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Genetic Diversity in Twenty Variants of the Avian Polyomavirus

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SUMMARY. To determine if different pathotypes of the avian polyomavirus (APV) exist and to compare the genomes of APVs originating from different geographic areas, dates, and species of birds, the partial sequences of 18 APVs were determined. New viral sequences were compared with three published APV sequences. Two of the new viruses had identical sequences. Forty point mutations were found at 31 loci. A 27-bp deletion was found in the VP2 and VP3 open reading frames of one virus. A duplication of the putative origin of replication and adjacent enhancer region was previously reported in one APV. Smaller duplications involving the origin in one APV and a second enhancer region in another were discovered. All duplications were in tissue culture–adapted viruses, suggesting they occurred during the isolation process. Excluding duplications and the deletion, maximum variation between viruses was small (11 bp). A maximum parsimony tree was constructed that contained three major branches. The three earliest isolates were on separate branches. The European viruses were confined to branch I, but APVs from the United States were on all three branches. Lovebird, budgerigar, and macaw APVs were also on each of the three branches, suggesting that species-specific pathotypes have not developed. Most nonsynonymous mutations occurred in a small portion of the VP2 and VP3 open reading frames, demonstrating a selection for these mutations. That a glycine at VP2 221 will inhibit virus replication in chicken embryo fibroblasts (CEFs) has been previously reported. In contrast, six of seven of the new APVs isolated in CEFs had a glycine at VP2 221.

RESUMEN. Diversidad genética en veinte variantes de poliomavirus aviar.

Se determinó la secuencia parcial de 18 variantes de poliomavirus aviar con el fin de determinar si existían diferentes patotipos de poliomavirus aviar y de comparar los genomas de poliomavirus aislados de diferentes zonas geográficas, fechas y especies de aves. Se compararon secuencias virales recientes con tres secuencias publicadas de poliomavirus. Dos de los nuevos virus tenían secuencias idénticas. Se encontraron 40 mutaciones puntuales en el locus 31. Se detectó la deleción de un fragmento de 27 pares de bases en los marcos de lectura abierta de las proteínas VP2 y VP3 de un virus. Una duplicación de la replicación de origen putativo y adyacente a la región multiplicadora fue reportada previamente en una variante de poliomavirus aviar. Se descubrió una duplicación más pequeña que involucra el origen en una variante de poliomavirus aviar y una segunda región multiplicadora en otro virus. Todas las duplicaciones ocurrieron en virus adaptados a cultivo de tejido, sugiriendo que las duplicaciones ocurrieron durante el proceso de aislamiento. Excluyendo las duplicaciones y las deleciones, las variaciones máximas entre los virus eran pequeñas (11 pares de bases). Se construyó un árbol de máxima simplicidad con tres ramas importantes. Los tres aislamientos más tempranos estaban situados en diferentes ramas. Los virus Europeos fueron colocados en la rama I, pero los poliomavirus de Estados Unidos se situaron en todas las tres ramas. Los poliomavirus de agopornis, periquitos australianos y guacamayas se situaron también en todas las ramas, sugiriendo que no se han desarrollado patotipos específicos de especie. La mayoría de las mutaciones que no fueron sinónimas ocurrieron en una porción pequeña de los marcos abiertos de lectura de la VP2 y VP3, demostrando la ocurrencia de una

selección por estas mutaciones. Se ha reportado previamente que la glicina situada en el sitio 221 del gen VP2 inhibe la replicación viral en fibroblastos de embrión de pollo. En contraste, seis de siete de las cepas de poliomavirus aisladas recientemente tenían glicina situada en el sitio 221 del gen VP2.

Key words: avian polyomavirus, psittacine birds, variants, maximum parsimony tree

Abbrevations: APV = avian polyomavirus; BFDV = budgerigar fledgling disease virus; CEF = chicken embryo fibroblast; PCR = polymerase chain reaction

The budgerigar fledgling disease virus (BFDV) I, the prototype avian polyomavirus (APV), is a nonenveloped, icosohedral virus (4,5,10) with a double-stranded, circular (19,22) 4981-bp genome (27,31). The APV genome has a similar organization to that of the mammalian polyomavirus SV40. The early region of APV appears to encode two regulatory proteins, the large T antigen and the small t antigen (27). The large T antigen is a large complex protein with multiple functional domains. The late region of APV encodes three capsid proteins (VP1, VP2, and VP3) and a smaller nonstructural protein, the agno protein. VP1 is the major structural protein. Pentamers of this protein form the surface of each face of the virus capsid. VP3 is a truncated protein identical to the carboxyl terminal portion of VP2 (27). VP2 and VP3 assist in packaging the minichromosome and anchoring it to the capsid. In the SV40, nucleic acid (8) and VP1 binding domains (13) and the nuclear localization signal (9) are present in the carboxyl terminus of these proteins. The amino termini of the VP2 and VP3 proteins are believed to form a folded spike that is inserted into the center of each VP1 pentamer (15). These domains also appear to be present in the VP2 and VP3 reading frames of APV (27).

