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Genetic Analysis of Orbiviruses by Using RNase T_1 Oligonucleotide Fingerprints

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Corresponding double-stranded RNA segments of the related orbiviruses Wallal and Mudjinbarry produced distinctly different RNase T_1 fingerprint patterns. No extensive sequence reiteration was observed between segments of Mudjinbarry virus. Fingerprint analysis of the genome of recombinant orbiviruses confirmed segment reassortment as a mechanism of interchange of genetic information. When temperature-sensitive mutants of each virus were crossed in mixed infection, a consistent pattern of segment reassortment was correlated with generation of the wild-type phenotype. Thus, the temperature-sensitive lesion of group II Wallal serogroup mutants was mapped to segment 1. The group I mutant lesion appears to be located in segment 2.

Wallal and Mudjinbarry viruses are serotypes of the Wallal serogroup of orbiviruses which were isolated from *Culicoides* spp. in northern and central Australia (5, 6). The viruses share complement-fixing antigens but may be distinguished by the plaque reduction neutralization test (6). Each contains 10 segments of double-stranded RNA which range in size from approximately 2.3×10^5 to 2.3×10^6 daltons. The molecular weight distribution of their segments, as determined by polyacrylamide gel electrophoresis, shows similar patterns with distinct differences in three segments (13).

Generation of recombinant progeny by reassortment of RNA segments during mixed infections has been demonstrated for a number of segmented genome viruses (8, 26, 33). It has been suggested that such a mechanism is responsible for the appearance of new pandemic strains of influenza A virus (22).

Reassortment of RNA segments during mixed infection with orbiviruses has been reported (13). Temperature-sensitive mutants of Wallal and Mudjinbarry viruses have been divided into two groups according to their ability to generate wild-type progeny during mixed infection. A series of 60 recombinant clones was isolated from one such mixed infection. The RNA genome of each clone was examined for exchange of segments by polyacrylamide gel electrophoresis. Fifty-seven displayed electrophoretic migration patterns that could not be distinguished from that of the Wallal parental mutant. In the three remaining clones, various substitutions of Mudjinbarry segments 4, 5, 6, and possibly 10 had occurred (13).

Although this work provided evidence of segment reassortment, several points required further investigation. First, in the absence of supportive data, equivalence of electrophoretic mobility cannot be interpreted as indicating segment identity. Of the four possible reassortment events reported, two were in segments in which the parental viruses could barely be distinguished. Second, 57 of the 60 clones could not be distinguished from possible intragenic recombinants or mutational revertants on other than theoretical grounds. Finally, it was not demonstrated that the consistent reassortment of a single defective segment had resulted in generation of the wild-type phenotype.

In this paper we show that corresponding segments of Wallal and Mudjinbarry viruses can be clearly distinguished by using RNase T_1 oligonucleotide fingerprints. We used this technique to confirm electrophoretic evidence of segment reassortment and establish the genotype of recombinant viruses which could not be distinguished from the Wallal parental mutant by polyacrylamide gel electrophoresis. The locations of temperature-sensitive lesions characteristic of group I and group II Wallal serogroup mutants were investigated.

MATERIALS AND METHODS

Cells and viruses. Growth and plaque assay of viruses in pig kidney (PS-EK) cells were by methods described previously (10).

The origin of temperature-sensitive mutants of Wallal virus and Mudjinbarry virus has been described (13). The following mutants were used: group I, Wallal *ts*30 and Mudjinbarry *ts*3; group II, Wallal *ts*101 and Mudjinbarry *ts*5. Temperature-sensitive mutants and recombinant viruses were grown routinely at the permissive temperature (32°C).

Mixed infections and isolation of recombinant viruses. Mixed infections at high multiplicity were

performed in 96-well Linbro microculture plates as described by Gorman et al. (13) except that a combined input multiplicity of 10 PFU/cell was used. Progeny virus was titrated at the nonpermissive temperature (37°C) and well-isolated, end-dilution plaques were selected. Plaques were each ground in 1 ml of Eagle minimum essential medium (MEM), and 0.5 ml of each was used to inoculate monolayer cultures of 10^6 cells. Infected cultures were incubated at 32°C until maximal cytopathic effect occurred and stored at 4°C until required for further passage.

Preparation of viral double-stranded RNA. Virus-infected cell cultures were harvested at maximal cytopathic effect and RNA was extracted by using the diethyl pyrocarbonate-sodium dodecyl sulfate method of Summers (35). Nucleic acids were precipitated from the extract with ethanol and suspended in 1 M sodium perchlorate. The solution was adjusted to 1.25% sodium dodecyl sulfate and extracted with 1/3 volume of chloroform-isoamyl alcohol (5:2) until no white interphase remained (20). The ethanol precipitate of this extract was suspended in buffer (0.01 M NaCl, 0.05 M Tris-hydrochloride, 0.001 M EDTA, pH 6.9) and adjusted to 2 M LiCl. Single-stranded RNA was removed by precipitation at 4°C (1). Double-stranded RNA was collected from the supernatant by cellulose (Whatman CF11) chromatography (7).

