Analysis of the DNA Produced during Infection of Monkey Kidney Cells by Adenosatellite Virus Type 4 and Adenovirus SV15

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SUMMARY

Radioisotope labelling, autoradiography, electron microscopy and DNA–DNA hybridization have been used to analyse the DNA species produced in a stable line of monkey kidney cells after infection with defective adeno-associated satellite virus (ASV), in presence of and without simian helper adenovirus (SV 15). Extraction of DNA by the Hirt (1967) procedure and CsCl equilibrium density gradient sedimentation indicated that a newly synthesized low mol. wt. DNA was readily isolated from cells infected with the defective satellite virus alone, within 6 h after inoculation. Sedimentation analyses of this DNA through neutral sucrose gradients showed that it was heterogeneous, with a sedimentation coefficient in the range 11 to 23 S. The DNA was non-infectious when titrated with helper adenovirus. Sedimentation analyses of DNA extracted from cultures infected with satellite virus and helper adenovirus showed linear 32 S adenovirus DNA and a 26 S linear DNA which may represent a replicative intermediate of satellite virus. Electron microscopy showed that the latter DNA was linear with marked propensity for clumping and concatenation. Molecules with side chains were seen. Autoradiography showed that newly synthesized DNA isolated from cells infected with satellite alone was nuclear in origin and DNA–DNA hybridization indicated that the synthesized DNA not extracted by the Hirt procedure was cellular rather than virus DNA.

INTRODUCTION

Parvo- or picodnaviruses are small DNA animal viruses with icosahedral symmetry (Mayor & Melnick, 1966). The adeno-associated satellite viruses (ASV) are unique members of this group because they appear to be non-conditionally defective and cannot replicate unless there is co-infection with competent adenovirus. The DNA of satellite virus also has unique properties. The virus genome is single-stranded, but individual virus particles contain either a ‘plus’ or a ‘minus’ single strand (Mayor et al. 1969; Rose et al. 1969; Rose & Koczot, 1971; Berns & Adler, 1972). Upon extraction, these complementary strands from separate particles unite rapidly to form double-stranded DNA helices.

As one approach to the problem of satellite defectiveness at the molecular level we have undertaken an analysis of the low mol. wt. DNA species produced during a single cycle of

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infection of stable monkey kidney tissue culture cells with adenosatellite virus type 4 and simian helper adenovirus SV 15. These experiments have been aimed at determining the properties and possible origin of these intracellular species of DNA produced in the system, and whether any circular molecules are involved in satellite virus replication.

METHODS

Cells and viruses. BSC-1 cells, a stable line of African green monkey kidney cells, were grown in 16-oz bottles in Eagle's medium with 1 % glutamine and 10 % foetal calf serum. When the cells became confluent they were maintained in medium with 2 % calf serum for at least 3 additional days, until they were well established in stationary phase. Type 4 adenovirus associated satellite virus (ASV 4) with a titre of 10^7.5 haemagglutinin (HA) producing units/ml was used at a 10^-2 dilution. Contaminating adenovirus was inactivated by heating at 60 °C for 15 min. Simian helper adenovirus SV 15, titre 10^7.5 p.f.u./ml, was used at a 10^-3 dilution. In some experiments with satellite virus alone, particles purified by isopycnic sedimentation in CsCl with a density of 1.43 g/cm^3 were used as inoculum (10^10 particles/ml).

Infection of cells and [aH]-thymidine ([aH]-dT) labelling of DNA. Usually from six to twelve 16-oz bottles of BSC-1 monolayers were used for each experiment. Of these bottles, two or three were inoculated with virus, or were uninoculated, following the schedule shown in Table I. After 1 h adsorption of ASV 4, the cells were washed well and pulsed with 2 ml [aH]-dT (50 μCi/ml in Eagle's medium) for 2 h. Thymidine was poured off and the monolayers were washed and maintained in Eagle's medium for 3 or 5 additional h. They were washed once with ice-cold (4 °C) 0.01 M-tris, pH 7.8, before extraction of DNA.

