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Polymorphism of the Migration of Double-Stranded RNA Genome Segments of Avian Reoviruses

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A number of field isolates of avian reovirus were characterized by analysis of the migration pattern of their genomic double-stranded RNA (dsRNA) segments upon polyacrylamide gel electrophoresis. Comparison of the various isolates has demonstrated (i) no relationship between serotype and migration of any individual dsRNA segment, (ii) marked polymorphism of migration patterns of all dsRNA segments among isolates of the same serotype as well as among different serotypes, (iii) no correlation between genotype and disease state, (iv) less marked variability in migration pattern from isolates within a restricted geographic locale compared to isolates from distant locales, (v) the presence of a single genotype in local outbreaks of disease, and (vi) the relative invariant migration of several dsRNA segments among the avian reoviruses, one of which (S1) may serve to distinguish the avian from the mammalian reoviruses.

Reoviruses have been isolated from an extremely wide range of animals, including humans, cattle, mice, chickens, ducks, and turkeys (10). Those viruses derived from mammalian species have been classified into three distinct serotypes and have undergone extensive seroepidemiological and biochemical investigations (21, 29). The avian reoviruses have been primarily the subject of detailed pathological investigations (2; C. H. Kircher, Ph.D. thesis, University of Connecticut, Storrs, 1977). These viruses are known to be endemic in many bird populations and have been repeatedly associated with several different naturally occurring diseases (viral arthritis and tenosynovitis, gastroenteritis, and respiratory illnesses) in a variety of avian species (18, 31). A number of different serotypes of the avian reoviruses have been described, and it has been suggested that different disease states may arise as a consequence of infection by specific serotypes, a situation not unlike that reported for experimental murine infection with the mammalian reoviruses (6).

Although the avian reoviruses share many properties with their mammalian counterparts (a double-capsid structure 70 to 80 nm in diameter and a genome consisting of 10 segments of double-stranded RNA [dsRNA]), their lack of a hemagglutinin and their inability to infect mammalian cells productively are distinguishing features (7, 20). Only recently have the avian reoviruses undergone more detailed biochemical investigation and characterization. Studies in this laboratory (V. S. Gouvea and T. J. Schnitzer, J. Gen. Virol., in press) have demonstrated that, as with their mammalian counterparts, polyacrylamide gel electrophoresis (PAGE) of the avian reovirus genomic RNA does reveal significant differences in the migration patterns of the double-stranded segments among different isolates. This finding has permitted the characterization of several individual isolates of avian reoviruses and allowed comparison with standard mammalian serotypes.

The availability of a large number of individual field isolates of avian reoviruses, coupled with the demonstrated ability of this method of genotype analysis to discriminate among isolates, now permits a number of questions to be addressed, as follows. (i) Within any given serotype, how much variability in the migration pattern of the genomic dsRNA segments does occur? (ii) What correspondence exists between migration patterns of the avian reovirus dsRNA segments and serotype? (iii) What correlation exists between any given disease state and the migration pattern of the genomic RNAs? (iv) How much variability exists in the migration pattern of the genomic RNAs of isolates obtained from any single outbreak of disease? (v) How much variability can be seen among isolates obtained from similar, as well as widely separated, geographical locales? (vi) Are there any features of the migration patterns of the avian reovirus dsRNA segments that distinguish the avian reoviruses from their mammalian counterparts? Utilizing a number of mammalian reovirus isolates, Hrdy et al. have recently ad-

dressed several of these issues (8) for the mammalian system. However, the avian reoviruses differ from their mammalian counterparts in two important respects: (i) the avian viruses have been directly associated with clinical disease syndromes, and (ii) there is a reported association of specific serotypes with different naturally occurring disease states. It was therefore of interest to undertake the examination of the available avian reovirus isolates to discern what correlations, if any, could be demonstrated.

MATERIALS AND METHODS

Viruses and cells. Thirty-two strains of avian reovirus were studied. Thirty-one represent field isolates obtained in different locations and from birds suffering from a variety of clinical symptoms. They were kindly supplied by L. van der Heide, University of Connecticut, Storrs; R. C. Jones, University of Liverpool, Wirral, England; and J. K. Rosenberger, University of Delaware, Newark. Table 1 lists the strains and their source, country of origin, and animal of origin and the disease affecting the bird from which the virus was isolated.