The APV is a highly infectious virus that can apparently infect most, if not all, parrot species (4,14,17). Disease, however, predominates in nestling captive-raised budgerigars (*Melopsittacus undulatus*), conures, macaws, eclectus parrots (*Eclectus roratus*), and ring-necked parakeets (*Psittacula krameri krameri*). Both nestling and young adult lovebirds (*Agapornis* sp.) also are susceptible to APV disease (24). Two forms of APV disease are recognized. In the budgerigar, nestlings 1–3 wk of age are affected. Viral cytopathic effects are found in a wide variety of tissues and apparently account for the clinical signs of disease (4,10). In other parrot species, disease predominates in 3-to-14-wk-old nestlings. Cytopathic effects are often limited to Fc receptor-bearing cells such as splenic histocytes. Additionally, the disease is characterized by hepatic necrosis, a generalized hemorrhagic diathesis, and an immune complex glomerulopathy, lesions not seen in the budgerigar (14,26).

Serologically (14,36), and by limited genetic analysis (25), the viruses infecting the budgerigar and nonbudgerigar parrots have been demonstrated to be very similar, but the precise relationship between them has not been shown conclusively. Therefore, if the different disease manifestations observed in different species of parrots result from different viral pathotypes or host factors is not known. Recently, the sequences of two additional APVs have been reported. One, BFDV II, renamed CHEU93 for the purpose of this manuscript, was isolated from the drinking water and feces of chickens. The other, BFDV III, referred to as BGEU93 in this manuscript, was isolated from a nestling blue and gold macaw (*Ara ararana*) with APV disease (31). Fifteen point mutations were identified in one or both of these viruses when compared with a previously sequenced budgerigar isolate (BFDV I, referred to as BDEU84 subsequently). In contrast to the other two isolates, BGEU93 was found to have a reduced capacity to grow in chicken embryo fibroblasts (CEFs). Subsequently, in transfection experiments with various chimeric constructs of each of these viruses, a single nonsynonymous point mutation in the combined VP2 and VP3 reading frame resulted in an abortive infection of CEFs (30). These data suggested that other host-limiting mutations might also be present in the capsid coding regions of other APVs.

CHEU93 had a 151-bp duplication and a 2-bp deletion in the noncoding regulatory region. Significant alterations in the regulatory region of SV40 (6,7,16) and the human JC (21) and BK polyomaviruses (28,35,37) have been documented to occur in cell culture and may also occur in nature (1,3,33). These rearrangements

may occur in as few as one cell culture passage and may significantly alter tissue specificity (28,33,37).

For the control of APV infection in parrots, it will be critical to know if there are one or more APV pathotypes. It will also be essential to know if tissue culture–adapted viruses to be used for infection and immunization studies are significantly altered from the original wild-type viruses. To this end, portions of the APV genome of 18 viruses were amplified by polymerase chain reaction (PCR), either directly from diseased tissues or from tissue culture media, and sequenced. Sequences of the new viruses and those of three previously reported APV sequences were compared (27,31).

MATERIALS AND METHODS

Sources of APV variants. The sources and species of origin of the viruses used in this study are listed in Table 1. The sequence of the three European virus isolates has been previously reported (27,31). The remaining 18 virus sequences were derived from virus DNA amplified directly from tissue removed from carcasses of birds with natural APV infections (nine birds) or from tissue culture media containing APV isolated from naturally infected birds (eight birds). On the basis of histopathologic examination, 16 new avian polyomaviruses caused the death of their host. The 17th, MCFL87, was isolated from the tissues of a Moluccan cockatoo with concurrent psittacine beak and feather disease virus infection. Specific histopathologic evidence of APV infection in this bird was not observed. The 18th, BDGA81B, contained a duplication that represented an *in vitro* rearrangement. Of the three European isolates, BDEU84 and BGEU93 were isolated from birds with classical lesions of APV disease. CHEU93 was isolated from the water and feces of a flock of chickens; its ability to cause disease in the chicken or other birds is not known.

