VIRAL DNA SYNTHESIS IN ISOLATED NUCLEI FROM ADENOVIRUS-INFECTED KB CELLS

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1. Introduction

The application of permeabilized cells and membrane fractions, which possess DNA-synthesizing capacity, has stimulated considerably the study of the mechanism of DNA replication in procaryotic systems [1, 2]. A similar approach for the replication of DNA in higher organisms has hardly been investigated. Nuclei, isolated from mammalian cells, can incorporate deoxyribonucleoside triphosphates into DNA [3], while Winnacker et al. [4] have shown that isolated nuclei from polyoma-infected cells can perform synthesis of viral DNA. These nuclear systems may be very helpful to unravel the problem of DNA replication in mammalian cells.

We are interested in the replication of adenovirus DNA. This DNA has a linear duplex structure and the absence of circular permutation and terminal redundancy [5, 6] suggests that the mechanism of replication of adenovirus DNA differs from the known mechanisms, in which circular intermediates are involved [7].

To investigate the mechanism of replication of adenovirus DNA we have developed a DNA-synthesizing system, based on isolated nuclei from cells infected with adenovirus type 5 (Ad5). This paper describes the properties of this system and the characteristics of the viral DNA synthesized.

Abbreviations:
Ad5: adenovirus type 5
TCA: trichloroacetic acid
Hepes: N-2-hydroxyethylpiperazine-$N^1$2-ethanesulfonic acid
Sarkosyl: sodium *N*-lauroyl-Sarcosinate (Geigy, Basel, Switzerland).

2. Methods

The growth of KB cells in monolayer and the purification of Ad5 and Ad5 DNA have been described [8, 9]. Cells in suspension cultures were infected in the presence of 4 μg/ml mitomycin C to suppress cellular DNA synthesis (m.o.i. about 3000 physical particles per cell). Cells were harvested 15 hr post infection and washed with hypotonic Hepes buffer (20 mM Hepes, pH 8.1, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 1 mM dithiothreitol) [4].

After washing, cells were resuspended in hypotonic Hepes buffer at a concentration of $5-10 \times 10^6$ cells/ml. After 10 min, cells were ruptured by 20 strokes with a Potter homogenizer with a clearance of 0.005 cm, after which the nuclei were sedimented at 800 *g* for 10 min, washed in isotonic Hepes buffer (50 mM Hepes, pH 8.1, 110 mM NaCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 1 mM dithiothreitol), again sedimented at 800 *g* and finally resuspended in isotonic Hepes buffer. Under these conditions mitochondria and other small cell organelles as well as soluble cell components do not sediment and are removed from the nuclear preparations. Repeated washings and centrifugations of the nuclei under the conditions described did not influence the synthesizing capacity per nucleus, although a slight loss of nuclei after each washing was observed.

Further purification involving dense sucrose solu-

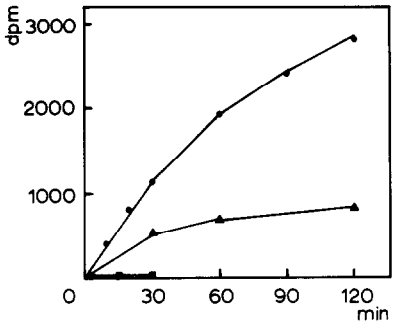

Fig. 1. DNA synthesis in isolated nuclei (4.4 × 10^5) from Ad5-infected cells (●–●–●), in nuclei (4.4 × 10^5) from uninfected cells (▲–▲–▲) and in intact infected cells (5 × 10^5) (■–■–■).

tions or detergent treatments, which are successfully used for uninfected nuclei, could not be applied to nuclei of infected cells, since these nuclei are very fragile. Treatment with the detergent Nonidet P40 and centrifugation on sucrose gradients resulted in an extensive aggregation and loss of nuclei. The final reaction mixture contained 40 mM Hepes, pH 7.1, 90 mM NaCl, 8 mM $MgCl_2$, 0.4 mM $CaCl_2$, 8 mM phosphoenolpyruvate, 5 mM ATP, 0.8 mM dithiothreitol, 0.05 mM each of dATP, dGTP, dCTP, 0.001 mM 6-^{3}H-TTP (7 Ci/mM) and 1 U/ml pyruvate kinase (Worthington, New Jersey, USA). The concentration of nuclei varied between 5–10 × 10^6 per ml and the incubation was performed at 30°. TCA-insoluble radioactivity was measured by filtration on nitrocellulose filters.

For the isolation of newly synthesized DNA the reaction was stopped by addition of EDTA up to 10 mM, sodium dodecylsulphate (0.1%) and pronase (500 μg/ml) were added and after 10 min at 30° the sodium dodecylsulphate concentration was raised to 1%. After 5 min NaCl was added to a concentration of 1 M and viral DNA was isolated [10].

DNA–DNA hybridization was performed according to Aloni et al. [11].