Extraction and analysis of low mol. wt. DNA. Low mol. wt. DNA was extracted from control and infected cells using the SDS–1-M-sodium chloride precipitation technique of Hirt (1967). Low mol. wt. satellite virus DNA has been isolated in two other tissue culture systems using the Hirt procedure (Boucher, Melnick & Mayor, 1971; Mayor & Ratner, 1972). In most cases, pronase (2 mg/ml, self-digested for 1 h at 37 °C) was added 10 min 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 SSC (0.15 M-sodium chloride, 0.015 M-sodium citrate, 0.001 M-EDTA, pH 7.0) until the dialysate was phenol-free, as determined by checking the dialysis medium after frequent changes, until it was free from material absorbing at 260 nm. They were then re-assayed for radioactivity and centrifuged to equilibrium in density gradients of CsCl of mean density 1.705 g/cm^3 at 25 °C for 48 h at 100,000 g. The solutions were then fractionated either by top unloading, using an ISCO density gradient collector and analyser, or by a simple bottom puncture and dripping procedure. In most experiments 0.25 ml fractions were collected, of which 0.1 ml was added to 10 ml Aquasol (New England Nuclear, Boston, Mass.) 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 were pooled and dialysed against SSC overnight. The DNA fractions were then analysed by velocity sedimentation in neutral sucrose gradients (5 to 50 %, w/w). The sucrose solutions were prepared in TNE (0.01 M-tris buffer, 0.1 M-NaCl, 0.001 M-EDTA, pH 7.2). DNA samples in lamellae of 0.2 ml were layered on top of the sucrose gradients (4.8 ml) and centrifuged at 5 °C for 3.5 h at 100,000 g in the SW 50–1 rotor or the Spinco L 2 ultracentrifuge. Three or five drop fractions were collected by bottom
puncture and 0.1 ml of each fraction was assayed for radioactivity. The remaining material was used for electron microscopic examination or infective DNA assay when deemed advisable.

Either [14C]-labelled SV 40 Form I DNA or [3H]-labelled SV 40 Form I DNA was included as a marker during sedimentation analysis. The sedimentation velocities of DNA species were calculated using the method of Martin & Ames (1961).

DNA–DNA hybridization. In some experiments the high mol. wt. cellular DNA fraction prepared from uninfected BSC-1 cells (Hirt pellet) both cold and [3H]-labelled was extracted with 6 M sodium para amino salicylate (PAS) and phenol, treated with RNase and banded in CsCl gradients as described above. This DNA was used for DNA–DNA hybridization studies. In addition purified type 4 satellite virus DNA, both cold and [3H]-labelled, was prepared from virus particles by lysis with sodium lauroyl sarcosinate and phenol, as described by Mayor et al. (1969). DNA–DNA hybridizations were performed on nitrocellulose membrane filters using the procedure of Denhardt (1966) as modified by Smith (1968).

Radioactivity assay. Radioactive samples were prepared as 0.1 or 0.05 ml mixtures with 10 ml Aquasol and counted in a Beckman liquid scintillation counter.

Assay of satellite virus DNA infectivity. BSC-1 monolayers were prepared as coverslip cultures in Petri dishes. Usually, four coverslips were introduced into each dish. After infection 12 to 16 h previously with adenovirus SV 15 (input multiplicity of 1 p.f.u./cell), they were inoculated with dialysed DNA samples, using the diethylamine ethyl (DEAE)-dextran method of Pagano, McCutchan & Vaheri (1967) as modified by Kit et al. (1968). The coverslips were harvested 48 h later, fixed in acetone and assayed for satellite type 4 virus, using the direct fluorescent antibody assay as described by Ito, Melnick & Mayor (1967). This method is capable of detecting as little as 0.02 μg of DNA as inoculum/coverslip and DNA extracted from purified satellite virus particles was routinely infective by this technique (Boucher et al. 1971).

Electron microscopy of DNA. Crude supernatant fluids were checked for the presence of contaminating virus particles or empty capsids by negative staining with phosphotungstic acid. For examination as DNA, fractions were diluted to 1 to 2 μg/ml in a hypophase of 2 M-ammonium acetate. They were prepared for microscopy using the microtechnique developed by Mayor & Jordan (1968). The molecules were captured on collodion membranes and dehydrated in absolute ethanol and isopentane. The grids were then rotary shadowed with completely evaporated platinum–carbon pellets. Measurements of contour lengths were obtained on photographic enlargements using a calibrated map measurer.

