Properties of a Novel DNA Virus from the Tsetse Fly, *Glossina pallidipes*

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SUMMARY

Virus particles were isolated from hypertrophied salivary glands of the tsetse fly, *Glossina pallidipes* collected near Mombasa, Kenya. Purified virus particles were rod-shaped, 57 nm wide by 700 to 1300 nm long. Particle lengths fell into two size classes, with 'short' particles averaging 869 nm and 'long' particles 1175 nm. The virus particles morphologically resembled elongated baculovirus nucleocapsids although, unlike baculoviruses, no fully enveloped virions were found in purified preparations. The particles contained double-stranded DNA which appeared to be linear when analysed by electrophoresis in agarose gels, ethidium bromide–cesium chloride gradient centrifugation or electron microscopy (EM). There was some evidence for the DNA being heterogeneous in size from EM studies and from the observation that restriction enzyme analysis failed to provide a clear profile of DNA fragments. Protein from purified virions contained at least 12 polypeptides with a major component of 39000 mol. wt. These results suggest that the virus cannot be placed in any of the existing taxonomic groupings of DNA viruses.

INTRODUCTION

Tsetse flies (*Glossina* spp.) are well known as vectors of trypanosomes, the causal agents of nagana in cattle and sleeping sickness in humans. Although *Trypanosoma* spp. and related protozoans were the only pathogens known to multiply in these vectors, a number of reports have suggested that virus-like particles (VLPs) may also be associated with the salivary glands and midgut epithelium of *G. morsitans*, *G. fusipes* and *G. pallidipes* (Jenni, 1973; Jenni & Steiger, 1974a, b; Jaenson, 1978). There was no clear indication from these studies whether these VLPs were pathogens of the tsetse flies or virus diseases of other hosts, which were transmitted by these insects.

In field studies of trypanosome infection in tsetse in Zululand, South Africa, Whitnall (1934) noted that some individuals of *G. pallidipes* had grossly enlarged salivary glands. Jaenson (1978) first observed VLPs in such hypertrophied glands and described the particles as baculovirus-like although their average length in thin section (597 to 708 nm) was much in excess of that normally associated with members of the baculovirus group (Matthews, 1982). More recent reports have confirmed Jaenson's observations and have shown that the prevalence of hypertrophied salivary glands among wild populations of *G. pallidipes* in Kenya ranges from 0.3% to 15.6% of the fly population (Otieno et al., 1980; Odindo, 1982). The infectious nature of the virus to adult *G. pallidipes* has been implied by the induction of salivary gland hypertrophy following oral or haemocoelic inoculation with partially purified virus (Odindo et al., 1981). In addition, the virus appears to induce sterility in male flies although adults can survive for long periods after infection (Jaenson, 1978; M. O. Odindo, unpublished observations).

In the present study we report the purification and partial characterization of the virus from *G. pallidipes*. From these studies we conclude that the virus cannot be placed in any of the existing taxonomic groups of DNA viruses.
Collection of tsetse flies and extraction of salivary glands. Large numbers of adult tsetse were trapped at sites in the Shimba Hills Game Reserve near Mombasa, Kenya during October and November, 1982 using pale blue biconical traps (Challier & Laveissière, 1973). The traps were emptied daily and the flies transported to the ICIPE Muhaka Outreach Station, Mombasa, where they were stored at −20 °C for no longer than 3 days before dissection. The main species collected was G. pallidipes, with much smaller numbers of G. brevipalpis and G. austeni. Flies were dissected and the incidence of salivary gland hypertrophy was recorded. Hypertrophied glands were removed and stored at −20 °C in distilled water.

Purification of virus. Hypertrophied salivary glands from G. pallidipes were mixed with buffer to a final concentration of 0.03 M-Tris-HCl, 0.025 mM-KCl, pH 7.5 (TK buffer). The mixture was triturated in a ground glass tissue homogenizer and the homogenate was centrifuged at 3000 r.p.m. for 20 min. The supernatant was decanted and centrifuged at 42000 g for 1 h at 10 °C. Pellets were resuspended in TK buffer at 4 °C overnight. The resuspended material was centrifuged again at low speed (2000 r.p.m., 5 min) and the supernatant fraction was layered onto continuous 30 to 65% (w/w) sucrose gradients prepared in TK buffer. The gradients were centrifuged at 94000 g for 2 h at 10 °C. Semi-pure virus, contaminated with some membranous material, banded approximately midway down the gradient. This band was harvested, diluted with an equal volume of TK buffer and centrifuged at 94000 g for 2 h at 10 °C to pellet the virus. The pellet was resuspended in TK buffer and mixed with an equal volume of 0.25% sodium deoxycholate (DOC) in TK buffer. The mixture was left at 20 °C for 1 h with occasional shaking. The sample was then layered on a further 30 to 65% (w/w) sucrose gradient and centrifuged as above. A band of purified virus was recovered at a position in the gradient corresponding to 45% (w/w) sucrose (as measured by refractometry). Virus was pelleted by centrifugation as described above and resuspended in distilled water.