For preparation of tritium-labeled RNA, infected cultures were incubated in [^{3}H]uridine (10 μCi/ml) (Radiochemical Centre, Amersham, England) in MEM throughout infection. For preparation of ^{32}P-labeled RNA, cultures were incubated in phosphate-free MEM at 32°C for 7 h before infection. Throughout infection cells were treated with [^{32}P]orthophosphoric acid (360 μCi/ml) (Australian Atomic Energy Commission) in phosphate-free MEM. Contamination with single-stranded RNA was estimated by hydroxylapatite (Bio-Rad, Bio-Gel HTP) thermal elution chromatography to be less than 1% (25). A specific radioactivity of 1.5×10^6 to 2.0×10^6 cpm/μg was generally achieved for ^{32}P-labeled RNA.

Electrophoresis of double-stranded RNA. Electrophoresis of RNA in 7.5% polyacrylamide slab gels has been described (13). Tritium-labeled RNA was detected by fluorography by using the method of Laskey and Mills (21).

Preparation of genome segments. Individual double-stranded RNA segments were isolated essentially by the method of Schuerch et al. (32). A 10- to 20-μg amount of viral RNA per tube (10 by 0.6 cm) was electrophoresed in Tris-phosphate buffer (pH 7.8) (24) at 4°C in gels consisting of 7.5% acrylamide and 0.2% bisacrylamide. After completion of the run, gels were stained with 0.01% ethidium bromide in TAE buffer (0.04 M Tris-acetate, 0.02 M sodium acetate, 0.002 M EDTA, pH 7.8) and visualized under UV light. Segments were sliced from the gel and soaked in 0.5 ml of TAE buffer before electrophoretic elution as described by Schuerch et al. (32). Pooled eluates of each segment were extracted three times with an equal volume of water-saturated phenol at room temperature. Samples were adjusted to 0.1 M sodium chloride and precipitated with two volumes of ethanol at −20°C in the presence of 100 μg of highly polymerized RNA carrier (Calbiochem). Precipitates were dissolved in 100 μl of sterile, deionized water and lyophilized.

RNase T_1 digestion of double-stranded RNA and two-dimensional gel electrophoresis. ^{32}P-labeled double-stranded RNA was denatured by heating at 100°C for 10 min and then chilled and adjusted to 5 mM Tris-hydrochloride, pH 7.3. Samples were digested with 50 IU of RNase T_1 at 37°C for 90 min (enzyme/RNA ratio = 0.25). The digest was subjected to two-dimensional polyacrylamide gel electrophoresis (4, 23). First-dimension gels (25 by 8 by 0.16 cm) consisted of 8% acrylamide and 0.1% bisacrylamide in 6 M urea adjusted to pH 3.3 with saturated citric acid. Gels were run at 600 V at 4°C until a bromophenol blue marker had migrated 12 cm. Second-dimension gels (40 by 25 by 0.16 cm) consisted of 22% acrylamide, 1.5% bisacrylamide, 2.5 mM EDTA in 0.1 M Tris-borate, pH 8.3. Gels were run at 600 to 700 V at 4°C in an upward direction until the bromophenol blue marker had migrated 20 cm. Autoradiography of gels was performed by using Fuji RX or Ilfix 90 X-ray film at 4°C for up to 2 weeks.

RESULTS

Oligonucleotide fingerprints of Wallal virus and Mudjinbarry virus genome segments. Fingerprints have been obtained for each of the 10 genome segments of Wallal and Mudjinbarry viruses. The RNase cleavage products of denatured double-stranded RNA were separated by two-dimensional polyacrylamide gel electrophoresis. Representative segments of each virus are shown in Fig. 1. Cleavage of several preparations of each segment reproducibly generated a unique pattern of large oligonucleotides. Although Wallal and Mudjinbarry viruses share a close serological relationship (6), we observed little similarity between fingerprints of corresponding genome segments. Segments 1, 7, 8, and 9, which are not distinguishable by coelectrophoresis of viral genomes, could be distinguished clearly by this technique. For segment 7 a distinct relationship could be recognized by visual comparison of fingerprints electrophoresed under identical conditions. Of the 25 largest oligonucleotides, 12 were of identical mobility (Fig. 1). The fingerprints of other segments were not sufficiently related to compare in this manner.

