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GENETIC RELATEDNESS OF THE KEMEROVO SEROGROUP VIRUSES: III. RNA-RNA BLOT HYBRIDIZATION AND GENE REASSORTMENT *IN VITRO* OF THE CHENUDA SEROCOMPLEX

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Summary. — The dsRNA polyacrylamide gel profiles of five Chenuda serocomplex viruses were distinct. Blot hybridization and gene reassortment *in vitro* studies demonstrated that the Chenuda serocomplex may be divided into three sets: Chenuda, Huacho, and Mono Lake. Genes were highly conserved among members of each set, whereas genes were not highly conserved between members of different sets. Gene reassortment was demonstrated in intra-set crosses, but inter-set crosses did not yield reassortant progeny. The taxonomic significance of these data to the Chenuda serocomplex is discussed.

Key words: blot hybridization; dsRNA; Chenuda serocomplex, genetic relatedness; Orbivirus; reassortment

Introduction

Complement-fixation (CF) test results led to the division of the Kemerovo serogroup into four antigenic complexes. The four serocomplexes are Chenuda, Cape Wrath-Great Island, Kemerovo, and Seletar-Wad Medani (Casals, 1971; Main *et al.*, 1976); and isolates within each serocomplex are similar biologically. Chenuda serocomplex viruses are associated with birds and softbodied argasid ticks, and four serotypes are recognized. They are Baku, Chenuda, Huacho, and Mono Lake (Taylor *et al.*, 1966; Yunker *et al.*, 1972; Gromashevsky *et al.*, 1973; Karabatsos, 1985). Sixgun City is a close serologic relative to Mono Lake because it is indistinguishable by intracerebral cross-neutralization tests in suckling mice (Yunker *et al.*, 1972). Two additional serotypes in the Chenuda serocomplex may also exist because Essaouria and Kala Iris which were isolated from *Ornithodoros maritimus* in Morocco were distinct when compared with Mono Lake in plaque-reduction neutralization tests (Jacobs *et al.*, 1987). Viruses from this serocomplex have been isolated from a variety of tick species collected in widely separated geographic areas; such as, North America, South America, Africa, and U.S.S.R.

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RNA-RNA blot hybridization and gene reassortment *in vitro* have been useful in clarifying the taxonomic relationships of members of other *Orbivirus* serogroups (Bodkin and Knudson, 1985*b*, 1986, 1987; Gonzalez and Knudson, 1987*a*, 1987*b*, 1988; Brown *et al.*, 1988*a*). Blot hybridization and gene reassortment *in vitro* have been used to assess the relatedness of members of two of the four Kemerovo antigenic complexes (Brown *et al.*, 1988*b*; 1988*c*). Recently, blot hybridization confirmed the serologic data which suggested that the Kemerovo serocomplex contained two virus types by the demonstration that these virus types were not highly related in the majority of their genes (Brown *et al.*, 1988*b*). Although the two virus types were isolated from different tick hosts collected in two different geographic regions, the two virus types were capable of genetic interaction via gene reassortment of their dsRNA segments *in vitro* (Brown *et al.*, 1988*b*). In contrast, the Cape Wrath-Great Island serocomplex viruses were highly related by both serology and blot hybridization. While Cape Wrath-Great Island serocomplex viruses were isolated from the same tick host collected from widely separated geographic regions, these viruses were close relatives because reassortment was also demonstrated (Brown *et al.*, 1988*c*). The data from these two studies on the intra-serocomplex relatedness of the Kemerovo viruses indicated that the tick host may be important to the evolution of tick-borne orbiviruses.

If the tick host is important in the evolution of orbiviruses, then the relatedness of the Chenuda serocomplex viruses warrants examination because they have been isolated from a variety of tick vectors. In this study, blot hybridization was used to assess the sequence conservation among members of the Chenuda serocomplex and gene reassortment *in vitro* was used to assess the functional relatedness between selected members of the serocomplex. These data are discussed in light of the antigenic and taxonomic relationships of the Chenuda serocomplex viruses.

Table 1. Chenuda serocomplex virus osolates

Virus isolate	Isolation source	Geographical origin	Year[a]
Chenuda Ar 1152	*Argas reflexus hermanni*	Egypt	1954
Baku LEIV-46A	*Ornithodoros capensis*	U.S.S.R.	1970
Huacho Ar 883	*Ornithodoros amblus*	Peru	1967
Mono Lake Cal Ar 861	*Argas cooleyi*	California	1966
Sixgun City USA RML52451	*Argas cooleyi*	Texas	1969

[a] Represents the year which the isolate was collected in the field.