TABLE 1. Origin of the avian reovirus isolates

Virus isolate	Disease[a]	Bird	Place	Source[b]	Reference
S1133	TS	Chicken	Connecticut	A	33
Lasswade 126/75	TS	Chicken	Scotland	B	15
Fahey-Crawley	Resp	Chicken	Canada	B	5
WVU 2937	TS	Chicken	West Virginia	B	19
Reo 25	CP	Chicken	Minnesota	A	3
R2	TS	Chicken	England	B	11
R10	Resp	Chicken	England	B	
R11	TS	Chicken	England	B	
R12	Resp	Duck	England	B	
R17	Diarrhea	Chicken	England	B	
R19	TS	Chicken	England	B	
R21	Normal	Duck	England	B	
Uchida	GI,Resp	Chicken	Japan	A	13
TS17	GI,Resp	Chicken	Japan	A	13
CS108	GI,Resp	Chicken	Japan	A	13
EK	Liver	Chicken	West Germany	B	
UCon1	FHN	Chicken	Connecticut	A	
UCon2	TS	Chicken	Connecticut	A	
UCon3	TS	Chicken	Connecticut	A	
UCon4	TS	Chicken	Connecticut	A	
UCon5	TS	Chicken	Connecticut	A	
UCon9	TS	Chicken	Connecticut	A	
UMI-203	TS	Chicken	Maine	A	9
2176-IL	TS	Chicken	Delaware	C	22
1733	FHN	Chicken	Delaware	C	
2035	TS	Chicken	Delaware	C	
2123-2	TS	Chicken	Delaware	C	
2151-5	TS	Chicken	Delaware	C	
2407-4	TS	Chicken	Delaware	C	
2177-2L	TS	Chicken	Delaware	C	
2408-3LS	TS	Chicken	Delaware	C	
2124-2	TS	Chicken	Delaware	C	

[a] TS, Tenosynovitis (viral arthritis); Resp, respiratory; CP, cloacal pasting; GI, gastrointestinal; FHN, femoral head necrosis.

[b] A, Obtained from L. van der Heide; B, obtained from R. C. Jones; C, obtained from J. K. Rosenberger.

Chicken embryo fibroblasts were prepared from 10-day-old embryos and grown in Dulbecco modified essential medium supplemented with 5% tryptose phosphate broth, 2% newborn calf serum, and 2% chick serum, as previously described (25). The first passage of chicken embryo fibroblasts grown in the same medium was used to prepare a stock pool of each strain of avian reovirus as well as to prepare labeled viral RNA.

Human reovirus type 1 (Lang) was obtained from B. N. Fields, Harvard Medical School, Boston, Mass. A stock pool and RNA of this virus were made in mouse L cells grown as monolayer cultures in Dulbecco modified essential medium supplemented with 10% fetal calf serum.

Preparation of labeled cytoplasmic dsRNA. Monolayers of chicken embryo fibroblasts containing 10^6 cells in 30-mm tissue culture plates (Nunc, Denmark) were infected with avian reovirus at a multiplicity of infection of 3 PFU/cell and incubated at 37°C for 1 h. After adsorption, the inoculum was removed, and 2 ml of Dulbecco modified essential medium containing 2% dialyzed fetal calf serum, 60 ng of actinomycin D per ml (Sigma Chemical Co., St. Louis, Mo.), and 100 μCi of [5,6-^{3}H]uridine (Amersham Corp., Arlington Heights, Ill.) was added per plate. The plates were then incubated at 37°C in a 5% CO_2 atmosphere. At 14 h postinfection, the cells were harvested. At this stage of viral growth, almost all virus dsRNA remains within the cell, and any virions formed are still cell associated. Cytoplasmic RNA was prepared as described by Sharpe et al. (27). The cells were scraped off the plates, washed with NMT (0.15 M NaCl, 0.015 M $MgCl_2$, 0.01 M Tris [pH 7.4]), and treated with 0.5% Nonidet P-40 (Shell Co.) in NMT. The suspension was briefly agitated to disperse cell clumps and incubated on ice for 30 min. Nuclei were removed by low-speed centrifugation, and the cytoplasmic extract was adjusted to 0.25 M NaCl. Three volumes of cold ethanol was added, and the solution was kept at −20°C overnight. Precipitated RNA was collected by centrifugation, dried under vacuum, and suspended in 200 μl of gel sample buffer (2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 10% glycerol, 0.05 M Tris [pH 6.8], and bromophenol blue). Labeled dsRNA from human reovirus type 1 was prepared by the same procedure in L cell monolayers except that 250 ng of actinomycin D per ml was used in the medium during infection, and the cytoplasmic extract was processed at 24 h postinfection.

