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Mature Form of the Deoxyribonucleic Acid from Chick Embryo Lethal Orphan Virus

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The deoxyribonucleic acid (DNA) of chick embryo lethal orphan (CELO) virus, an oncogenic avian adenovirus, had a biphasic denaturation profile indicating intramolecular base composition heterogeneity. This was confirmed by shearing the DNA and centrifuging it to equilibrium in Cs_2SO_4 in the presence of $HgCl_2$ when two bands were formed. No circular molecules formed when CELO virus DNA was annealed, although λ DNA formed circles under the same conditions. No circular molecules were found by sedimentation or electron microscopy when the DNA was digested with exonuclease III and then annealed, but 30 to 40% of T7 DNA molecules became circular under similar conditions. The complementary strands of CELO virus DNA both appeared to be continuous, and, when CELO DNA was denatured and then annealed under appropriate conditions, all of the renatured molecules were linear. It is concluded that CELO virus DNA consists of a unique rather than permuted collection of linear molecules that lack exposed single-strand complementary ends or duplex terminal repetitions. These results are discussed in relation to the replication of viral DNA and the transformation of host cells.

The deoxyribonucleic acid (DNA) of chick embryo lethal orphan (CELO) virus, an oncogenic avian adenovirus, is a linear double-stranded molecule with a molecular weight of 30 million daltons and an average base composition of 54% guanine plus cytosine (W. G. Laver et al., Virology, *in press*). This paper describes further studies on the mature form of CELO virus DNA to help in understanding the replication of viral DNA and also the transformation of cells by oncogenic viruses.

Double-stranded DNA from all bacteriophages that have been studied is either terminally repetitious or has exposed, single-stranded complementary ends (25). Many bacteriophages also contain circularly permuted DNA (25). The mature bacteriophage DNA is derived from a precursor DNA molecule in infected cells. The precursor molecule is either circular or concatmeric and gives rise to the mature viral DNA as a result of enzymatically introduced breaks which occur either at specific sequences in the molecule or at specific distances along the molecule (25). A study of the mature form of a viral DNA might suggest which mechanism is operative in its replication. This type of study may also be relevant to the mechanism of viral oncogenesis.

An attempt to identify terminal repetitions in human adenovirus DNA (9) yielded inconclusive results. Thomas (25) reported preliminary results suggesting that adenovirus DNA may contain a circularly permuted collection of sequences. Denaturation mapping by Doerfler and Kleinschmidt (7) showed conclusively that adenovirus type 2 DNA is not circularly permuted. Their methods were not applicable to the oncogenic adenovirus type 12, however, as its DNA does not have extensive base composition heterogeneity as does adenovirus type 2 DNA (7).

MATERIALS AND METHODS

Virus. The Phelps strain (29) of CELO virus grown in chick embryo kidney monolayers was used in these studies.

Media. Growth medium for cell cultures was modified Eagle's medium (22) supplemented with 10% tryptose-phosphate broth and 10% calf serum. The same medium was used during virus growth. When virus DNA was labeled with ^{3}H-thymidine, broth was omitted from the medium and serum which had been dialyzed against normal saline was used. When virus was labeled with ^{32}P, phosphate was also omitted from the medium and 1 mM sodium citrate was added. The agar overlay medium for plaque assay was that previously used for Semliki Forest virus (24), with the bicarbonate concentration doubled and tris (hydroxymethyl)aminomethane (Tris) buffer omitted.

Isotopes. ^{32}P ("carrier free") was obtained from the Australian Atomic Energy Commission, Lucas

Heights, Sydney. Thymidine-*methyl*-^3H (6 Ci/mmole) was purchased from Schwartz BioResearch, Inc., Orangeburg, N.Y.

Enzyme. Exonuclease III was a gift from I. R. Lehman, Department of Biochemistry, Stanford University Medical Center. The preparation contained 5×10^5 enzyme units and 3.7 mg of protein per ml. It had a trace of endonuclease activity, but this had no detectable effect under our experimental conditions.

Cell cultures. Kidneys were removed aseptically from 19-day chick embryos, minced with scissors, washed in phosphate-buffered saline, and incubated with 0.25% trypsin in phosphate-buffered saline for 1 hr at room temperature with occasional vigorous shaking. The cell suspension was filtered through wire gauze, sedimented at about $250 \times g$ for 10 min, resuspended in growth medium, again centrifuged, and resuspended in about 100 times the packed cell volume of growth medium. The cells were counted and diluted to 10^6 per ml in medium. Plastic cell culture dishes (50 by 15 mm, Falcon Plastic) were seeded with 5 ml of the cell suspension and incubated at 36 C in an atmosphere of 5% CO_2 in air. Monolayers became confluent 2 to 3 days later. At this time, an average of 4.6×10^6 cells per plate could be recovered by trypsinization.

Plaque assay. Medium was removed from confluent cell monolayers and replaced with 0.2 ml of virus diluted in gelatin saline (8). After 1 hr of incubation at 36 C, 7 ml of agar overlay medium was added. Plates were incubated for 6 or 7 days at 36 C, after which a further 3 ml of overlay medium containing 0.01% neutral red was added. Plaques were counted 10 to 20 hr later.

Virus growth and purification. Monolayers were inoculated with 5 to 10 plaque-forming units (PFU) per cell of virus in 1 ml of growth medium. After 1 hr at 36 C, an additional 2 ml of medium was added. When labeled virus was prepared, the medium was replaced 5 to 7 hr later with 1.5 ml of the appropriate medium containing either 2 μCi of ^3H-thymidine per ml or 10 μCi of ^{32}P per ml. At 40 to 48 hr after inoculation, the cultures were frozen and thawed three times, and the medium containing the disrupted cells was aspirated into a centrifuge tube. The suspension was centrifuged at about $2{,}000 \times g$ for 10 min, and virus was purified from the supernatant fluid by the method used previously for egg grown virus (W. G. Laver et al., Virology, *in press*).

