JOURNAL OF VIROLOGY, June 1991, p. 3131–3139
0022-538X/91/063131-09$02.00/0

Vol. 65, No. 6

Characterization of Cloned Chicken Anemia Virus DNA That Contains All Elements for the Infectious Replication Cycle

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Received 13 November 1990/Accepted 22 February 1991

Circular double-stranded replication intermediates were identified in low-molecular-weight DNA of cells of the avian leukemia virus-induced lymphoblastoid cell line 1104-X-5 infected with chicken anemia virus (CAV). To characterize the genome of CAV, we cloned linearized CAV DNA into the vector pIC20H. Transfection of the circularized cloned insert into chicken cell lines caused a cytopathogenic effect, which was arrested when a chicken serum with neutralizing antibodies directed against CAV was added. Chickens inoculated at 1 day of age with CAV collected from cell lines transfected with cloned CAV DNA developed clinical signs of CAV. The 2,319-bp cloned CAV DNA contained all the genetic information needed for the complete replication cycle of CAV. The CAV DNA sequence has three partially overlapping major reading frames coding for putative peptides of 51.6, 24.0, and 13.6 kDa. The CAV genome probably contains only one promoter region and only one poly(A) addition signal. Southern blot analysis using oligomers derived from the CAV DNA sequence showed that infected cells contained double- and single-stranded CAV DNAs, whereas purified virus contained only the minus strand. It is the first time that the genome of one of the three known single-stranded circular DNA viruses has been completely analyzed.

Chicken anemia virus (CAV) was first isolated by Yuasa et al. (51) during an investigation of Marek's disease outbreaks. The isolates were designated chicken anemia agent. The clinical signs of chicken anemia are caused by an agent which appears to be infectious and transmissible. Recently, other investigators have characterized this agent as an encapsidated virus (10, 42). In this article we describe the complete DNA genome of CAV, and we designate the agent CAV.

CAV causes severe anemia, subcutaneous and intramuscular hemorrhages, destruction of erythroblastoid cells in the bone marrow, and depletion of certain lymphoid organs in young chickens (46, 49, 51). Two weeks after infection of 1-day-old chickens that are specific pathogen free (SPF), the cortex of the thymic lobules becomes depleted. At that time the chickens are anemic. When the chickens are of 3 weeks old, the depleted areas of the thymus cortex are repopulated with thymocytes (18). The transient depletion of thymus may explain why chickens with CAV infections are not immunologically protected by certain vaccines, such as inactivated Newcastle disease vaccine (3) and infectious bronchitis vaccine (35a).

In the field, CAV infections per se seem to cause fewer deaths. Dual infections, however, are serious. Chickens with dual infections of CAV and Marek's disease virus, infectious bursal disease virus, reticuloendotheliosis virus, adenovirus, or reovirus may develop severe signs associated with the concurrent infection (9, 36, 47, 52). In an earlier investigation, we demonstrated that the pathogenesis of Marek's disease virus is enhanced by CAV (5, 6, 6a). Rosenberger and Cloud (37) have also reported that CAV aggravates the signs of infectious bursal disease. Exposing hens to CAV induces abundant maternal antibodies in chickens and largely prevents outbreaks of CAV infections and associated disease in the progeny of these chickens (43, 44).

CAV infections are distributed worldwide (23, 28, 37, 47, 51). We have recovered CAV, occurring in combination with Marek's disease virus of normal virulence, from materials originating from the United States, Israel, Tunisia, and The Netherlands (5, 6). CAV isolates thus far appear to have only one serotype (28, 29).

CAV particles have an average diameter of 25 nm (10) or 23.5 nm (42). In CsCl gradients, CAV has a buoyant density of 1.33 to 1.34 g/ml. Gelderblom et al. (10) and Todd et al. (42) have elegantly demonstrated circular single-stranded CAV DNA molecules. The length of the CAV genome was estimated at 2,300 nucleotides. One major polypeptide with an M_r of 50,000 has been described elsewhere (42).

In infected cells, viruses with a circular single-stranded DNA genome should generate a double-stranded DNA, which is needed for the correct transcription and replication of the virus DNA. In the present study, we isolated double-stranded DNA, cloned it in *Escherichia coli*, and determined its DNA sequence. The cloned material was characterized for its biological properties.

MATERIALS AND METHODS

Abbreviations. c-CAV, cloned CAV; CPE, cytopathogenic effect; ORF, open reading frame; PBFD, psittacine beak and feather disease; t-CAV, DNA extracted from CAV-infected 1104-X-5 cells; ATF, activating transcription factor.

Virus and cells. Two chicken lymphoblastoid cell lines were used: the B-cell line 1104-X-5 induced by avian leukemia virus (15) and the chicken T-cell line MDCC-MSB1 transformed by Marek's disease virus (48, 50). These cells were infected with the Cuxhaven-1 isolate of CAV (45, 46). Cells were infected with a multiplicity of infection of 0.1 to 1

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per cell. CAV was purified from infected MDCC-MSB1 cells as described by Gelderblom et al. (10).

Extraction of total and low-molecular-weight DNA. DNA was extracted from virus particles and virus-infected cells. Samples were suspended in 20 mM Tris-HCl (pH 7.5) containing 2 mM EDTA, 0.2% sodium dodecyl sulfate, and 0.6 mg of proteinase K per ml. The suspensions were incubated for 1 h at 37°C and subsequently extracted with phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was precipitated with ethanol and suspended in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA. Low-molecular-weight DNA was isolated from tissue culture cells as described by Hirt (16).

Southern blot analysis of DNA. The DNA was fractionated on agarose gels and blotted onto Biotrace RP filters (Gelman Sciences, Ann Arbor, Mich.) as described by Southern (39). The blots were hybridized with randomly primed ^{32}P-labeled CAV DNA and ^{32}P-end-labeled synthetic oligomers derived from the CAV DNA sequence reported in this article. The sequences of the synthetic oligomers were derived from the plus-DNA strand (5′-GCAGTAGGTATACGCAAGG-3′ [positions 349 to 366]) and the minus-DNA strand (5′-TTG CCCCTCTCGGCTAAGC-3′ [positions 445 to 427]).

Cloning of CAV DNA. The complete CAV DNA genome was cloned into the vector pIC20H, and fragments of it were cloned into the vector pIC19R (26). All plasmid DNA modifications were carried out as described by Maniatis et al. (25).

Sequence analysis of CAV DNA. Plasmid DNA was purified by centrifugation in a CsCl gradient and by column chromatography in Sephacryl-S500 (Pharmacia). Double-stranded DNA was sequenced with T7 DNA polymerase (Pharmacia), a modification (Pharmacia) of the method described by Sanger et al. (38). Some G/C-rich stretches of CAV DNA were sequenced by the Maxam and Gilbert technique (27). Oligonucleotides were 5′ labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Pharmacia).