Comparison of segments of Mudjinbarry virus showed that very few large oligonucleotides were common to more than one segment. The positions of oligonucleotides from neighboring segments could be derived from a low but detectable level of cross-contamination. By such analysis, it was determined that no segment was wholly or largely contained within any other segment. The cross-contamination patterns were used to assign segment origin to 65 of the largest oligonucleotides of the unfractionated genome of Mudjinbarry virus (Fig. 2). Only four of these

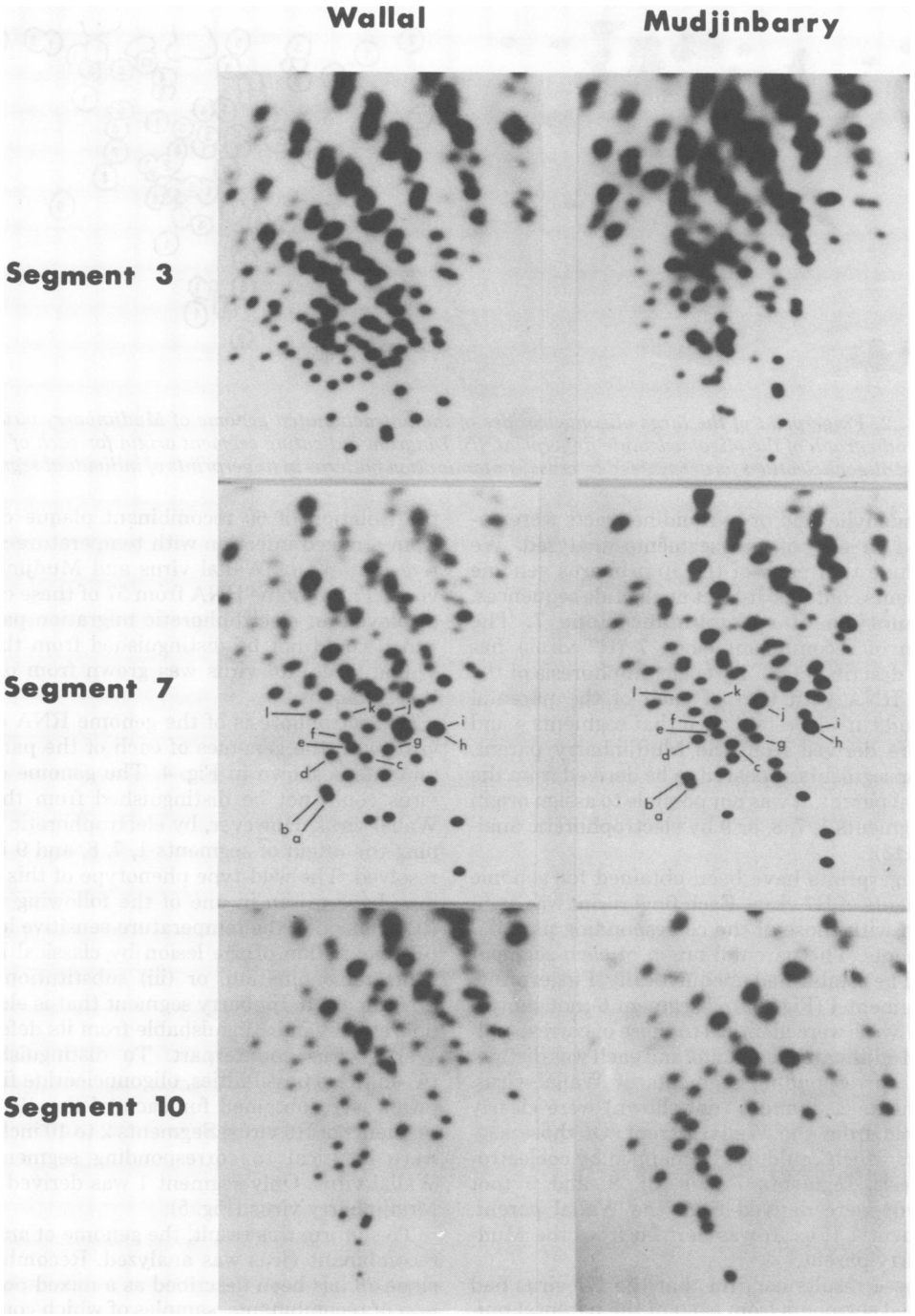

FIG. 1. *Two-dimensional oligonucleotide fingerprints of double-stranded RNA genome segments 3, 7, and 10 of Wallal and Mudjinbarry viruses. For segment 7 from each virus, a set of 12 large oligonucleotides (a–l) were of identical electrophoretic mobility as determined by visual comparison of fingerprints.*

oligonucleotides could not be at least partially resolved into unique spots. Two of these comigrating oligonucleotides were derived from segments 3 and 4. The other two were both derived from segment 2. Accurate segment origin allocation of smaller oligonucleotides was not possible because of the increasing density of the fingerprint pattern. No high-molecular-weight

