Densonucleosis Virus DNA: Analysis of Fine Structure by Electron Microscopy and Agarose Gel Electrophoresis

By D. C. KELLY and HARRIET M. BUD

N.E.R.C., Unit of Invertebrate Virology, 5 South Parks Road, Oxford OX1 3UB

(Accepted 10 January 1978)

SUMMARY

Double stranded DNA, created on extraction of the plus and minus strands, which are separately packaged in densonucleosis virus particles, exists predominantly as linear monomers, although circular monomers and concatamers and other less well defined structures were observed by electron microscopy and agarose gel electrophoresis. A limited nucleotide sequence permutation, which is probably non-random, comprises about 2.7% of the genome (160 base pairs) and is considered to be the structural feature causing circularization and concatamerization. Evidence of an inverted terminal repetition observed by circularization of single stranded DNA was obtained by electron microscopy and S1 nuclease digestion. Estimates of the size of the terminal repeats varied from 60 to 380 base pairs.

INTRODUCTION

Densonucleosis viruses (DNV) are small (22 nm) isometric viruses which contain linear single stranded DNA with a mol. wt. of about $1.95 \times 10^6$ (Barwise & Walker, 1970; Kurstak et al. 1971; Kelly et al. 1977). Two such viruses have been isolated from insects – DNV 1 from the greater wax moth (Galleria mellonella) and DNV 2 from the Trinidadian buckeye caterpillar (Junonia coenia; Tinsley & Longworth, 1973) and the two viruses are very closely related as determined by DNA hybridization studies (Kelly et al. 1977). Both viruses have been included within the Paroviridae (Bachmann et al. 1975) although their relationship to paroviruses as typified by minute virus of mice (MVM) and Kilham rat virus (KRV) and the adeno-associated virus (AAV) group is not clear. Densonucleosis viruses contain complementary strands of DNA separately encapsidated in virus particles (Barwise & Walker, 1970; Kurstak et al. 1971; Kelly et al. 1977) and in this respect resemble AAV (Rose et al. 1969; Berns, 1973) rather than MVM or KRV which contain linear single stranded DNA of one polarity, with small palindromic hair pin loops at both ends of the molecule (Bourguignon et al. 1976; Salzman, 1977). Biologically, DNV appears to resemble the autonomous paroviruses (KRV and MVM) since DNV can replicate without the aid of a helper virus whereas AAV requires an adenovirus to complete replication. We have examined the fine structure of densonucleosis virus DNA by electron microscopy to compare the genome structure of DNV with AAV and other paroviruses.

METHODS

DNA. Double stranded (ds) DNA was extracted from purified DNV 1 and DNV 2 as previously described (Kelly et al. 1977). AAV 2 ds DNA was a gift from Dr B. J. Carter
(National Institutes of Health, Bethesda, Maryland, U.S.A.). Supercoiled polyoma DNA was a gift from Dr Beverly Griffin (Imperial Cancer Research Fund Laboratories, London, U.K.). Bacteriophage λ DNA was a gift from Mr A. Jones (Department of Pathology, University of Oxford, Oxford, U.K.). Calf thymus nucleosomal DNA digested with micrococcal nuclease was a gift from Mr M. J. Butler (Department of Biochemistry, University of Oxford, U.K.).

Polyoma and bacteriophage λ DNAs were digested with the restriction enzymes EcoRI, Hpa II, and Hind III as described by Griffin et al. (1974). The restriction enzymes were purchased from Miles Laboratories Ltd, Stoke Poges, U.K.

Electron microscopy of DNA. DNA was spread for electron microscopy using a modification of the method of Inman (1973). Fifteen microlitres of buffer (0.065 M-Na₂CO₃, 12.03 % (v/v) formaldehyde, 0.0102 M-EDTA, and 0.04 M-tris-HCl pH 8.0) was added to 10 μl of viral DNA. The volume was made up to 50 μl with 20 mm-NaCl, 5 mM-EDTA. Fifty μl of formamide and 10 μl of cytochrome c (100 μg/ml) were added. Fifty μl of the mixture were spread on to water by running the droplet down a stainless steel slide. The DNA was picked up on carbon coated grids and fixed in absolute ethanol. The DNA was then shadowed with platinum. The molecules were examined on A.E.I. 6B or 801 electron microscopes. The magnification was calibrated with a cross grating replica grid (2160 lines/mm: Emscope Laboratories, London). The molecular lengths of DNA were determined by tracing enlarged images on to paper and measuring the traces with a Hewlett Packard 9820 calculator and digitizer.

