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Complementary Strands of CELO Virus DNA

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When alkali-denatured DNA from CELO virus (an avian adenovirus) was annealed for 15 min at 37 C in 0.1 M NaCl, 70% of the molecules formed single-stranded circles. This is probably due to base pairing of complementary sequences not more than 110 nucleotides long at the ends of the single strands and implies an inverted terminal repetition in the duplex DNA similar to that reported for the DNA from human adenoviruses. The circular molecules had a uniform length that was approximately the same as that of linear single-stranded molecules. The complementary strands of CELO virus DNA were separated on a preparative scale, and at least 40% of the heavy strands and 56% of the light strands were found to be intact as judged by the formation of single-stranded circles.

Preparative separation of the strands of the DNA of the human adenoviruses types 2 and 7 (8, 15) and adenovirus type 5 (14) has been reported, but to date this has not been achieved with DNA from a highly oncogenic adenovirus. CELO virus is an avian adenovirus capable of inducing tumors when inoculated into baby hamsters (12) and of transforming hamster (1) and human (2) cells in vitro. DNA extracted from CELO virus by the use of Pronase, sodium dodecyl sulfate, and phenol is a linear duplex molecule of mol wt 28.3×10^6 (11). Neither duplex terminal repetitions nor complementary single-stranded ends can be demonstrated, and the molecules are not circularly permuted (17, 18). An inverted terminal repetition has been demonstrated for adenovirus types 1, 2, 3, 7, 18, and 31 (5, 16), and also for the plus and minus strands of adeno-associated virus 2 DNA (7).

We asked if the single strands of CELO virus DNA could circularize and this report confirms that they can. We give evidence that circularization is due to complementary sequences at the ends of the single strands of the DNA and that these arise from an inverted terminal repetition about 110 nucleotides long.

We also describe the preparative separation of the complementary strands of CELO virus DNA for use in investigations of transcription and DNA replication.

MATERIALS AND METHODS

Virus and DNA. The Phelps strain of CELO virus was grown and purified and the DNA was extracted as described previously (9, 17). ϕX174 virus was grown and the DNA was extracted by the method of Sinsheimer (13).

Radioactive labeling of DNA and scintillation counting. The methods used for DNA labeling and scintillation counting have been described elsewhere (17).

Ribopolymers. Polyuridylic acid·polyguanylic acid [poly(U·G)] (lot no. 7001, U:G ratio of 1:0.3) was purchased from Schwarz-Mann, Orangeburg, N.Y.

Cesium chloride. Sequanal-grade CsCl was purchased from Pearce Chemical Co., Rockford, Ill.

Circularization of the single strands of CELO virus DNA. CELO virus DNA (5 μg/ml) in a solution of 0.1 M NaCl, 0.01 M Tris (pH 7.2), 0.001 M EDTA (STE) was denatured by addition of $\frac{1}{10}$ volume of 1 M NaOH. After 15 min at room temperature, the mixture was adjusted to pH 7.9 by addition of $\frac{1}{11}$ volume of 1 M HCl and $\frac{1}{11}$ volume of 1 M HCl and $\frac{1}{11}$ volume of 1 M Tris (pH 7.9), incubated at 37 C for 15 min, and finally chilled in ice or stored at 4 C. Under these conditions, only 1% linear duplex molecules were formed.

Exonuclease III digestion of CELO virus DNA. For digestion of CELO virus DNA, the method of Rhoades et al. (10) was used with minor modifications. Digestion was done in a buffer containing 0.002 M MgCl, 0.01 M mercaptoethanol, and 0.07 M Tris (pH 7.2). Samples containing 10 μg of [^{3}H]dThD-labeled CELO virus DNA per ml (specific activity 10^5 to 3×10^5 counts/min per μg) and 20 to 25 U of exonuclease III per ml in the buffer were incubated at 37 C, and at various times two 100-μliter aliquots were removed for estimation of acid-soluble radioactivity, and 50-μliter aliquots were removed for electron microscopy. Each sample for electron microscopy was added to 50 μliters of buffer containing 0.05 M Tris (pH 7.9), 0.2 M NaCl, and 0.02 M EDTA. To estimate the proportion of single-stranded DNA molecules able to circularize, we treated the samples as described above. *Escherichia coli* exonuclease III was a gift from I. R. Lehman, Department of Biochemistry, Stanford University Medical Center.

