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# Investigation of the Attenuation Exhibited by a Molecularly Cloned Chicken Anemia Virus Isolate by Utilizing a Chimeric Virus Approach

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**Molecular cloning of the Cux-1 isolate of chicken anemia virus (CAV), which had been passaged 173 times in cell culture, resulted in the isolation of an attenuated strain, designated cloned isolate 10, which reverted to virulence following 10 passages in young chicks (D. Todd, T. J. Connor, V. M. Calvert, J. L. Creelan, B. M. Meehan, and M. S. McNulty, Avian Pathol. 24:171–187, 1995). The attenuated cloned isolate 10 differs from the molecularly cloned pathogenic Cux-1 isolate in that it possesses a 21-nucleotide insertion within the nontranscribed region of the CAV genome and 17 individual nucleotide substitutions dispersed throughout the genome. Comparative analyses with other published CAV sequences indicated that cloned isolate 10 was unique at nine nucleotide positions and at five amino acid positions. The molecular basis of the attenuation exhibited by cloned isolate 10 was investigated by evaluating the pathogenicities of two sets of complementary chimeric viruses. These sets were produced by transfection with chimeric double-stranded replicative-form (RF) DNA equivalents that contained DNA sequences derived from cloned isolate 10 and the pathogenic cloned Cux-1 isolate. The construction of the chimeric RFs exploited the occurrence of unique *Eco*RI, *Pst*I, and *Bam*HI restriction sites, which allowed their respective circular CAV RFs to be manipulated as three restriction fragments of 0.58, 0.93, and 0.71 kbp. Examination of the levels of anemia and gross pathology in the thymuses and bone marrows of 14 day-old specific-pathogen-free chicks following infection of 1-day-old chicks with the chimeric and cloned parental isolates indicated that nucleotide changes in each of the three genomic regions contributed towards attenuation. The significance of this result to the development and use of live attenuated CAV vaccines is discussed.**

Chicken anemia virus (CAV) is an economically important avian pathogen with a worldwide distribution (5). On the basis of its morphology and circular, single-stranded DNA genome, CAV has recently been classified in a newly recognized animal virus family, the *Circoviridae* (4). The virus can be grown in the Marek's disease virus-transformed chicken lymphoblastoid MDCC-MSB1 cell line (22). The double-stranded replicative form (RF) of CAV DNA that can be isolated from infected MDCC-MSB1 cells has been cloned, sequenced, and shown by transfection to contain all the elements necessary to produce infectious virus (1, 3, 10, 11).

CAV infections produce clinical and subclinical diseases (5). Transmission of the virus by eggs from infected breeder flocks can result in increased mortality in 10- to 14-day-old chicks that is associated with anemia, hemorrhages, and lymphoid depletion (5). Chicks that become infected when they are 2 to 3 weeks old, when maternally derived antibody levels are no longer protective, fail to develop clinical signs. Field studies, however, have shown that subclinical disease of commercial broiler chicks, resulting from infection with horizontally acquired virus, adversely affects growth and profitability (9).

All the naturally occurring isolates of CAV were pathogenic when they were tested experimentally in 1-day-old specific-pathogen-free (SPF) chicks (5). At 14 days after intramuscular inoculation, the majority of chicks showed anemia, thymus atrophy, and pale bone marrows. Regarding the attenuation of CAV, the 26P4 isolate has been partially attenuated by passage in chick embryos (19). This isolate has been developed as a commercially available live vaccine, administered to breeders before they come into lay, to control clinical disease. A long-term goal of our research is to produce a substantially attenuated CAV isolate for development as a live vaccine to protect against the clinical and subclinical disease forms. We have previously reported that the pathogenicity of the Cux-1 isolate of CAV can be progressively reduced by multiple passage in MDCC-MSB1 cells (20). Restriction analysis of CAV DNA amplified by PCR from cells infected with virus at different passage levels showed that a 21-nucleotide (nt) insertion became established within the virus population before 30 cell culture passages were completed (20). Recombinant DNA cloning and transfection procedures were used to select individual cloned isolates from the genetically diverse virus pool present after 173 cell culture passages (20). One such isolate, designated cloned isolate 10, was found to have reduced pathogenicity compared to those of low-number-passage virus pools. Plans to develop cloned isolate 10 as a potential live vaccine received a setback when it was shown that passage of this virus 10 times in young chicks allowed recovery of pathogenicity (20).

