Chemical and physical properties of adeno-associated satellite virus DNA produced during coinfection with herpes simplex virus.

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ABSTRACT

Infectious DNA from adeno-associated satellite virus (ASV) has been isolated from cells coinfecting with a temperature-sensitive mutant of herpes simplex virus (HSV) type 1 in the absence of contaminating HSV DNA. This satellite virus DNA does not appear to differ in its physical, chemical and biological properties from DNA isolated directly from virions or from cells co-infected with adenovirus. The DNA is double-stranded with a buoyant density of 1.718 gm/cm^3 . It sediments at 16S in both neutral and alkaline sucrose gradients. Single-stranded DNA from alkaline sucrose gradients has a modal length of $1.5 \mu\text{m}$ and demonstrates evidence of internal redundancies in the electron microscope.

INTRODUCTION

Production of adeno-associated satellite virus (ASV) type 4 structural antigens has been studied recently using selected temperature-sensitive (ts) mutants of herpes simplex virus (HSV) types 1 and 2.¹ It was found that a number of mutants of HSV, many of which were incapable of synthesizing detectable amounts of herpes virus DNA at their non-permissive temperature (40°C), were nevertheless able to complement ASV antigen production. Infectious satellite virus DNA was isolated from the HSV-1 ts-1 complementing system in amounts which appeared to make further physical and chemical categorization of the DNA feasible. Previous studies from our laboratory had established that infectious but non-encapsidated ASV DNA was produced in coinfections with herpes and ASV.² However, presence of contaminating herpes virus DNA made further dissection of these systems extremely difficult. The HSV ts-1 complementing system offered a direct approach to quantitative analysis of the ASV DNA in the presence of herpes DNA. The results of these experiments are presented here.

MATERIALS AND METHODS

The ASV-4 virus stock was grown in BSC-1 cells with SV15 simian adenovirus as helper as described previously.³ SV15 activity was inactivated by heating at 60°C for 15 minutes. Herpes simplex wild type strain KOS was provided by Dr. Kendall O. Smith. Herpes simplex mutant HSV ts-1 was provided by Dr. Priscilla Schaffer.⁴ The properties of this DNA-negative, BUDR-mutagenized herpes virus have been described.⁴ HSV ts-1 stocks were prepared in Vero cells.¹ HSV ts-1 was inoculated at a multiplicity of 5 plaque forming units (PFU) per cell. ASV-4 was used at a multiplicity of 100 hemagglutinating units (HAV) per cell.³

Confluent monolayer cultures of African Green monkey (VERO) cells in 16 oz. bottles (approximately 10^7 cells) were infected simultaneously with HSV-1 ts-1 and ASV type 4 at both 34°C and 40°C. In both cases virus adsorption was carried out for 1 hour at 37°C. Six hours later, 50 µC of ³H-thymidine was added to cultures and incubation continued. Thirty-six hours after coinfection cells which had detached from the monolayer were spun out of the supernatant and added back to the cells in the monolayer before proceeding with the DNA extraction. Low molecular weight DNA was selectively extracted from control and infected cells using the sodium dodecyl sulfate - 1 M sodium chloride precipitation technique of Hirt.⁵ In most cases, pronase (2 mg/ml, self-digested for 1 hour at 37°C) was added 10 minutes after treatment with SDS and maintained in the cultures until addition of 5 M NaCl. The crude supernatant fractions were assayed for radioactivity and then further deproteinized by extracting with an equal volume of 90% phenol followed by dialysis against 1 x SSC (0.15 M sodium chloride, 0.015 M sodium citrate, 0.001 EDTA, pH 7) until the dialysate was phenol-free as determined by absorption at 260 nm. They were then re-assayed for radioactivity and centrifuged to equilibrium in isopycnic gradients of CsCl of mean density 1.705 gm/cm³ at 25°C for 48 hours at 100,000 x g. The solutions were fractionated either by top unloading, using an ISCO density gradient collector and analyzer, or by a simple bottom puncture and dripping procedure. 0.025 ml of the fractions was added to 10 ml Insta-Gel (Packard Instruments) in scintillation vials for radioactive assay and a