Autoradiography. Cells were grown on 15 mm round coverslips in plastic Petri dishes. Monolayer coverslips were inoculated with virus, and Eagle's medium containing 1 μCi/ml of [3H]-thymidine was added after an adsorption period of 1 to 1.5 h. The coverslips were washed with tris-buffered saline and fixed with a modified Carnoy's solution (3 parts absolute ethanol and 1 part glacial acetic acid) 8 h after inoculation. The coverslips were air-dried and mounted, cell surface up, on a standard glass slide with permount. After a drying period of 3 days, the stripping film technique described by Schmidt (1965) was used. Slides stripped with Kodak Autoradiographic Stripping Plates AR.10 were developed with Kodak developer (D-19B) after 1- and 2-week exposure times at 4 °C. The developed slides were stained with the diluted and buffered Giemsa blood stain described by Schmidt (1965) and were examined with the light microscope.
RESULTS

It has been shown (Ito et al. 1967) that when helper adenovirus has been inoculated 12 to 16 h previously, satellite virus particles begin to appear from 4 to 6 h after subsequent inoculation with satellite virus. With this background and the fact that the genome of adeno-associated satellite virus in its single- or double-stranded configuration could be considered a low mol. wt. species, we decided first to examine the DNA species about 6 to 8 h after the inoculation of satellite virus.

Table 1. Inoculation and harvesting schedule for ASV 4 and SV 15 adenovirus

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<th>Series A: culture no.</th>
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<th>7 to 10</th>
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<td>SV 15</td>
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<td>-</td>
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<tr>
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<td>+6 h Harvest</td>
<td>3, 4</td>
<td>7, 8</td>
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<tr>
<td>+8 h Harvest</td>
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Fig. 1 illustrates a typical caesium chloride density-gradient analysis of [3H]-dT labelled low mol. wt. DNA obtained by the Hirt procedure from control uninoculated cells, cells infected for 18 and 20 h with adenovirus alone, cells infected 12 h with adenovirus and then additionally for 6 and 8 h with satellite virus, and cells inoculated with satellite virus alone for 6 h. In every case, the monolayers were pulsed with [3H]-dT for 5 or 7 h, as described in Table 1, series A. In the control cultures, there was no peak of radioactivity at a density of 1.699 g/cm³, the published density of African Green Monkey cell DNA (Rapp, Feldman & Mandel, 1966), indicating that the stationary phase cells were not actively synthesizing significant amounts of low mol. wt. DNA during the duration of the [3H]-dT label. In contrast, in the cultures infected with both adenovirus and satellite virus, there was a peak of [3H] radioactivity at a density of about 1.710 g/cm³ in samples obtained 6 h after addition of satellite virus and a more pronounced peak by 8 h after addition of satellite virus. Similarly, peaks of radioactivity at an identical density were visible in supernatant fluids of cultures inoculated with adenovirus alone 20 h previously. An unexpected finding was the appearance of a peak of radioactivity in the 6 h supernatant fluid from cultures infected with satellite virus alone.

Fractions with maximum radioactivities shown in Fig. 1 were dialysed against 1 x SSC to free them from CsCl and further analysed by sucrose velocity sedimentation as shown in Fig. 2. The DNA extracted from the cultures infected for 20 h with adenovirus alone gave a peak of radioactivity sedimenting at approximately 32 S. Electron microscopy revealed that this fraction contained linear double-stranded DNA with a modal contour length (12 μm) consistent with adenovirus DNA (Fig. 3 a). A small peak of radioactivity was found in the 7 S region, indicating occurrence of some DNA fragments in SV 15 infected cells possibly because of the presence of an endonuclease similar to that described in tissue culture cells infected with human adenoviruses (Burlingham & Doerffler, 1969). Sedimentation velocity analysis of the DNA obtained from the cultures infected for 20 h with both adenovirus and
satellite virus again revealed a peak population of 33 S molecules (adenovirus DNA) and indications of a population with 26 S characteristics. The 26 S species was again linear and double-stranded by electron microscopy. It was tested for DNA infectivity as a satellite virus DNA but was found to be noninfective. There was no evidence of circular or 'replicating' forms such as have been described for polyoma by Hirt (1969), for bacteriophage ΦX 174 by Knippers et al. (1969), and for SV 40 DNA by Levine, Kang & Billheimer (1970).