In this study, we compare the nucleotide sequence of cloned isolate 10 with that of the pathogenic cloned Cux-1 isolate and investigate the contributions that sequence changes that are present in different areas of the genome make to the attenuation using a series of chimeric virus constructs. The implica-

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TABLE 1. Characterization of virus pools used for experimental infections

| Group | Construct genotype or control | No. of passages posttransfection to produce infecting pool | Infectivity titer of pool ($log_{10}$ $TCID_{50}$/0.1 ml) | Inoculum used ($log_{10}$ $TCID_{50}$) |
|---|---|---|---|---|
| 1 | Isolate 10 BE-Cux isolate EB | 8 | 6.25 | 6.0 |
| 2 | Cux isolate BE-isolate 10 EB | 8 | 6.25 | 6.0 |
| 3 | Isolate 10 EP-Cux isolate PE | 9 | 7.0 | 6.0 |
| 4 | Cux isolate EP-isolate 10 PE | 8 | 6.5 | 6.0 |
| 5 | Cux isolate PB-isolate 10 BP | 8 | 5.75 | 5.75[a] |
| 6 | Isolate 10 PB-Cux isolate BP | 8 | 6.0 | 6.0 |
| 7 | Cloned isolate 10 | 10 | 6.0 | 6.0 |
| 8 | Cloned low-number-passage Cux isolate | 11 | 5.5 | 5.5[a] |
| 9 | Pathogenic control | NA[b] | 6.75 | 6.75 |
| 10 | Uninfected MSB1 control | 0 | 0 | 0 |

[a] Maximum titer reached by virus pool.
[b] NA, not applicable.

tions of these results for the stable attenuation of cloned isolate 10 for use as a CAV vaccine are discussed.

## MATERIALS AND METHODS

**Cells and virus.** The MDCC-MSB1 cells and the Cux-1 isolate of CAV, which had received 13 cell culture passages after preparation as a clinical specimen, were obtained from V. von Bülow (Free University, Berlin, Germany). Virus that had been passaged an additional 13 times in MDCC-MSB1 cells since its receipt in our laboratory was used as the pathogenic control in the chick inoculation experiments.

**Parental CAV plasmids.** The production of pCAA-5, which comprises the pGEM-1 vector containing a 2.3-kbp CAV (Cux-1 isolate) RF *Eco*RI insert, was described previously (21). The complete sequence of this cloned RF has been reported (10). Following transfection with *Eco*RI-restricted pCAA-5, this cloned RF produces a pathogenic Cux-1 isolate, which we have called cloned Cux isolate. Molecular cloning of CAV RF isolated from cells infected with Cux-1 virus passaged many times (passage number, 173) resulted in the production of the cloned RF of isolate 10 as an *Eco*RI insert in pGEM-1, from which the attenuated cloned isolate 10 was produced following transfection (20).

**DNA sequencing.** The complete RF of cloned isolate 10, initially cloned as a linearized 2.3-kbp *Eco*RI fragment in pGEM-1 (20), was subsequently subcloned into the bacterial plasmid pBluescript (pBS-SK; Stratagene). A series of deletions were then made with the exonuclease Erase-a-Base system (Promega). The nucleotide sequences of these overlapping clones were determined by the dideoxy termination method (16) with T3 and T7 primers and a commercial deaza T7 DNA polymerase sequencing kit (Promega) in accordance with the manufacturer's instructions. The sequence of the complete RF of cloned isolate 10 was assembled and analyzed with the computer program DNASIS (Hitachi).

**Transfection.** Virus pools of cloned isolate 10 and the cloned Cux isolate were produced by transfecting MDCC-MSB1 cells with CAV RF DNAs by the DEAE method (18) as described by Todd et al. (21). Linearized RF DNAs were prepared for transfection by *Eco*RI digestion of plasmid DNAs (1 μg) purified from bacterial cultures by the alkaline lysis method (15). Transfected cells were examined by indirect immunofluorescence (IIF) after 4 or 5 passages, and virus pools were prepared after 8 to 11 passages when cell death was apparent (20).

**Production of chimeric viruses.** Chimeric RF DNAs were constructed from the parental plasmid pCAA-5 and plasmids with the cloned RF of isolate 10 containing the respective, cloned parental RFs as 2.3-kbp *Eco*RI fragments. These cloned RFs were used as sources of virus DNAs for the subcloning of specific virus DNA fragments for subsequent use in chimeric virus construction.