PAGE of dsRNA. PAGE was carried out in slab gels in a discontinuous Tris-glycine buffer system as described by Cross and Fields (1). The concentration of acrylamide was 9%, and the conditions of the electrophoresis were as previously described (Gouvea and Schnitzer, in press). Within 30 min before loading on the gels, the samples were heated in a boiling-water bath for 0.5 min to disperse aggregates. Electrophoresis proceeded at constant voltage at room tempera-

ture. After the run, the gels were immersed in En^{3}Hance (New England Nuclear Corp., Boston, Mass.) for 1 h and in water for 45 min and then dried onto filter paper. Radioautography was performed by exposing X-Omat film (Kodak) to the dried gel at −70°C, and developing was done by standard photographic techniques.

RESULTS

Nomenclature of dsRNA segments. The genomic RNA of the S1133 strain of avian reovirus has been previously characterized (30; Gouvea and Schnitzer, in press) and demonstrated to contain 10 dsRNA segments. On the basis of their mobility upon PAGE, these segments have been segregated into large (L), medium (M), and small (S) size groups and numbered consecutively within each group in their order of migration (from slowest to fastest). Although the avian S1 band migrates more closely to the avian M bands than to the remaining S segments, to retain a similar nomenclature to that used for the mammalian reoviruses (28) the number of dsRNA segments allocated to the L, M, and S size classes for the avian reoviruses is 3, 3, and 4, respectively. Correlations between individual dsRNA segments and their protein products have not yet been made for the avian reoviruses; hence, it can only be inferred that the genomic segment designated S1 does code for one of the σ class proteins. Furthermore, since the gene coding assignments are not currently known, it is not possible to define the correspondence of the dsRNA segments of the avian reoviruses with any of their mammalian counterparts, nor to know what correspondence exists among the dsRNA segments of the different avian reovirus isolates themselves.

Variability of dsRNA migration pattern within one serotype. A number of different field isolates of avian reoviruses that are neutralized by antiserum to the S1133 avian reovirus strain or that can provide protection to challenge by this virus have been identified both in this laboratory and in that of L. van der Heide. Although many of these isolates originated in the New England area in proximity to the location of the S1133 isolate, others are available from different locations in the United States and abroad. The migration patterns of the dsRNA segments of a representative group are shown in Fig. 1C, D, E, and J. Considerable heterogeneity was found to exist within this single serotype with regard to the migration of most, if not all, of the dsRNA segments within each size group.

The S1 segment in the mammalian reoviruses is known to be responsible for determining serotype specificity (34). Therefore, particular attention was directed toward the mobility of the S dsRNA segments of the various avian reovirus isolates available. Although many of the isolates showed one or more S segments having identical mobility, there was no single S segment with the same mobility in all reovirus isolates within this serotype. Other serotypes of the avian reoviruses have been defined (35, 36; R. C. Jones, personal communication), and several isolates from a number of these were available. Analysis of the migration patterns of their dsRNAs (Fig. 2A, B, D, E, and H) further confirmed the fact that there was no single S band with the same migration in all isolates of a given serotype.