Viral DNA preparation. DNA from the liver of each clinical case was extracted and purified as previously described (21). Briefly, liver (0.5 g) was digested with sodium dodecyl sulfate and proteinase K. The digest was extracted twice with phenol and once with chloroform, and the DNA was precipitated with the addition of sodium acetate and two volumes of ethanol. The precipitate was washed once in 70% ethanol and resuspended in distilled water. Virions in tissue culture fluid were disrupted by boiling for 15 min, after which the fluid was centrifuged (16,000 rpm, 5 min) and the supernatant was passed through a 1.0-ml Sephadex G-50 column (29).

Oligonucleotide primers. Oligonucleotide primers used for amplification and sequencing reactions are listed in Table 2. The DNA segments amplified were chosen to include most of the mutations previously observed in the three European virus variants, to incorporate coding and noncoding sequences, and to incorporate sequences coding for structural and nonstructural proteins. Although nucleotides 4433 and 4504 have been reported to occur in the open reading frame of the large T antigen (30,31), they are within the large T antigen intron and code for part of neither the large T nor small t antigen and are considered noncoding for the purposes of this paper.

Viral DNA amplification. APV DNA was amplified either from 5 μl of boiled tissue culture media or 1 μg of tissue DNA by PCR. DNA amplification was done in a volume of 100 μl. The reaction mixture contained 3.125 mM $MgCl_2$, 1 IU Taq DNA polymerase (Promega, Madison, WI), 0.1 μM (each) cytosine triphosphate, thymine triphosphate, adenine triphosphate, guanine triphosphate, 10 μl of magnesium-free thermophilic buffer (Promega), and 86 μl of water. Reactions were carried out in a programmable heating block (Temp-Tronic; Thermolyne, Dubuque, IA). DNA was denatured by a 5-min incubation at 94 C and amplified through 40 cycles at an annealing temperature of 55 C, extension temperature of 72 C, and melting temperature of 94 C. DNA amplification products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide. Amplified viral DNA fragments were extracted from agarose slices by selective binding to a silica-based matrix (Prep-A-Gene; Bio-Rad, Hercules, CA).

DNA sequencing. PCR-amplified DNA fragments were sequenced by either the double-stranded DNA Cycle Sequencing System (Gibco BRL Life Technologies, Gaithensburg, MO) or the ABI® Dye Terminator Cycle Sequencer Ready Reaction Kit (Perkin Elmer, Norwalk, CT) and an Applied Biosystems Model 373A DNA Sequencer with the primers in Table 2. The sequences have been submitted to GenBank and have been given accession numbers AF054335–AF054351.

Consensus sequence. A consensus sequence was deduced from the 19 unique virus sequences (Table 1). Sequence numbering is based on the original sequence reported by Rott *et al.* (27) that was subsequently corrected by Stoll *et al.* (31).

Analysis. The viral sequences were analyzed cladistically by the maximum parsimony procedure (34). The ratio of synonymous *vs.* nonsynonymous mutations was determined by the method of Li (20). Differences were considered significant at $P \leq 0.01$.

RESULTS

Sequence variations. A total of 1046 bp of viral DNA representing 20.9% of the viral

Table 1. Species, location, and date of origin of avian polyomaviruses used in this study.

Abbreviation	Species of origin	Date of origin[A]	Location of origin
BDEU84 (BFDV I)[B]	Budgerigar[C] (*Melopsittacus undulatus*)	1984	Europe
CHEU93 (BFDV II)[D]	Chicken[C] (*Gallus gallus*)	1993	Europe
BGEU93 (BFDV III)[D]	Blue and gold macaw[C] (*Ara auranca*)	1993	Europe
MCFL87	Moluccan cockatoo (*Cacatua moluccensis*)[C]	1987	Florida
LBMI92	Peach-faced lovebird (*Agapornis personata*)[C]	1992	Michigan
LB85	Peach-faced lovebird[C]	1985	Not reported[E]
ECTX92	Eclectus parrot (*Eclectus roratus*)	1992	Texas
GCAZ92	Green-cheeked conure (*Pyrrhura molinae*)	1992	Arizona
YCFL87	Yellow-collared macaw[B] (*Ara auricollis*)	1987	Florida
SCIL88	Sun conure (*Aratinga solstitialis*)	1988	Illinois
BDTX88	Budgerigar	1988	Texas
BDTX89	Budgerigar	1989	Texas
RNIA91	Indian ring-necked parakeet (*Psittacula echo*)	1991	Iowa
BDGA81	Budgerigar[B]	1981	Georgia
BCFL92	Blue-crowned conure[B] (*Aratinga acuticaudata*)	1992	Florida
ECFL91	Eclectus parrot[B]	1991	Florida
LBFL93	Peach-faced lovebird[B]	1993	Florida
LBTX94	Peach-faced lovebird	1994	Texas
BGTX93	Blue and gold macaw	1993	Texas
RLTX93	Red lory (*Eos bornea*)	1993	Texas

[A]The date of origin is the actual year the tissues containing the virus were collected or is the year the publication describing the virus or its sequence was reported.