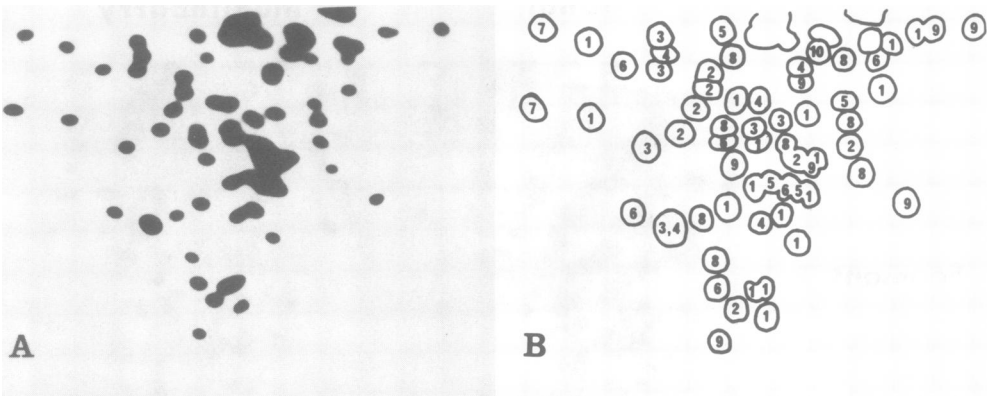

FIG. 2. *Fingerprint of the large oligonucleotides of the unfractionated genome of Mudjinbarry virus. (A) Autoradiograph of the oligonucleotide fingerprint. (B) Diagram indicating segment origin for each of the 65 largest oligonucleotides as determined by cross-contamination patterns in fingerprints of individual segments.*

polyadenylic acid or pyrimidine tracts were detected in any of the segments analyzed. We conclude that each of the 10 orbivirus genome segments contains distinct nucleotide sequences.

Genotype of recombinant clone 7. The origin of recombinant clone 7 (R7 virus) has been described (13). By coelectrophoresis of the viral RNA with that of each of the parental mutants, it was established that segments 4 and 6 were derived from the Mudjinbarry parent. Other segments appeared to be derived from the Wallal parent. It was not possible to assign origin to segments 1, 7, 8, or 9 by electrophoretic analysis (13).

Fingerprints have been obtained for genome segments of R7 virus. Each fingerprint was compared with those of the corresponding parental segments. The parental origin of each segment could be established unequivocally. Fingerprints of segment 4 (Fig. 3) and segment 6 (not shown) of R7 virus were identical to those of corresponding Mudjinbarry segments, and each was distinct from corresponding segments of Wallal virus. Segments 2, 3, and 5 (not shown) were clearly derived from the Wallal parent. Of those segments which could not be mapped by coelectrophoresis, segments 7 (Fig. 3), 8, and 9 (not shown) were derived from the Wallal parent. Segment 1 (Fig. 3) was derived from the Mudjinbarry parent.

These results confirm that the R7 virus had derived segments from each of the parental mutants and demonstrate that a combination of segment migration analysis and segment fingerprinting is a useful approach for defining the genotype of recombinant viruses.

Genotypes of recombinant clones electrophoretically indistinguishable from the Wallal parent. Gorman et al. (13) described the isolation of 60 recombinant plaque clones from a mixed infection with temperature-sensitive mutants of Wallal virus and Mudjinbarry virus. The genome RNA from 57 of these clones displayed an electrophoretic migration pattern which could not be distinguished from that of Wallal virus. R6 virus was grown from one of these plaques.

Coelectrophoresis of the genome RNA of R6 virus with the genomes of each of the parental mutants is shown in Fig. 4. The genome of R6 virus could not be distinguished from that of Wallal virus. However, by electrophoretic mapping the origin of segments 1, 7, 8, and 9 is not resolved. The wild-type phenotype of this virus may have arisen in one of the following ways: (i) reversion of the temperature-sensitive lesion, (ii) elimination of the lesion by classical intragenic recombination, or (iii) substitution of a functional Mudjinbarry segment that is electrophoretically indistinguishable from its defective Wallal virus counterpart. To distinguish between these possibilities, oligonucleotide fingerprints were obtained for each of the 10 RNA segments of R6 virus. Segments 2 to 10 inclusive were identical to corresponding segments of Wallal virus. Only segment 1 was derived from Mudjinbarry virus (Fig. 5).