Materials and Methods

Virus stocks and tissue culture. Chenuda serocomplex viruses used in this study are listed in Table 1. Viruses were grown in BHK-21 cells as described elsewhere (Gonzalez and Knudson, 1987*a*).

Plaque assay and in vitro reassortment. Plaque assay was used to titrate virus stocks and reassortant viruses (Buckley, 1974; Gonzalez and Knudson, 1987*b*). Procedures for the reassortment studies have been described previously (Gonzalez and Knudson, 1987*b*; Brown *et al.*, 1988*c*). Briefly, BHK-21 cells grown in microtest plates (96 well) were infected with viral suspensions which contained the parental virus stocks in varying multiplicity of infection (M.O.I.) ratios. The infected cells were incubated at 32° for 24 hr, harvested, and frozen at −70 °C. The M.O.I. on the day of the reassortment experiment was determined by a plaque assay titration. Progeny from the reassortment experiments were plaque-purified. Viral dsRNA was extracted from cells infected with reassortant progeny. The dsRNA was electrophoresed in a polyacrylamide gel which was stained in ethidium bromide. The dsRNA polyacrylamide gel profiles were scored for the parental origin of the segments. Viral dsRNA of selected reassortants was 3′ end-labeled and electrophoresed in a polyacrylamide gel.

Polyacrylamide gel electrophoresis (PAGE). Chenuda dsRNA was isolated from infected cells which were grown in 24-well cluster plates (Travassos da Rosa *et al.*, 1984). DsRNA from the additional isolates was purified from infected cell supernatants. Virus was pelleted from supernatant fluids (Beckman SW27.1 rotor, 25 000 rev/min, 1 hr), and the dsRNA was extracted from the virus-containing pellet (Gaillard and Joklik, 1982). Polyacrylamide gels used for electrophoretic transfer were electrophoresed for 20 hr at 20 mAmps. The labeled dsRNA of reassortants was electrophoresed for 20 hr at 10 mAmps in polyacrylamide gels (Gonzalez and Knudson, 1987*b*).

RNA probes and blot hybridization. The dsRNA was purified and 3′ end-labeled with 1.48 MBq of [5′-^{32}P] pCp (Donis-Keller, 1979; Knudson, 1981; Bodkin and Knudson, 1985*b*, 1986; Brown *et al.*, 1988*a*). An aliquot of the probe was electrophoresed in a polyacrylamide gel, and the probe was prepared for hybridization (Bodkin and Knudson, 1986). Procedures for the electrophoretic transfer of dsRNA and for hybridization at T_m (RNA-36) have been described previously (Bodkin and Knudson, 1985*a*, 1985*b*; Gonzalez and Knudson, 1987*a*).

Results

Gel electrophoresis and RNA-RNA blot hybridization

The dsRNA profiles of the Chenuda serocomplex viruses examined were distinct with an overall 2-4-3-1 pattern (Fig. 1). The Chenuda probe hybridized strongly to the Baku isolate, and it hybridized to a lesser extent to Huacho, Mono Lake and Sixgun City (Fig. 2). The Huacho probe hybridized strongly to itself and weakly to the rest of the isolates (Fig. 3). The Mono Lake and Sixgun City probes hybridized strongly to each other and weakly to Chenuda, Baku, and Huacho (Figs. 4 and 5). In reciprocal hybridizations, the signal intensities between Huacho and Mono Lake or Sixgun City were greater than the signal intensity between Huacho and Chenuda, between Mono Lake and Chenuda, or between Sixgun City and Chenuda.

PAGE of the 3′ pCp end-labeled radioactive probes revealed that Chenuda genes 7 and 8 and gene 8 of Mono Lake and Sixgun City did not label uniformly when compared to the other segments (data not shown). A similar phenomenon has been observed with the Cape Wrath-Great Island serocomplex viruses (Brown *et al.*, 1988*c*), and Chenuda (Fig. 2, Lane 2) represents a good example because genes 7 and 8 exhibit a lighter signal.

Light signals not due to inefficient end-labeling in the hybridization experiments identify variant genes, and sequences between variant genes approach

Table 2. Chenuda serocomplex genetic relatedness[a]

Virus	Hybridization Probes				
	Chenuda	Baku	Huacho	Sixgun City	Mono Lake
Chenuda	4+	—	2+	2+	2+
Baku	4+	—	2+	2+	1+
Huacho	2+	—	4+	2+	2+
Sixgun City	2+	—	2+	4+	4+
Mono Lake	2+	—	2+	4+	4+

[a] A summary of the reciprocal hybridization data is presented. A 1+ represents weak cross-hybridization in at least one gene, 2+ represents weak cross-hybridization in the majority of the genes, 4+ represents strong cross-hybridization in all genes, and — represents not done.

the lower limit of 74 % similarity at T_m (RNA)-36 hybridization conditions. While all the serotypes cross-hybridized in 10 genes, the hybridization signals between selected pairs of isolates indicated that their sequence relatedness approached the lower limit of 74 % similarity. The intensity of the hybridization signals divided the isolates into three sets within which intra-set variant and unique genes were not identified. One set contained Chenuda and Baku; another, Huacho; and the third, Mono Lake and Sixgun City. The intensity of the hybridization signals indicated that Huacho might be more closely related to the Mono Lake set than to the Chenuda set.

