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The Complete DNA Sequence and Genomic Organization of the Avian Adenovirus CELO

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The complete DNA sequence of the avian adenovirus chicken embryo lethal orphan (CELO) virus (FAV-1) is reported here. The genome was found to be 43,804 bp in length, approximately 8 kb longer than those of the human subgenus C adenoviruses (Ad2 and Ad5). This length is supported by pulsed-field gel electrophoresis analysis of genomes isolated from several related FAV-1 isolates (Indiana C and OTE). The genes for major viral structural proteins (IIIa, penton base, hexon, pVI, and pVIII), as well as the 52,000-molecular-weight (52K) and 100K proteins and the early-region 2 genes and IVa2, are present in the expected locations in the genome. CELO virus encodes two fiber proteins and a different set of the DNA-packaging core proteins, which may be important in condensing the longer CELO virus genome. No pV or pIX genes are present. Most surprisingly, CELO virus possesses no identifiable E1, E3, and E4 regions. There is 5 kb at the left end of the CELO virus genome and 15 kb at the right end with no homology to Ad2. The sequences are rich in open reading frames, and it is likely that these encode functions that replace the missing E1, E3, and E4 functions.

The large adenovirus family is divided by host range into adenoviruses that infect mammals (the *Mastadenoviridae*) and adenoviruses that infect avian species (the *Aviadenoviridae*). Chicken embryo lethal orphan (CELO) virus (reviewed in references 55 and 56) was first identified as an adventitious contaminant of embryonated eggs during efforts to propagate a bovine skin disease agent (71) and was identified as an infectious agent in 1957 (81). CELO virus is classified as a fowl adenovirus type 1 (FAV-1) and was the major subject of avian adenovirology for a number of years. This interest was based partially on the early observation that CELO virus was tumorigenic in baby hamsters (66). However, interest in CELO virus has waned in recent years, primarily because there are few serious health or economic consequences of CELO virus infection. The FAV-1 adenoviruses can be isolated from healthy chickens and do not cause disease when experimentally reintroduced into chickens (18). Their isolation from diseased birds is more likely to be an outcome of adenovirus replication in a host that has a weakened immune system as a result of other agents.

The general structural organization of CELO virus is similar to that of the mammalian adenoviruses, with an icosahedral capsid 70 to 80 nm in diameter, made up of hexon and penton structures (45). The CELO virus genome is a linear, double-stranded DNA molecule with the DNA condensed within the virion by virus-encoded core proteins (45, 48). The CELO virus genome has covalently attached terminal proteins (46) and has inverted terminal repeats (ITRs), although they are shorter than the mammalian ITRs (4, 68). CELO virus encodes a protease with 61 to 69% homology to the mammalian adenovirus proteases (12).

There are clear differences between CELO virus and the mastadenoviruses. CELO virus has a larger genome, with sequence homology to adenovirus type 5 (Ad5) (by hybridization) detected only in two short regions of the CELO virus genome (3). The CELO virus virion has been reported to have two fibers of different lengths at each vertex (34, 45, 47). CELO virus is not able to complement the E1A functions of Ad5, and CELO virus replication is not facilitated by Ad5 E1 activity (49).

We have undertaken a complete sequence determination of the CELO virus for several reasons. To further our understanding of adenovirus biology, it is useful to elucidate the genomic organization of an adenovirus distant from the commonly studied mammalian adenoviruses. Because the conditions of virus transmission and survival are likely to be different for a virus infecting an avian species, it is possible that the avian adenoviruses have acquired novel viral functions or show a greater extent of variability than the mastadenoviruses. The complete CELO virus sequence will also allow the generation of alterations in the CELO virus genome for functional analyses. Finally, adenovirus vectors are proving to be potent vectors for gene delivery (reviewed in references 28, 43, and 70). The complete CELO virus sequence will facilitate the generation of recombinant vectors for gene delivery or for vaccine applications.

The DNA sequence and the genomic organization of CELO virus are reported here. The sequence indicates a viral genome of 43.8 kb, nearly 8 kb longer than the 35.9-kb genome of human subgenus C adenoviruses Ad2 and Ad5. The genes for major viral structural proteins (hexon, penton base, IIIa, fiber, pVI, pVII, and pVIII) are present and in the expected locations in the genome. The early region 2 (E2) genes (encoding DNA-binding protein, DNA polymerase, and terminal protein) are also present. However, CELO virus lacks sequences homologous to the mammalian adenovirus E1, E3, and E4 regions. There is approximately 5 kb of sequence at the left end and 15 kb of sequence at the right end of the CELO virus genome with limited or no homology to the mastadenovirus genomes. These new sequences contain a number of open

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TABLE 1. Summary of CELO virus sequences, published or in the database

Accession no.	Reference	Size (bp)	Coordinates in our sequence	Differences between published sequences and our sequence	Comments
None	4	101	1–101	7 base differences	5′ ITR
K00939	68	68	1–68	7 base differences	5′ ITR
Z17216, S61107	1	3,576	1–3576	3 base differences, 1 missing base, 3 additional bases	Labeled 92–100% slight differences in these two versions
Z48167	2a	3,433	13597–17033	6 base differences, 4 missing bases, 1 additional base	Contains genes for penton base and core proteins
L13161	12	900	21023–21922	No differences	Contains protease gene
X84724	34	7,359	27060–34299	2 base differences, 6 missing bases, 3 additional bases, 11 (GCA) repeats (our sequence indicates 9), 6 ambiguous bases	Contains genes for pVIII, fiber 1, fiber 2
M12738	44	440	39584–40023	No differences	Contains VA gene
Z22864	2a	3,670	35235–38905	2 base differences, 4 missing bases, 2 additional bases	Assigned 11.2–19.2% by authors
X17217	2	4,898	38906–43804	2 base differences	Assigned 0–11.2% by authors
K00940	68	68	43741–43804	7 base differences	3′ ITR
None	4	124	43680–43804	2 base differences, 1 missing base	3′ ITR

reading frames (ORFs), and it is likely that these encode functions that replace the missing E1, E3, and perhaps E4 regions.

MATERIALS AND METHODS

Virus and virus DNA. The CELO virus used (FAV-1, Phelps strain) was a plaque-purified isolate obtained from G. Monreal, Free University of Berlin. The single isolate of CELO virus used as the source of DNA either for direct sequencing or for generation of bacterial plasmid clones was grown in 9-day-old pathogen-free chicken embryos as previously described (17). The FAV-1 isolates OTE (41) and Indiana C (13, 18) were obtained from B. Cowen, Pennsylvania State University, and were grown in chicken embryo kidney cells (see below). Virus was purified from allantoic fluid or from infected embryonic kidney cells by banding in CsCl gradients as previously described (17, 45). Viral DNA was isolated by treating purified virions with proteinase K (0.1 mg/ml) and sodium dodecyl sulfate (0.2%) at 56°C for 45 min followed by double banding (to equilibrium) of the DNA in a CsCl gradient in the presence of ethidium bromide. After the second gradient, the ethidium bromide was removed by extraction with CsCl-saturated isopropanol and the viral DNA was dialyzed extensively against 10 mM Tris–0.1 mM EDTA (pH 8).