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Virus preparations were examined in a JEOL JEM 100S electron microscope after negative staining with saturated aqueous uranyl acetate (approx. 2% or 2% potassium phostoglutamate). Particle sizes were measured in comparison with the crystalline lattice of catalase photographed at the same magnification.

Protein analysis. Protein concentration was measured by the Folin test (Lowry et al., 1951) using bovine serum albumin as a standard. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of virus proteins was carried out in vertical 10% (w/v) gel slabs (15 × 12.5 × 0.15 cm) (Crook, 1981) using the buffer system described by Laemmli (1970). Polypeptide mol. wt. were determined by comparison with the electrophoretic mobilities of myosin (200000), phosphorylase a (97400), transferrin (76600), bovine serum albumin (69000), ovalbumin (45000), lactic dehydrogenase (35000) and carboxypeptidase (29000). Myoglobin (17200) and cytochrome c (12400) were also included as standards but were not used in mol. wt. estimations.

Nucleic acid analysis. Colorimetric tests for the presence of DNA and RNA using diphenylamine and orcinol respectively were carried out as described by Shatkin (1969).

Viral DNA was analysed by ethidium bromide-caesium chloride gradient centrifugation. Purified virus particles from G. pallidipes were disrupted with 2% 'Sarcosine' (sodium N-lauroyl sarcosinate) in a final volume of 0.34 ml and gently layered over 2 ml 54% (w/w) CsCl in 10 mM-Tris–HCl pH 8.0, 1 mM-EDTA (TE buffer), containing 100 μg/ml ethidium bromide. The gradient was formed by centrifugation for 62 h at 65000 g (20 °C).

The electrophoretic mobility of viral DNA was compared with that of covalently closed and linear DNA of known size from an acrylamide fiber of the HD1 strain of Bacillus thuringiensis (Gonzalez et al., 1981; P. Jarrett, unpublished observations). Electrophoresis was in 0.5% horizontal agarose gels containing 89 mM-Tris-borate pH 8.3, 2.5 mM-EDTA (Gonzalez et al., 1981) plus 0.1% SDS. A sample of purified virus was applied directly to the gel and lysed in situ by the SDS present in the gel buffer. Another virus sample was first disrupted in 2% sarcosine in TE buffer for 20 min at 60 °C. Spheroplasts of B. thuringiensis, produced by the method of Gonzalez et al. (1981), were layered directly into the sample well and were lysed in situ as above. The gel was run at 0.5 mA for 45 min, 2 mA for 60 min and 20 mA for 90 min. Bands were visualized under u.v. light (302 nm) after staining in 2 μg/ml ethidium bromide.

DNA was purified from virus particles after these had been incubated with 1% SDS for 1 h at 37 °C. The sample was extracted three times with equal volumes of phenol saturated with TE buffer. The final aqueous phase was extensively dialysed against TE buffer and then against polyethylene glycol to concentrate the DNA to approximately 38 μg/ml (A260 = 0.76).

Restriction endonuclease digestion of 0.23 μg viral DNA with 1 to 130 units of EcoRI or with other enzymes (12 units PstI, 10 units HindIII or 6 units BamHI) was carried out in appropriate buffers (EcoRI, 100 mM-Tris–HCl, 50 mM-NaCl, 10 mM-MgCl2, pH 7.5; PstI, 50 mM-Tris–HCl, 50 mM-NaCl, 10 mM-MgCl2, pH 8.0; HindIII, 6 mM-Tris–HCl, 50 mM-NaCl, 6 mM-MgCl2, pH 7.5; BamHI, 100 mM-Tris–HCl, 10 mM-MgCl2, pH 7.5) for 4 h at 37 °C. Digestion was stopped by the addition of 2 μl 0.1 M-EDTA, 20% Ficoll, 0.05% bromophenol blue and heating at 60 °C for 10 min. Electrophoresis on 0.7% or 0.8% agarose gels was as described by Crook (1981). Sizes of DNA fragments were calculated with fragments of bacteriophage λ DNA generated by digestion with EcoRI and HindIII.
Fig. 1. (a) Hypertrophied (virus-infected) salivary glands in the dissected abdomen of Glossina pallidipes. Bar marker represents 2 mm. (b) Light micrograph comparison of the relative sizes of normal (N) and virus-infected (VI) salivary glands extracted from G. pallidipes. Bar marker represents 250 μm.
DNA virus Jhom tsetse fly

DNA was spread for electron microscopy by a modification of the technique described by Bud & Kelly (1977).