Analytical strand separation. CELO virus DNA (5 μg) in STE was denatured with alkali and neutralized as described. Poly(U·G) (5 to 10 μg) was added immediately after neutralization, and the sample was adjusted to the appropriate density with CsCl. Samples were centrifuged in a Spinco model E analytical ultracentrifuge for 20 to 24 h at 25 C and 44,770 rpm using a 4°sector KEL F centerpiece with a 1° negative wedge window. UV absorbtion photographs were traced by using a Joyce-Loebl microdensitometer.

Preparative strand separation. The method described by Landgraf-Leurs and Green (8) for adenovirus 2 DNA strand separation was used with minor modifications. CELO virus DNA (100 to 200 μg) in STE was denatured with alkali and neutralized as described above, and then chilled to 0 C. Poly(U·G) was added immediately before neutralization, at a DNA:poly(U·G) ratio of 1:1 or 1:2 (wt/wt). The mixture was adjusted to a density of 1.79 g/ml with CsCl and a final volumes of 6 ml. Centrifugation was carried out at 25 C. Fractions containing the light (L) and heavy (H) bands were collected and stored at 4 C. The H band was purified by centrifugation in a CsCl solution of density 1.79 g/ml and the L band was separately centrifuged in a CsCl solution of density 1.78 g/ml.

Electron microscopy. The methods of Davis et al. (4) were used for electron microscopy. Specimens were viewed and photographed in a Siemens Elmiskop II.

Length measurement of molecules. Molecules were photographed, the negatives were projected onto a screen, and the images were traced onto paper. Tracings were measured with a map measurer. φX174 DNA was spread with single-stranded CELO virus DNA to serve as an internal length standard. Single-stranded, linear CELO virus DNA molecules that were obviously shorter than half length were not included in the length measurements.

Annealing of separated strands. To separate samples (10 to 20 μg of DNA/ml) of the H band and the L band and a mixture of equal amounts of the H band and L band in CsCl, 1/10 volume of 1 M NaOH was added. After 1 h at 37 C, each sample was adjusted to pH 7.0 with HCl and Tris and incubated at 65 C for 14 h. Under these conditions, denatured CELO virus DNA (5 μg/ml) fully reannealed as determined by analytical ultracentrifugation.

RESULTS

Formation of single-strand circles. Up to 70% of the single strands of CELO virus DNA (5 μg/ml in STE) formed circles visible in the electron microscope (Fig. 1) when incubated in STE at 37 C for 15 min. Length measurements (Fig. 2) of linear and circular single-stranded molecules show that circles are of uniform length, but the mean length is slightly shorter than the mean length of the linears ($P < 0.02$ in a t test). This may be due to a difference in the way circular molecules behave in the cytochrome c film as compared with linear molecules, rather than a real difference in length. Only molecules above 10×10^6 daltons were included in the calculation of the mean of linear molecules. Both linear and circular forms had a mol wt of about 13×10^6. This is 1.2×10^6 daltons less than expected from the molecular weight (28.3×10^6) of the duplex CELO virus DNA calculated by using φX174 RF II molecules as an internal length standard. This result was reproducible. Possibly, single-stranded CELO virus DNA has more secondary structure than the φX174 DNA molecule.

Effect of exonuclease III digestion of duplex CELO DNA on single-strand circle formation. CELO virus DNA was digested by exonuclease III, and the number of strands able to form single-strand circles after denaturation and annealing was estimated. In three separate experiments (Fig. 3), circle formation was unaffected after 60 nucleotides had been removed and was abolished after 180 nucleotides had been removed from each 3′ end. From the curve in Fig. 3, 50% reduction in circle formation corresponded to an average length of 110 nucleotides removed from each 3′ end. This estimate is based on the assumption that the average thymidine content of the terminal sequence is not greatly different from that in the rest of the molecule.

To exclude the possibility that the result was due to an endonuclease activity during digestion, φX174 RF I DNA (a gift from G. D. Clark-Walker, Research School of Biological Sciences, Australian National University) was mixed with a portion of the digestion mixture (containing an equivalent amount of CELO virus DNA) from one of the experiments (closed squares in Fig. 3). After incubation at 37 C for 60 min, 14% of the φX174 RF I DNA molecules were converted to φX174 RF II molecules as determined by electron microscopy. This is equivalent to a maximum of one nick per 10 duplex CELO virus DNA molecules per 5 min. Under equivalent conditions, CELO virus DNA circle formation was abolished after 5 min of digestion. Nicking would have contributed a maximum of 5% reduction in circle formation in this time.