Agarose gel electrophoresis of ds DNA. Vertical agarose slab gels (1.4 % and 2.0 %, w/v) made up in E buffer (0.04 M-tris-acetate, pH 7.9, 0.005 M-sodium acetate, 0.001 M-EDTA) containing 0.5 μg ethidium bromide/ml were cast (20 × 20 × 0.3 cm) and run (constant voltage, 20 V/60 min, 100 V/210 min) as described by Sugden et al. (1975).

Circularization of the single strands of DNV DNA. This was performed essentially as described by Robinson & Bellett (1975) for CELO virus DNA. DNV ds DNA (3 μg/ml) in a solution of 0.1 M-NaCl, 0.01 M-tris-HCl (pH 7.2), 0.001 M-EDTA was denatured by the addition of a one-tenth vol. of 1 M-NaOH. After 15 min at room temperature the mixture was adjusted to pH 7.9 by adding a one-eleventh vol. of 1 M-HCl and a one-eleventh vol. of 1 M-tris-HCl (pH 7.9). This preparation was incubated at 37 °C for up to 30 min and finally chilled on ice. With these conditions less than 0.5 % linear duplex molecules were formed.

Isolation of inverted repeats of DNV DNA. This procedure was based on the method devised by Ohtsubo & Ohtsubo (1976) to isolate inverted repeat sequences in Escherichia coli plasmids. DNV ds DNA was denatured by adding 100 μl of 0.3 M-NaOH to 100 μl of DNA (20 μg). After incubation at room temperature for 15 min, 100 μl of 0.3 M-HCl was added, followed by 100 μl of 1 M-NaCl. The DNA was then incubated at 68 °C for 22 s and then chilled on ice. One hundred μl of 150 mm-sodium acetate, pH 4.6, 22.5 mm-ZnSO₄, 1.5 M-NaCl and a 1000 units of S₁ nuclease (Sigma Chemical Company Ltd, London) was added and the whole incubated at 37 °C for 90 min. The reaction was stopped by chilling on ice, and by adding 100 μl of 65 mm-EDTA. One hundred and twenty-five μl of 40 % sucrose in 5 × E buffer was added and 100 μl samples were immediately electrophoresed on 2 % agarose gels.
RESULTS

Agarose gel electrophoresis of DNV ds DNA

Analysis of DNV ds DNA by agarose slab gel electrophoresis demonstrated that at least three components are present in DNV ds DNA preparations as shown in Fig. 1. Calibration of the gels with bacteriophage λ DNA restriction endonuclease fragments (Hind III and EcoRI) and polyoma DNA restriction endonuclease fragments (Hind III, EcoRI, and Hpa II), shown in Fig. 2, demonstrated that the major component of DNV ds DNA (which has previously been shown to be a linear monomer by electron microscopy: Kelly et al. 1977) has an apparent mol. wt. of about 3·6×10^6 (Table 1). This is in good agreement with previous determinations by four independent methods (Kelly et al. 1977). Table 1 also shows that DNV DNA is larger than both AAV 2 ds DNA and linear polyoma ds DNA.

By analogy with AAV 2 ds DNA preparations which also contain at least three components – linear monomers, circular monomers, and linear concatamers (Berns, 1973; Gerry et al. 1973) it seemed probable that the three components resolved in DNV ds DNA preparations were also linear monomers, circular monomers and concatamers. A comparison of AAV 2 ds DNA with DNV ds DNA in Fig. 1 shows that the profiles are similar...
Fig. 2. Calibration of 1.4% agarose gel with bacteriophage λ DNA cut with restriction enzymes EcoR1 (△) and Hind III (▲), polyoma DNA cut with restriction enzymes EcoRI (○), Hind III (●), and Hpa II (■). The molecular weights are taken from the literature (Griffin et al. 1974; Murray & Murray, 1974; Thomas & Davis, 1975). The migration of densonucleosis virus DNA is indicated (○).