Gene reassortment

Gene reassortment was demonstrated in the crosses between Chenuda and Baku and between Mono Lake and Sixgun City (Table 2). Only three reassortants were detected in the cross between Chenuda and Baku, whereas 12 reassortants were detected from the cross between Mono Lake and Sixgun City. Selected reassortants from the cross between Mono Lake and Sixgun City are represented in Fig. 6. Gene reassortment was not demonstrated in the crosses between Chenuda and Huacho, Chenuda and Mono Lake, and Huacho and Mono Lake (Table 3). Since the negative crosses yielded equivalent numbers of both parentals in the progeny, these crosses represent *bone fide* tests of genetic relatedness. The intra-set gene reassortment *in vitro* results were consistent with the hybridization results, and inter-set reassortment was not detected.

Discussion

These hybridization and reassortment data correlate with serologic data and clarify the taxonomic relationships of viruses within the Chenuda serocomplex viruses. Huacho, Mono Lake, and Sixgun City were included in the Chenuda serocomplex based upon CF cross-reactivity between Mono Lake and Chenuda, between Mono Lake and Huacho, and between Mono Lake

Table 3. Chenuda serocomplex in vitro reassortment

Minimum number of segments distinguishable[a]	MOI ratio (PFU/cell) (P1 : P2)	Observed progeny (P1 : P2 : R)	Reassortant genotype (Segment number)[b] 1	2	3	4	5	6	7	8	9	10	N[c]
Chenuda (C) X Baku (B) Cross:													
4	60 : 60	16 : 1 : 1	B	C	C	C	C	X	C	X	B	C	
	30 : 30	6 : 10 : 0	B	B	C	B	X	B	C	X	B	B	
			C	X	X	C	X	X	C	X	B	X	
Chenuda (C) X Huacho (H) Cross:													
7	60 : 6	40 : 29 : 0											
	30 : 6	6 : 12 : 0											
	8 : 6	1 : 16 : 0											
Chenuda (C) X Mono Lake (M) Cross:													
7	60 : 75	18 : 0 : 0											
	30 : 75	18 : 53 : 0											
	15 : 75	2 : 16 : 0											
	8 : 6	7 : 12 : 0											
Huacho (H) X Mono Lake (M) Cross:													
7	5 : 6	9 : 8 : 0											
Mono Lake (M) X Sixgun City (S) Cross:													
9	6 : 7	0 : 5 : 12	S	S	S	S	X	S	M	M	S	M[d]	2
			M	S	S	S	X	M	S	S	S	S[d]	
			M	M	M	S	X	M	M	M	M	M[d]	
			M	S	M	S	X	S	S	S	S	M[d]	
			S	S	S	M	X	S	M	M	S	M[d]	
			M	S	M	S	X	M	M	S	S	M[d]	
			M	S	S	M	X	S	S	M	S	M[d]	
			M	S	M	S	X	S	M	S	S	S	
			M	M	M	M	X	M	M	M	S	M	
			S	S	S	S	X	S	S	S	M	M	
			M	S	S	S	X	S	S	S	M	M	

[a] Reflects the number of genes for which the parental origin could be determined in progeny virus. This represents a minimum number because resolution was often improved using end-labeled dsRNA.

[b] The parental origin of each segment is indicated by a single letter abbreviation, and segments of undetermined origin are indicated by the letter X.

[c] N is the number of each genotype observed greater than one.

[d] Polyacrylamide gel profiles of reassortants indicated are shown in Figure 3.

and Sixgun City. Chenuda, however, does not cross-react or only low level cross-reactions are observed with Huacho or Sixgun City. Chenuda is related to Baku, and Mono Lake is closely related to Sixgun City. Cross CF tests and neutralization tests indicated that Mono Lake and Sixgun City may be indistingsuihable (Yunker *et al.*, 1972). Thus, members of a hybridization set are also closely related in CF tests, and they reassort their genes. In contrast,

members of different sets are either distant serologic relatives, or not related in CF tests; and they do not reassort their genes.