Thirty-four ~tl of viral DNA (0.2 ~tg DNA) and 1 ~tl of bacteriophage PM2 DNA (0.05 ~tg ~ Boehringer) in 20 mM-NaCl and 5 mM-EDTA were added to 15 ~tl of a solution [prepared with AnalaR water (BDH)] containing 65 mM-Na,CO₃, 37% (v/v) formaldehyde, 10 mM-EDTA and 46 mM-HC1 pH 8. Fifty ~tl of formamide and 10 ~tl (1 mg/ml) cytochrome c solution were then added. Fifty ~tl of this mixture was run down a glass slide (placed at an angle of 45 °) into a hypophase of AnalaR water, delineated by a small amount of talc. The DNA was collected on carbon-coated (200-mesh) copper grids which were then fixed for 15 s in absolute ethanol and rotary shadowed with gold-palladium. The grids were examined in a JEOL JEM 100S microscope. The sizes of tsetse virus DNA molecules were compared with the lengths of relaxed circular molecules of PM2 DNA (9-7 kbp).

RESULTS

Incidence of salivary gland hypertrophy

Of 18410 adult G. pallidipes collected, 325 flies (1-8~o of the population) were found with salivary gland hypertrophy (Fig. 1). None of the G. brevipalpis (152 individuals) and G. austeni (163 individuals) collected exhibited salivary gland enlargement.

Purification and motT~hology of virus particles

Extracts of hypertrophied salivary glands contained large numbers of slightly flexuous, rod-shaped particles (Fig. 2). The particles did not appear to be enveloped although, during the early phases of purification, membranous material was present as an apparent contaminant of the virus recovered from the first sucrose gradient (Fig, 2a). On one occasion, a single particle was detected at an early stage of purification, which consisted of an incomplete envelope partly enclosing an elongated rod-shaped nucleocapsid (Fig. 2a; inset). After treatment with DOC a pure preparation of virus rods was obtained (Fig. 2b).

Measurement of virus particle size revealed that particle length (ranging from 700 to 1300 nm) fell broadly into two size classes (Fig. 2 b, 3). 'Short' particles ( < 1000 nm) averaged 869 ± 41 nm (18 measurements) and 'long' particles (> 1000 nm) averaged 1 l 75 ± 48 nm (33 measurements). The mean particle width was 57 ± 7 nm for both 'short' and "long" particles.

The virus rods showed a regular arrangement of structural units on the 'capsid" (Fig. 2) consisting of a series of bands about 7 to 10 nm wide, at approximately 90 ° to the longitudinal axis of the capsid. The ends of intact particles were characterized by the presence of rounded and thickened 'caps'.

Viral proteins

Following SDS-PAGE of protein from purified virus (the same preparation as illustrated in Fig. 2b), at least 12 polypeptide bands were resolved with tool. wt. in the range 22000 to 143000 (Fig. 4). The major component had a mol. wt. of 39000. In addition to these clearly resolved bands, a diffuse, densely staining area was observed in the lower part of the gel (< 20000 mol. wt.). Bands were not clearly defined in this region, and two separately purified batches of virus produced a similar result.

Viral nucleic acid

Purified virus gave a strong positive reaction with diphenytamine while only a faint reaction was obtained with orcinol. The low level of the orcinoi reaction was consistent with only DNA being present. The protein : DNA ratio was calculated as 7 : 1, after quantitative Folin reagent and diphenylamine tests using purified virus particles.

After centrifugation in an ethidium bromide-caesium chloride gradient, a single band of DNA was observed (Fig. 5a) in conditions in which baculovirus DNA produces two distinct bands of supercoiled and open circular/linear DNA (Payne, 1974).
DNA was spread for electron microscopy by a modification of the technique described by Bud & Kelly (1977). Thirty-four μl of viral DNA (0.2 μg DNA) and 1 μl of bacteriophage PM2 DNA (0.05 μg; Boehringer) in 20 mM-NaCl and 5 mM-EDTA were added to 15 μl of a solution [prepared with AnalaR water (BDH)] containing 65 mM-Na$_2$CO$_3$, 37% (v/v) formaldehyde, 10 mM-EDTA and 46 mM-HCl pH 8. Fifty μl of formamide and 10 μl (1 mg/ml) cytochrome c solution were then added. Fifty μl of this mixture was run down a glass slide (placed at an angle of 45°) into a hypophase of AnalaR water, delineated by a small amount of talc. The DNA was collected on carbon-coated (200-mesh) copper grids which were then fixed for 15 s in absolute ethanol and rotary shadowed with gold–palladium. The grids were examined in a JEOL JEM 100S microscope. The sizes of tsetse virus DNA molecules were compared with the lengths of relaxed circular molecules of PM2 DNA (9.7 kbp).