3. Results

Isolated nuclei from Ad5-infected cells appeared to be able to synthesize DNA, when incubated in an appropriate reaction mixture in the presence of the four

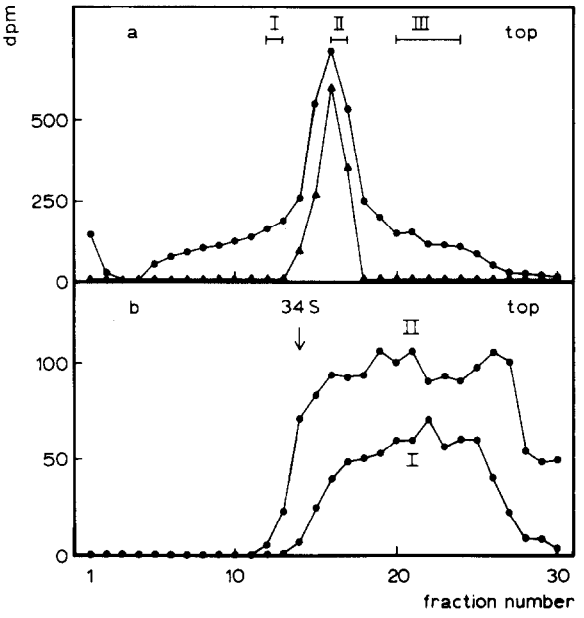

Fig. 2a. Preparative sucrose gradient centrifugation of new ^{3}H-DNA (●–●–●) on a isokinetic sucrose gradient containing 0.2 M NaCl, 0.01 M Tris pH 8.1, 0.001 M EDTA and 0.1% sarkosyl. Centrifugation was performed for 16 hr at 19,000 rpm at 5° in the Spinco SW25 rotor after which the distribution of TCA-insoluble radioactivity in aliquots of each fraction was determined. Fractions 12 and 13 (I), 16 and 17 (II) and 20–24 (III) were combined and I, II and III were each further investigated. ^{14}C-labeled Ad5 DNA (▲–▲–▲) sedimented as 31 S material. b) Alkaline sucrose gradient centrifugation of fractions I and II on isokinetic sucrose gradients containing 0.3 M NaOH, 0.9 M NaCl, 0.01 M Tris, 0.001 M EDTA and 0.1% sarkosyl. Centrifugation was performed at 37,000 rpm for 4 hr at 15° in the Spinco SW 41 rotor with ^{14}C-Ad5 DNA (34 S) as a marker.

deoxyribonucleoside triphosphates, including ^{3}H-TTP. A linear increase in TCA-insoluble radioactivity was observed during the first 30 min (fig. 1) and the reaction proceeded at least for 2 hr, although at a lower rate. Synthesis was not due to remaining whole cells as infected KB cells did not incorporate significant amounts of ^{3}H-TTP. Nuclei, isolated from uninfected cells synthesized some DNA and the reaction almost stopped after 30 min.

To distinguish between nuclear and extranuclear DNA synthesis, nuclei from infected cells were preincubated in the standard reaction mixture, omitting ^{3}H-TTP. After 15 min at 30° the nuclei were centri-

Table 1

^{3}H-DNA in solution	DNA on filter	^{3}H-DNA hybridized (%)
Ad5 DNA	none	0
Ad5 DNA	KB DNA (10 μg)	1.2
Ad5 DNA	Ad5 DNA (4 μg)	49.5
New DNA	none	0.4
New DNA	KB DNA (10 μg)	1.3
New DNA	Ad5 DNA (4 μg)	47.0

Viral DNA (1350 dpm) or newly synthesized DNA (1831 dpm) were incubated with nitrocellulose filters containing unlabeled DNA as indicated. Hybridization was performed in 0.3 M NaCl–0.03 M trisodium citrate for 16 hr at 66°.

fuged at 800 *g* for 10 min, to the supernatant ^{3}H-TTP was added and the nuclei were resuspended in the reaction mixture containing ^{3}H-TTP. All DNA synthesis appeared to be restricted to the nuclei. When the nuclear suspension was centrifuged after incubation, all TCA-insoluble radioactivity was present in the pellet.

New DNA, synthesized in isolated nuclei from Ad5-infected cells, was further characterized. Nuclei were incubated for 60 min and viral DNA was isolated as described in Methods. The isolated DNA was compared to authentic Ad5 DNA by DNA–DNA hybridization (Table 1). New DNA hybridized to Ad5 DNA with the same efficiency as DNA isolated from virions, while no significant hybridization with KB DNA was observed. The newly synthesized DNA was subjected to preparative sucrose gradient centrifugation. Fractions (0.9 ml) were collected and the distribution of TCA-insoluble radioactivity was determined (fig. 2a).

Besides molecules co-sedimenting with the 31 S marker (Ad5 DNA from virions), material could be detected which sedimented faster and slower than the marker, respectively. DNA, isolated from uninfected nuclei, sedimented heterogeneously and not faster than 12 S.

The different types of molecules in infected nuclei were further characterized by recentrifugation of some fractions (I, II and III) combined as indicated in fig. 2a. The results of neutral and alkaline CsCl density centrifugation are presented in fig. 3.