Embryonic chicken kidney cells. The kidneys from 14-day-old chicken embryos were collected, washed in PBS, and digested with pancreatic trypsin (2.5 mg/ml in phosphate-buffered saline) at 37°C. Dispersed cells were mixed with an equal volume of fetal calf serum, and the cells were collected by centrifugation, washed once with FCK medium, and resuspended in the same medium. FCK medium is medium 199 with Earle's salts (Sigma M2154) supplemented with 10% tryptose phosphate (Sigma T8159), 10% fetal calf serum, 2 mM glutamine, 100 μg of streptomycin per ml, and 100 IU of penicillin per ml. The cells were plated in 175-cm^2 tissue culture flasks (two embryo kidneys per flask) maintained at 37°C under 5% CO_2 and were infected 24 to 48 h later. The cells were infected with approximately 1,000 virus particles per cell and harvested 3 to 4 days postinfection when the cytopathic effect was complete.

Pulsed-field gel electrophoresis. Aliquots of purified adenovirus DNA (10 to 20 ng) were loaded onto a 1% agarose gel (PFC agarose; Bio-Rad) gel and separated with a Bio-Rad CHEF Mapper pulsed-field gel electrophoresis system in field inversion gel electrophoresis mode for 24 h in 0.5× TBE (0.045 M Tris-borate, 0.001 M EDTA [pH 8.0]) chilled to 14°C. The switch time in both the forward and reverse direction was logarithmically ramped from 0.22 to 0.92 s with a ramp factor of 0.357 (21%). The forward voltage gradient was 9 V/cm (300 V), and the reverse voltage gradient was 6 V/cm (200 V). After the run, the gel was stained for 25 min in 0.5 μg of ethidium bromide solution per ml in water and then destained for 1 h before the DNA pattern was visualized by UV illumination.

Sequencing methods and data analysis. For sequencing, *Eco*RI and *Hin*dIII restriction fragments of CELO virus DNA were cloned into pBlueScript SK(−). Three of the *Eco*RI clones (containing the *Eco*RI C, D, and E fragments [see Fig. 1b]) and five of the *Hin*dIII clones (containing the *Hin*dIII F, A, G, B, and E fragments [see Fig. 1b]) were selected for constructing unidirectional deletions with exonuclease III. These deletion clones were then sequenced by using the *Taq* Dyedeoxy Terminator system with an ABI 373 automatic sequencing apparatus as specified by the manufacturer. Sequence analysis of the terminal 2,000 bp at the left end and 1,000 bp at the right end of the CELO virus genome, sequencing to close the gaps between *Eco*RI-C–*Hin*dIII-G and *Hin*dIII-B–*Eco*RI-D, and confirmatory sequencing at various parts of the genome were performed by sequencing the viral DNA directly. All of the reported sequence is the result of at least three sequencing reactions.

Sequence data were assembled with the SeqEd (ABI) and SeqMan (Lasergene) programs. Sequence analysis was performed with the University of Wisconsin Genetics Computer Group programs.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned accession number U46933.

RESULTS

Portions of the CELO virus sequence have been previously reported, and these are listed in Table 1, as are the differences between the database sequence and the sequence reported here. In studies concentrating on particular viral genes, a homolog of the mastadenovirus VA RNA gene was reported (44) and a portion of the genome sequence bearing the endoprotease was described (12). Furthermore, fragments of the CELO virus genome have been published (1, 2, 34). The penton base sequence from the related FAV-10 has also been reported (67). Several additional sequence fragments have been deposited in the database, and these are also listed in Table 1. In total, approximately 50% of the CELO virus genome was previously available in fragments reported from separate groups (ca. 24 kb in total). The sequence reported here is complete and has the advantage that it was obtained from a single isolate.

General properties and organization of the CELO virus genome. The complete sequence of CELO virus reveals a large number of striking differences between Ad2 and CELO virus, and these are detailed below. The organization of the recognizable ORFs of the CELO virus genome, based on our sequence determination, is presented in Fig. 1a, and that of Ad2 is given for comparison.

Genome size. The sequenced CELO virus genome is 43,804 bp in length and has a G+C content of 54.3%. There are previous indications that the genome is much larger than the 34- to 36-kb mastadenovirus genomes. CELO virus DNA was found to have a molecular weight of 30×10^6, determined from its sedimentation coefficient (45), compared with 24×10^6 for Ad2 (31). The CELO virus genome size determined by the addition of restriction fragment sizes is approximately 43 kb (12, 20). A pulsed-field gel electrophoresis analysis of the CELO virus genome isolated from purified virions is shown in Fig. 2 and compared with the DNA isolated from Ad5 *dl*1014