Correspondence between serotype and migration of individual dsRNA segments. A number of different serotypes of avian reoviruses have been identified (35, 36). Prototype strains and additional isolates of several of these serotypes were available from England, Japan, the United States, and Europe. Examination of the migration pattern of dsRNA segments from these

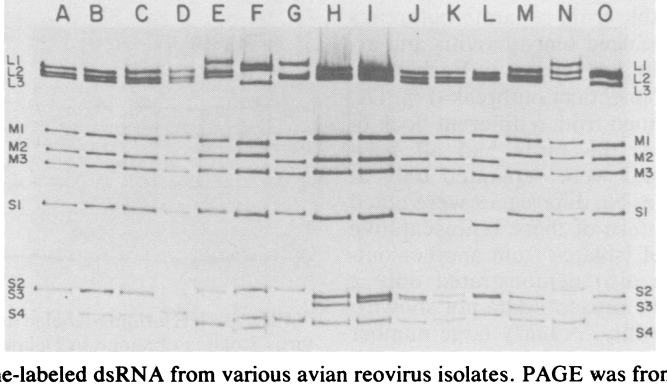

FIG. 1. [^{3}H]uridine-labeled dsRNA from various avian reovirus isolates. PAGE was from top to bottom in a single gel under conditions described in the text. (A) UCon5, (B) UCon4, (C) UCon3, (D) UCon2, (E) UCon1, (F) Lasswade 126/75, (G) Reo 25, (H) WVU 2937, (I) R19, (J) S1133, (K) R10, (L) R21, (M) 2177-2L, (N) Uchida, (O) R2.

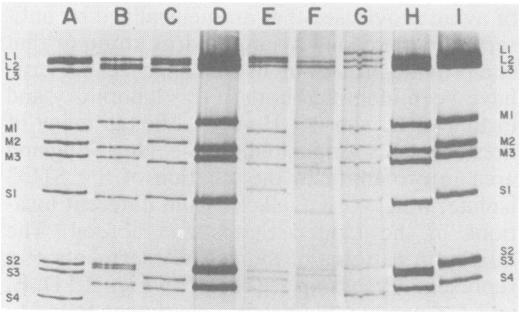

FIG. 2. [^{3}H]uridine-labeled dsRNA from various avian reovirus isolates. Page was from top to bottom in a single gel under conditions described in the text. Lanes have been rearranged for comparison. (A) R2, (B) R10, (C) R11, (D) R12, (E) R17, (F) R21, (G) Uchida, (H) R19, (I) EK.

viruses (Fig. 1F, G, H, I, J, N, and O) failed to demonstrate a unique pattern within any given serotype that would permit discrimination among different serotypes. No single band or pattern of S, M, or L bands was distinctive for any serotype examined.

Correlation between disease state and migration pattern of dsRNA segments. A number of different isolates were available from both healthy (asymptomatic) birds and birds with a variety of different clinical illnesses, including gastroenteritis, tenosynovitis, and respiratory disease. Since previous reports have suggested that certain serotypes of virus are associated with particular disease presentations (24, 35), the migration patterns of the dsRNA segments of isolates from birds with different disorders were analyzed and compared. These data (Fig. 1F, H, I, J, and O and 2B, D, and G) demonstrated no specific association between migration pattern and disease presentation.

Variability in migration pattern of dsRNA segments within a single outbreak of disease. Several isolates were available from separate outbreaks of avian reovirus-induced tenosynovitis and arthritis in Connecticut and Delaware. Each of the isolates from the Connecticut outbreak (Fig. 1A, B, and C) was obtained from a different flock of chickens. However, the birds had all been hatched together and were separated only at several weeks of age. No differences were noted in the migration pattern of these representative isolates. Analysis of isolates from another outbreak in Delaware also demonstrated only a single genotype to be present (data not shown).

