Genetic Relatedness of Palyam Serogroup Viruses by RNA–RNA Blot Hybridization

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SUMMARY

Cognate genes of members of the Palyam serogroup of orbiviruses have been identified previously, and their relatedness to the prototype virus was determined by blot hybridization of the genome segments of members of the serogroup using Palyam genomic RNA and isolated Palyam RNA segments as probes. In this study, the genetic relatedness of nine Palyam serogroup isolates was determined by reciprocal blot hybridizations of genomic RNA from each virus to the segments of all members of the group. The number and identity of highly related genes varied between isolates. For example, CSIRO Village and Palyam were related in genes 2 and 6, while Bunyip Creek and Vellore were related in genes 2 and 6. However, CSIRO Village and Bunyip Creek were highly related to D’Aguilar in all genes except 2 and 6, suggesting that there may have been genetic reassortment of Palyam serogroup dsRNA segments. Genes 2 and 6 were correlated consistently with serotype specificity. Genes 5, 7 and 9 were highly related among all members of the group. The Indian strains, Palyam and Vellore, were highly related in genes 1, 3 and 8, and they exhibited weak homology to genes 1, 3 and 8 of the Australian and African strains. However, one Indian isolate, Kasba, was more closely related to strains from Africa and Australia than it was to other Indian strains. There was little evidence which indicated that geography was predictive of the genetic relationships of the strains. Thus, immunological pressure may be the most important factor affecting the Palyam serogroup gene pool.

INTRODUCTION

Viruses of the Palyam serogroup of orbiviruses have been isolated in Africa, Australia and Asia over the past 20 to 25 years (Knudson et al., 1984). They have been associated with a variety of arthropod vectors, such as mosquitoes, ticks and Culicoides midges, and with large mammals, principally cattle. Like other members of the genus Orbivirus within the family Reoviridae, their genomes consist of ten segments of double-stranded RNA. The dsRNA polyacrylamide gel profiles and serological relationships of nine Palyam serogroup strains have been described (Knudson et al., 1984). Members of the serogroup cross-react in complement fixation tests, and serotypes within the serogroup have been defined by neutralization tests (Knudson et al., 1984).

RNA–RNA blot hybridization demonstrated that nine out of ten Palyam virus genes share > 74% sequence homology with their cognates in the other members of the group (Bodkin & Knudson, 1985a). Gene 2 was correlated with serotype specificity. Differences in the intensities of hybridization signals indicated that there was significant divergence between Palyam and the other strains in genes 1 to 4, 6, 8 and 10. The question arose as to whether Palyam virus segments had diverged while the other isolates remained highly related in these seven genes, or whether these genes exhibited sequence variability throughout the entire serogroup.
In order to identify divergent and highly related genes between the remaining strains, genomic RNA from each isolate was hybridized to the segments of all members of the Palyam serogroup. Conditions of RNA–RNA blot hybridization were identical to those used previously (Bodkin & Knudson, 1985a). These data indicate which genes exhibit the highest degree of sequence variability in this serogroup. In addition, they illustrate the relative importance of immunological pressure, geographical boundaries and host in determining the phylogenetic relationships of the Palyam serogroup viruses.

METHODS

Virus and tissue culture stocks. The Palyam serogroup viruses used in this study have been described elsewhere (Knudson et al., 1984); their abbreviations are given in the legend to Fig. 1. Tissue culture stocks were prepared directly from field isolates as described previously (Knudson et al., 1984).

Preparation of RNA probes. Probes were prepared as described previously (Bodkin & Knudson, 1985a, b). Briefly, dsRNA extracted from CsCl-purified virus (Smith et al., 1969) or from infected cells (Knudson et al., 1984) was 3’-end-labelled with 40 μCi [5’-32P]pCp to a specific activity of 5 × 10^5 to 10^6 c.p.m./μg. Segments were electrophoresed in 0.83% (w/v) low-melting-point agarose (Sea Plaque, Marine Colloids, Rockland, Me., U.S.A.) to separate genomic dsRNA from low molecular weight RNA which hybridized with cellular material. Genomic dsRNA was excised from the gel and extracted with phenol. Probes were denatured by heating to 95°C for 5 min prior to hybridization.