Thus, the formation of single-strand circles depends on a sequence with an average length of 110 nucleotides at the 3′ ends of the intact single strands of the DNA.

Analytical strand separation. Figure 4 shows UV absorption profile of a typical analytical CsCl gradient of CELO virus DNA after it was denatured and annealed with poly(U·G) at a DNA/poly(U·G) ratio of 1:1 or 1:2 (wt/wt). The major bands of density, 1.766 g/ml and 1.781 g/ml, were assumed to represent the com-

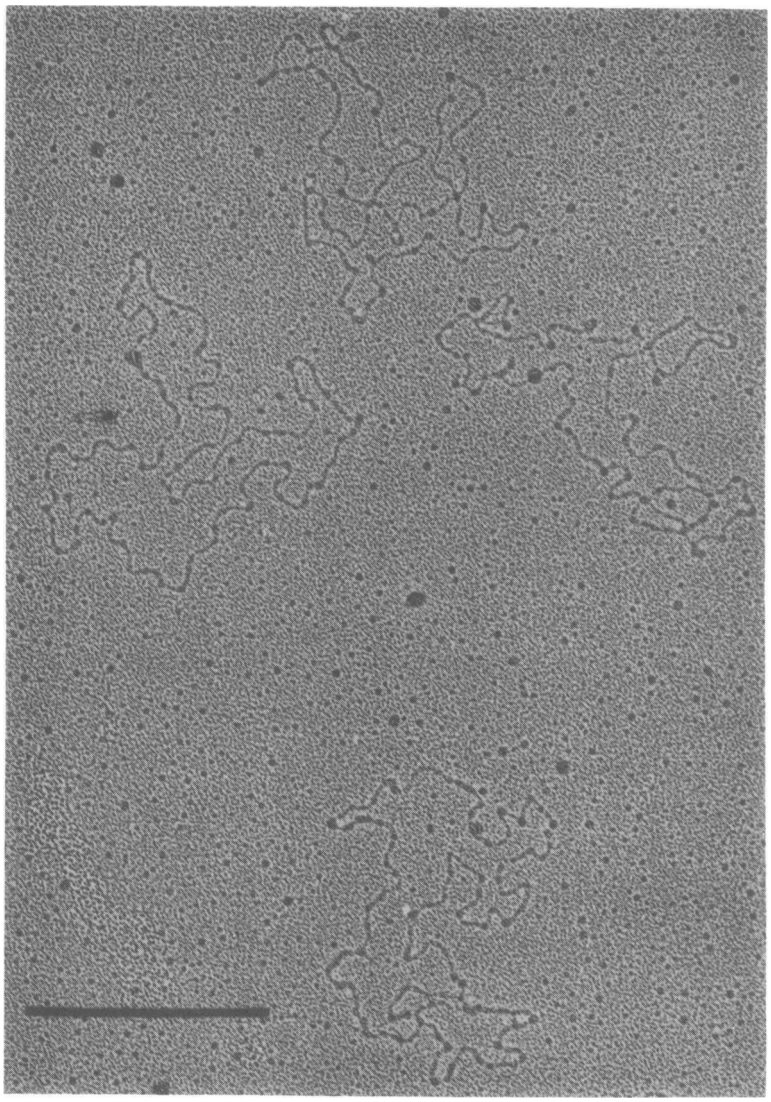

FIG. 1. *Typical circular forms of single-strand CELO virus DNA seen after annealing. Samples for electron microscopy (0.5 μg of DNA/ml) were spread in 50% formamide onto a 20% formamide hypophase. The grids were rotary-shadowed with platinum-palladium (80:20) at an angle of 7°. The bar represents 1 μm.*

plementary strands complexed with poly(U·G). A separation of 14 to 15 mg/ml between the two bands was achieved routinely.

Preparative separation of strands. The absorption (260 nm) profile of a preparative CsCl gradient of denatured CELO virus DNA annealed with poly(U·G) is shown in Fig. 5a. The H and L bands from two such gradients were separated as indicated, and the pooled H bands and the pooled L bands were again centrifuged in CsCl as described above. The A_{260} absorption (260 nm) profiles of these gradients are shown in Fig. 5b and c. The fractions corresponding to the H and L bands were collected as indicated.