Preparation of viral DNA. A drop of 0.1% bovine serum albumin was added to the purified virus to prevent aggregation and disruption during dialysis. The preparation was dialyzed against 0.15 M NaCl, 0.015 M trisodium citrate (SSC), and DNA was prepared as described previously (W. G. Laver et al., Virology, *in press*). DNA was dialyzed extensively against STE consisting of 0.1 M NaCl, 0.05 M Tris (*p*H 7.2), 0.001 M ethylenediaminetetraacetic acid (EDTA). DNA concentration was estimated from absorbance (*A*) at 260 nm, assuming that 1 A_{260} unit corresponded to 50 μg of DNA.

Measurement of radioactivity. Samples (usually 0.5 ml) were diluted to about 1.5 ml with water, mixed with 10 ml of Triton X scintillation fluid (62.5% toluene, 37.5% triton-X-100, containing 5 g of 2,5-diphenyloxazole per liter and 0.3 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter), and counted in a Packard 3000 series liquid scintillation spectrometer. When ^3H and ^{32}P were counted simultaneously, standards of each isotope in the same batch of scintillator were also counted. Counts were converted to counts per minute and corrected for background and ^{32}P contamination of the ^3H channel (usually about 2.5%) by the use of a PDP 8/I computer. To measure acid-soluble radioactivity, a 0.1-ml sample was mixed with 0.4 ml of calf thymus DNA (1 mg/ml) and 0.5 ml of 10% trichloroacetic acid at 0 C. After 15 min in ice, samples were centrifuged for 30 min at $17{,}000 \times g$ and 0 C. A 0.5-ml amount of the supernatant fluid was mixed with 1 ml of water and 10 ml of Triton X scintillation fluid and counted for 50 min. Untreated samples were counted for total radioactivity. Acid-soluble counts were corrected for background and slight quenching due to trichloroacetic acid and expressed as a percentage of total radioactivity. Acid-soluble radioactivity of DNA preparations before digestion with exonuclease was between 0 and 0.09%.

Exonuclease III digestion and annealing of DNA. The reaction conditions were those used by Rhoades et al. (18). The reaction mixture (1 ml) was prepared at 0 C, and samples were removed for measurement of total and initial acid-soluble radioactivity. The mixture was then transferred to a water bath at 37 C. Samples were removed at intervals. A 0.1-ml amount of each was used to determine acid-soluble counts, and the remainder was diluted to about 1 μg of DNA per ml in 2× SSC, heated to 65 C for 30 min, and allowed to cool slowly to room temperature.

Denaturation and annealing of DNA. DNA was denatured for 15 min at room temperature in 0.1 M NaOH. It was then diluted to about 1 μg/ml in 2× SSC and neutralized with 0.1 M Tris and sufficient HCl to bring the *p*H to 7.2. Annealing conditions were normally for 45 min at 65 C. Alternatively, the DNA was diluted after neutralization to reduce the concentration of NaCl to 0.05 M, and an equal volume of formamide was added. Annealing took place for about 16 hr at room temperature and was stopped by dialysis against STE. In some experiments, the DNA was finally heated to 75 C for 1 min and then allowed to cool to 40 C to encourage complete renaturation of imperfectly annealed complexes (23, see below).

DNA thermal denaturation profiles. Melting curves were measured on solutions of CELO virus DNA at an approximate concentration of 20 μg/ml in 0.1× SSC by the use of a Unicam SP500 series 2 spectrophotometer fitted with an electrical heating block (A. Adkins & Sons Ltd). The temperature of the DNA solution was monitored with a vinyl-coated thermistor fitted through the cuvette lid and connected to a Tele-Thermometer (YSI model 42SC). The A_{260} measurements were corrected for thermal expansion of the solution and plotted as described by Mandel and Marmur (16).

Shearing CELO virus DNA. A 2-ml solution of

CELO virus DNA at 50 μg/ml in SSC (containing a trace of ^{32}P-labeled CELO virus DNA to serve as a sedimentation marker) was sheared in a VirTis homogenizer at 0 C with a stainless-steel razor blade rotating at 8,000 rev/min for 2 hr. Sucrose density gradient centrifugation of a portion of this material, with untreated ^{3}H-labeled CELO virus DNA as a marker, gave an average molecular weight of about 30×10^5 daltons (1).

Hg (II) Cs_2SO_4 density gradient centrifugation. Sheared CELO virus DNA was centrifuged in Hg (II) Cs_2SO_4 density gradients in the Spinco model E analytical ultracentrifuge by the method of Nandi et al. (17). The sheared DNA (5 μg in 0.25 ml) in 0.025 M Na_2SO_4 was added to 0.7558 g of Cs_2SO_4 (Harshaw Chemical Co., optical grade), 0.1 ml of 0.05 M NaB_4O_7 (*p*H 9); 5 μliters of 10^{-3} M $HgCl_2$ was added; and the mixture was made up to 1.6075 g with water (ρ = 1.605 g per cm^3). The solution was centrifuged for 24 hr at 44,770 rev/min and 25 C. Ultraviolet photographs were traced with a Joyce-Loebl recording microdensitometer, and densities were calculated by using a value for α of 1.40×10^9 (17).