Polyacrylamide gel electrophoresis. One-half to 1.0 μg of viral dsRNA extracted from 5 × 10^5 infected BHK cells (Knudson et al., 1984) was electrophoresed through Tris–glycine-buffered polyacrylamide gels (Laemmli, 1970) for 20 h at 20 mA. Polyacrylamide gels were stained with ethidium bromide (0.5 μg/ml) and examined under u.v. light to ensure that all lanes contained roughly equal amounts of dsRNA. Viral RNAs to be used as markers were labelled using 4 μCi pCp and electrophoresed in the lane adjacent to the respective unlabelled lane.

RNA–RNA hybridization. The transfer of dsRNA genome segments from polyacrylamide to Zeta-Probe membrane (Bio-Rad) has been described previously (Bodkin & Knudson, 1985a, b). Blots were prehybridized for 3 to 4 h at 42°C in 50% (v/v) formamide, 5 × SSC, 50 mM-sodium phosphate pH 6.5, sonicated salmon testes DNA (250 μg/ml), 0.1% (w/v) SDS and 0.1% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone. Hybridization took place overnight at 52°C with the probe in fresh prehybridization buffer. Assuming a (G + C) content of 44% similar to reovirus (Bellamy et al., 1967), the calculated effective temperature (Howley et al., 1979) of RNA–RNA hybridization under these conditions was T_m = 36 (Bodkin & Knudson, 1985a). Blots were washed four times for 10 min at ambient temperature in 2 × SSC, 0.1% (w/v) SDS, and once for 1.5 h in 0.1 × SSC, 0.1% SDS, at 50°C.

Probes were removed from membrane-bound RNA (Bodkin & Knudson, 1985b) by washing the membranes twice for 20 min at 95°C in 0.1 × SSC, 0.1% SDS. The samples were re-hybridized beginning with prehybridization.

RESULTS AND DISCUSSION

Genetic relatedness of the Indian strains

In the discussion of all hybridization data, segments are consistently referred to according to their cognates in Palyam virus (Bodkin & Knudson, 1985a). Since the sixth segment from the top of the D’Aguilar, Bunyip Creek, Kasba and Petevo dsRNA polyacrylamide gel profiles cross-hybridized to gene 5 of Palyam (Bodkin & Knudson, 1985a), it will be referred to as gene 5. Likewise, the fifth segment from the top of the gel in these isolates will be referred to as gene 6.

Based on the intensity of the hybridization signals, it was revealed that of the three Indian strains, Palyam and Vellore were more closely related than were Palyam and Kasba (Bodkin & Knudson, 1985a). In order to confirm that the intensities of the hybridization signals were consistent in reciprocal reactions, Vellore and Kasba genomic RNAs were used as probes (Fig. 1a, b). Palyam genes 1, 3, 5 and 7 to 9 exhibited strong signals when hybridized to radiolabelled Vellore genomic RNA (Fig. 1a, lane PAL), while genes 4, 5, 7, 9 and 10 of Palyam exhibited strong signals when hybridized to Kasba genomic RNA (Fig. 1b, lane PAL). These results were consistent with the previous experiments (Bodkin & Knudson, 1985a). Of the 30 hybrid molecules which can be compared reciprocally in the Palyam, Vellore and Kasba blots (Bodkin & Knudson, 1985a; Fig. 1a, lanes PAL and KAS; Fig. 1b, lanes PAL and VEL), only genes 1 and 3 of Kasba and Vellore were not consistent in reciprocal reactions. Kasba genes 1 and 3 exhibited dark signals when Vellore genomic RNA was the probe (Fig. 1a, lane KAS), but
Fig. 1. Autoradiograms depicting hybridization of (a) Vellore and (b) Kasba genes to their counterparts in the Palyam serogroup viruses. Total genomic dsRNA was end-labelled with [5'-32P]pCp and hybridized to the genome profiles of nine members of the serogroup. Lanes are uninfected cellular control (U), Palyam (PAL), Kasba (KAS), Petevo (PET), Marrakai (MAR), Abadina (ABA), CSIRO Village (CV), Bunyip Creek (BC), D’Aguilar (DAG) and Vellore (VEL). The hybridization in (a) was performed by removing the Kasba probe from the membrane (Bodkin & Knudson, 1985b) used in (b) and re-hybridizing the samples to Vellore genomic RNA. The position of segment 2 (which does not cross-hybridize in some isolates) may be determined by examining the control labelled lanes for each virus in Fig. 2.
Vellore genes 1 and 3 exhibited light signals when Kasba genomic RNA was the probe (Fig. 1b, lane VEL).