Mono Lake and Sixgun City viruses were highly related, and these viruses were isolated from *Argas cooleyi* ticks collected in North America. Twelve additional isolations of Sixgun City virus have been made from pools of *Argas cooleyi* ticks collected in Texas and Colorado during 1969 and 1970 (Yunker *et al.*, 1972). Huacho was related distantly to members of the other two sets, and it was the only virus examined isolated from *Ornithodoros amblus* ticks which were collected in South America.

Chenuda and Baku were related, but they were isolated from two different geographic areas, Egypt and the U.S.S.R. Chenuda was isolated from ticks collected in a pigeon house, and Baku was isolated from ticks collected in the nesting ground of *Larus argentatus* (Taylor *et al.*, 1966; Gromashevsky *et al.*, 1973). Two closely related Kemerovo serocomplex viruses have also been isolated from ticks collected from the U.S.S.R. and Egypt, and the data available implicated migratory birds in the dispersal of the virus between the U.S.S.R. and Egypt (Schmidt and Shope, 1971; Brown *et al.*, 1988*b*).

Chenuda and Baku belong to the same set, and yet, these viruses were isolated from two different tick hosts, *Argas reflexus hermanni* and *Ornithodoros capensis*. Similarly, the Kemerovo serocomplex contains two viruses which belong to the same genetic set, but they were isolated from different tick hosts. These two Kemerovo viruses also represent two virus populations which may have evolved from a common ancestor (Brown *et al.*, 1988*b*). Only two of the Chenuda serocomplex viruses examined belonged to the Chenuda set, Chenuda Ar 1152 and Baku; and CF and neutralization test differences have been detected (Gromashevsky *et al.*, 1973). Reciprocal hybridizations were not done between Chenuda and Baku because the latter virus, Baku, grew poorly in cell culture. The small number of reassortants between Chenuda and Baku may be due to the fact that only 4 genes were distinguishable, or it may reflect true differences in their functional relatedness. Additional Chenuda and Baku strains need to be examined for systematic and reproducible differences by blot hybridization and gene reassortment to determine the extent of divergence between Chenuda and Baku viruses.

Further, a better understanding of the ecology of the Chenuda and Baku viruses is required before any correlation can be made between the tick host and likely genetic relatedness of isolates. For example, serological studies have shown that a Chenuda isolate Ar 3441 isolated from *Argas peringueyi* collected in South Africa was identical to Chenuda isolate Ar 1152. However, Ar 3441 was not closely related to an additional Chenuda isolate Ar 1170 which was isolated from *Argas r. hermanni* ticks collected from a pigeon house in Chenuda (Taylor *et al.*, 1966).

An *Orbivirus* serogroup has been equated recently with the gene pool (Brown *et al.*, 1988*a*; Gonzalez and Knudson, 1987*b*, 1988). The serogroup has been defined as a collection of isolates which exhibit high levels of cross-reactivity in CF tests, which have high sequence conservation in the majority of their genes, and which reassort cognate genes *in vitro*. Considering these criteria, the three sets of viruses or gene pools identified within the Chenuda

serocomplex would belong to three separate serogroups. The recognition of Chenuda sets as serogroups would also better reflect the biology of the virus-host system. Since Chenuda, Mono Lake, and Huacho are distant serologic relatives to members of the other three Kemerovo serocomplexes (Borden *et al.*, 1971), the taxonomy of the Chenuda serocomplex within the Kemerovo serogroup should be reconsidered.

