Granulosis Virus Deoxyribonucleic Acid: a Closed, Double-Stranded Molecule

M. D. SUMMERS AND D. L. ANDERSON

The Cell Research Institute and The Department of Botany, The University of Texas at Austin, Austin, Texas 78712

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Studies on deoxyribonucleic acid purified from the granulosis virus of *Trichoplusia ni* have revealed the presence of a closed, double-stranded superhelix which sediments at 95S relative to relaxed circles (74S) and linear (60S) forms. Molecular weight estimates show that this insect virus deoxyribonucleic acid has a size of 100×10^6 daltons.

Covalently closed, circular deoxyribonucleic acid (DNA) molecules isolated from viruses and other sources are now a common occurrence (6). In this paper, we report the preliminary characterization of a large superhelical molecule isolated from the insect granulosis virus (GV) of *Trichoplusia ni* and estimate its molecular weight as 100×10^6 daltons. Not only is this one of the largest superhelical DNA molecules reported to date, but it is easily available in large quantities.

At the present time, although there is a paucity of information on the molecular biology of the insect occluded viruses, much of it appears confused and contradictory in nature. The GV are singly occluded, enveloped viruses which exhibit a narrow range of host and tissue specificity as compared to the nuclear and cytoplasmic polyhedrosis viruses (14). The cytopathology of infection has been studied in detail (16), and the virus is now known to exhibit distinctly, and notably, different cytopathologies in midgut and fat body cells. In midgut cells, uncoating apparently occurs by interaction with the nuclear pore with replication confined to the nucleus; a comparison of fat body cells shows that extensive replication takes place in the cytoplasm. As isolated from infected larvae, the virus is embedded in a large protein crystal in which the virion remains stable and viable at room temperature for years. In addition to this, the crystal allows for easy purification of the virus free from host cell and other contaminating factors. The enveloped virus is easily released from the proteinic crystal by mild alkali treatment and purified on sucrose (17) or CsCl gradients.

From purified virus, DNA was released and purified by the following two procedures. (i) The method of Gafford and Randall (5) was employed without variation, except that the DNA was not precipitated with ethanol but was extensively di-

alyzed against 0.15 M NaCl plus 0.015 M sodium citrate and 3 mM ethylenediaminetetraacetic acid, pH 7.2. A rapid method of DNA purification which produced GV DNA with sedimentation properties like those of Gafford and Randall extracted DNA employed 2.0% sodium lauryl sarcosine (final concentration) and a heat treatment of 60 C for 20 min. The latter technique was used with several variations dependent upon the requirements of the experiment: (i) the sarcosine-heat-treated virus preparation could be layered directly on top of a sucrose gradient without deproteinizing the sample or (ii) the virus was lysed directly on the top of a gradient with only a final concentration of 2.0% sarcosine to lyse the virus. In addition, DNA was released by various detergent, organic solvent systems, or both (5, 7, 9). With or without deproteinization by these procedures, the GV DNA exhibits similar sedimentation profiles to those reported in this paper.

Centrifugation to equilibrium in ethidium bromide-cesium chloride gradients produced two fluorescent bands in ultraviolet light (Fig. 1) as compared to a single band with sheared GV DNA and T4 and T7 DNA controls (not shown). The two GV DNA bands were separated by a distance of 0.38 cm (approximate density difference = 0.04 g/cm^3) as expected from Radloff et al. (12). To further confirm the presence of covalently closed DNA, centrifugation to equilibrium in alkaline CsCl, pH 13.0 (3), produced two bands with densities of 1.754 and 1.778 g/cm^3 . Densities were estimated relative to a single band of *Clostridium perfringens* DNA taking into consideration the correction factors for preparative ultracentrifugation (20, 21). Sheared GV DNA produced only a single band corresponding to the lower density fraction in alkaline gradients. On neutral CsCl, GV DNA purified by the method of Gafford and Randall (5) appears as a single homogeneous zone

