Adenovirus DNA replication in vitro is stimulated by RNA from uninfected HeLa cells

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Adenovirus DNA replication was studied in a partially reconstituted system consisting of purified viral proteins (DNA-binding protein, precursor terminal protein and Ad DNA polymerase) and a nuclear extract from uninfected HeLa cells. Optimal DNA replication required the presence of a heat-stable, ribonuclease-sensitive fraction from the cytosol of uninfected cells. This fraction stimulated the initiation about 3-fold and the replication of origin fragments 5–10-fold. Sedimentation analysis indicated the presence of a fast-sedimenting and a slow-sedimenting component which complemented each other. At least part of the stimulation was caused by low-molecular-mass RNA.

Adenovirus *DNA replication in vitro* *Reconstitution* *Small RNA*

1. INTRODUCTION

The linear double-stranded DNA from adenoviruses contains about 36000 base pairs and has a 55-kDa protein, the terminal protein (TP) covalently bound to both 5′-termini. The development of a cell-free DNA replication system performing both initiation and elongation in vitro [1] has revealed many features of both the mechanism and enzymology of the replication process (review [2–5]). Initiation proceeds by a novel mechanism in which the 80-kDa viral precursor of the terminal protein (pTP) binds a dCMP residue which serves as the first nucleotide of the nascent DNA chain. Subsequent elongation of the pTP-dCMP complex occurs by a displacement mechanism. In addition to the pTP, DNA synthesis requires two other virus-encoded proteins, the 72-kDa DNA-binding protein (DBP) and the 140-kDa Ad DNA polymerase (pol). Two cellular proteins isolated from uninfected HeLa cell nuclei are also involved in DNA replication. These are the 47-kDa nuclear factor I which binds specifically to the origin of DNA replication [6] and nuclear factor II, a type I DNA topoisomerase required for elongation [7].

We present evidence that in addition to these two nuclear proteins at least one, and possibly two, heat-stable factors from the cytosol of uninfected HeLa cells stimulate adenovirus DNA replication in a partially reconstituted system. One of these has the properties of low-molecular-mass RNA.

2. MATERIALS AND METHODS

2.1. *Replication components*

The purification of DBP [8], of the complex of pTP-pol [9] and of DNA–TP from virions [10] has been described. Extraction of nuclei from uninfected cells was performed with 0.3 M NaCl followed by dialysis against 25 mM Hepes–KOH (pH 7.5), 10% sucrose, 40% glycerol, 1 mM dithiothreitol. Nuclear factor I was purified as in [6] up to the glycerol gradient step. Crude nuclear extracts from Ad5 infected HeLa nuclei were prepared essentially as in [1].

2.2. *Incubation conditions*

Reaction mixtures (30 μl) contained 40 mM Hepes–KOH (pH 7.5), 4 mM $MgCl_2$, 0.4 mM dithiothreitol, 1.7 mM ATP, 5 mM creatine

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phosphate, 5 μg/ml creatine kinase, 17 μM each of dATP, dGTP and dTTP, 8 μM [α-^{32}P]dCTP (3–7 Ci/mmol), 2.5 μl (2 mU) pTP-pol, 1.25 μl (1.25 μg) DBP, 3 μl nuclear extract, 3 μl cytosol, and 40 ng DNA–TP previously digested with *Xho*I. After 60 min at 37°C the mixture was analysed by agarose gel electrophoresis in 1% gels containing 0.1% SDS [10]. For analysis of initiation [α-^{32}P]dCTP was added as the only deoxynucleotide triphosphate, while for partial elongation 2′,3′-dideoxy GTP replaced dGTP [10].

2.3. *Isolation of cytosol and RNA*

From 5 × 10^9 HeLa cells nuclei were prepared [1] and the cytoplasm (15–20 ml) was centrifuged for 45 min at 30000 rpm at 4°C in an SW41 rotor. The supernatant was heated in 1-ml portions for 15 min at 80°C, cooled and centrifuged for 10 min in an Eppendorf centrifuge. The resulting cytosol was used for further studies. For the isolation of RNA, cytosol was centrifuged for 60 min at 40000 rpm in an SW50.1 rotor. The supernatant was brought to 0.5% SDS, extracted with phenol and precipitated with 80% ethanol. Incubations of cytosol with pronase (100 μg/ml) or pancreatic ribonuclease (100 μg/ml) were for 2 h at 37°C.