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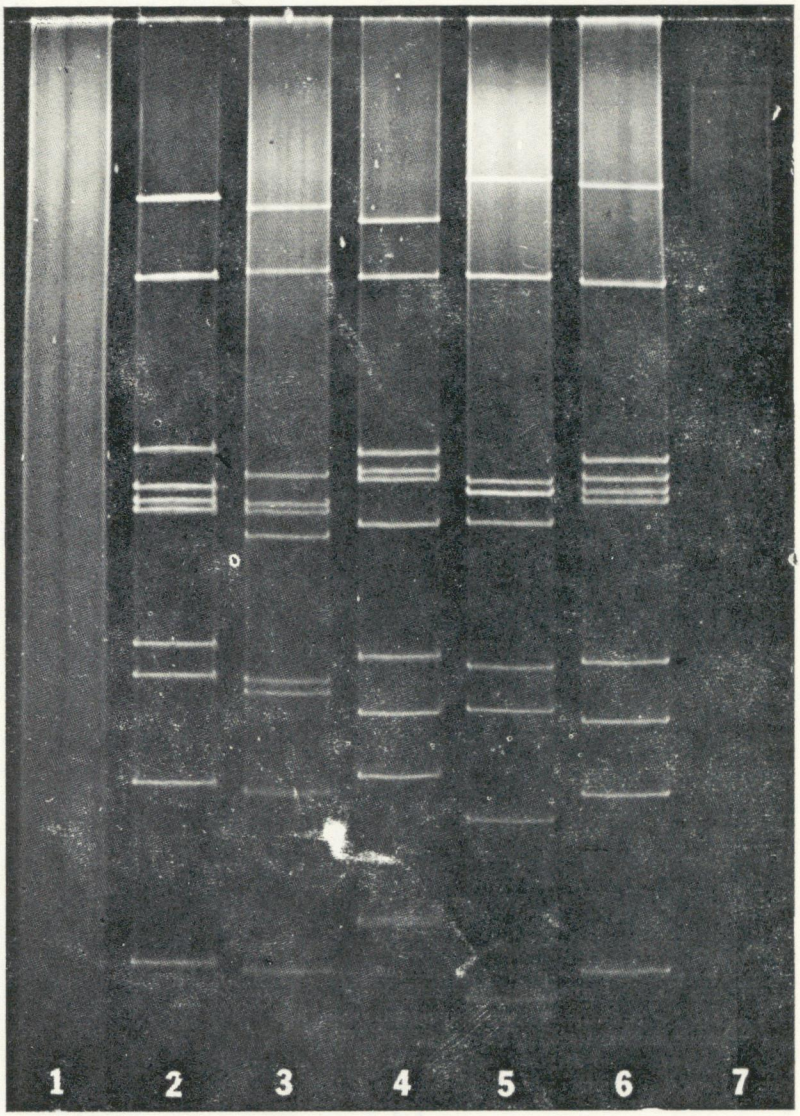

Fig. 1

The dsRNA profiles of the Chenuda serocomplex isolates in a 10 % polyacrylamide gel stained with ethidium bromide. Lanes are from left to right uninfected BHK-21 control (Lane 1), Chenuda (Lane 2), Baku (Lane 3), Huacho (Lane 4), Sixgun City (Lane 5), Mono Lake (Lane 6), and unifected BHK-21 control (Lane 7).