Circularization of c-CAV DNA. Plasmid DNAs from pCAV/E and pCAV/P containing the complete CAV DNA genome (10 μg each) were digested with *Eco*RI or *Pst*I, respectively. T4 DNA ligase treatment resulted in circularization of the insert DNA. Ligation products were analyzed on a 0.8% agarose gel stained with ethidium bromide.

DEAE-dextran transfection. For transfections, the DEAE-dextran method of Luthman and Magnusson was used (24), but the chloroquine treatment was replaced with a dimethyl sulfoxide boost. Two micrograms of circularized CAV DNA was used for transfection of the lymphoblastoid cell line MDCC-MSB1 or 1104-X-5. The transfected cells were observed for CPE. Up to six serial passages were made in both cell lines. Lysates of cells inoculated with supernatant from passage 6 were analyzed for the presence of intracellular CAV DNA.

Neutralization of c-CAV. MDCC-MSB1 cells were infected with supernatants of MDCC-MSB1 or 1104-X-5 cells that had been transfected with c-CAV DNA or control DNA. About 2×10^6 cells were infected with undiluted supernatants of the transfected cells. Polyclonal chicken serum with neutralizing antibodies directed against CAV was diluted 1:100 and added to half of the infected cell cultures. The absence of CPE in MDCC-MSB1 cells infected with CAV was considered positive neutralization.

Preparation of monoclonal antibody CVI-CAV-85.1. For each immunization, antigen was prepared by freeze-thawing and sonicating 3×10^7 MDCC-MSB1 cells infected with CAV. First, BALB/c mice were immunized intraperitoneally with incomplete Freund's adjuvant. Then, 4 weeks later they were immunized again intraperitoneally with incomplete Freund's adjuvant. Finally, 4 weeks later they were immunized intravenously without adjuvant. Four days later, spleen cells of immunized mice were fused with P3X63-Ag8.653 myeloma cells. Antibodies directed against CAV were detected by three different methods: a microneutralization test was used for the first screening, fluorescence staining was used on CAV-infected MDCC-MSB1 cells, and immunoperoxidase staining was used for the last screening to screen bone marrow and thymus samples of CAV-infected chickens as described for chicken tissues by Jeurissen et al. (17). The monoclonal antibody CVI-CAV-85.1 stained specifically CAV-infected MDCC-MSB1 cells. In SPF chickens inoculated at 1 day of age with a field strain of CAV, the monoclonal antibody CVI-CAV-85.1 stained cells in the thymus cortex and bone marrow at day 6 after infection.

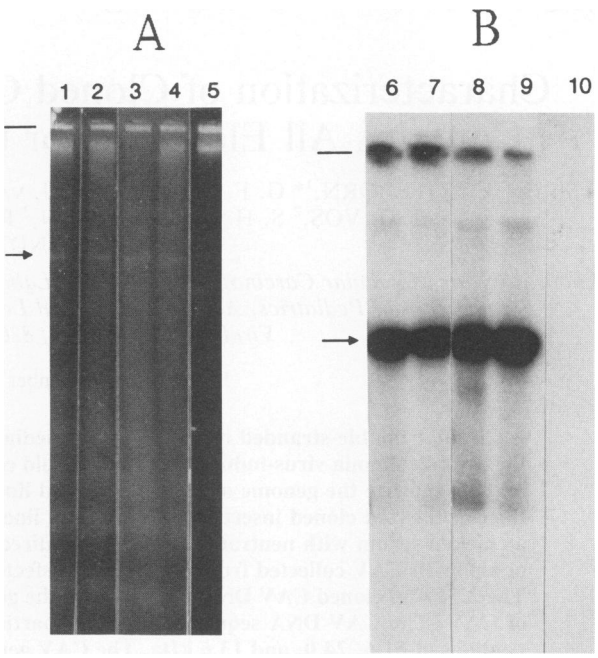


FIG. 1. Identification and cloning of 2.3-kb DNA isolated from 1104-X-5 cells infected with CAV. (A) Low-molecular-weight DNA isolated from infected 1104-X-5 cells was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Pst*I (lane 3), or *Xba*I (lane 4); DNA from uninfected 1104-X-5 cells was digested with *Eco*RI (lane 5). DNA fragments were size fractionated on a 0.8% agarose gel and stained with ethidium bromide. (B) Southern blot. Low-molecular-weight DNA from infected 1104-X-5 cells digested by *Bam*HI, *Eco*RI, *Pst*I, or *Xba*I (lanes 6 through 9) and *Eco*RI-digested DNA from uninfected 1104-X-5 cells (lane 10) were hybridized with ^{32}P-labeled c-CAV DNA. Symbols: —, slots; ⟶, 2.3-kb DNA.

Infection of 1-day-old SPF chickens with c-CAV. Supernatants of MDCC-MSB1 and 1104-X-5 cells that were transfected with c-CAV DNA were intramuscularly inoculated into 1-day-old SPF chickens. Fourteen days after infection, the hematocrit level and total body weights were measured. Samples of heparinized peripheral blood, bone marrow, spleen, bursa of Fabricius, and thymus were collected. Cryostat sections of lymphoid organs were stained by using monoclonal antibody CVI-CAV-85.1 directed against CAV, monoclonal antibody CVI-ChT-74.1 (18) directed against chicken thymocytes, a general marker for chicken leukocytes, monoclonal antibody HIS-C7 (17), and rabbit anti-mouse peroxidase conjugate.

TABLE 1. CPE of c-CAV DNA and its progeny on lymphoblastoid cell lines MDCC-MSB1 and 1104-X-5

Procedure on CAV and c-CAV DNA[a]	CPE after 2 to 3 serial passages on:	
	MDCC-MSB1 cells	1104-X-5 cells
Transfection		
c-CAV/P	+	+/−[b]
c-CAV/E	+	+
t-CAV[c]	+	+/−[b]
Control[d]	−	−
Infection		
c-CAV/P[e]	+	NA[f]
c-CAV/E[g]	+	NA
t-CAV	+	NA
c-CAV/P + antiCAV[h]	−	NA
c-CAV/E + antiCAV	−	NA
t-CAV + antiCAV	−	NA

[a] CAV/P and CAV/E DNAs contain complete CAV DNA. They were cloned into the *Pst*I (CAV/P) site and the *Eco*RI (CAV/E) site of pIC20H, respectively.
[b] Transfected 1104-X-5 cells showed no CPE after six blind passages. The supernatants of passage 3, however, yielded CPE 2 days after infection of MDCC-MSB1 cells.
[c] DNA was extracted from 1104-X-5 cells infected with CAV.
[d] pIC20H plasmid DNA.
[e] c-CAV progeny of the transfected DNA was added to chicken cells.
[f] NA, not applicable.
[g] DNA sequence of c-CAV/E is shown in Fig. 5.
[h] Chicken serum with neutralizing antibodies against CAV.