[B]This sequence was first reported by Rott *et al.* (27). The corrected sequence was reported by Stoll *et al.* (31). They refer to this sequence as BFDV I.

[C]These viruses have been grown in tissue culture. BGEU93 is reported to grow in Muscovy duck embryo fibroblasts but not chicken embryo fibroblasts (Stoll *et al.* [31]).

[D]These sequences were reported by Stoll *et al.* (31) and were referred to as BFDV II and BFDV III.

[E]The geographic origin of the bird from which this virus was isolated is no longer known.

genome was sequenced for each of the 18 new viruses. These sequences were compared with the sequences of the three previously reported APVs (27,31). Only two viruses, RLTX93 and RNIA91, had identical sequences. In the 17 new sequences, 40 point mutations were found at 31 loci (Table 3). Identical mutations were found to arise independently twice at seven loci. At nucleotide 1638, point mutations resulting in a base change from G to C arose independently twice, and a base change from G to T was also identified at this locus. Two mutations resulting in a change from T to A and T to G were also found to occur at nt 2289. BGTX93 had a 27-bp deletion in the VP2 open reading frame (position 1727 to 1753).

In addition to the duplication previously reported in CHEU93 (31), two new duplications were found (Fig. 1). ECFL91 had a 92-bp insert between bp 212 and 213. This insert began with an additional G and was followed by an exact duplication of positions 122 to 212. BDGA81 was found to be a mixture of two

Table 2. Primers used to amplify and sequence APV DNA.

Primer	Location	Region	Primer sequence (5′ to 3′)	Nucleotides sequenced
VP2/3-1	1398–1418	VP2/3	CGTTGTATGAACAAGTAGGCCC	1500–1768[A]
VP2/3-2	1541–1560	VP2/3	TTAGAAACCGCACGTTGGA	
VP2/3-3	1809–1790	VP2/3	CGTTGCATTGCACCTCCTGG	
VP1-1	2182–2207	VP1	CTTATGTGGGAGGCTGTCAGTGTT	2277–2399[B]
VP1-2	2733–2710	VP1	TACTGAAATAGCGTGGTAGGCCTC	
T-ag-1	3361–3342	Large T	AGTGTACCCGTAAACCTCGA	3204–3320, 3375–3477[C]
T-ag-2	3624–3604	Large T	CACCGAAGCGGCGATACTATA	
T-ag-3	3748–3770	Large T	CCGGACTGTGCTACGTAACATTC	
T-ag-4	4241–4260	Large T	CGTCGATATACGCGTCGGTT	4420–4558[D], 4820–235[DE]
T-ag-5	4390–4409	Large T intron	AATCAGGGCAGCTCTGCACG	
T-ag-6	4887–4903	Large T small t	TTCAGTGCCGTGCGACG	
NC-1	395–375	NC	GGCGTTCCGTTAGGCATTGT	

[A] Sequence data are in the combined open reading frame for the VP2 and VP3 capsid proteins.
[B] Sequence data are in the open reading frame for VP1 capsid protein.
[C] Sequence data are in the open reading frame of the large T antigen.
[D] Sequence data are in the combined open reading frame for the large T and small t antigens.
[E] Sequence data are in the noncoding region believed to contain the origin of replication and enhancer regions.

viruses (BDGA81A and BDGA81B) in approximately equal proportions. The viruses were identical except that the larger one (BDGA81B) had a 114-bp insert between positions 229 and 230. This insert was an exact duplication of positions 4976 to 77 and 199 to 229.

Phylogenetic analysis. The newly described point mutations, duplications, and deletion combined with the sequence data from the European isolates were used to construct a maximum parsimony tree (Fig. 2). For the purpose of this analysis, the duplications and the deletion were considered to have occurred as a single event and were given the same weight as a point mutation. Excluding the insertions and deletion, the greatest difference between viruses was between BDEU84 and LBTX94 and BGTX93, which differed by 11 nucleotides.