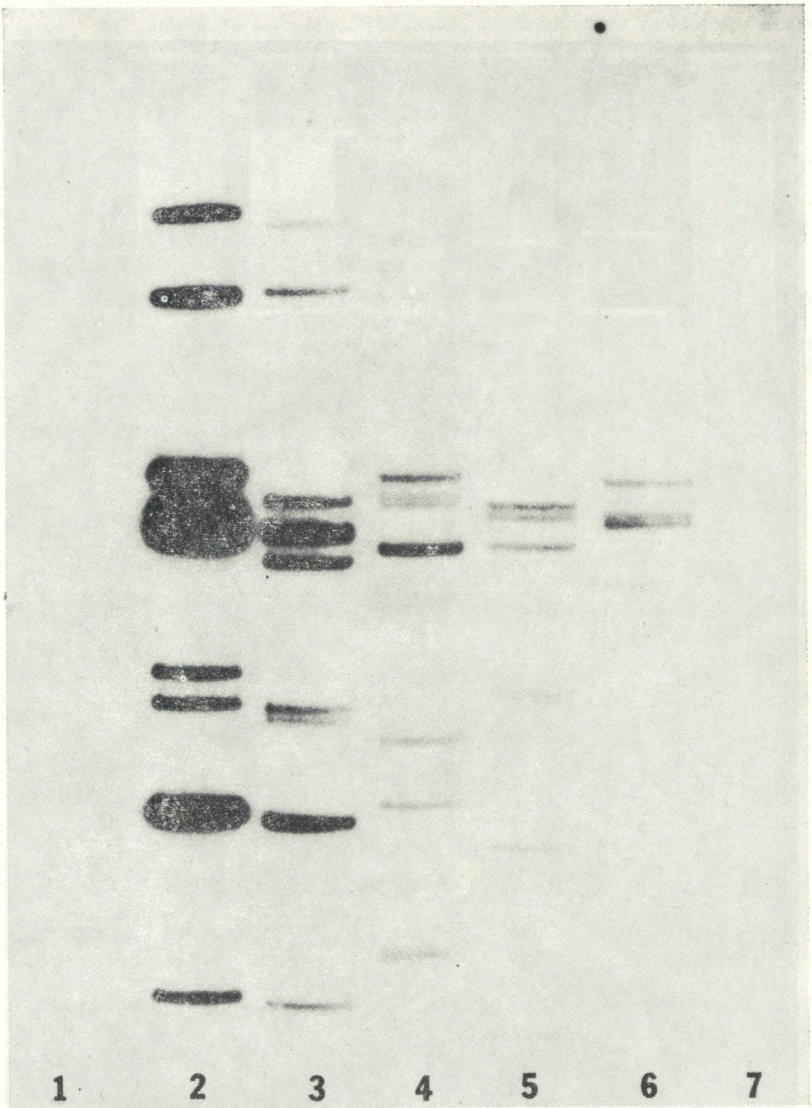

Fig. 2

Figs. 2—5. Autoradiogram depicting the hybridization of the 3′ end-labeled genomic dsRNA probes Chenuda (Fig. 2), Huacho (Fig. 3), Sixgun City (Fig. 4), and Mono Lake (Fig. 5) to a Zeta-Probe membrane containing the profiles of the Chenuda serocomplex viruses. Lanes are from left to right uninfected BHK-21 control (Lane 1), Chenuda (Lane 2), Baku (Lane 3), Huacho (Lane 4), Sixgun City (Lane 5), Mono Lake (Lane 6), and uninfected BHK-21 control (Lane 7).

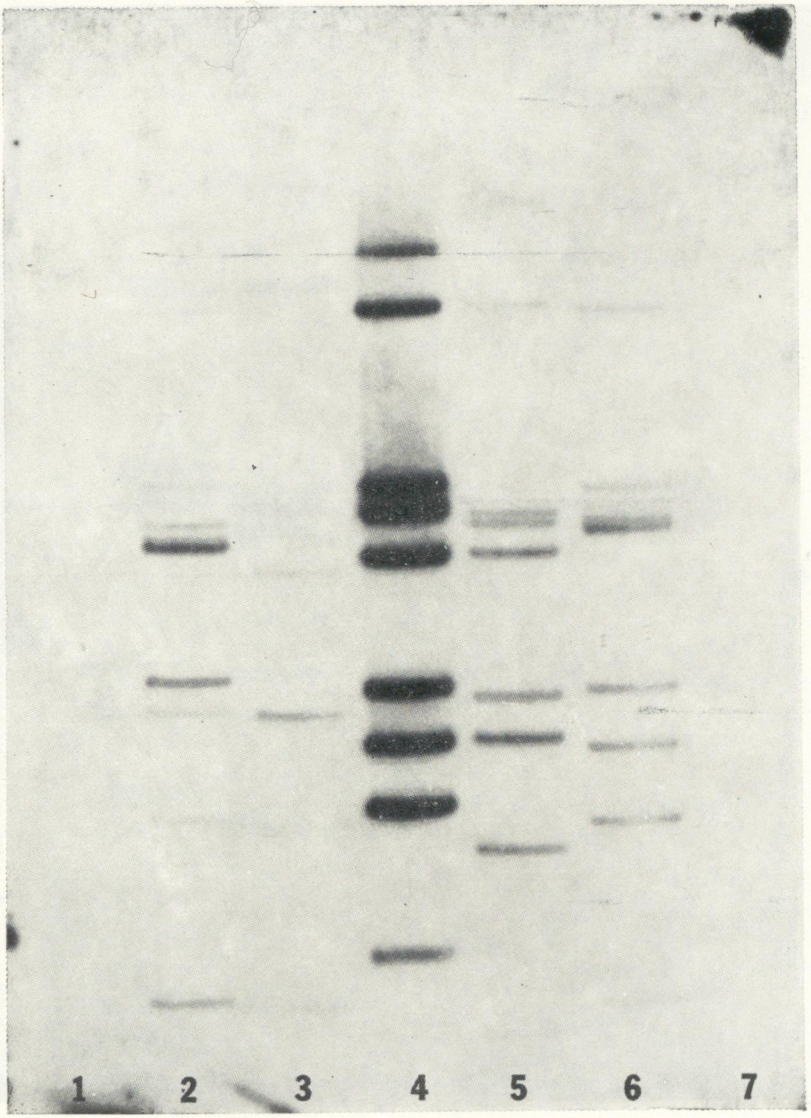

Fig. 3

For legend see page 230.