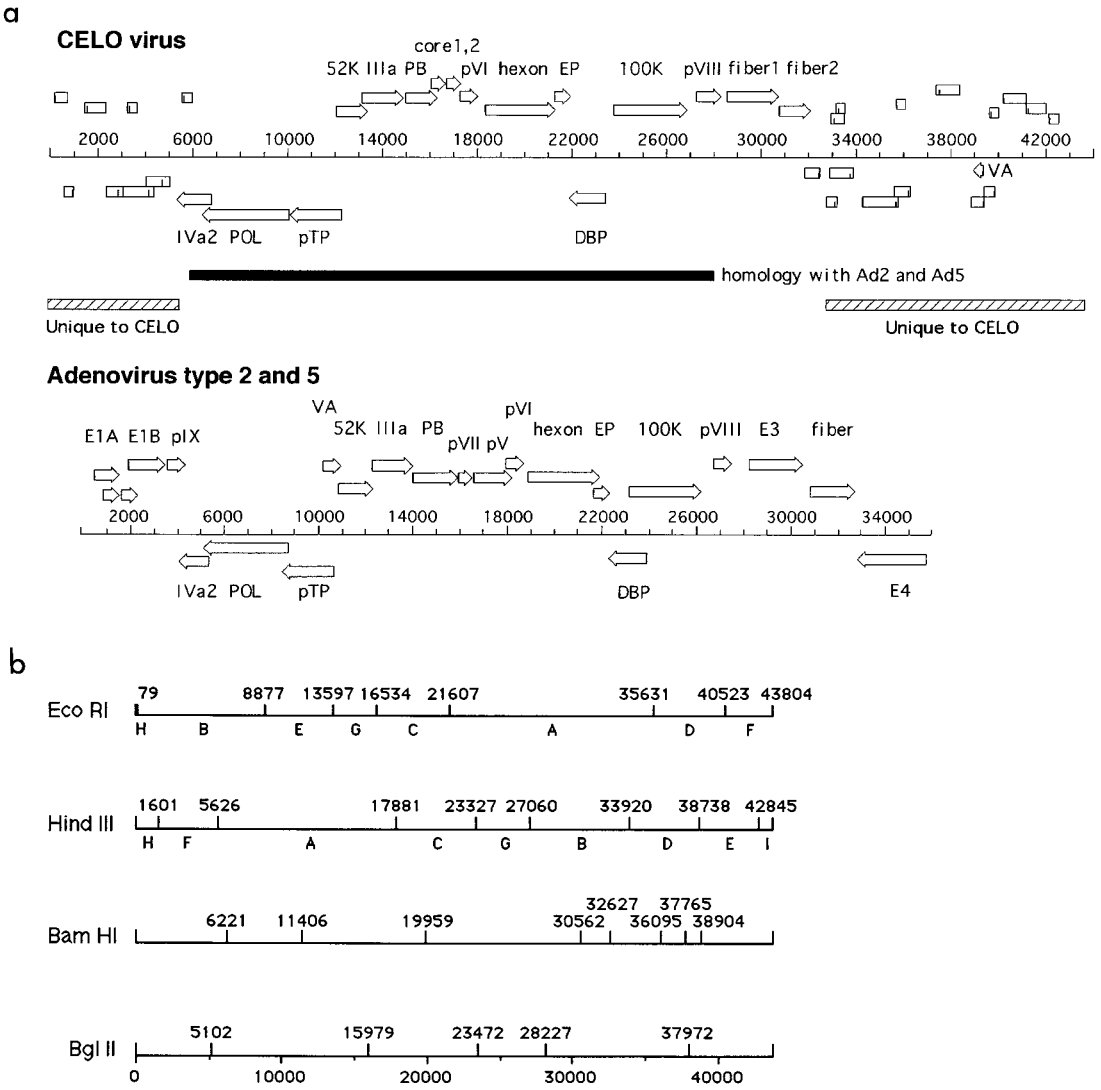

FIG. 1. (a) Comparison of Ad2 and Ad5 genomic organization with that of CELO virus. A summary of the Ad2, Ad5, and CELO virus genomic organization is presented. The arrows indicate the locations of coding regions but not the precise splicing patterns of the gene products. No indication of reading frame is implied by the positions of the ORF arrows. The CELO virus pattern also indicates (in the first 6,000 bp and the last 13,000 bp) all unassigned ORFs commencing with a methionine and encoding more than 99 amino acid residues (Table 3). The central region of the two genomes that show homology by dot matrix analysis (Fig. 4), as well as regions at the ends of the CELO virus genome with no homology to other adenoviruses (Unique to CELO), are indicated. Abbreviations: PB, penton base; EP, endoproteinase; DBP, DNA-binding protein; pTP, preterminal protein; pol, DNA polymerase. (b) Restriction map of the CELO virus genome. Cleavage sites for restriction endonuclease *Eco*RI, *Hin*dIII, *Bam*HI, and *Bgl*II are indicated. The alphabetic notation of the *Eco*RI and *Hin*dIII fragments (based on relative sizes) is also shown.

(34,600 bp [11]) or wild-type Ad5 virions (35,935 bp; *wt*300 [15, 39]). It is apparent from this analysis that the CELO virus genome is indeed substantially larger than the mammalian virus genome. Calculations based on the migration of bacteriophage lambda marker fragments give a size of 43 kb for the CELO virus genome. The DNA extracted from two additional FAV-1 isolates, Indiana C and OTE, comigrate with the CELO virus species, providing additional evidence for the expanded size of the CELO virus genome.

(i) Early region 1. There is no identifiable E1 region. No significant homology (at either the DNA or protein level) can be detected between the CELO virus genome and the first 4,000 bp of Ad2. A clear homology between the leftward ORF starting at nucleotide (nt) 6685 with the Ad5 IVa2 gene demonstrates the beginning of the CELO/Ad homologies. However, a number of small ORFs are found in the first 5,000 bp of CELO virus, and we are currently testing their function in some of the E1 tasks. These include an ORF at nt 794 that encodes a protein with dUTPase homologies (previously allocated a position at the right end of the genome) (2) and an ORF at nt 1999 with parvovirus REP homologies. An ORF at the right end of the viral genome (GAM-1) has been found to replace the E1B 19,000-molecular weight protein (19K protein) in a number of functional assays (14a). However, there is no significant protein or DNA sequence homology between GAM-1 and the E1B 19K protein.

Once the absence of E1 sequence homology became apparent, we had concerns that the sequenced genome could represent an aberrant viral species and that a second form of CELO virus, which bears a conventional adenovirus E1 region, might exist. The bulk of our sequencing reactions were performed with bacterially cloned fragments of the CELO virus genome.

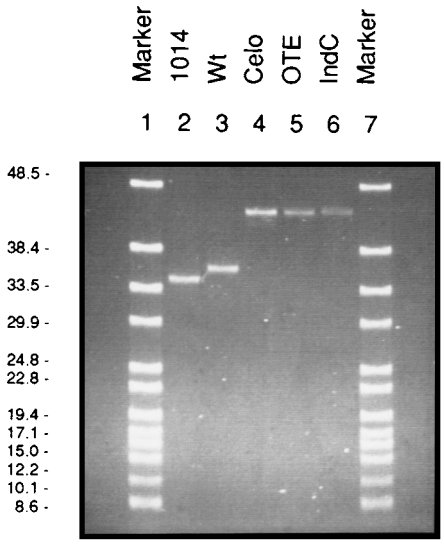

FIG. 2. Pulsed-field gel electrophoretic analysis of adenovirus genome sizes. Lanes: 1 and 7, molecular weight markers (a mixture of uncleaved bacteriophage lambda DNA and bacteriophage lambda DNA cleaved with a mixture of five restriction enzymes [Bio-Rad]); 2, Ad5 *dl*1014 DNA; 3, Ad5 *wt*300 DNA; 4, CELO virus DNA; 5, OTE DNA; 6, Indiana C DNA. Electrophoresis parameters are described in Materials and Methods. The sizes of some of the markers are indicated (in kilobase pairs).

To verify that the left end is representative of the wild-type CELO virus genome and not the sequence of a cloned variant, we performed the following three analyses.

The first involves direct sequencing reactions on DNA isolated directly from CELO virions, which generated sequences identical to the cloned sequences at three different locations within the first 5,000 bp.

In the second, Southern analysis was performed on DNA isolated from CELO virus, as well as from two other FAV-1 isolates, OTE (a Japanese FAV-1 isolate) and Indiana C (an American FAV-1 isolate). Using a probe isolated from the left end of CELO virus (the cloned *Eco*RI B fragment, nt 79 to 8877 [Fig. 1b]), we detected the same restriction digest fragments from all three FAV-1 isolates (Fig. 3). If a subpopulation of the virus carried an altered left end with an E1 sequence, we would expect to find heterogeneity in either the size or the intensity of the smaller *Hin*dIII fragments F and H. In fact, we found the expected three *Hin*dIII fragments in the expected ratios. There are two submolar bands present in the CELO virus and the OTE samples. The lower of the two, migrating at 5.5 kb, could be the product of a partial *Hin*dIII digestion. The band migrating at 8 kb is unexplained and could possibly represent a heterogeneity in the virus population. However, the bulk of the DNA presents the expected pattern. Thus, three independent isolates of FAV-1 possess the same organization of the left 9,000 bp of the genome, indicating that the viral genome that we sequenced is representative of FAV-1.

In the third type of analysis, pulsed-field gel electrophoresis of CELO virus, OTE, and Indiana C genomes revealed no heterogeneity in genome sizes of DNA extracted from purified virions, within the resolution capacity of this electrophoresis system, approximately ±500 bp (Fig. 2). Southern analysis of the same gel with either a left-end probe (nt 79 to 8877) or a right-end probe (nt 35630 to 40522) generated the same pattern of genomes, demonstrating that within the resolution of the gel system, there are no viral genome species in any of these three isolates possessing the right end with an alternate left end (results not shown).