In another series of experiments (Table 1, series B), a very sharp DNA peak was obtained in the CsCl density gradient at a density of about 1.700 g/cm³ after extraction from cultures infected for 20 h with satellite virus alone. This fraction on subsequent velocity analysis gave a heterogeneous sedimentation profile ranging from 25 to 33 S, suggestive of a cellular species of DNA (Ritzi & Levine, 1970). In the samples derived from cultures infected with

**Fig. 1.** CsCl equilibrium gradients of the 1 M-NaCl–SDS dialysed supernatant fluids. [³H] ct/min/0.1 ml of fractions from cultures infected with (a) satellite plus adenovirus (△), (b) adenovirus (○) and (c) satellite virus (□ – □). The control is shown in (c) (■ – ■). Time schedule is shown in Table 1, series A. Sedimentation was for 48 h at 100,000 g in a SW 50.1 rotor at 25 °C. Fractions were collected by top unloading and counted as described in Methods.
adenovirus and satellite virus the 32 S peak was again indicative of adenovirus DNA, while electron microscopic examination of the 26 S peak revealed that the majority of the DNA molecules were linear although there was some evidence of branching molecules and forks reminiscent of those reported for replicating linear DNAs (Fig. 3b; Huberman, 1968). There was some evidence of strand separation but these regions were infrequent and no biological activity of adenosatellite DNA was detected in any of these fractions. Contour lengths of typical molecules which could be measured ranged from 1.5 to 3.0 μm. There were many fragments and many clumps present. The sample derived from cultures infected with adenovirus alone again gave clear evidence of 32 S adenovirus DNA and a typical electron microscopic image (Fig. 3a). In no specimens was there evidence for faster sedimenting circular or concatenated mitochondrial DNA (Hudson, Clayton & Vinograd, 1968).

In order to detect whether low mol. wt. DNA could be found by the Hirt extraction procedure 6 to 8 h after inoculation of cultures with adenovirus alone, a series of similar analyses as in Table 1, series C, were carried out. The density profile of DNA derived from cultures infected for 8 h with adenovirus had a broad peak at 1.69 g/cm³ and similarly the low mol. wt. DNA in the companion fraction obtained from the cultures inoculated with
DNA in adeno-satellite virus replication

Fig. 3. (a) Electron micrograph of DNA from sucrose-gradient fractions 12 to 16 of Fig. 2(b), 20 h adenovirus 32 S peak. Linear double-stranded molecule is approximately 11 μm long, consistent with an adenovirus genome.

(b) Electron micrograph of DNA from sucrose gradient fraction 12 of adenovirus + satellite (●) profile in Fig. 2a. Some evidence of branch points is shown by white arrows.

(c) Electron micrograph of DNA from sucrose gradient fractions 11 to 23 S, of satellite alone. In the lower right hand corner a multi-branched molecular aggregate similar to those reported by Mayor et al. (1969) can be seen. Two molecules (white arrows) show clear side branches.
satellite virus alone was broad but peaked at 1.70 g/cm³. Sucrose velocity analyses showed that these newly synthesized low mol. wt. DNAs were a heterogeneous species suggestive of host cell DNA (Ritzi & Levine, 1970). The sedimentation range was between 11 and 23 S for the satellite species and 11 and 21 S for the adenovirus species.