Table 1. Molecular weights of densonucleosis, AAV 2 and polyoma virus linear ds DNA determined by agarose gel electrophoresis

<table>
<thead>
<tr>
<th>DNA</th>
<th>Mol. wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNV 1</td>
<td>(3.64 ± 0.01) × 10^6</td>
</tr>
<tr>
<td>DNV 2</td>
<td>(3.64 ± 0.01) × 10^6</td>
</tr>
<tr>
<td>AAV 2</td>
<td>(2.85 ± 0.01) × 10^6</td>
</tr>
<tr>
<td>Polyoma*</td>
<td>(3.17 ± 0.01) × 10^6</td>
</tr>
</tbody>
</table>

* Linear polyoma DNA was obtained by digesting polyoma DNA with the restriction enzyme EcoR1 which cuts the DNA at one site (Griffin et al. 1974).

(allowing for the intrinsic difference in genome size). The minor component entering the gel behind the linear monomer of DNV ds DNA co-runs approximately with the relaxed circular form II of polyoma DNA and runs appreciably slower than the supercoiled form I of polyoma DNA. This minor component also runs faster than anticipated for a linear ds dimer, so it is probable that it represents a circular monomer. The three forms of DNV DNA have been shown to be genetically equivalent material from (1) restriction enzyme analysis, (2) S1 nuclease digestion (which converts the postulated concatamers and circles to material which co-electrophoreses with the linear monomers) and (3) denaturing and reannealing pure linear monomers which re-creates all three forms (D. C. Kelly, unpublished observations). Exposure of DNV ds DNA to extended annealing conditions (i.e. 68 °C in 0.1 M-Na+ for various times) failed to alter the relative proportions of the three forms.

Electron microscopy of DNV ds DNA preparations

Since agarose gel electrophoretic analysis of densonucleosis virus DNA showed that the preparations were heterogeneous, we re-examined DNV ds DNA preparations by electron microscopy. Contrary to an earlier report (Kelly et al. 1977) we now find in carefully spread DNA that about 1 molecule in 400 monomers was circular (Fig. 3). Concatamers were also
observed, as well as other less well defined and tangled structures. The molecular lengths of the linear and circular forms of both DNV 1 and DNV 2 were similar (Table 2). Denso-nucleosis virus DNA was significantly larger than both AAV 2 ds DNA and polyoma DNA. The derived mol. wt. are also shown in Table 2, determined by using a molar linear density of \(2.03 \times 10^{10}\) daltons/cm which was obtained for bacteriophage T7 DNA under identical DNA spreading conditions (Bud & Kelly, 1977). The presence of circular monomer and concatameric forms of ds DNA are consistent with a limited nucleotide sequence permutation present in the DNV genome.
Table 2. Molecular weight of densonucleosis virus ds DNA determined by electron microscopy

<table>
<thead>
<tr>
<th>DNA*</th>
<th>Length (µm)</th>
<th>Mol. wt. (× 10^6)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNV 1, linear</td>
<td>2.011 ± 0.163</td>
<td>4.08 ± 0.33</td>
</tr>
<tr>
<td>DNV 1, circular</td>
<td>2.043 ± 0.192</td>
<td>4.14 ± 0.38</td>
</tr>
<tr>
<td>DNV 2, linear</td>
<td>1.976 ± 0.204</td>
<td>4.01 ± 0.41</td>
</tr>
<tr>
<td>DNV 2, circular</td>
<td>2.066 ± 0.173</td>
<td>4.19 ± 0.35</td>
</tr>
<tr>
<td>AAV 2, linear</td>
<td>1.692 ± 0.141</td>
<td>3.43 ± 0.28</td>
</tr>
<tr>
<td>Polyoma, circular</td>
<td>1.651 ± 0.074</td>
<td>3.35 ± 0.15</td>
</tr>
</tbody>
</table>

* Thirty molecules of each DNA were measured.
† Calculated from a molar linear density of 2.03 × 10^10 daltons/cm (Bud & Kelly, 1977).