Abadina was the only strain whose genes 2 and 6 were closely related to their Kasba cognates (Fig. 1b, lane ABA). Marrakai gene 6 exhibited strong homology with its Kasba cognate (Fig. 1b, lane MAR). Since Kasba, Marrakai and Abadina were included in the same antigenic complex (Knudson et al., 1984), these data were consistent with genes 2 and 6 being correlated with the immunological specificity of the viruses (Bodkin & Knudson, 1985a). Genes 2 and 6 of Bunyip Creek exhibited homology with their Vellore cognates (Fig. 1a, lane BC) as would be expected from the cross-reactivity of these viruses in neutralization tests (Knudson et al., 1984; Bodkin & Knudson, 1985a).

It has been demonstrated that viruses isolated within the same geographical area may be closely related genetically. For example, Gorman et al. (1981) found that there was little sequence homology between Australian and South African bluetongue virus strains, whereas South African strains have been shown to exhibit considerably higher levels of sequence homology (Huismans & Howell, 1973). In the present study, geographical boundaries appeared to be of little importance in determining the genetic relatedness of the isolates. For example, Kasba was isolated in Vellore, India in 1957, while Abadina was isolated in Africa in 1967, and Marrakai was isolated in Australia in 1975 (Knudson et al., 1984). Yet, Kasba exhibited a higher degree of relatedness to these two isolates than it exhibited with Palyam, which was isolated in Vellore, India in 1956 (Knudson et al., 1984). In certain instances, strains isolated in distant geographical locations may be more closely related than some of those isolated within close proximity of one another.

Genetic relatedness of the Australian strains

Previous biochemical and biological investigations have indicated that Bunyip Creek, D'Aguilar and CSIRO Village might represent a gene pool of closely related strains. For example, several combinations of two of these three have been isolated from individual cattle (Cybinski & St. George, 1982). Knudson et al. (1984) observed that genes 1 and 3 to 10 of these strains exhibited identical electrophoretic mobilities in polyacrylamide gels.

D'Aguilar genomic RNA was hybridized to the genome segments of Palyam serogroup viruses which had been transferred from polyacrylamide gels to membranes (Fig. 2a, b). D'Aguilar gene 2 did not hybridize to the heterologous samples. Gene 6 of D'Aguilar (which is the fifth segment from the top of the gel) did not cross-hybridize to its cognates in seven of the eight isolates, while the cross-hybridization signal of the remaining isolate, Palyam virus, was light when compared to the other Palyam genes. In contrast, Palyam gene 6 exhibited a high degree of relatedness to its cognate in CSIRO Village (Bodkin & Knudson, 1985a). Therefore, D'Aguilar gene 6 appeared to share minimal sequence homology with its cognates in the other members of the serogroup.

CSIRO Village and Bunyip Creek (Fig. 2a, lanes 4 and 6) appeared to be most closely related genetically to D'Aguilar. The consistently strong signals exhibited by the genes of these two isolates contrasted with the relatedness of D'Aguilar to Palyam and Vellore whose genes 1, 3, 4, 6, 8 and 10 exhibited light signals (Fig. 2a, lanes 2 and 10). Among the remaining isolates genes 1, 3 and 8 exhibited light signals, with the exception of Kasba genes 1 and 3 (Fig. 2b, lane 4).