Electron microscopy of DNA. The methods used were essentially those of Davis and Davidson (3) with minor modifications. The hyperphase consisted of 0.5 μg of DNA per ml, 0.1 mg of cytochrome *c* per ml (Sigma Chemical Co.), and 10 μliters of saturated aqueous solution of isobutyl citrate per ml in 0.5 M ammonium acetate, 10^{-3} M EDTA. This mixture was run down a wet stainless-steel ramp on to a solution of 0.15 M ammonium acetate. The film was picked up on carbon-Formvar-coated grids, stained as described by Davis and Davidson (3), and rotary-shadowed with carbon-platinum pellets from an angle of 7°.

RESULTS

Tests for base sequence heterogeneity. Thermal denaturation profiles for CELO virus DNA had a broad hyperchromic shift with an average T_m of 75.6 C which corresponds to 53% guanine plus cytosine (16), in good agreement with the previously reported value of 54 to 55% based on buoyant density (W. G. Laver et al., Virology, *in press*). A small but reproducible step was observed in the curve at about 74 C (Fig. 1). The biphasic nature of the profile is more readily apparent when the curve is differentiated as shown in Fig. 2. This method of displaying the data demonstrates two components: (i) a minor peak at about 73 C and (ii) the major component which has a T_m of about 76.5 C.

It was previously shown (W. G. Laver et al., Virology, *in press*) that CELO virus DNA preparations were homogeneous in buoyant density and sedimentation velocity. The DNA preparations used in the present studies were centrifuged to equilibrium in CsCl gradients in the analytical ultracentrifuge to determine their purity. Only one sharp band was observed (Fig. 3a). The DNA had a ratio of absorbance at 260 nm to 280 nm of 1.92 and a ratio of absorbance at 260 nm to 230 nm of 2.34.

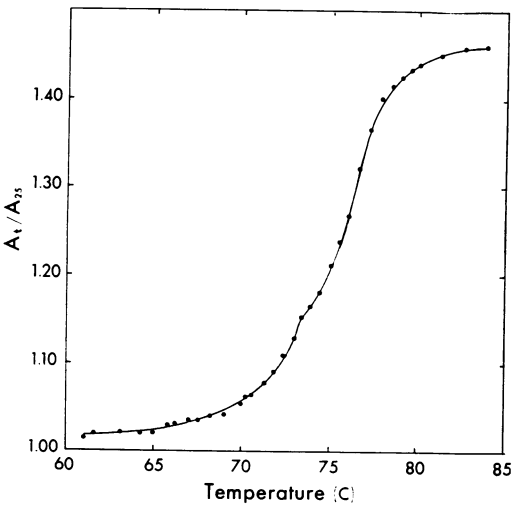

FIG. 1. *Thermal denaturation profile for CELO virus DNA in 0.1× SSC.*

Since the DNA preparations appeared to be homogeneous, the denaturation results indicate that the molecules contain adenine·thymine-rich regions which denature at a lower temperature than the major portion of the DNA (16). If this interpretation is correct, it should be possible to separate fragmented CELO virus DNA on the basis of differing base composition.

CELO virus DNA was broken by hydrodynamic shear (11) and centrifuged to equilibrium in a gradient of Cs_2SO_4 in the presence of $HgCl_2$ as described above. The densitometer tracing (Fig. 3b) shows that the fragments of CELO virus DNA have been separated into two components. This result supports the conclusion that CELO virus DNA contains regions rich in adenine·thymine base pairs.

Tests for exposed single-strand complementary ends. When DNA having complementary single-strand terminals is exposed to annealing conditions, many of the molecules circularize and can be detected in a zone sedimenting 12 to 14% faster than linear DNA (12). CELO virus DNA labeled with ^{32}P was exposed to annealing conditions either by heating for 35 min at 65 C in 0.1 M NaCl or by cooling from 75 to 45 C at 0.4 C/min in 2× SSC. The DNA was then mixed with untreated, ^{3}H-labeled CELO virus DNA and sedimented in a sucrose gradient. There was no detectable difference in the sedimentation velocities of annealed and untreated DNA (Fig. 4a). As a control, it was shown that λ DNA would

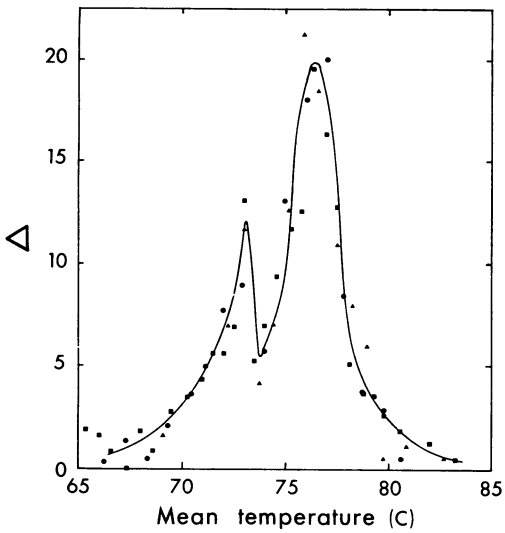

FIG. 2. *Rate of change in absorbance with increasing temperature for CELO virus DNA, expressed as a percentage of the total hyperchromic shift. The values on the ordinate (△) represent the increments in absorbance on raising the temperature from t_1 to t_2 and are given by the equation: $\triangle = 100\ [(At_2/A_{25} - At_1/A_{25})/(t_2 - t_1)]/[(A_d/A_{25}) - 1]$, where A_{25} is the absorbance at 25 C, At_1 is the corrected absorbance at temperature t_1 and At_2 that at t_2, and A_d is the corrected absorbance for the completely denatured DNA. The mean temperature on the abscissa is given by $(t_2 + t_1)/2$. The data from three separate experiments were plotted (note different symbols), and the line was drawn by eye.*

