+c)/2 (bp) ¹		a + (b+c)/2 (bp)		N ²
	mean	sd ³	mean	sd	mean	sd	
L-M1 (P1)	1097	104	371	46	1468	114	22
L-M2+3 (P4)	687	69	379	48	1066	84	27

¹ The branches b and c were indistinguishable in length.

² N is the number of heteroduplexes measured.

³ sd is standard deviation.

measured as 1548 bp with a s.d. of 128 bp. Control experiments of denatured and renatured L or M1 alone showed no heteroduplex forms. We conclude that P1 L is entirely derived from one end of P1 M1. Since L is necessary for immunity to toxin, it must encode a functional product. Consequently, both L and M1 encode the same gene product.

In each viral subtype, L is related to one of the M dsRNAs. If the relationship between L and M determined for P1 is general, then heteroduplexes of L with the M to which it is related should take the same form in each viral subtype. This is the case for P4. Heteroduplexes of P4 M2+3 with P4 L gave the same results as those with the homologous P1 dsRNAs (Table 3). Since we have not used separated M2 and M3, we cannot say from which P4 L is derived, but the control experiment in which M2+3 was denatured and renatured showed no forms similar to those of the heteroduplex preparation with L. The standard deviation of the length of M2+3 is quite small, which is consistent with previous measurements estimating that M2 and M3 differ by at most 50 bp (Table 1).

We have yet to perform similar experiments with P6, but the strong hybridization between P6 L and P6 M probably indicates a similar relationship in this subtype also. In UmV then, in contrast

Table 4. 3' end sequences of UmV dsRNAs

RNA	Strain		
	P1	P4	P6
H1	GAAAAUCA _{OH}	GAAAAUCA _{OH}	GAAAAUCA _{OH}
	GAAAAUCG _{OH}	GAAAAUCG _{OH}	GAAAAUCG _{OH}
	GUUUUUCA _{OH}	GUUUUUCA _{OH}	GUUUUUCA _{OH}
	GUUUUUCG _{OH}	GUUUUUCG _{OH}	GUUUUUCG _{OH}
H2	GAUAUUAAAUCA _{OH}	GUAAUAUCAAUAUCA _{OH}	
	GAUAUUAAAUCG _{OH}	GUAAUAUCAAUAUCG _{OH}	
	GAUAUUUUUAUCA _{OH}	GAUAUCAAUAUCA _{OH}	
	GAUAUUUUUAUCG _{OH}	GAUAUCAAUAUCG _{OH}	
H3+4		GCA _{OH}	
		GCG _{OH}	
		GCUUUUUUAUCA _{OH}	
		GCUUUUUUAUCG _{OH}	
M1	GCA _{OH}		
	GCG _{OH}		
	GUUUUUUCA _{OH}		
	GUUUUUUCG _{OH}		
M2+3	GCA _{OH}	GCA _{OH}	GUCA _{OH}
	GCG _{OH}	GCG _{OH}	GUCG _{OH}
	GAAAUUUUAUCA _{OH}	GAAAUUUUAUCA _{OH}	GUUUUUUCA _{OH}
	GAAAUUUUAUCG _{OH}	GAAAUUUUAUCG _{OH}	GUUUUUUCG _{OH}
	GCUUUUCUUCUUCA _{OH}		GAAAUUUUCA _{OH}
L	GCA _{OH}	GCA _{OH}	GCA _{OH}
	GCG _{OH}	GCG _{OH}	GCG _{OH}

to the ScV system, and in contrast to the reoviruses, two dsRNAs of different size code for the same product.