The samples were hybridized to pCp-labelled genomic RNA from Bunyip Creek (Fig. 3a). Although the exposure was light when compared to the other experiments, differences in the intensities of hybridization signals were discernible. Genes 1, 3 to 5 and 7 to 10 of Abadina, CSIRO Village and D'Aguilar exhibited strong signals (Fig. 3a, lanes ABA, CV, BC; gene 5 in D'Aguilar is the sixth segment from the top of the gel). Kasba genes 1, 3, 7, 9 and 10 exhibited strong signals (Fig. 3a, lane KAS). The genes of Palyam, Vellore, Petevon and Marrakai appeared to be more distantly related to their end-labelled cognates in Bunyip Creek than did those of the other strains. In the hybridization experiment using pCp-labelled genomic RNA from CSIRO Village as the probe, the relative intensity of the signals again indicated a high degree of relatedness between Bunyip Creek, CSIRO Village and D'Aguilar (data not shown).
Fig. 2. Autoradiogram depicting hybridization of D’Aguilar genomic RNA to the segments of Palyam serogroup viruses. $^{32}$P-labelled dsRNA from each virus was electrophoresed in polyacrylamide next to the respective unlabelled dsRNA. Comparison of labelled (even-numbered) and unlabelled (odd-numbered) lanes allows identification of the genes in the unlabelled lanes that hybridized with the probe. Abbreviations of virus names are as given in the legend to Fig. 1.
Fig. 3. Autoradiograms depicting hybridization of (a) Bunyip Creek and (b) Marrakai genes to their counterparts in the Palyam serogroup viruses. Lanes are designated as described in the legend to Fig. 1. The hybridization in (a) was performed by removing the Marrakai probe from the membrane (Bodkin & Knudson, 1985b) used in (b), and re-hybridizing the samples with Bunyip Creek genomic RNA. The position of segment 2 (which does not cross-hybridize in some isolates) may be determined by examining the control labelled lanes for each virus in Fig. 2.
Relatedness of Palyam serogroup viruses

Table 1. Conservative estimates of genetic relatedness of Palyam serogroup viruses*

<table>
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<th>Cognate segment</th>
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<th>PET</th>
<th>MAR</th>
<th>ABA</th>
<th>CV</th>
<th>BC</th>
<th>DAG</th>
<th>VEL</th>
<th>PAL</th>
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<td>PET</td>
<td>MAR</td>
<td>KAS-ABA</td>
<td>CV-PAL</td>
<td>BC-VEL</td>
<td>DAG</td>
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<tr>
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<tr>
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<td>pet</td>
<td>kas-aba</td>
<td>kas-aba</td>
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<td>dag</td>
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<td>vel-pal</td>
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* Membranes containing the genome segments of nine Palyam serogroup isolates were hybridized to [5'-32P]pCp-labelled genomic RNA from D'Aguilar (DAG) (Fig. 2), Kasba (KAS) (Fig. 1b), Vellore (VEL) (Fig. 1a), Marrakai (MAR) (Fig. 3b), Bunyip Creek (BC) (Fig. 3a), Palyam (PAL) (Bodkin & Knudson, 1985a), Abadina (ABA) (not shown), Petevo (PET) (not shown) and CSIRO Village (CV) (not shown). The genes were scored as described previously (Bodkin & Knudson, 1985a).
† Segments which exhibited strong hybridization signals are designated psg (Palyam serogroup gene). Unique segments, identified by the virus name in capital letters, did not cross-hybridize to the heterologous samples. Variant segments, designated by the virus name in lower case letters, hybridized weakly to their cognates when they were used as probes. Moreover, they exhibited weak signals in all reciprocal reactions when hybridized to radiolabelled genomic RNA from the other viruses.
‡ Gene 1 of Kasba exhibited a strong signal when hybridized to Vellore genomic RNA. Genes 3 of Kasba and Abadina exhibited strong signals when hybridized to Vellore genomic RNA. However, Vellore genes 1 and 3 were classified as variant because of their high degree of relatedness to Palyam genes 1 and 3.
§ If two viruses exhibited sequence homology in segments which were unique or variant with respect to the remaining viruses, the gene in question is identified by both virus names.