circularize when annealed under the conditions which left CELO DNA unchanged. ^{32}P-λCI857 DNA and ^{3}H-CELO DNA were mixed in 2× SSC and heated to 75 C for 1 min. Half of the preparation was chilled immediately in ice, and the remainder was annealed by slow cooling to 45 C; both samples were sedimented in sucrose gradients. The ^{32}P-λ DNA in the rapidly cooled sample sedimented 1.028 times faster than the ^{3}H-CELO DNA (Fig. 4b), in good agreement with the difference of 1 to 2 million in molecular weight reported (W. G. Laver et al., Virology, *in press*). The sedimentation of ^{3}H-CELO DNA was not changed by annealing, but most of the ^{32}P-λ DNA now sedimented 1.164 times faster than CELO DNA (Fig. 4c), that is 13% faster than linear λ DNA, consistent with circularization of the λ DNA molecules (12). λ DNA was also found in positions in the gradient expected for linear dimers and either circular dimers or linear trimers, all of which may be formed when DNA with exposed complementary ends is annealed. Less than 15% of the λ DNA remained as linear monomers. (^{32}P found at the top of the gradient was ignored in this calculation.) Thus, exposed complementary ends were not detected in CELO DNA by a method that easily detected those of λ DNA, which are only 12 nucleotides long (28).

Tests for terminal repetitions of DNA sequences. CELO virus DNA was tested for duplex terminal repetitions by limited digestion with exonuclease III followed by annealing. Exonuclease III re-

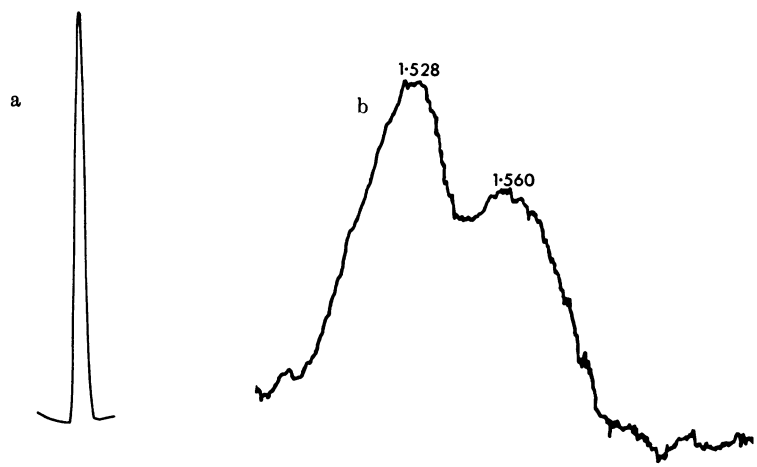

FIG. 3. *Microdensitometer tracings of analytical ultracentrifuge ultraviolet photographs of (a) a CsCl density gradient centrifugation of the CELO virus DNA preparation shown in (b) before shearing. The density of the band was 1.713 g per cm^3. (b) Hg (II) Cs_2SO_4 density gradient centrifugation of the same preparation after shearing. The densitites of the two bands are indicated in g per cm^3.*

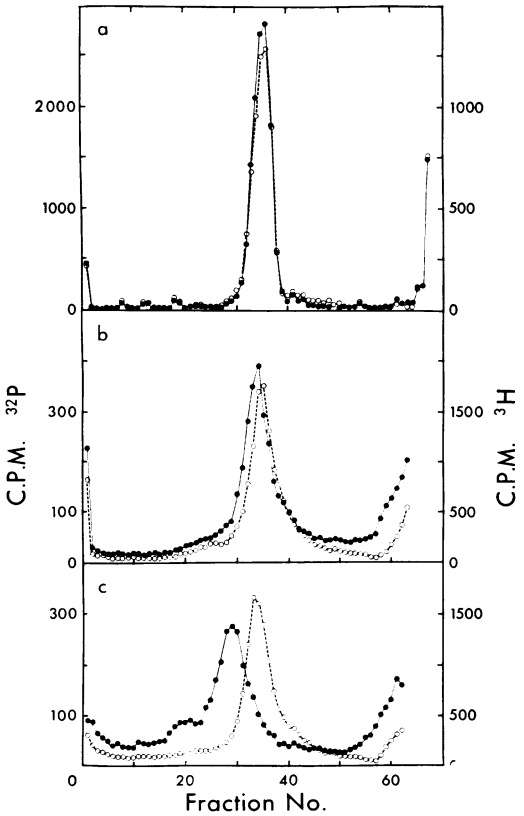

FIG. 4. *Tests for exposed complementary single-strand terminals in CELO virus DNA. (a) ^{32}P-labeled CELO virus DNA (2.5 μg/ml) was annealed by heating to 75 C for 1 min in 2× SSC and then cooling slowly (0.4 C/min at 65 C) to 45 C. The sample was then mixed with ^{3}H-labeled, untreated CELO virus DNA and sedimented in a 5 to 20% sucrose gradient in STE for 90 min at 50,000 rev/min in the SW65 rotor. (b) and (c) ^{32}P-λ CI857 DNA (4 μg/ml) was mixed with ^{3}H-CELO virus DNA (3 μg/ml) in 2× SSC and heated to 75 C for 1 min. Half (b) was immediately chilled in ice and the remainder (c) was annealed by slow cooling (0.4 C/min at 65 C) to 45 C. Each was then sedimented in a sucrose gradient for 120 min at 45,000 rev/min in the SW65 rotor. Symbols: ●, ^{32}P-DNA; ○, ^{3}H-DNA. Sedimentation is from right to left in this and all other figures.*

moves nucleotides stepwise from the 3′ ends. If the DNA has duplex terminal repetitions, the enzyme will expose complementary 5′-terminated single chains at the ends of the molecules, which can anneal under appropriate conditions to yield cyclic molecules detectable by sedimentation or electron microscopy (15, 18, 20).