Three pairs of chimeric viruses were produced. Their design exploited the occurrence of three unique restriction sites in the CAV RF, *Eco*RI, *Pst*I, and *Bam*HI, which divided the cloned CAV genomes into fragments of approximately 0.58, 0.93, and 0.71 kbp that make up the entire 2.3-kbp CAV RF. Fragments corresponding, in a clockwise manner on the circular RF, to the *Eco*RI-*Pst*I (EP), *Pst*I-*Eco*RI (PE), *Bam*HI-*Eco*RI (BE), *Eco*RI-*Bam*HI (EB), and *Pst*I-*Bam*HI (PB) regions of both the cloned Cux isolate and cloned isolate 10 were generated from the appropriately digested 2.3-kbp *Eco*RI parental RF clones and subcloned into plasmid pBS-SK for subsequent manipulation. The *Bam*HI-*Pst*I (BP) fragments of both the cloned Cux isolate and cloned isolate 10 were generated by endonuclease digestion of 2.3-kbp *Bam*HI clones, produced as a result of cloning the respective recircularized parental *Eco*RI 2.3-kbp inserts. These BP fragments were again subcloned into plasmid pBS-SK for subsequent manipulation. All plasmid constructs were verified by restriction endonuclease analysis.

Chimeric RFs were produced by ligation of approximately equimolar amounts of the two complementary RF fragments released from appropriately digested plasmid preparations. Chimeric viruses were selected on the basis of their abilities to replicate after transfection with the ligation mixtures. For example, to generate the Cux EP-isolate 10 PE chimeric virus, plasmid preparations containing the cloned Cux EP and cloned isolate 10 PE inserts were digested with *Pst*I and *Eco*RI to release each of the virus-specific fragments and linearized plasmids. Following ligation in equimolar amounts, the chimeric RF equivalents were selected from the other ligation products on the basis of their ability to produce infectious virus following transfection. Following IIF analysis (7), pools of each of the chimeric viruses were produced by transfection as described above for the cloned Cux isolate and isolate 10.

**Experimental infection.** One-day-old (SPF) chicks hatched from eggs from a CAV-free source (Lohmann Tierzucht, Cuxhaven, Germany), shown to be free of maternal antibody to CAV as determined by IIF (7), were used throughout. Each virus pool was titrated to endpoint as described by Todd et al. (20) prior to experimental infection. The pathogenicities of the cloned and chimeric viruses were evaluated as described previously (20). Briefly, groups of SPF chicks, ($n$ = 11 to 14) housed in separate negative-pressure isolators, were inoculated intramuscularly (0.1 ml) with selected CAV virus preparations. At 14 days posthatching, the birds were bled, hematocrit values were determined, and a postmortem examination was carried out. Birds were considered to be anemic if the hematocrit values were less than 27. The gross pathology examination of the thymuses and bone marrows was performed in a blind manner by two personnel (D.T. and J.L.C.) as described previously (8, 20), and a clinical score was estimated by taking the severity of the thymus atrophy and paleness of bone marrow, scored as 0, 1+, and 2+, into consideration.

Details of the 10 treatment groups are shown in Table 1. The infectivity titers of the inoculated parental cloned isolates and the chimeric viruses were standardized at as high an infectivity titer as practically possible to ensure meaningful pathogenicity comparison. Inocula of the chimeric viruses and cloned isolate 10 were standardized at $10^{6.0}$ 50% tissue culture infective doses ($TCID_{50}$)/0.1 ml, except for those of group 5, which were used at $10^{5.75}$ $TCID_{50}$/0.1 ml, the maximum titer of this chimeric virus pool. The cloned Cux isolate was also inoculated at its maximum (undilute) infectivity titer, $10^{5.5}$ $TCID_{50}$/0.1 ml. The pathogenic control virus, with an infectivity titer of $10^{6.75}$ $TCID_{50}$/0.1 ml, was included to test the susceptibility of the chicks and to facilitate comparison with results of earlier experiments (20).

**Nucleotide sequence accession number.** The nucleotide sequence of CAV cloned isolate 10 has been deposited in the GenBank nucleotide sequence database under the accession no. U66304.

## RESULTS AND DISCUSSION

This study was designed to elucidate the nature and significance of nucleotide sequence differences present in the genomes of molecularly cloned CAV isolates of differing pathogenicities. This paper contains the first published nucleotide sequence of an attenuated CAV isolate and the first report of an investigation into the molecular basis of CAV attenuation in vivo.