Computer analyses. The GCG software package (7) was used to compare DNA sequence data. The EMBL and GenBank data banks were screened for sequences homologous to CAV DNA by means of the FASTA and AFASTA programs (32). ORFs and protein similarities were established on an ATARI ST with the help of our own software (C.K.).

Nucleotide sequence accession number. The GenBank accession number for the sequence given in this paper is M55918.

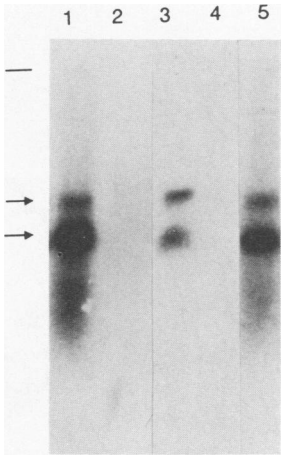


FIG. 2. Southern blot analysis of DNA isolated from cells infected with c-CAV. Cultures of MDCC-MSB1 cells (lanes 1 and 2) or 1104-X-5 cells (lanes 3 and 4) were inoculated with c-CAV (lanes 1 and 3) or supernatant derived from control cells transfected with DNA (lanes 2 and 4). MDCC-MSB1 cells infected with CAV (Cuxhaven-1 isolate) were used as a positive control (lane 5). Symbols: —, slots; ⟶, CAV DNA products.

TABLE 2. Clinical signs at 14 days after chickens were infected with virus released from lymphoblastoid cell lines transfected with c-CAV DNA

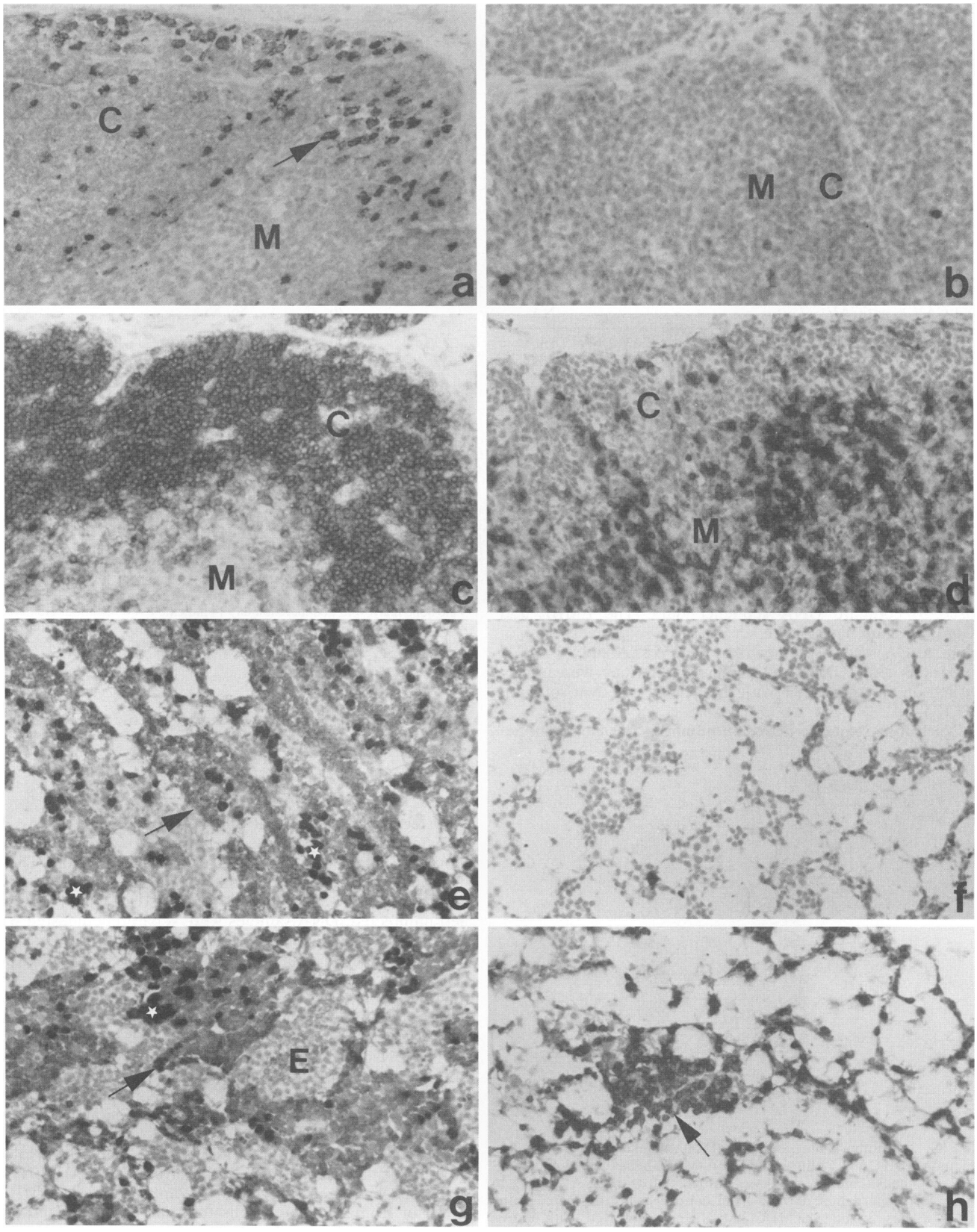
Inoculum virus[a]	Clinical signs of CAV in inoculated chickens[b]		
	Hematocrit value (%)	Body wt (g)	Thymus atrophy and pale bone marrow (no. affected/no. examined)
c-CAV/P	21, 27, 19, 26, 20, 14, 22, 30, 16, 27	116, 126, 81, 119, 92, 108, 99, 98, 106, 111	10/10
c-CAV/E	19, 21, 21, 26, 19, 16, 10, 16, 17, 20	111, 133, 137, 132, 116, 73, 102, 83, 86, 85	10/10
t-CAV	17, 18, 24, 21, 19, 16, 19, 24, 24, 20	113, 103, 118, 116, 99, 114, 130, 128, 107, 104	10/10
Uninfected control	33, 33, 35, 34, 34	114, 124, 135, 148, 186	0/5

[a] c-CAV/P, c-CAV/E, and t-CAV were collected after six serial passages in MDCC-MSB1 or 1104-X-5 cells. c-CAV/P and c-CAV/E were grown in cells transfected with CAV DNA cloned in the *Pst*I or *Eco*RI site of pIC20H. The DNA sequence of c-CAV/E is given in Fig. 5. t-CAV was grown in cells transfected with DNA isolated from CAV-infected 1104-X-5 cells.
[b] Hematocrit values below 27% in combination with thymus atrophy and pale bone marrow at postinfection day 14 indicate CAV infection. Hematocrit value and body weight of a chicken are given at the same position in the relevant columns; e.g., a c-CAV/P-infected chicken with a hematocrit value of 21% has a body weight of 116 g.