Three major branches were identified. Branch I was defined by a synonymous mutation (C to T) at position 4972. Branch II was defined by a nonsynonymous mutation (G to T) at position 3300, resulting in substitution of valine for alanine at large T antigen amino acid 451. Branch III was defined by a synonymous mutation (T to C) at position 3242. All three European viruses mapped to branch I. Viruses of budgerigar, lovebird, and macaw origin were present on all three branches of the tree. Although not the most divergent, BDGA81 differed from BDTX88 and BDEU86 by 9 and 11 bp, respectively. Tissue culture–adapted viruses were also found on all three branches. The earliest virus isolates, BDGA81, LB85, and BDEU84, were each found on separate branches.

Amino acid substitutions. Twenty-eight point mutations were identified in the coding sequence. Eighteen of these resulted in an amino acid substitution. Eleven amino acid substitutions were identified within a 55-amino acid stretch of the common amino acid portion of the VP2 and VP3 protein. All but one of the amino acid substitutions resulted in an amino acid with a larger or more polar side chain. Three amino acid substitutions were found in the VP1 protein. Of the remaining four amino acid substitutions, three were in the carboxyl terminus of the large T antigen and one was in the amino terminus of the overlapping large T and small t antigens. When synonymous mutations were considered, ECFL91 was found to be essentially identical to RLTX93 and RNIA91 because it differed from them by only two synonymous mutations. LB85 and GCAZ92 also differed by two synonymous mutations.

The rate of nonsynonymous mutations was significantly higher than the rate of synony-

Table 3. Nucleotide (nt) substitutions in 19 APV variants.

nt no.[A]	Consensus nt[B]	Location	Aa no.	Aa	BDTX89	RNIA91	BDGA81	BCFL92	ECFL91	LBFL93	LBTX94
19	G	NC	NC	NC	—	—	—	—	—	—	—
34	T	NC	NC	NC	—	—	—	—	—	—	—
199	C	NC	NC	NC	—	—	—	—	—	—	—
1572	C	VP2/VP3	199/93	A–E	—	—	—	—	—	—	—
1598	A	VP2/VP3	208/102	K–Q	—	—	—	—	—	—	—
1599	A	VP2/VP3	208/102	K–R	—	—	—	C	—	—	—
1601	A	VP2/VP3	209/115	S–G	—	—	—	—	—	—	—
1621	G	VP2/VP3	215/121	SYN	—	—	—	—	—	G	—
1637	G	VP2/VP3	221/115	G–S	—	—	—	—	—	—	—
1638	G	VP2/VP3	221/115	G–A	—	—	A	—	—	—	—
				G–V	—	—	—	—	—	—	—
1649	C	VP2/VP3	225/119	R–S	—	—	—	—	—	—	—
1667	G	VP2/VP3	231/125	A–T	A	—	—	—	—	—	—
1694	T	VP2/VP3	240/134	S–P	—	—	—	—	—	—	—
1734	A	VP2/VP3	253/147	K–T	—	—	—	—	—	—	—
1741	T	VP2/VP3	255/149	SYN	—	—	—	—	—	—	C
2289	T	VP1	131	F–I	—	—	—	—	—	—	—
				F–V	—	—	—	—	—	—	—
2372	A	VP1	158	K–N	—	—	—	—	—	—	—
3224	C	T-ag	476	SYN	—	—	C	—	—	—	—
3242	T	T-ag	471	SYN	—	—	—	—	—	—	—
3248	C	T-ag	469	SYN	C	C	C	C	C	C	C
3258	A	T-ag	466	Q–R	—	—	—	—	—	—	—
3300	G	T-ag	451	G–V	G	—	—	—	—	—	—
3382	C	T-ag	424	SYN	—	—	—	—	—	—	—
3413	T	T-ag	413	SYN	—	—	—	—	—	—	—
3434	T	T-ag	406	SYN	—	—	—	—	—	—	—
3443	A	T-ag	403	SYN	—	—	—	—	C	—	—
3468	A	T-ag	395	Y–F	—	—	—	—	—	—	G
4433	A	Intron	NC	NC	—	—	—	—	—	—	—
4504	T	Intron	NC	NC	—	—	—	—	—	—	—
4948	G	T/t-ag	12	G–C	—	—	—	—	—	—	—
4972	C	T/t-ag	4	SYN	—	—	—	—	T	—	—

[A]Numbering scheme is according to Stoll *et al.* (31).
[B]Nucleotides in open reading frames are from the coding DNA strand.