**Nucleotide sequence of cloned isolate 10: comparison with that of cloned Cux isolate and those of other sequenced isolates.** The nucleotide sequence (2,319 nt) of the attenuated cloned isolate 10 is given in Fig. 1. The most striking difference between this sequence and that of the pathogenic cloned Cux isolate is the 21-nt insertion which is located within the noncoding, transcription-regulatory region of the genome (12, 13) (Fig. 1 and 2). In an earlier paper describing the molecular cloning of cloned isolate 10, we reported that this isolate con-

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   1 CGCAGAAAAT AGATTTATCG CACTATCGAA TTCCGAGTGG TTACTATTCC ATCACCATTC TAGCCTGTAC ACAGAAAGTC AAGATGGACG AATCGCTCGA
 101 CTTCGCTCGC GATTCGTCGA AGGCGGGGGG CCGGAGGCCC CCCGGTGGCC CCCCTCCAAC GAGTGGAGCA CGTACAGGGG GGTACGTCAT CCGTACAGGG
             NNNNNNNNNNNNNNNNNNNN N
 201 GGGTACGTCA TCCGTACAGG GGGGTACGTC ACAAAGAGGC GTTCCCGTAC AGGGGGGTAC GTCACGCGTA CAGGGGGGTA CGTCACAGCC AATCAAAAGC
                               C                A
 301 TGCCACGTTG CGAAAGTGAC GTTTGGAAAA TGGGCGGCGC TAGCCTCTCT ATATATTGAG CGCACATACC GGTCGGCAGT AGGTATACGC AAGGCGGTCC
 401 GGGTGGATGC ACGGGAACGG CGGACAACCG GCCGCTGGGG GCAGTGAATC GGCGCTTAGC CGAGAGGGGC AACCTGGGCC CAGCGGAGCC GCGCAGGGGC
           C
 501 AAGTAATTTT AAATGAACGC TCTCCAAGAA GATACTCCAC CCGGACCATC AACGGTGTTC AGGCCACCAA CAAGTTCACG GCCGTTGGAA ACCCCTCACT
                                 G
 601 GCAGAGAGAT CCGGATTGGT ATCGCTGCAA TTACAATCAC TCTATCGCTG TGTGGCTGCG CGAATGCTCG CGCTCCCACG CTAAGATCTG CAACTGCGGA
                          C                                                    T
 701 CAATTCAGAA AGCACTGGTT TCAAGAATGT GCCGGACTTG AGGACCGATC AACCCAAGCC TCCCTCGAAG AAGCGATCCT GCGACCCCTC CGAGTACAGG
                                                                   A
 801 GTAAGCGAGC TAAAAGAAAG CTTGATTACC ACTACTCCCA GCCGACCCCG AACCGCAAGA AGGCGTATAA GACTGTAAGA TGGCAAGACG AGCTCGCAGA
 901 CCGAGAGGCC GATTTTACGC CTTCAGAAGA GGACGGTGGC ACCACCTCAA GCGACTTCGA CGAAGATATA AATTTCGACA TCGGAGGAGA CAGCGGTATC
1001 GTAGACGAGC TTTTAGGAAG GCCTTTCACA ACCCCCGCCC CGGTACGTAT AGTGTGAGGC TGCCGAACCC CCAATCTACT ATGACTATCC GCTTCCAAGG
1101 GGTCATCTTT CTCACGGAAG GACTCATTCT GCCTAAAAAC AGCACAGCGG GGGGCTATGC AGACCACATG TACGGGGCGA GAGTCGCCAA GATCTCTGTG
                                                  A
1201 AACCTGAAAG AGTTCCTGCT AGCCTCAATG AACCTGACAT ACGTGAGCAA ACTCGGAGGC CCCATCGCCG GTGAGTTGAT TGCGGACGGG TCTAAATCAC
     A          C
1301 TAGCCGCGGA GAATTGGCCT AATTGCTGGC TGCCGCTAGA TAATAACGTG CCCTCCGCTA CACCATCGGC ATGGTGGAGA TGGGCCTTAA TGATGATGCA
1401 GCCCACGGAC TCTTGCCGGT TCTTTAATCA CCCAAAGCAG ATGACCCTGC AAGACATGGG TCGCATGTTT GGGGGCTGGC ACCTGTTCCG ACACATTGAA
1501 ACCCGCTTTC AGCTCCTTGC CACTAAGAAT GAGGGATCCT TCAGCCCCGT GGCGAGTCTT CTCTCCCAGG GAGAGTACCT CACGCGTCGG GACGATGTTA
                                        G                                A
1601 AGTACAGCAG CGATCACCAG AACCGGTGGC AAAAAGGCCA ACAACCGATG ACGGGGGGCA TTGCTTATGC GACCGGGAAA ATGAGACCCG ACGAGCAACA
                                                                                                              A
1701 GTACCCTGCT ATGCCCCCAG ACCCCCCGAT CATCACCGCT ACTACAGCGC AAGGCACGCA AGTCCGCTGC ATGAATAGCA CGCAAGCTTG GTGGTCCTGG
                                              C G                  A
1801 GACACATATA TGAGCTTTGC AACACTCACA GCACTCGGTG CACAATGGTC TTTTCCTCCA GGGCAGCGTT CAGTTTCTAG ACGGTCCTTC AACCACCACA
1901 AGGCGAGAGG AGCCGGGGAC CCCAAGGGCC AGAGATGGCA CACGCTGGTG CCGCTCGGCA CGGAGACCAT CACCGACAGC TACATGTCAG CACCCGCATC
2001 AGAGCTGGAC ACTAATTTCT TTACGCTTTA CGTAGCGCAA GGCACAAATA AGTCGCAACA GTACAAGTTC GGCACAGCTA CATACGCGCT AAAGGAGCCG
2101 GTAATGAAGA GCGATGCATG GGCAGTGGTA CGCGTCCAGT CGGTCTGGCA GCTGGGTAAC AGGCAGAGGC CATACCCATG GGACGTCAAC TGGGCGAACA
2201 GCACCATGTA CTGGGGGACG CAGCCCTGAA AAGGGGGGGG GGCTAAAGCC CCCCCCCCTT AAACCCCCCC CTGGGGGGGA TTCCCCCCCA GACCCCCCCT
     T
2301 TAATATAGCA CTCAATAAA
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FIG. 1. Nucleotide sequence of CAV cloned isolate 10. The nucleotide sequence differences between cloned isolate 10 and the parental Cux-1 isolate are shown above the presented sequence. The position of the additional 21-nt insertion in the cloned isolate 10 genome is shown as N's.