^{3}H-labeled CELO virus DNA was digested with exonuclease III; the reaction was followed by measuring the production of acid-soluble radioactivity and the DNA was then annealed. Many DNA preparations digested to different extents were examined. The DNA was annealed in 2× SSC at 65 C for 1 hr and then allowed to cool slowly to room temperature (initial rate 0.4 C/min). This DNA was then mixed with ^{32}P-labeled untreated CELO virus DNA and sedimented in sucrose density gradients. DNA digested to 0.0, 0.2, 0.3, 0.4, 0.5, 1.1, 1.6, 2.4, 3.2, 3.5, 3.6, 3.9, 4.2, and 7.2% acid-soluble radioactivity, annealed, and slow-cooled was indistinguishable in sedimentation behavior from untreated DNA (Fig. 5a-g). Several hundred molecules from preparations annealed after digestion to give 0.4, 0.5, 3.6, and 7.2% acid-soluble radioactivity were also examined by electron microscopy. All molecules on well stained and shadowed grids were unquestionably linear and the majority of the molecules in the 7.2% digested sample had collapsed single-stranded regions at both ends, indicating that the exonuclease III had removed nucleotides from one strand only at each terminus (Fig. 6). In DNA annealed after digestion to 7.2% acid solubility, one questionable circle was seen on a poorly shadowed grid. Thus, no definite circles and only one questionable circle were seen in several hundred treated DNA molecules.

These results are in contrast to those obtained with all bacteriophage DNA preparations that have been studied (25), in which digestion and annealing under appropriate conditions yielded 30% or more circular molecules. Circles were first seen in annealed bacteriophage DNA after less than 1% to about 2.5% digestion, depending on the length of the repetition, and were frequent after 3 to 5% digestion (15, 18, 20). As a control, we repeated our experiments with bacteriophage T7 DNA, which has a terminal duplex repetition of 0.7 ± 0.2% of its total length (20). T7 DNA digested to 0.3% acid-soluble radioactivity with exonuclease III and then annealed under the conditions used for CELO virus DNA sedimented as a single zone in a sucrose density gradient (Fig. 5h), and no circular molecules were detected by electron microscopy. When digested to an average of 0.5% acid-soluble radioactivity and then annealed, about 40% of the T7 DNA sedimented as a sharp zone 12% faster than the main zone of linear molecules (Fig. 5i). From this preparation, 98 molecules were examined with the electron microscope; 23 were definitely circular and 8 more probably circular (Fig. 7).

Thus, by a technique which readily detected terminal repetitions in a bacteriophage DNA of

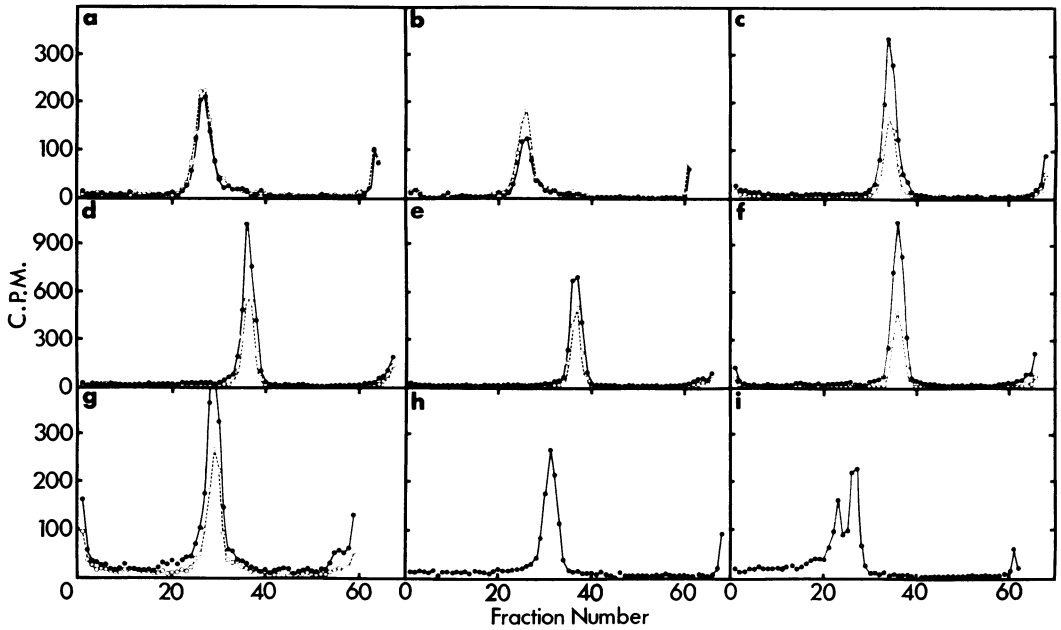

FIG. 5. *Tests for terminal repetitions in CELO virus DNA and T7 DNA. The DNA was digested with exonuclease III to give for ^{3}H-CELO virus DNA (a) 0.3%, (b) 0.5%, (c) 1.1%, (d) 2.4%, (e) 2.6%, (f) 4.2%, and (g) 7.2% acid-soluble radioactivity and for ^{32}P-T7 DNA (h) 0.3% and (i) 0.5% acid-soluble radioactivity. Samples were annealed as described in Materials and Methods. A sample of each was then sedimented in a 5 to 20% sucrose gradient in STE. ^{3}H-CELO virus DNA samples contained untreated ^{32}P-CELO virus DNA as marker. Sedimentation was (a to b) 120 min at 50,000 rev/min in the SW65 rotor; (c to g) 960 min at 20,000 rev/min in the SW25.3 rotor; (h to i) 960 min at 21,000 rev/min in the SW25.1 rotor. Symbols: ●, treated ^{3}H-DNA; ○, marker ^{32}P-DNA.*

comparable size, we were unable to detect any such repetitions in CELO virus DNA.