An analytical ultracentrifuge UV absorption profile of the preparative gradient in Fig. 5a is shown with and without a density maker in Fig. 6a and b. Similarly, Fig. 6c and d and e and f are analytical ultracentrifuge UV absorption profiles of the L and H bands collected from the preparative gradients shown in Fig. 5b and c. The band of density 1.765 g/ml in Fig. 6b corresponds to the L band in Fig. 5a, and the band of density 1.779 g/ml corresponds to the H band. As can be seen, purification of the L and

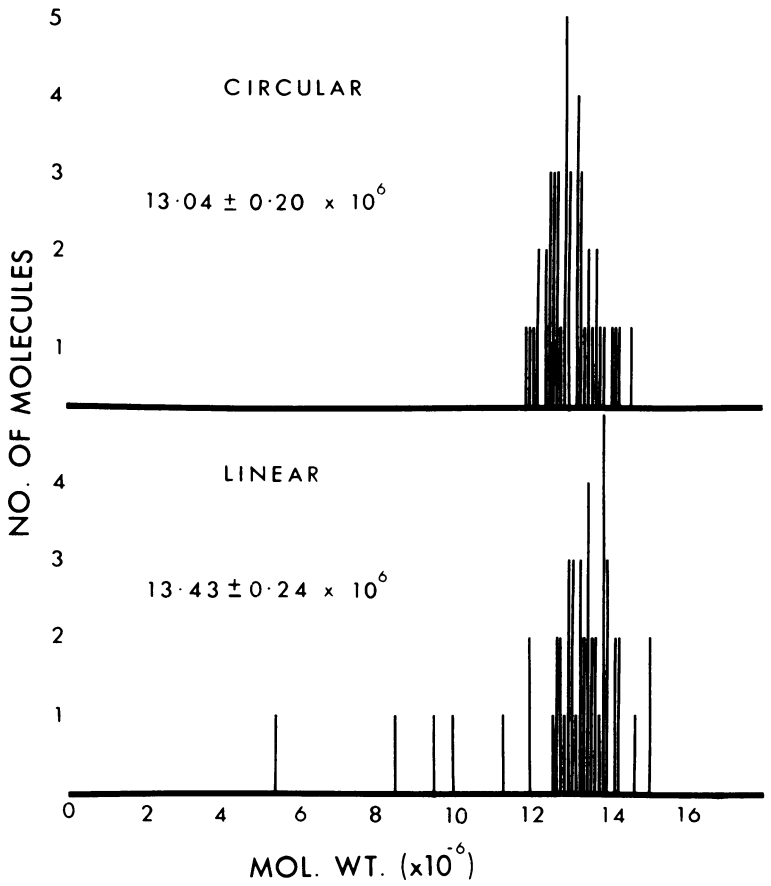

FIG. 2. *Histograms of length measurements of single-strand circular and linear molecules of CELO virus DNA. φX174 DNA (1.7 × 10⁶ daltons) was used as an internal length standard. Measurements were made from electron micrographs.*

H bands in CsCl gave an L band free from contaminating H band and an H band free from contaminating L band. A third UV-absorbing band could be seen in the analytical gradient in Fig. 6b at a density of approximately 1.748 g/ml. This band probably corresponds to the band seen in fractions 50 through 53 in Fig. 5a and again in fractions 28 through 35 in Fig. 5c. A small amount (less than 5% of the main band) of this contaminating band (P band) can be seen in Fig. 6d to the lighter side of the main L band. The nature of this P band is discussed below.

In a further series of analytical ultracentrifuge experiments, the H and L bands were shown to correspond to the complementary strands of the DNA complexed with poly(U·G). Poly(U·G) was removed from the H and L bands by incubation in alkali. When annealed, the L band had a density of 1.729 g/ml in CsCl (Fig. 7b), and no duplex DNA of density 1.713 g/ml could be detected (Fig. 7a). Similarly, the annealed H band had a density of 1.726 gm/ml in CsCl (Fig. 7d), and no detectable duplex material of density 1.713 g/ml could be seen (Fig. 7c). When equimolar amounts of the H and L bands were annealed together, a single peak of duplex DNA (density 1.716 g/ml) was obtained (Fig. 7e and f). The slightly higher density of the annealed strands compared with native CELO virus DNA (density, 1.713 g/ml) was probably due to imperfect annealing of fragmented strands.