Table 3. Continued.

nt no.[A]	BGTX93	GCAZ92	ECTX91	LB85	YCFL87	SCIL88	BDTX88	LBMI92	MCFL87	BGEU93	CHEU93	BDEU84
19	—	—	—	—	—	—	—	—	A	—	—	—
34	—	—	—	—	—	A	A	—	—	—	—	A
199	—	—	—	—	—	—	—	—	—	T	—	—
1572	—	—	—	—	—	A	—	—	—	—	—	—
1598	—	—	—	—	—	—	—	—	—	—	—	—
1599	—	—	—	—	—	—	G	—	—	—	—	—
1601	—	—	—	—	—	—	—	—	—	G	—	—
1621	—	A	—	—	—	—	—	—	—	—	—	—
1637	—	—	—	—	—	—	—	—	—	—	—	—
1638	—	—	—	—	—	C	C	C	—	—	C	—
	—	—	—	—	—	—	—	—	—	—	—	T
1649	—	—	A	—	—	—	—	—	—	—	—	—
1667	—	—	—	—	—	—	—	—	—	—	—	—
1694	—	—	—	—	C	—	—	—	—	—	—	—
1734	—	—	—	—	—	—	—	—	—	—	—	—
1741	—	—	—	—	—	C	—	—	—	—	—	—
2289	—	—	—	—	—	A	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	—	—	G	—
2372	—	—	—	—	—	—	—	—	—	—	—	—
3224	—	—	—	—	—	—	—	—	—	—	—	T
3242	C	—	—	—	—	—	—	—	—	—	—	—
3248	—	—	—	—	—	—	—	T	T	—	—	—
3258	—	—	—	—	—	—	G	—	—	—	—	—
3300	—	T	T	T	T	T	T	—	—	—	—	—
3382	—	—	—	—	—	—	—	—	—	T	—	—
3413	G	—	—	—	—	—	—	—	—	—	—	—
3434	—	—	—	C	—	—	—	—	—	—	—	—
3443	G	—	—	—	—	—	—	—	—	—	—	—
3468	—	—	—	—	—	T	T	—	—	—	T	T
4433	—	—	—	—	—	—	—	—	—	—	—	T
4504	—	—	—	—	—	—	—	—	—	—	—	C
4948	—	—	—	—	—	—	—	—	—	—	—	T
4972	—	—	—	—	—	—	—	T	T	T	T	T

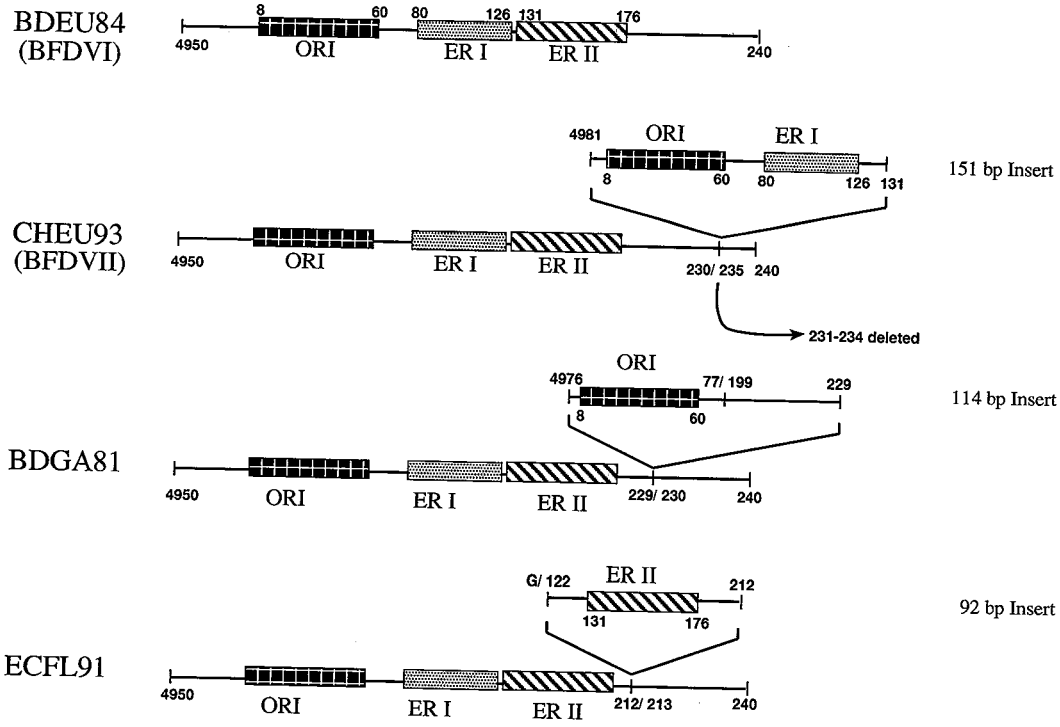

Fig. 1. Insertions within the putative origin of replication (ORI) and adjacent regulatory regions (ER I and ER I) of three cell culture–adapted APVs. The sequence numbers are those reported by Rott *et al.* (27) and subsequently corrected by Stoll *et al.* (31).