RESULTS

Detection and cloning of double-stranded CAV DNA. We reasoned that CAV-infected cells should contain a circular double-stranded DNA needed for virus replication. Therefore, we isolated low-molecular-weight DNA from 1104-X-5 cells infected with CAV (Cuxhaven-1 isolate) and from cells left uninfected. DNA samples were separately digested with restriction enzyme *Bam*HI, *Eco*RI, *Pst*I, or *Xba*I, all of which recognized a unique site on CAV DNA (see below). The DNA samples were size fractionated on a 0.8% low-melting-point agarose–ethidium bromide gel. The DNA from CAV-infected cells had a clearly visible DNA band of 2.3 kb that was absent in the DNA of uninfected cells (Fig. 1A).

The DNA fragments of 2.3 kb were isolated and cloned into the vector pIC20H, which had been cleaved with the appropriate restriction enzyme and treated with calf intestine alkaline phosphatase. The plasmids containing all four differently integrated DNA fragments were ^{32}P labeled. The labeled plasmids were hybridized to a Southern blot of low-molecular-weight DNA either from infected 1104-X-5 cells that had been digested with *Bam*HI, *Eco*RI, *Pst*I, or *Xba*I or from uninfected cells that had been digested with *Bam*HI. A 2.3-kb band of low-molecular-weight DNA isolated from infected cells hybridized with all four probes.


C
M
a
M C
b
C
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c
C
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e
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FIG. 3. Histopathology of thymus and bone marrow of 1-day-old chickens inoculated with c-CAV/E cultured on MDCC-MSB1 cells. Monoclonal antibodies used were CVI-CAV-85.1 against CAV, CVI-ChT-74.1 against thymocytes, and HIS-C7 against leucocytes (17). Magnification, ×250. C, cortex; M, medulla; E, erythrocytes. (a) After 6 days, many CVI-CAV-85.1-positive cells (arrow) were detected in the cortex of the thymus. (b) After 14 days, CVI-CAV-85.1-positive cells were absent from the thymus. (c) After 6 days, CVI-ChT-74.1 stained thymocytes in the whole cortex and scattered cells in the medulla. (d) After 14 days, CVI-ChT-74.1 could not stain thymocytes in the depleted cortex. CVI-ChT-74.1-positive cells were still detected in the medulla. (e) After 6 days, large areas of CVI-CAV-85.1-positive cells (arrow) were detected in the bone marrow. In addition, some endogenous peroxidase-containing granulocytes (★) could be seen. (f) After 14 days, CVI-CAV-85.1-positive cells were absent from the bone marrow. (g) After 6 days, monoclonal antibody HIS-C7 stained leukocytes in the bone marrow between areas of HIS-C7-negative erythrocytes. Endogenous peroxidase-containing granulocytes (★) could also be seen. (h) After 14 days, bone marrow consisted only of HIS-C7-positive leukocytes.

DNA isolated from uninfected 1104-X-5 cells did not hybridize. The Southern blot hybridized with ^{32}P-labeled DNA derived from clone pCAV/E, which contains CAV DNA in the *Eco*RI site, is shown in Fig. 1B.

Sites recognized by the four restriction enzymes were mapped on the CAV genome at different positions (see Fig. 4). Digestions of CAV DNA with these enzymes always yielded linear DNA fragments of equal lengths (Fig. 1). Therefore, we concluded that the double-stranded CAV genome is circular.

CPE of c-CAV DNA. To investigate whether the cloned DNA contained all sequences required for virus replication, we studied the effect of transfected cloned DNA in lymphoblastoid cell lines. CAV DNA was excised from pCAV/E or pCAV/P, circularized by self-ligation, and transfected into MDCC-MSB1 and 1104-X-5 cells. The circular DNA insert derived from pCAV/E (c-CAV/E DNA) caused swelling and granular degeneration of cells in both lymphoblastoid cell lines after two to three serial passages. This CPE was specific, because the alterations did not occur in those wells to which neutralizing antibodies directed against CAV were added. DNA from pCAV/P produced a similar CPE in MDCC-MSB1 cells but altered 1104-X-5 cells only slightly. The latter cells, however, were also successfully transfected, because the supernatants of the third blind passage caused CPE in MDCC-MSB1 cells. The virus derived from c-CAV DNA-transfected cells will subsequently be called c-CAV. In the negative control (cells transfected with pIC20H plasmid DNA), no CPE was observed. t-CAV DNA caused a CPE in MDCC-MSB1 cells; its virus progeny is designated t-CAV. Polyclonal neutralizing antibodies directed against CAV prevented a CPE in MDCC-MSB1 cells infected with c-CAV and t-CAV (Table 1). Figure 2 shows the presence of intracellular CAV DNA in cells infected with c-CAV and wild-type CAV. The DNA molecules of the c-CAV are similar to those of wild-type CAV, suggesting that our c-CAV DNA contains all elements necessary for replication. The double-stranded and single-stranded forms of the CAV DNAs will be discussed below.

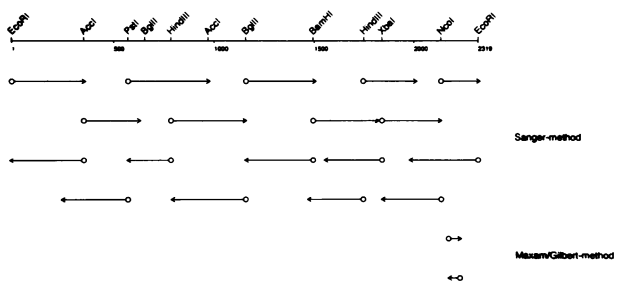

FIG. 4. Sequencing strategy. CAV DNA was sequenced in both directions.