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**Fig. 2.** Morphology of virus particles extracted from hypertrophied salivary glands of *G. pallidipes*. (a) Semi-purified virus particles recovered from a band produced in the first sucrose gradient centrifugation, showing rod-shaped virus particles (V) and membranous material (M). Inset: a partially enveloped virus rod. (b) Purified virus particles including both 'short' (S) and 'long' (L) particle types. Bar markers represent 200 nm.
When particles were gently lysed with SDS or sarcosine and electrophoresed in a 0·5 % agarose gel, the DNA migrated as a single band to the same position as chromosomal (linear) DNA from *B. thuringiensis* (Fig. 5b). Supercoiled *B. thuringiensis* plasmids of known size ranging from 14 to approximately 182 kbp migrated more slowly than the virus DNA, while a linear DNA molecule of 16·7 kbp migrated more rapidly.

These results suggested that (i) the viral DNA could be present as intact supercoiled DNA, smaller than 14·1 kbp or (ii) that the DNA was linear and larger than 16·7 kbp. In an attempt to resolve the size and conformation of the DNA, purified viral DNA was spread and examined by electron microscopy. Under conditions where circular forms of PM2 DNA were readily observed, only linear molecules of the DNA from *G. pallidipes* virus were found. There was some
Fig. 5. Analyses of DNA of *G. pallidipes* virus. (a) Ethidium bromide–cesium chloride gradient of virus disrupted with 2% sarcosine. A single band of DNA can be seen. (b) Comparison of the electrophoretic mobility of *G. pallidipes* virus DNA (lanes 1 and 2) with the DNA from an acrystalliferous mutant of *Bacillus thuringiensis* strain HD1 (lane 3), in 0.5% agarose gels. The sample in lane 1 was treated with 2% sarcosine to disrupt the virus before electrophoresis. Samples in lanes 2 and 3 were lysed *in situ* by SDS in the electrophoresis buffer. Sizes in kbp and conformation of the *B. thuringiensis* bands (l, linear; s, supercoiled) are shown in the figure. Xmal, chromosomal DNA. (c) Electrophoresis in a 0.8% agarose gel of (1) phage λ DNA digested with HindIII, (2) *G. pallidipes* virus DNA digested with EcoRI, (3) undigested *G. pallidipes* virus DNA, (4) undigested phage λ DNA. The estimated sizes of the main bands of the digested viral DNA samples are given in kbp.

heterogeneity in the length of the viral DNA; linear molecules were observed between 2.1 and 3.5 times the length of the PM2 DNA standard, consistent with a size range between 20.4 and 34.0 kbp (Fig. 6).

Electrophoretic analysis of two batches of purified DNA treated with EcoRI failed to provide a clear profile of the restriction fragments (Fig. 5c). A similar effect was observed after treatment with *PstI*, *HindIII* or *BamHI*. This could have occurred if the DNA was heterogeneous in size or if the enzymes had only partially digested the DNA. There was some evidence for DNA size heterogeneity from the electron microscope studies and the observation that purified DNA undigested by restriction enzymes did not migrate as a sharp band on the gel, unlike undigested λ DNA (Fig. 5c). Treatment of the viral DNA with amounts of EcoRI up to 130 units did not modify the profile, suggesting that enzyme digestion was complete.
DISCUSSION

The low incidence of salivary gland hypertrophy in *G. pallidipes* collected during October and November 1982 was similar to the results obtained in earlier studies (Jaenson, 1978; Otieno et al., 1980; Odindo, 1982). The virus particles associated with these hypertrophied glands were long, unenveloped rods, containing linear DNA. It has not yet been possible to test the infectivity of the purified virus as no laboratory culture of *G. pallidipes* was available during the course of this investigation.

In a previous description of the virus, based on thin sections of infected cells, Jaenson (1978) considered that there was some morphological resemblance between the *G. pallidipes* virus and baculoviruses. Three distinct subgroups of baculoviruses have been defined to date (Matthews, 1982). Two groups, the nuclear polyhedrosis and granulosis viruses are characterized by the production during infection of large proteinaceous inclusion bodies containing the enveloped rod-shaped virus. No such inclusion bodies have been detected in cells infected with the *G. pallidipes* virus (Jaenson, 1978). The third baculovirus group, typified by the *Oryctes* baculovirus (Payne, 1974; Payne et al., 1977) does not produce inclusion bodies; the virus particles are non-occluded with a rod-shaped nucleocapsid surrounded by an envelope. Although Jaenson (1978) suggested that the tsetse virus possessed an outer membrane when examined in thin section, close examination of his micrographs does not reveal the characteristic membrane structures.