Confirmation that this E1-minus adenovirus is fully replication competent will come from the establishment of virus from a bacterially cloned copy of the CELO virus genome, and these experiments are in progress.

(ii) Early region 3. There is no identifiable E3 region. The E3 region is found between the protein VIII gene and the fiber gene in all mastadenoviruses. The CELO virus genome has 476 bp between the stop codon of pVIII and the initiation codon of the first fiber gene. In the mammalian adenoviruses, this region shows great variability in both size and sequence (8), ranging from ca. 2.5 kb in the human adenoviruses (25, 80) to 0.5 kb in the murine adenovirus (63). There are two small ORFs in this region of CELO virus, but the predicted protein sequences have no significant homology with either the murine E3 gene product (63) or any of the other described E3 proteins. The ovine adenovirus has only 197 bp separating the pVIII and fiber genes (73), although in this case the possibility remains that an E3 region is located elsewhere in the genome.

(iii) Early region 4. There is a group of small leftward ORFs between positions 36000 and 31000. The position of these ORFs is suggestive of the mammalian E4 region but with an additional 8 kb of sequence added to the right end of CELO virus. However, there are no sequence homology or functional data to support such a designation. The left-end ORF beginning at nt 1999 shows homology to various dUTPases as well as limited homology to the E4 ORF 1 protein of Ad2, suggesting as an alternate model that a general rearrangement of the genome has resulted in an E4 region at the left end of the genome and a VA gene at the right end (see Discussion).

(iv) Protein IX. No protein IX-like sequence has been iden-

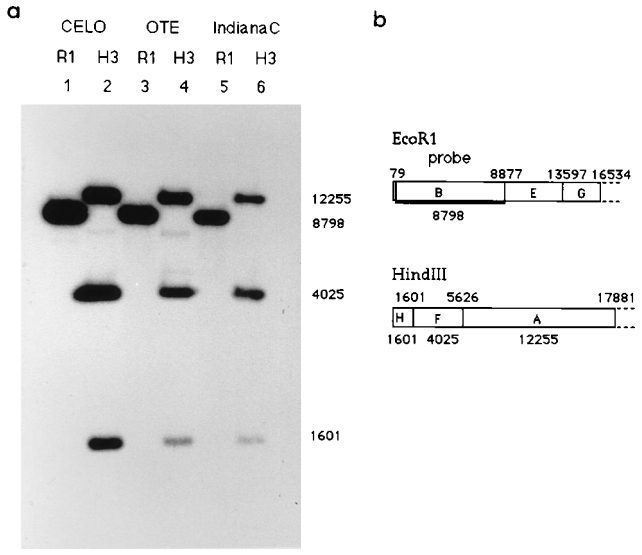

FIG. 3. Analysis of the genomic left end of three FAV-1 isolates. (a) DNA purified from the virions of CELO virus, OTE, and Indiana C was digested with the restriction enzyme *Eco*RI (R1) or *Hin*dIII (H3), resolved on an 0.8% agarose gel, transferred to Nucleobond paper, and probed with a radioactively labeled *Eco*RI fragment (nt 79 to 8877). After hybridization and washing, the radioactive pattern was revealed by autoradiography. Lanes: 1, CELO virus DNA digested with *Eco*RI; 2, CELO virus DNA digested with *Hin*dIII; 3, OTE DNA digested with *Eco*RI; 4, OTE DNA digested with *Hin*dIII; 5, Indiana C DNA digested with *Eco*RI; 6, Indiana C DNA digested with *Hin*dIII. (b) Description of the restriction map of this portion of the CELO virus genome (based on the sequence) and the sizes of the expected products. The probe fragment should hybridize to itself in an *Eco*RI digest (8,798 bp), and it should hybridize to fragments of 12,255, 4,025, and 1,601 bp in a *Hin*dIII digest.

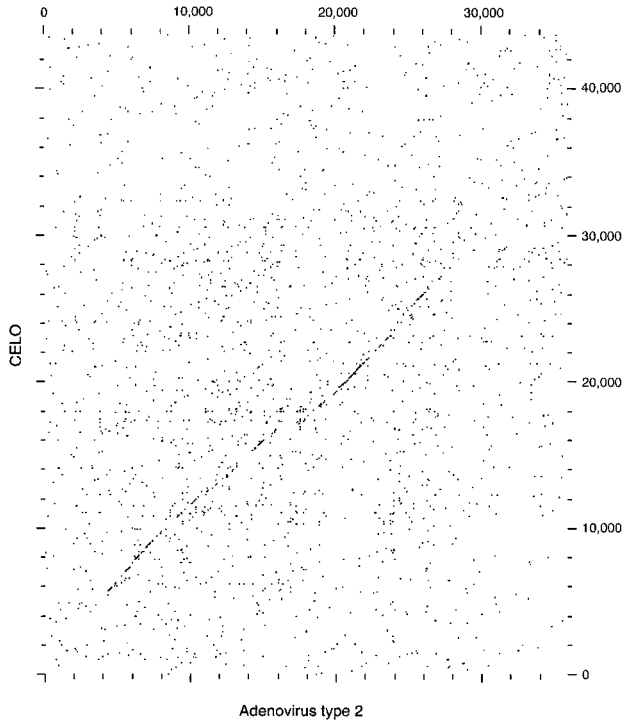

FIG. 4. Dot matrix analysis of DNA sequence homology between CELO virus and Ad2. Analysis was performed with the University of Wisconsin Genetics Computer Group program Compare with a window of 30 and a stringency of 20.

tified. Protein IX is essential for hexon-hexon interactions (10, 21, 22, 50) and for the stability of the mammalian adenovirus virion (16), especially when bearing a full genome (14, 24). Consistent with an absence of pIX in CELO virus, Laver et al. (45) reported that CELO virus virions do not yield group-of-nine hexon assemblies when dismantled under conditions that generate these hexon structures with Ad5. On the basis of the Ad5 behavior, one might expect pIX-negative virus to have decreased heat stability. However, CELO virus is quite heat stable (56), suggesting that if the virus lacks a pIX equivalent, it has adopted an alternate method of stabilizing the virion.

(v) Protein V. It was previously noted that the CELO virus core lacked the higher-molecular-weight core protein V found in the mammalian adenoviruses (48). Although genes encoding basic proteins that may correspond to pVII and mu are present (see below), a protein V gene has not been identified.

Regions conserved between CELO virus and Ad2. The central portion of the CELO virus genome, from the IVa2 gene (from approximately nt 5000) on the left strand through to the fiber genes on the right strand (to approximately nt 33000) is organized like the mastadenoviruses, and most of the major viral genes can be identified both by position and by sequence homology. Previous studies on CELO-Ad2 homology (3) indicated two regions of CELO virus that cross-hybridize with the Ad2 sequence. These two fragments are nt 5626 to 8877 (which encodes IVa2 and the carboxy terminus of the DNA polymerase) and nt 17881 to 21607 (which encodes the hexon). A dot matrix analysis indicates that all DNA sequence homology between CELO virus and Ad2 maps to the central region of the CELO virus genome (Fig. 4; summarized in Fig. 1a). This might be expected in that the capsid proteins are encoded in this central region and the gross structure of the CELO virus virion is comparable to that of the mammalian adenovirus capsid (45, 47). The genes for proteins equivalent to the human adenovirus hexon, IIIa, penton base, protein VI, and protein VIII are present and are in the expected order and positions (Fig. 1a; Table 2). Unassigned ORFs larger than 99 amino acids are listed in Table 3. A compilation of the amino acid sequence similarity and identity between CELO virus and other known adenoviruses is given in Table 4.