3. RESULTS

Ad5 DNA-TP was digested with *Xho*I which gave 7 fragments (CEFGADB) containing 15.8, 6.8, 3.9, 1.6, 41.9, 12.8 and 17.2% of the genome, respectively. Incubation of this mixture under DNA replication conditions gave almost exclusively labeling of the origin fragments B and C (fig.1). In addition slower-migrating fragments (B-RI, C-RI) are observed which constitute replicating intermediates consisting of new DNA to which a full length displaced single-stranded arm is connected [5]. Also fast-migrating single-stranded B and C fragments are labeled which originate from second rounds of replication [11,12]. Reconstitution could be performed by combining the viral DBP, pTP-pol and nuclear extract from uninfected cells (fig.1, lane 2). Omission of any of these components leads to less than 1.5% incorporation compared to the complete system. Nuclear extract could be substituted by partially purified nuclear factor I protein (not shown). The replication level in this reconstituted system was only 10–20% of

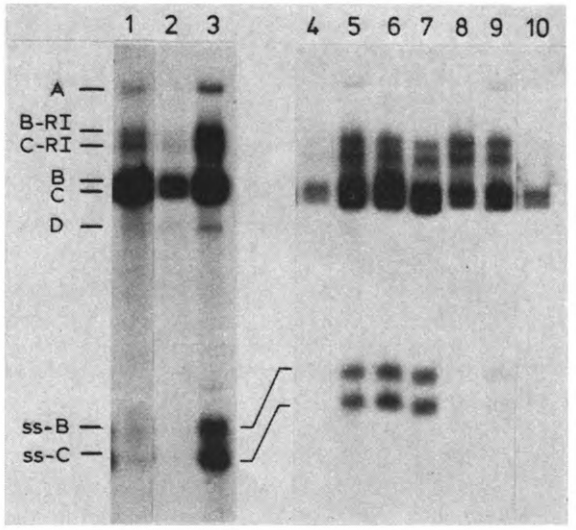

Fig.1. Stimulation of DNA replication by cytosol and RNA. Ad5 DNA-TP was digested with *Xho*I and incubated under various conditions. B and C are the origin containing fragments. An autoradiogram of a 1% agarose gel is shown. Lanes 1–3 and 4–10 are from two different experiments. Lane 1, crude nuclear extract from Ad5 infected HeLa nuclei. Lanes 2–10, reconstituted system. Lanes 2,4, no cytosol. Lanes 3,5, plus cytosol. Lane 6, cytosol heated for 15 min at 80°C. Lane 7, cytosol preincubated with pronase. Lane 8, cytosol preincubated with ribonuclease. Lane 9, RNA (20 μg/ml) added. Lane 10, RNA preincubated with ribonuclease.

that observed for crude extracts from infected HeLa nuclei (cf. fig.1, lanes 1 and 2). Restoration to, or even above this level was possible by addition of cytosol with an optimum of 3 μl per 30 μl incubation (fig.1, lanes 3,5). This cytosol factor was heat-stable, insensitive to pronase and partially sensitive to ribonuclease (fig.1, lanes 6–8), suggesting that the active component contains RNA. This was substantiated by isolation of RNA from cytosol after high-speed centrifugation. RNA (20–50 μg/ml optimum) stimulated also (fig.1, lane 9) but only 3-fold compared to 5–10-fold stimulation by crude cytosol.

To study the level at which cytosol increased the replication efficiency the synthesis of an initiation complex (pTP-dCMP) and the partial elongation until nucleotide 26 in the presence of ddGTP were analyzed. As shown in fig.2, cytosol stimulated both processes. This indicates that cytosol acts at