The summed sizes of the four major fragments obtained after *EcoRI* digestion totalled 39 kbp compared with the estimated maximum size of 34 kbp obtained from electron microscope studies.

Fig. 6. Electron micrograph of purified viral DNA from *G. pallidipes* virus in comparison with (inset) DNA from phage PM2. Bar markers represent 200 nm.
observed in baculovirus infections (Payne, 1974; Harrap et al., 1977). The morphology of the G. pallidipes virus does, however, superficially resemble a baculovirus nucleocapsid such as that of the Oryctes virus though it is much longer than the latter (> 700 nm compared with 160 nm; Payne et al., 1977).

Whereas the virions of all baculoviruses so far described are enveloped, we did not isolate any particles with intact envelopes from the tsetse fly salivary glands. Although the DOC treatment used in the final stages of virus purification would have removed any virus envelope, extracts prior to DOC treatment also contained naked rods. We must therefore conclude that the virus is non-enveloped.

The polypeptide composition of the tsetse fly virus was complex and, like baculovirus particles or nucleocapsids (Harrap & Payne, 1979) contained a large number of distinct proteins. However, no precise similarities can be drawn between the tsetse virus profile and published profiles of baculoviruses. It is not clear why a diffusely staining region of low mol. wt. polypeptides was present in purified virus preparations.

Our observations on the conformation of the tsetse virus DNA are distinct from those obtained with baculoviruses, as the evidence suggests strongly that the viral DNA is double-stranded but linear. The evidence for its double-stranded nature comes not only from its digestion by restriction endonucleases but also from the electron microscope studies. Single-stranded DNA would not have been readily visible under the conditions used. The single band in ethidium bromide-caesium chloride gradients could represent either supercoiled or open circular/linear DNA. Baculovirus DNAs treated under identical conditions produce two bands on such gradients as it is almost inevitable that some supercoiled DNA is damaged during handling (Payne, 1974; Harrap et al., 1977). Electron microscope spreading of the tsetse virus DNA revealed only linear DNA molecules with the largest size observed of approximately 34 kbp. Agarose gel electrophoresis provided results consistent with a linear molecule larger than 16-7 kbp. Electron microscopical studies and restriction enzyme analysis of purified DNA suggested that the size could be heterogeneous. Although we were unable (with the limited amount of material available) to exclude the possibility that the DNA was damaged during storage and extraction, the range of virus particle sizes observed may mean that the DNA itself is present in different molecular sizes (> 16-7 kbp) within the virus population. Further studies on freshly purified virus are needed to investigate the genome structure in more detail.

The virus described here from G. pallidipes does not, therefore, have the characteristics of any of the recognized subgroups of baculoviruses. It does not resemble the unclassified filamentous DNA-containing virus of the honey bee (Bailey et al., 1981) which is enveloped and has a nucleocapsid up to 3000 nm long. The closest affinities would appear to be with another unclassified virus causing salivary gland hypertrophy in the syrphid Merodon equestris (Amargier et al., 1979). This latter virus has several biological properties in common with the G. pallidipes virus as it was isolated from salivary glands where it induced gland enlargement and gonadal atrophy. It was described as a rod-shaped, DNA-containing particle with a 650 nm nucleocapsid. The main discrepancy is that the syrphid virus was enveloped. It therefore seems that the tsetse virus is a member of a new group of DNA viruses.

Although the tsetse virus has only been isolated from G. pallidipes, the possibility cannot be excluded that the virus has alternative, and possibly vertebrate, hosts. Virus particles can be detected in the saliva of infected flies (C. C. Payne & M. O. Odindo, unpublished observations) and could be transmitted to vertebrates during feeding. However, at present we have no evidence to suggest that the virus is other than a pathogen of G. pallidipes. An attempt to detect viral antigen or virus-specific antibody in the sera of animals (camels, cattle and goats) reared in areas known to have the highest incidence of virus-infected tsetse (Odindo, 1982) gave negative results (C. C. Payne & M. O. Odindo, unpublished observations).

Although the virus could have some potential in the biological control of G. pallidipes [as it is known to induce male sterility and is efficiently transmitted from female to offspring (Jaenson, 1978; M. O. Odindo, unpublished observations), further studies on the biochemical and epizootiological properties of the virus are required before the possibility of biological control can be given serious consideration.
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