Integrity of the separated strands. Electron microscopy of the self-annealed H strand revealed 40% circular, 58% linear, and 2% oligomeric molecules. Similarly, 56% of the L-strand molecules were circular, 46% were linear, and 3% were oligomeric. Thus, at least 40% of the H-strand molecules and 56% of the L-strand

molecules were intact. In this preparation, 12.5% of the initial duplex DNA was recovered as separated complementary strands.

DISCUSSION

The complementary strands of CELO virus DNA, like those of human adenoviruses (5, 16) and adeno-associated virus 2 (7), will form single-strand circles under annealing conditions. The simplest interpretation of this observation is that they arise from base pairing of complementary sequences at the ends of the single strands of the DNA. This would imply an in-

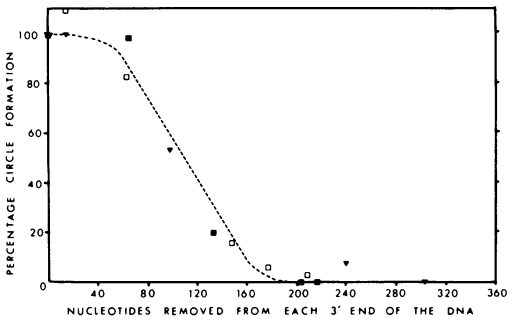

FIG. 3. *CELO virus DNA (10 μg/ml) was digested with exonuclease III, and at various times samples were removed for electron microscopy and determination of acid-soluble radioactivity (from which the number of nucleotides removed from each 3' end of the DNA was calculated). The samples for electron microscopy were denatured and annealed, and the proportion of circles to linears was assessed by counting 100 molecules at random. The circle formation was expressed as a percentage of the proportion of circles formed by untreated DNA under the same conditions. In the three experiments shown, the proportion of circles formed without digestion was 48% (□), 42% (▼), and 50% (■).*

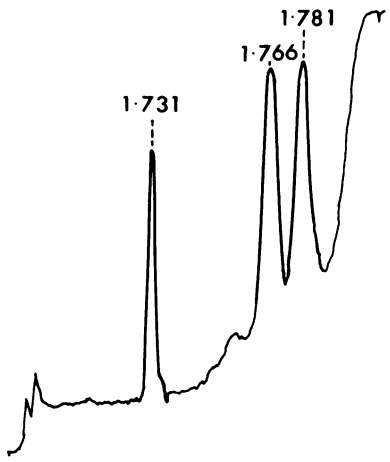

FIG. 4. *CELO virus DNA was denatured with alkali, neutralized, and mixed with poly(U·G). To the mixture was added Micrococcus lysodeikticus DNA (density, 1,731 g/ml) and CsCl to a final density of 1.755 g/ml. A tracing of a UV photograph from a Spinco model E analytical ultracentrifuge is shown. Centrifugation was at 44,770 rpm for 24 h at 25 C. The bands shown at 1.766 and 1.781 g/ml represent the L and H bands, respectively.*

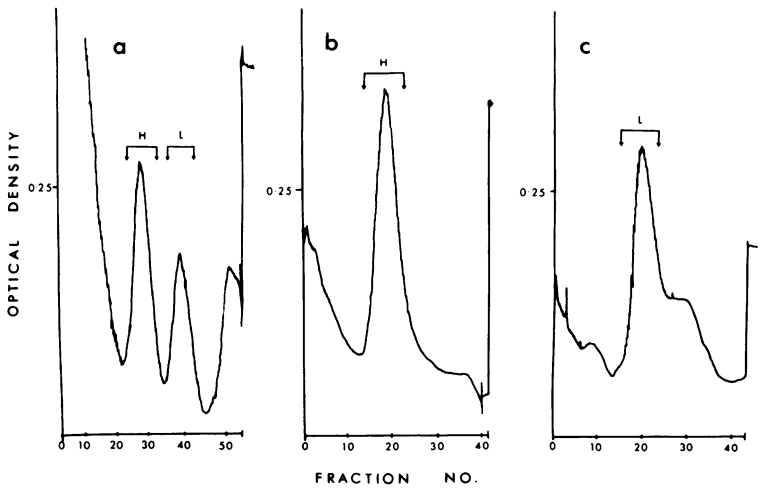

FIG. 5. *Gilford recording spectrophotometer tracings of preparative CsCl gradients containing the strands of CELO virus DNA complexed with poly(U·G). The optical density (at 260 nm) values given are from a 2-mm light path flow cell. (a) CELO virus DNA was denatured, complexed with poly(U·G), and centrifuged. The fractions representing the H and L bands from two such gradients were collected (arrows) and separately centrifuged in CsCl (b, c). The fractions containing the purified H and the purified L bands were collected (arrows).*