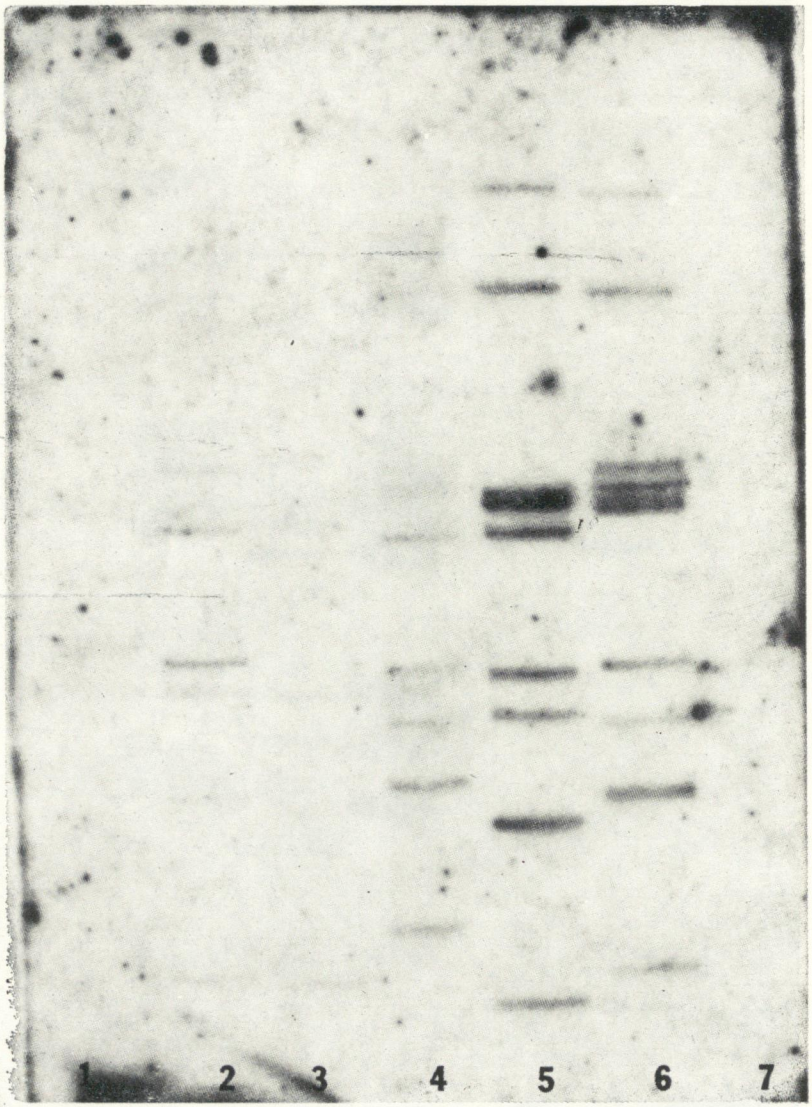

Fig. 4

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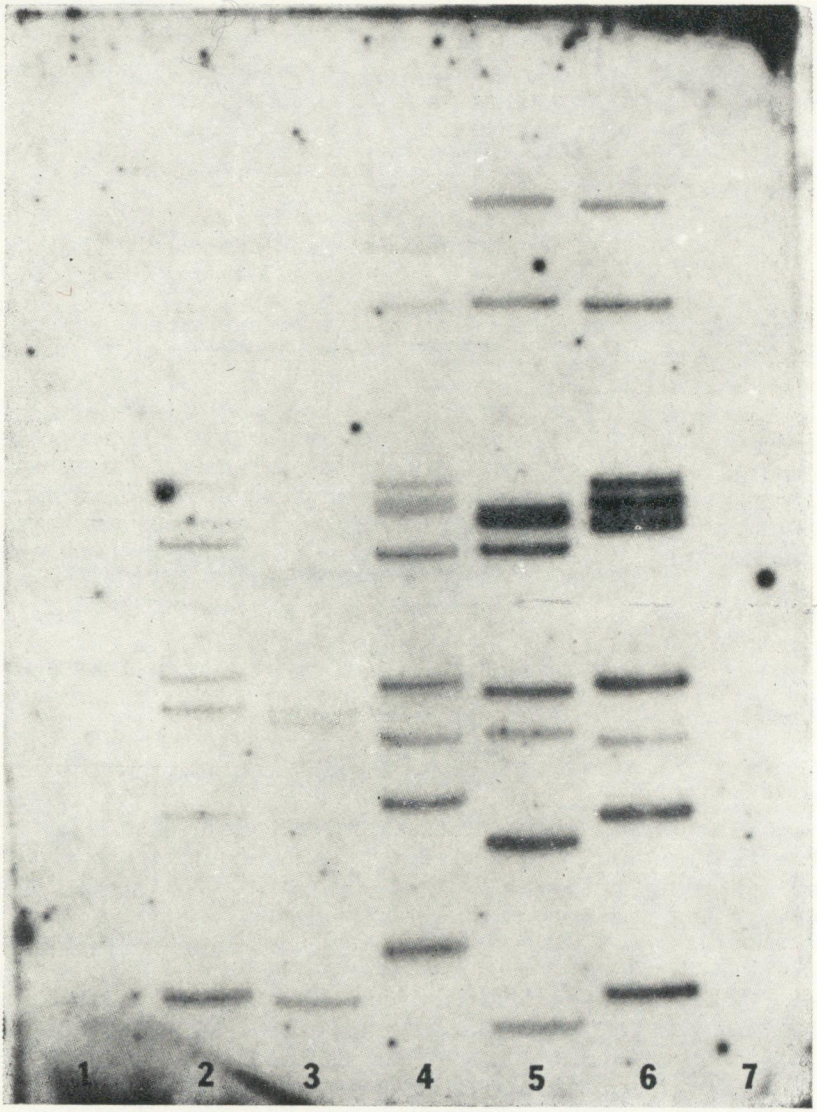