FIG. 1. Ethidium bromide-cesium chloride equilibrium centrifugation with gradient components by the method of Radloff et al. (12) and centrifugation at 40,000 rev/min for 24 hr (23°C) with a SW 65 rotor and L2-65B ultracentrifuge. Linear gradients (5.0-ml volume) were performed with an average density = 1.55 g/cm^3 containing $100 \text{ } \mu\text{g}$ of ethidium bromide (Calbiochem) per ml. Gradients were fractionated from the top of the tube in two-drop fractions (approximately 200 total fractions) with the ISCO model D fractionator. Symbols: (□) approximately 18 to 20 μg of tritium-labeled GV DNA purified by treatment of the virus with 2.0% sodium lauryl sarcosine and exposure to 60°C for 20 min. The sarcosine-treated preparation was subsequently extracted three times with chloroform-butanol, and the aqueous fraction was extensively dialyzed against 0.15 M NaCl plus 0.015 M sodium citrate (pH 7.2) containing 3 mM ethylenediaminetetraacetic acid. (●) A sample of purified, labeled GV DNA sheared by forcing the DNA solution six times through a 26-gauge needle. Approximately 20% of the counts/min are found in the higher density band of DNA.

with a density of 1.6994 g/cm^3 (39.5% GC) using an analytical ultracentrifuge and the procedure of Mandel et al. (8).

The size of the linear form of GV DNA was estimated by sedimentation in neutral and alkaline sucrose gradients relative to T4 and T7 DNA with a preparative ultracentrifuge (Fig. 2 and 3). Alkali denaturation and centrifugation were performed by the method of Freifelder (3) but with 2 to 40% sucrose gradients which produced superior resolution of peaks as compared to the broad peaks obtained on 5 to 20% sucrose gradients. At the low concentrations of DNA applied on these gradients (1 to 5 μg), sedimentation was determined to be concentration independent. In all, three sedimenting bands of GV DNA were found in alkaline sucrose. During short periods of centrifugation, however, only two zones of GV DNA were observed (Fig. 2). Zone I_{a1k} which

corresponds to interlocked single-strand rings sedimented approximately three times faster than a slower zone. The $3 \times$ -GV DNA was not detected on neutral gradients run simultaneously with alkaline gradients. The slower sedimenting species of GV DNA were not resolved in the short run, but, after longer periods of centrifugation, zones II_{a1k} and III_{a1k} were easily resolved corresponding to single-strand rings and single-strand linear forms of denatured GV DNA, respectively. A neutral sucrose gradient (not shown) exhibited only a single band of GV DNA sedimenting at 74S. In regions of these gradients in which sedimentation was determined to be approximately linear, these two DNA species are separated by about 10% of the distance sedimented with de-

FIG. 2. Alkaline sucrose gradients (5.0 ml) prepared with 0.3 M NaOH-1.0 M NaCl-0.01 M ethylenediaminetetraacetic acid (pH 13.0) but with 2 to 40% sucrose gradients to improve band resolution of the 3 to 10% differences in sedimentation. Approximately 5 to 8 μg of purified GV DNA was denatured in 0.3 M NaOH (final concentration) for 15 min at 25°C. The denatured DNA (0.15-ml samples) was gently layered on alkaline sucrose gradients and centrifuged for (A) 20 min or (B) 60 min at 62,500 rev/min (23°C) with a SW65 rotor and Beckman L2-65B ultracentrifuge. (A) The $3 \times$ GV DNA (I_{a1k}) form; (B) the resolved single-strand circular (II_{a1k}) and single-strand linear (III_{a1k}) forms. Arrow designates the level of sedimentation of denatured T4 DNA which was cosedimented with GV DNA, in parallel gradients, or both. Gradients were fractionated with an ISCO model D fractionator and an ISCO UA-2 ultraviolet analyzer (10-mm flow cell) set at maximum sensitivity scale. Accurate measurements of distances of sedimentation were made with a Gilford-Honeywell 10-inch chart recorder tied in with the UA-2 analyzer.

FIG. 3. Neutral sucrose gradient sedimentation of GV DNA. Five to 30% sucrose gradients (5.0 ml) containing 10 mM tris(hydroxymethyl)aminomethane-hydrochloride + 10 mM ethylenediaminetetraacetic acid (pH 7.0), and NaCl concentration as shown (I). Ionic strength = 1.05 for the 1.0 M NaCl gradients and 0.042 for the 2.0 mM NaCl gradients. Centrifugation was conducted at 5°C and 62,500 rev/min for 60 min with the SW 65 rotor. Gradients were fractionated and monitored as described in Fig. 2. Arrows indicate the relative level of sedimentation of phage DNA standards. The relative ratios of sedimentation shown for the linear forms of phage DNA and GV DNA in this figure were confirmed to be like sedimentation on gradients prepared according to Burgi and Hershey (2). All gradients shown contain approximately 5 to 8 μ g of GV DNA. (A and B) GV DNA purified from nonlyophilized virus. (C) GV DNA from lyophilized virus showing the absence of superhelix DNA and the presence of double-stranded linear GV DNA (III).

natured T4 sedimenting between, and slightly nearer to, the linear single strands of GV DNA. The S-value is estimated as 70S relative to denatured T4 DNA (73S) with a molecular weight estimate of 50×10^6 daltons [using alkaline S-values from Studier (15) and molecular weight estimates calculated by the method of Freifelder (4)]. These calculations are quite close to molecular weight estimates from neutral sucrose studies (later) in view of the anomalous sedimentation behavior of very high molecular weight DNA species on alkaline gradients (10).