Fiber genes. Each vertex of the mastadenovirus virion contains a pentamer of the penton base protein associated with a single fiber consisting of three copies of the fiber polypeptide. Ad2 (like most of the mastadenoviruses) has a single fiber gene. Ad40 and Ad41 each have two fiber genes (19, 61), but each vertex contains only the short or the long fiber (42). CELO virus is reported to have two fibers at each vertex of the virion (23, 45, 47). When exposed to low-ionic-strength solutions, CELO virus virions release vertices containing penton bases associated with two types of fibers, a long structure (425 Å [42.5 nm]) often with a sharp kink, and a short structure (85 Å [8.5 nm]) (45). The two fiber types are distinct proteins (as indicated by partial peptide mapping [47]).

In common with the enteric adenoviruses Ad40 and Ad41, which have fibers of different lengths, the CELO virus genome encodes two fiber polypeptides of distinct lengths and sequences. In contrast to the arrangement in Ad40, in which each fiber exon is followed by a polyadenylation site, in CELO virus there is only one signal, at the end of the second fiber gene.

Stouten et al. (69) have described a general model for the adenovirus fiber structure based on an earlier model of Green et al. (32). The shaft is a three-stranded helix stabilized by interstrand hydrogen bonds between short β-sheets. Each β-sheet contains three residues; the outer two are invariably hydrophobic. The three-residue β-sheet is followed by a five-residue turn sequence invariably terminating in a proline or glycine, followed by a second three-residue β-sheet and a second turn sequence which is less stringent in length and in the requirement for a proline or glycine terminator. We find that the two CELO virus fiber sequences also contain this pattern of amino acid residues. The general features of the model of Stouten et al. are depicted in Fig. 5 for the Ad2 fiber and the two CELO virus fibers. The long fiber 1 adheres to the Stouten pattern more closely than does the short fiber 2. Furthermore, it appears from the repeat pattern that fiber 2 lacks the typical knob element.

There is a reported feature of the CELO virus penton base-fiber assembly that is difficult to reconcile with the accepted structure of adenovirus fibers. If both of the CELO virus fibers adopt a trimeric form (and the protein sequence is consistent with this [Fig. 5]), it is difficult to understand how a single vertex can accommodate both a long and a short fiber. This would require either that a penton base associate with two trimeric fibers or that a completely different penton-fiber organization be present in CELO virus, such that two dimeric fibers associate with each penton base. Because the penton base sequence is one of the most highly conserved (65% homology and 48% identity with the Ad2 penton), it appears unlikely that CELO virus has adopted a different vertex organization. However, all other chicken adenoviruses examined by electron microscopy appear to have two fibers at each vertex, albeit of the same length (23). Thus the two-fiber vertex is not a unique feature of CELO virus but a common feature of chicken adenoviruses. EDS, which, although it infects chickens, has been designated a duck adenovirus, has only one fiber at each vertex (23).

Recently, an analysis of the CELO virus fiber genes was published (34), and a similar concern about the two fiber pen-

TABLE 2. CELO virus genome organization

Protein	ATG position	STOP position	Cap, splice, poly(A) site(s)	Mol wt	No. of residues	Comments[a]
L1						
52K	12193	13329		42,094	378	
IIIa	13316	15043		63,771	575	Protease cleavage site at aa 551
L2						
			15080			Penton base SA
Penton base	15110	16657		56,719	515	No arginine-glycine-aspartic acid motif
			16196			Poly(A) site
pVII	16679	16897		8,562	72	Protease cleavage sites at aa 27, 40
mu (pX, 11K)	16929	17495		19,787	188	Protease cleavage sites at aa 125, 144
			17526			Poly(A) site
L3						
pVI	17559	18230		23,890	223	Protease cleavage sites at aa 28, 212
Hexon	18289	21117		106,704	942	
			18261			Hexon SA
Protease	21134	21754		23,763	206	
			21102 or 21123			Protease SA
			21767, 21836			L3 poly(A) sites
L4						
			23608 or 23649			100K SA
100K	23680	26634		109,905	984	
pVIII	27149	27886		26,876	245	Protease cleavage sites at aa 40, 115, 130, 141, 166
			27920			L4 poly(A) site
L5						
			28315 or 28341			Fiber SA
Fiber 1	28114	30495		81,526	793	
			30509			$[GCA]_9$ reiteration
			30511			Fiber SA
Fiber 2	30536	31768		42,939	410	
			31771			L5 poly(A) site
VA RNA			39841–39751			
E2 and IVa2						
IVa2	6685	5366		50,366	439	
E2b pol	10268	6501		144,984	1,255	
E2b pTP	11996	10269		66,089	575	Protease cleavage sites at aa 116, 141, 260, 264
DBP	23224	21899		49,272	441	
			23292			DBP cap site
			21824 or 21882			DBP poly(A) site

[a] aa, amino acids; DBP, DNA-binding protein.

ton was raised. Based on the EM appearances of vertices, Hess et al. (34) proposed that the longer fiber associates with the penton base at a site different from the short fiber-penton interaction.

Early region E2. (i) DNA-binding proteins. Li et al. (49) identified DNA-binding proteins produced in CELO virus-infected chicken embryonic kidney cells in the presence of an inhibitor of viral DNA replication (cytosine arabinoside). Four proteins of 74, 64, 56, and 52 kDa that showed similar peptide maps were described, suggesting a single precursor that is processed (or degraded) into several forms. The leftward ORF starting at nt 23224 is positioned in the expected DNA-binding protein location and has sequence homologies with the Ad2 72K DNA-binding protein. The predicted CELO virus protein is only 49 kDa (441 residues), compared with 529 residues for the Ad2 DNA-binding protein. More detailed analysis is required to determine if protein modification (e.g., phosphorylation) or splicing (perhaps to another exon) generates proteins of the sizes determined by Li et al. (49).

(ii) Other E2 genes. The genes encoding DNA polymerase, pTP, are present and in the expected positions (Fig. 1a; Table 2). The CELO virus DNA polymerase has 39% amino acid sequence identity with its Ad2 counterpart. The CELO virus pTP has 37% sequence identity with the Ad2 pTP, and it contains three cleavage sites for the adenovirus protease in the equivalent regions of the protein, consistent with the importance of this cleavage event in regulating adenovirus DNA replication (77).