To confirm this result, the genome of another recombinant virus was analyzed. Recombinant clone 28 has been described as a mixed population of recombinants, samples of which could be isolated by subcloning. Among the subclones were a number with genomes that were electrophoretically indistinguishable from the Wallal parent (13). Serial passage of primary plaque 28 isolate has rapidly established one of these subclones as the dominant type. After only five passages in PS-EK cells, the electrophoretic mi-

gration pattern of double-stranded RNA isolated from the pool could not be distinguished from that of Wallal virus. Contamination of the sample with other RNA segments could not be detected by staining with methylene blue. The dominant RNA genome was stable during sub-

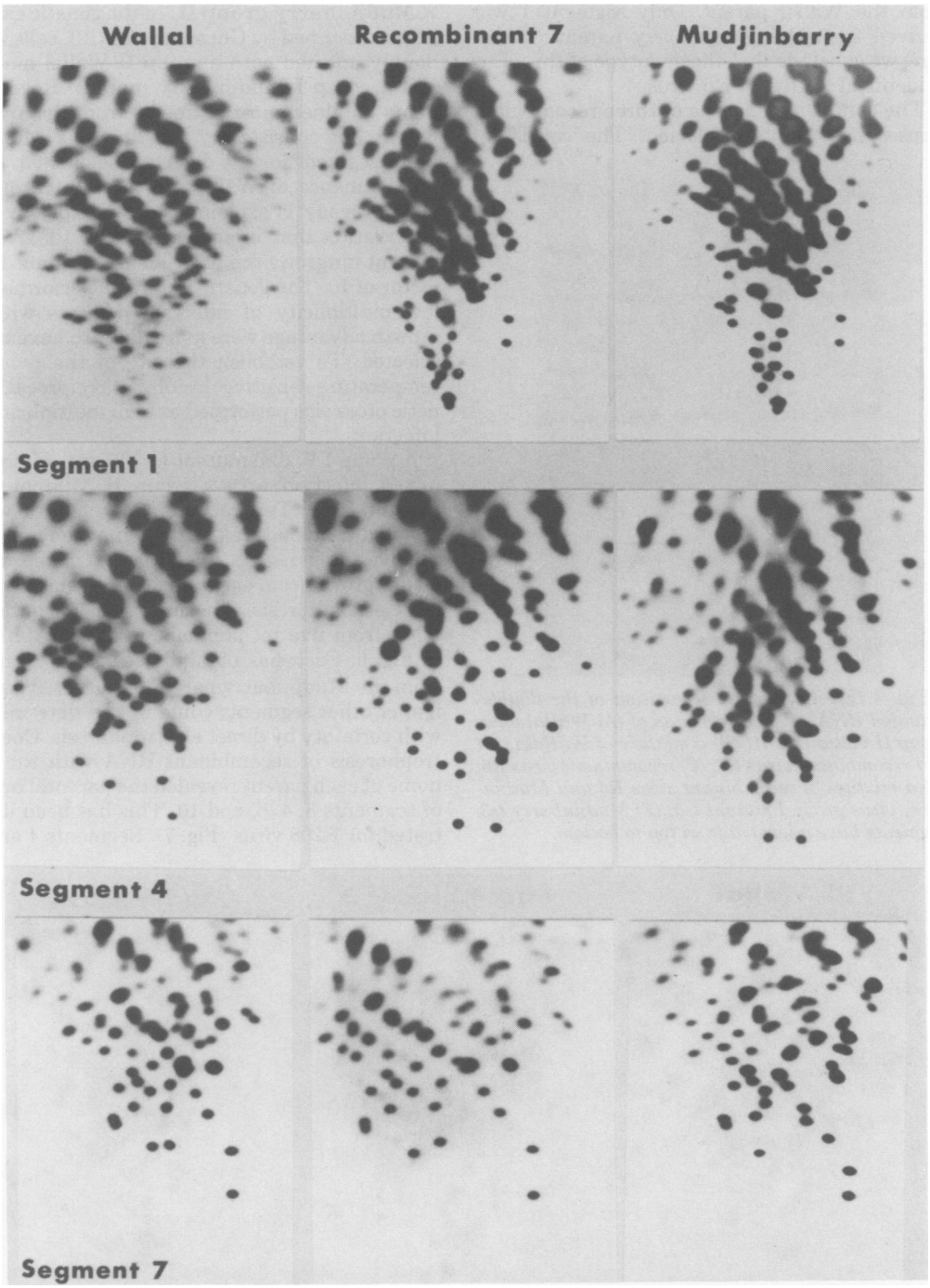

FIG. 3. *Fingerprints of segments 1, 4, and 7 of recombinant orbivirus R7 and corresponding segments of parental mutant viruses, Wallal ts101 and Mudjinbarry ts3. Wild-type recombinant virus R7 was derived from a mixed infection cross: Wallal group II mutant ts101 × Mudjinbarry group I mutant ts3.*

sequent passaging. Genome RNA segments were isolated after the fifth passage of the culture. Nine segments could be recognized from oligonucleotide fingerprints as being derived uniquely from the Wallal parent. Only segment 1 was derived from the Mudjinbarry parent. Therefore, we conclude that the genotype of this virus is identical to that of R6 virus.