tained a fifth 19-nt repeat (contained within the 21-nt insertion) in addition to the four repeats present in the Cux-1 isolate populations at low passage numbers (20). The insertion, which became established within the Cux-1 CAV population after between 20 and 30 passages in MDCC-MSB1 cells, has also been detected in another pathogenic Cux-1 isolate (11) and in the T82-2 isolate (3) but is not present in other sequenced isolates (26P4 [1], CIA-1 [14]) (Table 2). We have previously speculated that isolates possessing the additional repeat may be better suited to replication in MDCC-MSB1 cell cultures (20). However, the contribution of this insertion to the attenuation of cloned isolate 10 is questionable since partial sequencing analysis showed that pathogenic cloned isolates, molecularly cloned from the same RF preparation as cloned isolate 10 (20), also contain the insertion (data not shown).

In addition to the insertion, 17 individual nucleotide sequence differences were identified between cloned isolate 10 and the cloned Cux isolate (10). These differences were dispersed throughout the genome (Fig. 2), with three being located in the nontranscribed region. The number of nucleotide differences between cloned isolate 10 and the cloned Cux isolate is relatively small considering the levels of sequence variation already identified in previous analyses of published sequences (2, 14). Cloned isolate 10 is most closely related to the Cux-1 isolate sequenced by Noteborn et al. (11), differing at 14 nucleotide positions and most distantly related to the T82-2 isolate (3) (Table 2). Close examination indicated that the genome sequence of cloned isolate 10 differs from the other published complete genome sequences at nine unique nucleotide positions. We have previously reported that the CAV (Cux-1 isolate) population that has received 173 cell culture passages is genetically diverse (20). However, the level of sequence diversity resulting from cell culture passage and the associated mutation rate have yet to be established.

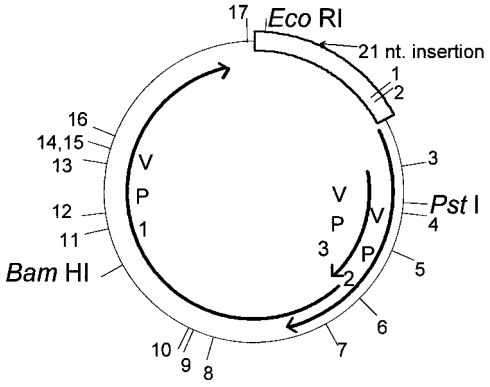


FIG. 2. Genome organization of CAV showing the positions of the 17 nucleotide sequence changes present in the genome of cloned isolate 10, compared to that of the cloned Cux isolate. The transcription-regulatory region containing the 21-nt insertion is boxed. The positions of the three restriction sites used for construction of the chimeric viruses are also shown.