Core structure. It is of interest to identify the mechanisms used by CELO virus to package nearly 44 kb of DNA in a virion of similar size to the human adenoviruses, which have strict limits on their packaging capacity (9, 14, 24). One possibility is that the CELO virus virion, although nearly identical in size to the Ad2 and Ad5 virions as determined by light-scattering analysis (16a), has actually enough of an expanded structure to accommodate the larger genome. An alternate hypothesis is that CELO virus has an altered mechanism of DNA condensation and hence that CELO virus would show

TABLE 3. Unassigned ORFs larger than 99 amino acid residues

ATG position	STOP position	No. of residues
Rightward ORFs		
794	1330	178
1999	2829	276
3781	4095	104
5963	6373	136
33030	33476	148
33169	33483	104
35629	36024	131
37391	38239	282
40037	41002	321
41002	41853	283
41958	42365	135
Leftward ORFs		
5094	4462	210
4568	3549	339
3374	2892	160
1514	1191	107
39705	39286	139
39256	38717	179
36144	35536	202
35599	34238	453
33707	32892	271
33058	32735	107
32429	31812	251

differences in the set of core proteins responsible for DNA packaging. Laver et al. (45) identified two proteins in the CELO virus core and noted the absence of a protein V-like molecule. Using higher resolution electrophoresis, Li et al. (48) reported a viral core structure with three polypeptides (20, 12, and 9.5 kDa). From both of these reports, CELO virus appears to lack the larger basic core protein V (41 kDa) found in the mammalian adenoviruses. Perhaps the absence of protein V and/or the presence of smaller, basic proteins is involved in the extra packaging capacity of the CELO virus virion. The smallest of the CELO virus core proteins identified by Li et al. (9.5 kDa [48]) associates most closely with the viral DNA, similar to the human adenovirus protein VII. An ORF predicting a 72-residue protein of 8,597 Da is present at nt 16679; the encoded protein is arginine rich (32.9 mol%) and contains two protease cleavage sites (Ad2 pVII has only one cleavage site). An ORF predicting a 188-residue protein of 19,777 Da is present at nt 16929. The predicted protein has adenovirus protease cleavage sites after residues 22, 128, and 145, and the carboxy-terminal residues have homology with the mastadenovirus pX (Fig. 6). The 19-residue basic mastadenovirus DNA-binding protein mu is generated by two protease cleavages of the pX precursor (5, 35, 74). Cleavage of the 188R protein after residues 128 and 145 would yield a mu-like basic 17-residue peptide (41% arginine, 12% lysine). The uncleaved form of the 188R protein is also highly basic, and perhaps uncleaved copies of this protein are the 20-kDa core protein observed by Li et al. (48). A third major core protein of 12 kDa identified by Li et al. (48) cannot yet be assigned.

CELO virus protease. The mastadenoviruses encode a protease which is essential for virus maturation (reviewed in reference 75). Cai and Weber (12) previously sequenced and characterized the protease encoded by CELO virus. The CELO virus protease has 43% amino acid sequence identity with the Ad2 protease. We found that all the viral proteins that are substrates for the protease in the mastadenovirus, i.e., pVI, pVII, pVIII, IIIa, pMu (pX), and pTP, have maintained protease cleavage sites in the CELO virus homologs (listed in Table 2). In particular, the Ad2 carboxy-terminal pVI cleavage releases a peptide that can function to activate the Ad2 protease (53, 76). A similar peptide sequence is present in the CELO virus pVI, with two of the arginine residues and the essential cysteine residue conserved.

Novel or unassigned ORFs. A number of novel or unassigned ORFs are present in the CELO virus genome. A compilation of these ORFs is presented in Table 2, and these ORFs are also indicated in Fig. 1a. We have limited this compilation to the sequences at nt 0 to 6000 and 31000 to 43804, and only ORFs containing a methionine residue and encoding a protein of >99 amino acid residues are indicated. As men-

TABLE 4. Protein sequence homologies between CELO virus and other adenoviruses[a]

Protein	FAV-10		Ad2		Ad12		Ad40		Ovad		Mav1		Bav3		Cav1	
	%id	%sim	%id	%sim	%id	%sim	%id	%sim	%id	%sim	%id	%sim	%id	%sim	%id	%sim
52K			31	55	31	55	32	54								
IIIa			29	53	29	50	30	51								
PB	78	88	48	65	46	64	46	65								
pVII (core 2)	74	76													37	50
pX (core 1)	69	81														
pVI			26	50	29	50	27	51			27	46				
Hexon			51	67	50	67	51	66			48	66	49	66		
Protease			43	67	40	63					44	70	41	67	41	65
100K	57	70	35	55	32	53	33	51	38	59	32	53				
22K			21	41	23	46	24	42								
pVIII			29	50	25	44	28	46	30	48	26	46	27	49	28	47
Fiber 1			20	42	24	48	25	45	25	50	21	44	26	49	26	49
Fiber 2			22	46	21	45	25	45	25	50			26	50		
DBP			29	52	29	53	29	51			30	50				
pol			39	60	40	60	40	60								
pTP			37	59	36	58	35	57								
IVa2			33	56	33	58	34	57								

[a] The percent identity (%id) and similarity (%sim) were determined by using the University of Wisconsin Genetics Computer Group Bestfit program with default parameters (gap weight 3.0, gap length weight 0.1). The sequences were obtained from GenBank: Ad2, J01917; Ad12, X73487; Ad40, L19443; FAV-10, P32538, L08450, and L07890; ovine adenovirus (Ovad), U18755; bovine adenovirus type 3 (Bav3), K01264, X53990, U08884, and D16839; and canine adenovirus type 1 (Cav1), M73811, M72715, U09195, and M60937. Additional homologies include 25% identity and 48% similarity with the Bav2 pVIII (S75673); 37% identity and 61% similarity with the Bav7 protease (X53989); 28% identity and 50% similarity with the Pav3 pVIII (U10433); and 26% identity and 47% similarity with the Pav3 fiber (U10433).