Fig. 4. Electron micrographs of DNV 2 ss DNA allowed to circularize after denaturation. The panhandles created on 'snap back' are arrowed.

Electron microscopy of circularized DNV ss DNA

Densonucleosis virus ds DNA was denatured and allowed to re-anneal under conditions which would promote intramolecular (if complementary sequences exist within the molecule) rather than intermolecular annealing. This was achieved by using low DNA concentrations and suboptimal temperature (Robinson & Bellett, 1975). Samples were then after various times of annealing; by 5 min, 80% of the single strands were circular and by 10 min over 95% of the single strands were circular. 'Panhandles', i.e. apparent ds DNA protrusions, were observed on about 20% of these molecules as shown in Fig. 4. The lengths of these protrusions varied from 1 to 2.5% of the genome length, equivalent to 60 to 150 base pairs. The lengths of the single stranded circles and linear single stranded monomers were approx. 2 microns for both DNV 1 and DNV 2.

The circularization of the single stranded DNA to create monomers with ds DNA protrusions is most simply explained by the presence of inverted terminal repetitions in the genome of DNV.
Isolation of inverted repeats of DNV DNA

The presence of palindromic sequences in the DNV genome was confirmed by allowing the strands of DNV DNA to anneal under conditions which promoted intramolecular rather than intermolecular annealing. In this experiment the procedure devised by Ohtsubo & Ohtsubo (1976) was employed in which a reasonable concentration of DNA was used, permitting the isolated fragments to be visualized on an agarose gel. Annealed DNA was digested with S₁ nuclease (which specifically digests single stranded DNA: Vogt, 1973).
leaving double stranded DNA available for analysis. Figure 5 shows ds DNA fragments produced in these experiments resolved on 2% agarose gels. Fragments of similar size were produced for both viruses. The fragments had an apparent mol. wt. of 470,000, which after correction (determined empirically) for the Zn²⁺ ions present which retarded electrophoresis, was equivalent to a mol. wt. of 250,000, i.e. about 380 base pairs. The gels were calibrated with nucleosomal DNA which had been extensively digested with micrococcal nuclease (Fig. 5 and Fig. 6).

**DISCUSSION**

Our results are consistent with the proposal that densonucleosis virus DNA, like AAV 2 DNA, comprises a genome containing both a natural nucleotide sequence permutation and inverted terminal repetitions. DNV 1 and DNV 2 have an identical fine structure.

The demonstration that DNV ds DNA created by the annealing of complementary strands of ss DNA on release from virus particles can exist primarily as linear monomers but also as circular monomers and concatamers indicate that the termini of the DNV genome are cohesive and this probably results from a natural limited nucleotide sequence permutation within the genome. As yet we have been unable to estimate the size of the permutation by exonuclease digestion because we are unable to produce DNA with radioactivity of high specific activity *in vivo* and the virus will not replicate *in vitro*. The ratio of circular to linear monomers was about 1:400 by electron microscopy, and a similar though not so reliable figure is derived from scans of agarose gels. Using the formula \( f = \frac{2r}{(1 + 2r)} \), where \( f \) is the fraction of circles and \( r \) is the fractional length of the repeated region (Rhoades *et al.* 1968), one can estimate that to produce 0.25% circularization a repeat of 0.13% is required, which is equivalent to the remarkably low figure of 8 nucleotides. This is probably a vast underestimate because concatamerization was not considered. The extent of concatamerization is, however, difficult to estimate. It is impossible to estimate the number ratio of concatamers to monomers from electron microscope data because of the presence of considerable amounts of ill-defined and tangled material. About 45% of the material remains at the top of 1.4% agarose gels and though it is considered to represent
Densonucleosis virus DNA

concatamers it also probably contains a considerable proportion of ill-defined monomers. Circularization and concatamerization of 45% of monomers would require a permutation of about 40% (Rhoades et al. 1968). From analytical ultracentrifugation (Barwise, 1969; Kelly et al. 1977) at least 95% of the material sedimented with a $s_{20, w}$ of about $16.15^{-1} \times 10^{-13}$; if one assumes that the residual 5% represents concatamers and circles then the size of the permutation in the DNV genome is about 2.7% (about 160 base pairs).