In the electron microscope the molecular species in all these fractions were linear and double-stranded. However, there was marked evidence of branching and end-to-end aggregation (concatenation). The image was somewhat reminiscent of denatured satellite virus DNA during rapid denaturation (Fig. 3c; Mayor et al. 1969). Many molecules showed clear evidence of side chains and appeared markedly similar in morphology to the replicative intermediate form shown by Granboulan & Franklin (1968) for the RNA bacteriophage R17. However, these specimens were again non-infective as satellite virus DNA in the fluorescent antibody assay system. Identical results were obtained when the inoculum comprised purified satellite virus rather than stock from which any contaminating adenovirus had been removed by heat. In agreement with these findings, J. A. Rose (personal communication in Johnson, Blacklow & Hoggan, 1972) has reported that, in the absence of helper virus, satellite virus does not initiate detectable replication of virus DNA.

**DNA–DNA hybridization**

As can be seen in Table 2, the newly synthesized low mol. wt. DNA from cells infected with satellite virus alone shows that 35% hybridization with DNA from BSC-1 cells but has little reaction with DNA from purified satellite virus. This is a clear indication that the ‘turned on’ DNA is of cellular rather than virus origin. DNA from satellite virus particles gave a 90% hybridization reaction with its homologous DNA and essentially no reaction with BSC-1 cellular DNA. These results indicate that the DNA ‘turned on’ by satellite virus alone is not encapsidated in satellite particles.

**Autoradiography**

The above experiments were designed to focus attention on the low mol. wt. DNA species in the adenovirus–satellite virus system. However, in an attempt to detect the sites of origin of all the newly synthesized DNA in these systems, cell monolayers which had been treated in an identical way to those destined for extraction by the Hirt procedure (Table 1) were studied by autoradiographic examination of all DNA species labelled during the [³H]-dT pulse period. An important factor in these experiments was the condition of the cells during the radioactive pulse. In our experience, the cells should be confluent monolayers almost to the point of being overgrown, i.e. about 2 weeks after seeding and being in
DNA in adeno-satellite virus replication

Fig. 4. (a) Autoradiograph of uninfected BSC-1 monolayer from stationary cultures labelled with [³H]-thymidine as described in Methods. (b) Autoradiograph of BSC-1 monolayer 8 h after infection with satellite alone. Culture was labelled with [³H]-thymidine as described in Methods.

maintenance medium with 1 % serum for at least 1 week. Under these conditions, there were no heavily labelled cells in the controls; in fact, very light labelling was usual in less than 0·1 % of the nuclei (Fig. 4a). In specimens inoculated with satellite virus alone and harvested at 8 h, approximately 30 % of the cells showed heavy nuclear labelling. The label appeared to be all over the nucleus and usually included the nucleolus (Fig. 4b). In specimens infected with adenovirus alone 8 h previously, 27 % of the cells were labelled while, of those which had been treated with adenovirus 20 h before and also inoculated with satellite virus 8 h previously, 55 % of the cells were heavily stained. There was no visible cytoplasmic staining in any of the cells. Clearly, newly synthesized DNA is apparent in the nuclei of cells inoculated with adeno-satellite virus without helper as early as 6 to 8 h after infection. The sites of origin of this DNA were nuclear and widely distributed.
DISCUSSION

Our results have shown that, without helper virus, defective satellite virus can induce DNA synthesis in non-growing BSC-1 cells shortly after inoculation. Tennant & Hand (1970) have previously found that Kilham rat virus (RV), an autonomous picornavirus, was unable to replicate in cells which had been u.v.-irradiated prior to infection. More recently, R. W. Tennant (personal communication) has shown that other autonomous picornaviruses, H-1 and MVM, also require some radiosensitive cellular function but that adenovirus-associated satellite viruses replication, like that of SV 40, polyoma and adenovirus, is not affected by irradiation of the cells prior to infection. In its ability to promote synthesis of presumably cellular DNA, satellite virus may be grouped with SV 40, polyoma and adenovirus. Thus, perhaps in their requirements for a cellular function not needed by other DNA viruses, it may be reiterated in agreement with Tennant that the ‘autonomous’ picornaviruses are defective while satellite virus at least in this respect is not.