The complete genotype of three recombinant viruses has been established. The consistent reassortment of segment 1 would suggest that the temperature-sensitive lesion of the group II Wallal mutant (*ts*101) lies in that segment.

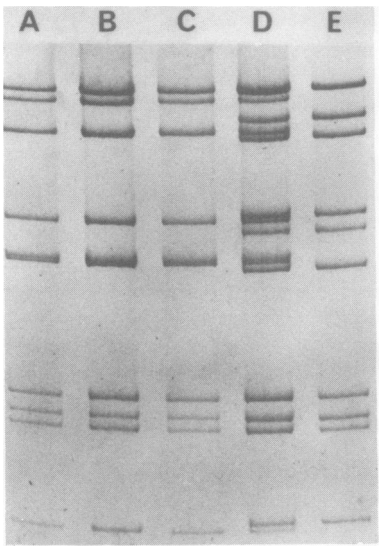

FIG. 4. *Electrophoretic separation of the double-stranded RNA genome segments of (A) Wallal virus group II mutant ts101; (B) a mixture of Wallal ts101 and recombinant virus R6; (C) recombinant virus R6; (D) a mixture of recombinant virus R6 and Mudjinbarry virus group I mutant ts3; (E) Mudjinbarry ts3. Segments have migrated from top to bottom.*

Reciprocal genetic cross: Wallal group I × Mudjinbarry group II. In the genetic experiment described by Gorman et al. (13), cells were doubly infected with a group II Wallal mutant and a group I Mudjinbarry mutant. Recombinants obtained most segments from the Wallal parent but consistently substituted a Mudjinbarry segment for the defective segment 1. The predominance of Wallal segments in recombinant progeny is explained most simply by the observation that in single infection the Wallal mutant outgrows the Mudjinbarry mutant by a factor of 10. The genetic cross was performed at low multiplicity of infection. Viruses with a growth advantage were more likely to have been selected. To establish the site of the group I temperature-sensitive lesion, a reciprocal genetic cross was performed at high multiplicity of infection.

A group I Wallal mutant (*ts*30) was crossed in mixed infection with a group II Mudjinbarry mutant (*ts*5). Ten recombinant plaques were selected from cultures incubated at the nonpermissive temperature. RNA was extracted from clones grown from each plaque and analyzed by gel electrophoresis. The migration patterns of RNA from five recombinant viruses are shown in Fig. 6. Each has obtained segments 2 and 5 from the Mudjinbarry parental mutant. The origin of other segments could not be determined with certainty by direct electrophoresis. Coelectrophoresis of recombinant RNA with the genome of each parent revealed the parental origin of segments 3, 4, 6, and 10. This has been illustrated for R208 virus (Fig. 7). Segments 4 and 6

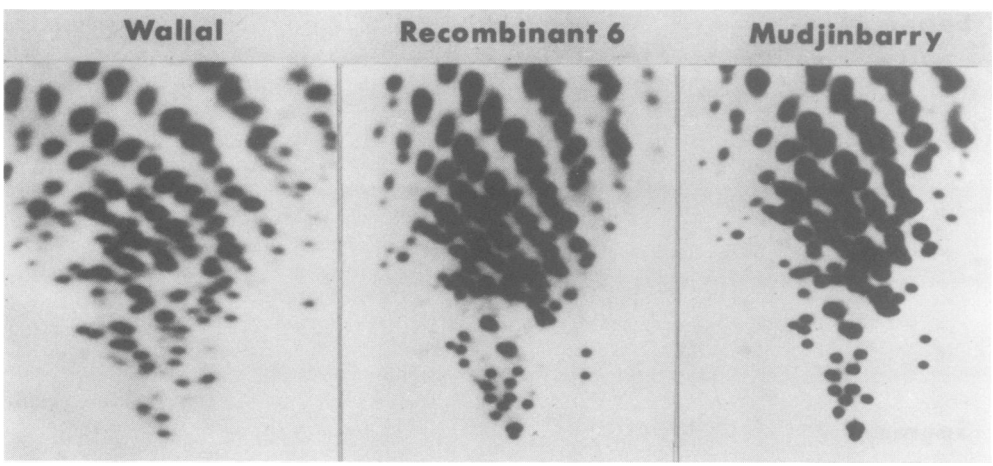

FIG. 5. *Fingerprints of segment 1 of recombinant virus R6 and of parental mutant viruses Wallal ts101 and Mudjinbarry ts3.*