Tests for permutations of DNA sequences. When a unique collection of linear duplex DNA molecules such as that from bacteriophage T7 or T3 is denatured and annealed, only linear renatured molecules are formed (20). However, if permutations exist as in DNA from bacteriophage T2 or P22, molecules having different permutations can anneal together, resulting in the formation of circular molecules (13, 14, 18, 25, 27). We have tested CELO virus DNA for permuted sequences by searching for circular molecules in denatured annealed DNA.

When denatured and sedimented in a sucrose gradient containing 0.1 M NaOH, 0.9 M NaCl, CELO virus DNA sedimented in a sharp zone at about the same rate as native DNA in neutral sucrose (Fig. 8a, b). This indicated that most of the single strands were intact (19). Comparisons with native DNA indicated that only 8 to 9% of the duplex molecules contained single-strand breaks. Thus, CELO DNA does not have regular single-chain interruptions of the type found in bacteriophage T5 DNA. In neutral sucrose density gradients, denatured CELO virus DNA sedimented much faster than native DNA, as expected, and under the conditions used for analysis of renatured DNA formed a pellet at the bottom of the tube.

When CELO virus DNA was denatured and then annealed, most of the DNA sedimented at the same rate as native DNA and presumably consisted of duplex linear molecules (Fig. 8c). No peak sedimenting 13% faster than marker DNA was found, indicating that the DNA molecules had not circularized.

However, a variable amount of heterogeneous, faster sedimenting material was present. This was probably due to imperfect renaturation (23). Similar results were obtained with bacteriophage T7 DNA, which is not permuted (20).

The sedimentation results suggested that denatured CELO DNA anneals to form linear duplexes of the same size as native molecules, although some are imperfectly renatured. This was confirmed by electron microscopy. Nearly 300 molecules of renatured CELO DNA were examined and none was circular. The renatured molecules were linear duplexes, although many had collapsed single-stranded regions. Under similar conditions, permuted bacteriophage DNA