small portion of the remainder was used for measurements of refractive index. After scintillation analysis the fractions judged to contain the major peaks of DNA activity were pooled and dialyzed against 1 x SSC overnight. The DNA fractions were further analyzed by velocity sedimentation in neutral and alkaline sucrose gradients. Gradients were made by layering 5% to 30% sucrose in TNE (.01 M Tris, .15 M NaCl, .001 M EDTA, pH 7.2). For alkaline gradients, sucrose was brought to pH 12.5 before layering by addition of 10 N NaOH to each of the layering solutions. SV 40 form I DNA (21s) was used as a marker in all neutral sucrose experiments. SV 40 form II DNA (16s) was used in alkaline velocity gradients.⁶ Sedimentation was continued at 4°C for 3.5 hours at 100,000 x g and fractions were collected as described above. Selected DNA samples were used for electron microscopic examination and infectivity assay as described previously.²

As a control for DNA activity ASV-4 DNA was extracted from purified particles⁷, adjusted, following measurements of optical density at 260 and 280 nm, to the same μ gm/ml DNA content as that of viral DNA isolated directly from infected cells and assayed as above. Infectivity was assessed by the appearance of ASV-specific immunofluorescent antigens developed in BSC-1 coverslip cultures in presence of SV15 helper adenovirus. DNA samples were inoculated at 1×10^{-1} and 1×10^{-2} dilutions using the DEAE-dextran method² and the percentage of cells developing specific immunofluorescence was determined. There was no difference between the specific activity of the ASV DNA isolated directly for infected cells and that of DNA prepared directly from virions.

DNA was prepared and examined in the electron microscope following the microtechnique of Mayor and Jordan.⁸ For molecules where a single-stranded structure was anticipated 33% formamide was included in the hypophase. Contour lengths were measured on photographic enlargements using a calibrated map measurer. Temperature and optical density measurements of DNA samples in 1 x SSC were made in a Beckman spectrophotometer.

RESULTS

The results of a typical equilibrium centrifugation run are shown in Fig. 1. Profiles of radioactivity were obtained in all cases except the control from which essentially no low molecular weight DNA was isolated. Reasonably homogeneous

Fig. 1. Labeled ASV-4 DNA from 1 M NaCl SDS-pronase treated cells. Supernatant fluids were sedimented to equilibrium in CsCl gradients for 48 hours at 35,000 rpm in a SW 50.1 rotor at 25°C. Labeled ASV-4 DNA from CsCl density gradient above were dialysed against 1 x SSC and sedimented in neutral sucrose at 35,000 rpm for 3.5 hours at 4°C. Similar material was held at pH 12.5 at room temperature for 15 minutes and then sedimented in alkaline sucrose at 35,000 rpm for 3.5 hours at 4°C. Vertical arrows indicate the marker of ^3H -SV 40 DNA form I (21S), II (16S).

* Incubation temperature
Adsorption temperature

Brackets indicate fractions harvested and pooled for further analysis by electron microscopy and velocity sedimentation.