It is well recognized that segments with identical electrophoretic mobilities may not have identical sequence composition (Walker et al., 1980). However, the hybridization analysis supported the electrophoretic data which suggested high levels of relatedness among eight genes of these three strains. Given that CSIRO Village and Bunyip Creek exhibit Palyam- and Vellore-'like' genes 2 and 6, the relatedness of CSIRO Village, Bunyip Creek and D'Aguilar genes 1, 3 to 5 and 7 to 10, may be evidence for reassortment of genome segments. Taken together with the surveys demonstrating that the strains co-circulate in nature (Cybinski & St. George, 1982), the hybridization analysis may indicate that these three Australian isolates represent a common gene pool through which functionally equivalent, but genetically distinct segments 2 and 6 circulate.

Hybridization of Marrakai genomic RNA to the genome segments of Palyam serogroup viruses is depicted in Fig. 3(b). Marrakai gene 2 did not cross-hybridize to the heterologous samples. Kasba and Abadina genes 1 and 3 to 10 (Fig. 3b, lanes KAS and ABA) exhibited a high degree of relatedness to their Marrakai cognates. Genes 1, 3 to 5, 7 and 9 of Bunyip Creek and D'Aguilar exhibited strong signals (Fig. 3b, lanes BC and DAG). Genes 4, 5, 7, 9 and 10 of Vellore and Palyam exhibited strong signals (Fig. 3b, lanes VEL and PAL), while genes 5, 7, 9 and 10 of Petevo exhibited strong signals (Fig. 3b, lane PET).

The Australian isolate, Marrakai, does not exhibit the same host and geographical range as the three other isolates. It is the only Australian serotype which has not been isolated from cattle in which one of the other strains had been isolated previously (Cybinski & St. George, 1982). The Marrakai hybridization data supported the conclusion that a gene pool defined by three of the four Australian isolates exists. The mechanism by which Marrakai remains outside this gene pool has not been elucidated.

Genetic composition of Palyam serogroup viruses

Genomic RNA from Petevo and Abadina was hybridized to membranes containing the segments of Palyam serogroup viruses (data not shown). The results of the blot hybridization
experiments (Bodkin & Knudson, 1985a; Fig. 1 to 3 and data not shown) are summarized in Table 1. Table 1 is a more conservative estimate of the extent of sequence variation exhibited by Palyam serogroup viruses than is seen in Fig. 1 to 3, which represent pairwise relationships.

Only segment 2 exhibited more than one unique gene, and segment 6 exhibited the greatest number of variants. These data were consistent with the previous suggestion that gene 2 may encode the neutralization antigen of the Palyam serogroup (Bodkin & Knudson, 1985a). Likewise, gene 6 was also associated more clearly with serotype specificity than had been demonstrated previously.

It would appear that among Palyam serogroup strains isolated in nature, the evolutionary pressure of the vertebrate immune system is exerted on both genes 2 and 6, such that sequence divergence in gene 2 is accompanied by sequence divergence in gene 6. A similar situation may exist with respect to evolutionary pressures acting on genes 1, 3 and 8 as a unit (Table 1). In vitro reassortment studies have not yielded evidence of genetic linkage of dsRNA segments (Mustoe et al., 1978; Ramig et al., 1978; Kahlon et al., 1983; E. F. McCance & D. L. Knudson, unpublished results). Nor do these data provide evidence of physical linkage of dsRNA segments. However, the data presented here imply that a given subset(s) of the viral genes may be involved in related functions such that they respond to evolutionary pressures interdependently.

The evolutionary relationships of orbiviruses are topics of considerable interest as indicated by a number of studies on this subject (Gorman et al., 1978, 1981; Huismans & Bremer, 1981; Rao et al., 1983). The results of this study imply that the vertebrate immune system is the major cause of sequence divergence among members of an orbivirus serogroup. Geographical boundaries are less important in determining sequence divergence between strains. However, a comparison of three Australian isolates suggested that locale may be critical for the maintenance of common gene pools which enable viruses to interact genetically. Moreover, a comparison of the amount of sequence variability exhibited by individual genes indicated that certain subsets may maintain similar levels of sequence variability. Continued investigation of the relatedness of orbiviruses by RNA-RNA blot hybridization may provide further insight into the mechanisms by which these viruses respond to evolutionary pressures.

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