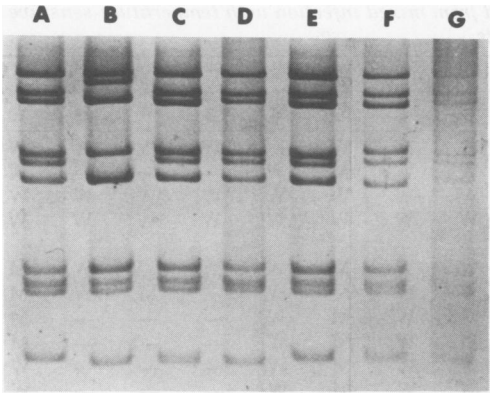

FIG. 6. *Electrophoretic separation of double-stranded RNA genome segments of (A) recombinant virus R207; (B) Wallal virus group I mutant ts30; (C) recombinant virus R201; (D) recombinant virus R204; (E) recombinant virus R208; (F) Mudjinbarry virus group II mutant ts5; (G) recombinant virus R203.*

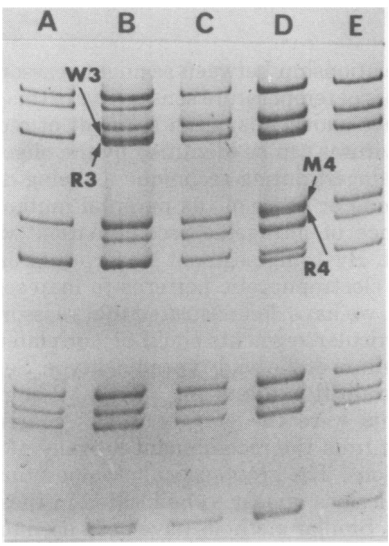

FIG. 7. *Electrophoretic separation of double-stranded RNA genome segments of (A) Wallal virus group I mutant ts30; (B) a mixture of Wallal ts30 and recombinant virus R208; (C) recombinant virus R208; (D) a mixture of recombinant virus R208 and Mudjinbarry virus group II mutant ts5; (E) Mudjinbarry ts5. W3, Wallal virus segment 3; R3, R208 virus segment 3; R4, R208 virus segment 4; M4, Mudjinbarry virus segment 4.*

were each derived from the Wallal parent. Segments 3 and 10 were derived from the Mudjinbarry parent. The origin of segments 1, 7, 8, and 9 could not be determined by this method. Coelectrophoresis of five other recombinant viruses (R201, R202, R205, R209, and R210) with each of the parental mutants revealed the partial genotypes present in Table 1.

The genome of R202 virus was fractionated into segments. Fingerprints of segments 1, 7, and 9 showed each was derived from Wallal virus. Fingerprint analysis of segment 8 revealed a mixture of Wallal segment 8 and Mudjinbarry segment 8. The Mudjinbarry segment was in molar excess as determined by the intensity of spots on the fingerprint. R202 virus was plaque cloned twice more, and the genotype of the new clone (R202C) was determined by comparative electrophoresis and by fingerprinting. Fingerprint analysis showed that the R202C clone derived segment 8 from Mudjinbarry virus. The genotype of each clone is shown in Table 1. It is likely that the original R202 clone was a mixture of two viruses that differed only in the origin of segment 8. A similar observation has been reported by Gorman et al. (13) for recombinant clone 28.

The results presented in Table 1 indicate only two segments were consistently derived from the Mudjinbarry parent, segment 2 and segment 5. The group I temperature-sensitive lesion would appear to be located in one of these segments. Gorman et al. (13) have shown recombinant 27 virus to have obtained segment 5 from the group I Mudjinbarry parent, *ts*3 (Table 1). This would eliminate segment 5 as a possible site. The group I lesion appears to be located in segment 2.

DISCUSSION

Wallal and Mudjinbarry viruses share a close serological relationship and display similar patterns of segment electrophoretic migration with distinct differences in only three segments. Genome electrophoretic patterns have been used to compare orbivirus isolates of the Wallal, Warrego, and Eubenangee serogroups (11, 12, 14). It was suggested that differences in migration of segments coding for surface polypeptides may reflect serological differences between closely related isolates. Similar methods have been used to compare isolates of other segmented RNA viruses (15, 18, 19, 27, 28, 31). Payne et al. (27) claimed that electrophoretic profiles were more useful for grouping cytoplasmic polyhedrosis viruses than were serological methods. Rodger and Holmes (31) have proposed the use of genome electrophoretic analysis for both diagnosis and strain identification of rotaviruses.