Pathogenic effect of c-CAV in chickens. When 1-day-old chickens were intramuscularly inoculated with c-CAV, they exhibited all clinical signs associated with infection with field strains of CAV. At day 14, their bone marrow was pale and the hematocrit values were extremely low because of destruction of erythroblastoid cells. The infected chickens exhibited reduced total body weights and retarded growth rates (Table 2).

Microscopic examination of c-CAV-infected chickens demonstrated lesions in thymus and bone marrow identical to those caused by infection with a CAV field strain. No differences were seen between c-CAV/E and c-CAV/P or between clones multiplied on MDCC-MSB1 or 1104-X-5 cells. At day 6 after infection, monoclonal antibody CVI-CAV-85.1 demonstrated numerous CAV-positive cells in the thymus cortex (Fig. 3a) and the bone marrow (Fig. 3e). At day 14, CVI-CAV-85.1 no longer detected any CAV-positive cells in thymus (Fig. 3b), bone marrow (Fig. 3f), or other organs (data not shown). Thymocytes were still in evidence in the thymus cortex 6 days after infection (Fig. 3c) but had disappeared after 14 days (Fig. 3d), as could be demonstrated with antibody CVI-ChT-74.1 (18). Fourteen days after infection, bone marrow consisted only of HIS-C7-positive leukocytes (Fig. 3h), whereas bone marrow 6 days after infection consisted of equal numbers of HIS-C7-negative erythrocytes and HIS-C7-positive leukocytes (Fig. 3g). Apparently, erythrocytes are severely depleted at day 14 postinfection. Uninfected chickens in the control group had none of the clinical and histopathological signs described above. In view of the above-mentioned observations, we consider the CAV sequences cloned by us to represent the complete CAV genome.

DNA sequence of CAV. CAV DNA was sequenced by the Sanger method (38), with M13 sequencing and reverse-sequencing primers that hybridized to the vector DNA. One region contained a strong stop site and was therefore analyzed by the Maxam-Gilbert method (27). Both strands of the CAV DNA genome were sequenced completely by the strategy shown in Fig. 4.

Figure 5 shows the sequence of the strand arbitrarily called plus-DNA strand. The numbering starts at the first base of the *Eco*RI site of CAV DNA. The complete CAV DNA genome is 2,319 nucleotides long. The CAV DNA genome contained three major ORFs on the plus-DNA strand (Fig. 6). A computer search with the FASTA program (32) revealed no significant homology with sequences of known proteins.

A search for known transcription factor-binding sites showed that only one region in the genome had potential promoter-enhancer properties (positions about 150 to 350) (Table 3). A cap site was determined to lie at positions 352 to 356 (data not shown). The whole sequence contained only one polyadenylation signal (position 2287). The poly(A)

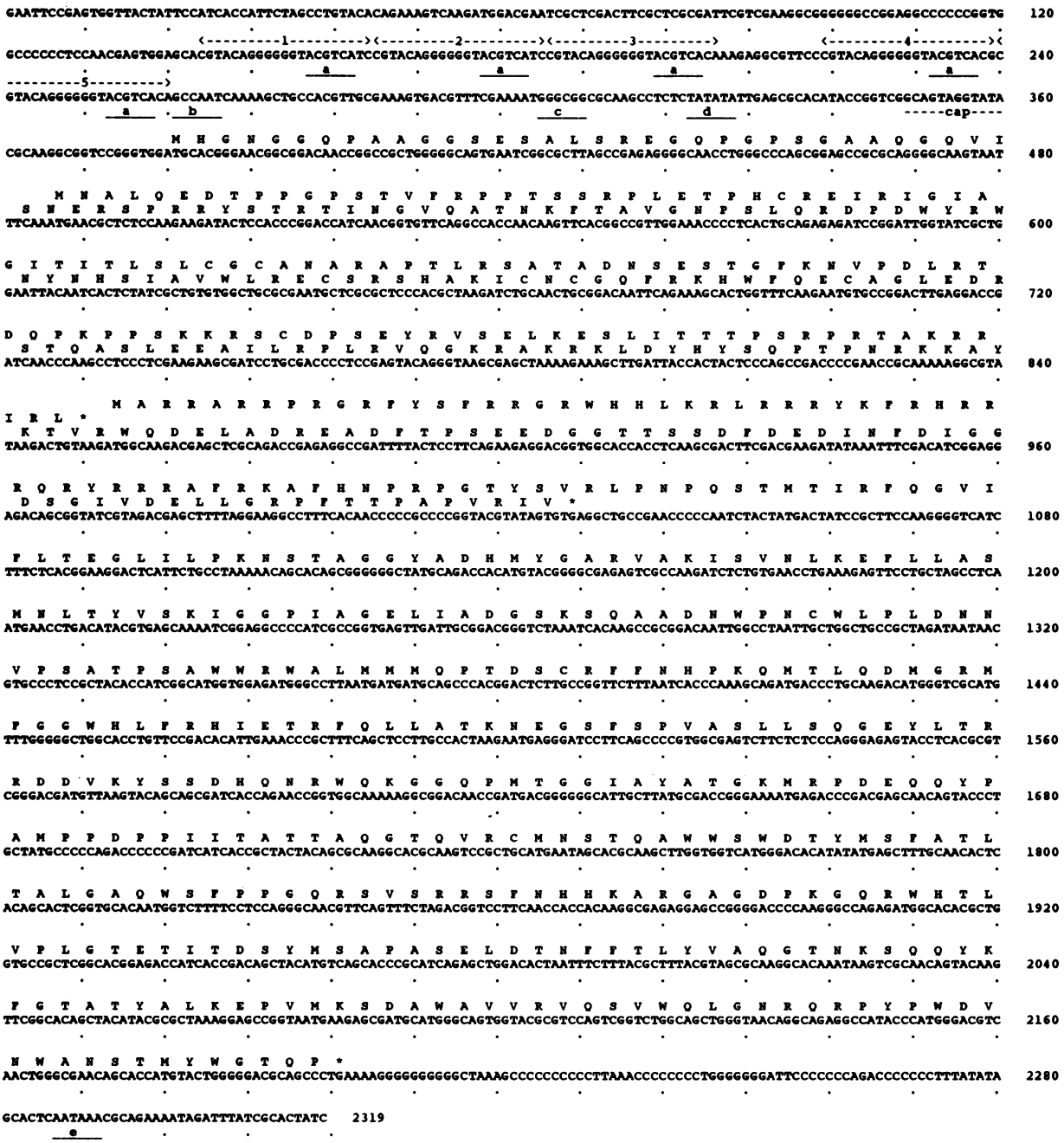

FIG. 5. Complete nucleotide sequence of CAV DNA (2,319 bp). The first nucleotide of the *Eco*RI site is considered to be residue +1. The plus-DNA strand is shown. Above the DNA sequence, the amino acid sequence of three potentially encoded polypeptides is given. 1 to 5, direct repeats; a, ATF-binding element; b, CCAAT-F-binding element; c, SP1-binding element; d, TFIID-binding element; e, poly(A) signal.

addition site was located at positions 2315 to 2319 (data not shown). Both transcription elements were located on the plus-DNA strand. A remarkable feature of the putative promoter-enhancer region was a sequence of five near-perfect 21-bp direct repeats situated between positions 144 and 260 with a gap of 12 bp between units 3 and 4 (Fig. 5).