Neutral CsCl centrifugation showed that the slowly

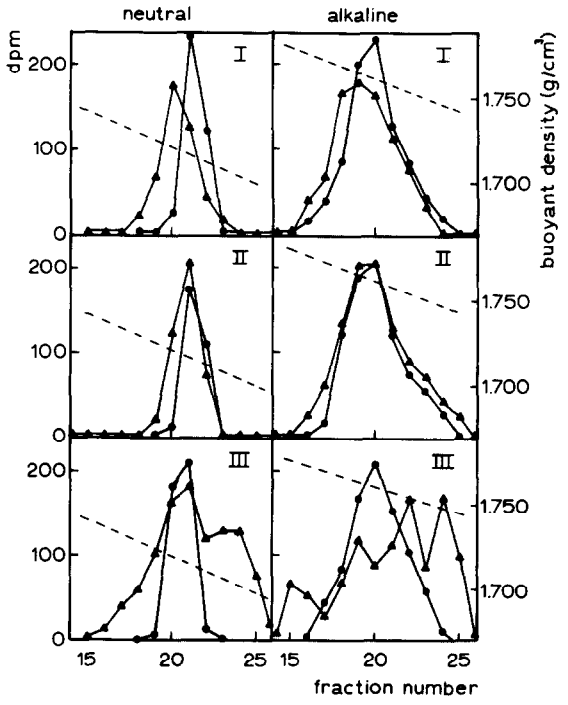

Fig. 3. Buoyant density of new ^{3}H-DNA (▲–▲–▲) in neutral and alkaline CsCl gradients. Fractions I, II and III were obtained as indicated in fig. 2a. The gradients consisted of CsCl in 0.15 M NaCl–0.015 M trisodium citrate, 0.1% sarkosyl, pH 7.2 for neutral and pH 12.5 for alkaline gradients. Centrifugation was performed at 38,000 rpm for 76 hr at 10° in a Spinco R50 rotor. ^{14}C-labeled Ad5 DNA was used as a density marker (●–●–●).

sedimenting material (III) consisted of 60% viral (1.716 g/cm^3) and 40% cellular DNA (1.700 g/cm^3). The amount of cellular DNA was calculated to be less than 5% of total new DNA. The "31 S" fraction (II) mainly banded at the same position as Ad5 DNA and contained also heavier material. The fast sedimenting molecules (I) were distinctively heavier (1.722 g/cm^3) than Ad5 DNA. Incubation of fraction I with 100 μg/ml RNase or 80 μg/ml pronase for 1 hr at 37° did not affect the buoyant density. The alkaline CsCl centrifugation revealed that molecules of fractions I and II were both of viral origin, while the distribution of radioactivity of III was rather complex, probably because of the presence of cellular DNA fragments.

Fractions I, II and III were also subjected to neutral and alkaline sucrose gradient centrifugation.

The results of the neutral gradients confirmed the existence of rapidly sedimenting molecules in fraction I. Centrifugation on alkaline gradients showed that new DNA in fraction I sedimented heterogeneously with a broad peak at about 15 S and did not contain molecules of 34 S (single strands of Ad5 DNA) (fig. 2b). Fraction II showed a heterogeneous distribution too with molecules ranging from 34 S to very small fragments (fig. 2b). Under alkaline conditions no molecules longer than genome size were observed.

4. Discussion

Our results indicate that isolated nuclei from Ad5-infected cells synthesize viral DNA during incubation with the four deoxyribonucleoside triphosphates. The amount of viral DNA synthesized after 30 min was calculated taking into account the specific activity of ^{3}H-TTP added and a pool size of 66 pmoles TTP per 10^6 nuclei. The latter was determined by isotope dilution. Under the reaction conditions described 4.5 Ad5 DNA equivalents per nucleus were synthesized. In experiments in which 5-bromodeoxyuridine triphosphate was substituted for TTP during 120 min of incubation the buoyant density of product DNA in neutral CsCl increased from 1.716 to 1.727 g/cm^3. This means that a considerable amount of new DNA was present per molecule as the hybrid density of Ad5 DNA is 1.754 g/cm^3. This result in combination with the number of Ad5 DNA equivalents synthesized per nucleus, suggests that a limited number of DNA molecules is involved in DNA synthesis, which is in favour of replication.

A preliminary interpretation of the sedimentation results is that the rapidly sedimenting fraction I represents replicating intermediates, while the "31 S" fraction (II) consists partially of completed molecules. In addition, fraction II contains molecules which may have originated from disruption of intermediates during isolation, which may also account for fraction III. This view is supported by the alkaline sucrose gradient centrifugation of fraction II, in which molecules of genome size were observed as well as smaller pieces. Fraction I contained molecules which sedimented between 31 S and 60 S in neutral gradients and slower than 34 S in alkaline gradients.

In neutral CsCl gradients fraction I showed an increase in buoyant density of 6 mg/cm^3 as compared to Ad5 DNA. Recently Pearson and Hanawalt [12] observed DNA molecules in Ad2-infected cells after pulse labeling with ^{3}H-thymidine with similar characteristics. From chase experiments they concluded that these molecules are replicative intermediates. Therefore it seems likely that the mechanism of viral DNA synthesis in isolated nuclei reflects the *in vivo* situation. Detailed investigations on viral DNA synthesis in isolated nuclei are in progress.

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