TABLE 2. Nucleotide and protein sequence differences between cloned isolate 10 and other reported CAV isolates

| CAV isolate (GenBank accession no.) | No. of nucleotide sequence differences from cloned isolate 10 | Additional repeat | No. of amino acid differences from cloned isolate 10 | | |
|---|---|---|---|---|---|
| | | | VP1 | VP2 | VP3 |
| Cloned Cux-1 (M81223) | 17 | − | 7 | 4 | 3 |
| Kato (D31965) | 58 | + | 10 | 4 | 3 |
| Noteborn (M55918) | 14 | + | 5 | 2 | 2 |
| Claessens (D10068, D01218) | 54 | − | 7 | 4 | 5 |
| Renshaw ≈ Soiné (L14767) | 46 | − | 9 | 4 | 3 |
| Renshaw L-028 (U69549) | | | 9 | | |
| Renshaw ConnB (U69548) | | | 10 | | |
| Pallister (S71488) | | | 8 | | |

Nucleotide differences between cloned isolate 10 and the cloned Cux isolate resulted in 14 amino acid changes, of which 7 were found in VP1, 4 were found in VP2, and 3 were found in VP3. Comparison of the amino acid sequences of cloned isolate 10 with those of other sequenced isolates indicated the levels of amino acid diversity (Table 2). Closer examination indicated that cloned isolate 10 possessed five amino acid changes which were not present in isolates for which total or partial (VP1) sequences were available. Of these changes, two were found in VP1, two were found in VP2, and one was found in VP3.

Renshaw et al. (14) have recently identified a hypervariable region (amino acids 139 to 152) within VP1, which corresponds to the major capsid protein, and have suggested that this region may have the potential to affect in vitro cell cytopathogenicity. The possible function of this hypervariable region in host pathogenesis was not, however, investigated. Of the seven amino acid differences that exist between cloned isolate 10 and the cloned Cux isolate, two amino acid differences (Q → L [position 141] and D → E [position 144]) were located in the hypervariable region. One of the five amino acid positions (L, position 141), unique to cloned isolate 10, was located within the hypervariable region.

Such differences may be of questionable significance, because we cannot be certain that the isolates T82-2 (3), CIA-1 (14), and 26P4 (1) possess pathogenic phenotypes in vivo. Only in the case of the Cux-1 isolates (10, 11) has it been shown that viruses produced by transfection with the RF DNAs sequenced are pathogenic.

**Production of chimeric viruses and cloned parental isolates.** Three pairs of chimeric viruses were produced. Their designs exploited the occurrence of three unique restriction sites in the CAV RF: *Eco*RI at nucleotide position 29, *Pst*I at nucleotide position 604, and *Bam*HI at nucleotide position 1535. This numbering system is based on the nucleotide sequence of cloned isolate 10 (which includes the 21-nt insertion). The positions of these restriction sites in relation to those in the genomes of the parental CAV RFs, along with a diagrammatic representation of the resultant chimeric viruses generated following transfection with cloned parental genome fragments, are shown in Fig. 2 and 3.

The six chimeric viruses and the cloned parental isolates (Cux isolate and isolate 10) were produced by transfecting MDCC-MSB1 cells. The number of cell culture passages required to produce infecting virus pools together with their respective infectivity titers are shown in Table 1. The cloned Cux isolate attained an infectivity titer less than that of cloned isolate 10 and those of the chimeric viruses. This finding is not surprising given the fact that the cloned Cux isolate was derived from a virus pool at a low passage number and that we have previously shown that the infectivity titer of the Cux-1 isolate of CAV increases markedly with multiple cell culture passages (20). The infecting inocula of cloned isolate 10 and selected chimeric viruses were adjusted to $10^{6.0}$ $TCID_{50}$ to achieve a meaningful comparison (Table 1). Previous work in this laboratory indicated that virus inocula with infectivity titers of less than $10^{5.75}$ $TCID_{50}$ failed to exhibit maximum pathogenic effect (6).

**Pathogenicity evaluations of chimeric viruses and cloned parental isolates.** The results of the pathogenicity evaluation for each treatment group are given in Table 3. A number of points are noteworthy. The cloned Cux isolate resembled the pathogenic control virus in being highly pathogenic. Despite the smaller virus inoculum used, chicks infected with the cloned Cux isolate exhibited a low hematocrit value similar to that found with the pathogenic control virus (group 9) and, based on the severity of the thymus and bone marrow lesions,