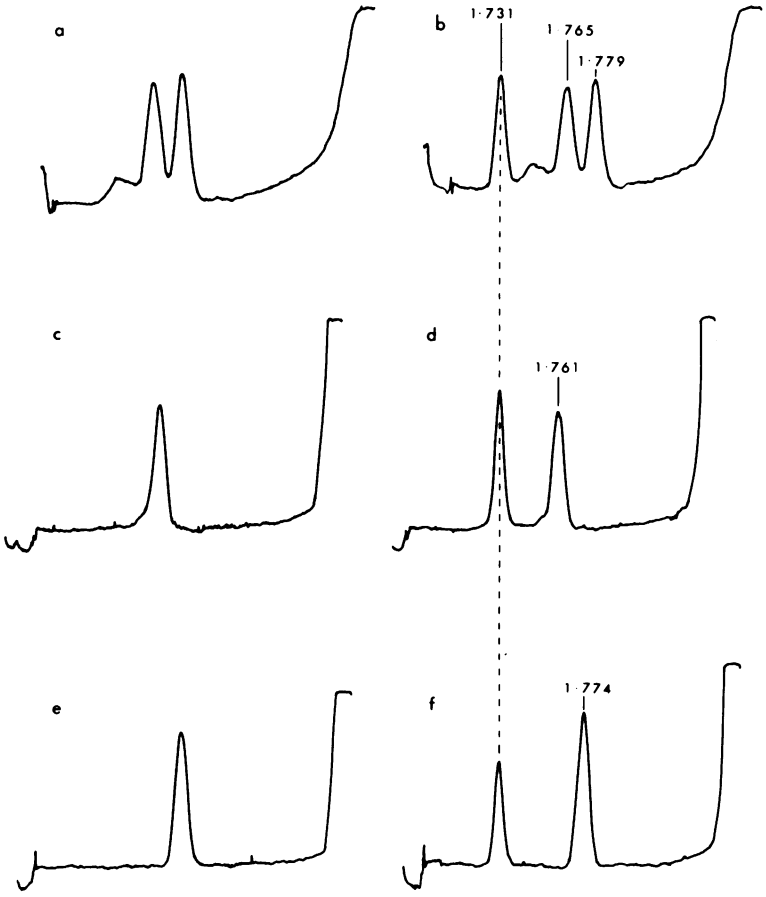

FIG. 6. *Tracings of UV photographs taken in a Spinco model E analytical ultracentrifuge. All centrifugations were at 44,770 rpm., 25 C, for 20 to 24 h. (a) Portion of the preparative gradient (Fig. 5a) in CsCl. (c) Purified L band in CsCl. (e) Purified H band in CsCl. (b, d, f) Gradients a, c, and e, respectively, with M. lysodeikticus DNA (density, 1.731 g/ml) added as a density marker. The calculated densities of the other bands are given in grams per milliliter.*

verted terminal repetition in the duplex DNA of the type proposed by Wolfson and Dressler (16) for human adenoviruses. Each strand would have terminal sequences of the type ABCD and D'C'B'A' (where A' is complementary to A, etc.), and the single-stranded circles formed by annealing would have a projecting linear duplex "panhandle." The alternative structure proposed by Garon et al. (5) for human adenovirus DNA would require a parallel "in line" duplex segment in the single-stranded circle. We favor the panhandle model. Structures interpreted as panhandles have been observed in the electron microscope with adenovirus 18 DNA (C. F. Garon, K. H. Berry, and J. A. Rose, personal communication) and adeno-associated virus 2 DNA (3) but were not seen with CELO virus DNA.

The length of the inverted terminal repetition of CELO virus DNA is about 110 base pairs as determined by exonuclease III digestion. This could not be seen in the electron microscope as a panhandle. The length of the repetition is shorter than the estimates given for adenovirus type 2 by Wolfson and Dressler (16), adenovirus types 1, 2, 3, 7, and 31 by Garon et al. (5), and adenovirus type 18 DNA (Garon, Berry, Rose, personal communication). The terminal repeats in the plus and minus strands of adeno-associated virus 2 DNA have been estimated as 40 to 100 nucleotides long (3).