Fig. 5

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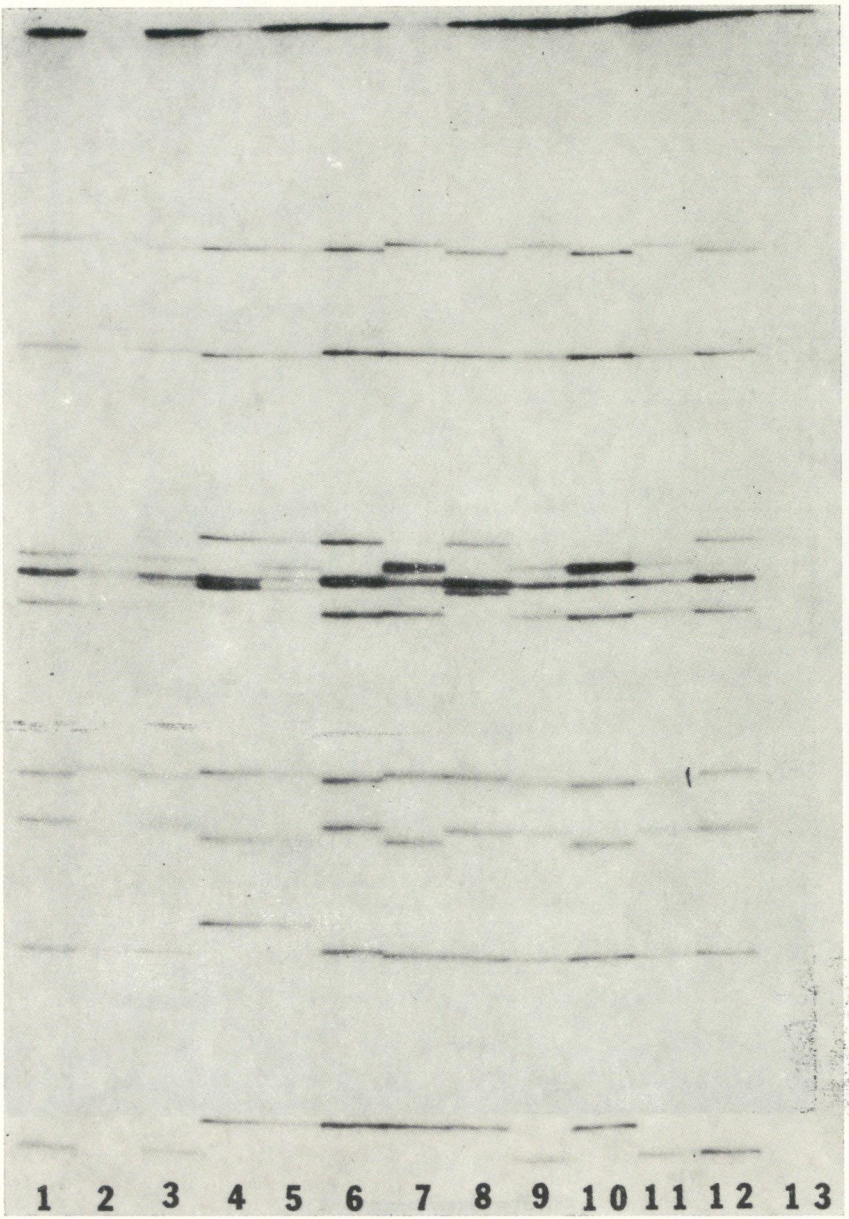

Fig. 6

Autoradiogram depicting the resolution of the dsRNA genomes of Mono Lake, Sixgun City, and selected reassortant progeny. Labeled dsRNA was electrophoresed through a 10 % polyacrylamide gel. The genotypes of the reassortants are listed in Table 3. The lanes are from left to right Sixgun City (Lane 1), reassortants (Lane 2—4), Mono Lake (Lane 5), reassortants (Lane 6—8), Sixgun City (Lane 9), reassortant (Lane 10), Sixgun City (Lane 11), reassortant (Lane 12), and Mono Lake (Lane 13).