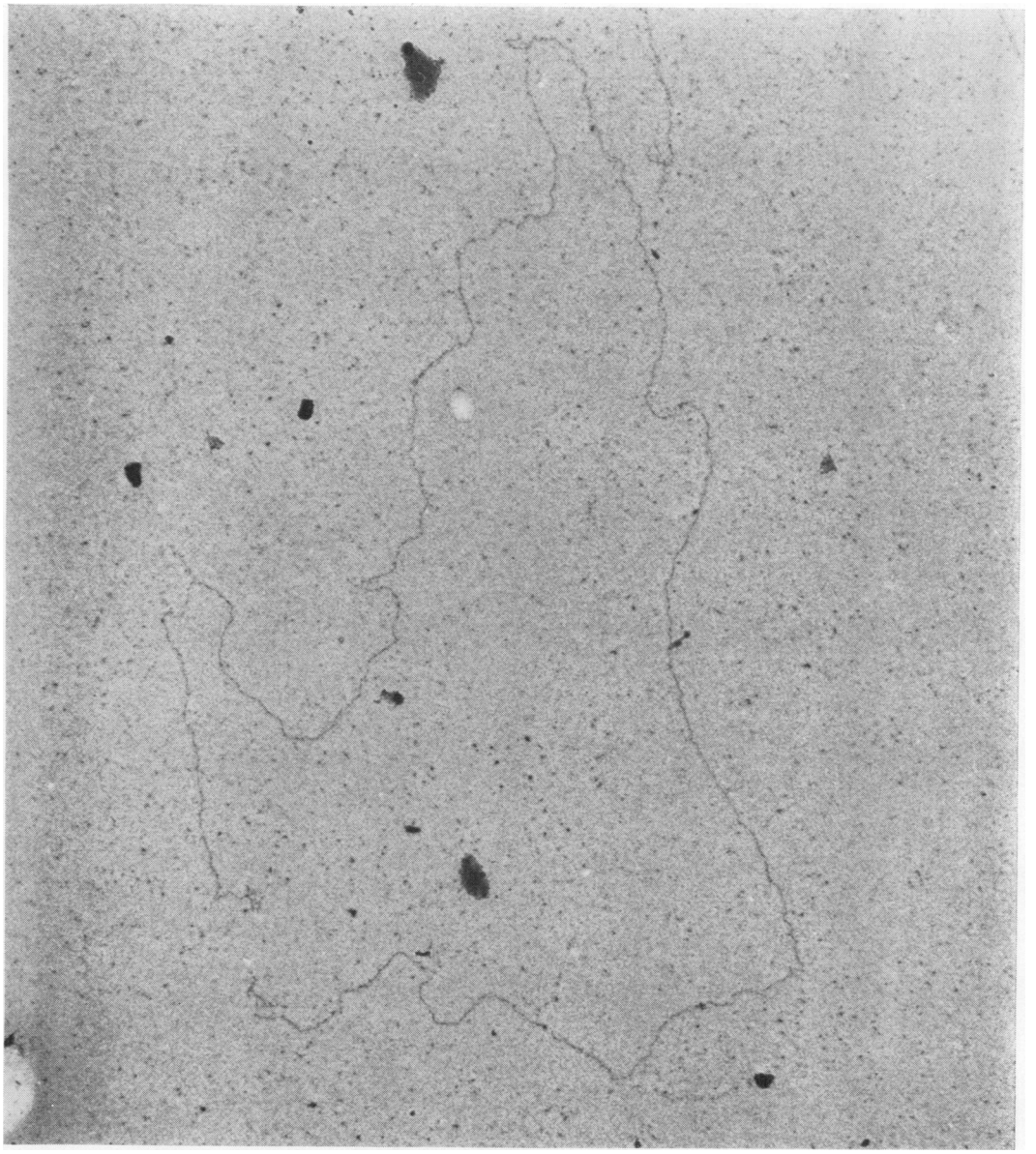

FIG. 6. *Electron micrograph of a CELO virus DNA molecule from a sample digested by exonuclease III to 7.2% acid-soluble radioactivity and annealed (Fig. 5g). Note the "bushes" at each end of the molecule, indicating that the enzyme had exposed single-stranded termini.*

yields 40 to 50% circular molecules (14, 18). The absence of circular molecules in renatured CELO DNA was not due to breakage, which could prevent the formation of circles during annealing of a permuted collection of DNA molecules. Many preparations of denatured, annealed CELO DNA (including that used for electron microscopy) were examined by sucrose gradient sedimentation and none showed evidence of degradation (Fig. 8c).

DISCUSSION

Based on the results of this study, it is possible to make the following conclusions about the mature form of the DNA molecules of CELO virus: (i) they contain regions of differing base

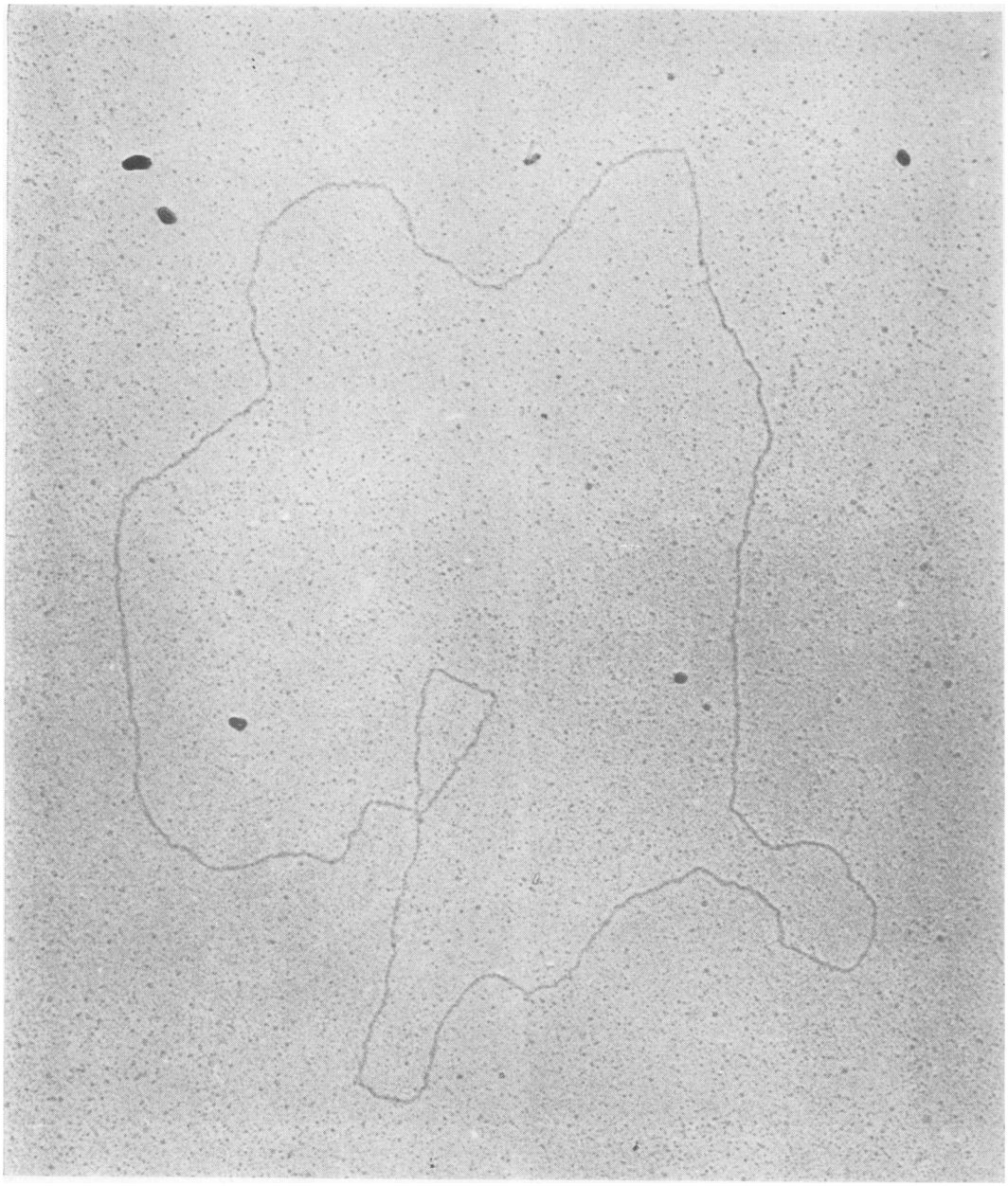

FIG. 7. *Electron micrograph of a T7 DNA molecule circularized by annealing after digestion with exonuclease III to 0.5% acid-soluble radioactivity (Fig. 5i).*

composition, (ii) they have neither complementary single-chain nor duplex terminal repetitions, (iii) they do not have regular single-strand interruptions of the type found in T5 DNA, and (iv) they are a unique rather than a permuted collection of sequences.