mous mutations in the VP2 and VP3 open reading frame. In the large T and small t antigen reading frames, synonymous mutations were found to occur at a significantly higher frequency than nonsynonymous mutations. Only three mutations were identified in the VP1 open reading frame; all were nonsynonymous. Because only 123 bp were sequenced, the data were considered insufficient to analyze statistically. When the rates of synonymous and nonsynonymous mutations were compared over the entire sequenced virus, these rates did not differ significantly.

DISCUSSION

Prior to this study, APV sequence information was limited to three European isolates (27,31). To compare viruses from different parrot species, time periods, and geographic locations, we expanded this database by partially sequencing 18 additional APVs. The amount of variation found between viruses was small. Excluding deletions and duplications, only 31 variable nucleotide positions, representing 3.0% of the sequenced viral genome, were found. The maximum number of nucleotide substitutions between divergent viruses was 11 (1.1%). Each of the three major branches of the maximum parsimony tree was defined by a single point mutation, two of which were synonymous mutations. Because these parrot APVs contain such a limited amount of variation, we propose that they all be considered variants of a single APV.

Viruses from multiple regions of the United States were found on each branch of the maximum parsimony tree. These data are consistent with current and past avicultural practices of interstate sale and transport of birds. Branch I contained all of the European variants, suggesting a single introduction of APV into Europe and subsequent evolution. Branch I also contained two United States variants, demonstrating that the original European variant was also introduced into the United States or, conversely, it may have originated in the United States.

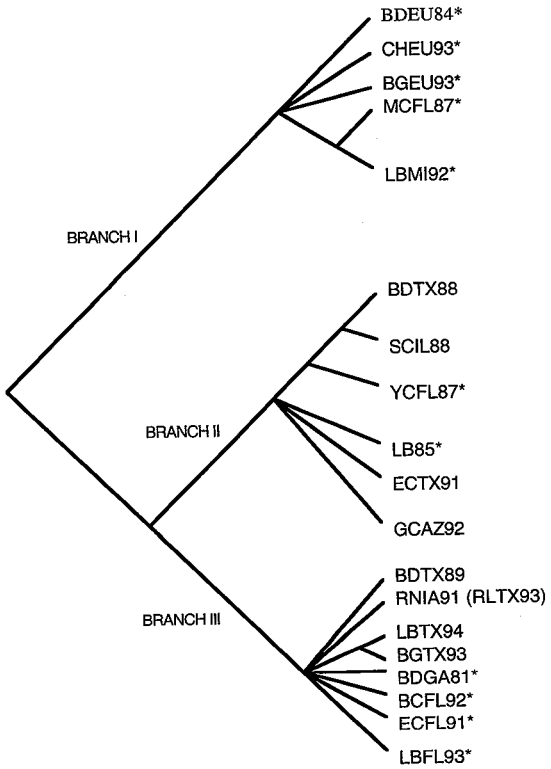

Fig. 2. Maximum parsimony tree of 20 APV variants. *Tissue culture adapted viruses.

The three virus variants originating from the earliest sources (BDGA81, BDEU84, and LB84) were found on separate major branches of the maximum parsimony tree. This finding suggests that these branches were defined by 1984. Because of parallel evolution, the paucity of early virus isolates, and the uncertainty of when APV was first introduced to parrots, determining the evolutionary rate of APV is impossible. Our data suggest however, that APV is a relatively stable virus because 1) 11 of 18 viruses had two or fewer amino acid substitutions compared with the RLTX93/RNIA91 virus and 2) two of these viruses, BDGA81 and LB85, were isolated 12 and 8 yr before RLTX93. Additionally, LB85 and GCAZ92, although separated by 7 yr, differed by only two synonymous mutations. The greatest variation between viruses within one branch occurred among the three European viruses. Whether this indicates a more rapid evolutionary rate in this group or evolution over a longer period of time is not known.