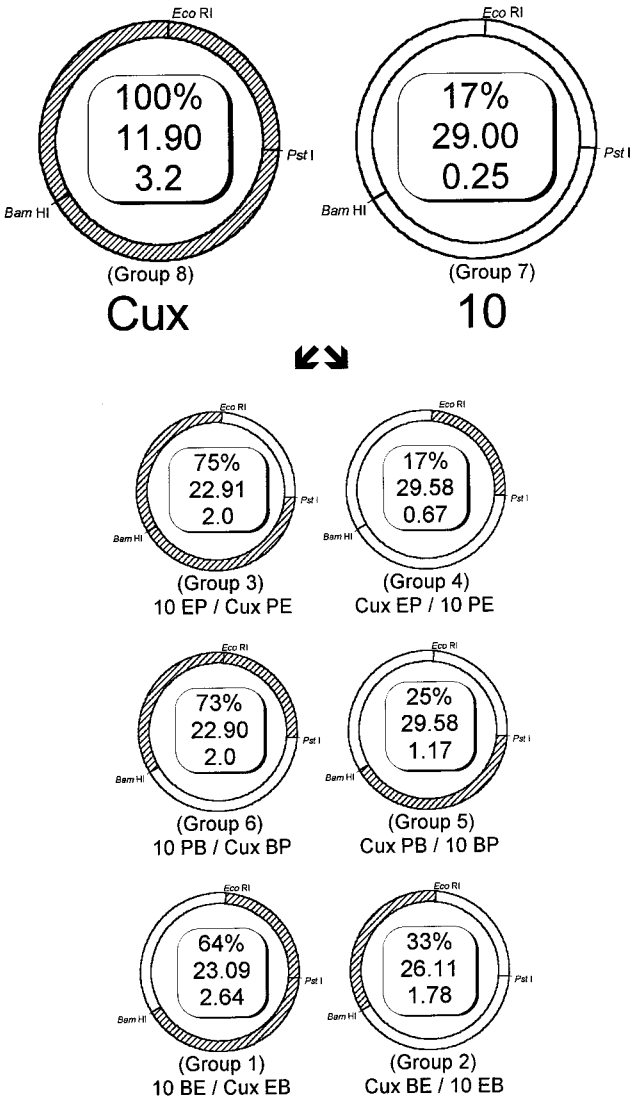


FIG. 3. Diagrammatic representations of the genotype of each of the experimental groups. The percentage of chicks with anemia, mean hematocrit value, and mean clinical score for each group, in that order, appear within the diagrams.

TABLE 3. Pathogenicity evaluations of cloned parental isolates and chimeric viruses

| Group | Description | No. of chicks | Hematocrit values | No. of chicks with anemia[a] (%) | Mean hematocrit value ± SD | No. of chicks (score) with: | | Mean clinical score |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Thymus atrophy | Bone marrow paleness | |
| 1 | Isolate 10 BE-Cux isolate EB | 11[b] | 33, 15, 37, 17, 20, 19, 18, 12, 32, 21, 30 | 7 (64) | 23.09 ± 8.37 | 4 (1+)<br>4 (2+) | 1 (1+)<br>8 (2+) | 2.64 |
| 2 | Isolate 10 EB-Cux isolate BE | 9[b] | 15, 30, 30, 34, 32, 28, 28, 12, 26 | 3 (33) | 26.11 ± 7.55 | 6 (1+) | 4 (1+)<br>3 (2+) | 1.78 |
| 3 | Isolate 10 EP-Cux isolate PE | 12 | 37, 21, 25, 17, 37, 18, 16, 27, 16, 23, 13, 25 | 9 (75) | 22.91 ± 7.85 | 6 (1+)<br>1 (2+) | 4 (1+)<br>6 (2+) | 2.00 |
| 4 | Isolate 10 PE-Cux isolate EP | 12 | 32, 29, 30, 32, 30, 33, 28, 25, 22, 31, 33, 32 | 2 (17) | 29.58 ± 4.32 | 3 (1+) | 3 (1+)<br>1 (2+) | 0.67 |
| 5 | Isolate 10 BP-Cux isolate PB | 12 | 24, 32, 26, 31, 33, 23, 31, 30, 39, 30, 29, 27 | 3 (25) | 29.58 ± 4.32 | 7 (1+) | 3 (1+)<br>2 (2+) | 1.17 |
| 6 | Isolate 10 PB-Cux isolate BP | 11[b] | 24, 29, 30, 17, 25, 16, 29, 18, 24, 20, 20 | 8 (73) | 22.90 ± 5.05 | 3 (1+)<br>2 (2+) | 5 (1+)<br>5 (2+) | 2.00 |
| 7 | Cloned isolate 10 | 12 | 30, 30, 31, 26, 30, 27, 30, 24, 30, 30, 30, 30 | 2 (17) | 29.00 ± 2.13 | 1 (1+) | 2 (1+) | 0.25 |
| 8 | Cloned low-number-passaged Cux isolate | 10[b] | 7, 6, 15, 12, 11, 15, 14, 7, 20, 12 | 10 (100) | 11.90 ± 4.38 | 2 (1+)<br>5 (2+) | 10 (2+) | 3.20 |
| 9 | Pathogenic control | 11[b] | 15, 21, 15, 19, 10, 9, 10, 26, 9, 12, 15 | 11 (100) | 14.64 ± 5.5 | 2 (1+)<br>7 (2+) | 1 (1+)<br>9 (2+) | 3.18 |
| 10 | Uninfected MSB1 control | 14 | 32, 33, 33, 34, 33, 33, 35, 35, 32, 33, 33, 33, 32, 32 | 0 (0) | 33.07 ± 0.99 | None | None | 0.00 |