Since CELO virus DNA is unique and not terminally repetitious, it could not mature by a "headful" mechanism from a concatemeric molecule like the T-even bacteriophages (25, 26). Similarly it could not form a circular replicative form by ligase action or by recombination as is likely for the temperate bacteriophages λ and P22. A "rolling-circle" type of replication also seems unlikely. These conclusions are supported by our failure to find circular or concatemeric

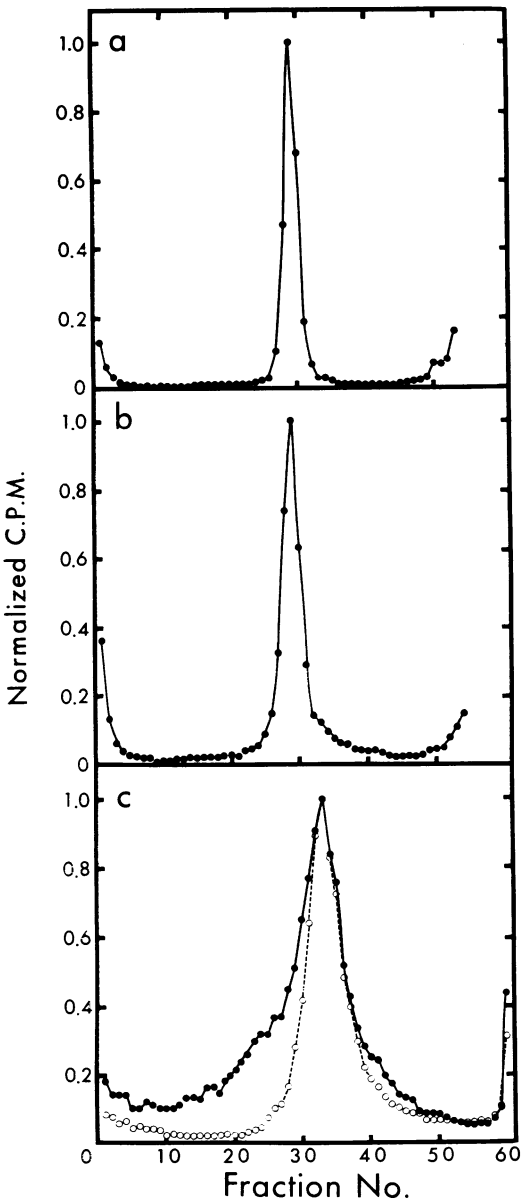

FIG. 8. *Tests for single-strand interruptions and permuted sequences in CELO DNA. (a) Sedimentation of untreated ^{32}P-CELO DNA in a neutral sucrose gradient in STE and (b) sedimentation of ^{32}P-CELO DNA, denatured in 0.3 M NaOH, in a sucrose gradient in 0.1 M NaOH, 0.9 M NaCl; both gradients were centrifuged for 16 hr at 18,000 rev/min in the SW25.1 rotor. (c) ^{3}H-CELO DNA (●) was denatured, neutralized, and annealed in 0.05 M NaCl and 50% formamide for 16 hr at room temperature (14). The reaction was stopped by dialysis against STE. The DNA was heated to 75 C for 1 min and cooled to encourage complete renaturation (23), mixed with untreated ^{32}P-CELO DNA (○), and sedimented in a neutral sucrose gradient for 2 hr at 45,000 rev/min in the SW65 rotor. Counts were normalized to simplify comparison of the curves by dividing each by the appropriate peak count. The peak counts were (a) 2,043 counts/min; (b) 1,493 counts/min; (c) ^{3}H, 361 counts/min; ^{32}P, 1,119 counts/min.*

forms in replicating CELO virus DNA (Bellett and Younghusband, *in preparation*). Among the bacteriophages, CELO virus DNA most resembles the DNA from T3 and T7, but unlike these it lacks terminal repetitions.

These results may be relevant to the mechanism of viral oncogenesis. There is some evidence that DNA from viruses of both the major groups of DNA-containing oncogenic animal viruses, the papovaviruses and adenoviruses, is inserted into the host DNA during transformation of cells (5, 6, 21). DNA from papovaviruses is cyclic and possibly has sequence homology with host DNA. Although there is no evidence for a specialized, site-specific integration system, the papovavirus DNA could be inserted intact into the host DNA by recombination between the circular viral DNA and the host DNA, by analogy with the Campbell model (2) for bacteriophage λ.

The difference in base composition between CELO DNA and host DNA does not rule out recombination as the mechanism of integration. Our results show that some regions of the viral DNA are similar in base composition to host DNA and could therefore contain short sequences homologous to host sequences, although these have not been demonstrated. Moreover, it is now known that the regions of λ and *Escherichia coli* DNA involved in integrative recombination have no homology detectable by genetic or physical methods (4, 10), so that detectable sequence homology is not necessarily required for this type of recombination.

We have found that the mature form of CELO DNA is linear and lacks the terminal repetitions or cohesive ends that would allow intracellular cyclization. Also we have been unable to find circular viral DNA in infected cells (Bellett and Younghusband, *in preparation*). It seems that if CELO DNA is integrated into host DNA by recombination, the viral DNA would be in the linear form. This recombination could take several forms, depending on the number of regions of viral and host DNA able to recombine with each other, but would always cause interruption in the sequence of both viral and host DNA. Integration of intact linear viral DNA could not occur by recombination. Therefore, either the viral DNA sequence is interrupted during integration or integration occurs by some process other than recombination.

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