Characteristics of CAV DNA. DNA from CAV-infected cells and partially purified CAV were blotted onto nylon filters and analyzed in a Southern assay with two synthetic oligomers derived from the CAV DNA plus and minus strands. The plus oligomer hybridized with two DNA products in the cellular DNA and one in the viral DNA. The minus oligomer, however, hybridized only with the upper band from cellular DNA (Fig. 7). The upper DNA band from CAV-infected cells was resistant to S1 nuclease, whereas the lower band was sensitive to it (data not shown). It was deduced that the lower band contained single-stranded CAV DNA and the upper band contained double-stranded CAV DNA. The virus particle contained the minus-DNA strand of CAV, which was complementary to the transcribed RNAs.

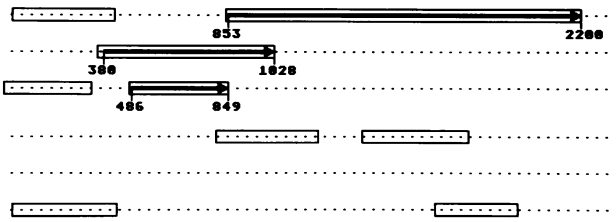

FIG. 6. Schematic representation of CAV ORFs longer than 300 nucleotides. The top three lines show the plus strand, and the bottom three lines show the minus strand. Open boxes are ORFs. The coding regions, whose translations are shown in Fig. 5, are indicated by arrows.

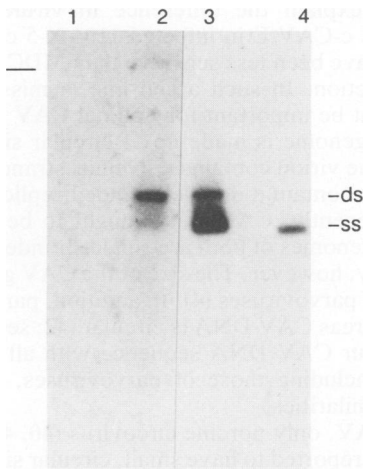

FIG. 7. Identification of the DNA strand in CAV particles. DNA from CAV-infected cells (lanes 2 and 3) and CAV particles (lanes 1 and 4) was isolated and blotted onto a nylon filter. The blotted DNA was hybridized with a ^{32}P-labeled synthetic DNA oligomer of the minus-DNA strand (lanes 1 and 2) or the plus-DNA strand (lanes 3 and 4). Symbols and abbreviations: —, slots; ds, double-stranded DNA; ss, single-stranded DNA.

DISCUSSION

We cloned a 2.3-kb circular DNA from CAV-infected chicken lymphoblastoid cells. The cloned material apparently represents the complete CAV DNA genome and contains all the elements required for the CAV replication cycle and pathogenicity. Transfection of c-CAV DNA into chicken cells caused the release of infectious virus that could be neutralized by antibodies directed against CAV. In addition, c-CAV caused the characteristic clinical signs of CAV infections: low hematocrit values, pale bone marrow, thymus atrophy, and retarded growth. The viral spread and histopathological changes observed in c-CAV-infected chickens were also similar to those caused by field isolates of CAV: depletion of erythroblastoid cells in bone marrow and moderate to severe depletion of cortical thymocytes (11–13, 18, 23, 31, 51).

Todd et al. (42) have reported that CAV contains one major capsid polypeptide with a molecular weight of 50,000. Our sequence data showed that CAV DNA contained three partially overlapping ORFs potentially encoding peptides of 51,600, 24,000, and 13,600 Da. Allowing for alternative start codons (e.g., CUG, GUG, and ACG [1, 14, 30]), one can construe additional coding regions from the CAV DNA sequence. The largest ORF may encode the CAV capsid peptide of 50 kDa described by Todd et al. (42). Since the sequences encoded by these ORFs do not resemble those of known viral and cellular proteins, CAV may be a unique virus.

The three ORFs starting at AUG partially or completely overlapped each other. We have evidence that CAV has one major start for RNA transcription and one polyadenylation site. These findings suggest that the use of different ORFs may be regulated by alternative splicing, as described for simian virus 40 (34) and polyomavirus (33), or that CAV encodes a polycistronic mRNA, as described for the large E1b mRNA of adenovirus (2) or the P/C mRNA of Sendai virus (8). However, we cannot exclude the possibility that, apart from the very obvious promoter region described in the next paragraph, less-prominent promoters are also used for regulating CAV transcription.

The putative CAV promoter-enhancer region contains a TATA box considered to be the RNA polymerase II transcription factor-binding sequence element. Other promoter elements (Table 2) can also be designated. The promoter-enhancer region of CAV contained five direct repeats, which are unique for CAV DNA. Each repeat contained a sequence that was homologous to the one that binds the ATF site. Lee et al. (22) reported that some ATF sites are activated by the E1A protein of adenovirus. Moreover, in dual infections of chickens, adenovirus is enhanced by CAV, as is shown by higher virus titers (47).

Preliminary data from the sequence analysis of pCAV/P show that this clone contains four instead of five direct repeats in the promoter-enhancer region. The missing direct

TABLE 3. Transcription factor-binding sequence elements[a]

Binding factor	Consensus sequence	CAV sequence	Position(s) in CAV sequence
TFIIID[b]	GTATA(A/T)A(A/T)	CTATATAT	321–330
CCAAT-TF[b]	AGCCAAT	AGCCAAT	260–266
SP1[b]	GGGCGG	GGGCGG	305–310
ATF[b]	ACGTCA	ACGTCA	253–258, 232–237, 199–204, 178–183, 157–162
CREB	(T/G)(T/A)CGTCA	GACGTTT	291–297
PEA-I[c]	GGAAGTGACTAAC	GAAAGTGACTTTC	286–298
GTII	G(G/C)TGTGGAA(A/T)GT	CGTTGCGAAAGT	279–290
MLTF	GGCCACGTGACC	TGCCACTGTCGA	274–285
CACCC-F	CACCC	CAGCC	259–263, 236–240
		CATCC	182–186, 161–165

[a] Binding factors and consensus sequences of the binding sites are reviewed in references 19, 20, and 21.
[b] Perfect homology between CAV and consensus sequence.
[c] Polyomavirus.

repeat may explain the difference in virulence between c-CAV/P and c-CAV/E in infected 1104-X-5 cells, which in our studies have been less sensitive than MDCC-MSB1 cells to CAV infection. In such a cell line, a missing enhancer element might be important for optimal CAV replication.