[a] Anemia is defined as a hematocrit value of <27.
[b] Smaller group size as a result of chick death unrelated to CAV infection prior to experiment completion.

a similar high clinical score. Although 2 of 12 chicks inoculated with cloned isolate 10 were anemic, a higher proportion than those found previously (20), in comparison to results with the cloned Cux isolate, this cloned isolate exhibited substantially reduced pathogenicity, especially in terms of its very low clinical score.

To allow easier comparison of the results obtained with the chimeric viruses with those obtained with the parental cloned isolates, values for selected pathogenicity criteria, namely, the percentage of chicks with anemia, the mean hematocrit value, and the clinical score, have been included together with virus genotype in Fig. 3. The pathogenicity evaluations of the chimeric viruses are considered in two different ways. First, comparison of the results obtained with groups 1, 3, and 6 with those obtained with group 8 indicated that replacement of each of the three restriction fragment components in the cloned Cux isolate by its cloned isolate 10 counterpart had the effect of reducing the pathogenicity of the parental cloned Cux isolate. This result was evident from the reductions in the proportions of anemic chicks, the higher mean hematocrit values, and the lower clinical scores. On the basis of the broadly similar natures of the results achieved with these three chimeras, it is apparent that nucleotide changes present in each of the three genomic regions contribute substantially to the attenuation exhibited by cloned isolate 10. Second, in the complementary scenario, comparison of the results obtained with groups 2, 5, and 4 with those obtained with group 7 have allowed us to assess the effects of each of the three restriction fragment components specified by the pathogenic cloned Cux isolate on the attenuated behavior of cloned isolate 10. In this case, the restriction fragment replacement had the effect of increasing the pathogenicity of cloned isolate 10. Although the levels of changes in the proportions of anemic chicks and in the hematocrit values were small or nonexistent, examination of the clinical scores indicated that each of the three chimeric viruses was more pathogenic than the parental cloned isolate 10, with the chimeric virus containing the *Bam*HI-*Eco*RI fragment specified by the cloned Cux isolate (group 2) contributing most to pathogenicity and the *Eco*RI-*Pst*I fragment (group 4) contributing least to pathogenicity.

The results from both of these comparisons support the view that nucleotide changes controlling the attenuation exhibited by cloned isolate 10 do not reside in a single area of the CAV genome. Rather, it appears that its attenuation is a consequence of the cumulative effects of nucleotide changes in the three genomic regions investigated. This finding suggests that alterations in more than one of the three CAV-specified proteins as well as in the noncoding region of the genome may influence pathogenicity.

In their recent paper, Renshaw et al. (14) speculated that the hypervariable region of the capsid protein, which was identified as having an effect on in vitro cytopathogenicity, may also have a role in the in vivo pathogenicity of CAV. Our results indicated that this region, contained in the *Pst*I-*Bam*HI restriction fragment, does not contribute disproportionately to pathogenicity compared to other regions. Pathogenicity evaluations of additional chimeric viruses constructed to contain changes in the hypervariable region only would resolve this issue.

The results of this study have implications for the development and use of live attenuated CAV vaccines. Our finding that changes in a number of CAV genome areas contribute cumulatively to the attenuation of cloned isolate 10 contrasts with that found with other virus examples such as rabies, where lack of pathogenicity has been shown to result from a single amino acid substitution (17). As mutations throughout the genome of cloned isolate 10 may result in a more pathogenic phenotype, the prospect of generating a stably attenuated derivative of cloned isolate 10 is remote.

The molecular basis of the attenuation possessed by the partially attenuated live vaccine based on the 26P4 isolate has yet to be determined (19). We therefore do not know whether the 26P4 isolate, from which the vaccine was derived by chick embryo passage, has become attenuated through the acquisition of one mutation or, like cloned isolate 10, a number of mutations or whether the changes selected for by embryo pas-

sage are similar to those introduced by passage in MDCC-MSB1 cell culture.

Investigations are under way to determine whether the reversion to virulence exhibited by cloned isolate 10 following passage in chicks is due to back mutations in one or more of the 17 nucleotide differences identified above or whether compensating mutations elsewhere in the genome are responsible.

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