Our results with SV 15 alone in non-growing BSC-1 cells show that a low mol. wt., heterogeneous DNA of 11 to 21 S is synthesized from 6 to 8 h after infection but that by 20 h after infection a homogeneous 32 S peak of adenovirus DNA is clearly detectable. In a recent publication, Yamashita & Shimojo (1969) showed that cellular DNA synthesis was induced by adenovirus type 12 in non-growing human embryo kidney cells (HEK) in which the virus replicated efficiently. Cellular DNA was synthesized first, beginning at about 8 h after inoculation. However, by 24 h a definite shift had occurred and most of the DNA being synthesized was virus. Similar shifts from cellular to virus DNA synthesis in other cell systems have been described (Green, Pina & Chagoya, 1964; Ginsberg, Bello & Levine, 1967). However, it is not clear from these publications whether or not the induction of cellular DNA synthesis is a prerequisite for the replication of virus DNA. In fact, in general, a mandatory requirement for cell DNA synthesis prior to virus DNA synthesis would not appear to be the case, e.g. the results of Ritzi & Levine (1970) in a comparative study of DNA replication in SV 40-infected monkey cells. They showed that, in primary cells (AGMK) and in CV-1 cells, cellular DNA was induced and infectious virus was later produced. However, in BSC-1 cells, where there was no detectable induction of cellular DNA, SV 40 still replicated well, although the eclipse period was longer than in AGMK or CV-1 cells.

In our velocity gradients from joint infections of cultures with adenovirus and satellite virus, 26 S peaks were resolved, but those molecules that could be measured by electron microscopy were linear and only 1.5 to 3.0 μm long. The reason for this discrepancy between the measured length and the sedimentation coefficient is not known and we intend to study this point further. In addition, aside from a few branching configurations, considered suggestive of replicative molecules (Huberman, 1968; Knippers et al. 1969), no circular replicative intermediates were observed and certainly no infectious DNA was isolated although the assay system used was certainly sensitive enough to do so (Boucher et al. 1971).

Perhaps the most helpful model at this stage is that postulated for single-stranded RNA phages such as R 17 (Granboulan & Franklin, 1968) and M 12 (Hofschneider, Amman & Francke, 1967). In these cases, double-stranded linear molecules and molecules with nascent side-chains (rather than circular forms) are believed to be intermediates in the process of replicating single-stranded RNA genomes (Fenwick, Erikson & Franklin, 1964). It is of interest that these replicative forms are not infective for E. coli spheroplasts unless they are denatured to single strands. There is also recent evidence for a linear replicating intermediate in bacteriophage T 7 DNA (Wolfson, Dressler & Magazin, 1971) and electron microscopy...
revealed Y-shaped rods whose length approached the length of the mature T 7 chromosome. These authors suggested that DNA viruses which contain their genetic information in duplex rods might replicate their DNA by a similar mechanism.

It is possible the final stages of satellite virus replication yielding ‘plus’ and ‘minus’ strands encapsidated in separate virus particles take place extremely rapidly. It is also possible, by analogy with the replicative RNA phage systems, that the linear double-stranded species isolated are true replicative forms which are non-infectious under the test conditions used. We intend studying the replication process further, using a more favourable detection system (see Boucher et al. 1971; Ito & Suzuki, 1971).

What then is the function of the newly synthesized DNA induced by infection with defective adenosatellite virus? The heterogeneous sedimentation profile coupled with the time of appearance, lack of infectivity and DNA–DNA hybridization pattern indicate that the DNA synthesized after inoculation with satellite virus alone is probably a cellular species. However, this does not preclude the possibility that it may be satellite-specific. The excision of pre-existing chromosomal DNA and its encapsidation into ‘pseudo’ virus particles is already well documented for polyoma and SV 40 (Michel, Hirt & Weil, 1967; Winocour, 1969; Levine & Teresky, 1970). However, polyoma or SV 40 ‘pseudo-virion’ DNA is detected in cells that have been labelled prior to infection with [3H]-dT and does not represent a new species of DNA. In the satellite virus system, the DNA is newly synthesized after infection. We are currently carrying out pulse-chase experiments using brief pulses of [3H]-labelled thymidine and examining satellite virus and adenovirus progeny after density-gradient centrifugation to determine the fate of this new species of DNA.

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