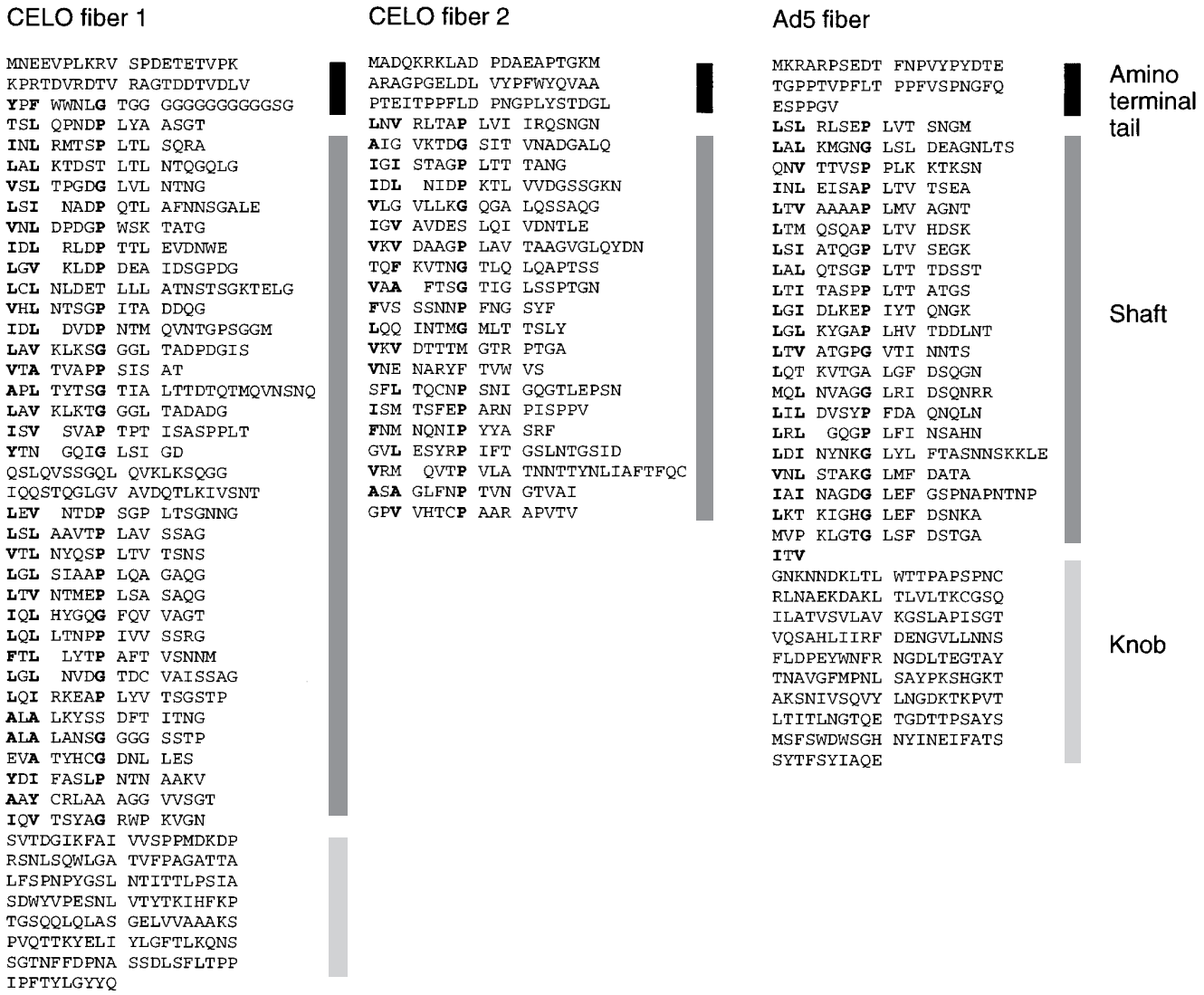

FIG. 5. Comparison of CELO virus fiber 1 and fiber 2 with the Ad5 fiber. The fiber amino acid sequences are arranged in the repetitive format described by Stouten et al. (69). Highlighted in bold are the hydrophobic residues of the first β-sheet triplet, as well as the proline or glycine residues that are proposed to mark the end of each four- or five-residue turn. The proposed amino-terminal penton interaction sequence, the repetitive shaft region, and the carboxy-terminal knob regions are also indicated.

tioned above, there is an ORF at nt 1999 that encodes a protein with parvovirus REP homologies and an ORF at nt 794 with dUTPase and Ad2 E4 ORF1 homology.

Sequence elements. (i) Major late promoter. A CELO virus sequence with strong homology to the mastadenovirus major late promoter is found near nt 7000, with a TATA box at nt 7488 (TATATAAGGG). The CELO virus major late promoter is located in the expected genomic location relative to the identified late genes.

(ii) Packaging signal. A series of AT-rich elements, resembling the Ad5 packaging signal (26, 27), is present between nt 70 and 200.

DISCUSSION

The adenovirus family comprises two genera, the mastadenoviruses and the aviadenoviruses (54) with this grouping largely based on antibody reactivities. From the CELO virus genome sequence and organization reported here, it is apparent that CELO virus is indeed distinct from the mastadenoviruses, with the E1, E3, and E4 changes and the 44-kb genome clearly setting CELO virus apart from other characterized adenoviruses.

The absence of an E1 region is a striking feature of CELO virus. The replication of this virus in embryonic tissues may obviate the need for E1A functions to induce a proliferative, S-phase response in the infected cell. However, although CELO virus is propagated in embryonic cells in the laboratory setting, the host tissue in the wild is the adult respiratory and alimentary tract (55, 56). It is possible that CELO virus has other genes that provide the proliferative E1 stimulus. Initial functional screens have failed to identify CELO virus regions that can complement growth of E1-defective Ad5 (16a).

Like a number of mammalian adenoviruses, CELO virus is capable of generating tumors when injected into baby hamsters (40, 51, 52, 66). CELO virus is also capable of transforming some cell types in vitro (6, 7). The transforming activity of the mammalian adenoviruses is a function primarily of the E1

A. pVII/Core 2

```
           1                                                  50
CELO core2  ....MSILIS PSDNRGWGA. .......... .......... ..NMRYRRRA
Fav10core2  ....MSILIS PNNNTGWGM. .......... .......... ..RRR.SRSS
    Ad12p7  ....MSILVS PSNNTGWGLG .AARMYGGAK TRSSQHPVRV RGHYRAPWGA
    Ad40p7  ....MSILIS PDNNTGWGLC .SAGMYGGAK RRSSQHPVRV RGHYRAPWGA
     Ad2p7  ....MSILIS PSNNTGWGLR FPSKMFGGAK KRSDQHPVRV RGHYRAPWGA
    Cav1p7  cavpMAILIS PSNNTGWGLG .THKLFGGAK QKSDQHPVYV QAHYRAPWSK

           51                                                 100
CELO core2  SMRGVGRRR. ...LTLRQLL GLGSRRR... RRSRPTTVSN RLVVVSTRRR
Fav10core2  SMRGVGMRRR ARPLTLRSLL GLGTRRRRGS RRSRPRTTS. RLVVVRTRTS
    Ad12p7  HTRGRTG.RT TVDDVIDSVV ADARKYRAPA ETAGSTVDAV IDEVVANARA
    Ad40p7  YTRGVISRRT TVDDVIDSVV ADAQRYTRP. .VATSTVDSV IDSVVANARR
     Ad2p7  HKRGRTG.RT TVDDAIDAVV EEARNYT.PT PPPVSTVDAA IQTVVRGARR
    Cav1p7  GRRRPGRARG VPLD...... .......... PKTEAEVVAT IDEVARNGPP

           101                                                150
CELO core2  SSRRRR.... .......... .......... .......... ..........
Fav10core2  SMRRRR.... .......... .......... .......... ..........
    Ad12p7  YARRRRRL.R .....RRRRP TTAMRAARAL VRRARRIGRR AMMRAARRAA
    Ad40p7  YAQRKRRLQR .....RRRRP TAAMTAARAV LRRAQRIGRR AMRRAA..AS
     Ad2p7  YAKMKRRRRR VARRHRRRPG TAAQRAAAAL LNRARRTGRR AAMRAARRLA
    Cav1p7  .......... .......... .....AARLV LEAARRVGAY NLRRARKLTP

           151                                                200
CELO core2  .......... .......... .......... .......... ..........
Fav10core2  .......... .......... .......... .......... ..........
    Ad12p7  TP....AGRA RRRAAAAAAT AIANLAAPRR GNVYWVRDSV TGTRVPVRTR
    Ad40p7  AS....AGRA RRQAARQAAA AIASMAQPRR GNIYWVRDA. SGVRVPVRSR
     Ad2p7  AGIVTVPPRS RRRAAAAAAA AISAMTQGRR GNVYWVRDSV SGLRVPVRTR
    Cav1p7  AGRAMAAMRA RQMVNQA... .......KRR KRRVRSK... ..........

           201
CELO core2  .....
Fav10core2  .....
    Ad12p7  PPHP.
    Ad40p7  PPRS.
     Ad2p7  PPRN.
    Cav1p7  .....
```