It is probable that the circular ds monomers and concatamers are created because the ends of the DNV genome are cohesive as a result of the nucleotide sequence permutation within the genome. This permutation is probably non-random though as yet no direct evidence that this is so has been obtained. The indirect evidence comes primarily from restriction enzyme analysis (D. C. Kelly, unpublished observations) and also from the facts that (i) about 50% of DNV ds DNA do not have cohesive termini (demonstrated by the experiments where prolonged annealing conditions do not alter the relative proportions of the DNA and (ii) the size of the permutation is about 2.7%. Denaturation and renaturation of linear ds monomers to recreate the three forms resolved on agarose gels in the same proportion as unfractionated DNA shows that each ds DNA species is equivalent.

The complementary strands of DNV DNA will, under appropriate conditions, anneal to create single stranded circles. The simplest explanation is that circles are created by complementary sequences present at the termini of the DNV genome. Since ‘panhandles’, i.e. projecting duplex DNA, are observed on some molecules it is likely that inverted terminal repetitions exist which are similar to those proposed for AAV 2 (Koczot et al. 1973; Berns & Kelly, 1974) and also human and avian adenoviruses (Wolfson & Dressler, 1972; Robinson & Bellett, 1975), rather than an ‘in line’ duplex repetition considered by Garon et al. (1972) and Koczot et al. (1973). The presence of the palindromic sequences demonstrated by electron microscopy does not indicate that the structures occur at the termini of the genome, especially since circles of SV40 ss DNA have been shown to possess inverted repeat sequences as ‘panhandles’ (Hsu & Jelinek, 1977; Shen & Hearst, 1977). However, since only one ‘panhandle’ per circular ss monomer was observed (unlike AAV 2 ss DNA; Berns & Kelly, 1974), and panhandles were not observed on linear or circular ds monomers in this and a previous study (Kelly et al. 1977) it seems probable that the ‘panhandles’ represent duplex DNA structures created by the annealing of terminal repeat structures.

The size of the ‘panhandles’ estimated by electron microscopy (60 to 150 base pairs) is appreciably smaller than the estimate obtained by electrophoresing S1 nuclease digestion products (380 base pairs). This is probably because the duplex structure is not a perfect linear duplex and so measurements in both systems are likely to be subject to considerable errors. A similar discrepancy exists between estimates for the terminal repeats in AAV 2 DNA by electron microscopy (Koczot et al. 1973; Berns & Kelly, 1974) and gel electrophoresis (Carter et al. 1972).

It appears that DNV and AAV are remarkably similar in their fine structure despite marked differences in their biology. DNV is, however, significantly larger than AAV 2 DNA, appearing about 12 to 13% larger as assessed by both electron microscopy and agarose gel electrophoresis. DNV DNA is also larger than estimates for the autonomous parvoviruses (Bourguignon et al. 1976; Seigl, 1976). DNV appears to be radically different in fine structure when compared with the autonomous parvoviruses MVM and KRV which comprise ss DNA of one polarity, with short palindromic sequences at the 5’ and 3’ ends; though there are no inverted terminals present in the genome since it is unable to circularize under annealing conditions (Bourguignon et al. 1976; Salzman, 1977).
Although DNV ss DNA is able to circularize under the appropriate conditions there is no evidence that the DNA exists as a covalently closed circle in the particle, nor that hydrogen bonded circles exist in situ. We have previously obtained evidence of considerable secondary structure to DNV DNA in situ (Kelly et al. 1977), though it is probable that this is mainly due to base stacking caused by the polyamines in the virus particles (Kelly & Elliott, 1977), rather than base pairing. Consequently we consider that the DNA exists as a highly ordered linear structure in virus particles.

We thank C. F. Rivers, M. H. Bew, and M. D. Ayres for help in growing the viruses; Beverly Griffin, B. J. Carter, A. Jones, and M. J. Butler for gifts of DNA; and A. J. Robinson and D. Kay for helpful discussion in the course of this work. H. M. Bud was in receipt of a N.E.R.C. post-graduate studentship.

REFERENCES


Densonucleosis virus DNA


(Received 12 September 1977)