Differences in histopathologic appearance of APV disease in budgerigars and parrots other than budgerigars suggested that different APV pathotypes may infect different species of parrots. It has been reported that a substitution of a glycine for alanine or valine at amino acid 221 of VP2 blocks virus replication in CEFs but not Muscovy duck embryo fibroblasts (30,31). Although a virus with this mutation was still capable of growth in chicken eggs, the authors suggested that this mutation might result in a restricted host range. Evidence for host range restriction was not found in the new APV variants reported here. The parsimony tree contained three branches, but viruses from lovebirds, budgerigars, and macaws were present on all of them. Though not necessarily the most diverse, viruses infecting the same species were never identical and often were separated by several mutations resulting in amino acid changes. These findings strongly suggest that the variations observed in the current population of APVs are minor and have not resulted in the development of species-specific pathotypes.

Seven of the new viruses we report were sequenced directly from culture supernatant. All seven of these viruses were isolated and grown in CEFs. In contrast to the findings of Stoll *et al.* (30) in which a glycine at VP2 221 blocked virus growth in CEFs, six of the seven cell culture viruses had a glycine at VP2 221. Although the explanation for this apparent discrepancy will require additional investigation and may have many possible explanations, it may be that a glycine at VP2 221 inhibits replication in only CEFs from certain strains of chickens.

Although there was no clear evidence for the existence of species-specific APV pathotypes, the significantly high rate of nonsynonymous mutations in a small portion of the combined VP2 and VP3 reading frame of these viruses, the independent occurrence of the same mutations in different viruses, and a large deletion in this same region suggested that mutations in this region are being actively selected for in nature. Stoll *et al.* (30) speculated that a mutation at VP2 221 may have interfered with virus uncoating or with nuclear localization of the viral DNA in their CEF culture system. These mutations might also affect these functions and alter the efficiency of viral replication at least in some species. However, why most of the mutations occurred at the terminal branches of the

maximum parsimony tree and why one or more of these mutations did not predominate remain to be explained. One possible explanation for these observations is that these mutations may enhance replication in one species but not in others. Because most parrots are housed in aviaries with numerous other parrot species, the improved ability to replicate in one species with the reduced ability to replicate in others would be of little benefit.

The noncoding region of the mammalian polyomaviruses contains the origin of replication, which includes binding sites for the large T antigen, enhancer elements, and binding sites for several cellular proteins. Protein interactions with these functional domains regulate both early and late protein synthesis and DNA replication. Specific transcriptional control elements and their organization impart tissue specificity (11) and cell line specificity (12,32) and alter transforming activity of the polyomaviruses (18,37). A consistent theme among mammalian polyomaviruses is their propensity for both *in vivo* and *in vitro* duplication and rearrangement of functional domains of the noncoding region. Studies show that enhancer duplication and rearrangement and, at least in the SV40 (7), origin duplication (32) increase the replication efficiency of these viruses *in vitro*. In the JC virus, duplications and deletions in this region have been identified in viruses causing progressive multifocal leukoencephalopathy (1,23).

The APV origin of replication has not been identified although it is thought to reside in an AT-rich sequence (positions 8–60). Two putative enhancer elements (positions 180–126 and 131–178) are also thought to be present in the APV noncoding region (27). Duplications of portions of the noncoding region were found in CHEU93, BDGA81, and ECFL87. The duplication within CHEU93 included the possible origin of replication and the putative first enhancer sequence. The duplication in BDGA81 contained the possible origin of replication but no enhancer sequences. ECFL87 had a duplication of a putative second enhancer element. Both the CHEU93 and BDGA81 duplications were inserted following bp 230, suggesting that the adjacent sequences may be important in the events that result in these duplications. All three of these viruses were tissue culture adapted, indicating that, like other polyomaviruses, *in vitro* passage of APV may select for variants with duplication of the origin of replication or enhancer sequences. It is also possible, however, that, similar to the JC virus, these duplications may have occurred in the host. In either case, alterations in the noncoding region of other polyomaviruses have profound effects on their biological activity. Therefore, it would seem prudent that, until the significance of these alterations in the noncoding region can be determined, APV variants possessing these alterations should not be used in infection and immunization studies.

In summary, although minor genetic variability appears to be common in APVs infecting parrots, the virus is relatively stable, and all the viruses sequenced have nearly identical sequences and few nonsynonymous mutations. Specific pathotypes were not identified, and no evidence was found to suggest that a virus infecting one species of parrot was incapable of infecting other parrot species. On the basis of these observations, we consider all the parrot APVs examined to date to be variants of a single APV. Within these variants, selection pressure for amino acid substitutions in a small region of the VP2 and VP3 proteins appears to exist. The biological significance of these substitutions remains to be determined.

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