There is general agreement that the structure of the terminal repeat in adenovirus DNA is inverted as compared with the repeat seen at the ends of the DNA of bacteriophages T3 and T7 (5, 16). The situation with the annealed plus

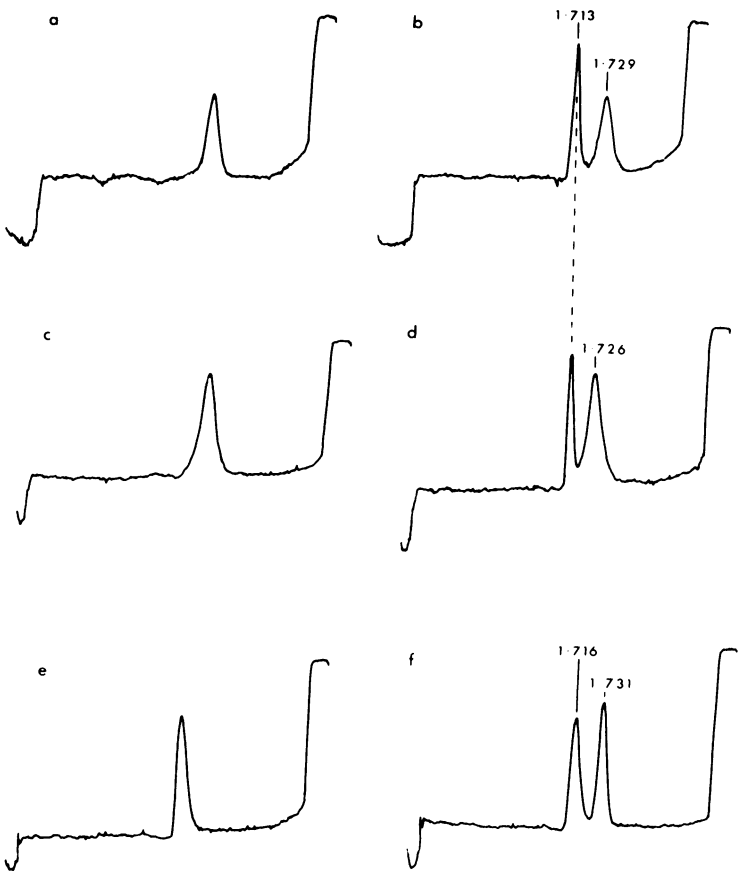

FIG. 7. *Tracings of UV photographs taken in a Spinco model E analytical ultracentrifuge. All centrifugations were as described in the legend to Fig. 6. (a) L band after removal of poly(U·G), annealing, and centrifugation in CsCl. (c) H band after removal of poly(U·G), annealing, and centrifugation in CsCl. (e) H band and the L bands after removal of poly(U·G), annealing together, and centrifugation in CsCl. (b, d) Gradients a and c, respectively, with native CELO virus DNA (density, 1.713 g/ml) added as a density marker. (f) Gradient e, with M. lysodeikticus DNA added as a density marker. The calculated densities are given in grams per milliliter.*

and minus strands of adeno-associated virus 2 DNA, however, may be more complicated, as Gerry et al. (6) suggest that both forms of terminal repeat are present.

The significance of the inverted terminal repetition remains obscure. It may have a role in replication (5, 11), and it is interesting to note that adeno-associated virus 2 which relies on a helper adenovirus for replication should also have this unusual repetition at the ends of its DNA.

The strands of CELO virus DNA can be separated preparatively by using poly(U·G). Up to 100 μg per 6-ml gradient can be processed, but above this level the amount of UV-absorbing material in the P band increases and can almost obscure the L band in some preparations. The nature of the material in the P band was not investigated by us, but Tibbetts et al. (15) found that it formed duplex DNA when the poly(U·G) was removed and the sample was annealed. A peak resembling the P band can be seen in the analytical gradients of adenovirus 2-poly(U·G) complexes of Landgraf-Leurs and Green (8), but they did not report any problems arising from such a band. We assume the P bands in the CELO virus DNA gradients are complexes of poly(U·G) with both strands of the DNA. Experiments using the separated complementary strands to investigate the transcription of CELO virus DNA are in progress.

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