B. Protein X/Core 1

```
           1                                                  50
CELO core1  MCAVAIHRSD VVMPSVLLTG GRTAKGKKRA SRRR....VK VPKLP.....
Fav10core1  .......... ..MPAVLLTG GRAASKRKFS TKQRRKKAVS VPKIRSRSGK
    Ad12px  .......... .......... .......... .......... ..........
    Ad40px  .......... .......... .......... .......... ..........
     Ad2px  .......... .......... .......... .......... ..........

           51                                                 100
CELO core1  KGARRKRASV TPVPTVATAT ASERAALTNL ARRLQRGDYA AWRPADYTSP
Fav10core1  RSGVRKRSSI .SVPVSGTAS ASERAALQNL AQRLQRGNYT AWRSAD.PSV
    Ad12px  .......... .......... .......... .......... .........M
    Ad40px  .......... .......... .......... .......... .........M
     Ad2px  .......... .......... .......... .......... .........M

           101                                                150
CELO core1  AVSEAARAAA SSGTPATARD LATGTLARAV PMTGTGGRRR KRTATRRRSL
Fav10core1  AASEAAKAAA ASGAAAYVRD LTTGTAAEAV PLTGTG..RR RRTGA.RRSM
    Ad12px  ALTCRMRIPI PGYRGRPRRR KGLTGNG... ......RFRR R...SMRRRM
    Ad40px  ALTCRFRIPV PSYRGRSRRR RGMAGSG... ........RR R...ALRRRI
     Ad2px  ALTCRLRFPV PGFRGRMHRR RGMAGHGLTG GMRRAHHRRR R...ASHRRM

           151                                                197
CELO core1  KGGFLPALIP IIAAAIGAIP GIAGTAVGIA NLKEQQRQFN KIYGDKK
Fav10core1  RGGFFPALIP LIAAAIGAIP GIAGTAVGIA SLKEQQRQFN KLYGNK.
    Ad12px  KGGVLPFLIP LIAAAIGAVP GIASVALQAS RKN..... .......
    Ad40px  KGGFLPALIP IIAAAIGAIP GVASVALQAA RKQ..... .......
     Ad2px  RGGILPLLIP LIAAAIGAVP GIASVALQAQ RH..... .......
```

FIG. 6. The amino acid sequences of pVII and pX of several mastadenoviruses in comparison with CELO virus and the FAV-10 core 2 and core 1 proteins. Sequences were aligned with the University of Wisconsin Genetics Computer Group Pileup program with a gap weight of 3.0 and a gap length weight of 0.1. The adenovirus protease cleavage sites are underlined. The GenBank accession numbers are listed in the footnotes to Table 4.

region (29, 65, 72; reviewed in references 58, 59, and 79) although in human Ad9, the E4 ORF1 protein also has a transforming function (36, 37). The absence of a discernible E1 region in CELO virus has led us to search for the genes responsible for the transforming activity of this virus. Although the left end of the genome that should contain the E1 region has no sequence homology to any of the E1A or E1B genes, at either the DNA or the protein level, there are a number of potential ORFs in this portion of the CELO virus genome and functional assays as well as deletion analysis will help identify their roles. It is also possible that the ends of the genome have undergone rearrangements, possibly including inversion around the central portion, such that the conventional E1, E3, and E4 regions are no longer discernible. The notion that there might be an inversion is supported by the observations that the only protein of recognizable function in the left end of the CELO virus genome has a very close homology to a family of dUTPases and the Ad2 E4 ORF1 gene product shows a degree of similarity to this family.

Another function of the E1 region in mammalian adenoviruses is to block apoptosis, performed both by the E1B 55K gene product, which binds to and alters the transcriptional properties of p53, and by the E1B 19K gene product, which blocks apoptosis similarly to the mammalian Bcl-2 (62, 79). An anti-apoptotic function in CELO virus has been identified in an ORF at the right end of the CELO virus genome, which encodes a protein designated GAM-1 with E1B 19K and Bcl-2-like properties in a number of functional assays (14a).

The mastadenovirus E4 ORF1 proteins all possess sequence similarity to the CELO virus dUTPase but, in contrast to the CELO virus protein, do not show enzyme activity (35a). The position of the dUTPase gene at the left end of the genome in CELO virus leads us to speculate that there might have been some rearrangement of the genome around the central block of structural genes in CELO virus, compared with the mammalian adenoviruses, such that a gene encoding an E4-related protein is located at the left end while the VA gene, characteristically located at the left end, is near the right end of the CELO virus genome. A similar relocation might have occurred for the right-end gene GAM-1, whose product has some attribute of an E1B protein. The homology of the nt 1999 ORF with the gene encoding parvovirus REP proteins prompts further speculation that rearrangement might have been a consequence of interaction between CELO virus and a help-dependent adeno-associated virus.

The E3 region is not required for virus replication in cell culture but functions instead (at least in the group C adenoviruses) to counteract the cellular antiviral immune responses (reviewed in references 25 and 80). Perhaps the replication strategy of CELO virus and passage in avian flocks does not depend on persistent infection and hence the survival functions provided by E3 (major histocompatibility complex down-regulation, tumor necrosis factor defense) are not required by CELO virus. Alternately, CELO virus may have different strategies to provide the E3 functions, and additional studies may clarify this.

From our data, it is clear that structural elements of the virion (e.g., hexon, penton base, IIIa) and the virus-encoded enzymatic functions (the protease and DNA polymerase) are well conserved. These proteins have definite functions that limit the changes that the virus can tolerate. However, genes encoding products that interact with the host (E1, E3, and E4) are not conserved in CELO virus, and this may be a consequence of replication in avian hosts or the use of alternate gene products to perform the same function. Because these host responses are in crucial areas of biology (cell cycle control and the immune response to viral infection), identification of the

E1, E3, and E4 functional counterparts in CELO virus should prove to be a rich source of novel and useful genes.

A number of immediate experiments are indicated by the CELO virus sequence. The absence of a discrete E1 region makes the identification of the transforming region of the CELO virus genome an exciting endeavor. We have identified an anti-apoptotic function in the GAM-1 gene (14a). According to one model of Ad5 transformation (79), both an anti-apoptotic gene (e.g., E1B 19K or GAM-1) and a gene that induces cell proliferation (e.g., E1A 12S) are required. We are currently working to identify E1A-like activity in CELO virus.

The complete sequence of CELO virus will also allow the generation of CELO virus mutants to aid in characterizing the novel genes in this virus. There are at least a dozen ORFs in the novel sequences carried by CELO virus, and deletion analysis will have to be performed to determine which of the sequences are essential. The strategy of inserting genes into a region like E3, used with success in Ad5 and bovine adenovirus vectors (28, 38, 57), cannot be used with CELO virus until such a cell culture-dispensable region is identified. Furthermore, the use of complementing cells lines has facilitated the characterization of the mammalian adenoviruses (e.g., 293 cells expressing E1 [30] and W162 cells expressing E4 [78]). Although the generation of immortalized avian cells is difficult (33, 60), similar strategies will be attempted with avian cells and CELO virus sequences.

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