Sequences at the 3' ends of the UmV dsRNAs. The sequences at the 3' ends of the UmV dsRNAs confirmed our model for the P1 and P4 M and L species. P1 M1 has two different 3' ends, only one of which is present in P1 L (Table 4). This is precisely what is expected if P4 L is entirely derived from one end of P1 M1. The same relationship holds for P4 L and P4 M2+3. Just as our heteroduplex mapping utilized a mixture of the poorly separated M2 and M3 species, so did the 3' end sequence analysis. The mixture has only 2 different 3' ends, allowing for the 3' terminal base ambiguity (see below) (Table

Figure 3. Two-dimensional gel electrophoresis of 3' terminal T_1 oligonucleotides of UmV H1 dsRNAs. T_1 fingerprints of the 3' end-labeled H1 dsRNAs of P1, P4, and P6 are shown. The sequences of oligonucleotides 1 to 4 are shown in Table 3 (first four oligonucleotides, in the order 1-4). A sequencing gel of two of them is shown in Fig. 4.

4). Consequently, M2 and M3 have the same 3' ends. The 3' sequence analysis does not help identify from which of these two species L is derived. However, since only one of the ends of P4 M2+3 is present in P4 L, the 3' sequence analysis confirms the heteroduplex analysis: L is derived from one end of M2+3. Only P6 L and M are not so simply related at one 3' end (Table 4).

The limited 3' sequence analysis is also consistent with the other homologies detected by Northern hybridizations. For instance, the two-dimensional fingerprints of the 3' end labeled T_1 oligonucleotides of the P1, P4, and P6 H1 segments are shown in Fig. 3. They have identical fingerprints, and, as shown in Table 3, their 3' termini are identical. The same is true of the three L segments as well, although here the complete T_1 products are very short (Table 4). The P1 and P4 H2 segments, which show sequence homology by Northern gels (Table 2) also have considerable 3' end sequence homology (Table 4).

One remarkable observation about the 3' termini of the UmV dsRNAs is the duality of the terminal nucleoside: most ends are present in two forms, one with a terminal A_{OH} and one with a terminal G_{OH} . This mirrors the situation of one of the yeast viruses, ScV- L_a , whose dsRNA terminates with either A_{OH} or G_{OH} at both ends. The sequencing gel of two such ends of P6 H1 is shown in Fig. 4. All terminal nucleosides (converted to nucleotides by pCp

Figure 4. Sequencing gel of the U-rich termini of P6 H1. This is a 20% polyacrylamide-7M urea gel of the partial chemical cleavage products of the U-rich 3' terminal T_1 oligonucleotides of P6 H1 - oligonucleotide 3 (left) and 4 (right) of Fig. 3. Terminal residues were verified as described (see text).

addition) were verified by high-voltage paper electrophoresis of complete alkali hydrolysates and of complete pancreatic RNase digests of isolated T_1 oligonucleotides. In analogy with the ScV system, the UmV dsRNAs should have 5' terminal pppG and 3' terminal post-transcriptionally added A_{OH} or G_{OH} . Like the ScV dsRNAs (6), the UmV dsRNAs show some additional 3' terminal heterogeneity (see for example the minor spots in Fig. 3). Some of the UmV dsRNAs then, like ScV-L (10), may be composed of more than one species of the same size.

In the yeast system, each of the viral dsRNAs has a "C-rich" terminus and a "U-rich" terminus. Actually, both ends are rather A-U rich, although the U-rich end is more so (7). Except in one case, all the ScV C-rich termini end in GCA_{OH} . Many of the UmV

dsRNAs also have one such end (e. g. all the L segments, P1 M1, P4 H3+4, P4 M2+3). Some of these dsRNAs seem to be like the ScV dsRNAs in that they have one end with GCA_{OH} (or GCG_{OH}) and another that is very A-U rich (e.g. P1 M1). There are, however, some UmV dsRNAs with no GCA_{OH} (or GCG_{OH}) end (e.g. P1 H2) or with no obvious A-U rich end (all the L segments).

Some homologies are evident from the 3' terminal sequences that are not detectable by Northern blots. For instance, P1 M2+3 has one species that also appears to be present in P4 M2+3 and in P6 M2: that is, a dsRNA one of whose ends is GAAAUUUUAAUCA_{OH} in P1, GAAAUUUUAAUCA_{OH} in P4, and GAAAUUUUAAUCA_{OH} in P6. These may have a relationship like that of ScV-L₁ and L₂, which are similar in 3' end sequence but whose homology is detectable by Northern blots only with one of many cloned cDNA probes (9, 10).