On neutral or high ionic strength sucrose gradients, two bands of GV DNA are observed sedi-

menting at 60S and 74S relative to T4 and T7 DNA (Fig. 3). If freshly prepared GV DNA is used, only a single, broad band of GV DNA is observed. However, on neutral gradients with low ionic strength salt conditions, all three bands of double-stranded GV DNA are distinguishable (I, superhelical closed circles; II, relaxed nicked circles; and III, intact linear form) as expected in comparison with the polyoma virus DNA and lambda virus DNA studies (1). The best estimates for a molecular weight for the linear duplex give a value of 100×10^6 daltons [using the gradient system of Burgi and Hershey (2) and the revised molecular weight estimates of Freifelder (4)]. At the low salt concentration (ionic strength = 0.042), the superhelix sediments at approximately 95S relative to 60S GV DNA and T4 DNA. Although the complete series of studies is not shown, the 60S and 74S GV DNA sedimentation behavior did not significantly vary with ionic conditions of the gradients. In freshly prepared GV DNA there were no slower peaks or shoulders evident, as could be produced however using artificial conditions. After exposure to pancreatic deoxyribonuclease I (0.5 μ g of pancreatic deoxyribonuclease/7.0 μ g of GV DNA; incubation at 25°C for 90 min in 50 mM potassium phosphate and 3 mM $MgCl_2$, pH 7.8), a broad band of GV DNA was detected sedimenting at 40S which is consistent with half-molecules of GV DNA. Deoxyribonuclease-nicking studies (with 1.0×10^{-3} to 100×10^{-3} μ g of pancreatic deoxyribonuclease/7 μ g of GV DNA), the complete context of which will be published shortly in comparison with studies on the characterization of *Spodoptera frugiperda* GV DNA, have shown that the faster sedimenting species can be converted to the 60S GV DNA without the presence of GV DNA sedimenting intermediate to the three forms described above (21). Shearing the GV-DNA preparation with a syringe and 26-gauge needle produced a fraction which sedimented at approximately 12 to 14S. In neutral gradients, as in alkaline gradients, T4 DNA usually sedimented slightly faster than the linear form (60S) of GV DNA; however, when T4 was cosedimented with GV DNA, it could not be distinguished from the 60S GV DNA on 5 to 20% or 5 to 30% sucrose gradients under conditions where sedimentation was determined to be concentration independent.

In conclusion, in this preliminary report, we have shown by use of hydrodynamic techniques that purified GV DNA from *T. ni* occluded virus consists, in part, of a covalently closed circle with superimposed tertiary structure and is a large molecule with the linear form comparable in size to T4 DNA. Although these studies have not

provided an absolute figure for the molecular weight of the GV DNA, it appears that we can confidently estimate its size in the range of $100 \times 10^6 \pm 10\%$ daltons. This size, compared with the relative ease of preparing large quantities for characterization, may allow this DNA to be used for studies where the accessibility to only small amounts of superhelix DNA is a limiting factor. In addition, when more accurate values for the size of this DNA are made (*manuscript in preparation*), this may provide another convenient molecular weight standard in the 100×10^6 dalton range.

A distinct difference, presently unexplained, was noted in the recovery of covalently closed DNA from lyophilized and nonlyophilized occluded virus preparations (Fig. 3). Covalently closed DNA was only recovered in significant quantities from nonlyophilized samples. However in our studies, this was used to advantage for additional proof in the characterization of a covalently closed species and to prepare GV DNA which contained only double-stranded linear and relaxed circular molecules.

The results and techniques provided by this study perhaps will bring clarity to other studies, some of which report infectious DNA purified from occluded viruses with molecular weight estimates ranging from 2.0×10^6 to 100×10^6 daltons (11, 13, 18, 22). Comparisons with structurally similar occluded viruses (nuclear polyhedrosis and granulosis viruses) with and without overlapping host ranges are in progress and will be reported in detail later; preliminary results suggest that these DNA species have physical properties similar to that of the GV DNA reported herein.

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