peaks were obtained from cultures incubated at both permissive and non-permissive temperature (40°C). The mean density of these peaks ranged from 1.72 gm/cm³ for gradients prepared from cultures incubated at the permissive temperature to 1.68 gm/cm³ for gradients harvested from non-permissive experiments. In the latter cases the amounts of DNA isolated were always about one log less as monitored by scintillation counting than in the permissive cultures. The relatively small amounts of cellular DNA always present in these supernatants, skewed the density profiles resulting in lower mean density readings. The majority of this cellular DNA was removed by subsequent sucrose velocity gradients. The results of subsequent neutral and alkaline sucrose velocity sedimentations on aliquots of selected CsCl peaks are also shown in Fig. 1. The major component of DNA from cells infected with ts-1 and ASV-4 invariably sedimented at 16S, a figure consistent with double-stranded linear satellite DNA. Isolation of viral DNA species appeared to be more efficient when cultures had been absorbed at 37°C for one hour prior to being placed at the relevant incubation temperature of either 34°C or 40°C. The 16S peaks from gradient ts-1 + ASV-4 contained infectious satellite virus DNA on subsequent titration by immunofluorescence. At the permissive temperature, gradients analysed using wild type KOS strain HSV-1 were indistinguishable from those using HSV-1 ts-1. Small amounts of low molecular weight DNA were isolated from specimens inoculated with HSV ts-1 alone at the non-permissive temperature. However this DNA occurred at a density of about 1.700 gm/cm³ and was markedly heterogeneous in a sedimentation characteristics. This DNA appears to be of cellular origin. A similar result was obtained by Mayor and Ratner⁹ in centrifugal analyses of supernatants prepared at the non-permissive temperature from cultures inoculated with a temperature sensitive mutant of adenovirus type 31, viz ts 13.

Electron microscopy. Linear double-stranded DNA molecules, many with lengths around 1.5 µm were observed in specimens prepared from the 16S peaks from neutral sucrose gradients (Fig. 2a). Single-stranded DNA prepared from 16S peaks from alkaline sucrose gradients also had a mean length of 1.5 µm. This DNA appeared in both linear and circular monomer forms (Fig. 2b, c, d). In some molecules small "pan handle"-like projections similar to those reported by Koczot et al.¹⁰ and Berns and Kelly¹¹ were observed.

DISCUSSION

In tissue cultures coinfecting with adeno-associated satellite virus and a temperature-sensitive mutant of herpes virus type 1 at the non-permissive temperature, satellite virus DNA can be isolated in the absence of detectable amounts of herpes virus DNA. Under the high salt condition of the extraction procedure (precipitation with 5 M NaCl, final concentration 1 M NaCl) the DNA is double-stranded indicating that both "plus" and "minus" strands have been synthesized. This satellite virus DNA does not appear to differ in its physical, chemical and biological properties from satellite virus DNA isolated directly from virions¹² or from cells coinfecting with helper adenoviruses where the end products include complete satellite virus particles⁹.

This DNA was markedly stable and did not suffer a significant loss in infectivity after storage for 9 months at 4°C. The G + C content of ASV-4 DNA in the double-stranded form predicted from the buoyant density in CsCl is 59%. Double-stranded ASV-4-DNA melts at 93°C^{7,15} consistent with a G + C content of 58%. The G + C content from chemical analysis is 58%.¹² This excellent agreement between the G + C content of the satellite virus DNA in its double-stranded form and its predicted values from melting temperature and buoyant density in CsCl suggests a reasonably homogeneous distribution of bases within the molecule. This result is in agreement with the recent findings of Koczot *et al.*¹⁰ and Berns and Kelly¹¹ that possibly palindromic DNA regions in the form of inverted terminal repetitions amounted to around 1.5% of the total genome length - an insignificant heterogeneity. It is highly probable that the majority of the DNA sequences in satellite virus are unique.

Satellite virus DNA extracted directly from cells coinfecting with satellite virus and a temperature-sensitive mutant of herpes simplex has identical properties to that extracted from virions. Why is this DNA then not encapsidated when the system also produces apparently ample amounts of satellite virus structural proteins?^{13,14} We are currently investigating this seeming anomaly.

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Fig. 2. a. Electron micrograph of linear double-stranded ASV DNA from neutral sucrose. X 50,000
b, c. Electron micrograph of circular monomeric single-stranded ASV DNA from alkaline sucrose. Regions suggesting internal complementarity are marked by arrows. X 40,000
d. Electron micrograph of a linear single-stranded DNA fragment from alkaline sucrose. A region suggesting internal complementarity is marked by an arrow. X 50,000

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