The CAV genome is made up of circular single-stranded DNA (42). The virion contains the minus strand of the DNA. Infected cells contain a double-stranded replicating form as well. Until recently, CAV was thought to be a parvovirus because the genomes of both are single stranded; this is their sole similarity, however. The size of the CAV genome differs from those of parvoviruses (4). In addition, parvovirus DNA is linear, whereas CAV DNA is circular (42; see above also). Comparing our CAV DNA sequence with all known DNA sequences, including those of parvoviruses, we found no significant similarities.

Besides CAV, only porcine circovirus (40, 41) and PBFD virus (35) are reported to have small, circular single-stranded DNA. The genomes of these viruses, however, are significantly smaller than that of CAV. Porcine circovirus particles contain one polypeptide of 36,000 Da; PBFD virus particles contain three proteins of 26,300, 23,700, and 15,900 Da. At the moment, it is unknown whether porcine circovirus and PBFD, like CAV, encapsidate the minus strand. These differences suggest that the three viruses do not belong to a single taxonomic group. However, until the other two viruses are more fully characterized, a close relationship between these viruses cannot be excluded.

CAV is the first of the viruses with circular single-stranded DNA to be fully characterized. The next step in this line of research will be to develop expression vectors derived from CAV DNA in avian cell culture systems. c-CAV DNA can now be used to develop diagnostic assays and vaccines against CAV infections. The development of a CAV vaccine will perhaps help to control not only CAV but also other diseases that are aggravated by CAV.

ACKNOWLEDGMENTS

We thank V. Thatcher for excellent editorial assistance and C. A. J. Verschueren and E. M. Janse for technical assistance.

REFERENCES

1. **Becerra, S. P., J. A. Rose, M. Hardy, B. M. Baroudy, and C. W. Anderson.** 1985. Direct mapping of adeno-associated virus capsid protein B and C: a possible ACG initiation codon. Proc. Natl. Acad. Sci. USA **82**:7919–7923.
2. **Bos, J. L., L. J. Polder, R. Bernards, P. I. Schrier, P. J. Van den Elsen, A. J. Van der Eb, and H. Van Ormondt.** 1981. The 2.2 kb E1b mRNA of human Ad12 and Ad5 codes for two tumor antigens starting at different AUG triplets. Cell **27**:121–131.
3. **Box, P. G., H. C. Holmes, A. C. Bushell, and P. M. Finney.** 1988. Impaired response to killed Newcastle disease vaccine in chicken anaemia agent. Avian Pathol. **17**:713–723.
4. **Cotmore, S. F., and P. Tattersall.** 1987. The autonomously replicating parvoviruses of vertebrates. Adv. Virus Res. **33**:91–174.
5. **De Boer, G. F., S. H. M. Jeurissen, D. J. Van Roozelaar, G. J. Vos, and G. Koch.** 1989. Enhancing effects of chicken anemia agent (CAA) on Marek's disease pathogenesis, p. 28. *In* Proceedings of the 38th Western Poultry Diseases Conference, Tempe, Ariz.
6. **De Boer, G. F., J. M. A. Pol, and S. H. M. Jeurissen.** 1988. Marek's disease vaccination strategies using vaccines made from three avian herpesvirus serotypes, p. 14–15. *In* Proceedings of the 1st International Poultry and Poultry Disease Symposium, Manisa, Turkey.

6a. **De Boer, G. F.** Unpublished data.

7. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
8. **Dillon, P., and K. C. Gupta.** 1989. Expression of five proteins from the Sendai virus P/C mRNA in infected cells. J. Virol. **63**:974–977.
9. **Engström, B. E.** 1988. Blue wing disease of chickens: isolation of avian reovirus and chicken anaemia agent. Avian Pathol. **17**:23–32.
10. **Gelderblom, H., S. Kling, R. Lurz, I. Tischer, and V. von Bülow.** 1989. Morphological characterization of chicken anaemia agent (CAA). Arch. Virol. **109**:115–120.
11. **Goodwin, M. A., R. C. Wellenstein, J. Brown, M. A. Smeltzer, W. L. Steffens, W. D. Waltman, and T. G. Dickinson.** 1989. Isolation and identification of a parvovirus-like virus, the so-called chick anemia agent (CAA) that causes infectious anemia in chicks, p. 21–23. *In* Proceedings of the 38th Western Poultry Diseases Conference, Tempe, Ariz.
12. **Goryo, M., Y. Shibata, T. Suwa, T. Umemura, and C. Itakura.** 1987. Outbreak of anemia associated with chicken anemia agent in young chickens. Jpn. J. Vet. Sci. **49**:867–873.
13. **Goryo, M., H. Sugimura, S. Matsumoto, T. Umemura, and C. Itakura.** 1985. Isolation of an agent inducing chicken anaemia. Avian Pathol. **14**:483–496.
14. **Hann, S. R., M. W. King, D. L. Bentley, C. W. Anderson, and R. N. Eisenman.** 1988. A non-AUG translational initiation in c-myc exon 1 generates an N-terminally distinct protein whose synthesis is disrupted in Burkitt's lymphomas. Cell **52**:185–195.
15. **Hihara, H., T. Shimizu, and H. Yamamoto.** 1974. Establishment of tumor cell lines cultured from chickens with avian lymphoid leukosis. Natl. Inst. Anim. Health Q. **14**:163–173.
16. **Hirt, B.** 1967. Selective extraction of polyoma DNA from injected mouse cell cultures. J. Mol. Biol. **26**:365–369.
17. **Jeurissen, S. H. M., E. M. Janse, S. Ekino, P. Nieuwenhuis, G. Koch, and G. F. de Boer.** 1988. Monoclonal antibodies as probes for defining cellular subsets in the bone marrow, thymus, bursa of Fabricius, and spleen of the chicken. Vet. Immunol. Immunopathol. **19**:225–238.
18. **Jeurissen, S. H. M., J. M. A. Pol, and G. F. de Boer.** 1989. Transient depletion of cortical thymocytes induced by chicken anaemia agent. Thymus **14**:115–123.
19. **Johnson, P. F., and S. L. McKnight.** 1989. Eukaryotic transcriptional regulatory proteins. Annu. Rev. Biochem. **58**:799–819.
20. **Jones, N.** 1990. Structure and function of transcription factors. Semin. Cancer Biol. **1**:5–19.
21. **Jones, N. C., P. W. J. Rigby, and E. B. Ziff.** 1988. Trans-acting protein factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. Genes Dev. **2**:267–281.
22. **Lee, K. A. W., T. Y. Hai, L. SivaRamen, B. Thimmappaya, H. C. Hurst, N. C. Jones, and M. R. Green.** 1987. A cellular transcription factor ATF activates transcription of multiple E1A-inducible adenovirus early promoters. Proc. Natl. Acad. Sci. USA **84**:8355–8359.
23. **Lucio, B., K. A. Schat, and H. L. Shivaprasad.** 1990. Identifications of the chicken anemia agent, reproduction of the disease, and serological survey in the United States. Avian Dis. **34**:146–153.
24. **Luthman, H., and G. Magnusson.** 1983. High efficiency polyoma DNA transfection of chloroquine treated cells. Nucleic Acids Res. **11**:1295–1308.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Marsh, J. L., M. Erfle, and E. J. Wykes.** 1984. The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene **32**:481–485.
27. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74**:560–564.
28. **McNulty, M. S., T. J. Connor, F. McNeilly, M. F. McLoughlin, and K. S. Kirkpatrick.** 1990. Preliminary characterization of isolates of chicken anaemia agent from the United Kingdom. Avian Pathol. **19**:67–73.
29. **McNulty, M. S., T. J. Connor, F. McNeilly, and D. Spackman.**