DISCUSSION

Unlike the ScV system, in which it is unusual to find two viral dsRNAs in the same cell with extensive sequence homology, this is routinely true for UmV: the L dsRNAs are entirely derived from one end of their respective M dsRNAs. This is unlike the origination of the ScV defective interfering (S) dsRNAs from M in several respects. Firstly, the S dsRNAs are derived by internal deletion (6, 26, 27). Secondly, the S dsRNAs are defective: they do not encode any functional viral polypeptides. The L dsRNAs of UmV are functional - they encode resistance to toxin (17). Finally, the S dsRNAs interfere with the replication of M (presumably by more rapid replication), replacing it when both are present in the same cell. The UmV L and M containing particles coexist in the same cell. Hence two genomic UmV dsRNAs of different size in each subtype encode the same function.

There are few straightforward sequence relationships among the UmV dsRNAs. The H1 segments, all of which apparently encode the major viral capsid polypeptide, are similar among the three subtypes. Similarly, the L segments share homology. On the other hand, P1 M1, rather than P1 M2+3 shares homology with P4 M2+3. The H3 and H4 segments, which are sufficient to encode the necessary capsid polypeptide(s) in strains with only H3 and H4, have no detectable homology with H1. Both the hybridization and the

sequencing results show that P1 and P4 are much more closely related to each other than either is to P6.

Like the ScV dsRNAs, the UmV dsRNAs have some 3' terminal heterogeneity in addition to the variable ultimate base. Some of the UmV dsRNAs may therefore be like ScV-L, which has more than one species of the same size in the same cells (10).

The 3' ends of the UmV and ScV dsRNAs fall into the consensus sequence A/U A/U A/U A/U A/U A/U A/U U/G C A/G_{OH}. This is true of both ends of the ScV dsRNAs and of each of the 50 3' ends of UmV dsRNAs determined (70-100% agreement at each position). Only one of the yeast dsRNAs terminates either with A_{OH} or G_{OH}, while each of the UmV dsRNAs appears to have such a choice. Since each UmV 3' terminal T₁ oligonucleotide appears with both A_{OH} and G_{OH} and since the last nucleoside of the ScV dsRNAs is post-transcriptionally added, we conclude that that is the case in UmV as well. Even though the UmV and ScV dsRNAs show no homology by hybridization, they do have recognizable sequence homology at the 3' termini. Therefore, there may be some fundamental similarities in the replication and transcription of the fungal virus dsRNAs. Some of the UmV dsRNAs have one end with unambiguous similarity to the ScV transcriptase initiation consensus sequence, which ends UUUUUA/G_{OH} (6,7). P1 H1, P1 M1, P4 H1, P6 H1, P6 M2, and P4 H3+4 all have only one such end, which terminates in this same consensus sequence. This may therefore be the initiation site for the UmV transcriptase.

If the UmV transcriptase does initiate here, then the 3' terminal portion of the resultant M mRNA would encode the resistance factor. In the ScV system, the analogous dsRNA (also called M) is transcribed to produce both a full-length transcript and a subgenomic mRNA, probably from the 5' end of the transcript. The current model for the ScV M also places the resistance factor at the 3' end of the message (K. Bostian, V. Burn, S. Jayachandran, and D. Tipper, pers. comm.). It is possible that in the UmV system, the 3' fragment of a such a processed mRNA from M, encoding the resistance factor, has been replicated to form a new dsRNA (L) encoding only the resistance factor. Such a mechanism of evolution of new, functional, genomic dsRNAs would be unique to the fungal viruses, since the dsRNA viruses of higher eucaryotes, e. g. reovirus, have

only full-length mRNAs for the dsRNA segments, each encoding a single polypeptide (28).

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