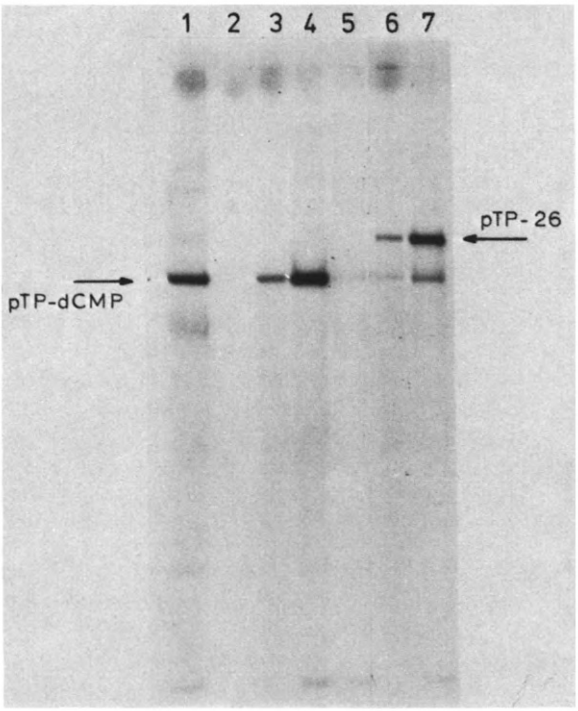

Fig.2. Effect of cytosol on pTP-dCMP formation. Ad5 DNA-TP was incubated under various conditions and the initiation (lanes 1–5) or the synthesis of a 26-mer (pTP-26, lanes 6,7) was studied. An autoradiogram of a polyacrylamide gel is shown. Lane 1, crude nuclear extract from Ad5 infected nuclei. Lanes 2–7, reconstituted system. Lane 2, nuclear extract and cytosol omitted. Lanes 3,6, cytosol omitted; lanes 4,7, complete system. Lane 5, nuclear extract omitted. The cytosol was preheated for 15 min at 80°C.

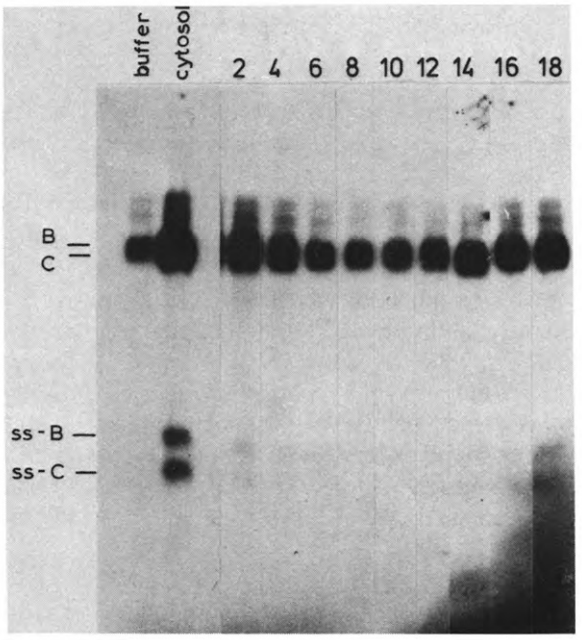

Fig.3. Sucrose gradient analysis of stimulatory factors from cytosol. Cytosol (300 μl) was heated for 15 min at 80°C and loaded on a linear 5–20% sucrose gradient in 20 mM Hepes–KOH (pH 7.5), 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol. Centrifugation was for 4 h at 35000 rpm at 4°C in an SW41 rotor. Eighteen fractions were collected and 12 μl of each fraction was analyzed in a partially reconstituted DNA replication system lacking cytosol. Only the results of the even fractions are shown.

the level of initiation. However, the stimulation is only 3-fold compared to 5–10-fold in the fragment replication assay. This suggests an additional effect of cytosol on the level of elongation. RNA from cytosol had only a marginal (1.3-fold) stimulating effect on initiation (not shown) suggesting that its main function is in elongation beyond nucleotide 26. This would indicate that more than one cytosol factor is required. To investigate this possibility the crude cytosol was separated by sucrose gradient centrifugation and the fractions were analysed for stimulatory activity (fig.3). Both fast- and slow-sedimenting fractions were able to enhance the replication about 3-fold. When these fractions (fraction no.2,18) were combined, 83% of the level of the input cytosol was reached which in this experiment amounted to a 9-fold stimulation. This shows that the fast- and slow-sedimenting components complemented each other. The slow-sedimenting material could be completely substituted by RNA.

4. DISCUSSION

So far, the replication of adenovirus DNA is the only system in mammalian cells capable of both initiation and elongation in vitro. Reconstitution studies have revealed that at least 3 viral and 2 cellular proteins are required. Our results indicate that in addition two heat-stable factors stimulate the reaction. The factors differ from the nuclear proteins since nuclear extracts are completely inactivated by heating for 15 min at 80°C. The slow-sedimenting factor consists most likely of small

RNA and may be required for elongation. The available evidence suggests a role for the faster component in initiation. However, this has not yet been demonstrated directly and the situation may be more complicated. Although we cannot exclude a general stimulatory effect of RNA this seems less likely since low concentrations of tRNA severely inhibit DNA replication (unpublished). Small RNA molecules have been implicated in various regulatory processes such as RNA splicing [13] and transcription termination [14], but we are not aware of any function of pre-existing RNA in eukaryotic DNA replication. Using affinity chromatography we recently observed that at least part of the small RNA can bind to the adenovirus DNA-binding protein. The physiological significance of this binding remains to be demonstrated.

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