Geographic variability. A fairly large number of different avian reovirus isolates were available from Connecticut (Fig. 1A through E and J) and Delaware (Fig. 3A through I) and represented field samples collected over a several-year period. In addition, reovirus isolates from other areas within the United States (Fig. 1G and H), England (Fig. 1I, K, L, and O and 2A through F and H), Scotland (Fig. 1F), and Germany (Fig. 2I) and prototype Japanese strains (Fig. 4B through D) were also analyzed. From these data, it appears that those viruses isolated from within the same geographic area (Fig. 3A through I) were most alike with respect to their dsRNA migration patterns. However, we occasionally found an isolate (Fig. 3G) with a migration pattern strikingly different from that of other isolates obtained within the same geographic area. As far as is known, the bird infected with this isolate originated from a breeding stock similar to that of birds infected by the other isolates. In addition, no birds from outside the immediate geographic vicinity had been recently introduced.

Avian versus mammalian dsRNA migration patterns. From a study of Fig. 1 through 4, considerable similarity in the migration of several of the dsRNA segments was seen to exist within the wide range of avian reovirus isolates examined. In particular, the M3 and S1 bands of many of the isolates had identical mobility under the PAGE conditions employed. Comparison of these migration patterns with those of the prototype mammalian viruses (Fig. 4), as well as with the patterns reported by others for additional mammalian reovirus isolates (8), demonstrated considerable overlap among most dsRNA segments of each size class. However, the S1 avian reovirus dsRNA segment had a mobility that appeared significantly different from that of either the S or the M band of the mammalian viruses, invariably running between the two dsRNA classes. As such, it provided a distinguishing feature for the avian reoviruses.

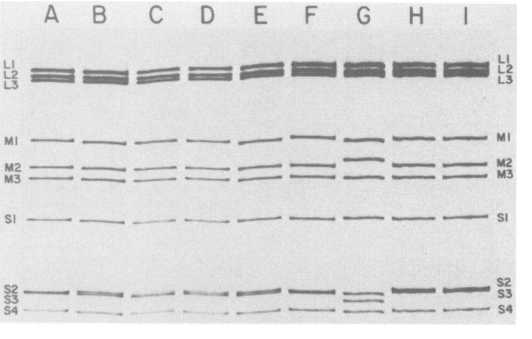

FIG. 3. [^{3}H]uridine-labeled dsRNA from avian reovirus isolates obtained in Delaware. PAGE was from top to bottom in a single gel under conditions described in the text. (A) 2176-IL, (B) 1733, (C) 2035-7A, (D) 2132-2, (E) 2151-5, (F) 2407-4, (G) 2177-2L, (H) 2408-3LS, (I) 2124-2.

DISCUSSION

Infection with avian reoviruses is endemic within many bird populations, and this virus has been directly implicated in the pathogenesis of a range of disease states, including gastroenteritis, tenosynovitis, and respiratory disease (18, 31). Previous studies have indicated that a number of distinct serotypes of avian reovirus exist and have suggested specificity between serotype and disease presentation (24, 35; Kircher, thesis). Biochemical characterizations of prototype viruses of different serotypes, or individual viral isolates within any one serotype, have not been reported, however, and what relationship, if any, exists among these viruses on a molecular level is not known.

The ability to discriminate individual reovirus isolates by the migration pattern of their dsRNA genome segments has permitted not only an analysis of the relationship between individual viral isolates and factors such as viral serotype and disease expression but also the opportunity to investigate in an epidemiological manner the spread of virus within given avian populations. In this investigation, extensive polymorphism has been demonstrated to exist in the avian reovirus population with respect to the migration patterns of their viral genomic RNAs. Even within a single serotype, no unique gene segment can be identified in all isolates, suggesting that dsRNA segments are capable of harboring alterations that are antigenically silent, a finding identical to that reported for the mammalian reoviruses by Hrdy et al. (8). Additionally, this study has demonstrated that there is no specific pattern of migration of the dsRNA gene segments that correlates with any given serotype or disease presentation.

The fact that viruses with identical genotypes may have different phenotypes suggests that a comparison of genome segment size among isolates is not an adequate measure of their genetic relatedness. This finding is not unexpected, as similar data have been reported for mammalian reoviruses (8) and rotaviruses (4, 12). Hence, other methods, such as nucleic acid hybridization (16) of individual genome segments, may need to be assessed to determine their usefulness in demonstrating genetic relatedness among different avian reovirus strains.