Results reported in this paper suggest that classification of viruses according to genome electrophoretic patterns could be misleading. Fingerprint analysis of corresponding segments of Wallal and Mudjinbarry viruses showed that substantial base sequence heterology is not restricted to segments with different electrophoretic mobility. Segments with identical mobility also produced dissimilar fingerprint patterns.

TABLE 1. *Genotypes of wild-type recombinants derived from mixed infection with temperature-sensitive mutants*

Wild-type recombinant	Parental mutants		Derivation of segment									
	Wallal	Mudjinbarry	1	2	3	4	5	6	7	8	9	10
R7	*ts*101[a]	*ts*3[b]	M[c]	W	W	M	W	M	W	W	W	W
R27[e]	*ts*101	*ts*3	—[d]	W	W	W	M	W	—	—	—	W
R6	*ts*101	*ts*3	M	W	W	W	W	W	W	W	W	W
R28A	*ts*101	*ts*3	M	W	W	W	W	W	W	W	W	W
R202	*ts*30[b]	*ts*5[a]	W	M	W	W	M	W	W	M/W[f]	W	M
R202C[g]	*ts*30	*ts*5	—	M	W	W	M	W	—	M	W	M
R208	*ts*30	*ts*5	—	M	M	W	M	W	—	—	—	M
R209	*ts*30	*ts*5	—	M	M	W	M	W	—	—	—	M
R205	*ts*30	*ts*5	—	M	W	W	M	W	—	—	—	W
R201	*ts*30	*ts*5	—	M	M	W	M	W	—	—	—	W
R210	*ts*30	*ts*5	—	M	M	W	M	M	—	—	—	W

[a] Group II.
[b] Group I.
[c] M, Mudjinbarry; W, Wallal.
[d] —, Not determined.
[e] Derivation of segments described by Gorman et al. (13).
[f] Fingerprint analysis revealed segments from each parent.
[g] Clone derived from R202 virus.

Only in segment 7 could any appreciable similarity among the large oligonucleotides be discerned. The observation of such variation between segments with identical migration raises an important question. How much more divergence in sequence (if any) would be required to cause a shift in segment electrophoretic mobility? Desselberger and Palese (2) have shown that heterogeneity in migration of influenza virus single-stranded RNA, for the most part, is due not to variations in segment molecular weight but to secondary structure effects. Since the order of migration of reovirus segments has been shown to vary in different buffer systems (33), it is not unlikely that secondary structure influences the migration of double-stranded RNA. Over a range of acrylamide concentrations, segments 2, 5, and 8 of Wallal and Mudjinbarry viruses display a similar variation in migration (P. J. Walker, J. Taylor, and B. M. Gorman, unpublished data). Until the basis of segment migration is more clearly understood, it cannot be assumed that orbivirus segments with different mobility are any less related than segments with equivalent mobility.

Shipham and De La Rey (34) divided temperature-sensitive mutants of bluetongue virus into six groups on the basis of "all or none" recombination frequencies. Gorman et al. (13) reported high-frequency recombination between temperature-sensitive mutants of Wallal and Mudjinbarry viruses and defined two recombination groups. Using comparative electrophoresis, they demonstrated physical reassortment in orbivirus genomes but were unable to establish a consistent relationship between segment reassortment and loss of temperature sensitivity. In this paper, we have shown that each segment of recombinant viruses can be identified by the oligonucleotide fingerprinting technique as being derived from one or other of the parental mutants. No evidence of intragenic recombination was detected. By using segment fingerprints and genome electrophoretic patterns to map segment origin, we have demonstrated that reassortment of particular segments could be correlated with acquisition of the wild-type phenotype. Segment 1 of group II mutants and segment 2 of group I mutants were the only segments consistently absent from the recombinant progeny of mixed infections. The group-specific temperature-sensitive lesions appear to be located in these segments. Similar methods have been used to identify defective segments in temperature-sensitive mutants of reoviruses (29), influenza viruses (30), and bunyaviruses (8). The polypeptide products of segments 1 and 2 of Wallal serogroup viruses have not yet been identified. For orbiviruses of the bluetongue serogroup, segment 2 is thought to code for a polypeptide located on the outer surface of the virion (36). This protein provides the major determinant of intertypic serological specificity (3, 16, 17). Segment 1 is thought to code for a minor component of the bluetongue virus nucleocapsid (36).

The role of genome segment reassortment in the natural evolution of orbiviruses has yet to be established. The frequency with which such an event could occur would depend upon the opportunity for simultaneous infection with suf-

ficiently related viruses. Gorman (9) has reviewed evidence suggesting a high rate of concurrent circulation of related orbivirus serotypes in insect and vertebrate populations. The frequent movement of insects from host to host should provide ready opportunity for multiple infection and subsequent generation of recombinant orbiviruses.

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