1989. Chicken anemia agent in the United States: isolation of the virus and detection of antibody in the broiler breeder flocks. Avian Dis. **33**:691–694.

30. **Mehdi, H., E. Ono, and K. Gupta.** 1990. Initiation of translation at CUG, GUG, and ACG codons in mammalian cells. Gene **91**:173–178.
31. **Otaki, Y., T. Nunoya, M. Tajima, H. Tamada, and Y. Nomura.** 1987. Isolation of chicken anaemia agent and Marek's disease virus from chickens vaccinated with turkey herpesvirus and lesions induced in chickens by inoculating both agents. Avian Pathol. **16**:291–306.
32. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85**:2444–2448.
33. **Rassoulzadegan, M., A. Cowie, A. Carr, N. Glaichenhaus, R. Kamen, and F. Cuzin.** 1982. The roles of individual polyoma virus early proteins in oncogenic transformation. Nature (London) **300**:713–718.
34. **Reddy, V. B., B. Thimmappaya, R. Dhar, K. N. Subramanian, B. S. Zain, J. Pan, P. K. Ghosh, M. L. Celma, and S. M. Weissman.** 1978. The genome of simian virus 40. Science **200**:494–502.
35. **Ritchie, B. W., F. Niagro, D. Lukert, W. L. Steffens, and S. Latimer.** 1989. Characterization of a new virus from cockatoos with psittacine beak and feather disease. Virology **171**:83–88.

35a. **Rosenberger, J. K.** Personal communication.

36. **Rosenberger, J. K., and S. S. Cloud.** 1989. The effects of age, route of exposure, and coinfection with infectious bursal disease virus on the pathogenicity and transmissibility of chicken anemia agent (CAA). Avian Dis. **33**:753–759.
37. **Rosenberger, J. K., and S. S. Cloud.** 1989. The isolation and characterization of chicken anaemia agent (CAA) from broilers in the United States. Avian Dis. **33**:707–713.
38. **Sanger, F., S. Nicklen, and A. R. Coulsen.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
39. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
40. **Tischer, I., H. Gelderblom, W. Vetterman, and M. A. Koch.** 1982. A very small porcine virus with circular, single-stranded DNA. Nature (London) **295**:64–66.
41. **Tischer, I., W. Mields, D. Wolff, M. Vagt, and W. Griem.** 1986. Studies on epidemiology and pathogenicity of porcine circovirus. Arch. Virol. **91**:271–276.
42. **Todd, D., J. L. Creelan, D. P. Mackie, F. Rixon, and M. S. McNulty.** 1990. Purification and biochemical characterisation of chicken anaemia agent. J. Gen. Virol. **71**:819–823.
43. **Vielitz, E.** 1989. Protect your chicks from infectious anemia. Poultry Sci. **68**:34–35.
44. **Vielitz, E., and H. Landgraf.** 1988. Anaemia-dermatitis of broilers: field observations on its occurrence, transmission and prevention. Avian Pathol. **17**:113–120.
45. **von Bülow, V., B. Fuchs, and M. Bertram.** 1985. Untersuchungen über den Erreger der Infektiösen Anämie bei Hühnerkücken (CAA) in vitro: Vermehrung, Titration, Serumneutralisationstest und indirekter Immunofluoreszenstest. J. Vet. Med. B **32**:679–693.
46. **von Bülow, V., B. Fuchs, B. Vielitz, and H. Landgraf.** 1983. Frühsterblichkeitssyndrom bei Küken nach Doppelinfektion mit dem Virus des Marekschen Krankheit (MDV) und eine Anämie-Erreger (CAA). J. Vet. Med. B **30**:742–750.
47. **von Bülow, V., R. Rudolph, and B. Fuchs.** 1986. Folgen der Doppelinfektion von Küken mit Adenovirus oder Reovirus und dem Erreger der aviären infektiösen Anämie (CAA). J. Vet. Med. B **33**:717–726.
48. **Yuasa, N.** 1983. Propagation and infectivity titration of the Gifu-1 strain of chicken anaemia agent in a cell line (MDCC-MSB1) derived from Marek's disease lymphoma. Natl. Inst. Anim. Health Q. **23**:13–20.
49. **Yuasa, N., and K. Imai.** 1986. Pathogenicity and antigenicity of eleven isolates of chicken anaemia agent (CAA). Avian Pathol. **15**:639–645.
50. **Yuasa, N., T. Taniguchi, M. Goda, M. Shibatanni, T. Imada, and H. Hihara.** 1983. Isolation of chicken anemia agent with MDCC-MSB1 cells from chickens in the field. Natl. Inst. Anim. Health Q. **23**:78–81.
51. **Yuasa, N., T. Taniguchi, and I. Yoshida.** 1979. Isolation and some properties of an agent inducing anaemia in chicks. Avian Dis. **23**:366–385.
52. **Yuasa, N., T. Taniguchi, and I. Yoshida.** 1980. Effect of infectious bursal disease virus infection on incidence of anaemia by chicken anaemia agent. Avian Dis. **24**:202–209.