The fact that such heterogeneity in the dsRNA genome segments exists among viruses of different serotypes and different disease presentations suggests that the identification of those viral genes responsible for these properties may be undertaken by the production and evaluation of appropriate "hybrid" viruses (reassortants containing individual gene segments from both parents). The value of such a genetic analysis has been amply demonstrated for the mammalian

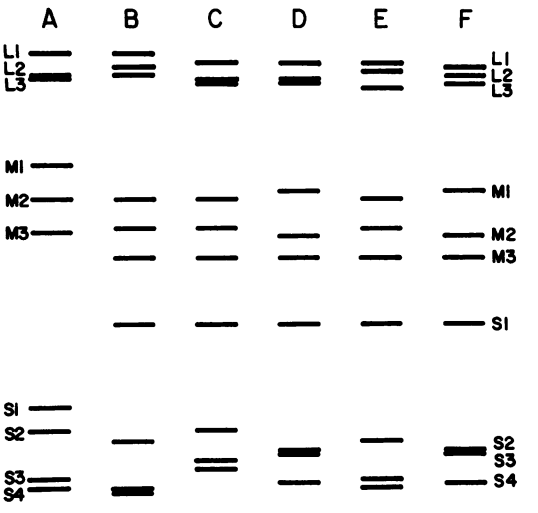

FIG. 4. Diagrammatic representation of migration patterns of [^{3}H]uridine-labeled dsRNA from various reovirus isolates under PAGE conditions described in the text. (A) Mammalian reovirus T1 (Lang), (B) Uchida, (C) Cs108, (D) TS17, (E) Fahey-Crawley, (F) S1133.

reoviruses, in which system the genes responsible for a variety of biological functions of the virus have been identified (6, 14, 23, 26). Additional characterization of such hybrid viruses on a molecular level has also permitted a correlation to be made between individual dsRNA segments or genes and their respective protein products (17). The production and characterization of similar reassortants in the avian reovirus system have not yet been reported but should prove feasible and will allow comparable analyses to be performed.

The ability to characterize individual viral isolates by analysis of their genome dsRNA migration patterns permits a number of epidemiological investigations of potential importance in avian disease. From the data presented here, it appears that under field conditions a single viral genotype is usually responsible for a given outbreak. The origin of the virus may be determined by characterizing the avian reovirus or reoviruses endemic in the avian population affected. By genotyping viruses from different outbreaks, it should prove possible to determine whether there has been transmission of a given agent from one flock to another or whether a different reovirus has been introduced from another source. Since in ovo transmission of avian reoviruses has been well documented (33), this method of epidemiological investigation has importance for monitoring not only vertical but also horizontal viral spread.

The factors responsible for the extensive polymorphism exhibited by the avian reoviruses is

not known. Repeated passage in vitro and in eggs does not result in such variability, at least in the single example studied (32; Gouvea and Schnitzer, in press). The importance of selective pressure, particularly antibody production, for alterations in the outer coat structural proteins, and hence changes in the corresponding dsRNA gene segments, is not known but may play an important role. In that regard, it would be important to determine which of the viral proteins are coded for by the individual dsRNA genome segments. Since the M3, L1, L3, and S4 segments show the least variability in the avian species, these may be responsible for the most highly conserved viral proteins, perhaps those involved with viral replication. The fact that the avian S1 segment has a mobility different from that of any of the mammalian S genes might also imply a protein product with very different specificity or structure and could help explain the different host range of the avian viruses.

It is theoretically possible that genetically "novel" avian reoviruses may be generated in vivo by coinfection of birds in the field by two different reoviruses, with the subsequent production of a virus containing some genes from each of the parental viruses. In a limited number of samples examined in this study, however, only a single viral genotype was isolated from any individual outbreak or from any given bird. However, the fact that in ovo transmission does occur and that viruses of different serotypes are endemic in the bird population, often resulting in subclinical infection, suggest that such an event could occur. Analysis of further isolates from individual birds in infected flocks should permit the detection of naturally occurring reassortants. In addition, laboratory infection of chickens with two different viruses permits direct testing of this possibility.

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